EFFECTS OF PERI-OPERATIVE STATIN TREATMENT ON ATRIAL ELECTRICAL PROPERTIES, POST-OPERATIVE ATRIAL FIBRILLATION AND IN-HOSPITAL CLINICAL OUTCOMES IN PATIENTS UNDERGOING ELECTIVE CARDIAC SURGERY

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I dedicate this thesis to all the past, present and future clinical trial participants selflessly taking part in the studies and contributing to the advancement of medical science and health of fellow human beings

‘Le bon Dieu est dans le détail’
(The good God is in the detail)
Gustave Flaubert (1821–1880)

ABSTRACT

Surgical myocardial revascularization remains the standard of care for patients with multi-vessel coronary artery disease. A growing body of evidence indicates that systemic inflammation and myocardial oxidative stress are associated with the development of postoperative atrial fibrillation (POAF) and low cardiac output syndrome in patients undergoing cardiac surgery. Statins have been shown to exert rapid anti-inflammatory and antioxidant effects by inhibiting myocardial NOX2 oxidases and by increasing the bioavailability of nitric oxide (NO). However, whether these so-called pleiotropic effects of statins result in improved patient outcomes remains to be established. To provide further insights into the mechanisms of action and impact on clinical outcomes of peri-operative statin treatment in patients undergoing cardiac surgery, I studied the molecular mechanisms underlying the myocardial nitroso-redox balance in samples of the right atrial appendages (RAA) obtained before (PRE) and after cardiopulmonary bypass (CPB) and reperfusion (POST) and setup two double-blind randomised placebo-controlled trials: 1) STARR (Statin Treatment on Atrial Refractoriness and Reperfusion injury), which tested the effect of Atorvastatin (80 mg once daily for up to 6 days before surgery and 5 days after) on the atrial effective refractory period (AERP, over 4 post-operative days) and superoxide production in paired PRE- and POST- RAA samples from 60 patients 2) STICS (Statin Treatment In Cardiac Surgery), which assessed the effects of peri-operative treatment with Rosuvastatin (20mg od) on POAF (assessed by continuous holter ECG monitoring for 5 days post-operatively) and myocardial injury (assessed by serial troponin I measurements) in 1922 patients undergoing elective cardiac surgery.

I observed that atrial superoxide production increased significantly after reperfusion due to increased mitochondrial and NOX2 oxidase activity and to uncoupling of NOS activity. NOS activity in RAA samples decreased significantly after reperfusion (by 60%), but this reduction was not prevented by BH4 supplementation (10 μM) or NOX2 inhibition. Instead, I identified increased endothelial NOS *S*-glutathionylation as the main mechanism responsible for NOS uncoupling after reperfusion.

In STARR, atorvastatin prevented increase in RAA superoxide production, maintained the functionally coupled status of NOS and NO bioavailability after reperfusion but had no measurable effect on postoperative AERP.

In STICS, treatment with rosuvastatin significantly reduced LDL-C concentration by 48 hours after surgery but had no effect on the incidence of POAF (203 (21%) of the Rosuvastatin-allocated patients *vs.* 197 (20%) of the placebo-allocated patients) or on perioperative myocardial damage (P = 0.80). Pre-defined subgroup analyses (age, sex, prior statin use, baseline troponin concentration, duration of randomized treatment before surgery, type of cardiac surgery, and postoperative use of anti-inflammatory drugs) did not identify any category of patient who benefited from perioperative rosuvastatin treatment. Nor were there beneficial effects on any of the other in-hospital clinical outcomes that were assessed.

In conclusion, cardiac surgery on CPB is associated with myocardial nitroso redox imbalance that is reversed by perioperative intensive therapy with statins. However, these effects have no beneficial effects on common in-hospital complications after elective cardiac surgery. Although the benefits of long-term statin therapy in patients requiring myocardial revascularization are well established, the work presented in this thesis does not support routine use of perioperative intensive therapy with statins for the prevention of postoperative complications in patients undergoing elective cardiac surgery.

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LIST OF ABBREVIATIONS

AA	Antimycin A
AERP	Atrial effective refractory period
AF	Atrial fibrillation
ANOVA	Analysis of variance
APD	Action potential duration
AV	Atrio-ventricular
AVR	Aortic valve replacement
B	Biopterin
BCA	Bicicholinic acid
7,8-BH2	7,8-Dihydrobiopterin
BH4	5,6,7,8-Tetrahydrobiopterin
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CPB	Cardiopulmonary bypass
CTSU	Clinical Trial Services Unit
Cu2+	Copper
DAHP	2,4-diamino-6-hydroxypyrimidine
DHE	Dihydroxy ethidium
DMSO	Dimethyl sulphoxide
D-NAME	Nω-Nitro-D-arginine methyl ester hydrochloride
DPI	Diphenylene iodinium
DTE	Dithio-erythritol
DTT	Dithiothreitol
DXM	Dexamethasone
E	Ethidium
EC	Excitation contraction
ECG	Electrocardiogram
eNOS	Endothelial nitric oxide synthase
ETC	Electron transport chain
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFRP	GTPCH Feedback Regulatory Protein
GSH	Glutathione
GSSG	Glutathione disulfide
GTPCH	GTP cyclohydrolase-1
HMG CoA	3-hydroxy-3-methyl-glutaryl-CoA
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
I/R	Ischemia/Reperfusion

KHB	Krebs-Hepes Buffer
LA	Left atrium
LAA	Left atrial appendage
LDL	Low density lipoprotein
L-NAME	Nω -Nitro-L-arginine methyl ester
LV	Left ventricle
MI	Myocardial infarction
mRNA	Messenger ribonucleic acid
MPTP	Mitochondrial permeability transition pore
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
nor-NOHA	Nω-Hydroxy-nor-L-arginine
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NT-proBNP	N-Terminal of the prohormone of brain natriuretic peptide
O2	Superoxide
2-OHE	2-Hydroxy ethidium
ONOO-	Peroxynitrite
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PKC	Protein kinase C
PLN	Phospholamban
PMI	Perioperative myocardial injury
POAF	Post-operative AF
PTM	Post translational modification
RA	Right atrium
RAA	Right atrial appendage
REC	Regional ethics committee
RLU	Relative light units
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
Rot	Rotenone
sCD40L	Soluble CD 40 ligand
SR	Sinus rhythm
STARR	Statin Therapy in Atrial Refractoriness and Reperfusion injury
STICS	Statin Therapy In Cardiac Surgery
Tg	Transgenic
WT	Wild type

CHAPTER 1

INTRODUCTION

This chapter reviews historical perspectives and current status of elective cardiac surgery, mechanistic aspects of post-operative atrial fibrillation and perioperative myocardial injury, current therapeutic strategies for prevention of these complications and finally summarizes the hypothesis and aims of the body of work presented in this thesis.

History of modern cardiac surgery

"An Experimental Investigation of the Treatment of Wounds of the Heart by Means of Suture of the Heart Muscle" [1]

It would, of course, be incorrect to attempt to draw conclusions as to the dangers and the chances of success of suture of cardiac wounds in man from the results obtained by animal experimentation. Animals are placed in very unfavorable conditions after the operation. They are very restless and cannot be kept quiet. Ideal cleanliness is impossible and the animals may infect their wound by rubbing the external wound against the dirt on the floor of their cage. From the animal mortality in these investigations no rigid inferences applicable to human beings can therefore be made. Some conclusions of importance can, however be drawn. Above all, my experiments seem to show that the mammalian heart will bear a much greater amount of manipulation than has hitherto been suspected. Very large wounds of the heart can heal and the healing process occurs in a manner entirely analogous to that in other muscular tissues. Even an extensive suture of the heart-wall of rabbits and dogs, although we know that thereby a large number of muscle fibers are destroyed and replaced by connective tissue, does not interfere with the function of the cardiac muscle as a whole. Can some of the results in the above-recorded experiments be, with some restrictions of course, applied to the human heart? I think that this question must be answered in the affirmative. If we compare the knowledge we possess of wounds of the heart in man, with that obtained from animal experiments, and find that they agree in all essential particulars, then we are justified in reasoning by analogy that suture of wounds of the heart in man will

give results similar to those obtained in the animal [1].

Earliest description of modern cardiac surgery can be traced to the late part of 19th century[1]. Despite intense focus on development of surgical treatment for angina pectoris, the only successful procedures performed in the first half of 20th century were closed techniques for mitral stenosis[2] and Blalock and Taussig shunt operation for "blue babies"[3]. Introduction of coronary angiography[4,5] and cardiopulmonary bypass[6] heralded a new era in the history of cardiac surgery with first coronary artery bypass surgery (CABG) for obstructive coronary artery disease being performed in 1967[7] and refined further by Favaloro in the following year[8]. Over the next decade, experience with CABG continued to grow reflected by the widespread adoption of the technique thereby establishing CABG as the standard of care for the treatment of coronary artery disease (CAD). Currently, it is the most common cardiac surgery performed in adults [9,10]. On the other hand, progress in the surgical management of valvular heart disease was made possible by the development of total valve replacement by two surgeons: Dwight Harken using a double-caged, ball-and-seat prosthesis[11] and Albert Starr by a caged ball-and-seat valve[12]. Commonest valve that requires surgical treatment is aortic valve and aortic valve replacement (AVR) is indicated in patients with symptomatic severe aortic stenosis (AS) or aortic regurgitation (AR) as it improves symptoms as well as life expectancy[13,14]. Prevalence of CAD is high in adults with severe symptomatic AS which may require concomitant CABG along with AVR[15]. Together, CABG, AVR or combined CABG and AVR constitute majority of adult cardiac surgery procedures and define the patient population presented in this thesis.

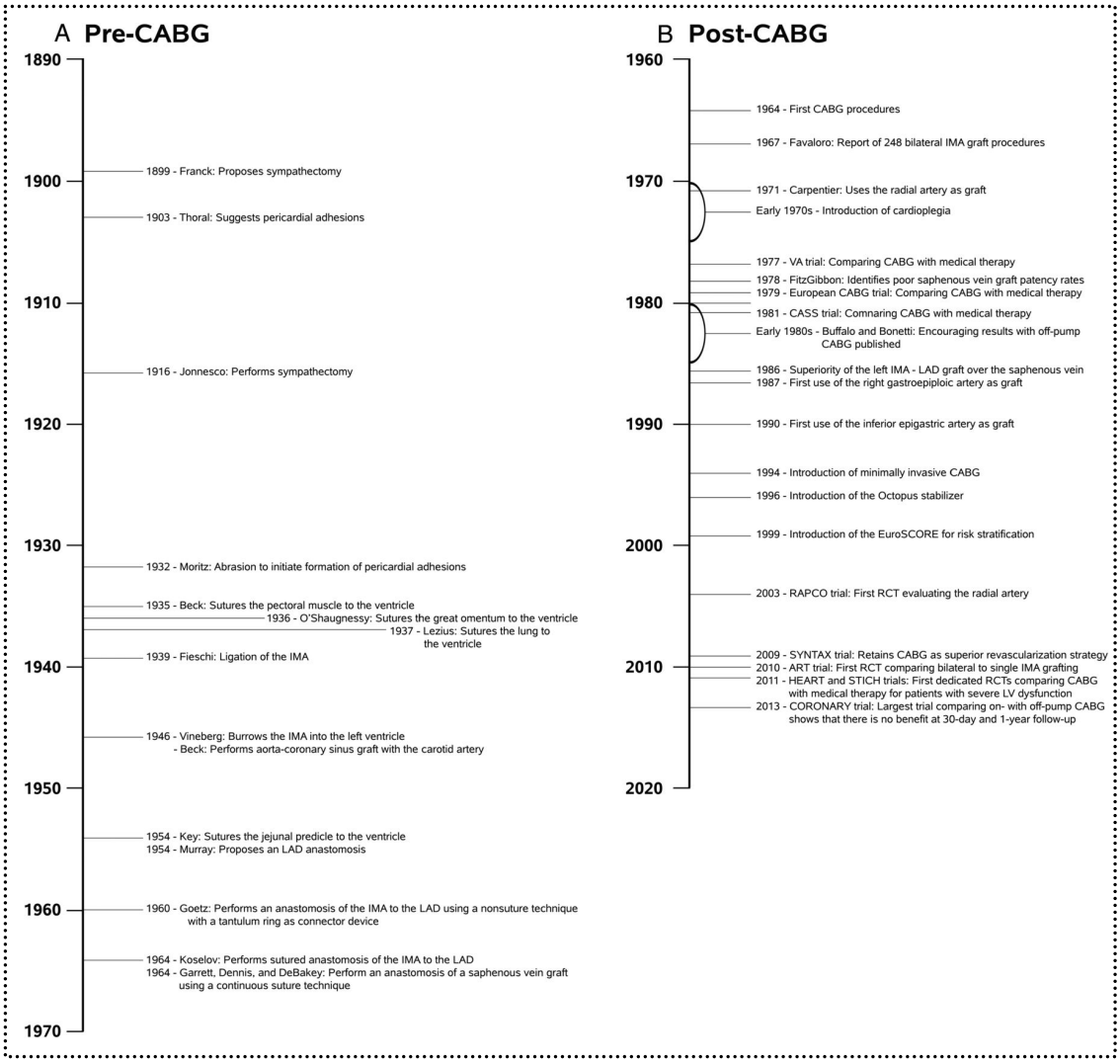


Figure 1.1: Timeline of developments that led to the first 'modern' coronary artery bypass grafting **(A)** and facilitated continuous improvements in surgical technique and outcomes during the first 50 years **(B).**

Reproduced from; Head S J et al. Eur Heart J 2013;34:2862-2872

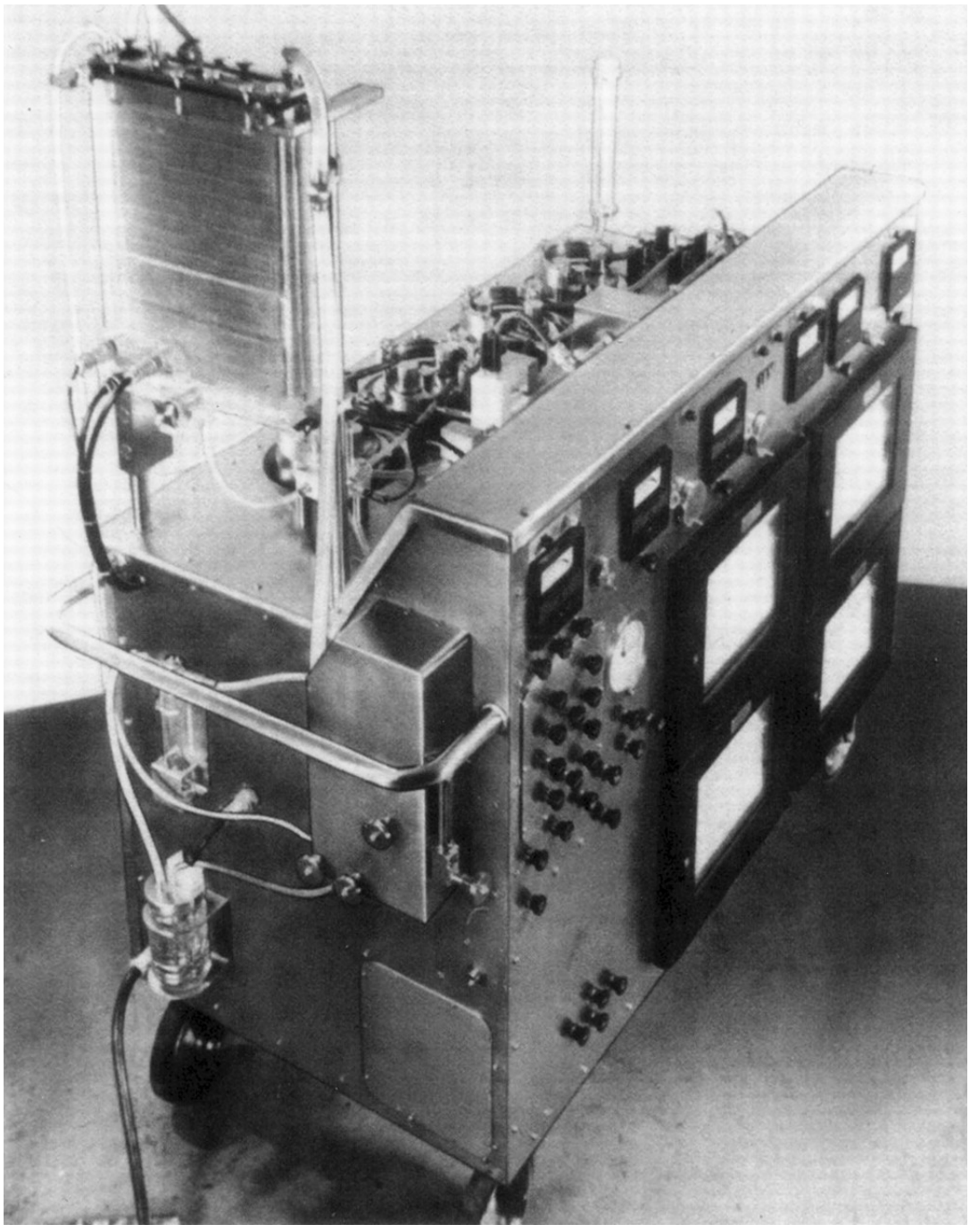


Figure 1.2: Gibbon heart-lung machine Model II[6].

Reproduced from; Cohn L H Circulation. 2003; 107:2168-2170

Patient population

The advent of percutaneous coronary intervention (PCI) in 1978[16] provided an alternative treatment option for CAD and the rapid development in the field of interventional cardiology expanded therapeutic indications of PCI. Currently, myocardial revascularization either by CABG or percutaneous coronary intervention is indicated for the relief of symptoms in persistent angina despite optimum medical treatment, left main coronary artery disease or on prognostic grounds for significant myocardial ischemia[17]. When compared to PCI, CABG is associated with better survival rates and remains the standard of care in patients with complex multivessel disease and co-morbidities such as diabetes mellitus[17]. Also, the number of patients who have undergone PCI in the past presenting for surgical myocardial revascularization is increasing[18-22]. The patients who underwent CABG in the 70s and 80s when technique was still evolving had a mean age of 50–55 years[23 24] with a prior history of smoking as an important risk factor followed by diabetes[25] and hypertension[26,27]. However, there is a clear shift in this risk factor profile as now the population is becoming increasingly older with more co morbidities[9,22,28]. Over the last two decades, the mean age of patients undergoing CABG has increased to 60–65 years with prevalence of risk factors such as diabetes, hypertension, hypercholesterolemia and co - morbidities such as chronic kidney disease, previous stroke showing an upward trend [29],[22] thereby elevating the overall risk profile of patients presenting currently for CABG, AVR or both [9,30,31].

Practice: on- and off pump techniques

Historically CABG was almost exclusively performed with the use of cardiopulmonary bypass (CPB) followed by cardioplegic arrest to provide a stable and bloodless operative field for performing coronary anastomoses. With increasing association of CPB with complications such as systemic inflammatory response, stroke, etc. [32,33], off-pump cardiac surgery was introduced as an alternative method for CABG [34] where extracorporeal circuit is avoided and using mechanical devices, heart is stabilized during coronary anastomosis.

However, large-scale clinical trials have been unable to demonstrate significant differences in post-operative clinical outcomes between the two techniques [35,36] [37,38]. Wide variations continue to exist in current practice with the majority of the CABG being performed on CPB in the western countries[9,29] whereas in developing countries especially in Asia, off pump technique appears to be the preferred option[39,40]. For surgical replacement of valves, however, use of CPB remains the default approach. The works presented in this thesis includes patients undergoing both on- and off pump cardiac surgery.

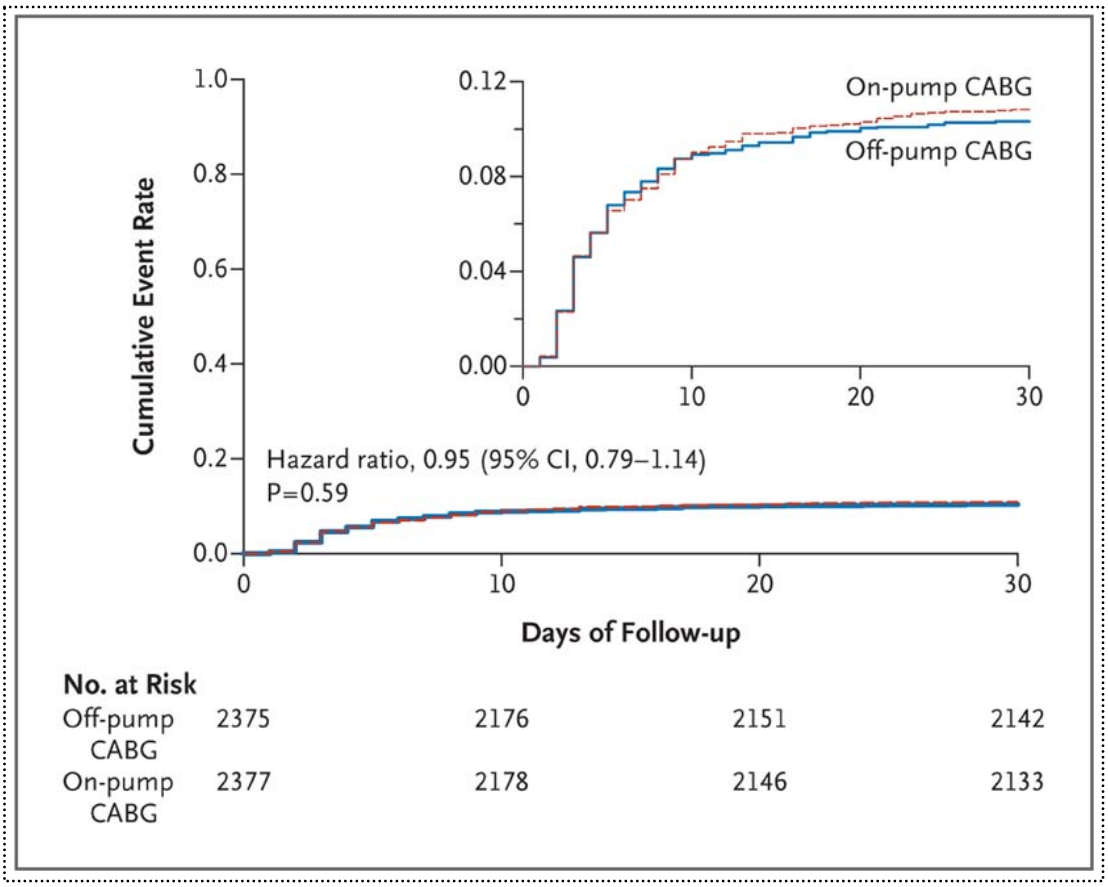


Figure 1.3: Kaplan–Meier Curves for the Primary Composite Outcome at 30 Days. The primary composite outcome was death, myocardial infarction, stroke, or new renal failure requiring dialysis.

Reproduced from; Lamy A et al. N Engl J Med 2012;366:1489-1497 "Off-Pump or On-Pump Coronary-Artery Bypass Grafting at 30 Days".

Postoperative outcomes

Despite the shift in risk profile of patients presenting for cardiac surgery as detailed earlier, the morbidity and mortality (defined as death within 30 days

after surgery) associated with CABG or AVR have fallen over a period of time[9,22]. Whilst the reasons for this are not very clear, improvements in pre-, intra and postoperative management of these patients may be contributory[22]. Nevertheless complications continue to happen and between 30 to 50% of patients develop new onset atrial fibrillation in the postoperative period [41]. Other significant complications include peri- or postoperative myocardial infarction (MI) or injury[42], renal failure[43], infectious complications such as mediastinitis[44] and intra procedural or early postoperative stroke[45]. As the number of elderly patients with co-morbidities undergoing cardiac surgery increases [46], the incidence of these complications are becoming increasingly common[47] and has been shown to be associated with increased morbidity, longer postoperative stays, higher costs of care and risk of early or delayed mortality[48-51].

Many of these complications, as well as risk of mortality, can be anticipated on the basis of the preoperative medical history, procedural details and patient demographics[52]. For instance, a history of cerebrovascular disease, atrial fibrillation, peripheral vascular disease, hypertension and severe atherosclerotic aorta are all predictors of stroke[45]. Likewise increasing age and perhaps obesity[53] has been associated with postoperative atrial fibrillation [54,55] whereas prior history of renal impairment, diabetes, and preoperative cardiogenic shock are linked to post operative renal failure[56]. However, therapeutic interventions to prevent post-operative complications after elective cardiac surgery have only been partially successful[57]. With the exception of beta-blockers for the prevention of new onset post-operative AF, no other pharmacological intervention or surgical technique has been shown to prevent postoperative complications after elective cardiac surgery[58] necessitating further research into the pathogenesis of these complications.

The work presented in this thesis focuses on two significant post-operative complications of elective cardiac surgery, post-operative atrial fibrillation and perioperative myocardial injury.

Postoperative atrial fibrillation

Epidemiology

Atrial fibrillation (AF) is the most common arrhythmia in the community and among the leading causes of stroke and heart failure[59]. Based on the electrocardiogram (ECG), AF is described by the replacement of consistent P waves by rapid oscillations or fibrillatory waves that vary in size, shape, and timing, associated with an irregular, frequently rapid ventricular response when atrioventricular (AV) conduction is intact[60]. In the hospital setting, AF is a common complication after cardiac surgery with reported incidence varying from 10% to 65% depending on the surgical procedure (CABG vs. AVR, on vs. off-pump), criteria used for diagnosis, and the method of post-operative monitoring for its detection [41,61]. Post-operative AF (POAF) tends to occur, usually within two to four days after the procedure, with a peak incidence on postoperative day 2[62]. In an observational multicentre study of 4657 patients undergoing CABG surgery in the United States (US), the main predictors of POAF were age and withdrawal of beta-blocker before or after surgery[63]. POAF has been associated with increased post-operative thromboembolic risk/ stroke[64], hemodynamic compromise[65], ventricular dysrhythmias[66] , perioperative myocardial infarction [67] and increased length of hospital stay period[68]. Moreover, in a series of 3,855 patients undergoing cardiac surgery, both hospital mortality (6% vs. 3%) and 6-month mortality (9% vs. 4%) were significantly higher in patients who developed POAF[67]. POAF has also been linked to increased long-term risk of mortality independent of patients' preoperative clinical status [69].The impact of POAF on hospital resources is substantial and was estimated to lengthen hospital stay by 4.9 days, with an extra cost of $10,000 to $11,500 in hospital stay expenses in the U.S[62].

Mechanisms

Inflammation:

It has been suggested that inflammation, both systemic as well as localized to atrial myocardium underlies pathogenesis of POAF[70]. This is mostly based on either association of pre – or postoperative elevation of inflammatory markers such as CRP, interleukin-6 (IL-6), interleukin -8 (IL-8)[71] with POAF [72 73,74] or the observation that during the postoperative period, the time course of changes in markers of systemic inflammation [75 76,77] parallels that of POAF. Likewise, a canine model of sterile pericarditis demonstrated inducibility of AF in a similar time scale as that of POAF[78] suggesting local inflammation may have a role in its pathogenesis. Supporting this notion further, in a canine model of pericardiotomy and atriotomy, the degree of atrial inflammation measured by myeloperoxidase activity (MPO) and neutrophil cell infiltration in the atrial myocardium was directly correlated with heterogeneity of atrial conduction, incidence as well as duration of AF[79]. Anti-inflammatory therapy with methyl prednisolone in this model significantly decreased both MPO activity and the inhomogeneity of atrial conduction, indicating neutrophil-derived inflammation creates an atrial substrate for the development of POAF[79]. Similar mechanistic studies in humans are difficult to perform; however, randomised trials of off-pump vs. on-pump cardiac surgery suggest that the latter might be associated with a reduced incidence of AF[80], even though in trials at low risk of bias, the difference was not significant. In particular, the CORONARY trial[35] that compared major clinical events 30 days after surgery between off-pump CABG (n=2375) and on-pump (n=2377) CABG, the incidence of POAF did not differ between the two groups (18.3% vs. 17.9%; hazard ratio1.02; 95% C.I 0.9 to 1.15; p=0.72). Of note, minimally invasive valve replacements and off-pump CABG compared to conventional surgery by a median sternotomy have less incidence of POAF in retrospective studies[81,82]. Moreover, perioperative administration of anti-inflammatory agents, such as steroids[83,84] and colchicine[85], also has been shown to decrease the incidence of POAF.

Together, these findings suggest that perioperative inflammation may play a role in POAF; however, a causal relationship between perioperative inflammation

and POAF after cardiac surgery in man remains to be conclusively demonstrated.

Oxidative stress:

Oxidative stress is defined as an imbalance in pro and antioxidant mechanisms implying disruption in redox signalling and its control[86]. In a study by Ramlawi *et al*, patients who developed POAF compared to those remaining in sinus rhythm had differential expression of genes linked to redox signalling after CPB in turn leading to increased myocardial oxidative stress[87]. Among the enzymatic sources of reactive oxygen species (ROS), NADPH oxidases (NOXs), mitochondrial electron transport chain (ETC) and dysfunctional NOS have been associated with AF and AF-induced atrial remodelling[88,89]. Postulated mechanisms include ROS-mediated alteration in myofibrillar energetics[90] and a reduction in the atrial effective refractory period (ERP)[91], which in turn increases the vulnerability to POAF[92,93] . Interventions that have been effective in reducing myocardial oxidative stress in animal models of atrial tachypacing have also prevented myocardial electrophysiological remodelling and AF vulnerability, at least in the short term[91,94]. These findings suggest that myocardial sources of ROS may be an important new target for therapeutic interventions aimed at preventing AF and associated atrial remodelling.

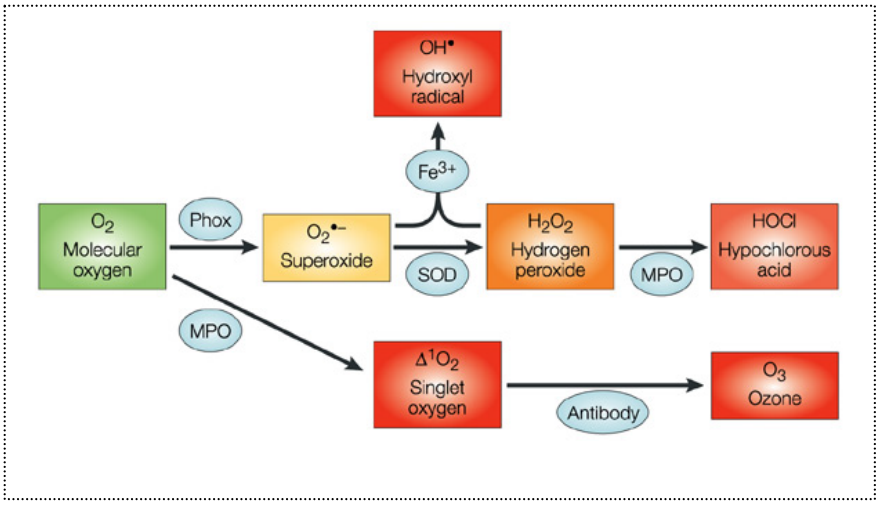


Figure 1.4: Reactive Oxygen Species (ROS)

Reproduced from; Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004 Mar;4(3):181-9.

Superoxide is generated from various sources and two molecules of superoxide can react to generate hydrogen peroxide (H_2O_2) in a reaction known as dismutation, which is accelerated by the enzyme superoxide dismutase (SOD). In the presence of iron, superoxide and H_2O_2 react to generate hydroxyl radicals. In addition to superoxide, H_2O_2 and hydroxyl radicals, other reactive oxygen species generated in biological systems include hypochlorous acid (HOCl), formed from H_2O_2 and chloride; singlet oxygen, which might be formed from oxygen and ozone.

The colour coding indicates the reactivity of individual molecules (green, relatively unreactive; yellow, limited reactivity; orange, moderate reactivity; red, high reactivity and non-specificity).

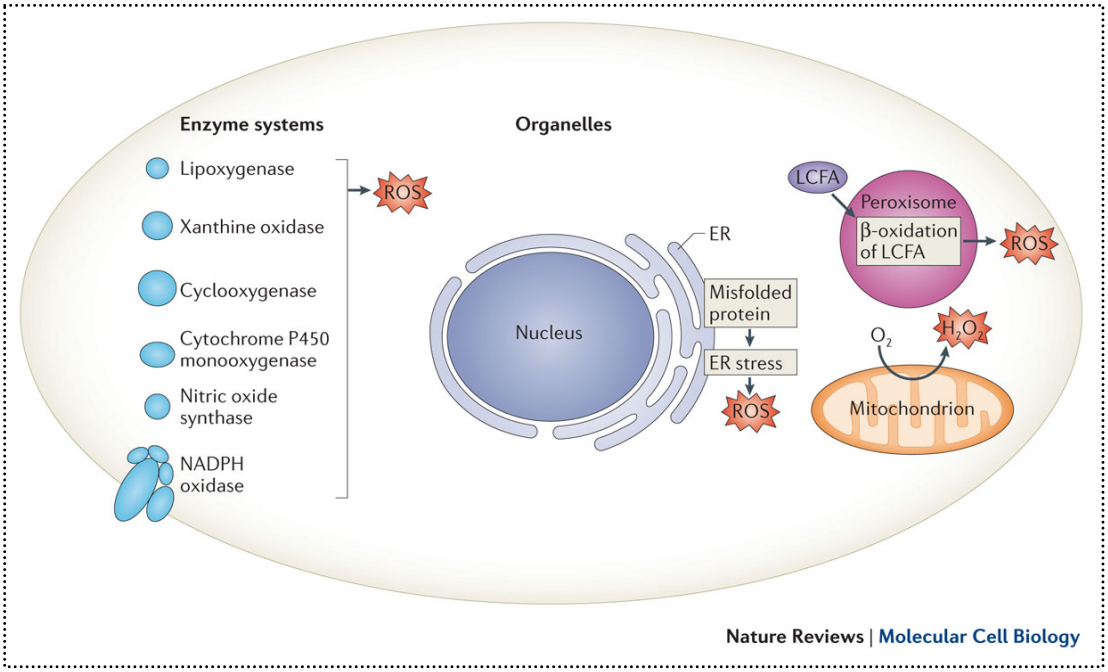


Figure 1.5: Intracellular sources of Reactive Oxygen Species (ROS)

Reproduced from; Holmstrom, Kira M, Finkel, Toren. *Nature Reviews Molecular Cell Biology* **15**, 411–421 (2014) " Cellular mechanisms and physiological consequences of redox-dependent signaling".

Various organelles within the cell can generate reactive oxygen species (ROS). These include mitochondria, the endoplasmic reticulum (ER; particularly in the setting of ER stress) and peroxisomes (as part of their role in metabolizing long-chain fatty acids (LCFAs)). In addition, various enzymes, including oxidases and oxygenases, generate ROS as part of their enzymatic reaction cycles.

NADPH Oxidase: NADPH oxidases function as ROS producing units. Based on the presence of a distinct catalytic subunit, seven isoforms have been described namely NOX1–5 and dual oxidase 1–2[95]. NOX2 and NOX4 have been described in cardiomyocytes, endothelial cells and fibroblasts[96]. NOX2 is activated by angiotensin II (AngII), endothelin-1, growth factors, cytokines and

mechanical forces. These stimuli induce posttranslational modifications in cytosolic subunits ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac1 and 2), which subsequently translocate to plasma membrane and docks with membrane bound cytochrome to initiate superoxide production by transferring an electron from NADPH in the cytosol to molecular oxygen. By contrast, NOX4 is constitutively active and regulated mainly by changes in its abundance induced by stimuli such as hypoxia, ischemia, pressure overload and endoplasmic reticulum stress[97].

Previous work from our group has shown that atrial NOX2-dependent superoxide production is independently associated with an increased risk of POAF and other in-hospital complications in patients undergoing cardiac surgery[98,99]. On the other hand, blood-based oxidative markers measured before and after surgery were not correlated with atrial NADPH oxidase activity, suggesting that the former are poor indicators of the redox state of the atrial myocardium and thus a poor surrogate of the efficacy of antioxidant interventions on the myocardium[98]. Delving into the mechanism behind NOX2 activation, murine cardiac-specific overexpression of a constitutively active Rac1 (a small G protein involved in the activation of NOX2 oxidases) leads to development of AF further reinforcing the role of NOX2 in arrythmogenesis[100]. Though the molecular mechanisms downstream of NOX2 that promotes AF remains unclear, the observation that NOX2-dependent calmodulin kinase II (CaMKII) oxidation promotes sinus node dysfunction may implicate this link in its pathogenesis[101]. Indeed, CaMKII-dependent phosphorylation of RyR2 has been shown to increase diastolic calcium leak in right atrial myocytes from patients with AF[102]. Interestingly as neuronal nitric oxide synthase 1 (nNOS1) co localizes with ryanodine receptor 2 (RyR2)[103], in murine models of Duchenne muscular dystrophy with deficient nNOS1 activity, it has been shown that dysfunctional RyR2 function leads to aberrant calcium release thereby increasing the incidence of arrhythmias[104]. Whether increase in bioavailability of nitric oxide under conditions of NOX driven oxidative stress might preserve RyR2 function and prevent AF remains to be demonstrated.

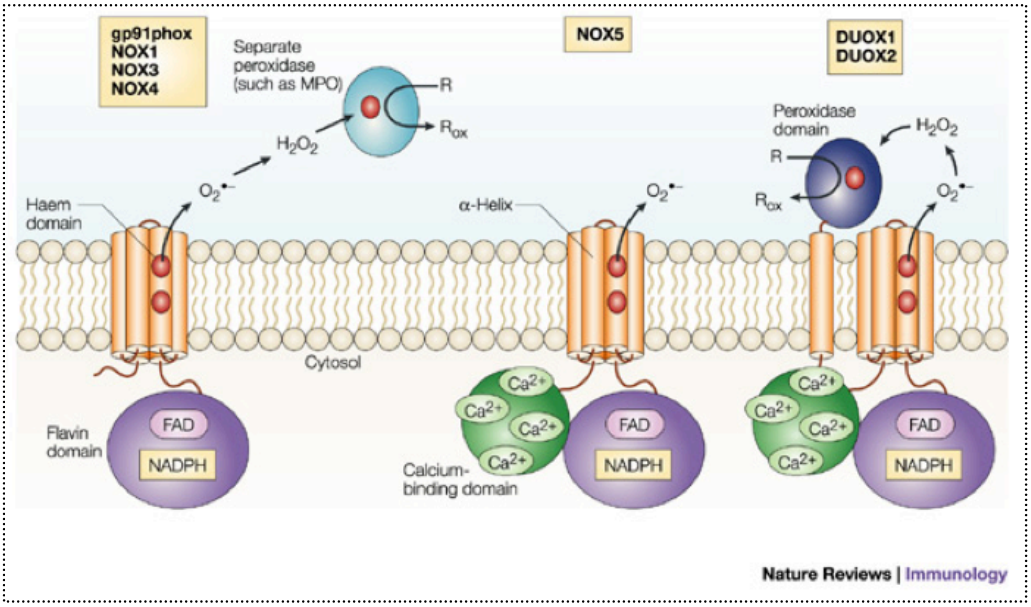


Figure 1.6: Transmembrane topology and domain structure of isoforms of NADPH Oxidase (NOX).

Reproduced from; Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol. 2004 Mar;4(3):181-9.

All NOX isoforms share six highly conserved transmembrane domains. Transmembrane domains III and V each contain two histidines, spanning two asymmetrical hemes. The cytoplasmic COOH terminus contains conserved flavin adenine dinucleotide (FAD) and NADPH binding domains. NOX enzymes are thought to be single electron transporters, passing electrons from NADPH to FAD, to the first heme, to the second heme, and finally to oxygen.

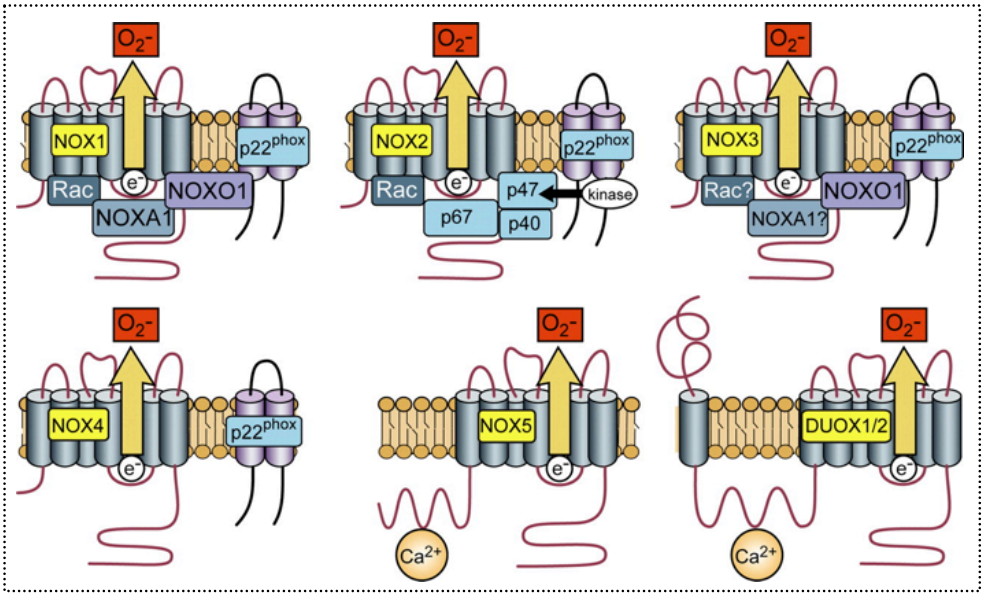


Figure 1.7: Activation of NADPH oxidase isoforms.

Reproduced from; Bedard K , and Krause K Physiol Rev 2007;87:245-313

Despite their similar structure and enzymatic function, NOX family enzymes differ in their mechanism of activation. *A*: NOX1 activity requires $p22^{phox}$, NOXO1 (or possibly $p47^{phox}$ in some cases) and NOXA1, and the small GTPase Rac. *B*: NOX2 requires $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac; $p47^{phox}$ phosphorylation is required for NOX2 activation. Although not absolutely required, $p40^{phox}$ also associates with this complex and may contribute to activation. *C*: NOX3 requires $p22^{phox}$ and NOXO1; the requirement for NOXA1 may be species dependent, and the requirement of Rac is still debated. *D*: NOX4 requires $p22^{phox}$, but in reconstitute systems it is constitutively active without the requirement for other subunits. However, in native NOX4-expressing cells, activation, possibly including Rac, has been described. *E* and *F*: NOX5, DUOX1, and DUOX2 are activated by Ca^{2+} and do not appear to require subunits.

Mitochondrial electron transport chain (ETC): Electron leakage from the ETC (predominantly from Complex 1) [105] causes one electron reduction of oxygen to superoxide instead of water. Excessive superoxide production can open the mitochondrial permeability transition (MPT) pore, which - in a feed-forward cascade - can lead to further ROS release, a phenomenon named ROS induced ROS release[106]. Oxidative stress that ensues may activate sarcolemmal K_{ATP} channels[107] and promote re-entry dependent arrhythmias by shortening action potential duration (APD) [108]. However, whether it can lead to POAF is unclear and merits further investigation.

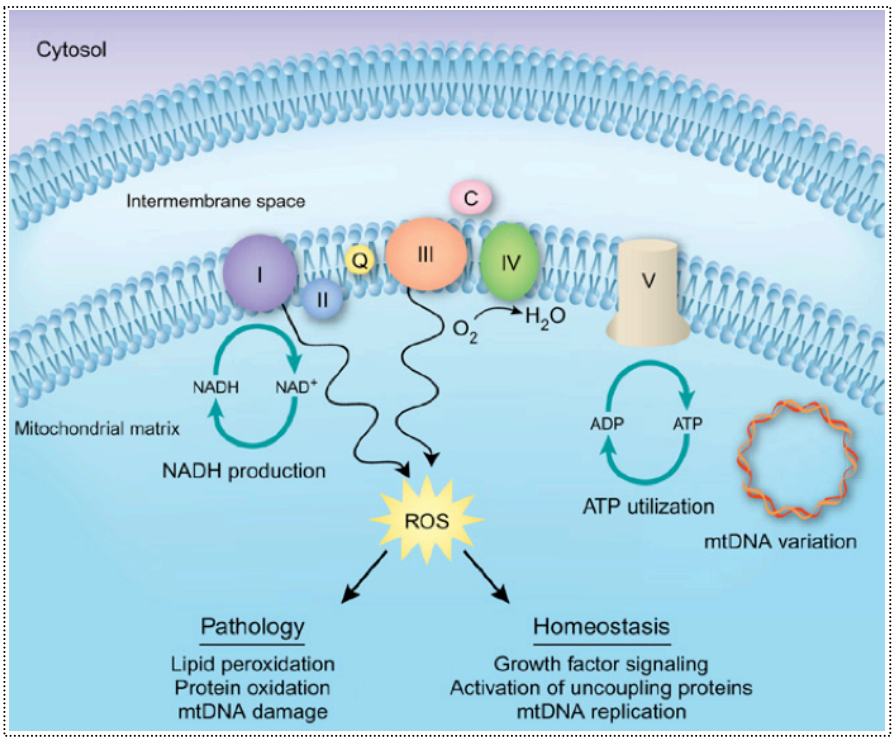


Figure 1.8: Generation and consequences of Mitochondrial ROS

Reproduced from; Baughman JM, Mootha VK. Buffering mitochondrial DNA variation. Nat Genet. 2006 Nov;38(11):1232-3

Quantitatively, complex I and complex III of the electron transport chain are the major sites of oxidant production, with the generation of superoxide anions occurring both on the matrix side of the mitochondrion and in the inner mitochondrial membrane space. Other contributors include metabolic enzymes in the mitochondrial matrix, such as OGDH (2-oxoglutarate dehydrogenase) and PDH (pyruvate dehydrogenase), and the mitochondrial membrane forms of GPDH (glycerol 3-phosphate dehydrogenase; also known as GPDM) and the FQR (electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial) system.

Dysfunctional Nitric Oxide synthase: Recent evidence indicates that nitric oxide synthases (NOS) can synthetize ROS rather than nitric oxide (NO) in the presence of enzyme S-glutathionylation [109], reduced availability of the NOS co-factor, tetrahydrobiopterin (BH4), or the substrate L-arginine [110-114], or as a result of altered enzyme phosphorylation[115]. Whilst NOS generated ROS has been associated with a diverse set of conditions[116-118] including permanent AF[89,119], whether it is linked to the onset of postoperative AF has not been investigated before.

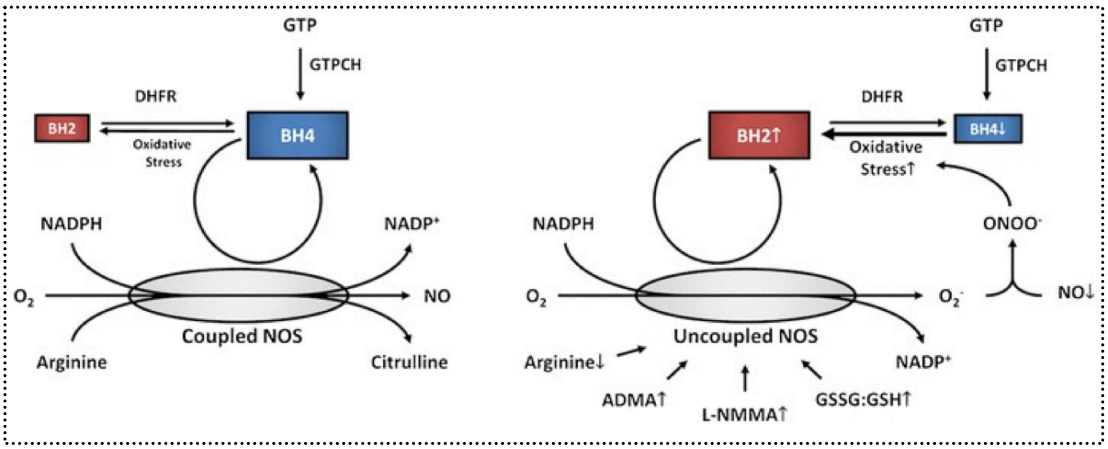


Figure 1.9: BH4 synthesis, recycling, and oxidation as determinants of NOS uncoupling.

Reproduced from; Alkaitis MS, Crabtree MJ. Recoupling the cardiac nitric oxide synthases: tetrahydrobiopterin synthesis and recycling. Curr Heart Fail Rep. 2012. Sep;9(3):200-10.

Left To produce nitric oxide (NO), nitric oxide synthase (NOS) enzymes require the substrates L-arginine and molecular oxygen (O2) and the cofactors tetrahydrobiopterin (BH4), reduced nicotinamide adenine diphosphate (NADPH), heme , flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD).

Right "Uncoupled" NOS is characterized by production of superoxide (O2−). NOS uncoupling is promoted by reduced BH4 bioavailability relative to either BH2 or NOS protein. In turn, O2− produced by uncoupled NOS reacts with NO, forming peroxynitrite (ONOO−), a highly reactive anion that rapidly oxidizes BH4. Therefore, a state of NOS uncoupling is stabilized by self-propagating oxidative stress. In addition to this primary BH4-mediated cycle, additional mechanisms have been shown to promote uncoupling,

including reduced arginine bioavailability, high levels of oxidized glutathione (GSSG) relative to reduced glutathione (GSH), or increased concentrations of the endogenous NOS inhibitors L-*N*-monomethylarginine (L-NMMA) and asymmetric dimethylarginine (ADMA)

Similarly, the role of other myocardial oxidases, such as monoamine oxidases[120] and xanthine oxidoreductases[121] in the pathogenesis of POAF remains unclear. Despite activation of inflammatory signalling pathways and increased myocardial oxidative stress after surgery, the fact that not all patients develop postoperative AF points towards the multifactorial nature of its pathogenesis.

Antoniades *et al* reported an association between preoperative serum levels of the soluble CD40 ligand (sCD40L; member of TNF-alpha superfamily[122]) and POAF in patients undergoing off-pump CABG surgery independent of markers of systemic inflammation or vascular oxidative stress[123]. There is, however, paucity of similar studies in the on-pump cohort (that comprises the majority of cardiac surgery patients). Platelets account for more than 90% of serum sCD40L[124], suggesting that platelet activation may be a common link between postoperative AF and thromboembolic complications after surgery[33 125 126]. sCD40L is also involved in the regulation of pro-inflammatory transcriptional pathways inducing myocardial oxidative stress and inflammatory signaling[122] and hence may be a link between two mechanisms implicated in the pathogenesis of postoperative AF.

Withdrawal from β-blockers is a powerful predictor of postoperative AF suggesting that sympathetic activation, by altering atrial refractoriness and promoting ectopic activity, may contribute to its pathogenesis[127]. However unlike in permanent AF, pre-operative changes in cellular Ca^{2+} and K^{+} channels do not appear to contribute to the occurrence of postoperative AF[127]. Advancing age is a strong independent predictor of postoperative AF[128] suggesting that a pre-existent atrial arrhythmogenic substrate may be required for the development of postoperative AF[129]. A link between postoperative AF and pre-existing atrial structural remodelling is further supported by the observation that expression of connexin 40 is significantly higher in patients who develop postoperative AF compared with sinus rhythm patients[130]. Increased connexin 40 may interfere

with the architecture of gap junctions, resulting in local conduction heterogeneity and arrythmogenesis[131]. Moreover angiotensin II–induced gap junction remodelling has been linked to arrythmogenesis primarily driven by mitochondrial oxidative stress[131].

Management

Based on the link between beta-blockers and the incidence of postoperative AF[132,133], current practice guidelines recommend perioperative beta-blocker treatment for minimising the incidence and sequelae of postoperative AF[17,134]. However in the guidelines focusing on perioperative beta-blockade [135], the recommendation meeting class 1 criteria is restricted to patients who are already treated with beta-blockers, in whom it is advocated that the treatment should be continued. In North American surgical practice, the incidence of beta-blocker use in patients presenting for CABG is around 60%[136] and has not changed significantly over the last decade[137]. In beta-blocker naive patients, the guideline recommends careful analysis of the risk-benefit ratio ad personam and cautions against routine administration of beta-blockers in the perioperative period. Given these practical considerations, further work needs to be done to define the role of other interventions investigated in this context based on their putative anti-inflammatory[84] and/or anti-oxidant effects[138,139].

Hydroxymethylglutaryl-CoA reductase inhibitors (Statins):

3-hydroxy-3-methylglutaryl (HMG-CoA) reductase inhibitors, commonly known as statins, reduce cardiovascular morbidity and mortality by virtue of their LDL-cholesterol lowering effects [140-154]. A secondary effect of HMG-CoA reductase inhibition is a decrease in the synthesis of proteins that are upstream in the cholesterol synthetic pathway such as farnesyl pyrophosphate (FPP) and geranyl pyrophosphate (GPP) [155]. These isoprenoid intermediates typically serve as lipid attachments for the posttranslational modification of proteins like Ras and the Rho GTPase family involved in oxidative stress, inflammation, thrombogenesis and metabolism [156-171]. A decrease in protein isoprenylation following treatment with statins may modify their function and affect

inflammation, platelet function, intracellular transport of signaling molecules, messenger ribonucleic acid (mRNA) stability and gene transcription [172-178].

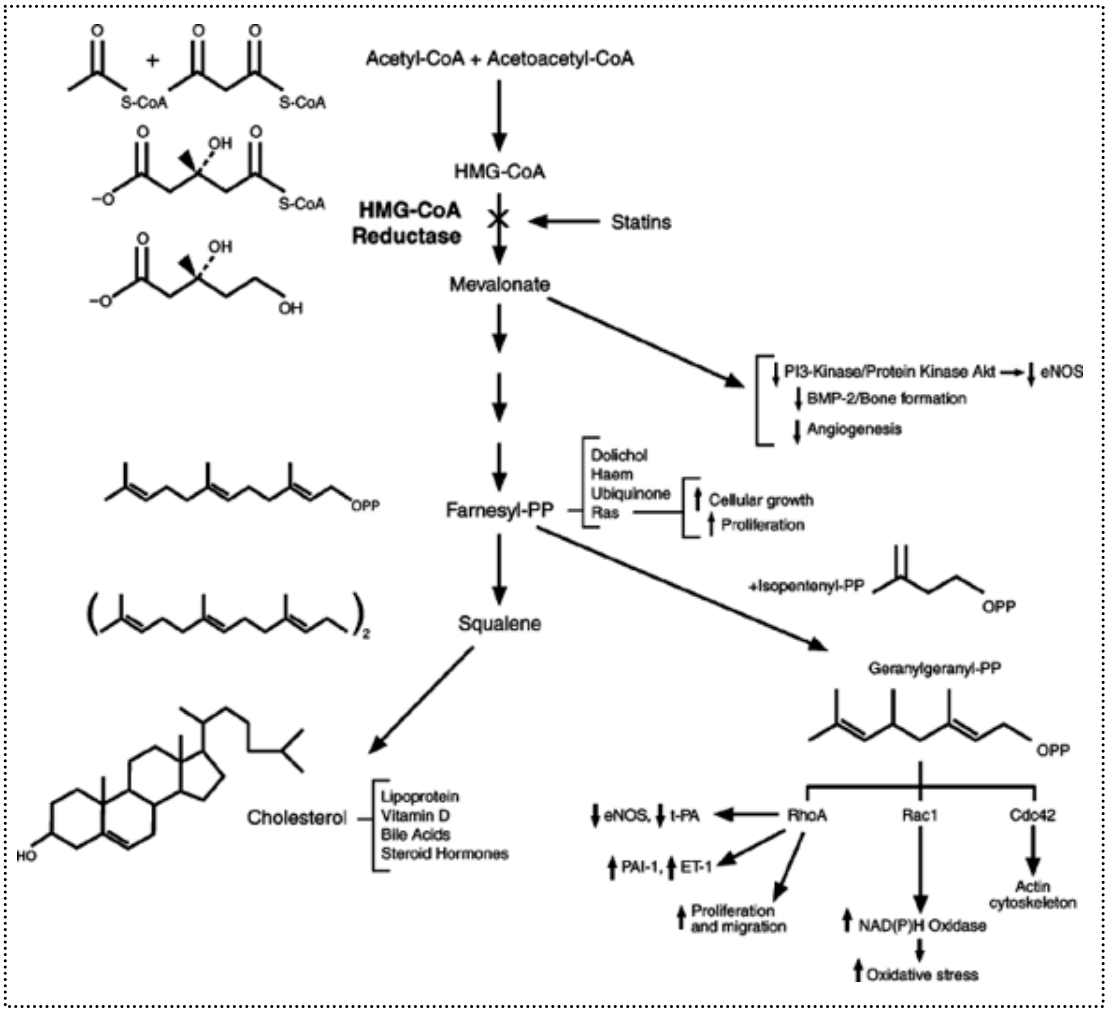


Figure 1.10: Mechanisms underlying the pleiotropic effects of statins.

Reproduced from; Liao JK and Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol **45**: 89–118

A schematic of the cholesterol biosynthesis pathway showing the effects of inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A reductase by statins. Decreased isoprenylation of signaling molecules, such as Ras, Rho and Rac, leads to modulation of various signaling pathways.

BMP2, bone morphogenetic protein 2; Cdc42, cell division cycle 42 GTP-binding protein; CoA, coenzyme A; eNOS, endothelial nitric oxide synthase; ET1, endothelin-1; HMG, 3-hydroxy-3-methylglutaryl; HO, hydroxy group; OPP, ether-linked pyrophosphate form of the isoprenoid; PAI1, plasminogen activator inhibitor 1; PI3 phosphatidylinositol 3; PP, pyrophosphate; tPA, tissue-type plasminogen activator.

Statins have been shown to reduce ROS formation by NADPH oxidases in human myocardial tissue by interacting with one of the principal components of

the NADPH oxidase complex, *i.e.*, the small G protein Rac1[179,180]. Statin-induced inhibition of Rac isoprenylation impairs its translocation to membranes, leading to suppressed superoxide formation[181,182]. In addition to this mechanism, statins are known to increase nitric oxide (NO) bioavailability by stimulating both the activity and the protein expression of NO synthases[183,184].

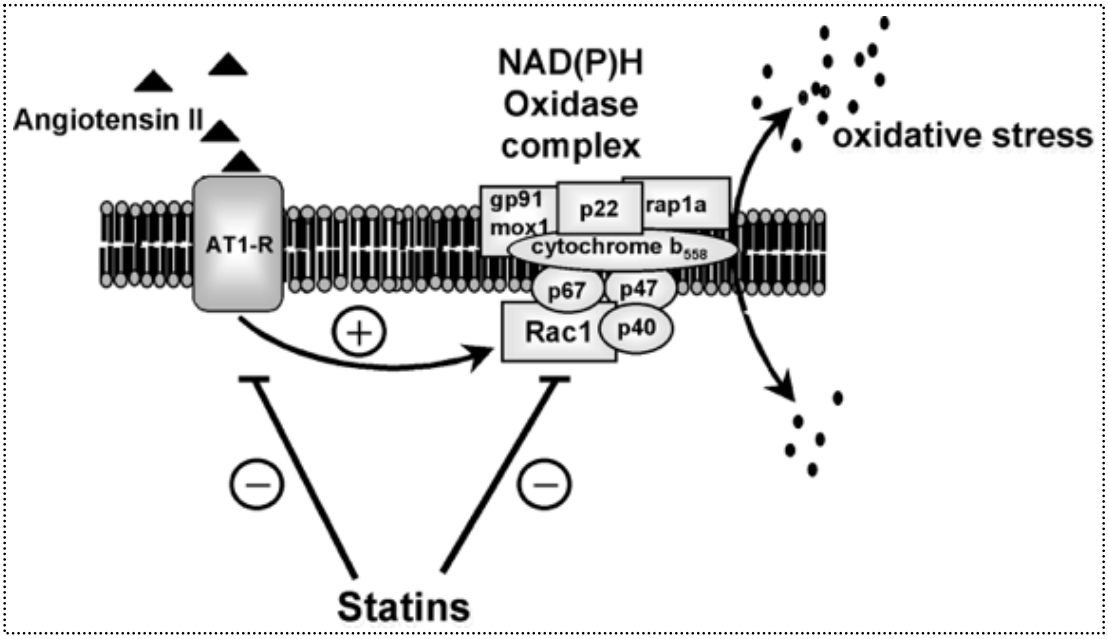


Figure 1.11: Antioxidative mechanisms of statins.

Reproduced from; Liao JK and Laufs U (2005) Pleiotropic effects of statins. Annu Rev Pharmacol Toxicol **45**: 89–118

The core NAD(P)H oxidase comprises five components: p40*phox* (PHOX for phagocyte oxidase), p47*phox*, p67*phox*, p22*phox*, and gp91*phox*. In the resting cell (*left*), three of these five components, p40*phox*, p47*phox*, and p67*phox*, exist in the cytosol as a complex. The other two components, p22*phox* and gp91*phox*, are located in the membranes. When it is stimulated by angiotensin, the cytosolic component becomes heavily phosphorylated and the entire cytosolic complex migrates to the membrane. Activation requires the participation not only of the core subunits but also of two low-molecular-weight guanine nucleotide-binding proteins, Rac and Rap. During activation, Rac binds GTP and migrates to the membrane along with the core cytosolic complex. Treatment with statin down regulates AT1-receptor expression and inhibits Rac1 GTPase, a necessary component of the NAD(P)H oxidase complex.

These rapid anti-oxidant effects of statins may contribute to early beneficial effects[185] and underpin recent findings indicating that short-term treatment with statins prevents AF-induced early electrical remodelling in a canine model of atrial tachypacing[94] and reduces atrial superoxide release by rac-1 mediated suppression of NOX activity in patients undergoing elective cardiac surgery[99]. Of

note, the latter findings were independent of changes in LDL cholesterol and reproduced after *ex vivo* incubation of right atrial tissue samples with atorvastatin. Finally, a number of small studies suggest that short-term peri- or pre-operative treatment with statins in statin-naïve patients undergoing cardiac surgery reduces the occurrence of postoperative AF [186] [139,187,188].

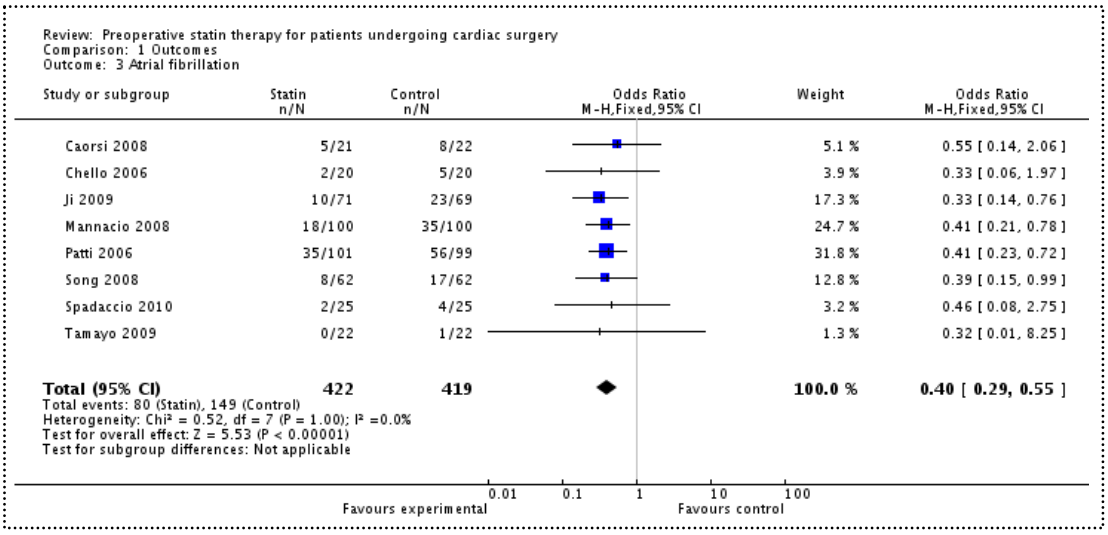


Figure 1.12: Pooled results of eight studies providing data on the incidence of AF from a total of 841 patients (85.5% of total patients).

Reproduced from: Liakopoulos OJ et al. Cochrane Database Syst Rev. 2012 Apr 18;4:

Eighty events of postoperative AF (19%) were observed in the statin group and 149 events (35.6%) were observed in the control group resulting in an odds reduction of 60% after pooled analysis with a fixed-effect model (OR 0.40; 95%-CI: 0.29 to 0.55; p<0.01).

In summary, antioxidant and anti-inflammatory effects of statins have been extensively studied in animal models and humans with encouraging trends as detailed above; however, the impact of perioperative statin treatment on postoperative outcomes in patients undergoing cardiac surgery remains to be conclusively demonstrated as most of the available data have significant limitations; i.e., they were obtained in single-center, small size clinical trials in statin-naïve patients and they are mostly based on “ancillary” findings” and not on continuous ECG monitoring. For these reasons, the recent guidelines for the management of AF have not given a strong recommendation for the use of statins in the prevention of POAF[134,189]. In the absence of evidence, whether statins are stopped or continued in patients undergoing cardiac surgery (majority would be already on statin therapy) remains at the discretion of the surgeons.

Perioperative myocardial injury

Background

Perioperative myocardial injury (PMI) in the context of elective cardiac surgery is a broad term that encompasses i) “Myocardial stunning” defined as the need for pharmacological and/or mechanical support (Intra-aortic balloon pump/left ventricular assist device) during the post-operative period[190] and ii) “type 5 myocardial infarction” as defined in the recently published 3rd universal definition[191]. PMI is an important determinant of the intermediate as well as long-term risk of mortality after CABG [192].

Diagnosis

In general, the elevation of troponins in the blood is attributed to myocardial injury secondary to surgical trauma, suboptimum myocardial perfusion/infarction[193], sepsis[194], and pulmonary embolism[195]. All these are relatively more common in the peri- and postoperative period of patients undergoing cardiac surgery. Hence, postoperative troponin rise is considered as a biomarker of PMI[196].

However, most published studies were done before the publication of standardised criteria detailed in the third universal definition of MI [197] [198,199] leading to considerable heterogeneity between the studies as highlighted in a recent meta-analysis[200]. It was noted that despite association of troponin levels with postoperative morbidity and mortality, estimations of actual effect size and cut-off values were difficult given the variability in patient population and troponin assays. In population based studies[201,202], isolated rise in troponin, i.e., without any concomitant clinical, ECG or imaging evidence of myocardial ischemia, is the commonest presentation in patients undergoing non-cardiac surgery. Together, there is a need for further investigations analyzing changes troponin levels during the peri- and postoperative period of patients undergoing cardiac surgery.

Mechanisms

Despite the advent of off-pump techniques, majority of CABG continues to be performed using CPB. [39,203]. Similarly, procedures such as valve replacements and surgical correction of congenital heart diseases are primarily carried out on CPB. Notwithstanding cardioplegia[204], hearts undergoing surgical procedures on CPB are subject to some degree of ischemia - reperfusion injury[205] leading to myocardial stunning[206,207], increased risk of arrhythmias[208 209] and death; indeed, autopsy findings have shown evidence of reperfusion injury in the myocardium in up to 25% patients who died after CABG[210]. Hence, it is conceivable that interventions that may limit or prevent this pathological process would have a significant impact on patient outcome. Several cardioprotective interventions have been tested; however, cardioprotective strategies that worked well in pre-clinical animal experiments has not translated into useful therapeutic intervention in man[211]. Hence apart from optimizing the determinants of coronary arterial perfusion in the perioperative period, no other approach or intervention is currently recommended to reduce the risk of perioperative myocardial ischemia and infarction[58]. This calls for better understanding of the molecular and biochemical changes associated with I/R in the human myocardium, which may provide direct evidence of molecular targets amenable for therapeutic modulation.

Oxidative stress and I/R:

In human cardiomyocytes, low levels of superoxide are continuously generated by electron leakage within the mitochondrial electron transport chain and from the membrane bound NOX2-containing NADPH oxidases [212-218]. By contrast, reperfusion after a brief period of ischemia elicits the generation of large amounts of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals, from multiple enzymatic sources [219-228]. However, the experimental evidence linking ROS with myocardial IR injury is mostly indirect and based on reports of reduced myocardial injury following treatment with ROS scavengers [229-235] or overexpression of antioxidant networks [236-241], and increased susceptibility to IR injury following targeted disruption of

genes encoding antioxidant enzymes[242,243]. The sources of ROS involved in mediating IR injury are also a matter of debate.

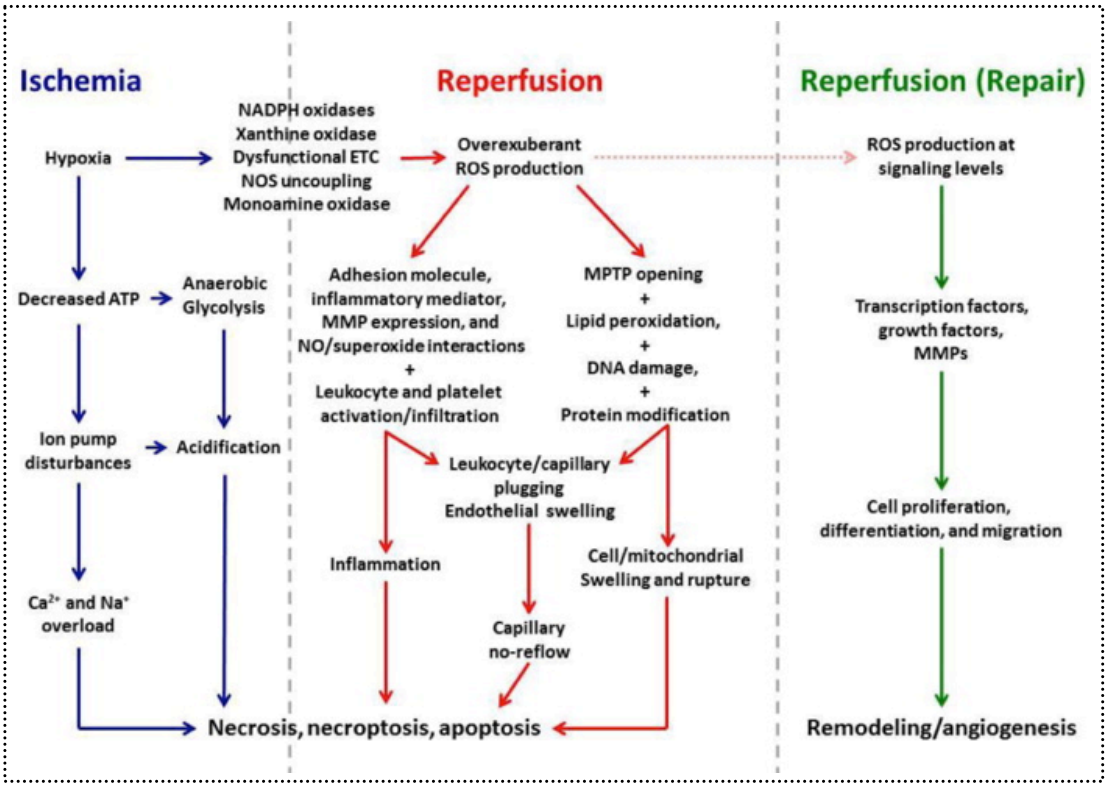


Figure 1.13: Mechanisms contributing to tissue injury in ischemia/reperfusion

Reproduced from; Theodore Kalogeris, Yimin Bao, Ronald J. Korthuis, Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning, Redox Biology, Volume 2, 2014, Pages 702-714

Mitochondrial electron transport chain (ETC): A growing body of evidence suggests that mitochondrial ROS determine the redox state of key proteins involved in processes such as mitophagy and apoptosis [244-248] and regulate the cellular response to hypoxia by maintaining the stability of the transcription factor HIF-1alpha [249-253] which may be beneficial following I/R. During ischemia, HIF-1 alpha regulates the expression of glycolytic enzymes and the metabolic switch to glycolysis from fatty acid oxidation[254,255,256-258]. However, excessive mitochondrial ROS generation can lead to redox imbalance, oxidation of mitochondrial lipids and protein thiols and opening of the mitochondrial permeability transition pore (MPTP) leading to mitochondrial dysfunction and ROS mediated cell death [259-266], suggesting that interventions able to maintain mitochondrial redox balance during IR could prevent cardio myocyte injury and

death [267,268]. Hence, the role of MPT in the context of I/R warrants further discussion.

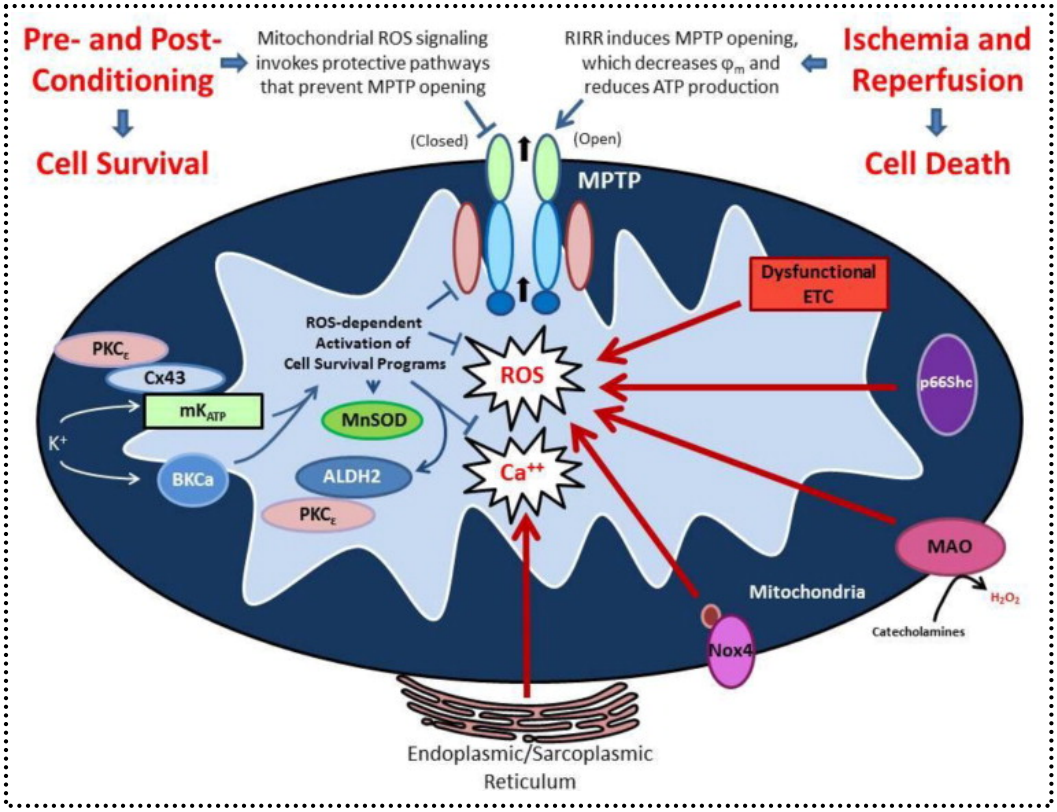


Figure 1.14: Generation of reactive oxygen species (ROS) by mitochondria is involved both in activation of cell survival programs that mediate the effect of conditioning stimuli to enhance tolerance to ischemia/reperfusion (I/R) and serves as a focal point for over exuberant ROS-induced ROS release that contributes to the pathogenesis of cell injury in I/R.

Reproduced from; Theodore Kalogeris, Yimin Bao, Ronald J. Korthuis, Mitochondrial reactive oxygen species: A double edged sword in ischemia/reperfusion vs preconditioning, Redox Biology, Volume 2, 2014, Pages 702-714

Opening of the mitochondrial permeability transition pore (MPTP)[269] enables free passage into the mitochondria of molecules of less than 1500 Daltons, including protons. This causes equilibration of H^+ across the inner membrane, which dissipates the inner mitochondrion potential ($\Delta\Psi_m$) and inhibits ATP production eventually leading to cell death. Several studies suggest that ROS signaling involved in cardioprotective strategies, such as preconditioning[270], prevents mitochondrial permeability transition (MPT) during prolonged ischemia[271,272]. Pro survival pathways such as RISK[273] and SAFE[274] kinases are thought to converge on mitochondria under such conditions. However, the precise molecular mechanisms that prevent MPT during reperfusion are not

evident. It has also been suggested that transient opening of MPTP is a physiological response[275] which might be cardioprotective. This is supported by the observation that superoxide originating from the mitochondria may activate protein kinase C (PKC) that acts as a major upstream preconditioning signal causing transient MPTP openings[276]. Whether there are additional cardioprotective mechanisms independent of MPT is unclear at present.

Dysfunctional nitric oxide synthase: Based on isolated heart studies, another proposed source of superoxide following ischemia is dysfunctional NOS (mechanisms underlying this functional uncoupling has been discussed in the section on postoperative AF) and interventions that maintain coupled NOS activity decreased myocardial injury [277]. BH4, one of the co factors of NOS, is synthesized de novo by the action of GTP cyclohydrolase-1 (GTPCH-1)[278-282] or by the salvage pathway that converts oxidized biopterins to BH4 via sepiapterin and dihydrofolate (DHFR) reductases [281]. BH4 depletion, secondary to oxidation and/or reduced synthesis is one of the important causes of functional uncoupling of NOS and ROS production during IR [277,282-294]. However exogenous supplementation of BH4 in patients undergoing CABG didn't improve vascular redox state and function[295], limiting its therapeutic potential at present [296 297] though further work needs to be done to explore the relevance of this finding in the human myocardium. It is, however, known that exogenous BH4 is first oxidized to BH2 in the plasma and then transported into the cell, where it can be reduced back to BH4 by dihydrofolate reductase[298]. Previous work from our group has shown that oral BH4 supplementation resulted in an increase in BH4 and biopterin content in isolated LV myocytes from C57BL/6 mice whereas biopterins synthesized in LV myocytes from mice with myocardial specific overexpression of GCH-1 (mGCH1-Tg) remained confined within this cell type, suggesting that biopterin transport across myocytes may be unidirectional[299]. It was also observed that the reduction in the ratio of BH4 to BH2+B was not associated with NOS dysfunction in the myocardium of mGCH1-Tg mice. This is in agreement with published work from our group indicating that in right atrial tissue samples from patients with atrial fibrillation, reduction in BH4: BH2+B ratio was not sufficient to cause NOS uncoupling in the absence of a significant decrease in tissue BH4 concentration[89].

Hence, strategies that can either increase BH4 synthesis[297] and/or augment BH4 recycling pathways might provide an alternative approach to increasing BH4 bioavailability in myocardium and preserve coupled status of NOS and its function [300-305].

NADPH Oxidase: Given the functional relevance of NOXs in the aged, hypertrophic and failing hearts (which are the most common substrate for IR injury associated with cardiac surgery [306-318]), the role of this enzyme complex in the setting of I/R merits further analysis.[319].

Despite earlier negative reports [319], the relevance of NOX-derived ROS in I/R was highlighted by the observations that in a rat model of myocardial infarction as well as in myocardial samples obtained from patients who died after MI, expression of NOXs was increased[320,321]. Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme for NADPH production through the pentose phosphate pathway, and its activity is critical for contractile function during I/R via its effects on myocardial calcium homeostasis [322]. On the other hand, an increase in G6PD-NADPH has also been associated with reductive stress (i.e., increased GSH/GSSG ratio) linked to increased glutathione levels in a mouse model of mutant αB - crystallin cardiomyopathy[323] suggesting that the relationship between NADPH and NOX mediated ROS levels may depend on the subcellular localisation of individual isoform.

Despite these complexities, NOX2-derived ROS have been proposed to mediate preconditioning through activation of PKC [324,325] and by facilitating protective HIF signaling [326],[327-329]. HIF activates key molecular pathways that are involved in the regulation of angiogenesis, survival pathways, antioxidant defense and metabolism thereby conferring cardioprotection. Indeed mouse models of enhanced HIF signaling show a reduction in infarct size and contractile dysfunction [330-332]. It has been recently shown that during I/R, low levels of ROS production from either NOX2 or NOX4 are required for activation of adaptive mechanisms essential for cardioprotection mediated by HIF signalling [333] whereas either absence or over production of NOX 2/4-derived ROS was associated with I/R injury.

It is possible that the source of ROS contributing to I/R may vary depending on the underlying pathology (such as myocardial failure and diabetes) and that, for this reason, treatment strategies may need to be "personalized," a paradigm that has not been explored in the context of IR injury in the human myocardium. This might also partly explain the general failure of antioxidant treatments in the prevention of complications after cardiac surgery[334,335] reinforcing the notion that targeting compartmentalized pools of ROS and their sources may be a more efficient strategy [336]. Given this possibility, the current lack of effective treatments and its potential role in cardioprotective strategies, as discussed above, investigation of enzymatic sources of ROS production in the human myocardium following I/R merits further investigation.

Nitric Oxide Redox balance during I/R:

NO is produced by oxidation of L-Arginine to L- Citrulline in a reaction catalyzed by NOS that requires the presence of O_2 and co-factors such as the tetrahydrobiopterin (BH4)[283,284,297,337-340]. Spatial confinement and co-localization of NOS with their target proteins within the myocardium ensures specificity of actions of NO, such as regulation of excitation-contraction coupling and ion channel function [341-347]. This is achieved by activation of cyclic guanosine monophosphate (cGMP) dependent-signaling pathways [348] as well as by direct modification of sulfhydryl residues of proteins by covalent attachment to cysteine thiol group, a process referred to as S-nitrosation [349-353]. This post-translational modification is thought to protect thiol residues from irreversible oxidation[354] and thus it may provide an important molecular mechanism by which NO mediates redox regulation of proteins involved in I/R injury[355]. Indeed it has been shown that the increase in NO production during ischemia may be cardioprotective [356-367]. On the other hand, when NOS is partially uncoupled, concurrent production of ROS as well as NO favors the formation of pro oxidant molecules such as "peroxynitrite" (ONOO-) [368-372] thereby increasing BH4 oxidation [373-376] and local oxidative stress further. ONOO- can aggravate myocardial reperfusion injury in isolated heart models [377] and lead to myocardial stunning and arrythmogeneis [113,370,378,379]. Hence, ROS may inflict cellular injury not only directly, but also by decreasing NO bioavailability and disrupting NO signaling. Consistent with this

hypothesis, transgenic murine models with conditional cardiomyocyte overexpression of NOS isoforms show decreased infarct size and preserved myocardial function following IR injury [380-382] whereas nNOS1 gene knockout significantly accelerates adverse LV remodeling in the murine myocardium after myocardial infarction [383] and increases the incidence of ventricular arrhythmias [384]. Similarly, decreasing ROS production and increasing ONOO- scavenging improved post I/R injury and ischemic myocardial contractile function in animal models [277,385-388].

Based on this it can be hypothesised that, in the context of I/R, interventions that increase NO bioavailability and decrease ROS production may be cardio protective. [277,380-388].

Hydroxymethylglutaryl-CoA reductase inhibitors and PMI:

Under experimental conditions, acute cardio protection with statins has produced favorable results[389 390]. The proposed mechanisms underlying these observations based on putative cholesterol-independent or "pleiotropic" effects include an increase nitric oxide (NO) bioavailability secondary to the activity and the protein expression of the endothelial NOS isoform (NOS3)[391], activation of the PI3K-Akt-eNOS pathway[392], and increase in GTP Cyclohydrolase I activity and BH4 synthesis [393-398]. However, as discussed earlier, current guidelines do not support the effectiveness of prophylactic pharmacological therapies or controlled reperfusion strategies aimed at inducing preconditioning or attenuating the adverse consequences of myocardial reperfusion injury[58] underscoring the need for further research in this context for development of interventions that can minimise PMI.

Thus, whether intensive statin treatment in the perioperative period is associated with acute cardio protection and improved clinical outcome remains to be demonstrated in adequately powered randomised controlled trials (RCTs).

HYPOTHESES

1) Elective cardiac surgery on cardiopulmonary bypass for myocardial revascularization, aortic valve replacements or both results in myocardial nitric oxide - redox disequilibrium.

2) Perioperative HMG-CoA reductase inhibition with statins preserves atrial nitric oxide - redox equilibrium and atrial electrical properties following on-pump cardiac surgery and prevents the new onset atrial fibrillation, myocardial damage and improves in-hospital outcomes after elective cardiac surgery

AIMS

1) To define the sources of ROS release, characterize NO-redox balance and analyze factors governing bioavailability of BH4 in the human atrial myocardium following ischemic cardioplegic arrest during cardiac surgery on cardio pulmonary bypass.

2) To evaluate the effect of perioperative treatment with high-dose atorvastatin (80 mg od) on atrial electrical remodelling and oxidative stress following elective cardiac surgery and cardio pulmonary bypass.

3) To establish whether perioperative administration of rosuvastatin (20 mg od) in patients undergoing elective cardiac surgery leads to a reduction in the incidence of post-operative atrial fibrillation (as assessed by Holter monitoring) and in perioperative myocardial injury (assessed by serial Troponin measurements).

4) To establish whether perioperative administration of rosuvastatin (20 mg od) in patients undergoing elective cardiac surgery affects in-hospital complications and duration of hospital, and intensive care unit stay period.

CHAPTER 2

METHODS

Section 1

General methods

This section outlines the principles and methods of core techniques used for the analysis of biological samples and their validation where applicable in human right atrial tissue homogenates.

Materials:

All chemicals used for this study were of analytical grade with more than 99% purity as certified by the manufacturer and were purchased as below.

- Chemicals - Sigma-Aldrich Inc.
- Western blot gels and consumables – Life technologies LTD (Invitrogen division).
- HPLC column and fittings – Hichrom LTD.
- GP91 ds tat peptides – Cambridge biosciences.

Solutions were prepared using Milli-Q ultra-purified water from the Millipore purification system (Massachusetts, USA) and/or HPLC grade organic solvents.

Sample preparation:

Based on the process validated in our lab before[89], 30-50 mg of frozen atrial tissue was homogenized for 30 seconds (using a pre-cooled Polytron® probe, PT2100 Kinematika®, Switzerland) in ice-cold Krebs-Hepes Buffer (KHB; 99 mM, NaCl; 4.7mM, KCl; 1.2 mM $MgSO_4$, KH_2PO_4; 1 mM, $CaCl_2$; 1.9 mM, $NaHCO_3$; 25 mM, Glucose; 11.1 mM and HEPES; 20 mM: pH-7.4; Volume depending on individual experiments) containing protease inhibitor cocktail (Roche Applied Science, USA; 1 tablet dissolved in 10 mL of KHB added prior to homogenization). Following homogenisation, the processed samples were spun at 4000 rpm in a cold room, and the supernatant was transferred to new eppendorf tubes kept on ice. A second cycle of spinning at 13000 rpm for 5 minutes done subsequently to clear any residual fat and tissue debris followed by collection of supernatant after passing through a 0.45 micron filter (MF-

Millipore™). Protein concentration was estimated as below, and appropriate amount of protein was used in a number of assays.

Estimation of protein concentration:

Protein concentration was measured using commercially available kit BCATM assay (Pierce, UK) which is based on the reduction of Cu2+ to Cu1+ by protein in an alkaline medium and the highly sensitive and selective coloriometric detection of the cuprous cation using bicinchoninic acid (BCA). Disulfide reducing agents, particularly dithiothreitol and 2-mercaptoethanol, were used in some experiments such as tissue biopterin measurement, are also capable of reducing Cu^{2+} to Cu^{1+}. To minimize the effect of these copper reducers, a compatibility reagent (iodoacetamide) that modifies disulfide reducing agents was added to the sample before adding the BCA reagents. A BCA™ protein assay kit was used in 1(Reagent B): 50 (Reagent A) dilutions to detect this reaction. Standards were prepared from bovine serum albumin (BSA, 2 mg/mL stock) achieving final concentrations: 0.25, 0.5, 0.75, 1, 1.5, 1.75 and 2 mg/mL (Table 2-1).

Eppendorf	Buffer Volume (µl)	BSA Volume (µl)	Final concentration (µg/ml)
1	0	40	2000
2	10	30	1500
3	20	20 of **1**	1000
4	20	20 of **2**	750
5	20	20 of **3**	500
6	20	20 of **5**	250
7	20	20 of **6**	125
8	20	0	0

Table-2.1: Serial dilutions of bovine serum albumin for preparation the standard curve for estimation of unknown protein concentration in test samples.

To estimate protein concentration in atrial homogenates, [x4] times sample dilution was used and the assay was performed in triplicate to ensure accurate measurement of protein content. Both the standards and the samples were incubated in a water bath at 37^0C for initially 15 minutes with iodocetamide (10 mg/mL) followed by 30 minutes incubation with a mixture of Reagent A and Reagent B prior to absorbance measurement at 562 nm in a spectrometer (Molecular Devices, kinetic plate reader, UK). The concentration of protein within each sample was calculated using a standard curve (Fig. 2-1).

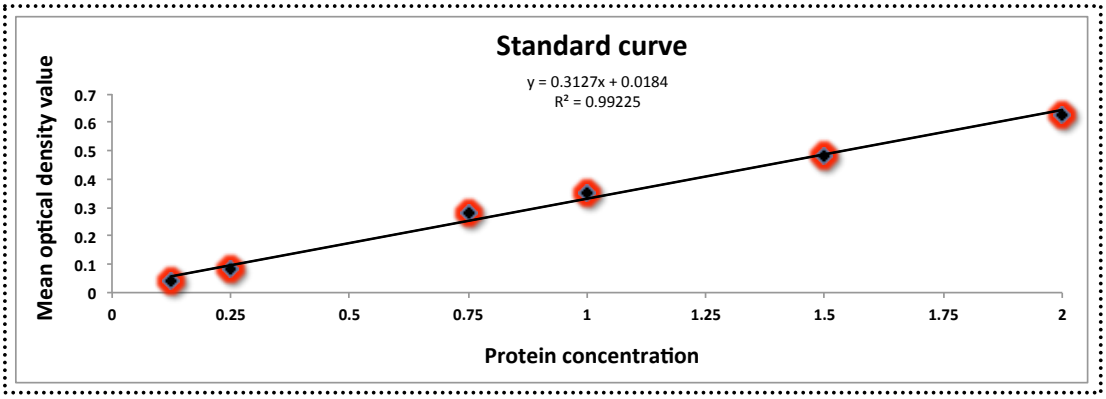


Figure 2.1 - A representative standard curve used in the protein assay for atrial homogenates using the BCA™ kit. Standards were prepared by sequential dilution of BSA stock solution achieving final concentrations of 0.125, 0.25, 0.5, 0.75, 1, 1.5 and 2 mg/mL.

Quantification of atrial superoxide production by Lucigenin enhanced chemiluminescence

Superoxide generation in atrial homogenates was measured using lucigenin-enhanced chemiluminescence in a single tube luminometer (Berthold FB12) modified to maintain the sample temperature 37°C, as validated in our laboratory before[399]. The principle of this method is reliant on the reaction between the chemiluminescence probe lucigenin (bis-N-methylacridinium nitrate) and superoxide to form an unstable dioxetane, which decomposes to form 2 molecules of methylacridone [400,401], one of which is in the excited state and emits a photon that can be measured in a luminometer. To prevent redox cycling and overestimation of superoxide, all luminometry experiments were performed using a low concentration (5 µMol/L) of lucigenin [402]. Since there is a

linear dose response within the range of 0.1 mg/mL to 0.4 mg/mL of protein content, as shown previously in our laboratory [403], experiments were performed using a protein concentration of 0.4 mg/mL wherever possible for optimum signal strength. Prior to each experiment, lucigenin (5 µMol/L) was added to KHB in a single 1.7 mL tube and incubated for 1 minute at 37^{o}C followed by recording for 200 seconds using the computer software FB-12. This background activity was later subtracted from sample measurements. Pre-cooled homogenate containing 0.4 mg of protein was then added to the mixture (lucigenin + assay buffer (KHB) + sample = 1 mL) followed by incubation for 1 minute at 37^{o}C after which basal superoxide chemiluminescence signal was recorded for 5 minutes. In order to identify the enzymatic sources of basal superoxide production, pharmacological inhibitors of atrial oxidases were used either in sequence or in a separate assay. To demonstrate specificity of the assay for superoxide, co-incubation (3 minutes at 37 °C) with the superoxide scavenger Tiron was performed (100 mMol/L) in the final step and the signal was subtracted from either basal or the signal following pharmacological inhibition depending on the sequence. Superoxide release was expressed as the Tiron-inhibitable fraction in relative light units per second per mg of protein (RLU/s/mg protein) after extrapolating the results for protein concentration of 1mg.

NOS Inhibitor:

N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) was used as an inhibitor of coupled and uncoupled NOS activity. After dissolving in milli Q water, L-NAME was incubated with the sample under analysis for 2 minutes at 37°C to achieve a final concentration of 1 mmol/L. Its dextro-isomer, N-nitro-D-arginine methyl ester (D-NAME) lacking properties of NOS inhibition was used (1 mmol/L) as a negative control. The difference (D-NAME – L-NAME) between either background subtracted lucigenin enhanced chemiluminescence signals or area under 2-OH- (E+) peaks detected by HPLC after incubation of samples with D-NAME and L-NAME was taken as the measure of uncoupled NOS activity.

Mitochondrial Inhibitors:

Rotenone (100 µMol/L) and Antimycin A (10 µMol/L) dissolved in 1% DMSO and 100% ethanol respectively were used to inhibit mitochondrial complex I and III. In this experiment, Tiron inhibitable fraction by lucigenin enhanced chemiluminescence was measured in samples under basal conditions with DMSO and also following incubation with rotenone or Antimycin-A for 30 min on ice. As shown in figure **2.2**, the difference between the two values were taken as respective inhibitable fractions representing ROS release from complex I (Rotenone) and III (Antimycin) of mitochondrial ETC (Electron transport chain).

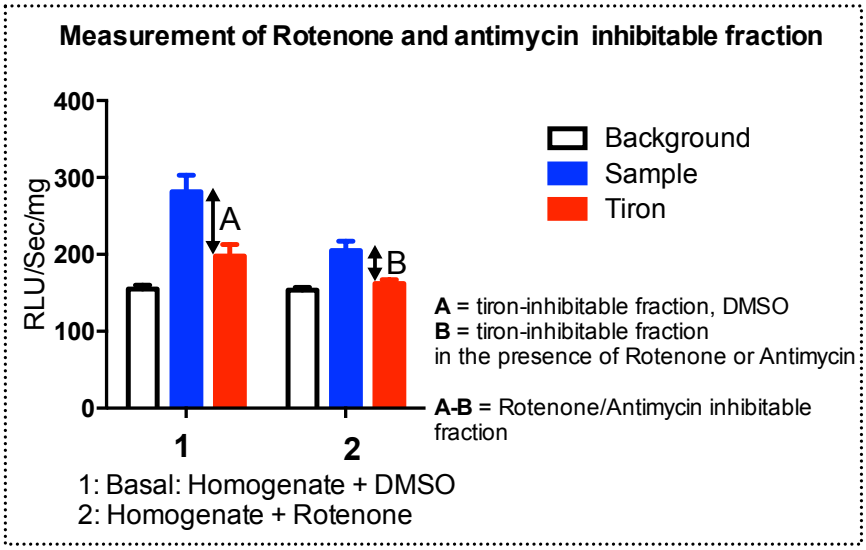


Figure 2.2 - Schema for measurement of ROS release from mitochondrial electron transport chain.

NADPH Oxidase inhibitors:

gp91 ds (docking sequence) tat peptide is a chimeric peptide that blocks the interaction between p47*phox* and the NADPH Oxidase enzyme complex thereby preventing its activation and generation of superoxide[404]. To measure the contribution of NOX2 containing NADPH oxidase to superoxide release, gp91 ds tat peptide and its scrambled sequence as a negative control was used based on a protocol validated as described later in this chapter. The NOX2-NADPH Oxidase derived ROS was defined as the difference between the areas under 2-OH- (E+) peaks of samples incubated with scrambled and test peptides.

NADPH-stimulated superoxide production was measured to evaluate the capacity of all homologues of the myocardial NADPH oxidase to produce superoxide. Following basal ROS measurements, atrial homogenates were pre-incubated with NADPH (100 µMol/L) for 3 minutes and the difference between basal and the stimulated ROS measurements were taken as an index of NADPH oxidase activity.

Quantification of superoxide production: 2-hydroxyethidium detection by HPLC

Principle:

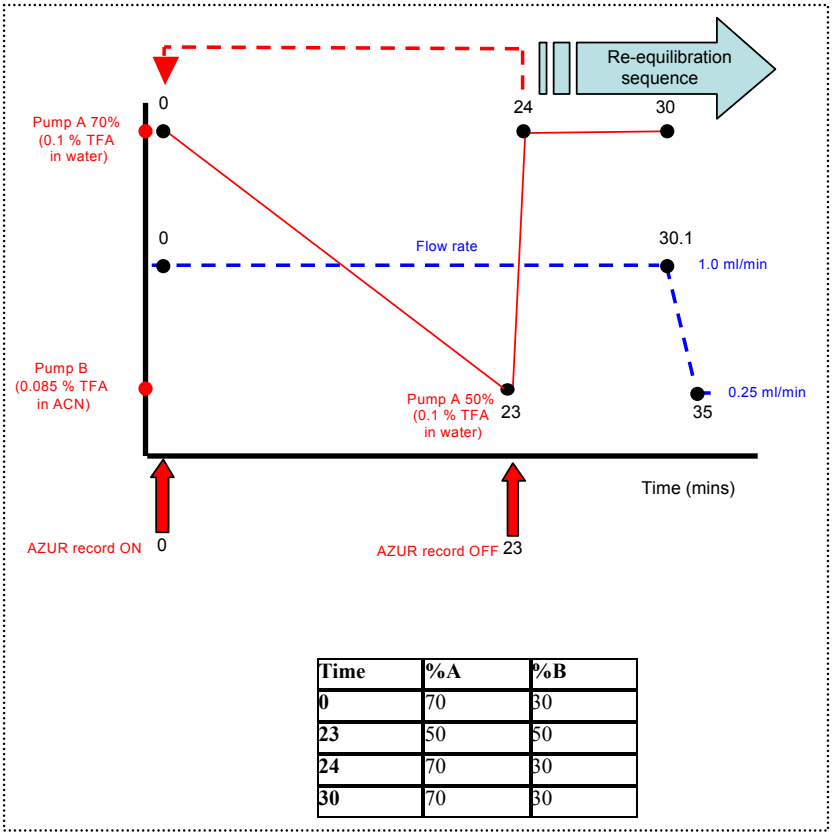
Dihydroethidium (DHE) is a redox-sensitive probe that is widely used to detect superoxide anion based on its exclusive conversion to 2-Hydroxyethidium (2-OH-E+), which is considered to be highly specific for detecting superoxide in biological systems[405]. The reaction chemistry between superoxide anion and DHE is unique in that 2-OH- (E+) formed is the only major product. The actual mechanism involves the formation of an intermediate radical cation that reacts with another molecule of superoxide anion to form the 2-hydroxylated cationic product. In a purely superoxide generating system, no other products are formed. However in right atrial homogenates, the presence of redox metal ions, heme proteins (cytochrome c) with peroxidase activity or other one-electron oxidants will oxidize DHE to several products including the fluorescent phenanthridinium cations (ethidium and analogs) and non-fluorescent or weakly fluorescent dimeric products. HPLC separation and analysis of corresponding chromatograms allows quantification of these oxidation products, and the method is currently considered as the gold-standard for detection of superoxide[406].

Sample preparation:

Stock solution of DHE (Molecular probes, Life technologies, UK) was prepared by dissolving in DMSO stored in a dark anaerobic chamber filled with argon gas to a final concentration of 5 mMol/L. After diluting 1/10 with KHB, aliquots were stored in dark eppendorf at -80^{o}C. On the day of the experiment, one aliquot

was thawed and used for the entire batch of samples. Stock solutions of all the other reagents and substrates were made up on the day of the experiment and were kept on ice until used. Samples were processed in batches of 40/day.

Protocol:



Time	%A	%B
0	70	30
23	50	50
24	70	30
30	70	30

Figure 2.3 – HPLC running protocol for measurement of ROS by 2-OH- (E+) detection.

In the protocol used in our lab for measuring ROS release in murine myocardial samples, homogenates in KHB are incubated with vehicle control (KHB), Tiron (100mmol/L), or pharmacological inhibitors of enzymatic sources of ROS release at 37^{o}C for 30 minutes. In the next step, samples are mixed with dihydroethidium (10 µMol/L) and incubated in the dark for 30 minutes at 37^{o}C. In the final step, reaction is terminated by adding 110 µl of ice-cold pure 100% methanol followed by deproteination with 110 µl of ice-cold 0.1 M HCl. Samples are vortexed and spun at 13,000 rpm for 15 minutes at 4^{o}C. 150 µl of the supernatant is subsequently transferred to dark glass amber vials for proceeding with HPLC analysis. Separation of ethidium, oxyethidium, and dihydroethidium are performed using a gradient HPLC system (Jasco) with an ODS3 reverse

phase column (250 mm, 4.5 mm; Hichrom), and quantified using a fluorescence detector set at 510 nm (excitation) and 595 nm (emission). A linear gradient is applied from Mobile phase A (0.1% tri fluoro acetic acid –TFA- (v/v) to Mobile phase B (0.1% TFA (v/v) in acetonitrile) over 25 minutes (30% acetonitrile to 50% acetonitrile) as shown in figure **2.3.** In comparison with external standards, 2-hydroxyethidium (2-OHE) peaks are identified in the sample chromatograms and Azure Software version 4.02 (Datalys, France) is used for calculating the area under the peaks.

As this protocol has not been validated in human RAA homogenates before, studies were done to determine optimum protein and DHE concentration.

Results:

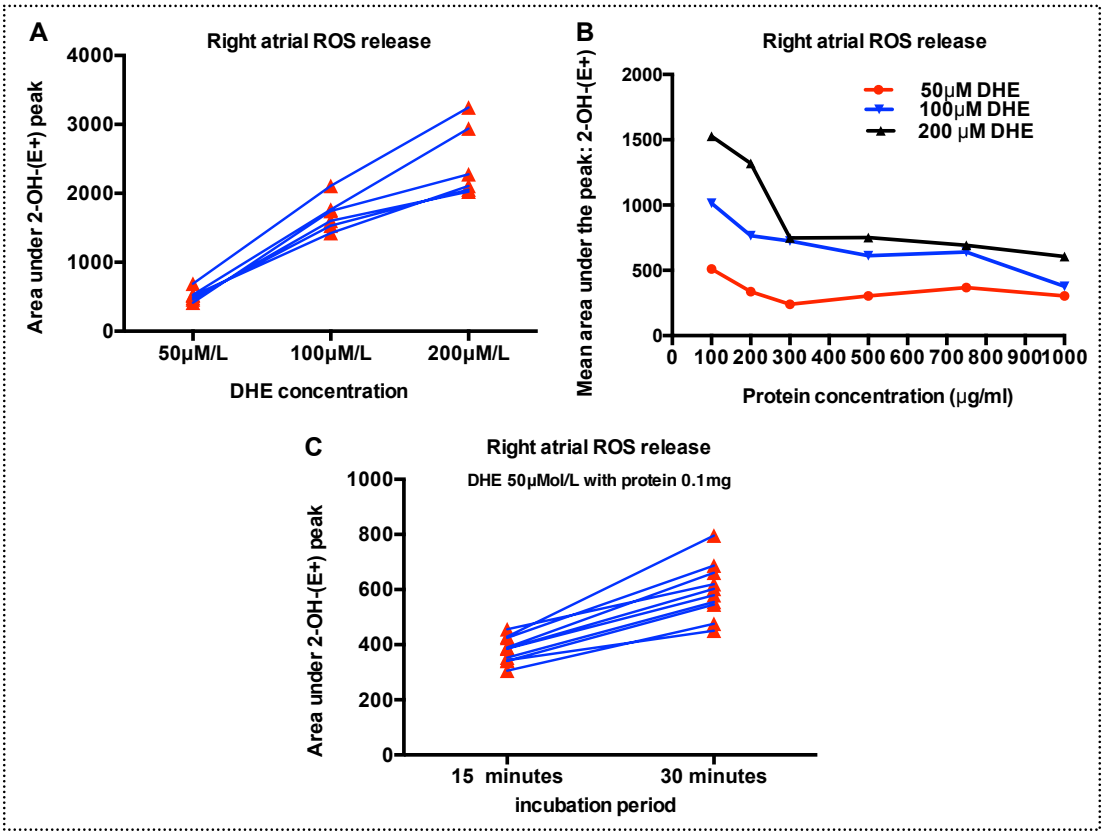


Figure 2.4 - ROS measurements in human atrial homogenates with varying DHE concentration (**A**), protein content (**B**) and incubation period (**C**) (n = 6-10 samples per each measurements).

To evaluate the effect of varying DHE concentration on ROS measurements, 2-OH- (E+) was detected in human RAA homogenates over DHE concentrations of 50 - 200 µMol/L. As shown in figure **2.4 A**, the relationship between DHE

concentration and 2-OH- (E+) fluorescence was linear whereas the protein concentration and 2-OH- (E+) fluorescence exhibited an inverse relationship (Figure **2.4B**) between 0.1 to 0.3 mg/ml of protein concentration. Based on these findings, for optimum signal strength to measure ROS generation in human RAA homogenates 0.1mg protein was loaded in KHB with 50 µMol/L of DHE and the method was compared between incubation period of 15 minutes and 30 minutes. It was observed that the relationship between the two measurements was linear (Figure **2.4C**). Accordingly, a representative chromatogram of 2-OH- (E+) detected by the protocol using protein 0.1mg incubated with 50 µMol/L of DHE for 30 minutes is shown in figure **2.5.**

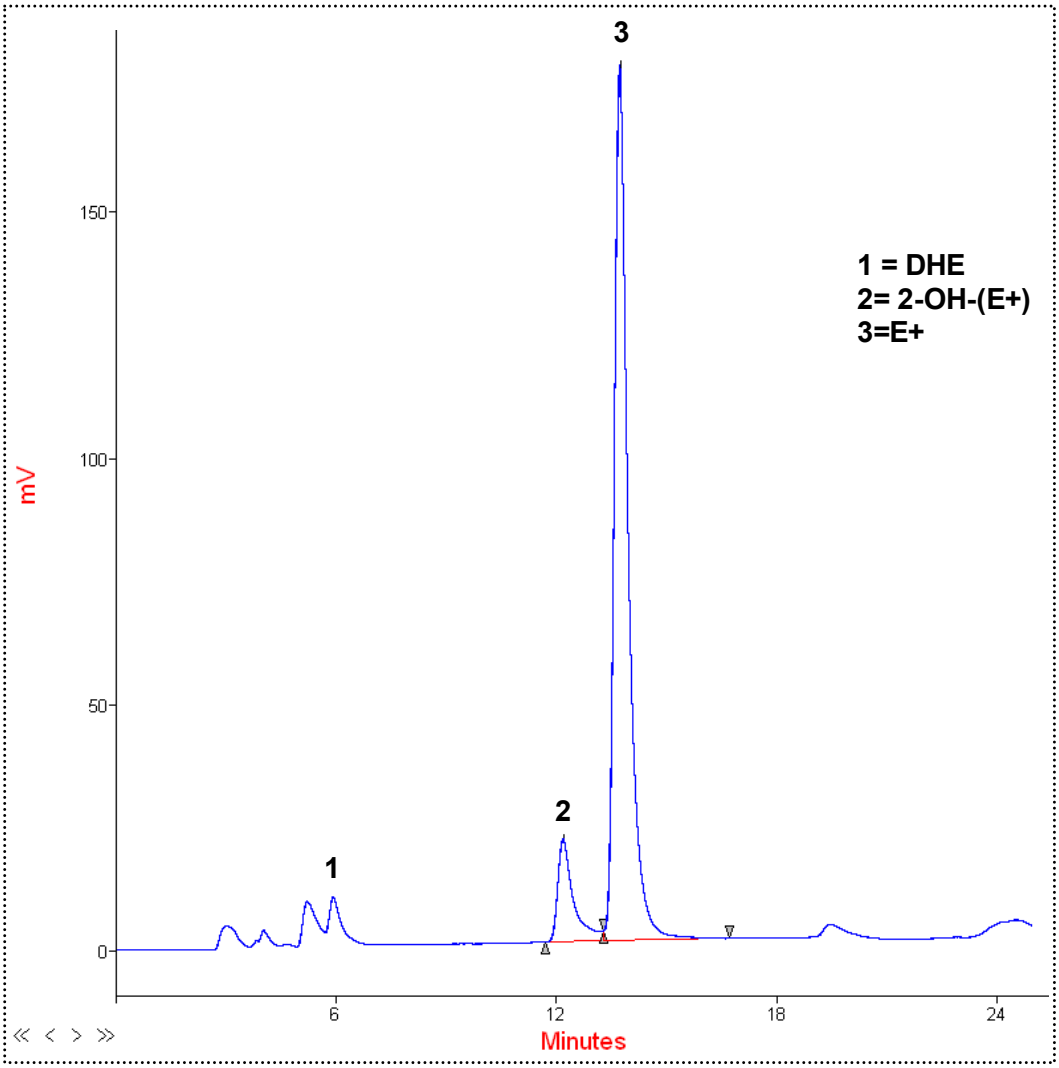


Figure 2.5 – Example of DHE (50 µMol/L) standard chromatogram showing eluting time points of dihydroethidium (DHE;1), 2-dihydroethidium (2-OH-E+;2) and Ethidium (E+;3) peaks.

Peroxynitrite measurements

Peroxynitrite (ONOO-) content of atrial homogenates prepared as described earlier was measured using luminol (100 µmol/L) [407] as a luminescent probe instead of lucigenin. Uric acid (1 mmol/L) was used as a specific peroxynitrite scavenger[408-410]. The difference between the basal and the signal after uric acid expressed as uric acid inhibitable fraction was considered as a measure of peroxynitrite content.

Biopterin Quantification by HPLC

Tetrahydrobiopterin (BH4), and its oxidized products 7,8-BH2 and B were measured by electrochemical (for BH4) and fluorescence detection (for 7,8-BH2 and B), following sample separation by HPLC as described previously[411-413]. Briefly, atrial tissue samples were homogenised in phosphate-buffered saline (50 mM), pH 7.4, containing dithioerythritol (1 mM) and EDTA (100 µM). Following centrifugation (15 min at 13,000 rpm and 4 °C), the samples were transferred to new, cooled micro tubes and precipitated with cold phosphoric acid (1 M), TCA (2 M), and dithioerythritol (1 mM). The samples were vigorously mixed and then centrifuged for 15 min at 13,000 rpm and 4 °C. The supernatant (150 µl) was transferred into a 96-well HPLC plate and placed in the cooled (4^{o}C) HPLC auto sampler and injected onto an isocratic HPLC system for quantification using sequential electrochemical (Dionex Coulochem III; Thermo scientific, Buckinghamshire, UK) and fluorescence (Jasco, Essex, UK) detection. All samples were processed in batches of up to 12 samples/day to prevent oxidation of BH4 and in duplicates during the measurements. The supernatant was injected into an isocratic HPLC system and biopterins were quantified using sequential electrochemical (Coulochem III, ESA Inc., UK) and fluorescence (Jasco Ltd, Dunmow, UK) detection, as described before [414]. HPLC separation was performed using a 250 mm, ACE C - 18 column (Hichrom, UK) with a mobile phase of 50 mM sodium acetate, 5 mM citric acid, 48 µM EDTA, and 160 µM dithioerythritol (pH 5.2, all ultrapure electrochemical HPLC grade) run at a flow rate of 1.3 mL/min. Background currents between +500 and +600 µA and -50 and -60 µA were used for the detection of BH4 on electrochemical cells electrode 1 (E1) and electrode 2 (E2) respectively. 7,8-

BH2 and B were measured using a FP2020 fluorescence detector (Jasco Ltd, Dunmow, UK). Quantification of BH4, 7,8-BH2 and B were made by comparison with external standards after normalizing for sample protein content. The standards (1 nMol/L – 1 µMol/L) were prepared from a stock solution containing 10 µMol/L BH4, 7,8-BH2 and B (Schircks, Zurich) in ice-cold re-suspension buffer. The results were expressed as picomoles per mg of protein.

Reproducibility of Biopterin measurements

To assess the reproducibility of the BH4 measurements in my hands, Bland Altman assessment for agreement was used for comparing measurements of BH4 and it's oxidized products (BH2 and B) in samples of mGCH1 transgenic murine myocardium on two separate days. Figure 2.6 shows the mean bias ± 2SD of BH4 measurements and of the ratio between BH4 and oxidised biopterins.

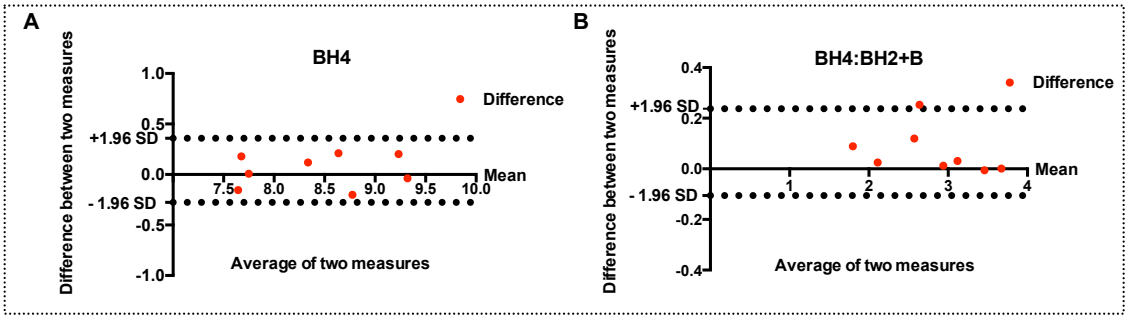
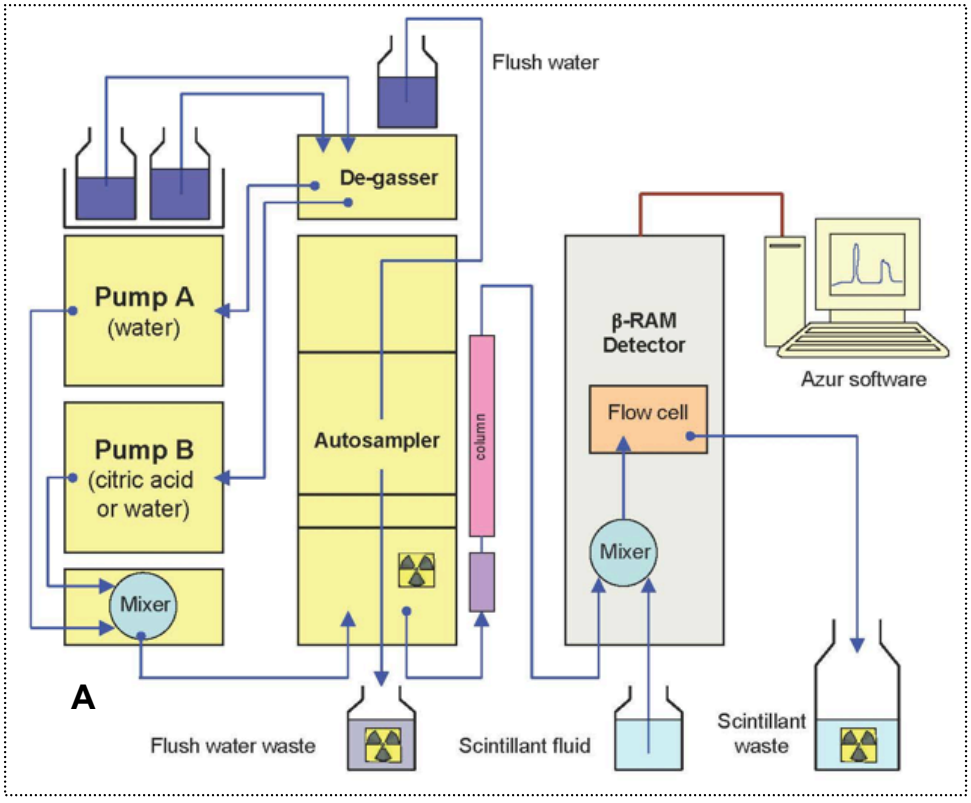


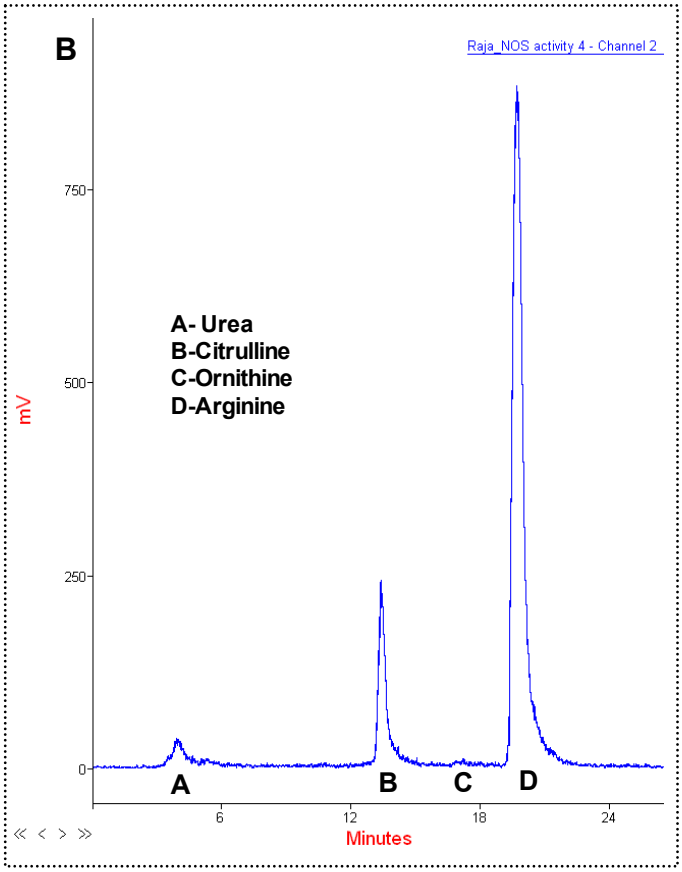
Figure 2.6: Bland-Altman analysis of reproducibility of BH4, BH2 and biopterins showing inter assay correlation of measurements performed on separate days (n=8 pairs).

Measurement of nitric oxide synthase (NOS) activity by real time radiochemical detection of 14^{C} labeled L- arginine to L-citrulline conversion

Enzymatic activity of NOS in atrial tissue homogenates was measured using radiochemical detection of 14C labeled L- arginine to L-citrulline conversion, with addition of exogenous cofactors (FMN, FAD and NADPH) as validated in our laboratory previously[415]. All experiments were performed in the presence of arginase inhibitor nor- NOHA (Calbiochem, UK) to prevent arginase activity and non-NOS sources of Citrulline synthesis. Samples were homogenized in 800 µl

of ice-cold KHB containing 5 µMol/L of nor-NOHA. 200 µl supernatants containing 0.5 mg protein each were then incubated for 30 minutes with cofactors (FAD - 100 µMol/L, FMN - 100 µMol/L, NADPH-100 µMol/L), in the presence or absence of NOS inhibitor L-NAME (1 mMol/L) followed by 4 hours incubation at 37^{o}C with 0.75 µl of ubiquitously labeled 14C L-arginine (1.85 MBq/mL, Amersham Biosciences UK Ltd., Chalfont St. Giles, UK) added to each tube. 60µl of 10% TCA was added in the next step to deproteinate samples followed by centrifugation at 13,000 rpm for 20 minutes at 4^{o}C. Supernatant (220 µl) was collected and added to a mixture of 450 µl of milli-Q water and 100 µl of 10% TCA in a plastic vial placed subsequently into the HPLC auto sampler rack cooled at 4^{o}C for chromatographic analysis. Recorded data were analyzed using Azure software package (Datalys, France). L- NAME inhibitable fraction of L-Citrulline as a percentage of total L-arginine activity was expressed as NOS activity.




B
Raja_NOS activity 4 - Channel 2
750
500
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mV
A- Urea
B-Citrulline
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D-Arginine
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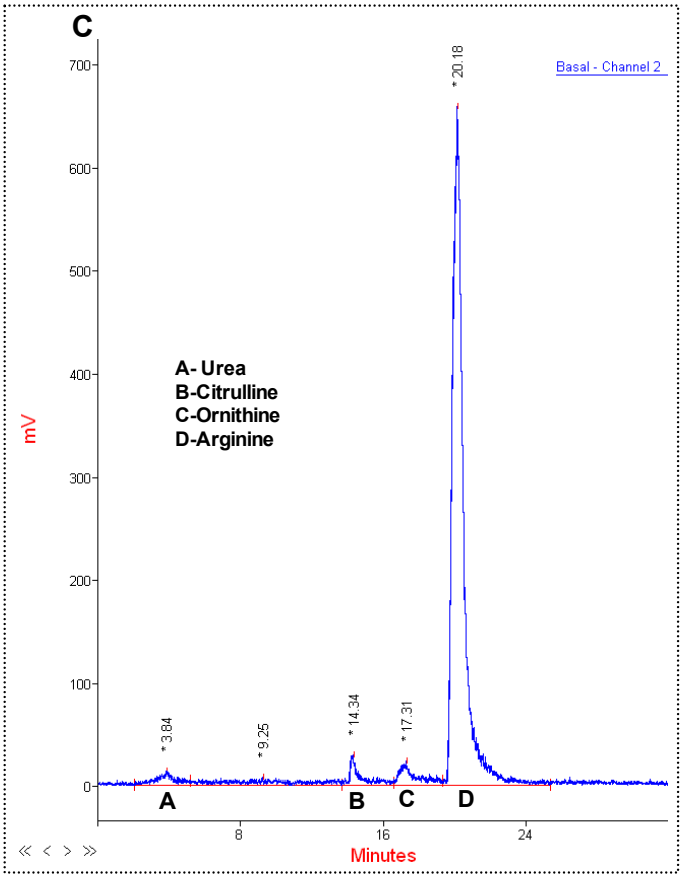
C
Basal - Channel 2
700
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300
200
100
0
mV
A- Urea
B-Citrulline
C-Ornithine
D-Arginine
* 20.18
* 3.84
* 9.25
* 14.34
* 17.31
A
B
C
D
8
16
24
Minutes

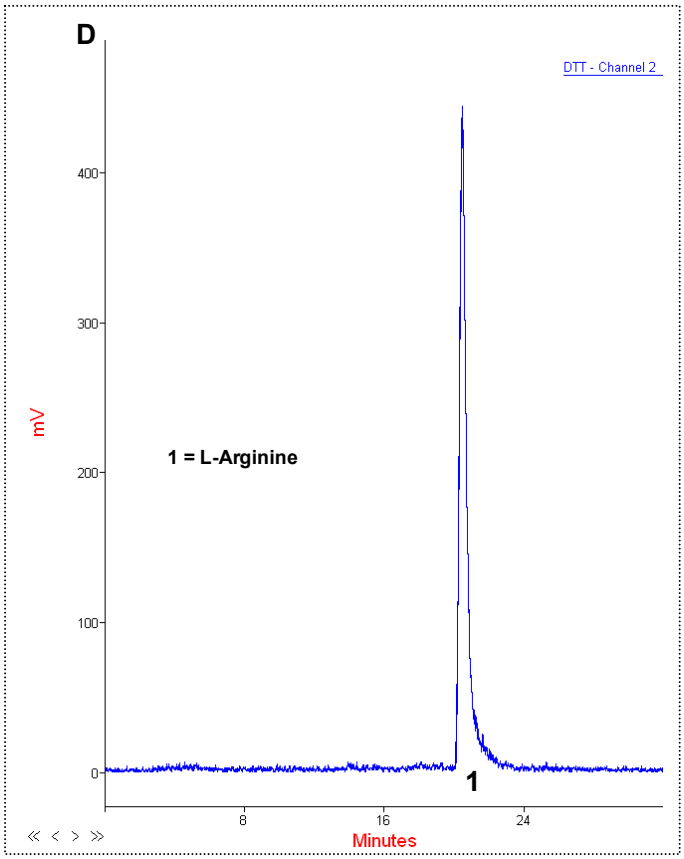


Figure 2.7: A: HPLC set up for measuring nitric oxide synthase (NOS) activity by real time radiochemical detection of 14^{C} labeled L- arginine to L-citrulline conversion **B:** Representative peaks of known standards of 14^{C} labeled Urea, Citrulline, Ornithine and Arginine showing elution time points **C:** Representative chromatogram of human atrial homogenate processed as per the protocol described with peaks eluting as in **'B'** thereby confirming identity of the peaks **D:** Representative chromatogram of dithiothreitol (DTT; 100 µMol/L used in experiments to reverse *S*-glutathionylation) in KHB buffer with 14^{C} arginine demonstrating that it is not eluted during the assay and hence has no confounding effects on identity and measurements of 14^{C} L citrulline peaks.

Detection of protein expression using immunoblotting

Atrial tissue samples (20-30 mg) were homogenized for 30 seconds using a pre cooled electric homogenizer (Polytron®) in 300 µl of KHB containing a protease inhibitor cocktail (1 tablet in 10 mL of lysis buffer; Complete, Mini, EDTA-free, Roche, UK). Homogenates were spun at 13,000 rpm for 10 minutes at 4^{o}C and the protein concentration of the supernatant was determined by BCA assay as described previously. NuPAGE® LDS Sample Buffer, NuPAGE® Reducing Agent (Invitrogen, UK) and urea lysis buffer were added to each sample incubated on ice for 30 minutes followed by heating at 95^{o}C for 5 minutes. 40µg protein per sample was aliquoted into labeled eppendorf and was frozen at -80^{o}C until analysis. On the day of gel electrophoresis, 20-40 µg of protein were resolved by SDS-PAGE using NuPAGE® Novex 4-12% Bis-Tris Gels and NuPAGE® MOPS SDS Running Buffer (Invitrogen, UK). Samples were then transferred onto a nitrocellulose membrane (Amersham hybond ECL) at 77V in cold room for 180 minutes. In the next step, membrane was blocked for an hour in room temperature using 5% non-fat milk in PBS containing 0.1% Tween-20 (PBS-T). Subsequently membranes were incubated with appropriate dilutions of primary antibody (as shown in **table 2.2**) in PBS-T with 5% milk either for 1 hour at room temperature or overnight at 4^{o}C followed by a wash cycle of 15 minutes * 4 with PBS-T. Afterwards washed membrane was incubated with matching secondary antibody (Table 1.2) in PBS-T with 5% milk for 1 hour at room temperature and wash cycle was repeated as above. Finally the HRP signal was visualized by enhanced chemiluminescence (Super signal West Dura Solution, PICO or ECL western blotting system) and imaged using a gel imaging system (Bio-Rad ChemiDoc XRS). The 2D density of the bands was normalised to a housekeeping protein and quantified using the Image J program (NIH).

To re-probe membranes with a different primary antibody, membranes were stripped using Re-blot plus (Millipore Inc) as per manufacturer's instructions followed by blocking in PBS-T with 5% milk and incubated with respective primary antibodies.

Target Protein		Dilution	Species	Source
NOS1	Monoclonal	1/2000	Mouse	Santa Cruz, USA
NOS3	Monoclonal	1/4000	Mouse	Santa Cruz, USA
NOS2	Monoclonal	1/1000 in 5% nonfat milk	Mouse	Millipore, USA
GTPCH	Monoclonal	1/500 in 0.1% BSA	Mouse	Kind gift from Dr.Mark crabtree Abnova, USA
GFRP	Monoclonal	1/250 0.1% BSA	Goat	Kind gift from Drs.Mark crabtree/Anna Starr Cell signalling, USA
NOX-2	Polyclonal	1/1000 in 2.5% nonfat milk	Mouse	BD Biosciences, UK
NOX-4	Monoclonal	1/1000 in 5% milk	Rabbit	AbCam, UK
3-Nitrotyrosine	Monoclonal	1/200	Mouse	Millipore, USA
Beta-Tubulin	Polyclonal	1/1000	Rabbit	Santa Cruz, USA
Alpha-actinin	Monoclonal	1/1000	Mouse	Sigma, UK
GAPDH	Monoclonal	1/5000	Mouse	Sigma, UK
GSH	Monoclonal	1/500	Mouse	Virogen, USA
Goat-anti-mouse, IgG	Polyclonal	1/5000	Secondary	Promega, USA
Goat-anti-rabbit, IgG	Polyclonal	1/5000	Secondary	Promega, USA
Donkey-anti-goat IgG	Polyclonal	1/5000	Secondary	Millipore, USA

Table 2.2 – The target protein, dilution, species type and source of primary and secondary antibodies used for immunoblotting.

Evaluation of loading control for immunoblotting

Background:

During immunoblotting, in order to confirm equal loading of protein of interest across gel lanes, an appropriate loading control that is constitutively expressed in the myocardium is necessary. It is also imperative that its expression should not change as a result of cardiac surgery, cardio pulmonary bypass, or both. To evaluate this further, I investigated GAPDH, Alpha actin and Beta tubulin as loading controls.

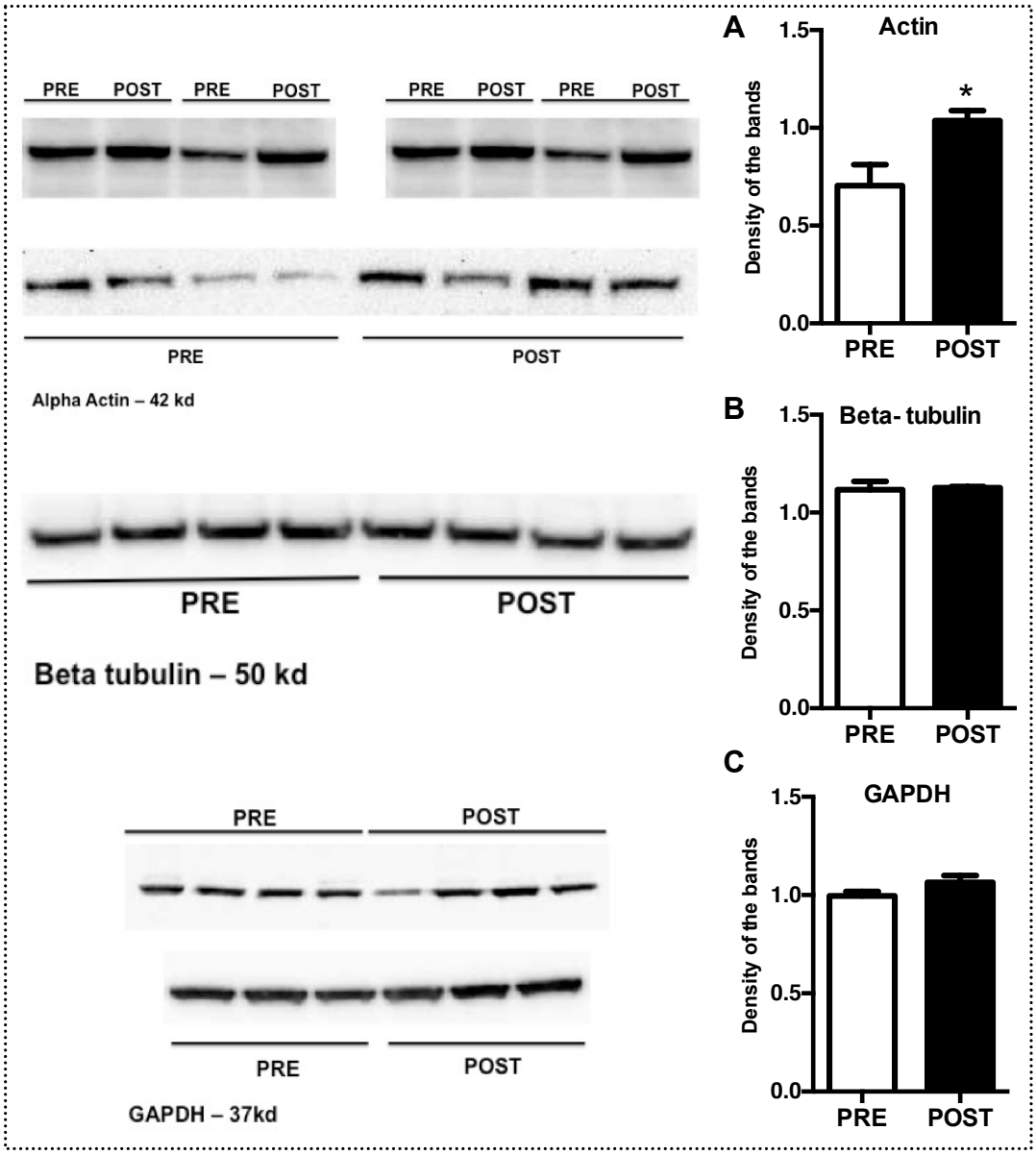


Figure 2.8 – Average protein expressions of Alpha-actin (**A**), Beta tubulin (**B**) and GAPDH (**C**) in homogenates of atrial samples collected before (PRE) and after CPB and reperfusion (POST) and corresponding representative immunoblots. *p < 0.05 vs. PRE by student's t test for paired samples. Data expressed as mean ± SEM.

Results

Protein expression of the multimeric protein complex actin increases (**Figure 2.8 A**) after CPB and reperfusion where as beta tubulin and GAPDH remains unchanged (**Figure 2.8 BC**).

Conclusion

For the evaluation of protein expression, GAPDH and beta tubulin were used as appropriate loading controls.

Quantitative RT-PCR

RNA was extracted from tissue by homogenization on ice in Trizol followed by isopropanol separation of the aqueous phase and clean up of RNA using RNEasy kit (Qiagen). RNA was reverse transcribed into cDNA using Quantitect RT kit (Qiagen) with the cDNA digestion step. Quantitative real-time PCR was then used to compare the expression of the primary transcripts of protein of interest as detailed in respective chapters. All primers and probes spanned two exons.

RT-PCR was performed using an iCycler IQ real-time detection system (Bio-Rad Laboratories, USA) in 96-well optical plates (Applied Bio systems, USA). For each 20µl reaction, 9µl of cDNA (100 ng/µl) was mixed with 10µl TaqMan universal master mix and 1µl of the appropriate TaqMan gene expression assay mix. Plates were cycled as follows: 50°C for 10 minutes, then 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Experiments were performed in triplicate as single-plex reactions. Results were expressed as Δ*Ct*, corresponding to the difference between the *Ct* of the gene of interest and the *Ct* of the gene used to normalize the results (here the GAPDH gene), and as a fold change between paired samples using ΔΔ*Ct.*

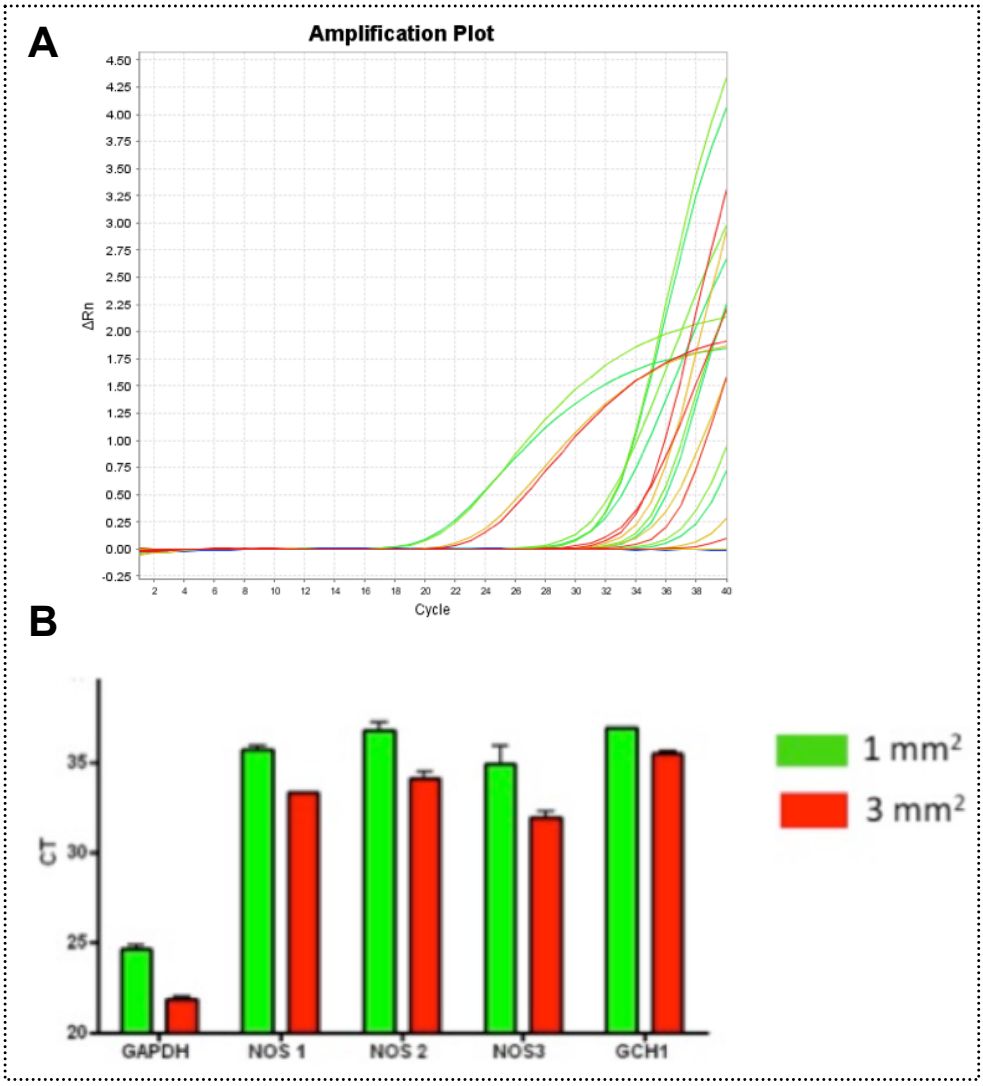


Figure 2.9: Amplification plot of GAPDH, NOS 1-3 and GCH1 genes (**A**) and corresponding threshold cycle values between two sizes of right atrial appendages (**B**) based on which for isolation of RNA in this study, 1mm^2 tissue sample was used.

Section 2

Mechanisms of Myocardial Nitroso-Redox imbalance Following Elective Cardiac Surgery on Cardiopulmonary Bypass

I was responsible for the operational management of the study and laboratory analysis of biological samples.

Between 2010-2011, 116 patients in sinus rhythm undergoing their first elective cardiac surgery for CABG ± aortic valve replacement on CPB were recruited in the Department of Cardiothoracic Surgery of John Radcliffe Hospital in Oxford, UK. The study flow chart, patient information leaflet, consent sheet, and case report form are listed in the appendix.

Inclusion criteria:

- Patient or legal representative is willing and able to give informed consent for participation in the study.
- Male or female, aged 18 years or above.
- Scheduled for the first time elective on- pump cardiac surgery.

Mid and South Buckinghamshire research ethics committee (merged with Berkshire REC in 2011) under the code 07/Q1607/38 provided ethical approval for the handling, storage and use of human tissue.

In all patients general anesthesia was induced by (Drug/total dose range in milligram); Midazolam (1-2 mg), Fentanyl (0.5-1 mg), Thiopentone (125-250 mg), Pancuronium bromide (8-12 mg) and maintained with oxygen in air/isoflurane at an inhaled concentration of 1%–1.5% with propofol infusion while on CPB. Under moderate hypothermia (34°C), CPB flow was non-pulsatile via a membrane oxygenator. For myocardial protection, 1 L of St Thomas's cold (4°C) crystalloid cardioplegia (Martindale Pharmaceuticals, Essex, United Kingdom) was delivered ante grade and repeated every 20 - 30 minutes. Protamine was administered to reverse heparinisation after CPB. Postoperative management was as practiced in the cardiac surgery directorate of the Oxford University Hospitals NHS trust, UK.

(http://www.ouh.nhs.uk/cardiac/surgery/ctccu/default.aspx#).

Sample collection and processing:

Samples of the right atrial appendage were taken at two time points: 1) Prior to the insertion of venous cannula in to the right atrium (PRE) and commencement of cardioplegia 2) Soon after venous decannulation and cardiac reperfusion (POST). Samples were received in ice-cold phosphate buffered saline, washed to clear off blood, blotted dry, cut into smaller pieces after removing fat tissue and transferred into bar-coded cryovials. After snap - freezing in liquid nitrogen, cryovials were stored at -80^{o} C.

In addition to right atrial appendages, blood samples (10 mls per time point fractionated into one EDTA and one serum vacutainer) were collected at baseline and after administration of protamine. Blood samples were processed within one hour of collection (1300g for 10 minutes at room temperature) and plasma/serum aliquots were stored at -80^{o} C.

Validation of the protocol for measuring GTPCH activity in human myocardium

GTPCH activity is measured in our lab in murine myocardial samples by HPLC analysis after iodine oxidation by a protocol described previously[416]. Briefly, paired RAA samples (30-50 mg) are homogenized in ice-cold assay buffer (0.1M Tris HCl, 0.3M KCL, 2.5mM EDTA, 50ml 10% glycerol, pH 7.8, 100 µM phenylmethylsulfonyl fluoride added per 50 ml of buffer). Equal amount of protein are incubated in the dark with 10 mm GTP followed by oxidization with 0.1 m potassium iodide/iodine and deproteination with 1 m HCl. The reaction is stopped by addition of 0.1M ascorbic acid, neutralized with sodium hydroxide and dephosphorylated with 16 units/ml of alkaline phosphatase by incubating samples for 1 h at 37 °C in the dark. Neopterin content is quantified by isocratic HPLC and fluorescence detection (JASCO) with an excitation wavelength of 350 nm and an emission wavelength of 450 nm after loading 100 µl of each sample into the column. Neopterin curve in the sample chromatogram is identified by comparison with external standards and Azure Software version 4.02 is used for

data analysis (Datalys, France). Values are normalized for sample protein content, and the activity of the enzyme is expressed as pmol per milligram of tissue per minute.

However, this protocol has not been tested in human myocardium previously, and it is also not clear whether Neopterin can be generated in human samples. To evaluate further, I validated the fidelity of the current protocol in human atrial homogenates.

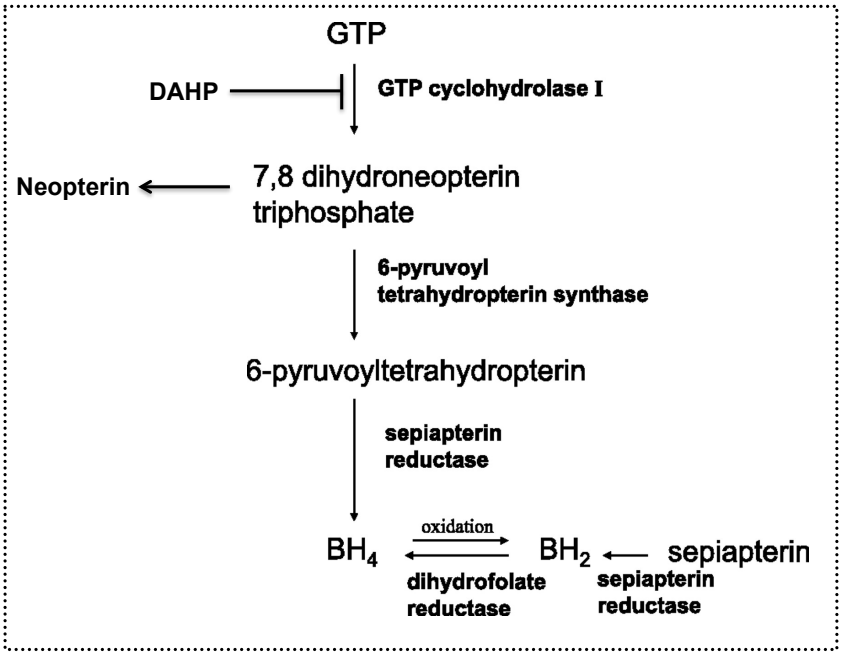


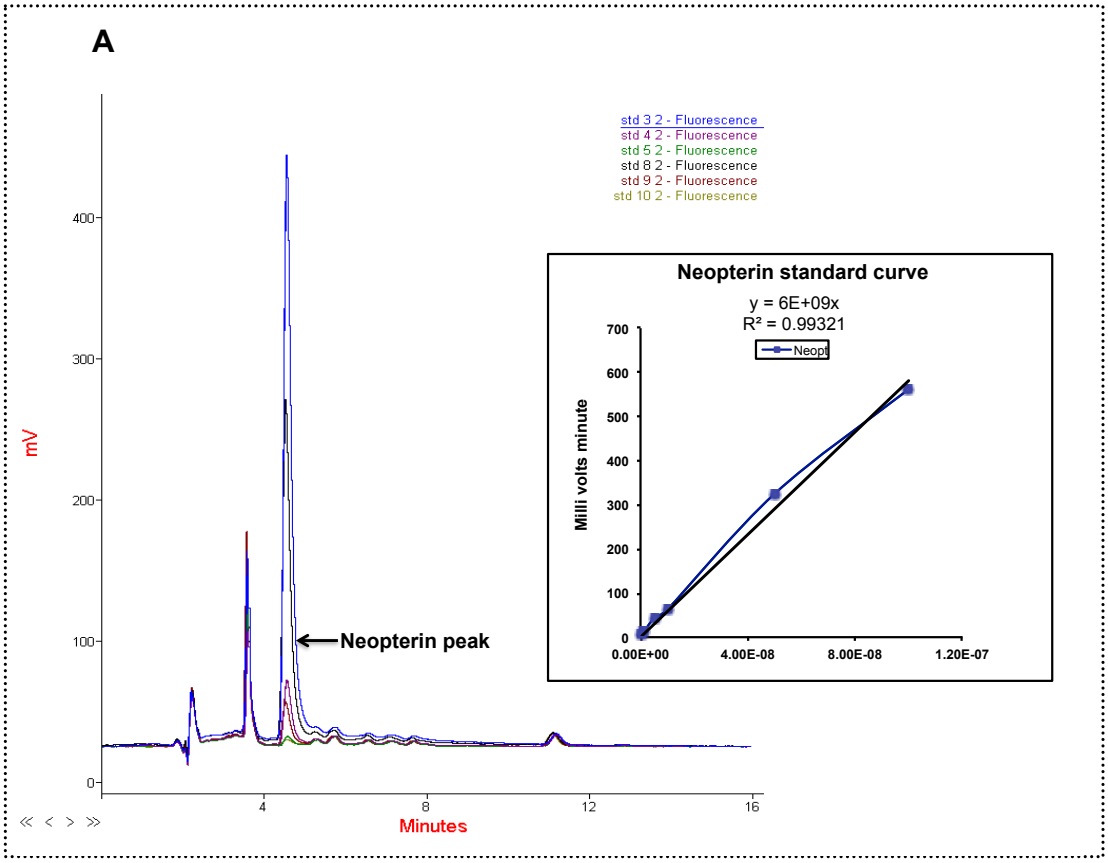
Figure 2.10 - BH4 synthetic pathway demonstrating catalytic function of GTP – cyclohydrolase 1.

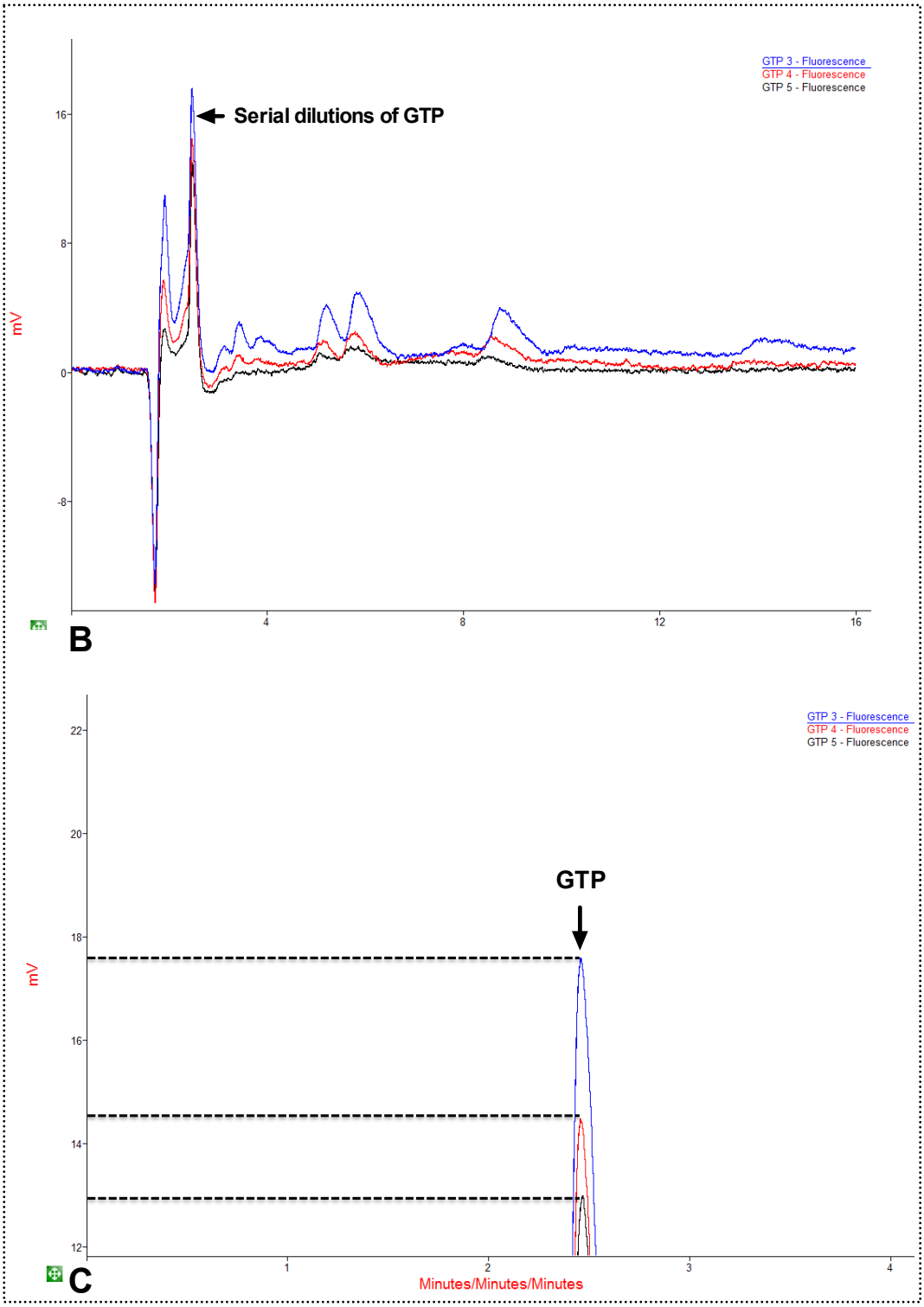
Rationale:

As a rate-limiting enzyme in the BH4 synthetic pathway (Figure **2.10**), GTP cyclohydrolase-1 (GTPCH-1) converts GTP into 7-8 dihydro Neopterin (7-8DN). In the protocol described earlier, in a series of subsequent chemical reactions, 7-8DN is oxidized to Neopterin that is detected and quantified by reverse phase HPLC. 2,4-diamino-6-hydroxypyrimidine (DAHP) is the prototype inhibitor of GTPCH [417]. Hence an approach based on pharmacological inhibition of GTPCH and measurement of Neopterin content in the right atrial homogenates as well as end products of the biopterin synthetic pathway will inform the presence, activity of the enzyme and fidelity of current protocol in the human myocardium.

Aim 1: To establish the correlation of peaks of authentic Neopterin standards with peaks from murine wild type left ventricle (LV), GCH-1 transgenic LV (GCH-1TG) and human right atrial appendage homogenates.

Results: Peak eluting after 4 minutes in figure **2.11A** represents Neopterin evidenced by the reducing trends in peak areas that correspond to serial dilutions of authentic Neopterin standards. The preceding peak is generated by elution of GTP as shown in figure **2.11B** with serial dilutions of GTP matching corresponding trends in the height and area under the peaks (Figure **2.11C**). In the absence of Neopterin, there were no peaks eluting between 4 and 5 minutes as shown in the GTP chromatogram (Figure **2.11D**). The Neopterin peak also superimposes over a set of sample peaks from all the three species types (Figure **2.11E**).




GTP 3 - Fluorescence
GTP 4 - Fluorescence
GTP 5 - Fluorescence
Serial dilutions of GTP
mV
16
8
0
-8
4
8
12
16
B
GTP 3 - Fluorescence
GTP 4 - Fluorescence
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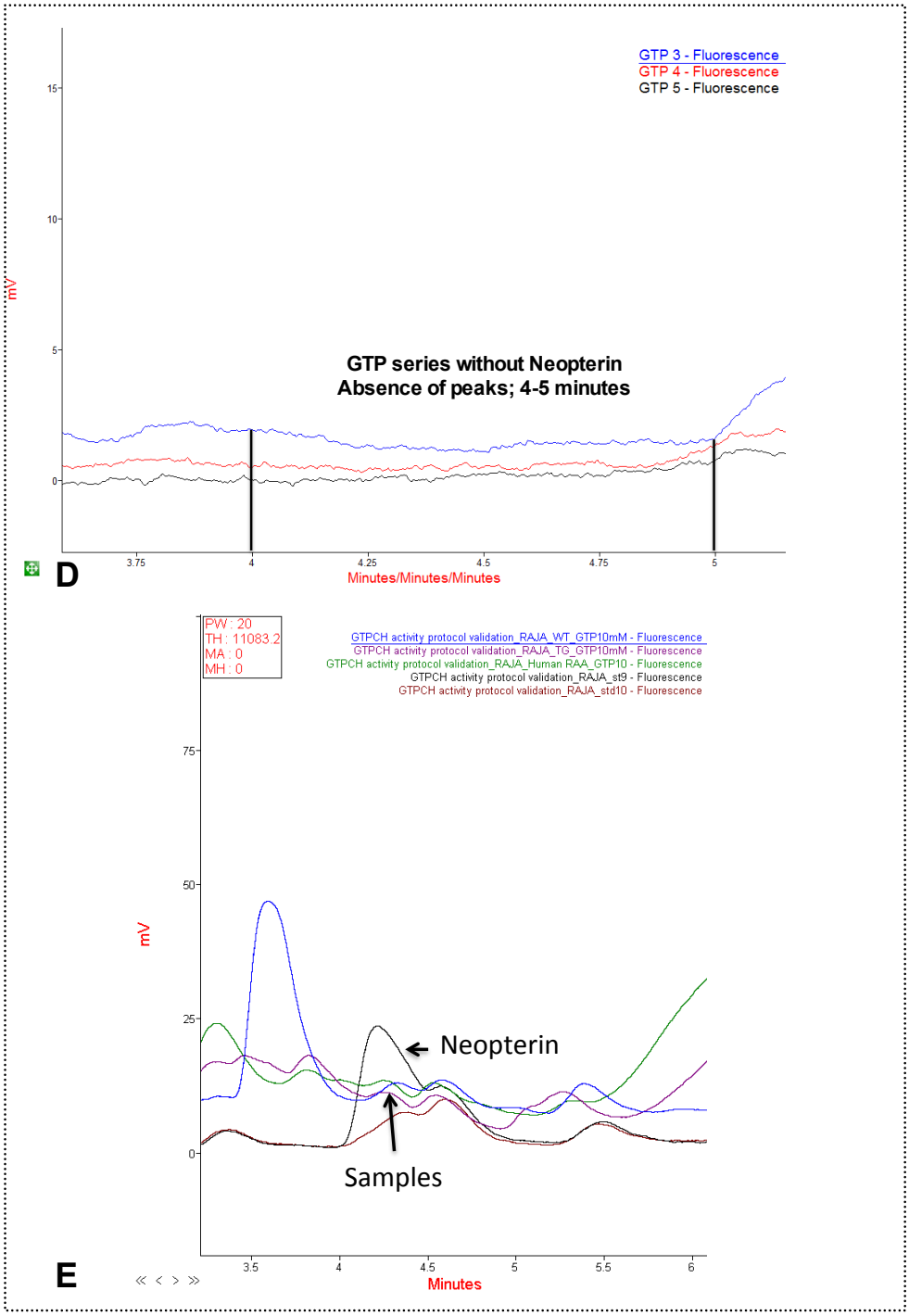


Figure 2.11 – Confirmation of Neopterin peaks.

A - Chromatogram of serial dilutions of authentic Neopterin standards and corresponding scatter plot **B, C** – Serial dilutions of authentic GTP (G8877; Sigma, UK) showing corresponding peaks eluting before 4 minutes **D** – Absence of peaks between four and five minutes in the GTP chromatogram **E** – Authentic Neopterin standards superimposed over the peaks eluting between four and five minutes in the samples.

Conclusion:

Peak eluting after 4 minutes represents Neopterin in human atrial homogenates.

Aim 2: To measure Neopterin content and corresponding GTPCH activity before and after DAHP (10mmol/L – dose based on previous published studies): GCH-1 Tg vs. WT Murine LV vs. Human RAA

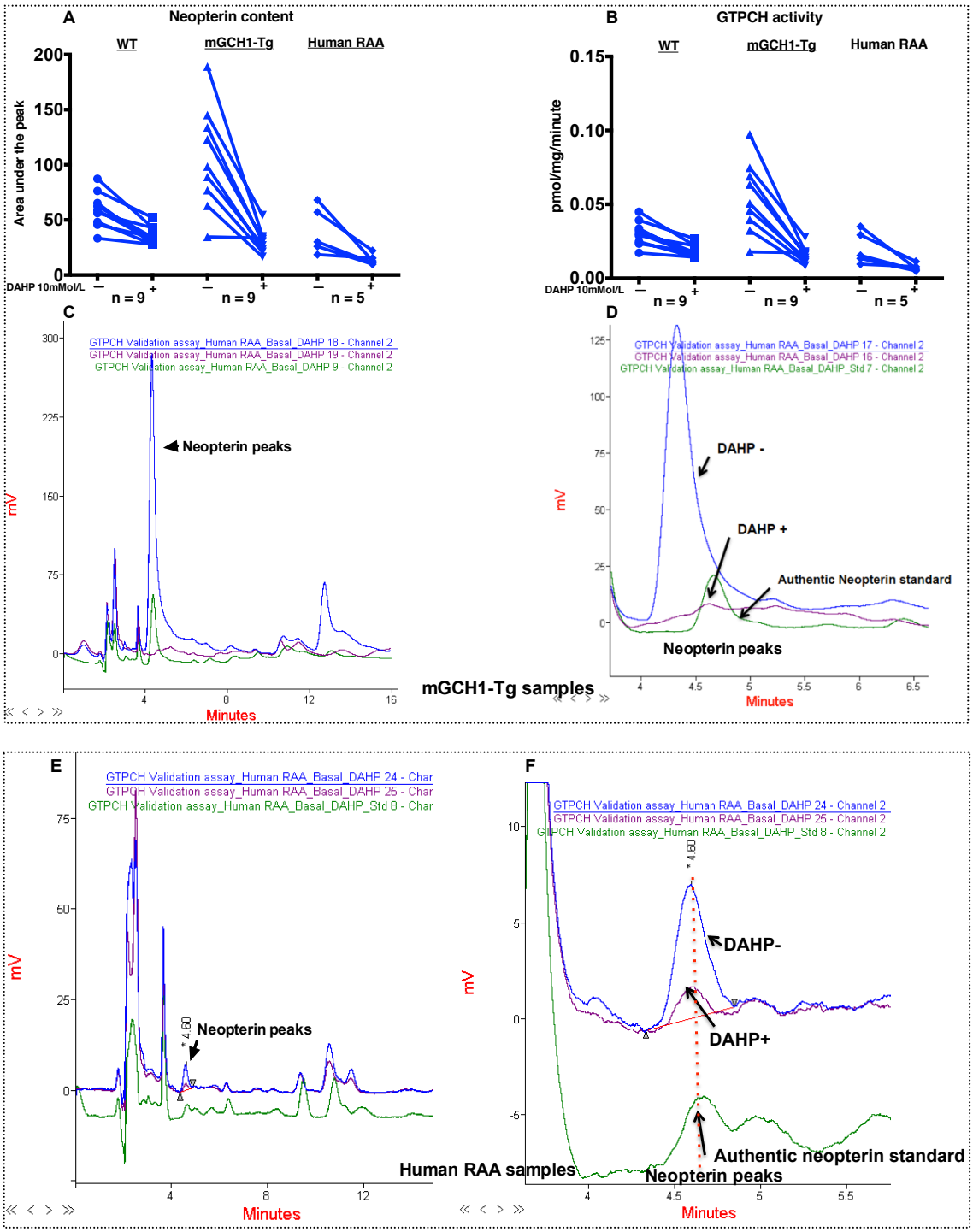


Figure 2.12: Confirmation of GTPCH-1 activity.

A) Samples incubated with DAHP 10mmol/L (Incubation time 1hr) demonstrates reduction in Neopterin peak area confirming inhibition of GTPCH-1 activity.

B) Reduction in GTPCH-1 activity after treatment of samples with DAHP 10mmol/L (Incubation time 1hr).

C and D) Representative chromatograms – mGCH-1Tg samples showing reduction in Neopterin peak areas (Identity of the peak confirmed by superimposition of authentic Neopterin peak) after treatment of samples with DAHP 10mmol/L confirming inhibition of GTPCH-1 activity.

E and F) Representative chromatograms – Human atrial homogenates showing reduction in Neopterin peak areas (Identity of the peak confirmed by superimposition of authentic Neopterin peak) after treatment of samples with DAHP 10mmol/L confirming inhibition of GTPCH-1 activity.

Result: Reduction in the Neopterin content of all sample types after DAHP confirms inhibition of GTPCH activity.

Conclusion: These results clearly confirm the fidelity of the current protocol for measuring GTPCH activity in human atrial homogenates.

Aim 3: To measure BH4 and BH2 content before and after DAHP (10mM/L): GCH-1 Tg vs. WT Murine LV vs. Human RAA

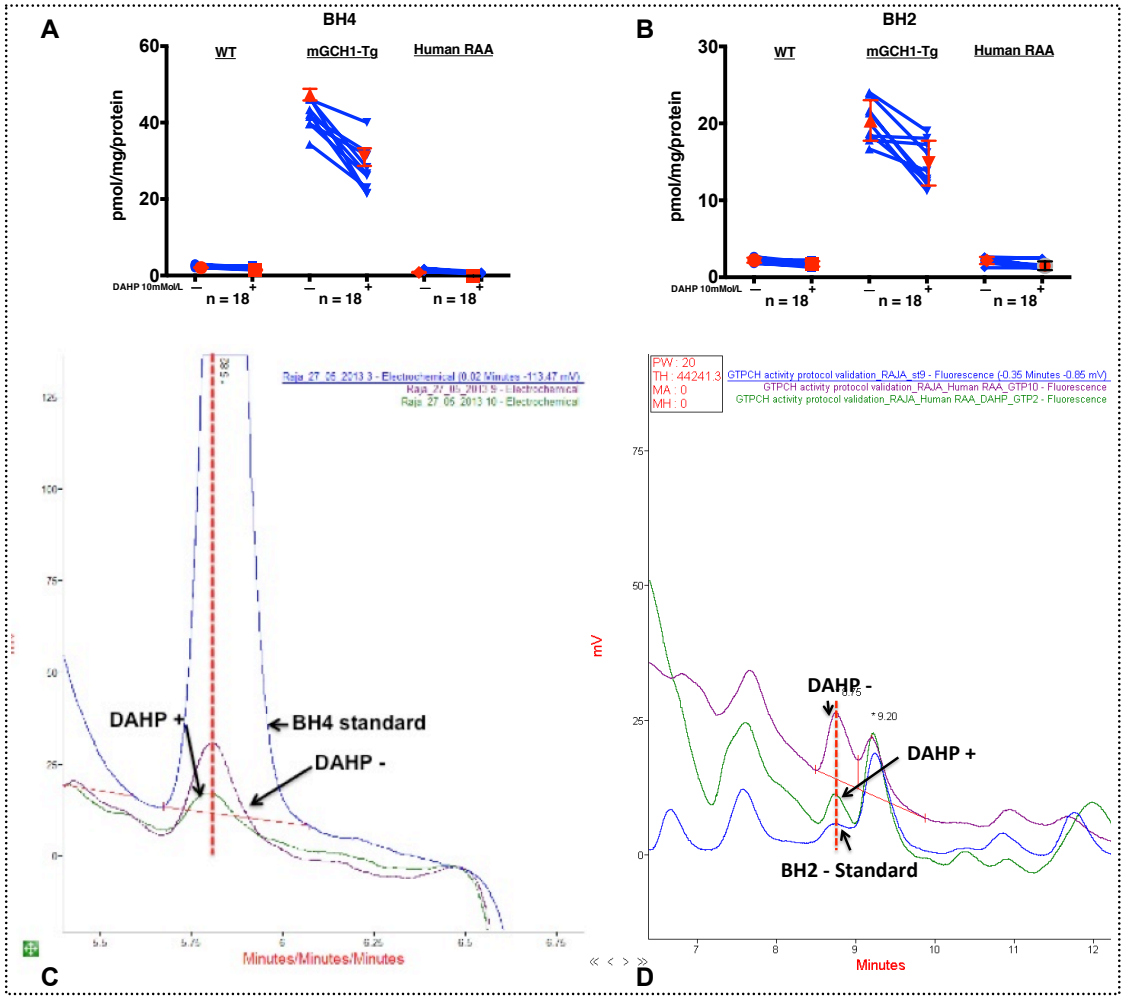


Figure 2.13: Correlation of GTPCH-1 activity and synthesis of biopterins.

A and B) Treatment of wild type murine LV, mGCH transgenic LV and human atrial homogenates with DAHP 10mmol/L (Incubation time 1hr) reduces BH4 and BH2 content.

C and D) Representative chromatograms from measurements in human atrial homogenates.

Result: Inhibition of GTPCH by DAHP results in decrease in the human atrial content of BH4 and BH2.

Conclusion: These results provide further evidence supporting the fidelity of the protocol to measure the activity of GTPCH-1 in human atrial homogenates.

Development and validation of the protocol for measuring superoxide production from NADPH Oxidase in atrial homogenates

Background: gp 91 ds - tat and its scrambled peptide (as a negative control) have not been previously tested for measuring superoxide production from NADPH oxidase in human atrial homogenates.

Objective: To establish the optimum **dose** and **incubation period** of the peptides for measuring superoxide production from NADPH Oxidase in human atrial homogenates.

Aim 1: To evaluate the optimum incubation period of gp 91 ds- tat peptide (10 µM/L; Dose as published previously in murine myocardium[418]) for inhibition of NADPH Oxidase.

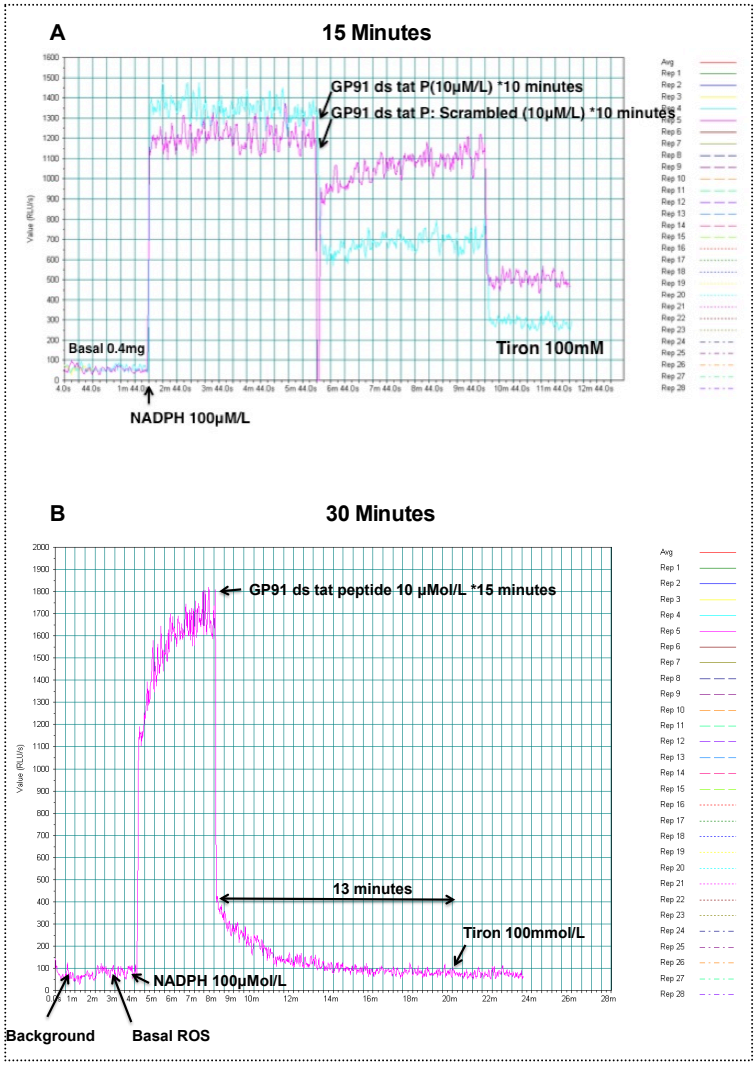


Figure 2.14: Lucigenin enhanced chemiluminescence following fifteen and thirty minutes treatment of human atrial homogenates with gp 91 ds- tat peptide (10 μM/L).

A) Representative luminometry trace showing partial inhibition of activity of NADPH oxidase (n = 4-6 samples).

B) Representative luminometry trace showing complete inhibition of activity of NADPH Oxidase at 30 minutes (n= 4 samples).

Results-1:

Treatment of human atrial homogenates with gp 91 ds tat peptide for 15 minutes (Figure **2.14A**), only partially reduced NADPH stimulated superoxide production whereas incubation period of 30 minutes resulted in complete inhibition (Figure **2.14B**). Addition of Tiron after 30 minutes reduced the signal further indicating ongoing superoxide production from other enzymatic sources.

Aim 2: To evaluate superoxide production from NADPH Oxidase in human atrial homogenates using gp 91 ds tat P and gp 91 ds tat P- scrambled over 10 -100 μM/L with an incubation period of 30 minutes**.**

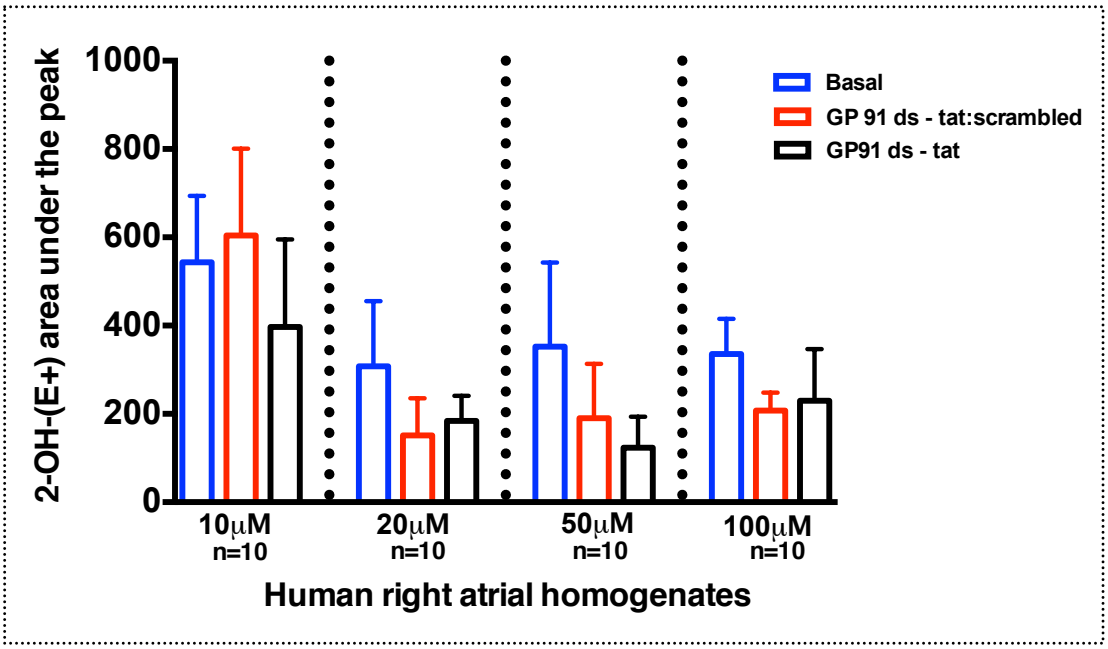


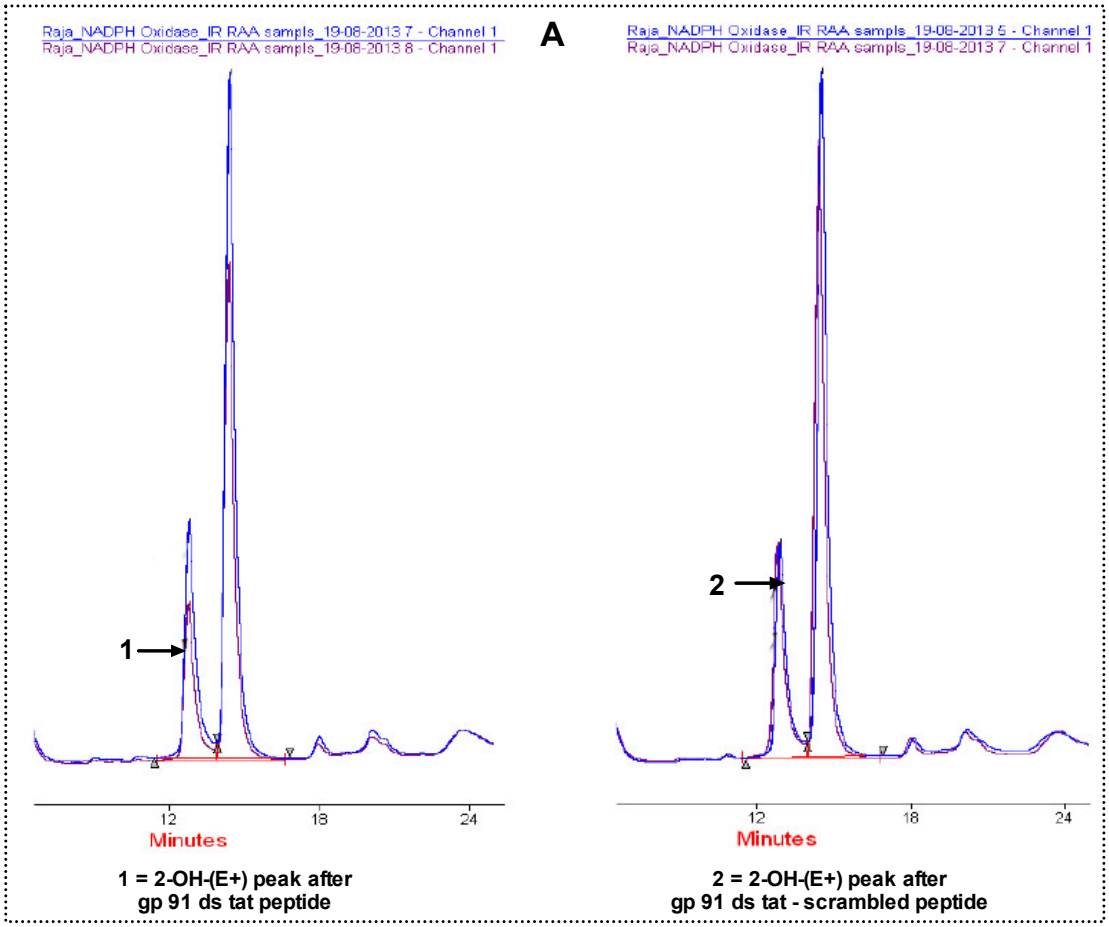
Figure 2.15: 2-OH- (E+) detected by HPLC after treatment of atrial homogenates with gp 91 ds tat and scrambled peptide (10-100 μM/L) for 30 minutes.

Results-2:

gp 91 ds tat peptide inhibited superoxide production from atrial homogenates at 10 µM/L as opposed to gp 91 ds tat scrambled P.

Conclusions:

Taken together, gp 91 ds-tat peptide and its scrambled sequence were used at **10 µM/L** with an incubation period of **30 minutes** for evaluating superoxide production from NADPH oxidase in human atrial homogenates. Accordingly, representative chromatograms of 2-OH- (E+) detected by HPLC and lucigenin enhanced chemiluminescence are shown (Figure **2.16**).



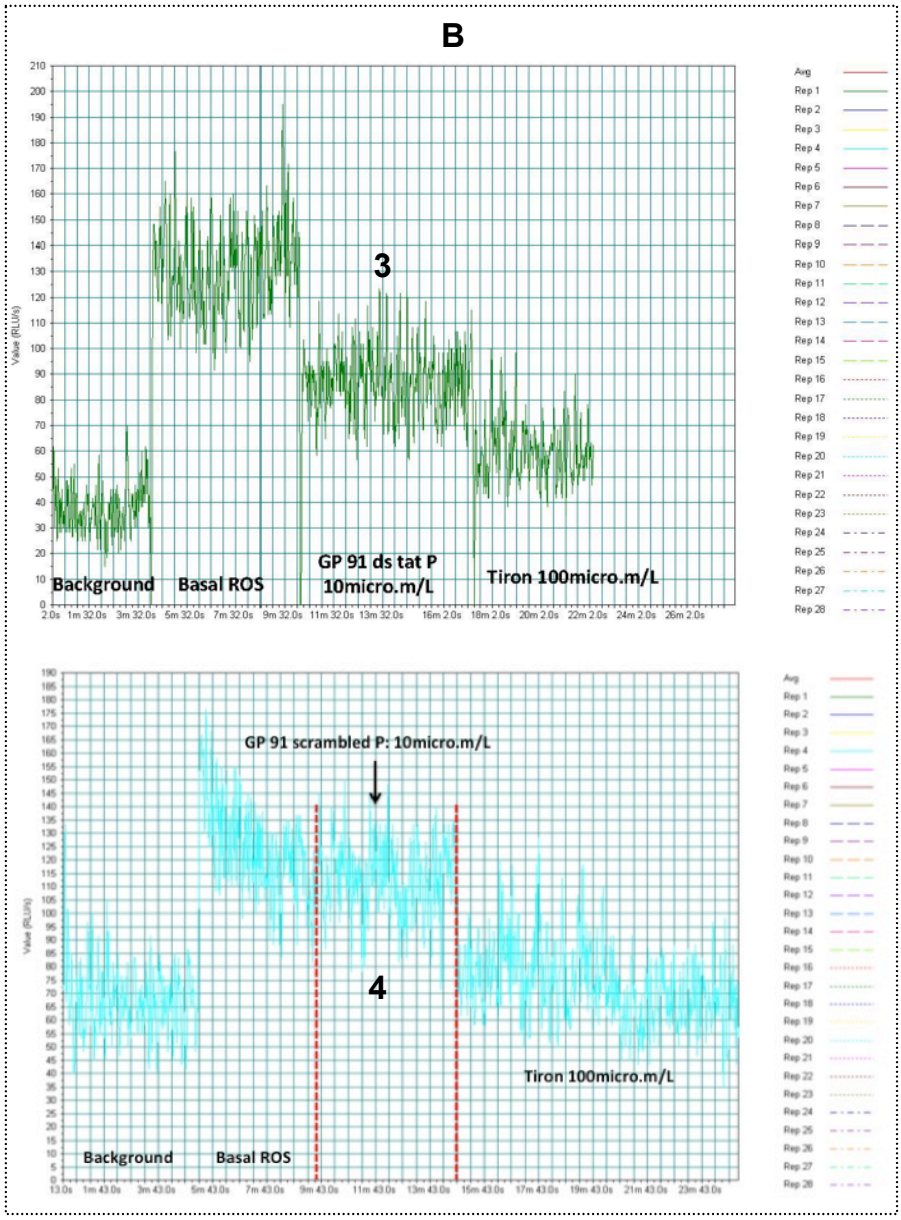


Figure 2.16: Representative chromatograms of 2 - OH+ (E+) detection (**A**) and lucigenin-enhanced chemiluminescence (**B**) following treatment of atrial homogenates with gp 91 ds tat and scrambled peptide (10 μM/L). In both the methods, gp 91 ds tat P is reducing superoxide production as opposed to the effects of its scrambled peptide (1 vs. 2 and 3 vs. 4) thereby demonstrating that the effects are secondary to inhibition of NADPH Oxidase.

Detection of protein s-glutathionylation by immunoprecipitation followed by immunoblotting

S-Glutathionylation is a post-translational modification of protein cysteine residues by reversible addition of glutathione and has been described as a potential mechanism for functional uncoupling of eNOS[26]. To evaluate this further in human atrial homogenates, samples were prepared in mammalian tissue lysis buffer (Cell LyticTM Sigma) with N-ethyl maleimide and a mixture of protease inhibitors (Roche Applied Science). For immunoprecipitation, in the presence (to remove the glutathionylation modification of target protein) or absence of DTT (100 µmol/L) in 1 mg protein/samples, eNOS was pulled down with agarose-conjugated anti-NOS3 antibody (Santa Cruz sc-654P). Immunoblotting was carried out as described in section 1- general methods with anti-glutathione monoclonal antibody (ViroGen; 1:1000) and anti-eNOS (Santa Cruz; 1:1000) antibodies. Final results were expressed as a ratio of glutathionylated eNOS3 to total eNOS. For probing glutathionylation of nNOS, in the absence of commercially available agarose conjugated antibodies, primary nNOS antibody (Santa Cruz: sc 5302) was incubated with agarose beads (Protein A/G plus; Santa cruz; sc 2003) for 24 hrs and rest of the sequence was performed as described for eNOS.

Rac1 activation assay

Rac1 is a small G –protein regulating activation of NADPH Oxidase[419] and superoxide generation from the enzyme complex. Rac1 activity, defined by the ratio of GTP-Rac1 to total Rac1, was evaluated by a commercially available affinity precipitation assay with p21-activated kinase (PAK)-1 fusion protein conjugated glutathione agarose beads (PAK1-PBD) according to the manufacturer's protocol (Millipore, Temecula, California, USA).

Principle:

This assay uses the downstream effector of Rac/Cdc42, p21-activated protein kinase (PAK1), to isolate the active GTP-bound form of Rac/Cdc42 from the sample. The p21-binding domain (PBD) of PAK1 is expressed as a GST-fusion protein and coupled to agarose beads. After precipitation, an immunoblot is

performed and the activated Rac/Cdc42 is detected with specific monoclonal antibodies, followed by HRP-conjugated secondary antibody and ECL reagent detection.

Process:

Right atrial tissue samples were homogenized in magnesium-containing lysis buffer provided by the manufacturer (125 mM HEPES, pH 7.5, 750 mM NaCl, 5% Igepal CA-630, 50 mM MgCl2, 5 mM EDTA and 10% glycerol) and centrifuged at 13,000g for 5 min at 4°C. Supernatant with equal amounts of protein were incubated with 10 µl of agarose labelled Rac/cdc42 Assay Reagent (PAK-1 PBD, agarose) at 4°C for 60 min. Beads were collected by centrifugation (14,000xg) and were washed 3 times with ice cold magnesium-containing lysis buffer to remove inactive Rac1. In the next step, beads were suspended in 40 µL of 4X NuPAGE® LDS Sample Buffer (Life technologies; Invitrogen division, UK) and were boiled for 5 minutes. In the final step, beads were collected by centrifugation and probed for bound Rac1 by immunoblotting using manufacturer supplied Anti Rac-1 antibodies (Protein G purified mouse IgG2b) as described in section 1. Total rac -1 in the samples (Same protein content as used in immunoprecipitation) was probed by immunoblotting and final result expressed as a ratio of bound rac-1 to total rac-1.

Section 3

STARR (Statin Therapy in Atrial Refractoriness and Reperfusion Injury)

Prevention of atrial oxidative stress and electrical remodelling in patients undergoing cardiac surgery: randomised placebo-controlled trial of perioperative atorvastatin (80 mg od). (Clinicaltrials.gov Identifier: NCT01780740).

Trial design:

The STARR trial was a randomised double blind comparison of atorvastatin (80 mg od) vs. placebo in the perioperative period (started from up to 6 days before surgery until the 5th post-operative day) in 80 patients undergoing cardiac surgery by a mixed randomisation approach. This method allocated treatments based on a pre-prepared sequence (of length 100). The sequence consisted of a deliberately uneven block of size 10 followed by random permuted blocks of size 8, 10 or 12 (three of each). Participants were randomised up to a maximum of 12 days. Drugs were obtained from Pfizer (UK) and packaged by Catalent Pharma Solutions (UK). I worked closely with the staff at Catalent to expedite quality control certification and release of the study medications in accordance with good clinical practice (GCP) guidelines. The medication bottles were sequentially numbered according to a randomisation schedule generated at the clinical trials services unit (CTSU), University of Oxford.

I was responsible for the operational management of the clinical trial and laboratory analysis of biological samples. South central Berkshire research ethics committee under the code 10/H0505/35 provided ethical approval for the clinical trial of an investigational medicinal product (CTIMP) including handling, storage and use of blood samples and human tissue. Clinical trial authorisation (CTA) was obtained from the “Medicines and Healthcare products Regulatory Agency (MHRA)” with Eudra CT ref no: 2009-013228-21. The study flow chart, patient information leaflet, consent sheet, and case report form are listed in the appendix.

Trial Participants:

Adult patients in sinus rhythm undergoing their first elective cardiac surgery (coronary bypass surgery and/or aortic valve replacement) on cardio pulmonary

bypass in the Dept. of Cardiothoracic surgery at John Radcliffe hospital, Oxford, UK.

Sample collection and processing:

Right atrial appendage was sampled at two time points: at the time of venous cannulation prior to the commencement of ischemic cardioplegic arrest (PRE) and soon after reperfusion (POST). RAA samples were received in ice cold phosphate buffered saline (PBS), washed three times to clear off blood, dried using blotting paper, cut into smaller pieces after removing the fat tissue and stored in bar coded cryovials in -80^{o} C until analysis.

In addition to RAA, blood samples (maximum of 10 mls per time point fractionated into one EDTA and one serum vacutainer) were collected at following time points.

- Baseline: Prior to the commencement of the study medications.
- Postoperative day 3.
- Postoperative day 5.

Blood samples were processed within one hour of collection (1300g for 10 minutes at room temperature) and plasma/serum aliquots in bar coded cryovials were stored at -80^{o} C.

Primary Endpoints:

- Post-operative right atrial effective refractory period (AERP).
- Production of reactive oxygen species (ROS) in the PRE and POST right atrial appendages (RAA).

Secondary Endpoints:

- Nitric oxide synthase activity in PRE/POST RAA
- Relative contribution of NADPH Oxidase and dysfunctional nitric oxide synthase to ROS release in PRE/POST RAA.
- Markers of oxidative stress and inflammation in PRE/POST RAA.

Inclusion criteria:

- Participant is willing and able to give informed consent.
- Male or Female, aged 18 years or above.
- Requiring elective cardiac surgery.
- Able (in the Investigators' opinion) and willing to comply with all study requirements.
- Willing to allow his or her General Practitioner and consultant, if appropriate, to be notified of participation in the study.

Exclusion criteria:

- Age>85yrs
- Pregnancy, lactation or planning pregnancy during the course of the study
- Women of childbearing potential without appropriate contraceptive measures.
- History of obstructive hepato biliary disease or other serious hepatic disease or pre-operative ALT >2-fold the upper limit of normal or alcohol abuse
- Creatinine >200 umol/L
- Untreated hypothyroidism
- Family history of hereditary muscle disorders
- Known intolerance to statins or history of muscle toxicity with fibrates or statins.
- Ongoing use of fibrates, niacin or of agents that are strong inhibitors of cytochrome P-450 or the P-glycoprotein within a month preceding randomization (cyclosporine, azole antifungals, such as itraconazole and ketoconazole, macrolide antibiotics, such as erythromycin and clarithromycin, protease inhibitors, nefazodone, verapamil, Amiodarone or large quantity of grapefruit juice (≥ 1L/day)
- Patients on treatment with antiarrhythmic agents, other than beta-adrenergic receptor blockers.

Atrial effective refractory period (ERP) measurements:

The right atrial effective refractory period (AERP) was measured daily up to post-operative day 5 (POD5). Prior to the closure of median sternotomy, one pair of epicardial pacing leads was inserted to the right atrial surface separated by few centimeters as a routine by attending cardiac surgeons (Figure **2.17A**). For delivering the programmed stimulation protocol for assessing atrial refractoriness, the epicardial leads were connected to a Medtronic pacemaker (Sensia: SESR01) using a lead adaptor kit (Medtronic: 5866-24M) and interrogated by a Medtronic pacing system programmer (PSA: 2090) via its programming head (Figure **2.17 B-C-D**).

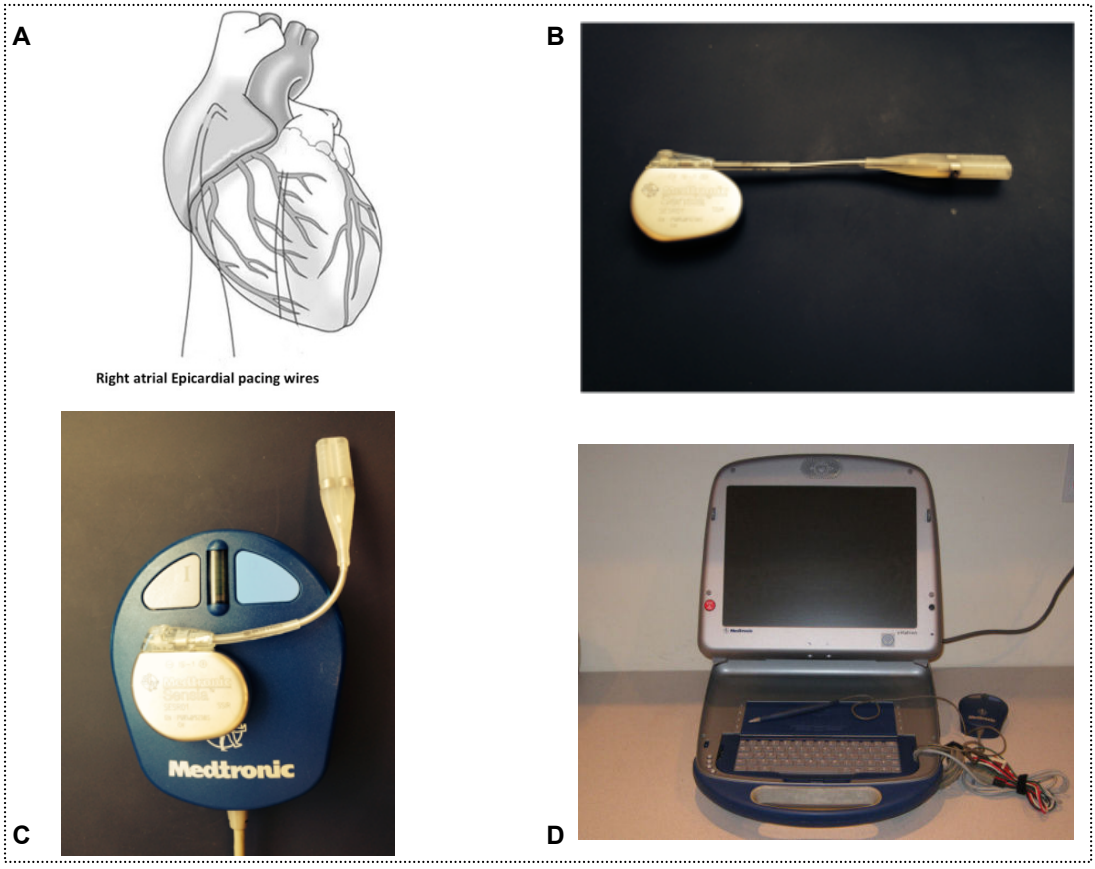


Figure-2.17: **A:** Diagram demonstrating the location of right atrial epicardial pacing wires **B:** Medtronic pacemaker connected to a lead adaptor **C:** Pacemaker on the programming head **D:** Medtronic pacing system programmer.

Protocol:

A conditioning train of 100 stimuli (S1) was delivered at 2 x diastolic threshold followed by continuous pacing over three pacing cycle length of 500ms, 600ms and 700 ms. An atrial extra stimulus (S2) was introduced after every eighth S1 with an initial coupling of 156 ms and with no pauses in the drive train. The coupling interval of the extra stimulus was increased in steps of 16 ms until atrial capture was achieved, and continued for another 3 coupling intervals. The atrial ERP was defined as the longest S1-S2 coupling interval that failed to result in atrial capture.

Measurements were performed in duplicates for the interval that resulted in atrial capture as well as for the interval before and after this measurement. Atrial fibrillation (AF) induced by the protocol that lasted >5 min was considered as sustained, and when this occurred, protocol was terminated and clinical management instituted as appropriate. A continuous 12 lead ECG was acquired throughout the protocol to demonstrate atrial capture and to measure AERP accordingly. ECG paper was run at 50 mm/second with twice the normal gain in order to augment ‘p’ wave amplitude.

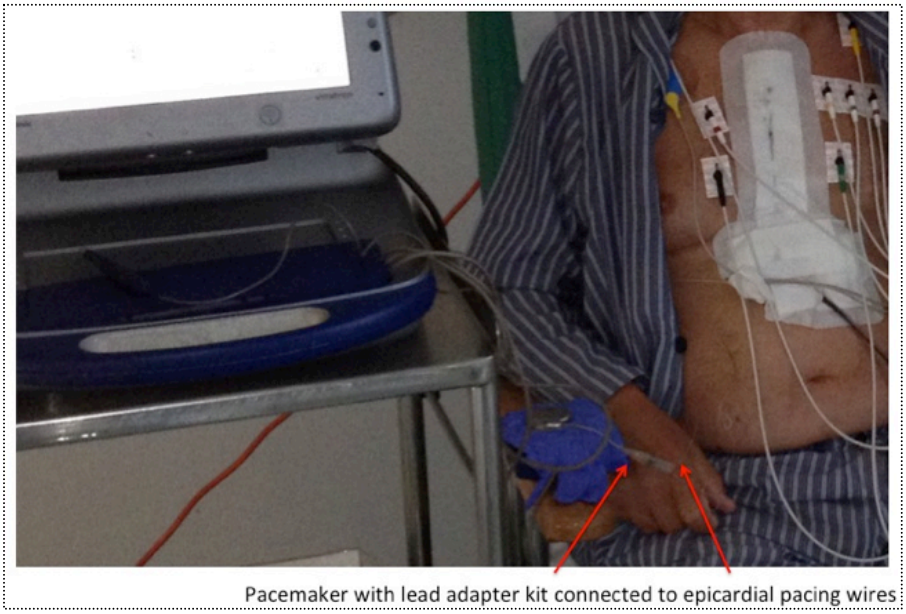


Figure 2.18: A and B - A study participant undergoing electrophysiology programmed stimulation protocol for measurement of right atrial refractoriness in the postoperative period after undergoing elective cardiac surgery on CPB.

Analysis:

Atrial capture was defined as either of the following;

- Presence of clearly visible ‘p’ wave on the continuous ECG after S2.
- A sinus cycle length of the first return intrinsic beat after S2.

In addition to my analysis, a consultant cardiologist with subspecialty experience in clinical electrophysiology analyzed the ECG recordings independently. In the event of any discrepancy in AERP measurements between the two investigators, recordings were referred to a third cardiologist with clinical electrophysiology experience for the final report.

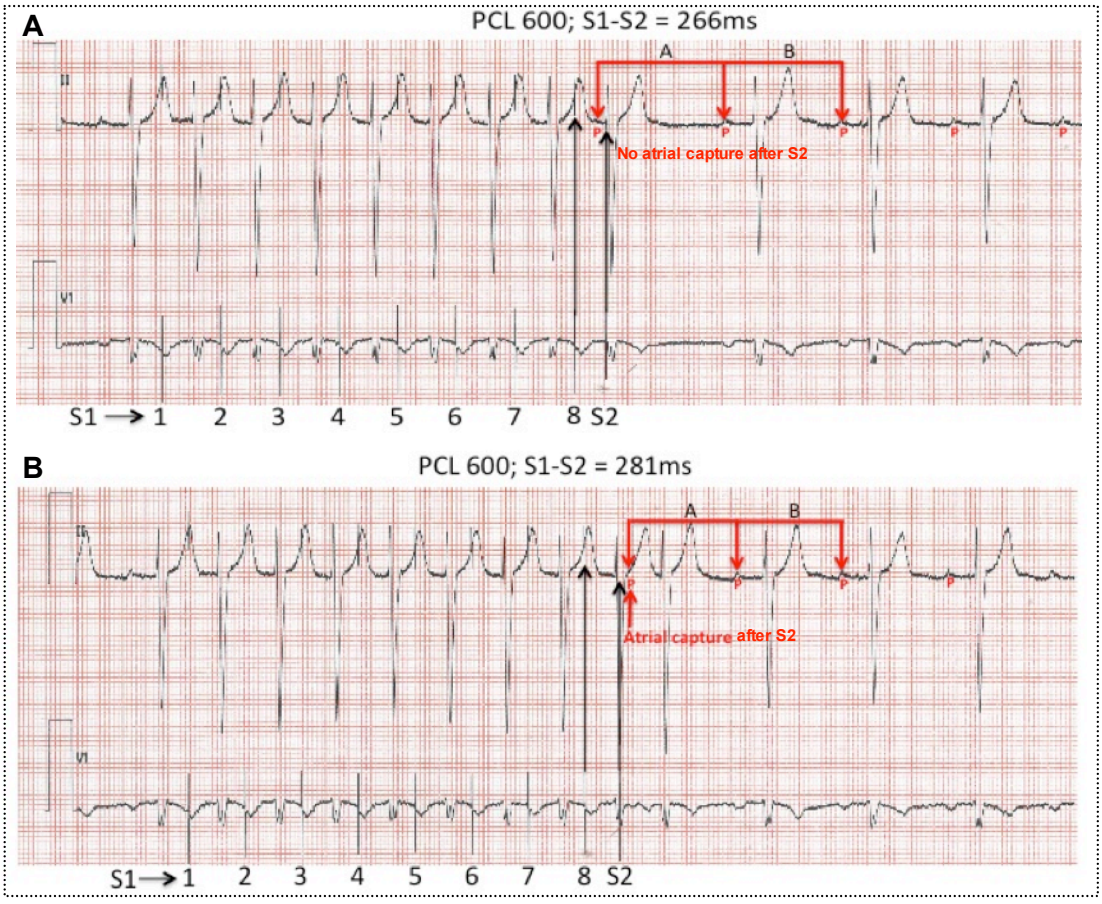


Figure – 2.19

A: A representative ECG recording obtained during programmed stimulation at 600ms pacing cycle length (PCL) with S1-S2 coupling interval of 266 ms showing failure of atrial capture following S2.

B: A representative ECG recording obtained during programmed stimulation at 600ms pacing cycle length (PCL) with S1-S2 coupling interval of 281 ms showing atrial capture following S2.

Echocardiography:

Conventional imaging:

Echocardiographic studies were performed using X5-1 transducer attached to Philips iE33 ultrasound system (Phillips medical systems) in the left lateral decubitus position with continuous ECG monitoring of the study participant. Images were obtained in the parasternal short- and long axis and apical 2- and 4-chamber views. Interventricular septal thickness (IVST), posterior wall thickness (PWT), left ventricular end-diastolic diameter (LVEDD), left ventricular end-systolic diameter (LVESD), left atrial size (LA) and left ventricular ejection fraction were calculated using standard echocardiographic 2D or M-mode measurements. From the 3D images, LA Volume and Ejection fraction were computed. LV mass was obtained from the 2D echocardiographic measurements using the M-mode formula and was normalized to body surface area as per ASE recommendations[420]. Peak early (E) and late (A) mitral flow velocities were measured at the tip of the mitral valve leaflets and the ratio of the early to late peak velocities (E/A) were derived accordingly.

Tissue Doppler imaging:

Tissue Doppler imaging (TDI) was performed in all patients with images taken based on the guidelines of the American Society of Echocardiography [421]. Using the 4-chamber apical view, TDI was done at the level of the mitral annulus on the lateral wall of the left ventricle and 2cm above AV junction on the inter ventricular septum. Early diastolic mitral annular velocity (E') and the late diastolic annular velocity (A') were determined from the average of septal and lateral wall data from the TDI recordings. The mitral E/E' ratio was also calculated.

Left atrial and left ventricular strain measurements:

2D image analysis was performed on digitally stored images using image analysis software "Tom tec" (Tomtec Imaging Systems, Munich, Germany). Myocardial longitudinal strain measurement was assessed on apical four chamber and short axis views with speckle tracking analysis. The traced

endocardium was divided into six segments from which the longitudinal strain was measured.

Holter monitoring:

Hardware and Software:

The Life card CF (Spacelabs healthcare, Washington, USA) is a compact ECG Ambulatory ECG Recorder utilizing a digital storage technique to store the ECG recording onto a Compact Flash (CF) card. In the extended mode, it provides continuous recording of ECG for 5 days after the surgery as planned in this study. The Life card CF comprises two sections, the 'Recorder Unit' and the 'Patient Cable Unit' (Figure 2.20 A and B). The recorder has a built in display to monitor the ECG and to verify the ECG quality before starting the recording. Menu options are selected using the 2 buttons on the front of the recorder unit. It requires one AAA battery for the duration (up to 6 days) of the study. On completion of recording, data from the CF card is downloaded onto a software system called “Sentinel” (Space Labs Healthcare; Figure 2.20C) and analysed using a diagnostic system “Pathfinder Digital” (Space Labs Healthcare) equipped with an automated AF detection algorithm.

Process:

Using “Sentinel”, the compact flash card was activated (Figure **2.21A**) with respective patient ID (1001- 1080) and a sticker was affixed to the card accordingly (Figure **2.22B**). Once the activated CF card was loaded into the recorder, it was ready for use (2 channel) and monitoring was commenced as soon as the patient was transferred to the intensive care unit after the surgery. 3 AMBU VL-00-S/25 electrodes were attached to the chest wall (Shaved, clean and dry skin over ribs) as shown below (Figure **2.23C**) and the patient cable unit (3 lead) of the recorder was connected to the electrodes.

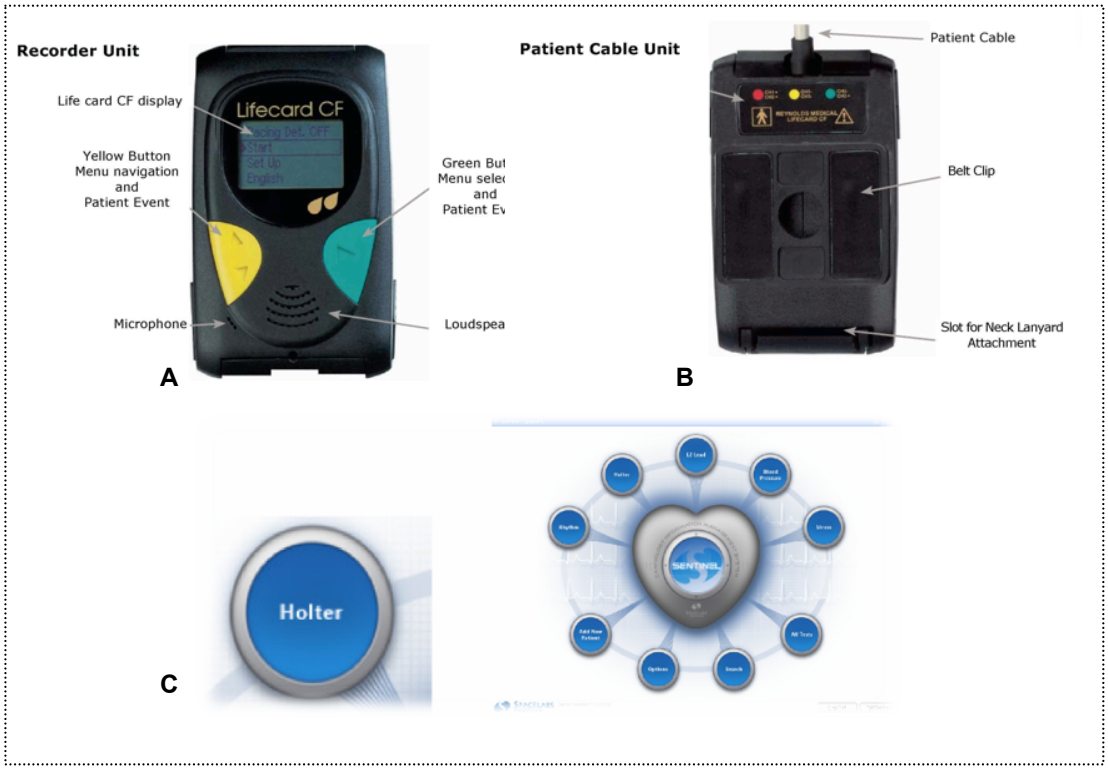


Figure 2.20 – Equipment for continuous holter monitoring after cardiac surgery and analysis of ECG recordings. **A)** Life card CF **B)** SENTINEL

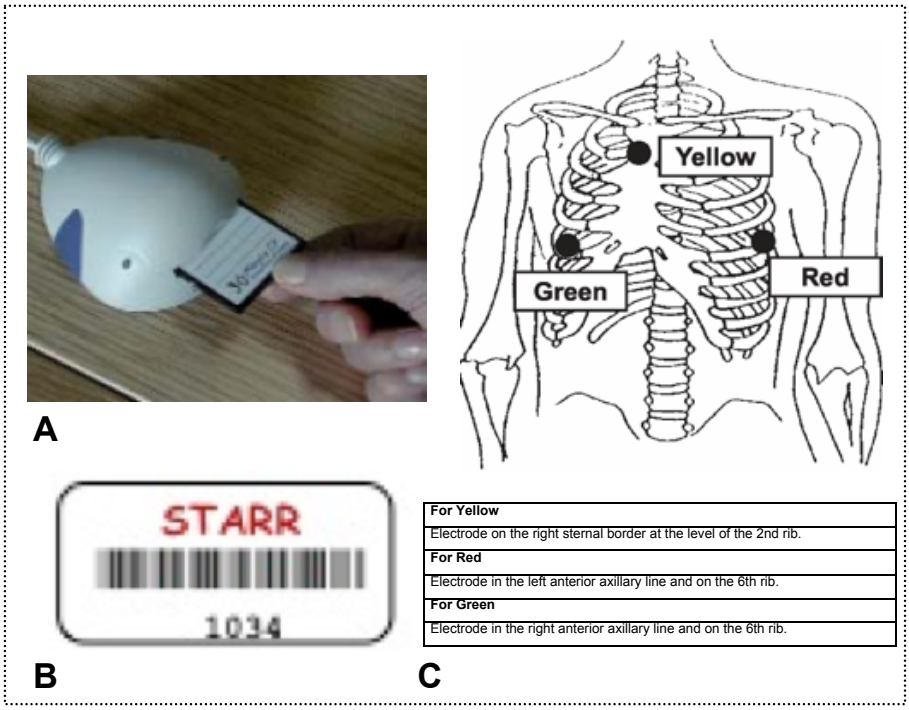


Figure 2.21 – **A)** Activation of Compact flash (CF) card **B)** CF card sticker **C)** Position of the ECG electrodes.

Monitoring was continued till the 6th day morning after surgery or until discharge from the hospital which ever was earlier. I monitored participants continuously during this period to ensure there was no disruption in the acquisition of the post-operative cardiac rhythms. Electrodes were changed twice during the period to ensure good quality of the recordings. Once the monitoring was completed, data from the CF card was downloaded into sentinel (Figure **2.20B**) where it was archived under the "pending analysis" section (Figure **2.22**).

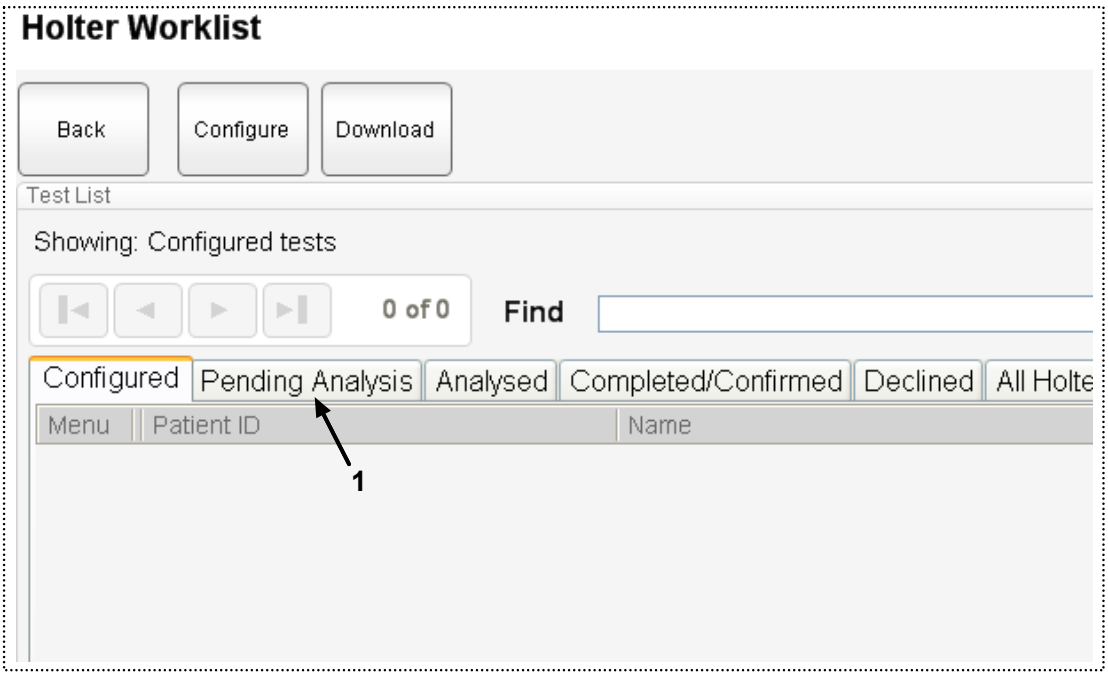


Figure 2.22 – Holter analysis sequence in the SENTINEL platform. Holter recordings imported into "SENTINEL" system are archived under "pending analysis".

Analysis:

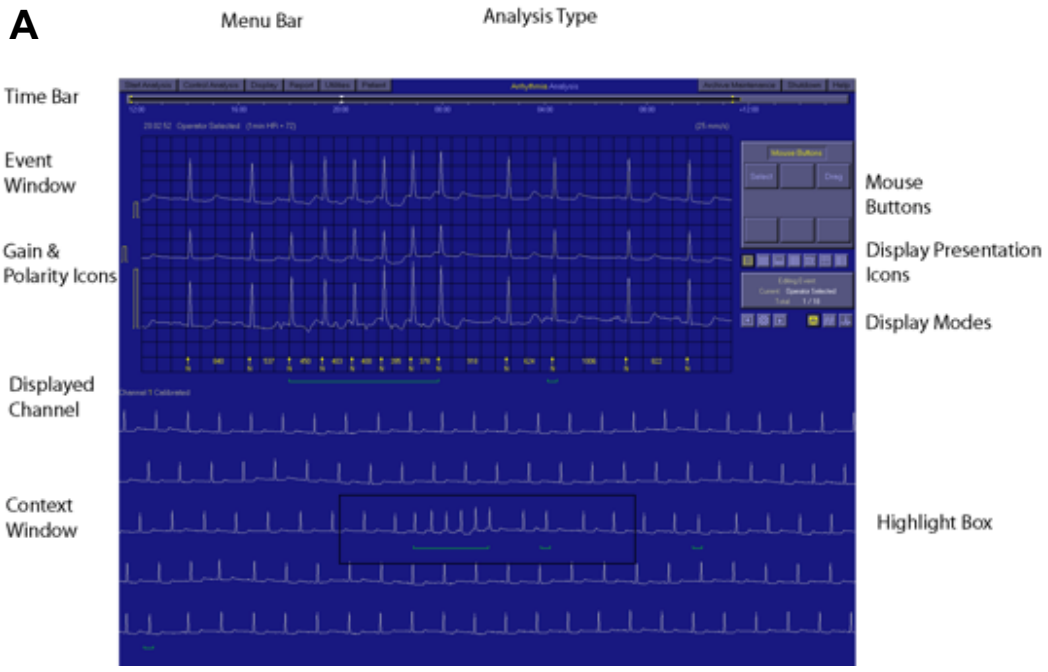
"The Pathfinder" is a system for analyzing continuous 3 lead holter monitoring recordings using the patented Neilson analysis principle. For detecting atrial fibrillation (AF), it uses an automated detection algorithm. On starting the Pathfinder program, operator is presented with a display on the screen, the framework of which will remain constant throughout the analysis (Figure **2.23A**). The upper area of the screen, the event Window, will show the first detected ECG event at 'full size', 25mm/s. The lower area of the screen, the context Window, will show the event in context at half size, 12.5mm/s. With two channel recordings as performed in the studies presented in this thesis, "Pathfinder"

analyzes each channel continuously and combines these values to determine the overall classification for the beat. The system divides the continuous ECG recordings in the entire post-operative monitoring period into 24-hour segments. “Sentinel” is interfaced with “Pathfinder” and loads the recordings into the analytical system. After loading each day’s recording, it automatically scans for non-AF arrhythmias. Once the analysis is completed, selection of the option “Automatically Detect AF” (Figure **2.23B**) from the control analysis window will diagnose AF in the recordings. In the summary table (Figure **2.23C**), in the most significant events column, pathfinder now lists the arrhythmias including total number of AF episodes if any and its duration in both HH:MM:SS and also as a % of the total recording. At this stage, if AF has been reported, diagnosis was confirmed by me based on the following criteria[422].

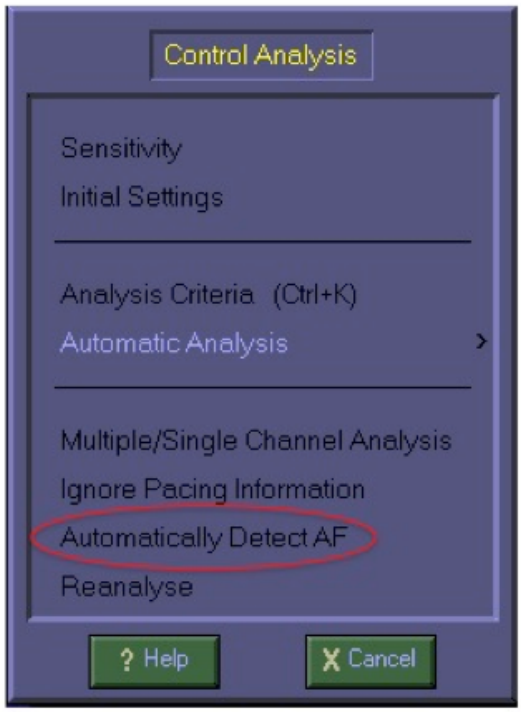
i) Absence of ‘P’ wave.
ii) Presence of fibrillatory waves.
iii) Adjacent R-R interval differing by > 50 ms.

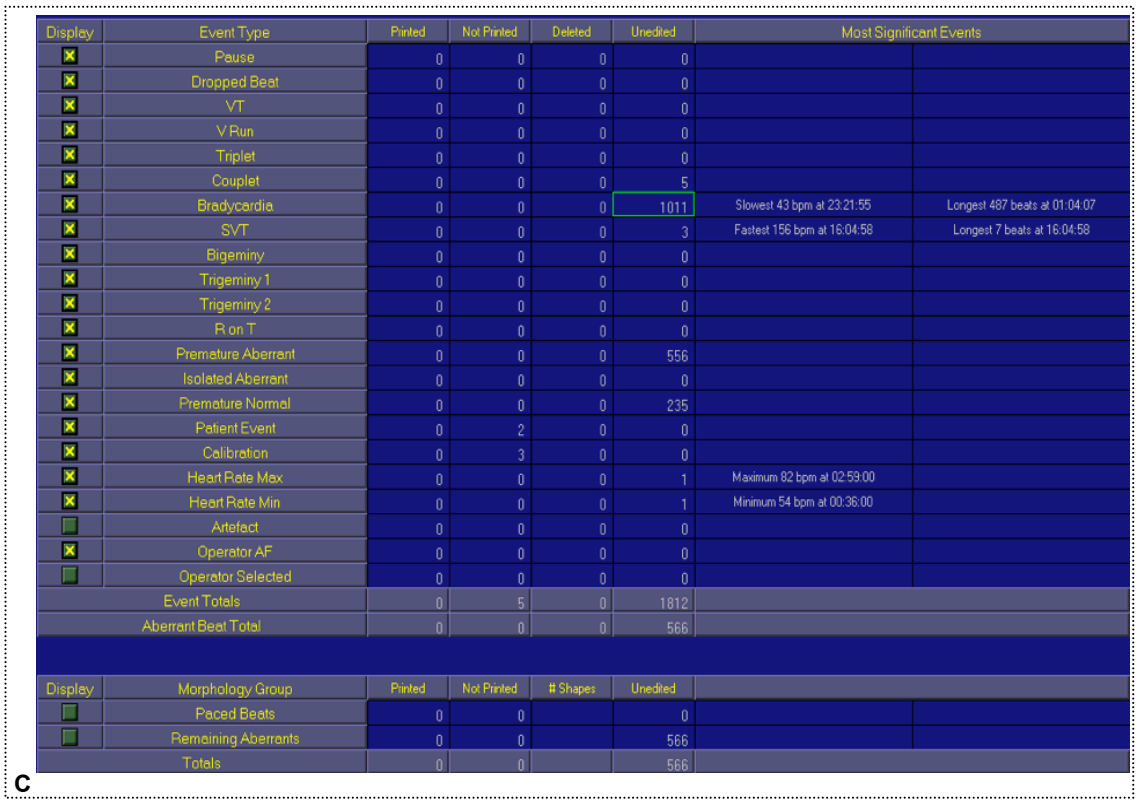
Clicking on the event count, analyzing the first episode of AF and moving forward till the last episode, completed the confirmation sequence. In addition to this manual exercise, all the recordings from this study were compared against clinical diagnosis of the rhythm in respective patients (Diagnosis based on either 12 lead ECG, cardiac monitor rhythm as documented during my follow up in the postoperative period, inputs by the attending cardiologists, intensive care physicians or by the cardiothoracic surgical team).

A


Menu Bar
Analysis Type
Time Bar
Event Window
Gain & Polarity Icons
Displayed Channel
Context Window
Mouse Buttons
Display Presentation Icons
Display Modes
Highlight Box

B


Control Analysis
Sensitivity
Initial Settings
Analysis Criteria (Ctrl+K)
Automatic Analysis
Multiple/Single Channel Analysis
Ignore Pacing Information
Automatically Detect AF
Reanalyse
? Help
X Cancel



Display	Event Type	Printed	Not Printed	Deleted	Unedited	Most Significant Events	
☒	Pause	0	0	0	0		
☒	Dropped Beat	0	0	0	0		
☒	VT	0	0	0	0		
☒	V Run	0	0	0	0		
☒	Triplet	0	0	0	0		
☒	Couplet	0	0	0	5		
☒	Bradycardia	0	0	0	1011	Slowest 43 bpm at 23:21:55	Longest 487 beats at 01:04:07
☒	SVT	0	0	0	3	Fastest 156 bpm at 16:04:58	Longest 7 beats at 16:04:58
☒	Bigeminy	0	0	0	0		
☒	Trigeminy 1	0	0	0	0		
☒	Trigeminy 2	0	0	0	0		
☒	R on T	0	0	0	0		
☒	Premature Aberrant	0	0	0	556		
☒	Isolated Aberrant	0	0	0	0		
☒	Premature Normal	0	0	0	235		
☒	Patient Event	0	2	0	0		
☒	Calibration	0	3	0	0		
☒	Heart Rate Max	0	0	0	1	Maximum 82 bpm at 02:59:00	
☒	Heart Rate Min	0	0	0	1	Minimum 54 bpm at 00:36:00	
☐	Artefact	0	0	0	0		
☒	Operator AF	0	0	0	0		
☐	Operator Selected	0	0	0	0		
	Event Totals	0	5	0	1812		
	Aberrant Beat Total	0	0	0	566		

Display	Morphology Group	Printed	Not Printed	# Shapes	Unedited
☐	Paced Beats	0	0		0
☐	Remaining Aberrants	0	0		566
	Totals	0	0		566

C

Figure 2.23 – Analysis of continuous postoperative holter recordings in the PATHFINDER platform. **A)** Pathfinder display format **B)** AF operation window **C)** Summary table.

Section 4

STICS (Statin Therapy In Cardiac Surgery)

Prevention of myocardial damage and post-operative atrial fibrillation in patients undergoing cardiac surgery: A double - blind, randomised, placebo-controlled trial of perioperative Rosuvastatin (20 mg od). (Clinical Trials.gov identifier: NCT01573143)

Study design:

The STICS trial was a double blind random allocation to Rosuvastatin (20mg od) or matching placebo via sequentially numbered treatment pack (started not earlier than 8 days before surgery and continued until the 5th postoperative day included; i.e, a maximum of 14 tablets) in patients undergoing elective cardiac surgery at the Fuwai Hospital in Beijing, China. Rosuvastatin (Crestor; AstraZeneca) tablets were purchased and matching placebo tablets were manufactured by Kaifeng Pharmaceutical Co. Ltd (China), who packed both the active and placebo tablets. The study treatment packs were labelled with sequential numbers, according to a randomisation schedule generated at the Clinical Trial Service Unit (CTSU), University of Oxford, by personnel in the China-Oxford Center for International Health Research of the Fuwai Hospital who had no further involvement in the trial. Subsequent retrieval of 52 randomly selected treatment packs found them all to contain the correct study treatment and, throughout the recruitment period, a total of only 4 packs were allocated out of sequence.

As a part of STICS collaborative group, I was responsible for,

- Initiating the site and setting up study in Fuwai hospital, Beijing, China.
- Preparation of standard operating procedures.
- Obtaining regulatory approvals.
- Staff training and trial oversight.
- Setting up the process and analysis of the ECG holter recordings.
- Training and supervision of two other colleagues involved in the analysis of holter recordings.
- Organization and analysis of samples of right atrial appendages as well as clinical database.

I worked closely with the CTSU Wolfson laboratory in the planning and analysis of plasma or serum for the biomarker assays.

The Fuwai hospital ethics committee in Beijing and the Oxford Tropical Research Ethics Committee (OXTREC) provided ethical approval for the study (Ref.no: 32-11). Under a separate approval from the Chinese Ministry of Health, permission was obtained for exporting biological samples to the UK for analysis. The Fuwai-Oxford Centre for international health research undertook operational management of the clinical trial. The entry- and discharge form used for data collection are listed in the appendix.

Primary outcomes:

- Postoperative atrial fibrillation; based on Holter monitoring and defined as "sustained" when the characteristic arrhythmia persists for more than 10 minutes and "non-sustained" when lasting between 10 beats and 10 minutes;
- Myocardial injury; based on the area under the troponin release curve derived from samples taken at 6, 24, 48 and 120 hours post-surgery.

Secondary outcomes:

- Duration of hospital and intensive care unit stay
- Major in-hospital serious adverse event reports:
    - Atrial fibrillation
    - Arrhythmias other than AF
    - Low cardiac output syndrome
    - Pleural effusion
    - Stroke
    - Myocardial infarction
    - Heart failure
    - Infection
    - Acute kidney injury
    - Death

- The ratio of the peak post-surgery troponin concentration to the baseline troponin concentration. That is; the maximum of the 6, 24, 48 and 120-

hour troponin concentrations divided by the baseline troponin concentration.

- Peak troponin concentration within first 24 hours (i.e., the maximum of the 6 and 24-hour concentrations).
- Plasma and serum biomarkers 48 hours post-surgery:
    - sCD40L
    - NT-Pro BNP
    - Creatinine
    - Directly measured LDL cholesterol
- Post-operative transthoracic echocardiography:
    - Left ventricular ejection fraction
    - Left atrial size
    - Left ventricular end systolic and diastolic diameters

Eligibility:

Inclusion criteria:

Adult patients awaiting elective cardiac surgery who are willing and able to give informed consent for participation in the study and who are in sinus rhythm and not taking any antiarrhythmic medication, other than beta-adrenergic blocking agents, at the time of surgery.

Exclusion criteria:

- Significant mitral valve disease (moderate or severe mitral regurgitation- eg. > grade II, and/or mitral stenosis and mitral annular calcification).

Sample collection and processing:

The right atrial appendage was sampled from patients undergoing surgery on cardiopulmonary bypass prior to the commencement of cardioplegia as well as from patients undergoing off pump surgery at the corresponding surgical time point. RAA samples were received in ice cold phosphate buffered saline (PBS), washed to clear off blood, dried using blotting paper, cut into smaller pieces

after removing the fat tissue and stored in cryovials in -80^{o} C until shipment to UK.

In addition to RAA, blood samples were collected prior to the commencement of study medications and at 6, 24, 48 and 120 hours after the surgery. Plasma and serum were separated by centrifugation at 1300g for 10 minutes at room temperature and were stored at -80^{o} C until shipment to UK.A bespoke software and bar code system managed the bio resource both at the Fuwai hospital site as well as in CTSU, Oxford.

Holter monitoring:

Methodology has been described in the previous section. Recordings were transferred from Fuwai site to Oxford in USB disks and by file transfer protocol (FTP) to the server at CTSU periodically. The flow chart sequence summarizing the analytical workflow for this study is listed in the appendix. Accuracy of the final spreadsheet, i.e., grouping respective patient IDs into sustained AF, non sustained AF and non-AF categories was checked by the following methods.

1) Random analysis of IDs in the folders archived as AF vs. non-AF and verifying corresponding rhythm in the pathfinder analysis report stored in a PDF format by two independent operators. This was done to confirm the accuracy of the archiving process after completion of the analysis.
2) Manual analysis of all the IDs in duplicates where there was discordance between clinical and automated analysis diagnoses. The sensitivity and specificity of the “automated AF detection algorithm” was calculated accordingly (Appendix).
3) Random reanalysis of the IDs reported previously by the first operator.

Study statistician provided final layer of scrutiny and error checks in the spreadsheet.

Biomarker assays:

Accredited staff at CTSU Wolfson’s laboratories performed the assays.

Clinical chemistry:

For the evaluation of following analytes, Beckman Coulter ACCESS 2 (Chemiluminiscent immunoassay; Troponin) and Beckman Coulter AU680 (Endpoint Assay for LDL and Jaffé Method A for Creatinine) were used. (https://www.beckmancoulter.com/wsrportal/WSR/diagnostics/clinical-products/immunoassay/index.htm). Assays were validated to confirm the accuracy, precision, linearity, recovery and reportable range. In addition, stability of troponin on room temperature was assessed prior to the analysis.

Troponin I (cTnI):

Sample type: Serum

Method details:

The Access AccuTnI assay is a two-site immunoenzymatic (“sandwich”) assay. A sample was added to the reaction vessel along with monoclonal anti-cTnI antibody conjugated to alkaline phosphatase and paramagnetic particles coated with monoclonal anti-cTnI antibody. The human cTnI binds to the anti-cTnI antibody on the solid phase, while the anti-cTnI antibody - alkaline phosphatase conjugate reacts with different antigenic sites on the cTnI molecules. After incubation in a reaction vessel, materials bound to the solid phase were held in a magnetic field while unbound materials were washed away. In the next step, the chemiluminiscent substrate Lumi-Phos* 530 was added to the vessel and light generated by the reaction was measured with a luminometer. The light production is directly proportional to the concentration of cTnI in the sample. The amount of analyte in the sample was determined from a stored, multi-point calibration curve adapted from the Beckman Coulter AccuTnI Chemistry Information Sheet (A34056E 2010 Beckman Coulter Inc).

LDL –Cholesterol (LDL-C):

Sample type: EDTA plasma

Method details:

The LDL-Cholesterol test is a two reagent homogenous system. The assay is comprised of two distinct phases. In phase one, a unique detergent solubilizes the cholesterol from non-LDL- lipoprotein particles. This cholesterol is consumed by cholesterol esterase, cholesterol oxidase, peroxidase and 4- aminoantipyrine to generate a colorless end product. In phase two, a second detergent in

reagent 2 releases the cholesterol from the LDL – lipoproteins. This cholesterol reacts with cholesterol esterase, cholesterol oxidase and a chromogen system to yield a blue color complex which can be measured bichromatically at 540/660nm. The resulting increase in absorbance is directly proportional to the LDL-C concentration in the sample. Results were printed out automatically by the platform for each sample in mg/dL at 37°C.

Creatinine:
Sample type: EDTA plasma
Method details: (Kinetic Jaffe compensated method A traceable to the IDMS reference method). Creatinine forms a yellow-orange colored compound with picric acid in an alkaline medium. The rate of change in absorbance at 520/800nm is proportional to the creatinine concentration in the sample.

ELISA based assays:
For the evaluation of following analytes in respective sample types, Meso scale discovery (MSD) sector Imager 6000 (Electrochemiluminescence Assay) platform (http://www.mesoscale.com/CatalogSystemWeb/WebRoot/) was used. Assays were validated to confirm the accuracy, precision, linearity, recovery and reportable range. In addition, stability of soluble CD40 ligand (sCD40L) following four cycles of freeze/thaw was assessed in the samples.

N-Terminal of the prohormone of brain natriuretic peptide (NT-proBNP):
Sample type: EDTA plasma
Method details:
Human NT-proBNP Assay detects NT-proBNP in a sandwich immunoassay format. MSD provides a plate that has been pre-coated with a capture antibody for the C-terminus of NT-proBNP. For analysis, sample and a solution containing the labeled detection antibody for the N-terminus of NT-proBNP (labeled with an eletrochemiluminescent compound, MSD SULFO-TAG™ label) were incubated so that NT-proBNP in the sample binds to the capture antibody immobilized on the working electrode surface. Recruitment of the labeled detection antibody by bound NT-proBNP completed the sandwich. In the next step, an MSD read buffer providing an appropriate chemical environment for electrochemiluminescence, was added, and the plate was loaded into an MSD

SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage was applied to the plate electrodes in order to emit light from the labels bound to the electrode surface. The instrument measured the intensity of emitted light to generate a quantitative measure of NT-ProBNP present in the sample.

Soluble CD 40 Ligand (sCD40L):
Sample type: Serum

Method details:

The Human sCD40L Assay detects sCD40L in a sandwich immunoassay format. This is a custom made assay prepared by MSD using the antibodies and reagents from RandD system. In the plate pre-coated with a capture antibody (goat anti-human CD40L), sample and a solution containing the labeled detection antibody (biotinylated goat anti-human CD40L labeled with an eletrochemiluminescent compound, MSD SULFO-TAG™ label) were incubated so that sCD40L in the sample binds to the capture antibody immobilized on the working electrode surface. Recruitment of the labeled detection antibody by bound sCD40L completed the sandwich. In the next step, an MSD read buffer providing an appropriate chemical environment for electrochemiluminescence was added and the plate was loaded into an MSD SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes emitted light from the labels bound to the electrode surface. The instrument measured the intensity of emitted light to generate a quantitative measure of sCD40L present in the sample.

Echocardiography:

The staff attached to the cardiovascular imaging department in Fuwai Hospital performed echocardiographic studies. Left ventricular end-diastolic diameter (LVEDD), left atrial size (LA) and left ventricular ejection fraction were calculated using standard echocardiographic 2D or M-mode measurements.

Section 5

Statistics

A: Mechanisms of Myocardial Nitroso-Redox imbalance Following Elective Cardiac Surgery on Cardiopulmonary Bypass

All continuous variables were tested for the normal distribution by Shapiro-Wilk test. Data were shown as mean ± standard error of mean (SEM). Comparisons of normally distributed variables between the PRE and POST groups were carried out using the paired t tests or two-way analysis of variance (ANOVA) for repeated measurements. Comparisons of non-normally distributed variables between groups were performed by using nonparametric tests (Eg: the Wilcoxon signed rank test). The Chi-square test was used to compare dichotomous variables between the groups.

All statistical analyses were performed using Graph Pad Prism version 6.00 for Mac (Graph Pad Software, San Diego California USA) and null hypothesis was rejected at two-tailed $p < 0.05$.

B: STARR

Sample size:

As there are no previous studies analyzing the primary and secondary end points defined in STARR, there was no data from which we were able to estimate the effect size of our intervention on the parameters under investigation or their standard deviation in the placebo arm. The sample size of n= 80 was chosen arbitrarily taking into account the study feasibility over the regulatory approval period.

Statistical Analysis plan:

A non- linear regression analysis using a straight-line model was fitted through the AERP data of both atorvastatin and placebo-treated patients over four postoperative days. The extra-sum-of-squares F test compared the goodness-of-fit of two alternative nested models; a global model where slope is shared among the data sets with a model where each dataset has its slope.

Comparisons of normally distributed tissue-based measurements between treatments and before and after reperfusion were carried out either by paired t-test or using two-way analysis of variance (ANOVA, repeated measurements x treatment) with Bonferroni correction. Non-normally distributed data were subjected to either logarithmic or square root transformation prior to analysis. The Chi-square test was used to compare dichotomous variables between the groups. Changes in LDL cholesterol levels in the perioperative period were analyzed by Analysis of covariance (IBM SPSS Statistics for Macintosh, Version 22.0.Armonk, NY: IBM Corp). Missing LDL cholesterol values were imputed using multiple imputation, generating 10 imputed data sets, with subsequent estimates of test statistics from each of the data sets being combined using the established methods of Rubin (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). All other statistical analyses were performed using Graph Pad Prism version 6.00 for Mac (Graph Pad Software, San Diego California USA). The null hypothesis was rejected at two-tailed $p < 0.05$.

C: STICS

Sample size calculation:

Assuming an AF rate of 35% in the placebo group, it was initially estimated that a study size of 1000 patients (500 in each arm) would have 95% power to detect a 30% relative risk reduction in AF at the 5% significance level (84% power at 2p=0.01) and a 84% power at 2p=0.05 to detect a relative risk reduction in AF of 25% at 2p=0.05. The study of this size was also powered to detect a 15% reduction in Troponin with >99% power at 2p=0.05 and 86% power at 2p=0.01.

However, following the recruitment of the first 500 patients, it was noticed that the AF rate in the trial participants was lower than originally anticipated (18%). It was, therefore, decided to increase recruitment to 1800 patients as this would ensure 80% power at 2p=0.05 to detect a relative risk reduction in post-operative AF of 25% (see Table 5.2). In order to account for protocol deviations,

study dropouts and other unforeseen circumstances, the actual recruitment target was set at 1900.

	Relative risk reduction		
Sample size	**30%**	**25%**	**20%**
1500	88% (71%)	70% (47%)	50% (26%)
1800	93% (81%)	78% (57%)	57% (33%)
2000	95% (86%)	83% (63%)	62% (38%)

Table 2.3: STICS - Power at 2p=0.05 (2p=0.01) to detect 30, 25 and 20% relative risk reductions given an overall AF rate of 18%.

Overview of the statistical analysis plan:

All analyses were by intention to treat, defined as patients were analyzed in the group to which they were randomized, no matter what treatment they received, and regardless of whether they deviated from the protocol in any way with missing data imputed as described later. All p-values were 2-sided and considered statistically significant, without allowance for multiple testing if less than 0.05.

Baseline characteristics:

In order to assess the balance of baseline characteristics between randomized arms, the Rosuvastatin and placebo groups were compared with respect to the following variables recorded at randomization:

- Age
- Sex
- Smoking status
- Past medical history (hypertension, myocardial infarction, stoke/TIA, peripheral arterial disease, heart failure, chronic obstructive pulmonary disease, diabetes mellitus, chronic kidney disease)
- Current/recent medication (beta-blockers, NSAIDs/steroids, insulin, antiplatelets, contrast agents [last 2 weeks], anticoagulants, calcium channel blockers, ACE inhibitors/ARBs, nitrates, potassium sparing diuretics, loop/thiazide diuretics, nephrotoxic antibiotics [last 2 weeks], statins)

- Height
- Weight
- Body mass index
- Pre-operative transthoracic echocardiography (left atrial size; ejection fraction; left ventricular end systolic diameter; left ventricular end diastolic diameter; left ventricular hypertrophy)
- Planned surgery (on/off pump procedure, CABG/AVR)

Imputation of missing outcome data:
All analyses were done according to the intention-to-treat principle and hence, where missing, primary and secondary outcome data were imputed.

For all adverse event outcomes (i.e., atrial fibrillation detected by Holter monitoring, all major in-hospital serious adverse events) any patient with missing data were assumed not to have had the outcome. Such patients will therefore, contributed to the denominator but not the numerator when the proportions of patients experiencing each outcome were compared between treatment groups.

For each of the plasma and serum biomarker outcomes, as well as post-operative transthoracic echocardiography measurements, any missing post-randomization results were imputed using multiple imputation, using 10 imputed data sets, with subsequent estimates of test statistics from each of the data sets being combined using the established methods of Rubin. For post-operative left ventricular end systolic diameter, however, analyses were limited to those with complete data. The imputation procedure for the other measurements took into consideration each patient's key baseline characteristics. For the relatively small proportion of patients who underwent surgery but for some reason missed subsequent blood results, the imputation procedure took into account of treatment allocation. However, for patients who did not undergo surgery, treatment allocation was not taken into account during the imputation procedure (to ensure that, for such patients, there was no systematic correlation between treatment allocation and imputed blood results). The results from these analyses

were compared with those from equivalent “complete-case” analyses, but primary emphasis was placed on the results after multiple imputations.

Primary outcome:

Main analysis of the co-primary outcomes:

The primary measure of the effect of allocation to Rosuvastatin on atrial fibrillation detected by Holter monitoring was the ratio of the odds of atrial fibrillation among Rosuvastatin-allocated patients to the odds of atrial fibrillation among placebo-allocated patients. This odds ratio was calculated and presented together with its 95% confidence interval and provided the statistic used to test the null hypothesis. However, due to the potential for the odds ratio to differ appreciably from the relative risk for an outcome that is not rare (and when there is an at least moderate treatment effect), the relative risk and its 95% confidence interval were also presented.

For the co-primary endpoint of postoperative troponin release, the logarithm of the area under each patient’s own troponin release curve (between 6 and 120 hours post-surgery) were calculated. The mean (and standard error) of the log area was estimated for each treatment arm and compared using a two-sample t-test.

Key subgroup analyses of the effect of treatment on co-primary outcomes:

This study was not designed to have real power to explore subgroup effects reliably, but further analyses to assess whether there was definite evidence of variations in the effect of Rosuvastatin on either or both of the co-primary outcomes in different subgroups of patient, when analyses were subdivided by:

- Age at randomization (≤60, >60)
- Prior statin use (Yes, No)
- Number of days’ treatment allocation prior to surgery (0-2, ≥3)*
- Actual surgery (on vs. off pump, ignoring any patients who had both types, and CABG vs. AVR, again ignoring any patients who had both)*
- Perioperative use of steroids (Yes, No)*
- Perioperative use of NSAIDs or steroids (Yes, No)*

* When presenting these analyses, it was noted that these characteristics were defined post-randomization.

Secondary outcomes:
Duration of hospital stay and, separately, duration of intensive care unit stay was compared between randomized groups using log-rank time-to-event methods. These analyses of “time to hospital discharge” and “time to intensive care discharge” yielded both a statistical test (the log-rank test) for the difference in discharge time between the treatment arms and estimates of the median time to discharge in both groups. Participants who did not have surgery were censored for these outcomes at the earliest opportunity (i.e., prior to any of the observed failure times).

For each of the significant in-hospital serious adverse events listed later, both the odds ratio and the relative risk of these outcomes for Rosuvastatin allocated patients compared with placebo-allocated patients were calculated. The test of each null hypothesis was based on the test statistic derived from the odds ratio. Between-group comparisons of the peak post-surgery troponin: baseline troponin ratio, as well as of the peak troponin concentration within the first 24 hours, were done on the log-scale using two-sample t-tests. For comparisons between treatment arms of mean plasma biomarkers collected 48 hours after surgery, analyses were done by analysis of covariance adjusted for each patient’s baseline value measured at randomization. Analyses were done on the log scale for sCD40L, NT- Pro BNP and creatinine. Between-group comparisons of mean left ventricular end systolic diameter were done using the t-test (due to the high proportion of missing data at both baseline and post-surgery). For the other post-operative transthoracic echocardiography measurements, analysis of covariance, adjusted for the baseline value, was used.

Other post-randomization comparisons:
Additional comparisons between patients allocated Rosuvastatin and patients allocated placebo:

- Duration between randomization and surgery.
- Compliance with treatment allocation.

- Details of surgery received (on/off pump, CABG/AVR, cardiopulmonary bypass and aortic cross-clamp times for on pump procedures, number of arterial/venous grafts for CABG operations).
- Perioperative interventions (intra-operative defibrillation, use of internal/external pacemakers, use of intra-aortic balloon pump, use of vasopressors/inotropes, surgical re-exploration).
- Postoperative outcomes (renal replacement therapy/dialysis, duration of ventilator support).
- Postoperative treatments received up to postoperative day 5 (beta-blockers; anti platelets, DXM, ibuprofen, any NSAID/steroid, potassium supplements, blood/blood products, non-study statin, nephrotoxic antibiotics, ACE inhibitors/ARBs, Amiodarone, digoxin, diuretics including potassium sparing drugs, calcium channel blockers, contrast agents).
- Collection of blood samples (6, 24, 48 and 120 hours post-surgery).
- Collection of urine samples (6 and 24 hours post-surgery).
- Fluid balance on postoperative days 1 and 2 (mean IV fluids, mean urine output, mean surgical drain and percentage receiving any blood/blood products).
- Proportion of patients from which a sample of the right atrial appendage was collected.

Protocol deviations:

Consent procedure:

These were tabulated and accompanied by a brief textual description.

Eligibility:

The numbers of randomized patients in each group failing each exclusion criterion were tabulated (but such patients were included in the analysis).

Patient withdrawals:

The numbers of patients withdrawing from the trial were tabulated according to reason and randomized treatment allocation. Information from such patients was included up until the point at which they withdrew and imputed subsequently.

CHAPTER 3

MYOCARDIAL NITROSO-REDOX BALANCE FOLLOWING ELECTIVE CARDIAC SURGERY ON CARDIOPULMONARY BYPASS

Background and rationale

Coronary artery bypass grafting (CABG) is currently recommended for myocardial revascularization in patients with multivessel coronary artery disease, significant stenosis of left main coronary artery or multivessel coronary artery disease associated with diabetes mellitus or chronic kidney disease [17]. Despite the advent of off-pump techniques, most CABG surgery continues to be performed using cardiopulmonary bypass [39,203] (CPB). Moreover, procedures such as valve replacements, surgical correction of congenital heart diseases, and heart transplantation, are also performed primarily on CPB. Notwithstanding cardioplegia, hearts undergoing surgical procedures on CPB are subjected to some degree of ischemia - reperfusion (I/R) injury[205,423] leading to myocardial stunning[206,207]and increased risk of arrhythmias[208 209] and death; indeed, reperfusion injury in the myocardium has been reported in up to 25% of patients who died after CABG[210]. It has long been known that reperfusion after a brief period of ischemia results in the production of large amounts of reactive oxygen species (ROS) from multiple enzymatic sources[219-228] including “uncoupled” nitric oxide synthase (NOS) activity. The latter is a phenomenon whereby, in response to *S* – glutathionylation or altered phosphorylation [109 115] or of reduced bioavailability of L-arginine or tetrahydrobiopterin (BH_4) [110-114], NOSs generate superoxide instead of nitric oxide (NO). In isolated rat hearts subjected to a prolonged I/R protocol, irreversible oxidation of BH_4 was associated with loss of endothelial NOS (eNOS) activity and post ischemic endothelial and myocardial dysfunction, that were partially reversed by BH_4 supplementation [277].

Whether this mechanism is also relevant to myocardial I/R injury associated with CABG surgery remains to be investigated. Since a synthetic formulation of the active 6R-isomer of BH_4 is already approved for the treatment of phenylketonuria, NOS uncoupling secondary to BH_4 deficiency could be a promising therapeutic target. To test this hypothesis, I investigated the regulation of myocardial NOS activity in patients undergoing on-pump cardiac surgery.

Methods

The study design can be found in appendix (**1**). The south central Berkshire research ethics committee provided ethical approval for the study, and all patients provided written informed consent.

Study participants

Between 2010 and 2012, 116 patients in sinus rhythm undergoing their first elective cardiac surgery (CABG, aortic valve replacement or both) on CPB in the Department of Cardiothoracic Surgery at John Radcliffe Hospital in Oxford were recruited in the study. During CPB, myocardial protection was achieved with cold blood cardioplegia and moderate systemic hypothermia.

Sample collection

Samples of the right atrial appendage were taken before (PRE) and soon after CPB and cardiac reperfusion (POST).

Measurements

Further details on the measurements below can be found in Chapter 2.

Atrial superoxide production was measured by lucigenin (5 µmol/L)-enhanced chemiluminescence[119,424] and 2-hydroxyethidium (2-OH- E+) detection by high-performance liquid chromatography and results shown as the tiron-inhibitable fraction (see Chapter 2). To elucidate the contribution of specific oxidases to basal atrial superoxide production, atrial homogenates were pre-treated with the following inhibitors: N_{ω} -nitro-L-arginine methyl ester and its dextro isomer (L-NAME and D-NAME; 1 mmol/L), gp91-ds-tat peptide and the respective scrambled peptide (10 µM/L), Rotenone (100 µMol/L) and Antimycin A (10 µMol/L).

Tetrahydrobiopterin (BH_4), and its oxidized products 7,8 dihydrobioterin (BH_2) and biopterin (B) were measured by electrochemical (for BH_4) and fluorescence

detection (for BH_2 and B) in homogenized atrial tissue, following sample separation by high-performance liquid chromatography.

NOS activity was measured in atrial tissue homogenates using radiochemical HPLC detection of ^{14}C labelled L- arginine to L-citrulline conversion and expressed as the L- NAME-inhibitable fraction of L-citrulline, as a percentage of total L-arginine.

The activity of the rate-limiting enzyme in the synthesis of BH4, GTP cyclohydrolase 1 (GTPCH) was measured in atrial homogenates by iodine oxidation and detection of neopterin content by HPLC.

For immunoblotting, primary antibodies raised against nNOS (Santa Cruz, 1/2000), iNOS (Millipore1/1000), eNOS (Santa Cruz, 1/4000), GTPCH (Abnova, 1/500), GTP cyclohydrolase feedback regulatory protein (Cell signalling, 1/250), NOX2 (BD Biosciences, 1/1000), NOX4 (AbCam, 1/1000) and 3-Nitrotyrosine (Millipore, 1/200) were used. Immunodetection of primary antibodies was performed using horse-radish -peroxidase (HRP) - conjugated secondary antibodies (Promega, USA). Bands were visualized with enhanced chemifluorescence (Amersham Bioscience UK Ltd.) and imaged using a gel imaging system (Bio-Rad ChemiDoc XRS). The 2D densities of the bands were quantified using the Image J program (NIH) and normalised to either beta-Tubulin (Santa Cruz, 1/1000) or GAPDH (Sigma, 1/1000). To re-probe with a different primary antibody, membranes were stripped using Re-blot plus (Millipore Inc) as per manufacturer's recommendations.

S-Glutathionylation of nNOS and eNOS were evaluated by using an anti-glutathione monoclonal antibody (1/1000; ViroGen) in NOS immunoprecipitates (Santa Cruz Biotechnologies) from atrial tissue homogenates. In some experiments, samples were incubated with the reducing agent DTT (100 μm/L) for 20 min before immunoprecipitation and immunoblotting. The 2D density of the GSH bands were quantified using the Image J program (NIH) and normalised to total nNOS or eNOS.

Quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR) was used to compare the expression of the primary transcripts of nNOS, eNOS, iNOS, NOX2, NOX4 and GTPCH.

Rac1 activity, defined by the ratio of GTP-Rac1 to total Rac1, was evaluated by an affinity precipitation assay with PAK1-PBD–conjugated glutathione agarose beads, according to the manufacturer's instructions (Millipore, Temecula, CA).

Statistics

Comparisons of normally distributed variables between treatments or before and after reperfusion were carried out using paired t-tests or two-way analysis of variance (ANOVA, repeated measurements x treatment) with Bonferroni correction. Non-normally distributed variables were log transformed prior to analysis or analysed by using the Wilcoxon matched-pairs signed rank test.

All statistical analyses were performed using Graph Pad Prism version 6.00 for Mac (Graph Pad Software, San Diego California USA).

The null hypothesis was rejected at two-tailed $p < 0.05$.

Results

Atrial sources of superoxide production before and after CPB and reperfusion

CPB and reperfusion was associated with increased atrial superoxide production measured both by lucigenin enhanced chemiluminescence and HPLC detection of 2 - hydroxy ethidium; **Figure 3.1)**. By contrast, atrial peroxynitrite content, measured by luminol-enhanced chemiluminescence, remained unchanged after CPB (**Figure 3.2)** indicating that nitrosative stress is not a major mediator of myocardial stunning after on-pump cardiac surgery.

Mitochondrial oxidases have been shown to contribute to superoxide release during myocardial ischemia, as well as after reperfusion in experimental animal models[425,426]. In my samples, the rotenone-inhibitable fraction of superoxide

release was higher after CPB in the absence of changes in protein abundance of mitochondrial complex I to IV (**Figure 3.3E and F**).

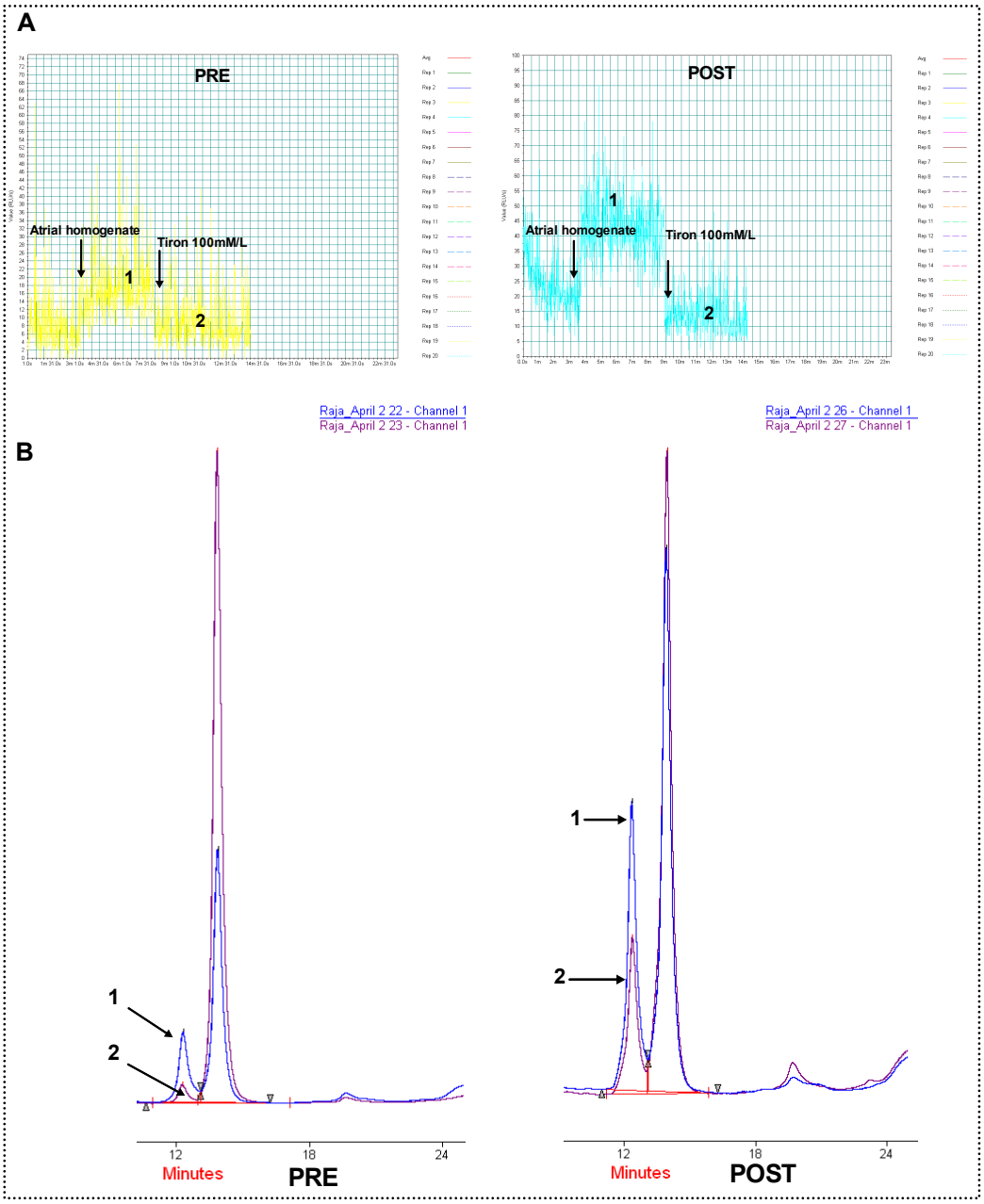


Figure 3.1: *Upper panel,* representative lucigenin-enhanced chemiluminescence (**A**) and chromatograms of 2- hydroxyethidium detection by HPLC (**B**) in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion (POST). 1 and 2 are the raw data traces obtained before and after treatment with tiron (100mM/L). The difference between 1 and 2 expressed as tiron-inhibitable fraction represents superoxide release in the respective samples.

Bottom panel, average superoxide production in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion measured by lucigenin-enhanced chemiluminescence (**C**) and by HPLC (**D**), respectively. Data were expressed as geometric mean ± 95% confidence intervals (CI), *** p <0.001 vs. PRE, Wilcoxon matched-pairs signed rank test. RLU - Relative light units.

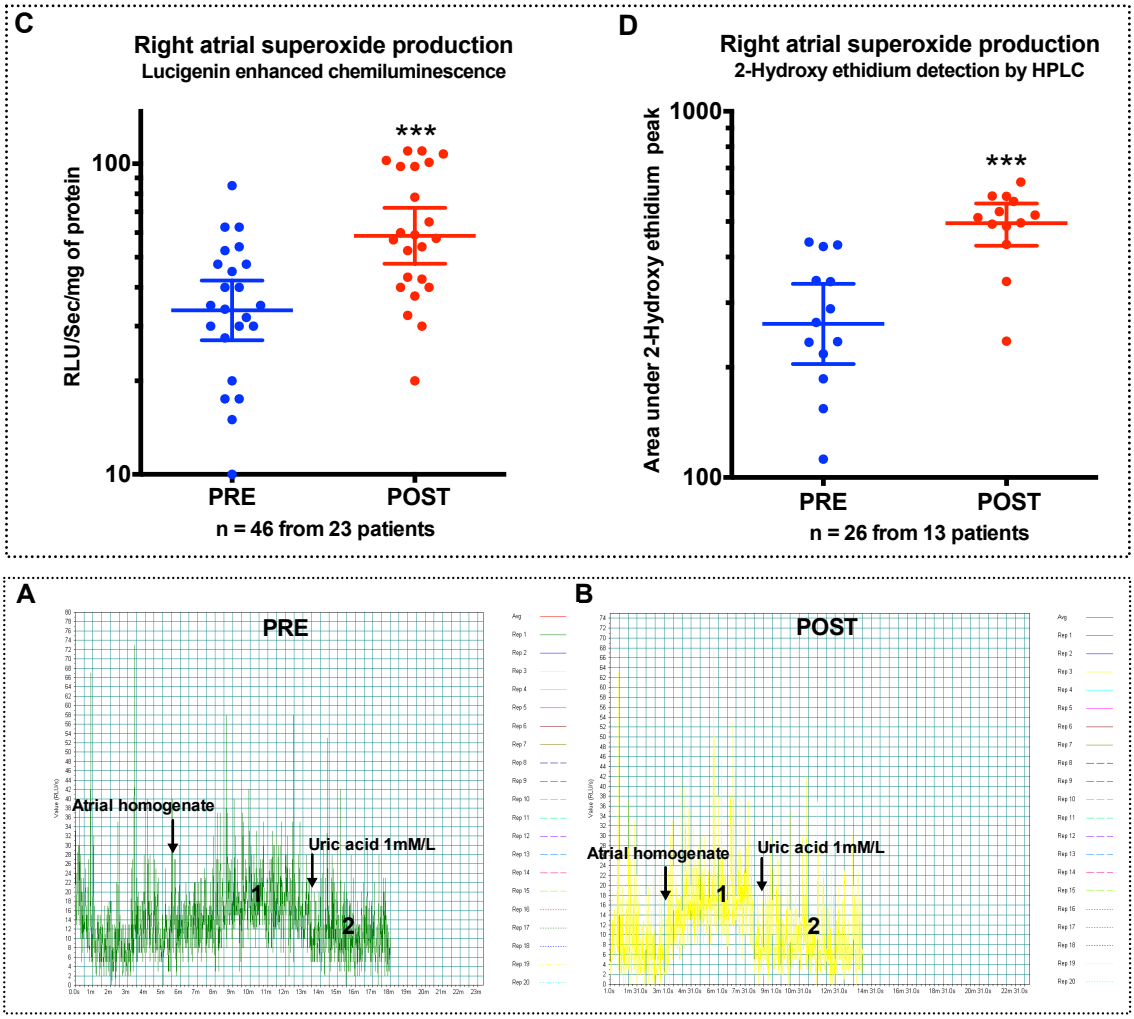
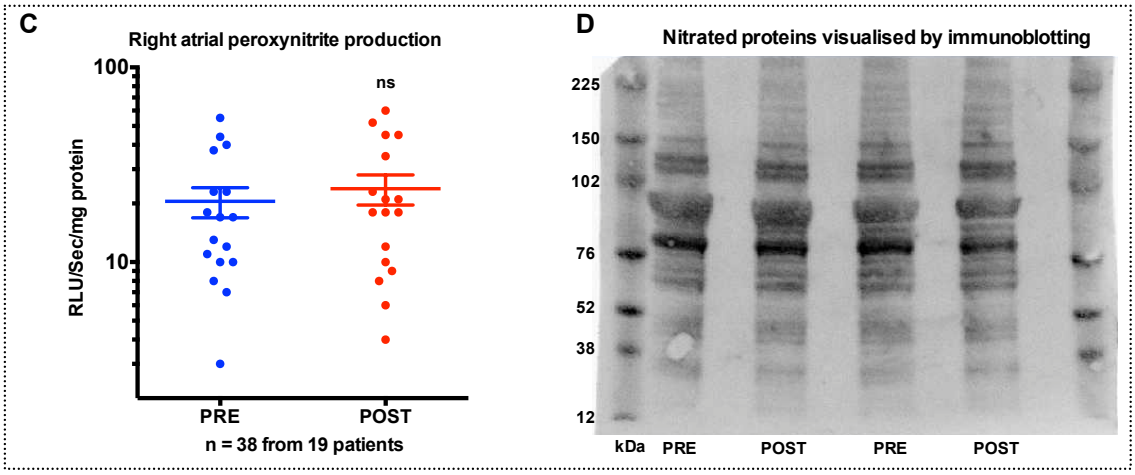
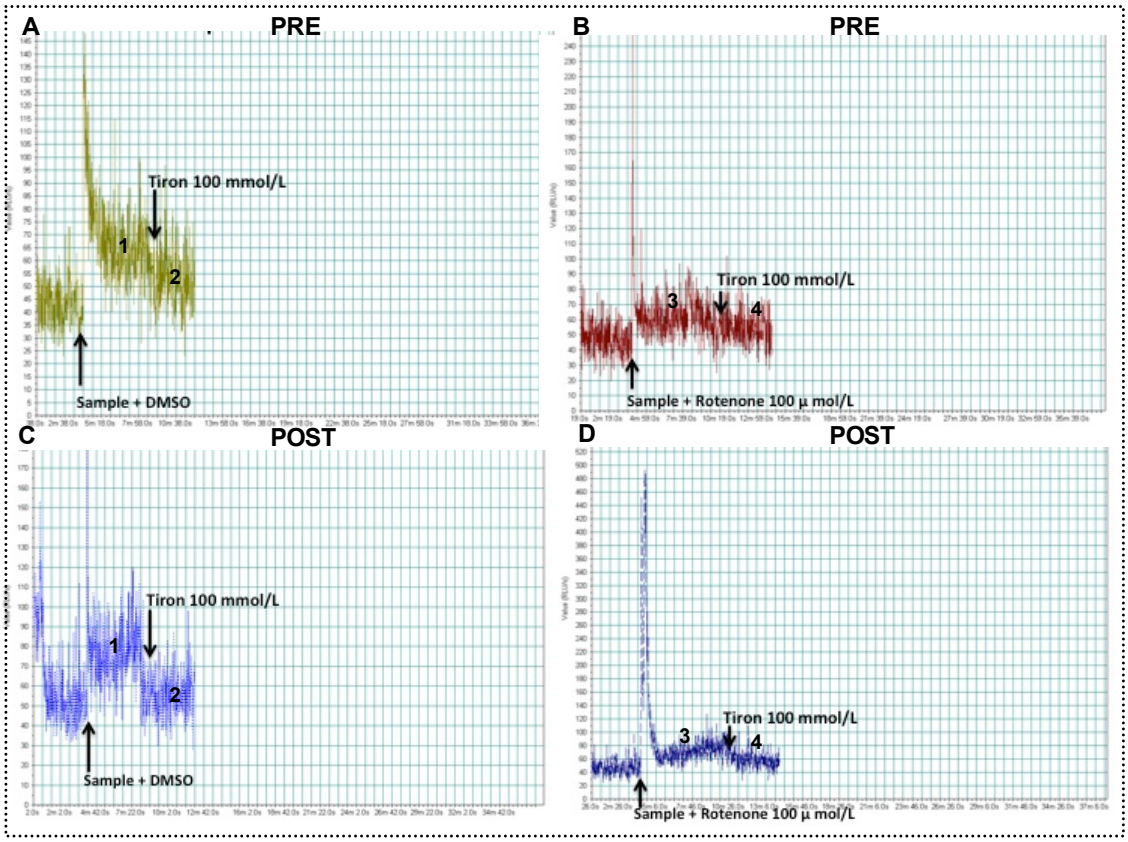


Figure 3.2: *Upper panel,* representative luminol - enhanced chemiluminescence (**A, B**) in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion (POST). 1 and 2 are the raw data traces obtained before and after uric acid (1mM/L) respectively. Results were expressed as uric acid-inhibitable fraction in respective samples.

Bottom panel, average peroxynitrite production in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion measured by luminol-enhanced chemiluminescence (**C**). Data were expressed as geometric mean ± 95% CI, ns = $p > 0.05$ vs. PRE, Wilcoxon matched-pairs signed rank test. **D.** Nitrated proteins detected by anti - nitro tyrosine antibodies not significantly changed after CPB and reperfusion.


C
Right atrial peroxynitrite production
RLU/Sec/mg protein
100
10
ns
PRE
POST
n = 38 from 19 patients
D
Nitrated proteins visualised by immunoblotting
225
150
102
76
52
38
12
kDa
PRE
POST
PRE
POST


A
PRE
Tiron 100 mmol/L
1
2
Sample + DMSO
B
PRE
Tiron 100 mmol/L
3
4
Sample + Rotenone 100 µ mol/L
C
POST
Tiron 100 mmol/L
1
2
Sample + DMSO
D
POST
Tiron 100 mmol/L
3
4
Sample + Rotenone 100 µ mol/L

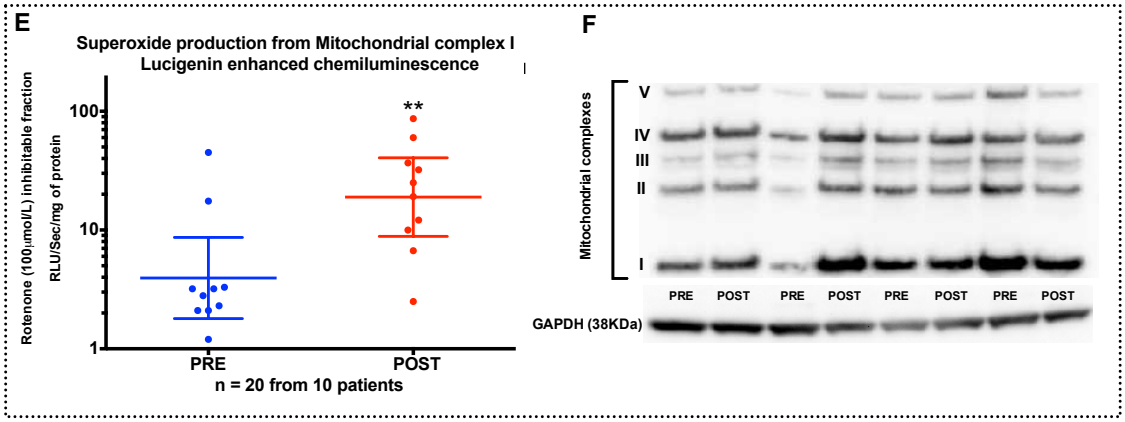

E
Superoxide production from Mitochondrial complex I
Lucigenin enhanced chemiluminescence
Rotenone (100µmol/L) inhibitable fraction
RLU/Sec/mg of protein
100
10
1
**
PRE
POST
n = 20 from 10 patients
F
Mitochondrial complexes
V
IV
III
II
I
PRE
POST
PRE
POST
PRE
POST
PRE
POST
GAPDH (38KDa)

Figure 3.3: Superoxide release from mitochondrial complex I before (PRE; **A** and **B**) and after CPB and reperfusion (POST; **C** and **D**) detected by lucigenin-enhanced chemiluminescence. 1 and 2 are the traces obtained from atrial homogenates in DMSO before and after tiron (100mMol/L) and 3 and 4 are the traces obtained from samples treated with rotenone (100μM/L) before and after tiron. The contribution of mitochondrial complex I to superoxide release represented by the rotenone inhibitable fraction, increased after CPB and reperfusion (**E**) in the absence of changes in protein expressions of mitochondrial complex I to IV (**F**; n = 16 samples from 8 patients). Data expressed as geometric mean ± 95% CI, ** p<0.01 vs. PRE, Wilcoxon matched-pairs signed rank test.

NADPH oxidases are an important source of superoxide release in the myocardium[427] and their activity is independently associated with an increased risk of post-operative AF[98]. **Figure 3.4** shows that NOX2 containing NADPH oxidases contribute to the increased superoxide production observed after CPB and reperfusion **(Figure 3.4,** ***bottom panel*****).** Rac, a small GTPase in the 'Rho' family, in its GTP-bound form activates NOX2-NADPH oxidase by binding to the cytosolic subunit p67 (phox), which in turn interacts with the enzyme complex leading to superoxide production[428]. However, the ratio of activated Rac-1 to total Rac-1 was unaltered after CPB (**Figure 3.5A**). By contrast, NOX4-containing NADPH oxidases are constitutively activated and transcriptionally regulated[429]. However, both protein and mRNA expressions of NOX2 and NOX4 containing oxidases remained unchanged after CPB (**Figure 3.5 B to D**). In agreement with this observation, NADPH-stimulated superoxide release, a measure of maximum capacity of NOX homologues to generate superoxide, was not different before and after CPB **(Figure 3.5, bottom panel).**

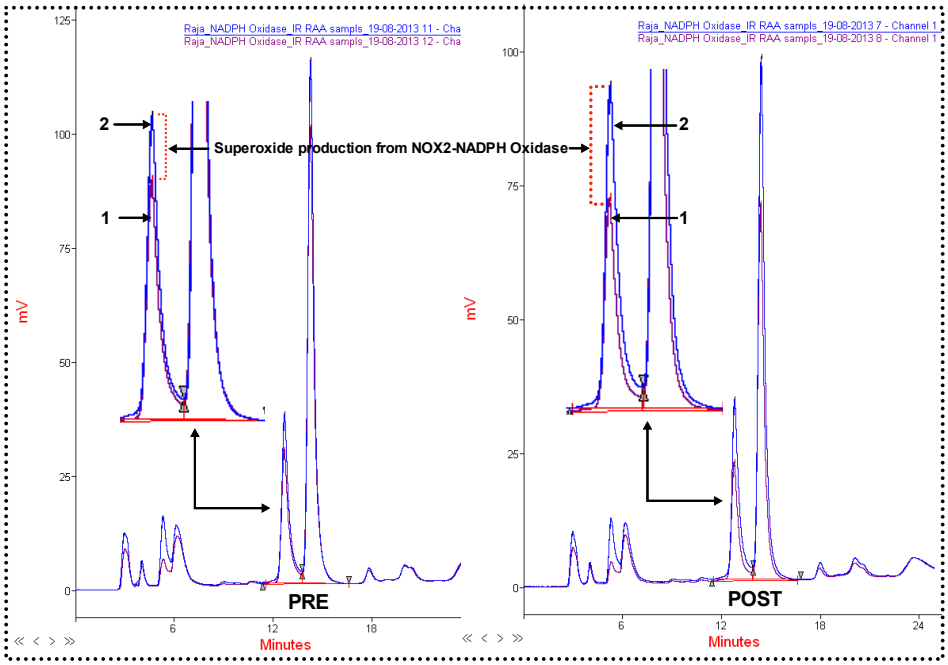
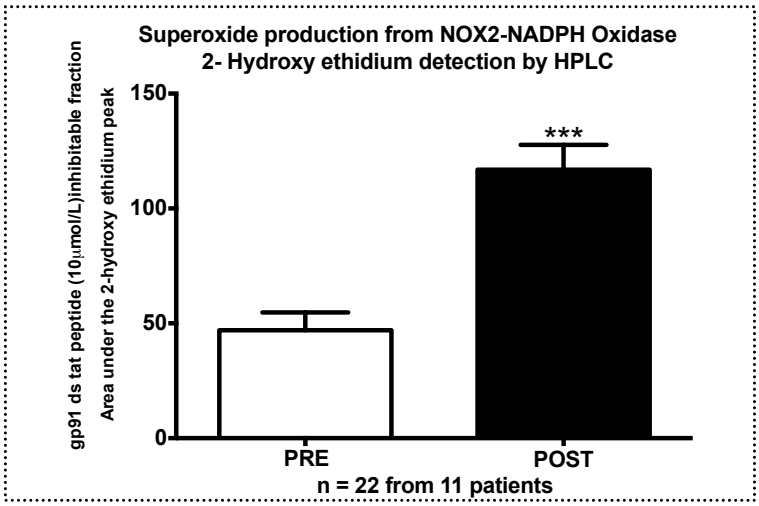


Figure 3.4: *Upper panel*, representative chromatograms of gp91-ds-tat peptide inhibitable fraction of superoxide release in homogenates of right atrial appendages taken before (PRE) and after CPB (POST) and reperfusion detected by HPLC**.** 1 and 2 are the peaks representing 2-OH - (E+) in the presence of the gp91-ds-tat peptide (10µm/L) or a scrambled peptide (10µm/L), respectively. The difference between the area under the 2-OH - (E+) peaks of samples incubated with the scrambled peptide and those incubated with the gp91-ds-tat peptide (i.e., the gp91-ds-tat peptide inhibitable fraction) represents the superoxide released by atrial NOX2 NADPH Oxidase.

Bottom panel, NOX2- derived superoxide production is increased POST CPB and reperfusion**.** *** p < 0.001 vs. PRE by student's t test for paired samples. Data expressed as mean ± SEM.



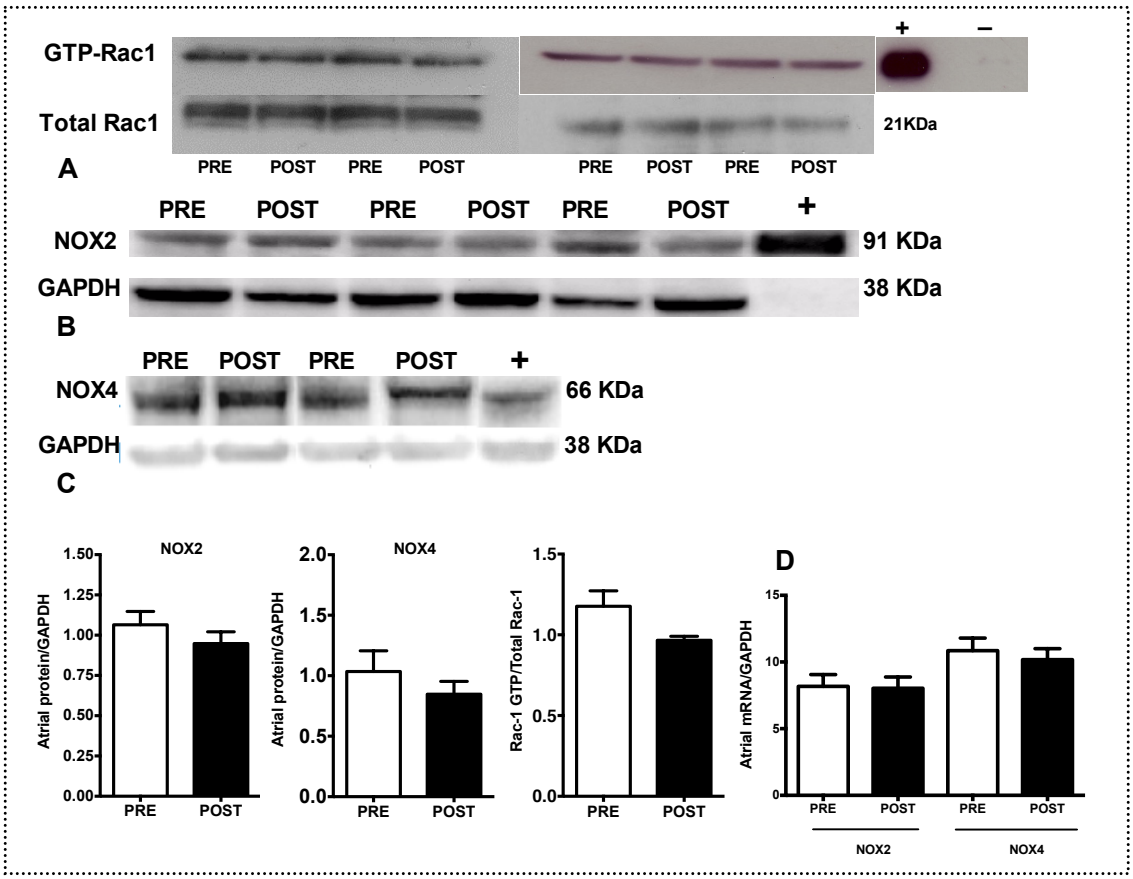
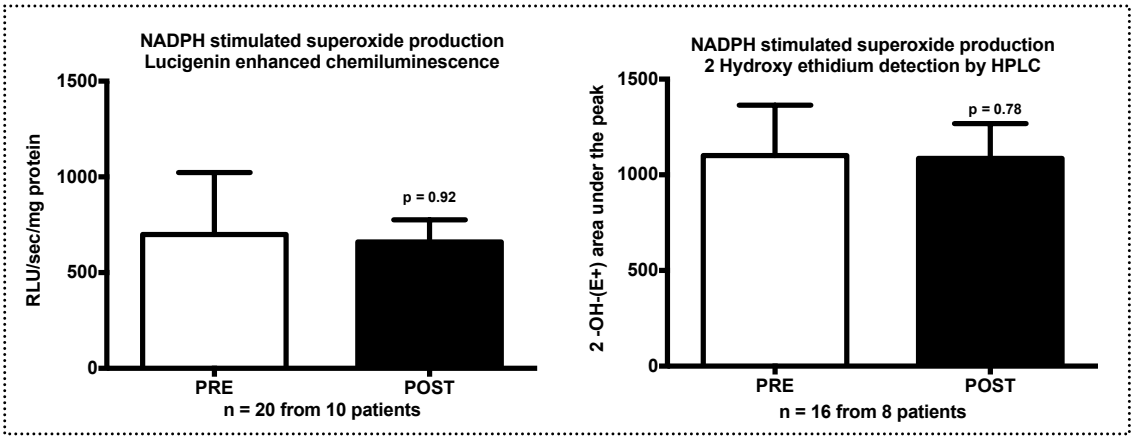


Figure 3.5: *Upper panel*, atrial protein content of activated Rac-1 **(A)**, NOX2 **(B), or** NOX4 **(C)** and NOX2 and NOX4 mRNA **(D)** were unaltered after CPB and reperfusion. NOX2 - transgenic murine left ventricle and murine kidney homogenates were used as positive controls (+) for NOX2 and NOX4 respectively. For activated Rac-1, the positive control was GTPγS, i.e., a non hydrolysable analog of GTP and the negative control (-) was GDP. Data are expressed as mean ± SEM (n = 16-20 samples from 8-10 patients). GTP – Guanosine triphosphate; GDP – Guanosine di phosphate.

Bottom panel, NADPH stimulated superoxide production (by lucigenin enhanced chemiluminescence and 2-hydroxy ethidium detection by HPLC) did not differ between PRE- and POST samples**,** by paired student's t test. Data expressed as mean ± SEM.



To assess whether functional uncoupling of NOS, contributes to increased superoxide production after CPB, samples were pre-treated with L-NAME or D-NAME (the inactive dextro-isomer of L-NAME). In the presence of L-NAME, superoxide was higher in PRE atrial samples and lower in POST atrial samples (**Figure 3.6, bottom panel**). These findings indicate that, before CPB and reperfusion, a significant fraction of atrial superoxide production is either scavenged or inhibited by NO; by contrast, after CPB and reperfusion, NOS appears to contribute to the overall increase in atrial superoxide production, suggesting that the activity of the synthase is uncoupled under these conditions.

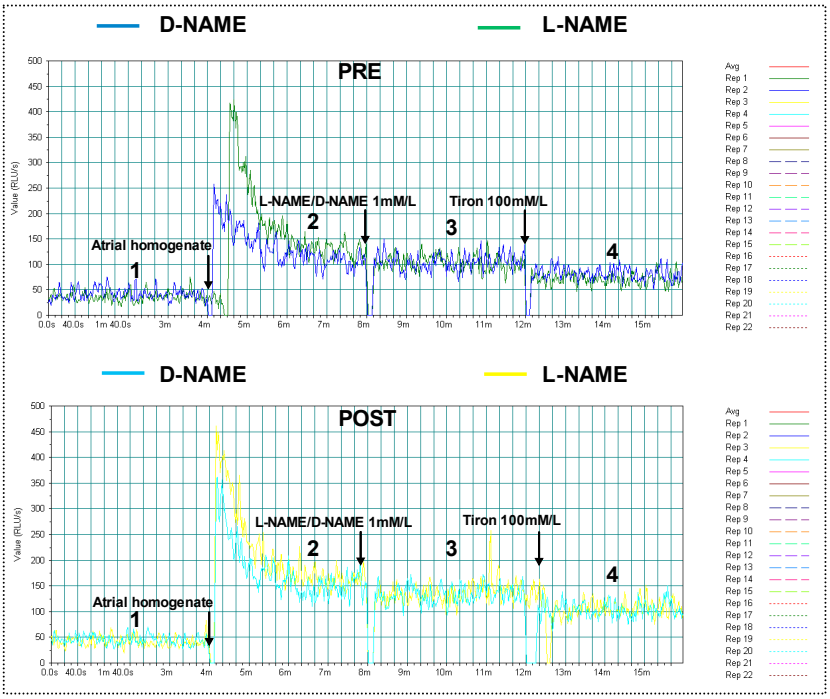
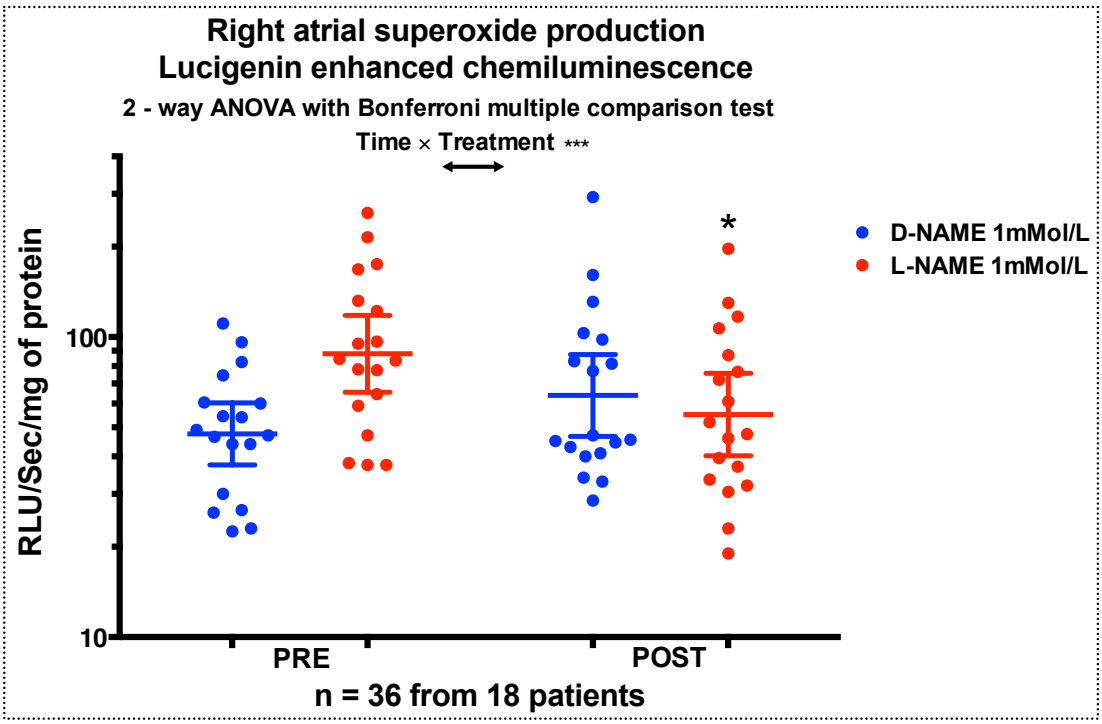


Figure 3.6: *Upper panel,* Examples of lucigenin-enhanced chemiluminescence in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion (POST) treated with D-NAME or L-NAME.

Bottom panel, Average superoxide production after pre-incubation with L-NAME or D-NAME in atrial samples obtained before (PRE) and after CPB and reperfusion (POST). * p <.05 vs. D-NAME. *** p<0.001 for the interaction between time (PRE and POST) and treatment. Two-way ANOVA for repeated measurements after log-transformation with Bonferroni correction. Data are expressed as geometric mean ± 95% CI. L-NAME ; N_ω - nitro-L-arginine methyl ester, D-NAME; N_ω -nitro-D-arginine methyl ester.



Together these data show that, after CPB and reperfusion, atrial superoxide production from mitochondria and NOX2 oxidases is significantly increased and NOS activity is uncoupled.

Atrial GTPCH-1 activity and BH_4 content are lower after CPB and reperfusion in the absence of concomitant changes in oxidized biopterins

In an isolated rat heart preparation subjected to a prolonged I/R protocol, oxidative depletion of BH_4 has been linked to functional uncoupling of eNOS[277]. By contrast, in human atrial samples, BH_4 content (**Figure 3.7B**) and the ratio of BH_4 and its oxidized products (BH_2 +B) were significantly lower (**Figure 3.7E**) after CPB in the absence of changes in BH_2 and B between the two groups. (**Figure 3.7CD**).

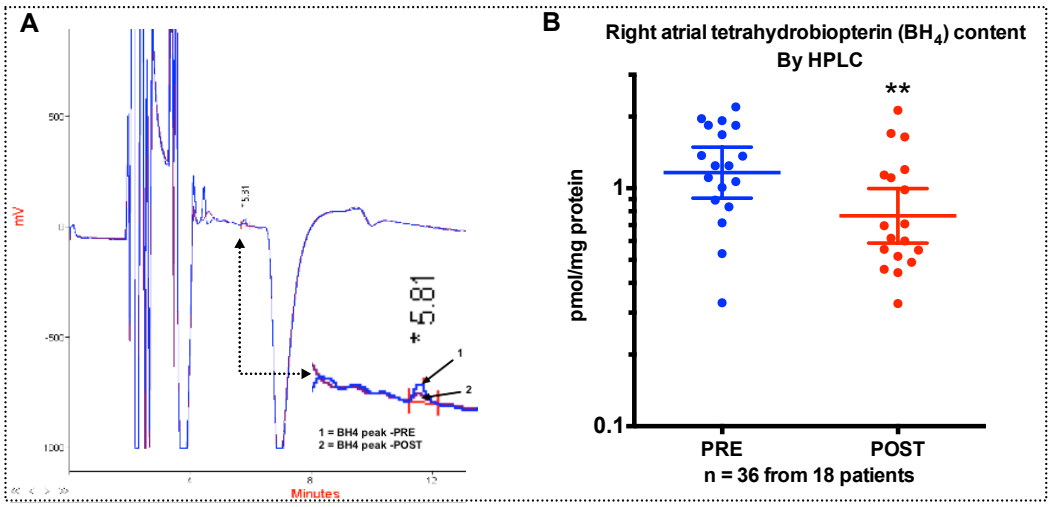
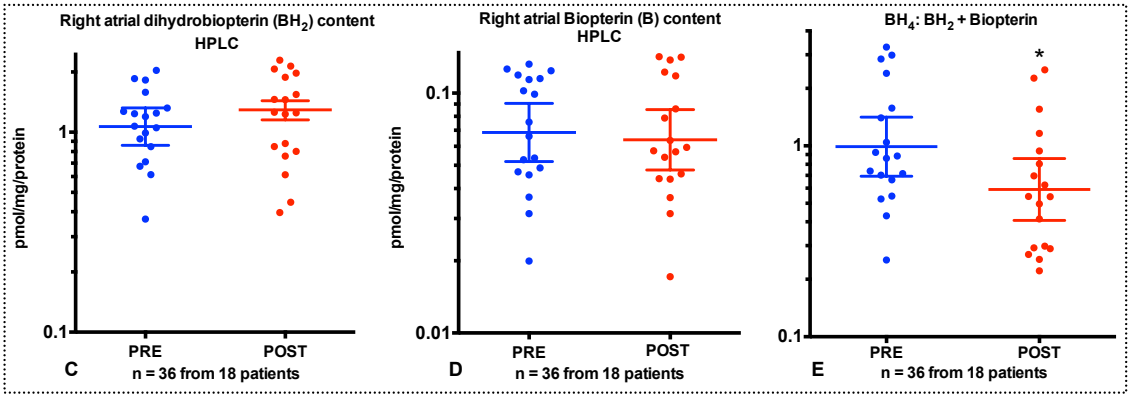


Figure 3.7: *Upper panel,* representative chromatogram of measurement of BH_4 by HPLC in homogenates of the right atrial appendage taken before (PRE) and after CPB and reperfusion (POST) **(A).** Atrial BH_4 content is decreased after CPB and reperfusion **(B)**.

Bottom panel, Atrial BH_2 **(C)** and Biopterin **(D)** levels were unaltered while the ratio of BH_4 to BH_2 + B **(E)** decreased after CPB and reperfusion. Wilcoxon matched pairs sign rank test. Data are expressed as geometric mean ± 95% CI. * p <.05 , ** p <.01 vs. PRE.



As shown in **Figure 3.8AB**, atrial GTPCH-1 activity was reduced after CPB suggesting that, in the absence of an increase in BH2 and B, a reduction in the rate of BH_4 synthesis may account for the lower BH4 content after CPB and reperfusion. Atrial protein and mRNA expression of GTPCH-1 remained unchanged after CPB and reperfusion (**Figure 3.8 bottom panel**). GTPCH-1 activity is inhibited by its interaction with GTPCH Feedback Regulatory Protein (GFRP); as shown in **Figure 3.8DG**, the atrial content of GFRP was significantly increased after CPB.

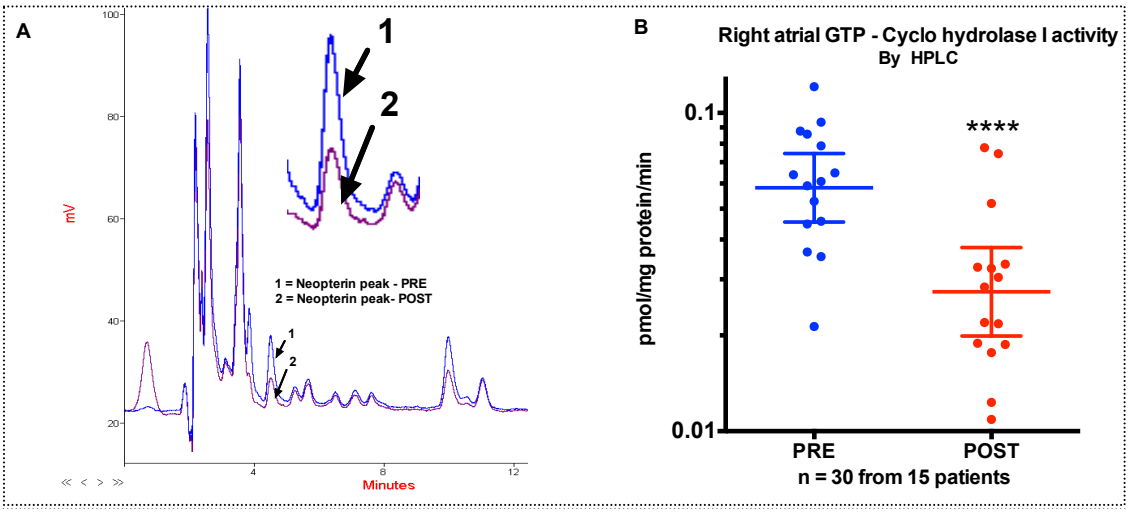
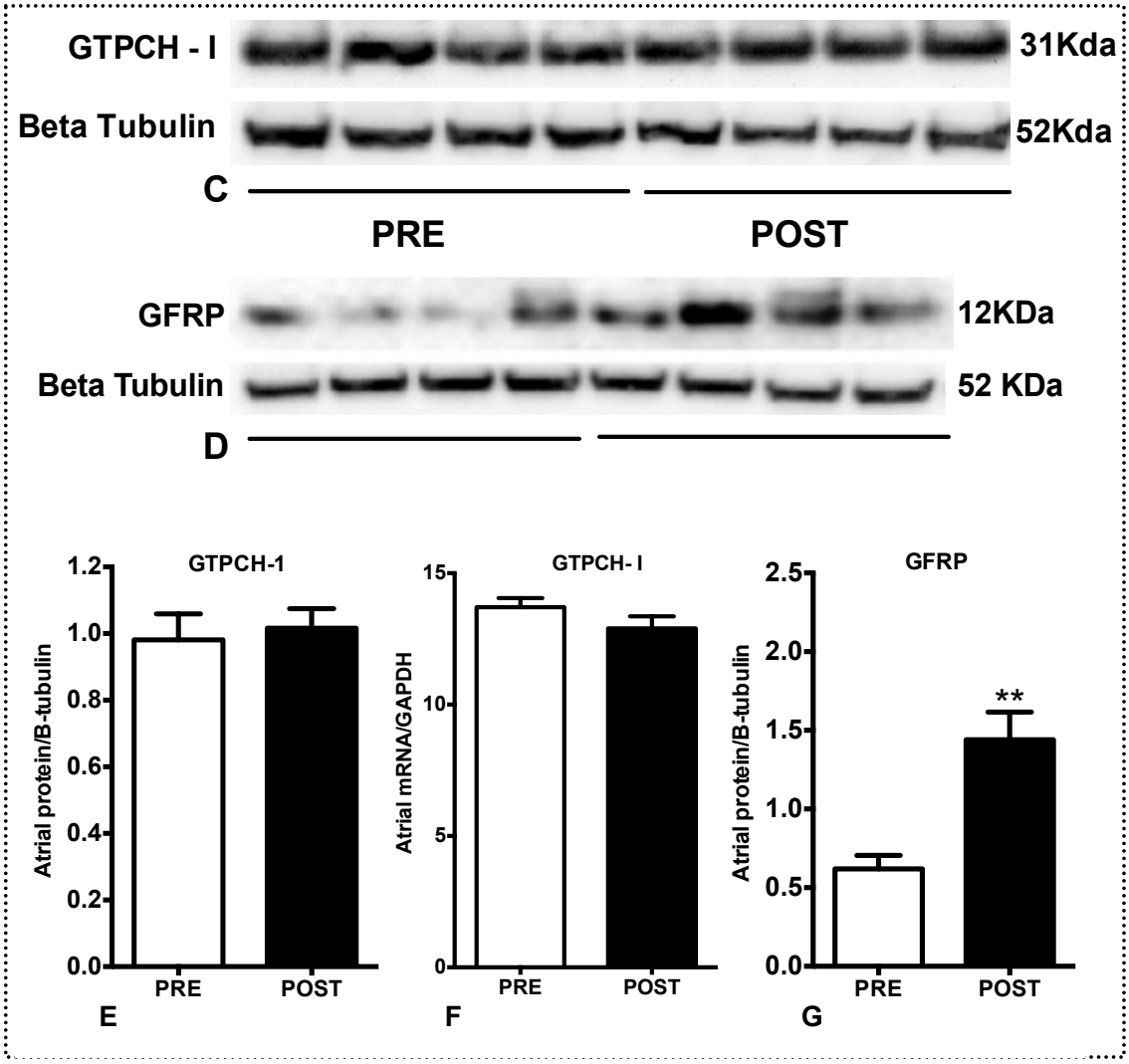


Figure 3.8: *Upper panel,* representative chromatogram of neopterin detected by HPLC for measurement of GTPCH-1 activity in homogenates of the right atrial appendages taken before (PRE) and after CPB and reperfusion (POST) **(A).** Atrial GTPCH-1 activity is decreased after CPB and reperfusion **(B)**, by Wilcoxon matched-pairs signed rank test. Data were expressed as as geometric mean ± 95% CI.**** p<0.0001 vs.PRE.

Bottom panel, atrial protein **(CE)** and mRNA levels **(F)** of GTPCH-1 were unaltered while atrial protein levels of GFRP were significantly increased after CPB and reperfusion **(DG)**, by paired student's t test. Data were expressed as mean ± SEM. ** *p* <.01 (n = 16 - 24 samples from 8 - 12 patients).


GTPCH - I
31Kda
Beta Tubulin
52Kda
C
PRE
POST
GFRP
12KDa
Beta Tubulin
52 KDa
D
GTPCH-1
Atrial protein/B-tubulin
1.2
1.0
0.8
0.6
0.4
0.2
0.0
PRE
POST
E
GTPCH- I
Atrial mRNA/GAPDH
15
10
5
0
PRE
POST
F
GFRP
Atrial protein/B-tubulin
2.5
2.0
1.5
1.0
0.5
0.0
**
PRE
POST
G

NOS activity is reduced in the atrial myocardium after CPB and reperfusion

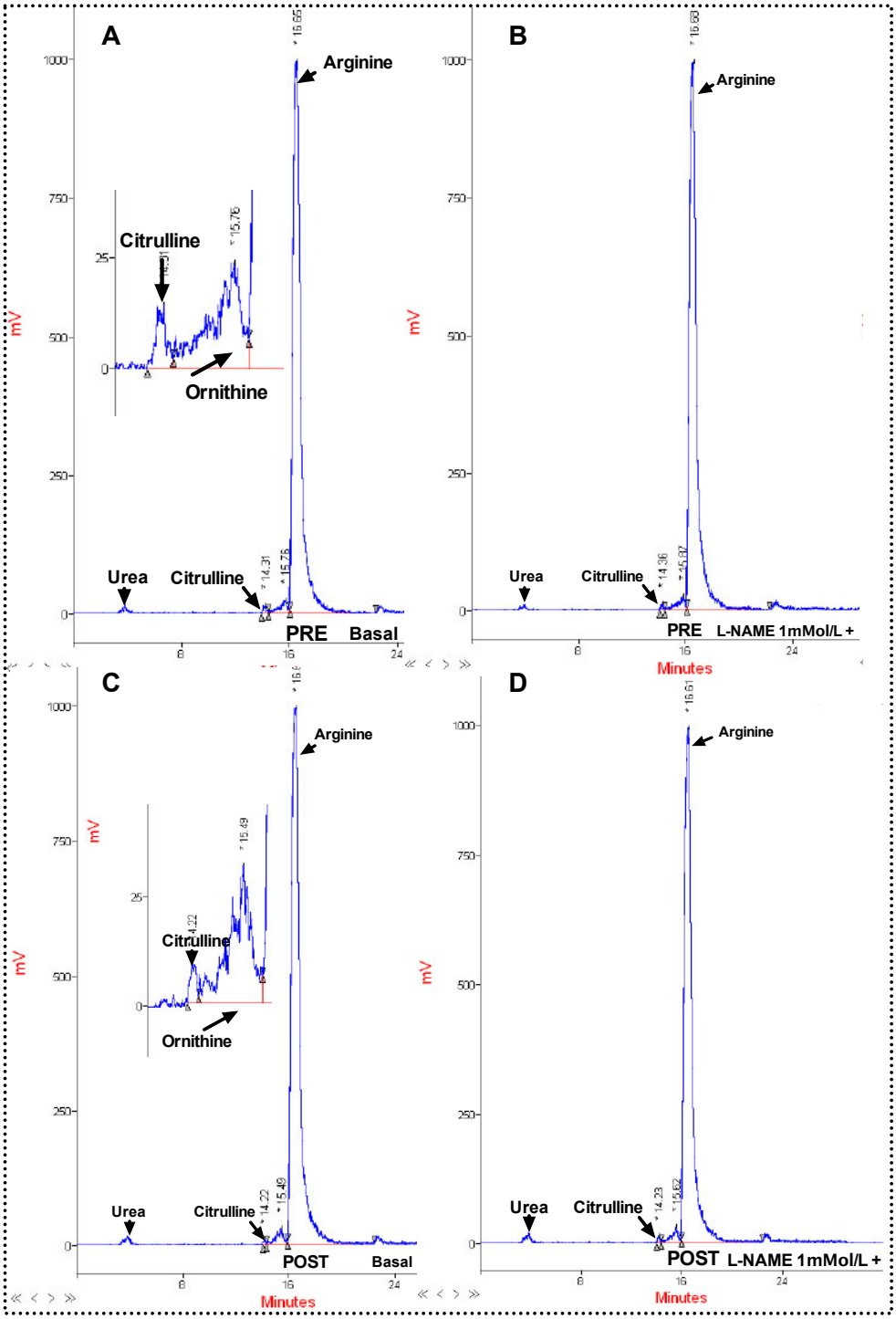


Figure 3.9: Representative chromatogram of measurement of activity of NOS by radiochemical HPLC detection of ^{14} C labelled L-Arginine to L-Citrulline conversion in homogenates of the right atrial appendage taken before (PRE) and after CPB and reperfusion (POST). Traces labelled A and B were obtained before and after L-NAME in PRE- atrial homogenates and similarly C and D were obtained from POST- atrial homogenates. The L-NAME inhibitable fraction of L-Citrulline expressed as a percentage of total L-Arginine represented activity of NOS.

In agreement with data indicating functional NOS uncoupling, atrial NOS activity decreased after CPB and reperfusion (**Figure 3.10A**), in the absence of changes in protein and mRNA expressions of eNOS and nNOS (**Figure 3.10 bottom panel;** iNOS protein was undetectable). To elucidate the link between BH_4 deficiency and reduction in NO production after CPB, NOS activity was also measured after incubating atrial homogenates with BH_4 (10 µmol/L). As shown in **Figure 3.10B**, BH_4 increased NOS activity overall but failed to abolish the reduction in NOS activity post CPB and reperfusion, indicating that mechanisms independent of BH4 bioavailability may contribute to NOS dysfunction under these conditions.

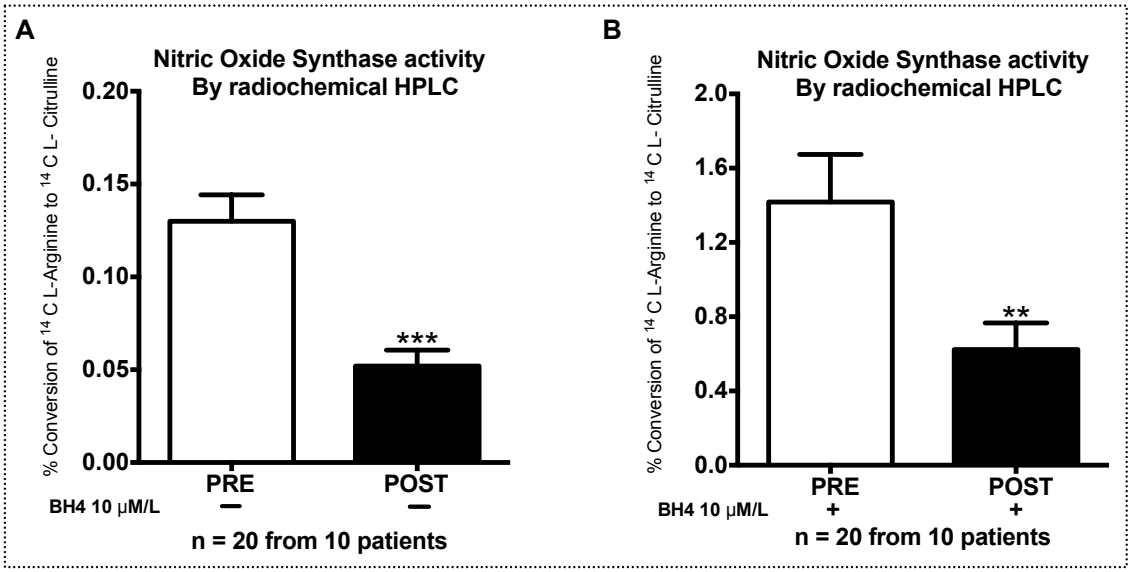
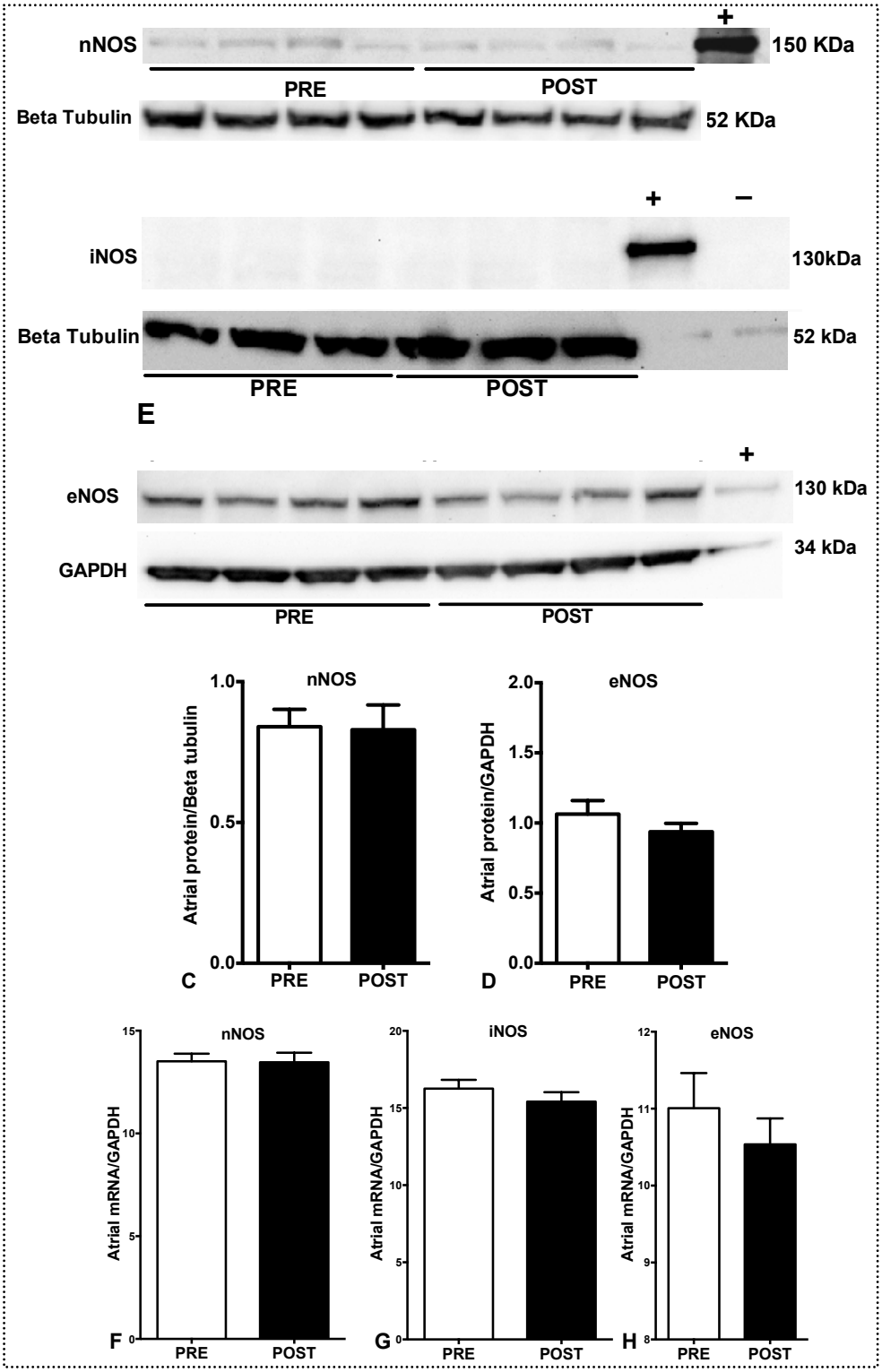


Figure 3.10: *Upper panel*, Reduction in atrial NOS activity associated with CPB and reperfusion **(A)** is not abolished by pre-treatment of samples with BH_4 **(B)**, by paired student's t test. Data expressed as mean ± SEM. ** *p* <.01, *** p<0.001 vs.PRE.

Bottom panel, atrial protein (**CD**) and mRNA levels of nNOS and eNOS (**FH**) along with mRNA levels of iNOS (**G**) were unaltered after CPB and reperfusion while iNOS protein was absent (**E**). nNOS positive control (+) - murine brain tissue; iNOS positive control - murine macrophage cell line stimulated by gamma interferon; Negative control (-) : murine macrophage cell line in the absence of cytokine stimulation. eNOS positive control - human saphenous vein homogenate.; n = 16 - 40 samples from 8 - 20 patients.


nNOS
+
150 KDa
PRE
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Beta Tubulin
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+
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130kDa
Beta Tubulin
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E
+
eNOS
130 kDa
GAPDH
34 kDa
PRE
POST
nNOS
Atrial protein/Beta tubulin
1.0
0.5
0.0
PRE
POST
C
eNOS
Atrial protein/GAPDH
2.0
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15
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Atrial mRNA/GAPDH
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eNOS
Atrial mRNA/GAPDH
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8
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H

Atrial levels of S - glutathionylated eNOS is increased after CPB and reperfusion

S-Glutathionylation is a redox-dependent post-translational modification whereby a glutathione tripeptide is reversibly bound to a protein thiol through the formation of a disulfide bond [430]. *S*-Glutathionylation of eNOS modifies the reactive cysteine residues at the interface of its FAD binding and FMN binding sites resulting in functional uncoupling and superoxide production from the reductase domain [431]. As shown in **Figure 3.11D**, atrial levels of *S* - glutathionylated eNOS increased after CPB and reperfusion and pre treatment with the reducing agent dithiothreitol (DTT) abolished both *S* - glutathionylation of eNOS as well as the reduction in atrial NOS activity after CPB and reperfusion **(Figure 3.11 bottom panel)**. Together these results indicate that enzyme S- glutathionylation is the main mechanism underlying functional uncoupling of NOS after CPB and reperfusion. The fraction of *S* - glutathionylated nNOS was also increased after CPB (**Supplement 4**), an observation that needs to be investigated further as *S* - glutathionylation of nNOS has not been reported before.

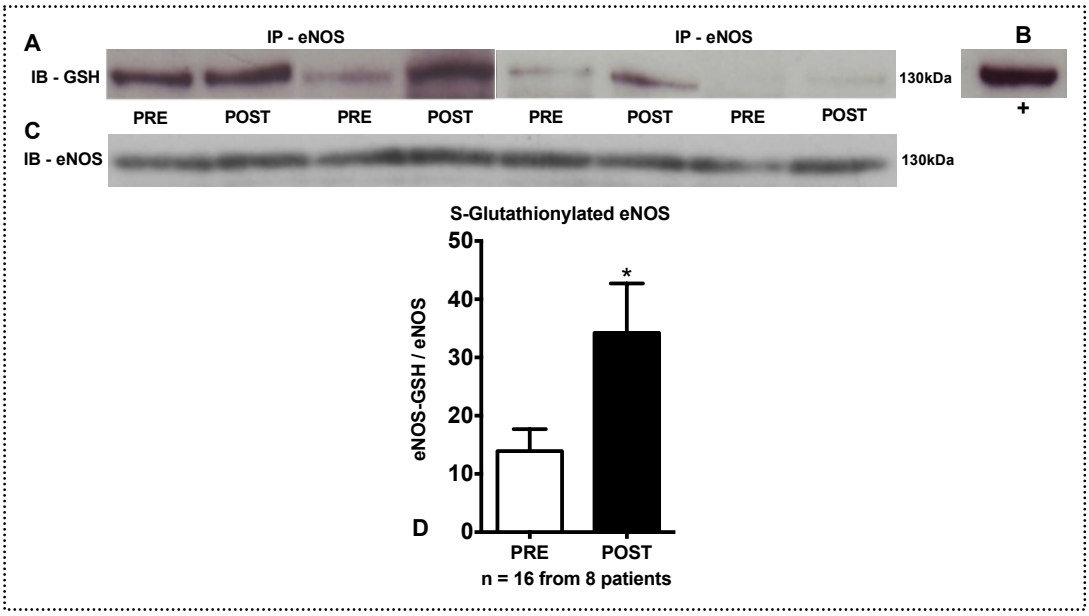
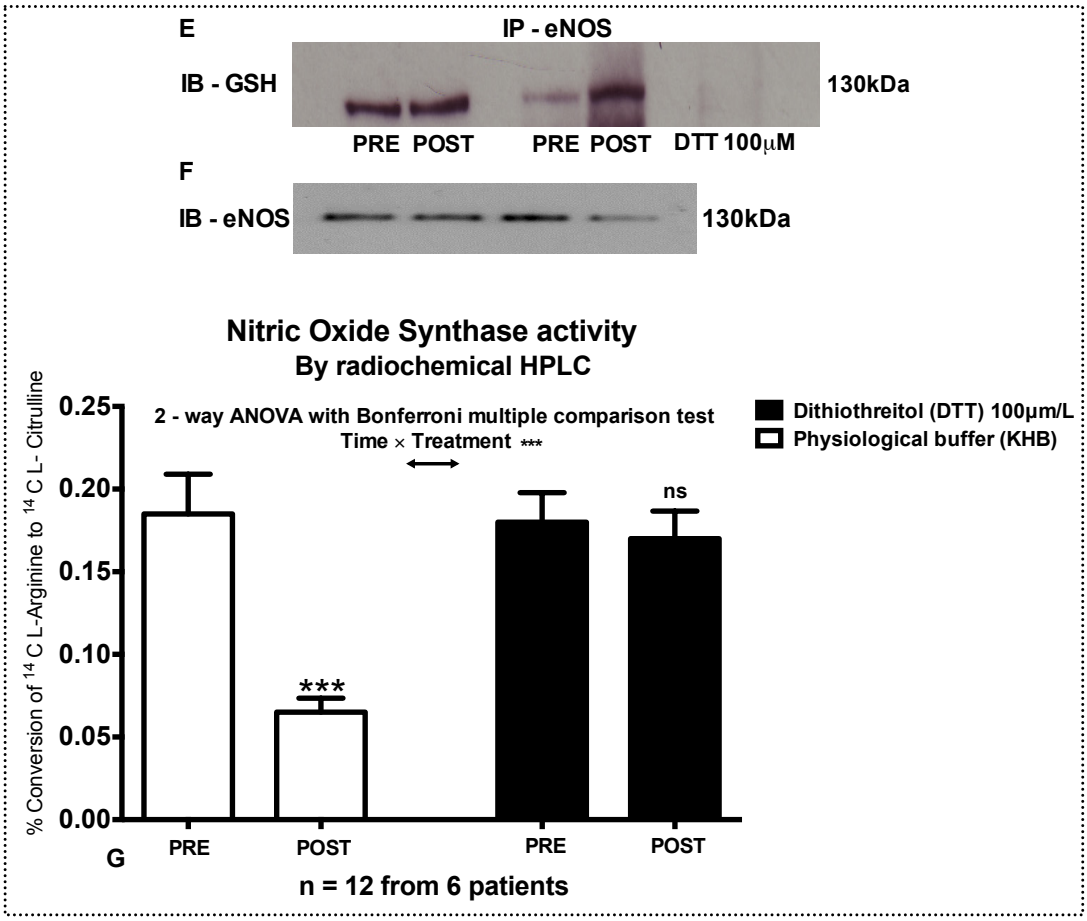


Figure 3.11: *Upper panel,* immunoblots of *S* - glutathionylated eNOS after immunoprecipitation (**A**) with $nNOS^{-/-}$ murine myocardial homogenate as positive control (**B**). Ratio of glutathionylated eNOS to eNOS (**C**) is increased after CPB and reperfusion **(D)**, by paired student's t test. Data expressed as mean ± SEM. * p <.05 vs.PRE. IP- Immunoprecipitate, IB – Immunoblot.

Bottom panel, Dithiothreitol reversed *S*-Glutathionylation and abolished GSH-eNOS band (**E**) as well as the PRE- POST difference in activity of NOS (**G**), providing further evidence for post translational modification of eNOS by *S*-Glutathionylation after CPB and reperfusion. Two-way ANOVA for repeated measurements with Bonferroni correction. *** p <.001 for the interaction between time (PRE and POST) and treatment. Data expressed as mean ± SEM. ns = p >0.05 vs.PRE. ns= not significant.



As *S*-Glutathionylation is a redox dependent post translational modification[432], decreasing the subcellular pools of superoxide release may reverse it and, therefore, recover NOS activity after CPB and reperfusion. As shown in **Figure 3.4**, NOX2 appears to be the main contributor to the increase in superoxide production after CPB and reperfusion; however, NOX2 inhibition had no significant effect on PRE and POST atrial NOS activity (**Figure 3.12**), suggesting that ROS generated by this oxidase system does not mediate *S* - glutathionylation of eNOS in patients undergoing on-pump cardiac surgery.

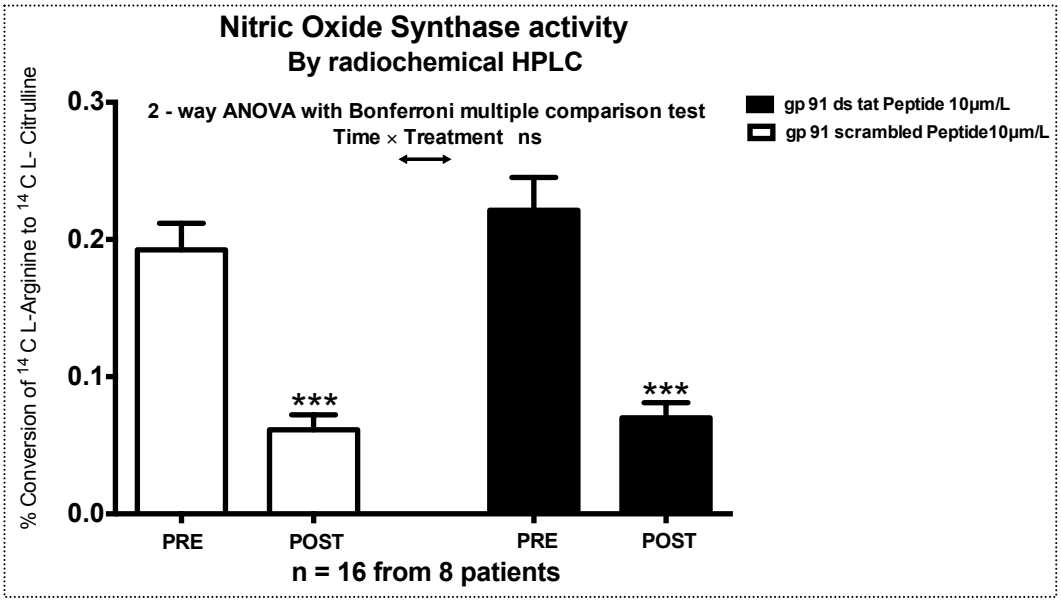


Figure 3.12: NOS activity in homogenates of right atrial samples obtained before (PRE) and after CPB and reperfusion (POST) following treatment with gp91ds tat and scrambled peptide. There was no significant effect of NOX2 inhibition on NOS activity before and after CPB and reperfusion confirming that POST atrial NOS activity remains significantly reduced. Two-way ANOVA for repeated measurements with Bonferroni correction. ns = p>0.05 for the interaction between time (PRE and POST) and treatment. *** *p* <.001. vs.PRE. Data expressed as mean ± SEM.

Discussion

The work presented in this chapter demonstrates that the mechanisms underlying myocardial NO- redox imbalance during elective on-pump cardiac surgery differ from the findings reported in an isolated rat heart preparation subjected to a prolonged I/R protocol[277]. In humans, *S* - glutathionylation of eNOS, rather than BH_4 deficiency, appears to be the main mechanism underlying the reduction in NOS activity after CPB and reperfusion. This conclusion is supported by a number of findings. First, CPB and reperfusion results in increased atrial superoxide production from mitochondrial complex I, functionally uncoupled NOS, and NOX2 containing NADPH oxidase. Second, the evaluation of mechanisms underlying functional uncoupling of NOS demonstrated a reduction in the bioavailability of BH_4 after CPB and reperfusion; this was due to down regulation of GTCPH-1 activity rather than to increased oxidation. Third, the reduction in NOS activity associated with CPB and reperfusion was not abolished by pretreatment of atrial homogenates with

BH_4. Finally, atrial levels of *S* - glutathionylated eNOS increased after CPB and deglutathionylation by DTT abolished the difference in both eNOS glutathionylated and eNOS activity associated with CPB and reperfusion.

Together, these findings identify NOS *S* - Glutathionylation as a target for interventions aiming to preserve NO atrial bioavailability in patients undergoing on-pump elective cardiac surgery.

Myocardial nitric oxide redox imbalance after CPB

Redox signaling refers to the process by which molecules involved in physiological, as well as pathological cellular signaling pathways, are modified by reactive oxygen species and reactive nitrogen species (ROS/RNS)[433]. Over the last two decades, data from experimental animal models implicate superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) formed by dismutation of O_2^- [433], NO and peroxynitrite[434] formed from the reaction of O_2^- with NO as the principal elements of redox signalling.

The physiological process of excitation-contraction (EC) coupling converts myocardial electrical activity to contraction and relaxation[435-437]. With the location of nNOS in distinct subcellular domains in proximity to the ryanodine receptor (RYR), SR Ca (2+) ATPase (SERCA2a) and the L-type Ca (2+) channel, NO modulates different facets of EC coupling such as signal transduction and ion channel function[438]. Under basal conditions, loss of NO from nNOS leads to prolongation of left ventricular myocyte relaxation by decreasing the phospholamban (PLB) phosphorylated fraction[439] whereas BH_4 dependent increase in myocardial nNOS activity leads to faster relaxation by increasing PLB phosphorylated fraction [440]. nNOS knockout mice show exacerbated adverse LV remodeling, increased arrhythmias, and depressed β-adrenergic reserve after myocardial infraction[384,441 442] whereas cardio myocyte-specific overexpression of nNOS was able to prevent the decline in myocardial contractility in a transverse aortic constriction model of heart failure[443]. On the other hand, there is accumulating evidence for the involvement of ROS in EC coupling both under physiological as well as pathological conditions by oxidative

modifications of ion channels, transporters and protein kinases[444]. Indeed, functional uncoupling of myocardial NOS and superoxide release can lead to disruption of normal cross-bridge kinetics, Ca2 + cycling, thereby prolonging myocardial relaxation time.[116,445]. Thus, an altered myocardial nitric oxide redox balance is associated with myocardial dysfunction under experimental conditions.

Following CPB, atrial superoxide production significantly increased with contribution from functionally uncoupled NOS. In conjunction with this, constitutive right atrial NOS activity and NO bioavailability decreased providing clear evidence of myocardial NO- redox imbalance associated with CPB and reperfusion. In animal models of I/R, peroxynitrite produced by the rapid reaction of NO and superoxide anion released after reperfusion appears to be the important oxidant source mediating post-ischaemic myocardial stunning and contractile dysfunction[446]. However, there was no change in right atrial peroxynitrite release or nitrotyrosine staining indicating that the nitrosative stress is not the primary mediator of myocardial dysfunction after CPB and reperfusion. Both mitochondrial and NADPH Oxidase have been implicated in I/R injury[333,433,447-452] and superoxide production from the two myocardial oxidases increased after CPB. It has been suggested that NADPH Oxidase derived ROS, may stimulate mitochondrial ROS production following many pathological processes[453-456]. In the same token, mitochondrial ROS also has also been shown to induce ROS production from NADPH Oxidase by a Protein kinase C dependent mechanism following hypoxia in pulmonary vessels [454,457,458]. In the absence of changes in well-defined mechanisms of activation of Rac-1 or protein expression of NOX isoforms, whether increase in mitochondrial ROS stimulated ROS production from NADPH Oxidase during CPB needs further investigation.

Mechanisms of myocardial nitric oxide redox imbalance after CPB

Tetrahydrobiopterin (BH_4) is synthesised de novo from guanosine triphosphate (GTP) in a rate limiting reaction catalysed by GTP cyclohydrolase I (GTPCH-1)[459]. By functioning as an allosteric modulator of arginine binding, facilitating

dimer assembly and electron transfer to haem iron, BH_4 catalyses NOS and synthesis of nitric oxide[460]. Oxidation of BH_4 to BH_2 and other oxidized biopterin species reduces its bioavailability for NOS, which results in functional uncoupling and superoxide release from the oxygenase domain[277].

Atrial BH_4 content decreased after CPB and reperfusion without a concomitant increase in oxidised biopterin species, BH_2 and biopterin. Given the lack of a measurable increase in atrial peroxynitrite release or nitrotyrosine staining, increased BH_4 oxidation is unlikely to explain the reduction in its bioavailability, although the side chain cleavage product, xanthopterin, was not measured in this study. Nevertheless in the work by Dumitrescu, C.*, et al.*[277]*,* where oxidation of BH_4 to xanthopterin was demonstrated following ischemia, BH_2 or Biopterin were undetectable. Moreover, a mean aortic cross clamp time of 43 minutes in my study resulted in only 32% reduction of BH_4 levels whereas global hypoxia for more than 30 minutes led to 95% loss of BH_4 by oxidation in the isolated rat heart preparation [277]. Furthermore, atrial GTPCH-1 activity was down regulated after CPB and reperfusion suggesting that the reduction in BH_4 content is likely to be secondary to decreased synthesis.

GTPCH - 1 activity is subject to feedback inhibition by BH_4 mediated by a regulatory subunit called GTP cyclohydrolase I feedback regulatory protein (GFRP) [461]. Under physiological conditions, GTPCH - 1 expression and activity determine the rate of BH_4 synthesis whereas GFRP may have a stronger regulatory role in pro inflammatory states and following oxidative stress[416,462]. In my samples atrial GFRP protein abundance increased after CPB and reperfusion in association with a reduction in GTPGH-1 activity. BH_4 deficiency is the most commonly reported mechanism associated with functional uncoupling of NOS, however, *ex vivo* supplementation with BH_4 (10 mmol/L) did not abolish the reduction in NOS activity associated with CPB, even though this concentration of BH4 is expected to saturate the binding site in the oxygenase domain of NOS [463]. In the absence of changes in atrial protein expressions of constitutive NOS isoforms, this observation provided evidence that mechanisms other than BH4 deficiency underlie functional uncoupling of NOS after CPB and reperfusion.

Under physiological conditions, the tripeptide (consisting of glycine, cysteine, and glutamic acid) glutathione (GSH) is the predominant non-protein thiol in the myocardium with less than 1% being present in the oxidised form (GSSG)[464,465]. *S*-Glutathionylation is a post-translational modification mediated by the insertion of GSH to thiolate anions of protein cysteines under physiological conditions as well as following oxidative and nitrosative stress or both [430,466,467]. The susceptibility of cysteine residues to *S*-Glutathionylation is determined by their location on the protein surface (and hence their accessibility) and by the proximity to amino acids such as arginine, histidine, lysine that determine its reactivity[468]. Thus, *S*-Glutathionylation is discrete, site specific and can regulate a variety of cellular signalling pathways by modulating protein function and preservation of thiol group function. Chen, C.A*., et al* reported that increased myocardial oxidative stress reflected by altered GSH/GSSG ratio induced *S*-Glutathionylation of eNOS resulting in functional uncoupling and superoxide release from its reductase domain[431]. Consistent with this finding, it was observed that *S*-Glutathionylation of atrial eNOS increased after CPB and reperfusion, providing evidence for an alternative mechanism underlying NOS dysfunction. Furthermore, deglutathionylation with dithiothreitol (DTT) abolished the difference in both eNOS glutathionylation and NOS activity associated with CPB. Idigo WO, et al.have shown that, nNOS null mice also exhibit eNOS uncoupling secondary to increased *S*-Glutathionylation [432]. Whereas eNOS coupled status was restored by inhibition of xanthine oxidase activity in the nNOS knockout myocardium, inhibition of NOX2 (the oxidase system accounting for most of the increase in atrial superoxide post CPB) did not recover NOS activity. The role of other myocardial oxidases in increasing atrial eNOS *S*-Glutathionylation after CPB and reperfusion needs to be explored further.

By the same token, in the presence of BH_4 deficiency, superoxide release from the NOS oxygenase domain can cause *S*-Glutathionylation of eNOS[469] whereas *S*-Glutathionylation of eNOS can lead to BH_4 oxidation by increasing superoxide release from the synthase's oxygenase domain[469] . These mechanisms, however, are unlikely to have played a significant role in the setting of my

experiments, since BH4 oxidation was not increased and BH4 supplementation did not prevent NOS uncoupling post-reperfusion.

Conclusions

This work presented in this chapter demonstrates that NOS *S*-Glutathionylation, rather than BH4 depletion, accounts for NOS dysfunction in patients undergoing cardiac surgery on cardiopulmonary bypass and imply that in this patient cohort, BH4 supplementation is not effective in restoring atrial bioavailability of nitric oxide. Currently, there are no class I recommendations to prevent functional consequences of I/R such as myocardial stunning during on-pump cardiac surgery[58]. Whether redressing atrial oxidative stress by deglutathionylation of eNOS will preserve NO-redox balance and prevent perioperative myocardial dysfunction remains to be established.

Supplement

1. Patient information leaflet

UNIVERSITY DEPARTMENT OF CARDIOVASCULAR MEDICINE
OXFORD UNIVERSITY HOSPITALS NHS TRUST, OXFORD, OX3 9 DU
Patient information
***(Version 3- May 2013)* REC Ref No: 07/Q1607/38**
Study title: Role of oxidative stress in the human myocardium

You are invited to take part in a research study. Before you make a decision it is important for you to understand why the research is being done and what it would involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

What is this study about?
The purpose of this study is to investigate the role of oxidative stress in the human heart. In other words we wish to study the cardiac effects of the imbalance between the production of reactive oxygen and the tissue ability to detoxify the reactive intermediates and repair the resulting damage. In humans, oxidative stress is involved in many diseases (e.g., atherosclerosis, diabetes, atrial fibrillation) and may also be important in ageing. However, reactive oxygen species can also be beneficial, as they contribute to the normal function of the cardiovascular system and are involved in protecting the heart from an ischaemic insult (i.e., from a "heart attack")

It has been shown that common cardiac conditions such as atrial fibrillation, heart failure and hypertension are associated with increased oxidative stress. However, a complete understanding of the mechanisms by which oxidative stress may contribute to the development of heart disease remains unclear.

To address these issues, we plan to examine the contribution of several sources of reactive oxygen species in the human myocardium and their role in regulating the function and excitability of cardiac cells, which we isolate from small surgical samples of cardiac tissue.

Which patients will take part in the study?
All male and female patients undergoing cardiac surgery, who are older than 18 years and able to give informed consent.

What will the study involve?
1. Your permission to collect samples of cardiac tissue that are routinely removed in the course of cardiac surgery. Normally, these samples are discarded and incinerated.

2. Your permission to collect a small sample of cardiac tissue from *i)* the left upper chamber of the heart (or left atrial appendage) or *ii)* a further sample from the right atrial appendage at the end of surgery or iii) samples from the upper chambers of the heart (as above) if you are scheduled to have off-pump cardiac surgery. These samples are not routinely removed in the course of surgery; however, if you were willing to donate this tissue, it would provide us with very valuable information on *i)* the mechanisms responsible for differences between the left and right side of the heart in response to stress and irregular heart rhythm and *ii)* on the impact of surgery on your heart muscle.

Note that you have the option of taking part in the study without accepting to donate samples of tissue that would be specifically removed for research purposes (as listed in 2.).

3. An ultrasound (Echocardiogram) of your heart done before the surgery. This is a safe and painless procedure that takes about 30 minutes. You would be asked to lie on a couch on your left side. A probe would be placed on your chest and a lubricating jelly would be used so that the probe makes good contact with the skin. Ultrasound waves then create images of your heart on the scanner monitor. We would be repeating this study 5 days after the surgery.

4. Collection of blood sample- We would collect approximately 20ml of blood (4 teaspoon) before and during surgery to analyse markers of inflammation/oxidative stress. We would also use this sample to analyse genetic variants and their relation to development of inflammation/ oxidative stress.
The study does not involve extra visits to the Hospital.

Are there any risks to taking part in this study?
No. As the extra risks in taking part in this research are very small, no specific compensation for injury is offered.

Will any genetic tests be done?
Yes, we intend to extract DNA from your blood sample and investigate how common genetic variants may affect the way in which the tissue produces or neutralises reactive oxygen species. These genetic variants are generally common and do not give rise to specific diseases.
We do not propose to test for specific inherited genetic diseases. It is also possible that some samples will be used for future genetic research. This research may be conducted by the study research team or by commercial research teams, however further ethical review would be sought prior to such testing. The samples will be stored in an anonymous format but a separate record of who donated the samples will be kept so that we can relate any findings to your medical history. These records also mean that if you decided to withdraw from the study at any time, we would be able to destroy your samples, if you wished. The results of these genetic studies are very unlikely to have significant personal implications.

Do I have to take part?
No. It is up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. After that, you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Will I benefit by taking part in this study?
This study will not benefit you directly but will help us to learn more about the role of oxidative stress in the human myocardium with the hope of improving the future management of atrial fibrillation and heart failure.

Will the information obtained be kept confidential?
If you join the study, some parts of your medical records and the data collected for the study will be looked at by authorised persons from the University of Oxford and relevant NHS Trust to check that the study is being carried out correctly. They all have a duty of confidentiality to you as a research participant and nothing that could reveal your identity will be disclosed outside the research site.

Will I be contacted in the future?

You will not be contacted in the future.

What will happen to the sample after the study has finished?
We expect all samples to be fully used by the end of the study. If not - as a part of a consent form - you will be asked if you agree to give your cardiac sample as a 'gift' to be stored by the University of Oxford for use in future research into common cardiac diseases, as new tests become available. You would not be contacted further about these samples, though approval for any new research would be obtained from an ethics committee. If you decide that you do not want your samples to be involved in further studies, they will be destroyed at this point.

Can I change my mind about being in the study?
Yes, if you decide to take part, you are still free to withdraw at any time without giving a reason. The samples would then be destroyed and the data obtained from the study would not be included in any reports. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive in any way.

What if there is a problem?
If you are harmed and this is due to someone's negligence you may have grounds for legal action for compensation against the University of Oxford (in respect of any harm specifically arising from the participation in this study) or the NHS (in respect of any harm which has resulted from the clinical procedure being undertaken).

Other information
There may be many questions you wish to ask either whilst considering to take part in the study or during the study itself; the research team and medical team will always be happy to answer your questions. You can also contact:

Dr. Raja Jayaram
Clinical Research Fellow in Cardiovascular Medicine
Raja.Jayaram@cardiov.ox.ac.uk

Professor Barbara Casadei
BHF Professor of Cardiovascular Medicine

Contact address:
University Department of Cardiovascular Medicine,
Level 6, West Wing, John Radcliffe Hospital
Oxford OX3 9DU
Tel +44 (0) 1865 234 671/660 ; Fax +44 (0) 1865 234667

2. Consent sheet

UNIVERSITY DEPARTMENT OF CARDIOVASCULAR MEDICINE

OXFORD UNIVERSITY HOSPITALS NHS TRUST, OXFORD, OX3 9DU

Role of oxidative stress in the human myocardium (07/Q1607/38); Researchers: Prof B Casadei, Dr R Jayaram, Dr S Reilly

CONSENT SHEET

Tick/Initial the boxes

1.I confirm that I have read and understand the information sheet for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactory. ☐

2.I agree to give tissue samples that are routinely removed during my surgery for research in this project. I understand that my participation is voluntary and that I am free to withdraw the consent at any time, without giving a reason, without my medical care or legal rights being affected. ☐

3.I understand that relevant sections of any of my medical notes and data collected during the study may be reviewed by the researchers named above and by authorised individuals from the University of Oxford and relevant NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4.I understand that the risk in taking part in this research is small and that the study will not benefit me directly. ☐

5.I understand that my blood & tissue samples will be used in research that aims to understand the role of common genetic variants on cardiac properties, but that the results of these investigations are unlikely to have any implications for me personally. ☐

6. I agree to take part in the study. ☐

The following is optional and will not affect your ability to take part in this study:

7.I also agree to give samples of my heart tissue that will be removed purely for research purposes (from the left upper chamber or an additional sample from right upper chamber after coming off the heart-lung machine or from the upper chambers during off pump cardiac surgery) along with blood samples, and to undergo an ultrasound examination of the heart. I understand that my participation is voluntary and that I am free to withdraw the consent at any time, without giving a reason, without my medical care or legal rights being affected. ☐

8.I agree for the samples to be given as a 'gift' to be held by the University of Oxford for research into the role of oxidative stress in the human myocardium as new tests become available. I understand that I would not be contacted further about this, though approval for any new research would be obtained from an ethics committee. ☐

Name of patient: _____ **Date** _____ **Signature** _____

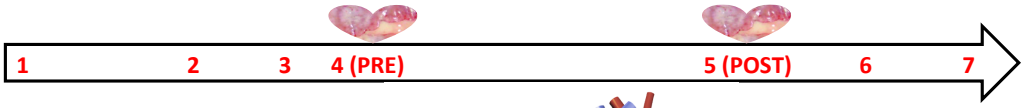
Name of person taking consent: _____ **Date** _____ **Signature** _____

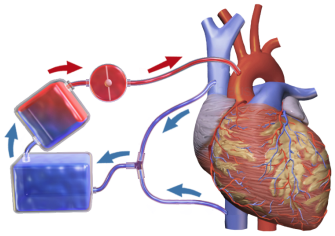
3. Study design

Study design

Elective Coronary surgery, Aortic valve replacement or both on cardio pulmonary bypass (CPB)

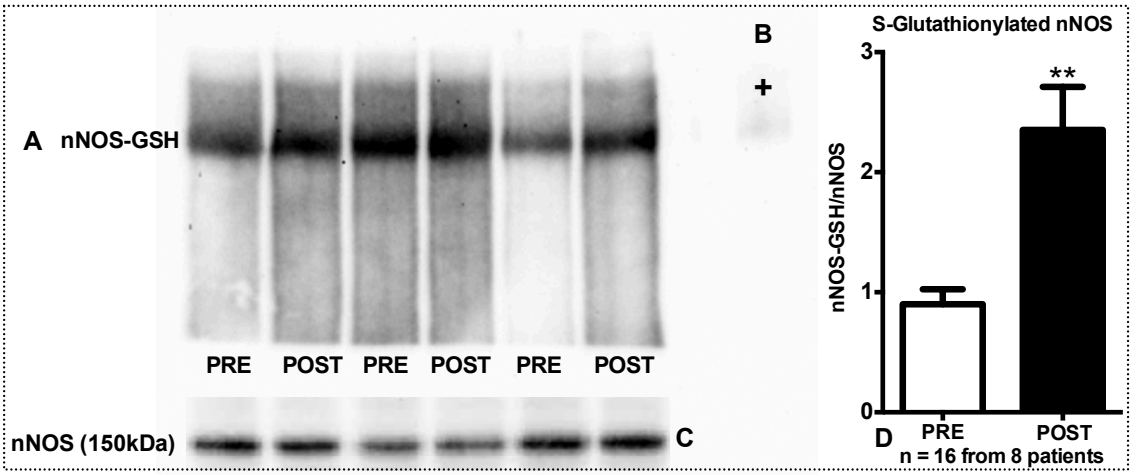
(n = 116)





1. Informed consent.
2. Record medical and medication history.
3. Blood sample for plasma and serum.
4. Right atrial appendage sample prior to commencement of CPB.
5. Right atrial appendage sample after CPB and coronary reperfusion.
6. Blood sample after protamine administration for plasma and serum.
7. Record aortic cross clamp and CPB time, details of surgical procedure and post operative outcomes.

4. Atrial levels of *S*-Glutathionylated nNOS is increased after CPB and reperfusion



Immunoblot of *S* - glutathionylated nNOS after immunoprecipitation (**A**) with murine brain homogenate as positive control (**B**). *S* - glutathionylated nNOS to nNOS (**C**) is increased after CPB and reperfusion **(D).** By paired student t – test. Data expressed as mean ± SEM. ** p <.01 vs.PRE.

CHAPTER 4

STARR

STATIN **T**HERAPY IN **A**TRIAL **R**EFRACTORINESS AND **R**EPERFUSION INJURY

Background and rationale

Atrial fibrillation (AF) is a common arrhythmia after cardiac surgery[63,470-473], associated with increased morbidity, mortality[474-476] and significant additional cost to patient care[470,477].Though multiple factors have been implicated in its pathogenesis[478], emerging evidence suggests that inflammation [75,79,479-483] and myocardial oxidative stress may play an important role[98,99].

Reactive oxygen species (ROS), particularly superoxide, are known to affect cardiac electrophysiological[104,484,485] and contractile properties[486]. Increased myocardial ROS production has also been associated with increased requirements for postoperative inotropic support and prolonged hospitalization after elective cardiac surgery[99]. Interventions that have been effective in reducing myocardial oxidative stress in animal models of atrial tachypacing have prevented myocardial electrophysiological remodelling and AF inducibility, at least in the short term[91,94], suggesting that myocardial sources of ROS may be an important target for therapeutic interventions. However, systemic antioxidant treatment for the prevention of AF has been unsuccessful to date[487,488]. Whilst the reasons for this failure are not clear, increasing evidence suggests that, because of the inherent high-reactivity and short half-life of ROS, myocytes could have evolved mechanisms for localizing ROS production to subcellular domains; similar to spatial localization of nitric oxide synthases and NO signaling pathways [489-492].Consistent with this hypothesis, NOX2-containing NADPH oxidase has been found to be the main source of ROS in the human atrial myocardium[493] and its activity (but not plasma markers of lipid and protein oxidation) is an independent predictor of the occurrence of postoperative AF in patients undergoing cardiac surgery[99,494]. These findings suggest that targeting specific pools of superoxide production may be a better therapeutic strategy than systemic antioxidant interventions for the prevention of new onset AF and low cardiac output syndrome after cardiac surgery.

Inhibition of 3-Hydroxy-3-methyl glutaryl-coenzyme A reductase by statins[495] reduces synthesis of both LDL cholesterol and isoprenoids that are involved in the activation of small guanosine triphosphate (GTP) ase signaling

molecules[496,497]. By preventing isoprenylation of the small G protein Rac1[179,180], statins exert antioxidant effects by suppressing superoxide release from the NOX2-NADPH Oxidases [99,181,182]. In addition, statins have been shown to increase nitric oxide (NO) bioavailability by stimulating both the activity and the protein expression of NO synthases[183,184], increasing intracellular BH_4 bioavailability[498] and, possibly, by reducing the plasma level of asymmetric dimethylarginine [499] and the expression of arginases[500] . In animal models, acute treatment with statins has been shown to have significant cardioprotective effects that are dependent on nitric oxide and ROS production [501-506]. While the beneficial effects of sustained LDL-cholesterol lowering by statins are well established[507], it is unclear whether their putative anti-inflammatory[508], antioxidant and cardioprotective properties[509] impact perioperative outcomes after elective cardiac surgery[510].

To gain further mechanistic insights in this matter, I have carried out a randomised, placebo controlled, double-blind study (STARR: Statin Treatment on Atrial Refractoriness and Reperfusion injury; clinicaltrials.gov NCT01780740) that investigated whether perioperative treatment with atorvastatin (80 mg daily, started up to 6 days before surgery and continued for 5 days after) affected atrial electrical properties (as evaluated by serial measurements of the atrial effective refractory period, AERP) and prevented the atrial nitroso-redox imbalance following reperfusion in 80 patients scheduled to receive elective cardiac surgery on CPB.

Methods

The south central Berkshire research ethics committee provided ethical approval for the study. Clinical trial authorisation (CTA) was obtained from the Medicines and Healthcare products Regulatory Agency (MHRA) with Eudra CT ref no: 2009-013228-21. All patients recruited in the study provided informed consent.

Trial Participants

Adult patients in sinus rhythm undergoing their first elective cardiac surgery (coronary bypass surgery, aortic valve replacement or both) on CPB in the Department of Cardiothoracic Surgery at John Radcliffe Hospital in Oxford were

screened for inclusion in the study. Exclusion criteria were: age >85 yrs, preoperative creatinine levels >200 umol/L, treatment with antiarrhythmic agents other than beta-adrenergic receptor blockers, pregnancy or lactation or planning pregnancy during the course of the study, women of childbearing potential without appropriate contraceptive measures, history of obstructive hepato-biliary disease or other serious hepatic disease or pre-operative ALT >2-fold the upper limit of normal or alcohol abuse, untreated hypothyroidism, family history of hereditary muscle disorders, known intolerance to statins or history of muscle toxicity with fibrates or statins, ongoing use of fibrates or niacin or of agents that are strong inhibitors of cytochrome P-450 or the P-glycoprotein within a month preceding randomization.

During CPB, myocardial protection was achieved with cold blood cardioplegia and moderate systemic hypothermia in all patients. Postoperative management was according to the standard protocols of the cardiothoracic critical care unit.

Randomisation

Consenting patients were randomised to atorvastatin or placebo following cessation of prior statin therapy, when appropriate, by using a mixed randomisation approach[511] that combines a simple with a permuted-block sequence of 8, 10 or 12 patients (three of each).The simple sequence comprised of an uneven block of 10 patients with a pre specified inequality between allocation to atorvastatin and placebo.

Outcomes

The primary outcome measures in STARR were postoperative changes in atrial effective refractory period (AERP) and superoxide production in paired samples of the right atrial appendage obtained before CPB and after reperfusion. Secondary endpoints of the study included: atrial nitric oxide synthases (NOS) activity, the relative contribution of individual atrial oxidases to atrial superoxide release, and systemic markers of inflammation or oxidative stress.

Sample collection

Samples of the right atrial appendage were taken before the onset of CPB (PRE) and commencement of cardioplegia and soon after cardiac reperfusion (POST). Blood samples were collected at randomisation and 72 and 120 hours after surgery. Plasma and serum were separated by centrifugation at 1300g for 10 minutes at room temperature.

Measurements

Further details on the measurements below can be found in Chapter 2.

The AERP was measured daily after surgery (up to post-operative day 4) using a programmed stimulation protocol delivered by the Medtronic Pacing System Analyser 2090 via a Medtronic pacemaker connected to the right atrial epicardial pacing wires that are routinely inserted by the surgeons at the time of surgery.

Holter ECG monitoring was carried out during the first five days after surgery (Life card CF; Space labs healthcare, Washington, USA). ECG recordings were downloaded using the "Sentinel" software (Space Labs Healthcare) onto the diagnostic system "Pathfinder Digital" (Space Labs Healthcare), which is equipped with an automated AF detection algorithm.

Echocardiographic studies were performed using X5-1 transducer and Philips iE33 ultrasound system (Phillips medical systems). From the 3D images, LA Volume and Ejection fraction were computed. Tissue Doppler imaging (TDI) was performed in all patients according to the guidelines of the American Society of Echocardiography [421]. In addition, on digitally stored images, myocardial longitudinal strain measurement was assessed with speckle tracking analysis using the image analysis software "Tom tec" (Tomtec Imaging Systems, Munich, Germany).

Atrial superoxide production was measured by 2 hydroxyethidium (2-OH- E+) detection by high-performance liquid chromatography and results are shown as the tiron-inhibitable fraction (see Chapter 2). To elucidate the contribution of specific oxidases to basal atrial superoxide production, samples were pre treated with the following inhibitors: N_{ω} - nitro-L-arginine methyl ester and its

dextro isomer (L-NAME and D-NAME; 1 mmol/L), gp91-ds-tat peptide and the respective scrambled peptide (10 μM/L).

Tetrahydrobiopterin (BH_4), and its oxidized products 7,8 dihydrobiopterin (BH_2) and biopterin (B) were measured by electrochemical (for BH_4) and fluorescence detection (for 7,8-BH_2 and B) in homogenized atrial tissue, following sample separation by high-performance liquid chromatography.

NOS activity was measured in atrial tissue homogenates using radiochemical HPLC detection of ^{14}C labelled L- arginine to L-citrulline conversion and expressed as the L- NAME inhibitable fraction of L-citrulline as a percentage of total L-arginine.

The activity of the rate-limiting enzyme in the synthesis of BH4, GTP cyclohydrolase 1 (GTPCH) was measured in atrial homogenates by iodine oxidation and detection of neopterin content by HPLC.

Plasma LDL cholesterol was measured using Beckman Coulter AU680.

Statistics

As there are no previous studies analyzing the primary and secondary outcomes of this study, an estimate of the effect size of the intervention on the parameters under investigation or their standard deviation in the placebo arm was not possible. The sample size of n= 80 was chosen arbitrarily taking into account the study feasibility over the regulatory approval period.

A non- linear regression analysis using a straight-line model was fitted through the AERP data of both atorvastatin and placebo-treated patients over four postoperative days. The extra-sum-of-squares F test compared the goodness-of-fit of two alternative nested models; a global model where slope is shared among the data sets with a model where each dataset has its slope.

Comparisons of normally distributed tissue-based measurements between treatments and before and after reperfusion were carried out either by paired t-test or using two-way analysis of variance (ANOVA, repeated measurements x treatment) with Bonferroni correction. Non-normally distributed data were subjected to either logarithmic or square root transformation prior to analysis.

The Chi-square test was used to compare dichotomous variables between the groups. Changes in LDL cholesterol levels in the perioperative period were analyzed by Analysis of covariance (IBM SPSS Statistics for Macintosh, Version 22.0.Armonk, NY: IBM Corp). Missing LDL cholesterol values were imputed using multiple imputation, generating 10 imputed data sets, with subsequent estimates of test statistics from each of the data sets being combined using the established methods of Rubin (StataCorp. 2013. *Stata Statistical Software: Release 13*. College Station, TX: StataCorp LP). All other statistical analyses were performed using Graph Pad Prism version 6.00 for Mac (Graph Pad Software, San Diego California USA). The null hypothesis was rejected at two-tailed $p < 0.05$.

Results

Study population

Between January 2012 and February 2013, 80 patients undergoing elective cardiac surgery for CABG, AVR or combined CABG and AVR on CPB were recruited in Oxford University Hospitals NHS Trust. Participants were assigned randomly to either Atorvastatin 80mg or placebo (**Figure 4.1**). There was no difference between the two groups in the number of days from randomisation to surgery. 79% of patients were on prior treatment with statins.
The median pre - and postoperative duration of randomised treatment in both the groups were two and five days, respectively (**Table 4.1**).

	Atorvastatin	Placebo
Preoperative treatment length		
Median (IQR)	2(1,5)	2(1,3)
Mean (SD)	2.5(1.8)	2.6(1.7)
Post operative treatment length		
Median (IQR)	5(2,5)	5 (2.8,5)
Mean (SD)	3.7 (1.6)	4(1.4)

Table 4.1: STARR **-** Pre- and post-operative treatment length**.**
IQR - Interquartile range; SD - Standard deviation.

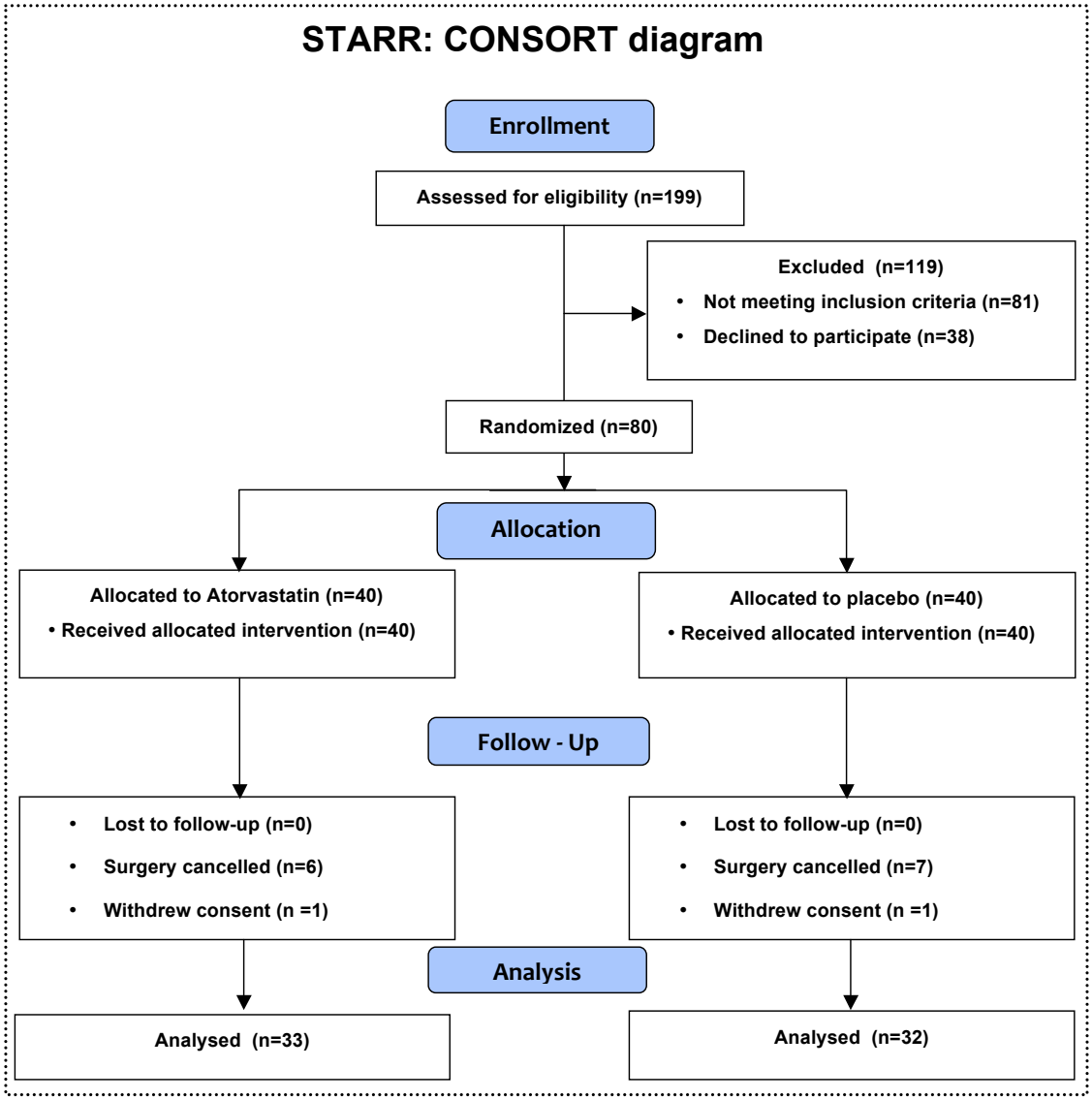


Figure 4.1: Consolidated standards of reporting trials flow diagram
STARR – Statin Therapy in Atrial Refractoriness and Reperfusion injury

As shown in **Table 4.2**, there was no difference in the demographic indices, risk factors profile (Logistic Euroscore), echocardiographic measurements, medical and medication history between the two groups. Peri- and postoperative management of patients in both groups was similar, and there was no difference in the recorded postoperative clinical outcomes (**Table 4.3**).

	Atorvastatin (40)	Placebo (40)
Demographic and clinical variables		
Age, years	64.9(10.9)	64.6(9.4)
Women	8(20%)	7(18%)
Current smoker	7(18%)	6(15%)
Height (cms)	172.3(9.2)	170.3(10.7)
Weight (kg)	82.9(16.4)	83.4(2.4)
Logistic Euroscore	3.1(2.2)	3.2(2.4)
Left ventricular ejection fraction in %	61.4(9.9)	61.9(10.7)
Left atrial volume index in mL/m2	29.7(11.1)	26.2(9.3)
E/A ratio	0.77(0.19)	1.03(0.66)
Medical history n (%)		
Hypertension	26(65)	34(85)
Myocardial infarction	5(12.5)	5(12.5)
Stroke/transient ischaemic attacks	1(2.5)	1(2.5)
Peripheral arterial disease	0	1(2.5)
Heart failure	0	1(2.5)
Chronic obstructive pulmonary disease	6(15)	2(5)
Diabetes mellitus	10(25)	10(25)
Chronic kidney disease	0	0
Medication history n(%)		
Beta blockers	20(50)	23(57.5)
NSAIDs/Steroids	1(2.5)	1(2.5)
Insulin	0	0
Antiplatelets	20(50)	24(60)
Anticoagulants	0	1(2.5)
Calcium channel blockers	8(20)	14(35)
ACEi/ARB	25(62.5)	26(65)
Nitrates	10(25)	6(15)
Potassium sparing diuretics	0	0
Loop/Thiazide Diuretics	7(17.5)	8(20)
Statins	30(75)	33(82.5)

Table 4.2: Baseline characteristics by randomized treatment allocation.
Data are mean (SD) or n (%) shown; E/A – Early to late ventricular filling velocities; NSAID- Non steroidal anti-inflammatory drugs; ACEi- Angiotensin converting enzyme inhibitors; ARB- Angiotensin receptor blocker.

	Atorvastatin (34)	Placebo (33)
Surgery n (%)		
CABG	16(47.1)	23(69.7)
AVR	13(38.2)	10(30.3)
CABG+AVR	5(14.7)	0
Intraoperative variables (mean/SD)		
Cardiopulmonary bypass time (minutes)	88.15(25.08)	81.09 (23.66)
Aortic cross clamp time (minutes)	57(21.70)	46.3(12.59)
Perioperative interventions n (%)		
Intra-operative defibrillation	4(12.1)	2(6.3)
Use of internal/external pacemaker	16(48.5)	17(53.1)
Use of intra-aortic balloon pump	0	0
Use of vasopressors/inotropes	8(24.2)	7(21.9)
Surgical re-exploration	2(6.1)	0
Renal replacement therapy/dialysis	0	0
Perioperative management (mean/SD)		
Duration of ventilatory support (hours)	5.9(0.84)	5.4(1.3)
Duration of ICU stay period (days)	2.1(0.51)	2.0 (0.39)
Postoperative treatments n (%)		
Beta blockers	29(87.9)	31(96.9)
Antiplatelets	1(3.03)	1(3.13)
NSAIDs/Steroids	2(6.06)	0
Potassium supplements	33(100)	30(93.4)
Blood/blood products	8(24.2)	4(12.5)
Non-study statins	0	0
ACEi/ARB	30(90.9)	31(96.9)
Amiodarone	12(36.4)	7(21.9)
Digoxin	1(3.03)	1(3.1)
Diuretics including potassium sparing drugs	30(90.9)	28(87.5)
Calcium channel blockers	9(27.3)	4(12.5)
Postoperative outcomes n (%)		
Atrial fibrillation*	14(42.4)	10(31.3)
Arrhythmias other than AF	4(12.1)	0
Low cardiac output syndrome	10(30.3)	8(25)
Pleural effusion	4(12.1)	5(15.6)
Stroke	0	1(3.1)
Myocardial Infarction	0	0
Heart failure	0	2(6.3)
Infection	4(12.1)	1(3.1)
Acute kidney injury	3(9.1)	2(6.3)
Death	0	0

Table 4.3: Peri-operative management and postoperative clinical outcomes
ICU - Intensive care unit. * diagnosed by analysis of continuous holter recordings.

Primary outcomes

Effect of Atorvastatin on postoperative right AERP:

Right AERP was measured serially over the first four postoperative days using a programmed electrical stimulation protocol at 3 different pacing cycle lengths (500, 600 and 700 milliseconds) in 29 patients. The details of the measurements including reasons for missing data are shown in **Table 4.4**.

Number of patients randomised to study medications	**80**
Number of patients who underwent surgery and followed up until discharge	**65**
Number of patients who underwent the AERP study protocol	**29**
Number of possible measurements over 4 post operative days/PCL	**116**
Final set of measurements	
PCL 500	**92/116**
PCL 600	**80/116**
PCL 700	**62/116**
Reasons for missing AERP measurements	
Basal heart rate higher than corresponding pacing cycle length	
Post operative atrial fibrillation	
Technical issues - Pacing wire dislodgement/non capture/? Ventricular capture	
Patient factors; Non availability, Non compliance, Premature discharge from Oxford University Hospitals NHS Trust and transfer to DGH	
Number of patients without AERP measurements	**36**
Non availability of Medtronic PSA and lead adaptor kit	**11**
Post operative atrial fibrillation	**21**
Technical issues - Pacing wire dislodgement/non capture	**2**
Complete heart block after AVR	**2**

Table 4.4: Measurement of atrial effective refractory period
PCL- Pacing cycle length; AERP – Atrial effective refractory period; PSA - Pacing system analyser; NHS- National health service; DGH – District general hospital.

As shown in **Figure 4.2**, the AERP tended to lengthen over the first four postoperative days and did not differ between the treatment groups. There was also no significant difference between the slopes of the linear regression lines of best-fit representing postoperative changes in AERP in the two groups across the three pacing cycle lengths. Together, these data indicate that inflammation and oxidative stress after cardiac surgery and CPB do not result in shortening of AERP and the latter is not affected by perioperative atorvastatin treatment.

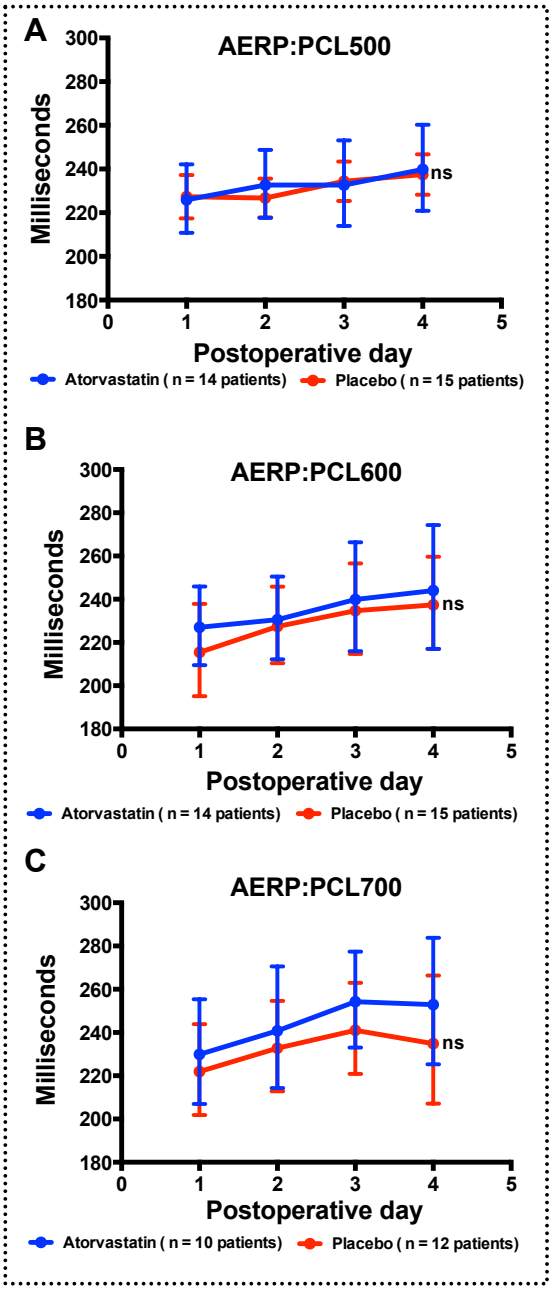


Figure 4.2: Average right atrial effective refractory period on first four postoperative days at three pacing cycle lengths (**A-C**). ns = p > 0.05 vs. Atorvastatin for the comparison of independent lines of fit with a global fit sharing slopes by extra-sum-of-squares F test. Data expressed as geometric mean ± 95% confidence interval.

Effect of Atorvastatin on superoxide release before and after CPB:

Superoxide release was measured in atrial samples before and after CPB and reperfusion by detecting 2 - OH - (E+) using HPLC. **Figure 4.3** show that the treatment with atorvastatin abolished the increase in myocardial superoxide production after CPB and reperfusion.

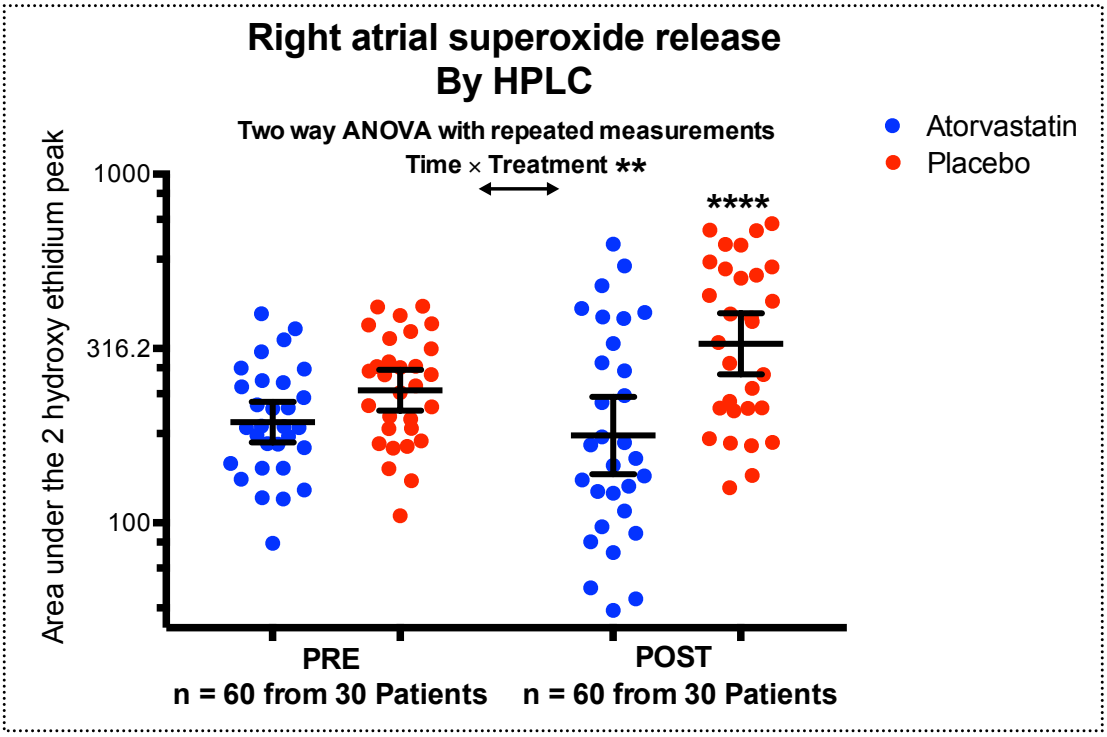


Figure 4.3: Average superoxide production in homogenates of the right atrial appendage taken before (PRE) and after CPB and reperfusion (POST) detected by HPLC.** *p* <.01 for the interaction between time (PRE and POST) and treatment, **** p <0.0001 vs. Atorvastatin. Two-way ANOVA for repeated measurements after log-transformation with Bonferroni correction. Data were expressed as geometric mean ± 95% confidence intervals.

Secondary outcomes

Effect of Atorvastatin on myocardial enzymatic sources of superoxide release:

In the previous chapter, I had shown that functionally uncoupled nitric oxide synthase (NOS) and NOX2-NADPH Oxidase derived superoxide contributed to increased superoxide production after CPB. To assess the effect of atorvastatin treatment on NOS function, superoxide release was measured in right atrial samples obtained before and after CPB and reperfusion following treatment with L-NAME and D-NAME (**Figure 4.4, AD).** In the presence of L-NAME, superoxide production was higher in atrial samples obtained before CPB and lower in samples obtained after CPB and reperfusion in the placebo group. (**Figure 4.4E**). These findings indicate that, before CPB and reperfusion, a significant fraction of atrial superoxide production is either scavenged or inhibited by NO; by contrast, after CPB and reperfusion, NOS appears to

contribute to the overall increase in atrial superoxide production, suggesting that the activity of the synthase is uncoupled under these conditions. On the other hand, treatment with Atorvastatin abolished the difference in atrial superoxide production observed in the presence of L-NAME in the placebo group after CPB and reperfusion (**Figure 4F**). This finding indicates that perioperative administration of Atorvastatin preserved coupled function of NOS during on-pump cardiac surgery.

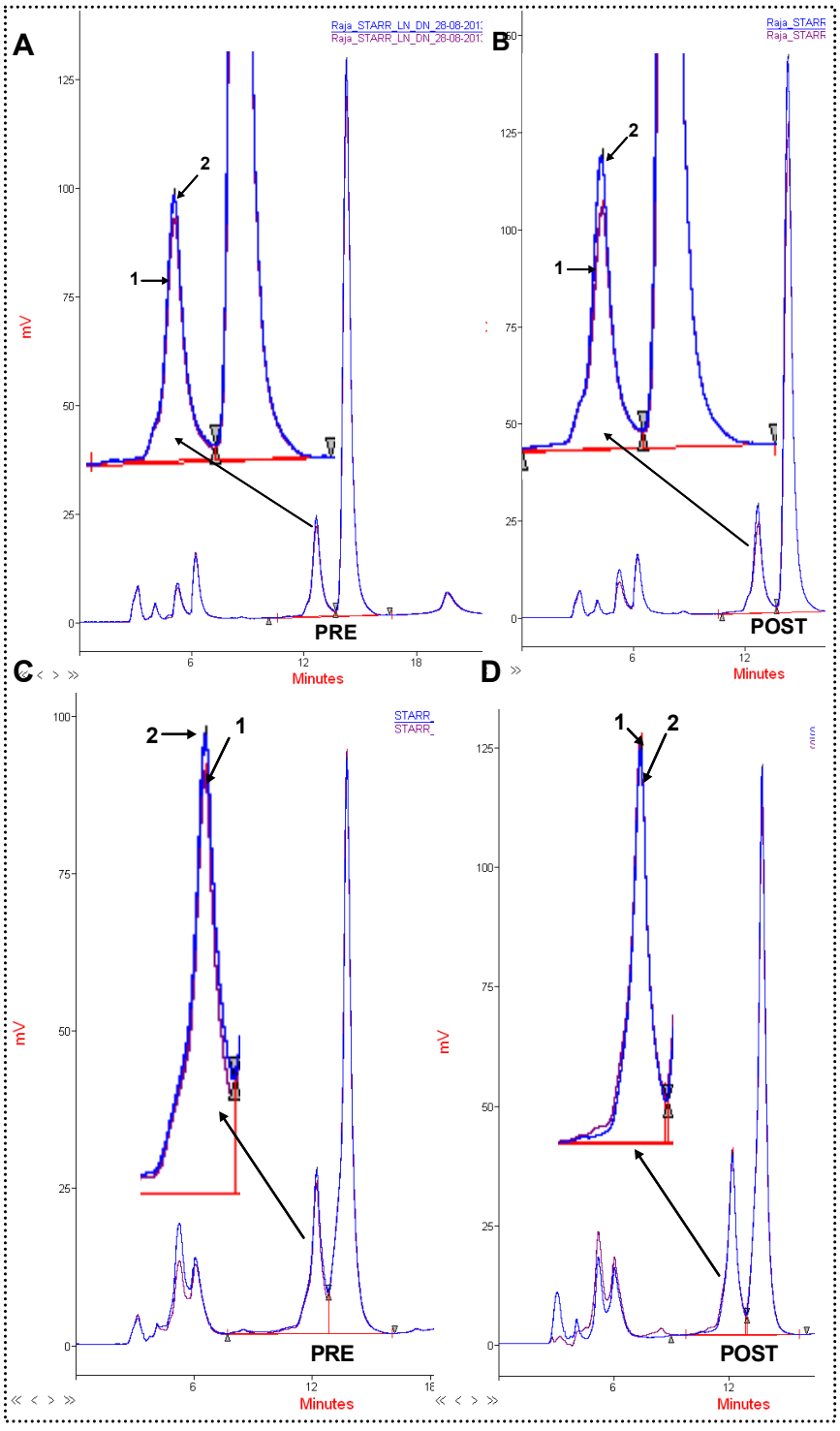
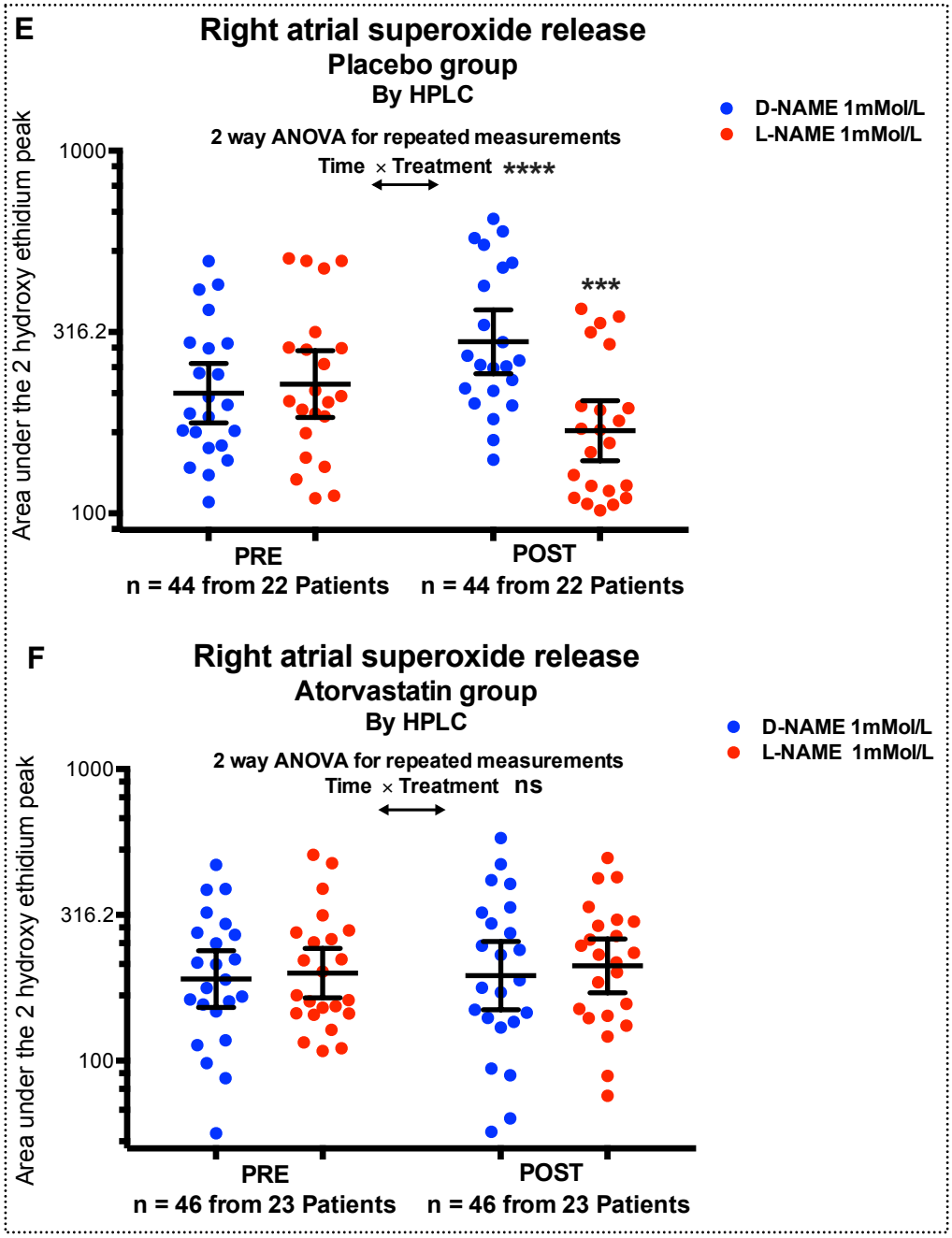
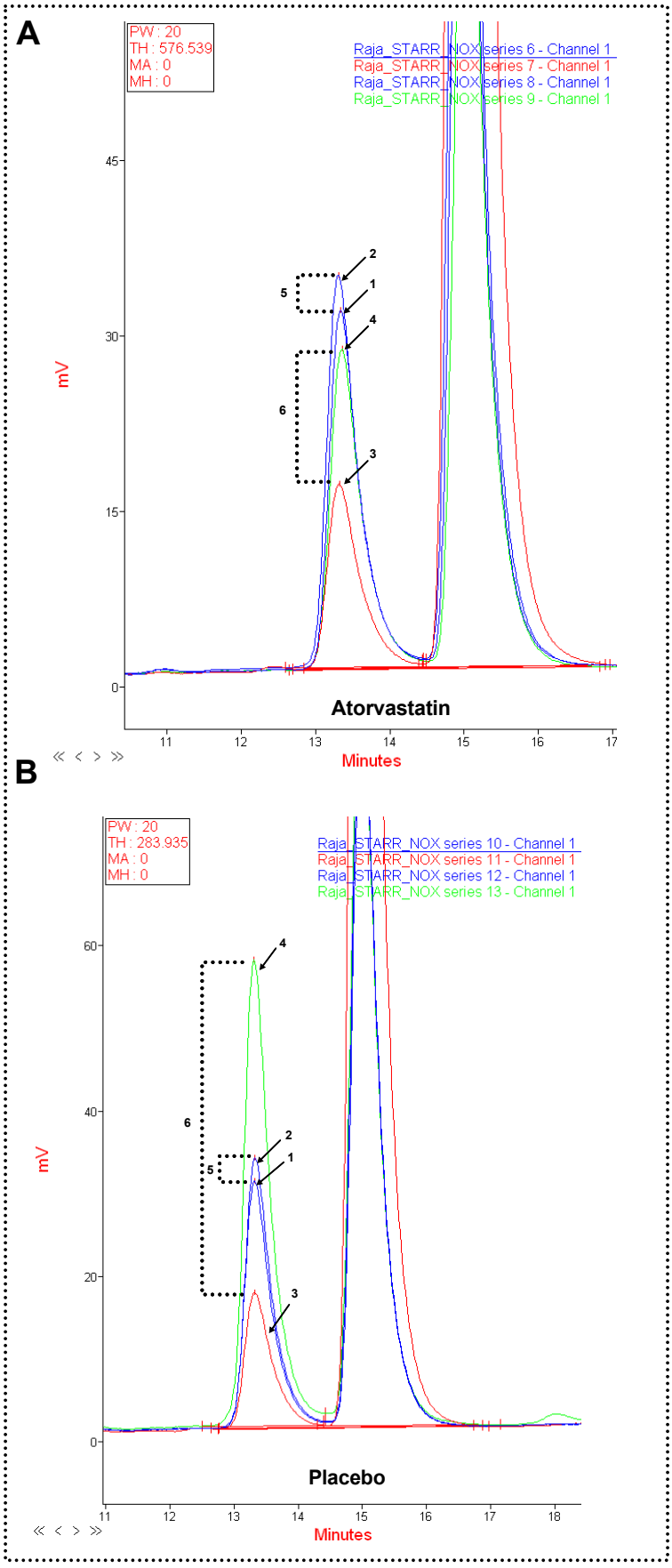


Figure 4.4: *Upper panel*, representative chromatograms of 2- hydroxyethidium detection by HPLC in homogenates of atrial samples obtained before (PRE) and after CPB (POST) and reperfusion. **A** and **B** were obtained from PRE- and POST atrial samples in one patient randomised to placebo. Similarly, **C** and **D** were obtained from PRE- and POST atrial samples in another patient randomised to Atorvastatin. 2-hydroxyethidium peaks labeled **1** and **2** were obtained in the presence of L-NAME (1mM/L) or D-NAME (1mM/L), respectively.

Bottom panel, Average superoxide production in atrial samples after pre-incubation with L-NAME or D-NAME, before (PRE) and after CPB and reperfusion (POST) detected by HPLC; Atorvastatin (**E**) and Placebo (**F**) groups. **** p <.0001, ns - not significant p > 0.05, for the interaction between time (PRE and POST) and treatment, *** p < = 0.001.vs D -NAME.Two-way ANOVA for repeated measurements after log-transformation with Bonferroni correction. Data were expressed as geometric mean ± 95% confidence intervals.




A
PW : 20
TH : 576.539
MA : 0
MH : 0
Raja_STARR_NOX series 6 - Channel 1
Raja_STARR_NOX series 7 - Channel 1
Raja_STARR_NOX series 8 - Channel 1
Raja_STARR_NOX series 9 - Channel 1
mV
45
30
15
0
1
2
3
4
5
6
Atorvastatin
11
12
13
14
15
16
17
Minutes
B
PW : 20
TH : 283.935
MA : 0
MH : 0
Raja_STARR_NOX series 10 - Channel 1
Raja_STARR_NOX series 11 - Channel 1
Raja_STARR_NOX series 12 - Channel 1
Raja_STARR_NOX series 13 - Channel 1
mV
60
40
20
0
1
2
3
4
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6
Placebo
11
12
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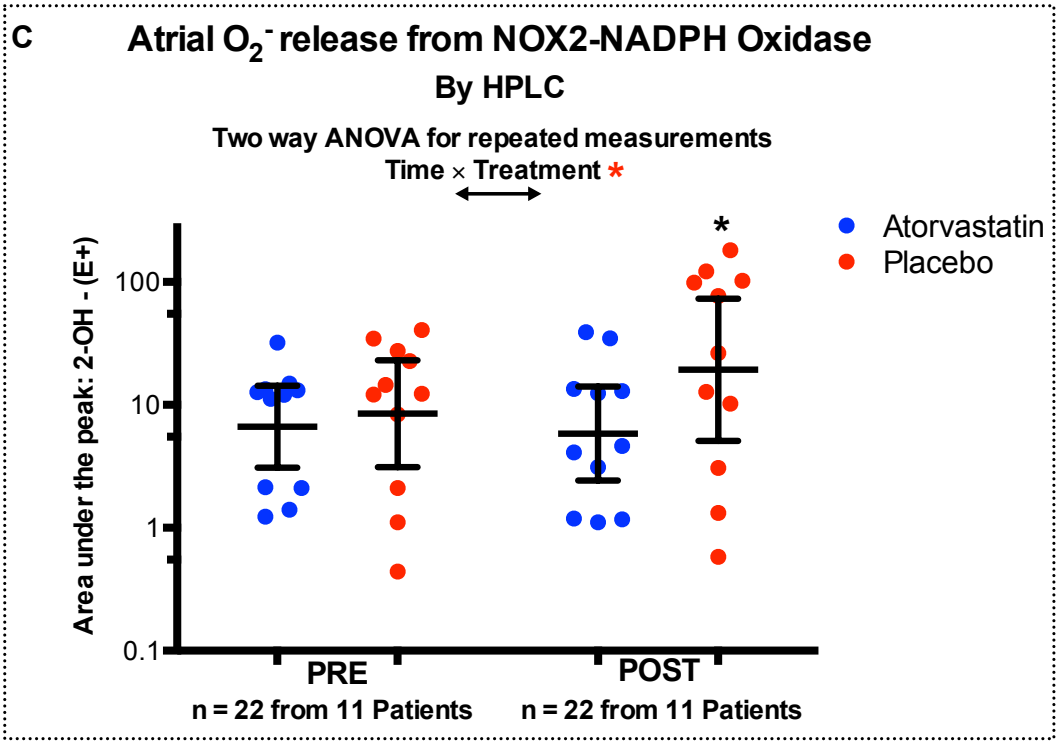
15
16
17
18
Minutes

Figure 4.5: *Upper panel*, representative chromatograms of 2- hydroxyethidium detection by HPLC in homogenates of atrial samples obtained before (PRE) and after CPB (POST) and reperfusion in two patients randomised to Atorvastatin (**A**) and Placebo (**B**). 1 and 2 are the peaks representing 2-OH - (E+) in the presence of, gp91 ds tat peptide (10µm/L) or scrambled peptide (10µm/L) respectively, in the atrial tissue obtained before CPB. 3 and 4 are the 2-OH - (E+) peaks in atrial samples obtained in the presence of, gp91 ds tat peptide or scrambled peptide after CPB and reperfusion. The difference (dotted line colored in black) between the area under the 2-OH - (E+) peaks of samples with gp91 scrambled peptide and gp91 ds tat peptide represents (i.e., the gp91 ds tat peptide inhibitable fraction) the superoxide released by atrial NOX2 NADPH Oxidase. (PRE: **5**; POST: **6**).

Bottom panel, average NOX2-NADPH Oxidase derived superoxide production in PRE- and POST atrial samples treated with Atorvastatin and Placebo (**C**). * p <.05 for the interaction between time (PRE and POST) and treatment, * p < 0.05 vs.atorvastatin.Two-way ANOVA for repeated measurements after square root transformation with Bonferroni correction. Data were expressed as geometric mean ± 95% confidence intervals. gp91 ds tat P;gp91 docking sequence tat peptide. O_2- ; Superoxide anion.



To investigate the effect of Atorvastatin on NOX2-NADPH Oxidase, gp91 ds tat peptide inhibitable fraction of superoxide release was measured in atrial tissue samples obtained before and after CPB and reperfusion (**Figure 5AB**). It was seen that perioperative treatment with Atorvastatin 80 mg was associated with abrogation of increased superoxide production from NOX2-NADPH Oxidase after CPB in patients undergoing on-pump cardiac surgery (**Figure 5C).**

Effect of Atorvastatin on atrial bioavailability of Nitric Oxide during CPB:

In the previous chapter, I had shown that cardiac surgery on CPB results in functional uncoupling of myocardial nitric oxide synthases due to post-translational modification of eNOS by *S*- glutathionylation. To investigate the effect of atorvastatin on activity of NOS, conversion of ^{14}C labeled L-Arginine to L-Citrulline was measured in atrial samples obtained before as well as after CPB and reperfusion (**Figure 6A**). As shown in the previous chapter, CPB and reperfusion resulted in reduction in activity of NOS in the placebo group. On the other hand, in patients allocated to atorvastatin, there was no difference in activity of NOS during CPB (**Figure 6C**) with preserved bioavailability of NO and together with the effect on superoxide production, perioperative atorvastatin therapy reversed myocardial nitric oxide redox imbalance in patients undergoing on-pump cardiac surgery. Of note, as opposed to placebo allocated samples, activity of NOS was lower in atrial samples obtained before the onset of CPB from patients allocated to atorvastatin. Whilst the reasons for this observation are not clear, under experimental conditions, post translational modifications associated with pleiotropic effects of statins such as S-nitrosylation or phosphorylation at threonine 495 residue of endothelial nitric oxide synthase has been shown to be associated with decreased enzyme activity and NO generation[512,513].

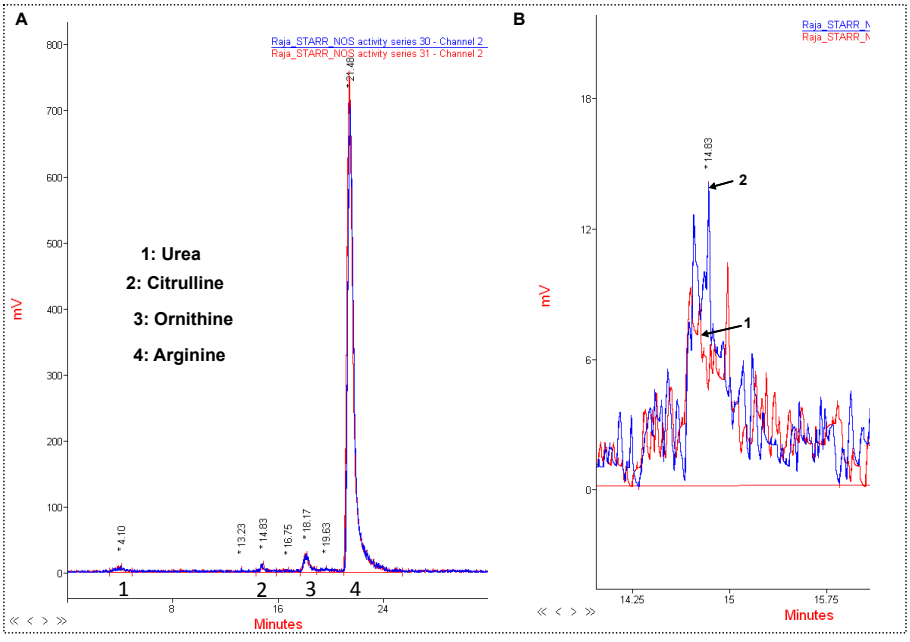
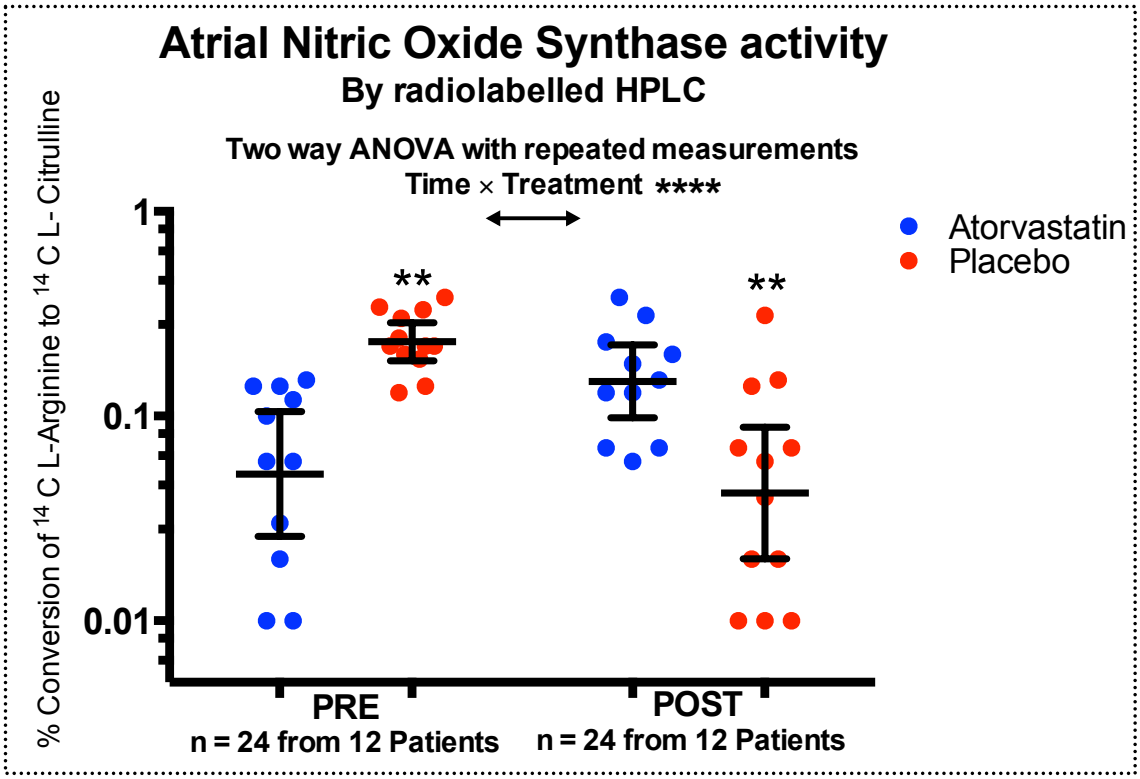


Figure 4.6: *Upper panel,* representative chromatogram obtained during radiochemical HPLC detection of ^{14}C labeled L-Arginine to L-Citrulline conversion in atrial samples obtained before (PRE) and after CPB (POST) and reperfusion. On the left (**A**), identity and elution time point of individual peaks are shown (**1 to 4**). On the right (**B**), L-Citrulline peaks obtained in the presence (Red: **1)** of L-NAME (1 mMol/L, incubation time 30 minutes prior to addition of co factors and ^{14}C labeled L-Arginine) or in the absence of L-NAME (Blue: **2)** in atrial homogenates are shown. The L-NAME inhibitable fraction of L-Citrulline expressed as a percentage of total L-Arginine represented activity of NOS.

Bottom panel, average activity of NOS in PRE- and POST atrial samples treated with Atorvastatin and Placebo (**C**). **** $p < 0.0001$ for the interaction between time (PRE and POST) and treatment, ** $p < 0.01$ vs. Atorvastatin. Two-way ANOVA for repeated measurements after square root transformation with Bonferroni correction. Data were expressed as geometric mean ± 95% confidence intervals.



Effect of Atorvastatin on GTPCH-1 activity and synthesis of biopterin species during CPB:

In the previous chapter, I had demonstrated that CPB is associated with down regulation of the activity of GTPCH-1 and reduced biosynthesis of the essential NOS cofactor, BH_4. In human umbilical vein endothelial cells, treatment with HMG Co-A reductase inhibitors increases GTPCH-1 gene expression and in parallel biosynthesis of intracellular BH_4 levels[514]. Accordingly, to dissect the effect of Atorvastatin on biopterin synthesis and elucidate factors governing bioavailability of NO during CPB, BH_4, its oxidized products (BH_2 and Biopterin)

and the activity of GTPCH-1 were measured in atrial samples obtained before and after CPB and reperfusion. While in the placebo group, CPB and reperfusion resulted in reduction in atrial GTPCH-1 activity and BH4 content, perioperative treatment with Atorvastatin maintained atrial BH_4 bioavailability (**Figure 4.7A**) and GTPCH-1 activity **(Figure 4.8)** during CPB. Measurement of the ratio of BH_4 to BH_2 and biopterin were also not different before and after CPB and reperfusion in atrial samples from patients allocated to with Atorvastatin (**Figure 4.7B**). Together with the effects on atrial GTPCH-1 activity, this finding suggests that the effect of Atorvastatin on atrial biopterins content after CPB and reperfusion is secondary to increased synthesis.

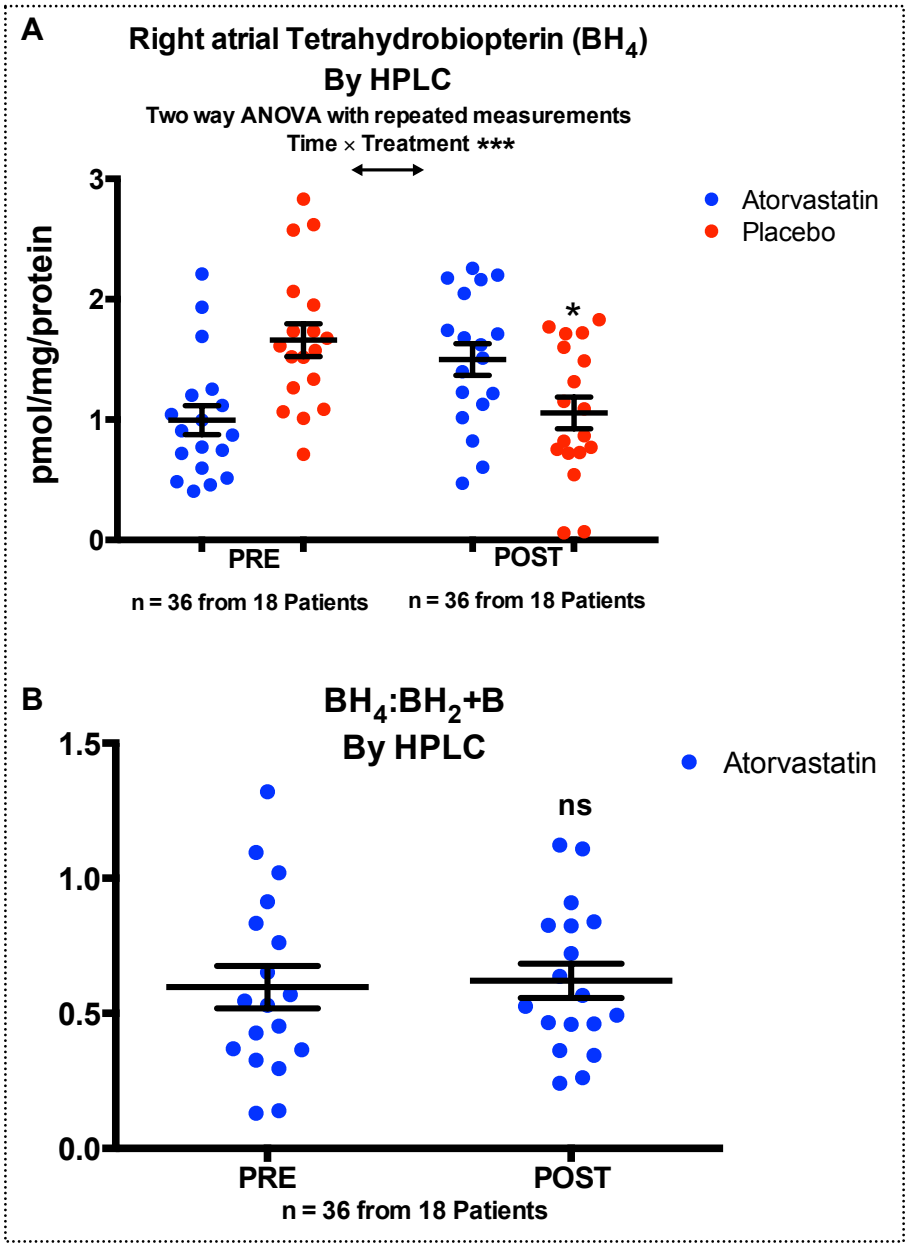


Figure 4.7: Average right atrial content of BH_4 (**A**) and its ratio to oxidized biopterins (**B**) in samples obtained before (PRE) and after CPB (POST) and reperfusion detected by HPLC. *** p <0.001 for the interaction between time (PRE and POST) and treatment, * p <0.05 vs. Atorvastatin. Two - way ANOVA for repeated measurements with Bonferroni correction. ns = p >*0.05* vs. PRE , paired student's t-test. Values expressed as mean ± SEM. ns = Not significant.

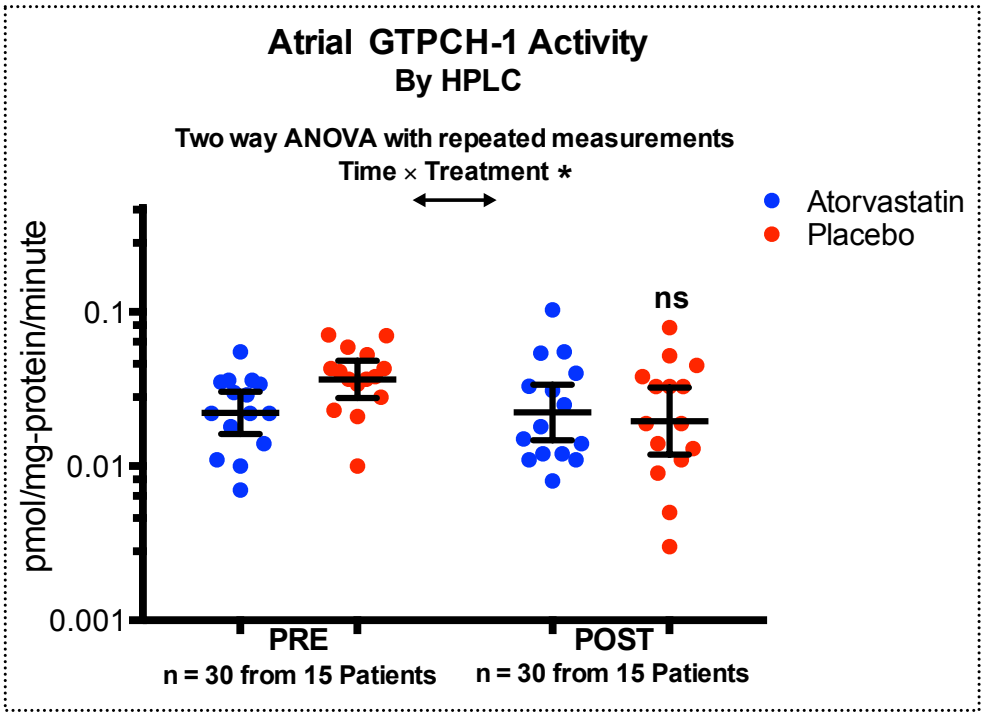


Figure 4.8: Average atrial GTPCH-1 activity detected by HPLC. * p <0.05 for the interaction between time (PRE and POST) and treatment. Two-way ANOVA for repeated measurements after square root transformation with Bonferroni correction. Data were expressed as geometric mean ± 95% confidence intervals. ns = p >*0.05* vs.Atorvastatin.

Effect of Atorvastatin on plasma low-density lipoprotein (LDL):

It has been suggested that both LDL-cholesterol dependent as well as independent effects of HMG-Co A reductase inhibition are associated with the putative anti-inflammatory and the antioxidant effects of statins[497,515-519]. Accordingly, to define the time course and magnitude of changes in LDL cholesterol levels, measurements were done in plasma samples collected before the commencement of study medications, on third and fifth post-operative days. There was no difference in LDL cholesterol levels between the randomised groups before surgery. In contrast, treatment with Atorvastatin was associated with significantly lower LDL cholesterol, 72 and 120 hours after the surgery confirming inhibition of HMG-CoA reductase (**Figure 4.9**).

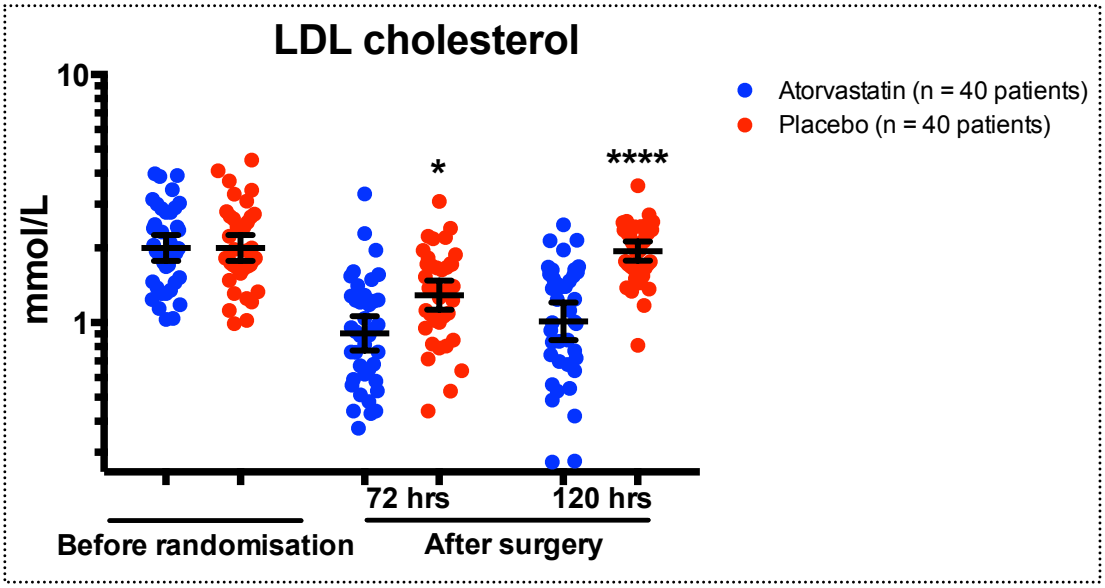


Figure 4.9: Average plasma LDL cholesterol levels before and at 72 and 120 hours after surgery. *$p < 0.05$, **** $p < 0.0001$ vs. Atorvastatin. Analysis of covariance with Bonferroni correction, missing data imputed by multiple imputation. Data expressed as geometric mean ± 95% confidence intervals. LDL - Low-density lipoprotein.

Discussion

The work presented in this chapter demonstrates that perioperative treatment with atorvastatin 80 mg in patients undergoing on-pump cardiac surgery prevents the atrial nitric oxide-redox imbalance associated with CPB and reperfusion. However, perioperative atorvastatin treatment had no measurable effect on right atrial AERP after surgery. These conclusions are supported by the following findings. First, treatment with Atorvastatin abolished the increase in superoxide production associated with CPB and reperfusion. Second, the suppression of myocardial superoxide production was related to the maintenance of coupled NOS function and inhibition of NOX2- activity after CPB and reperfusion. Third, treatment with atorvastatin maintained atrial NO bioavailability, right atrial BH_4 content and atrial GTPCH-1 activity during CPB. Finally, an average of two days of treatment with Atorvastatin before surgery decreased LDL cholesterol levels indicating that these effects are associated with HMG-CoA reductase inhibition. Together, these findings lend credence to the notion that perioperative treatment with atorvastatin 80mg maintains atrial

NO bioavailability during CPB in patients undergoing on-pump cardiac surgery. On the other hand, changes in postoperative atrial effective refractory period (AERP) did not differ between randomised groups.

Atorvastatin and atrial nitric oxide - redox balance during CPB

Evidence from large-scale clinical trials has clearly shown that statins have established role in the prevention of morbidity and mortality associated with cardiovascular disease[520-526].In addition to their LDL-lowering effects, number of studies in animal models and also in patients undergoing CABG have suggested that they exert antioxidant[89,498] and anti-inflammatory[508,527] actions referred to as "pleiotropic effects[515,528-530]." Statins by decreasing the synthesis of mevalonic acid, prevent the isoprenylation of Rac1 in the human myocardium[99]. In addition, in a double blind randomised placebo controlled trial, preoperative treatment with Atorvastatin 40 mg for three days reduced NADPH stimulated apocynin-inhibitable fraction of superoxide production in right atrial samples obtained before the onset of CPB from patients undergoing on-pump CABG[99]. Similarly in internal mammary arteries obtained at the time of CABG, statins have been shown to increase bioavailability of nitric oxide (NO) by up regulating GTP-cyclohydrolase-I gene expression leading to a tetrahydrobiopterin (BH_4) dependent improvement in endothelial NO synthase coupling[498]. Despite the evidence supporting the impact of statin treatment on myocardial or vascular redox state and bioavailability of NO, it is unclear whether these mechanisms confer cardio protection during CPB in patients undergoing on-pump cardiac surgery.

In the previous chapter, I had shown that on-pump cardiac surgery is associated with atrial NO-redox imbalance and identified *S* - glutathionylation of eNOS as a therapeutic target for maintaining atrial NO bioavailability during CPB. As *S*-glutathionylation is a redox sensitive post-translational modification, interventions redressing atrial oxidative stress during CPB may deglutathionylate eNOS and restore NO- redox balance. To investigate this further, I assessed the effect of short term statin treatment on atrial redox state and bioavailability of NO during CPB, in samples obtained before and after CPB and reperfusion and showed that perioperative therapy with atorvastatin

suppressed myocardial superoxide production after CPB and maintained NO bioavailability after CPB. This was primarily due to improved coupling status of NOS and inhibition of increased superoxide production from NOX2-NADPH Oxidase after CPB and reperfusion.

In STARR, I show that atorvastatin treatment prevents the reduction of BH_4 whilst the ratio of BH_4 to oxidized biopterins remained unchanged. These findings suggest that the effects of atorvastatin may be mediated by an increase in the biosynthesis of BH_4 (rather by a reduction in its oxidation). In agreement with this conclusion, atrial GTPCH-1 activity was not decreased after CPB in patients allocated to atorvastatin.

Regarding other putative mechanisms by which statin therapy may maintain bioavailability of NO during on-pump cardiac surgery, Almansob MA. et al[531] observed that, simvastatin treatment increased atrial eNOS expression and phosphorylation at serine 1177 site, decreased eNOS phosphorylation at Threonine 495 site with a concomitant increase in atrial eNOS activity and NO production after CPB. Evaluation of NOS posttranslational modifications in STARR are ongoing.

In STARR, an average of two days of preoperative treatment with atorvastatin produced a significant reduction in LDL cholesterol after the surgery compared with placebo. This observation confirms that the LDL cholesterol lowering effects were mediated by inhibition of HMG CoA reductase and not by systemic inflammatory response (SIRS) associated with cardiac surgery and CPB as SIRS has been linked to a reduction in cholesterol levels[532-534]. The antioxidant effects of Atorvastatin were observed in concurrence with reduction in LDL cholesterol levels confirming that two days of preoperative treatment was sufficient to significantly inhibit HMG-CoA reductase. Other putative biomarkers of effect of statins independent of LDL cholesterol lowering, i.e evidence supporting their pleiotropic effects include markers of inflammation, endothelial function or their antioxidant effects[518]. For instance, the NADPH oxidase is composed of several functional components, whose assembly and activity require the presence of the small G protein Rac1 at the plasma membrane[535].

In patients undergoing on-pump cardiac surgery, ex vivo incubation of right atrial samples obtained before the onset of CPB with atorvastatin induced a mevalonate-reversible and Rac1-mediated inhibition of NADPH oxidase[99]. However, as shown in previous chapter, I did not observe changes in Rac1 activity during CPB, suggesting that this mechanism is unlikely to have resulted in activation of NOX2 in patients undergoing on-pump cardiac surgery. Moreover, there was no difference in myocardial superoxide generation before the onset of CPB between randomised groups and hence measurement of Rac1 activity as an atrial tissue specific biomarker of HMG Co A reductase inhibition was not pursued further.

Atorvastatin and postoperative atrial electrical remodelling

The earliest changes in the fibrillating atrial myocardium involve rapid and potentially reversible alteration (upon the restoration of sinus rhythm) in the electrical properties of the atria, referred as electrical remodelling[536-538]. Atrial effective refractory period (AERP) is a measure of atrial refractoriness[539]. A short AERP is expected to promote functional re-entry and contribute to the atrial electrical substrate that promotes AF.

Although AF-induced atrial electrical remodelling and its role in permanent AF have been very well described[540], the mechanisms underlying pathogenesis of postoperative atrial fibrillation are only partially understood[478]. Among the various animal models used to study the pathophysiology of AF, only the canine model of sterile pericarditis represents the experimental surrogate of AF associated with cardiac surgery closely [78,541,542]. In this model, an inflammatory milieu evidenced by the elevation of C- reactive protein levels and alteration in gap junctions and connexin distribution, is associated with the pathogenesis of AF that peaks on second day after the surgery. However, due to the multifactorial nature, it is well accepted that the animal models of AF reproduces at best a very limited component of the underlying pathophysiological mechanisms[543]. Direct measurement of changes in postoperative AERP may overcome some of these limitations, and allow reliable assessment of postoperative atrial electrical remodelling after cardiac surgery.

Accordingly, I measured right AERP serially during the first four postoperative days, and it was seen that right AERP lengthened in the post-operative period in patients receiving placebo as well as Atorvastatin. The differences between right and left atrial electrophysiological properties[544] and the role of the left atrium in the pathogenesis of AF are well characterized. As routine surgical practice involves only insertion of the right atrial and right ventricular pacing leads, ethical considerations prevented insertion of the left atrial pacing leads for the sole purpose of AERP measurements. In view of this, whether the observed changes in right AERP mirror the changes in the left atrium is unclear.

Notwithstanding the limitation on paucity of data on postoperative changes in left AERP, the reported right AERP findings in STARR demonstrate that the effects of postoperative inflammation and oxidative stress on atrial myocardial electrical properties in patients undergoing on-pump cardiac surgery differ from the observations in animal models of AF treated with antioxidants or statins. Carnes et al., observed that high doses of vitamin C prevented myocardial oxidative injury and a reduction in atrial effective refractory period induced by 48 hours of rapid atrial pacing in dogs[545]. However, Shiroshita - takashita et al were not able to reproduce the “antioxidant” effects of high dose vitamin C alone or in combination with vitamin E and instead demonstrated that simvastatin treatment prevented AF induced atrial electrical remodelling in a canine model of atrial tachypacing[546]. Similarly, in a canine sterile pericarditis model, oral administration of Atorvastatin started one week before the surgery continued until the end of the study, prolonged AERP in comparison to control group on second day after the surgery[547]. In STARR, AERP increased after surgery but did not differ between placebo and atorvastatin-allocated patients.

Previous work from our group by Kim YM et. al., have shown that increased activity of NOX2 containing NADPH Oxidase and NADPH stimulated superoxide production from this enzyme complex are independently associated with higher incidence of postoperative atrial fibrillation after on-pump cardiac surgery[98]. Recently, other enzyme complexes such as mitochondrial ETC[548], mono amine oxidases (MAO)[477] have also been shown to be associated with POAF. The experimental evidence supporting the role of reactive oxygen species in

promoting electrophysiological changes in myocardium has been based on the association between redox sensitive post-translational modifications (such as nitrosylation) and the activity of ion channels [549] [550] [551].Despite these observations, whether superoxide released by the atrial oxidases is directly involved in the initiation of new onset AF by reducing AERP remains unclear.

Reilly SN et.al[89] from our group, compared atrial superoxide production and enzymatic sources of increased superoxide release in patients who developed new onset AF after cardiac surgery and those who had permanent AF. It was shown that the mechanisms responsible for the NO-redox imbalance in the fibrillating atrial myocardium evolve with the duration of AF and the development of atrial structural remodelling. Based on these findings, it was proposed that interventions that maintain atrial NO-redox balance may be effective only in the prevention of early AF-induced atrial electrical remodelling, for instance, in reversing pro-arrhythmic changes in atrial electrical properties that may ensue following inflammation and oxidative stress after cardiac surgery and cardiopulmonary bypass.

In STARR, Atorvastatin treatment prevented the increase in atrial superoxide production and maintained NO bioavailability but did not affect atrial electrical properties in patients undergoing on-pump cardiac surgery.

Conclusions

STARR showed that perioperative treatment with Atorvastatin in patients undergoing cardiac surgery prevents atrial nitroso-redox imbalance after CPB and reperfusion in the absence of measurable effects on postoperative AERP. No study has yet established convincingly whether these ancillary effects of statin therapy translate into beneficial clinical outcomes. Hence, the functional relevance of observations gleaned from STARR needs to be tested in an adequately powered randomised controlled trial assessing the impact of perioperative intensive statin therapy on postoperative outcomes in patients undergoing elective cardiac surgery.

Supplement

1. Patient information leaflet

Do statins prevent atrial rhythm disturbances in patients undergoing cardiac surgery?

You are being invited to take part in a clinical trial. Before you decide, it is important for you to understand why the trial is being done and what it will involve. Please take time to read the following information and to consider carefully your participation in the study.

• Part 1 tells you the purpose of this study and what would happen to you if you took part.
• Part 2 gives you more detailed information on how the study would be conducted

Please ask the study personnel if there is anything that is not clear or if you would like more information.

Thank you for taking the time to read this.

PART 1

What is the purpose of the trial?

Caring for people who undergo open heart surgery presents challenges to doctors. After the surgery, up to half of patients can develop an irregular heart beat (i.e., an arrhythmia) called “atrial fibrillation”. Post-operative atrial fibrillation is usually short-lived but can lead to complications requiring a longer hospital stay. Patients with coronary artery disease are often prescribed drugs called statins because research has shown that, by lowering cholesterol, they reduce the risk of having a heart attack or other complications in the long-term. Experimental studies have suggested that statins may also have rapid anti-inflammatory, anti-oxidant and anti arrhythmic actions; however, whether these effects are of any benefit to patients remains to be proven.

This study will test whether a short course of a commonly used statin (atorvastatin, 80 mg once a day) decreases cardiac inflammation and stabilizes the heart rhythm in patients undergoing cardiac surgery (either for valve replacement or for coronary artery bypass grafting operation). In addition it will examine whether this treatment has an impact on the biology of the vessels and the fat. In order to address this issue accurately, the trial has been designed such that 50% of participants will receive atorvastatin and 50% placebo (i.e., dummy tablets that looks like atorvastatin, but contains no active ingredients).Which participants receive atorvastatin and which receive placebo will be decided according to a series of computer-generated random numbers and the treatment allocation will be unknown to both doctors and participants (although, if members of the healthcare team needed to find out, they could do so). This type of study (known as a “randomized, placebo-controlled, double-blind trial”) provides a very powerful and unbiased way of assessing the effects of a given treatment.

Why have I been invited?
You have been invited because your doctors have advised that you need to have coronary artery bypass surgery (with or without associated valve surgery) at the John Radcliffe Hospital.

What if I am already taking a statin?
If you are already taking a statin (for example Simvastatin, Atorvastatin, Rosuvastatin, Pravastatin etc) we would ask you to stop it (for up to 6 days before surgery and 5 days after) and replace it with the study medication that would be provided by us. It is important to note that there is no proven risk to you in stopping your statin treatment for a short time. You would be able to return to your usual statin treatment 5 days after the surgery.

Do I have to take part?
It is up to you to decide whether or not to take part. If you decided to take part, you would be free to withdraw consent at any time without giving a reason. This would not affect the standard of care you receive. If – at a later date - you decided that you no longer wished to continue with the study, we would still retain any data already obtained from you unless you request otherwise. We hope that up to 80 patients will take part in this trial.

What would happen to me if I took part?
Trial procedures would take part alongside your routine visits to the JR Hospital so you would not have to make any extra visits to the hospital. Once you have read all the information and asked as many questions as you feel you need to, we would ask you to sign your written consent to take part in the study, after which we would proceed as follows:

Data collection: Record some aspects of your medical/medication history from your medical notes (for instance whether you have kidney or liver disease, diabetes or had a heart attack in the past).

Evaluation of your heart by an echocardiogram: Carry out an ultrasound examination of your heart (*i.e.*, an echocardiogram or "echo") once before your surgery and also on 5th day after the surgery .This is a safe, non-invasive and painless procedure that takes about 20 minutes. You will be asked to lie on a couch on your left side. A probe is placed on your chest and lubricating jelly is used so the probe makes good contact with the skin. Ultrasound waves then create images of your heart on the scanner monitor.

Blood sampling: Take some additional blood samples (once before and then 3 times after surgery to measure your cholesterol and markers of inflammation and cardiac injury). We will also be using the blood sample to analyse the importance of some genetic variants. The blood that we will be taking for the purpose of this research study will not be more than 50 mls (approximately 10 teaspoons) in total.

Randomisation/drug administration: Ask you to take atorvastatin 80 mg daily or an identical placebo tablet for up to 6 days before surgery and 5 days after (i.e., for a maximum of 12 days in total)which will be administered on the ward or posted to your house address, depending on your inpatient/outpatient status. You will be given clear instructions (in person, writing and/or by phone) as to when to start taking the study drug.

Collect samples of surplus tissue and biopsies in the course of cardiac surgery:
A study Investigator will attend the operation. Several types of tissue specimens will be collected:

- Blood samples: about 50ml of blood samples will be collected, using the venous line already placed for the procedure.

- Samples of blood vessels (this applies only to patients having coronary bypass surgery): The surgeons will need to harvest a long segment of your own artery/vein from either the chest, arm, or leg for the purpose of the bypass operation. A small, surplus segment of the artery/vein used for the bypass surgery will be retained for the purpose of the study.
- Samples of heart muscles: a small bit of the heart muscle is routinely excised as part the bypass operation, this bit of heart muscle will be retained for the purpose of the study.
- Fatty tissue samples: In addition to the above collected surplus tissue, the surgeon will provide the investigator small biopsies of fat tissue from various sites (from the chest and from the leg if a vein from the leg is used for the procedure).

We intend to collect such samples and use them to test whether short-term treatment with atorvastatin decreases tissue inflammation and improves functional characteristics of these tissues. Blood and tissue samples will be stored in a secure environment/licensed tissue bank and may be used for analysis in other ethically approved studies.

Evaluate the electrical properties and excitability of the heart's upper chambers (atria): Normally at the end of cardiac surgery, surgeons place wires next to the wall of the heart in case "pacing" is needed after surgery. "Pacing" means delivering a small electrical stimulation to the heart via a pacemaker to make it beat regularly at a given frequency; this procedure is not painful. As a part of this trial, participants will be asked to undergo a short period of pacing of the atria every day after surgery for 5 days by using an external pacemaker connected to the wires. As well as pacing the heart at a given set of frequencies, the pacemaker will introduce some extra stimuli in between heartbeats to measure the changes in atrial excitability and electrical properties that promote the development of atrial fibrillation after cardiac surgery.

The following table illustrates the key points/issues you need to be aware of:

Routine procedure	**Trial procedure**
One or two sets of pacing wires loosely attached to the heart's surface.	One or two sets of pacing wires loosely attached the heart's surface.
Unstable patients may require pacing based on the heart rhythm.	All patients would receive a daily short pacing protocol after surgery to test the electrical properties of the atria.
Wires removed on 4th day after surgery	Wires removed on 4th day after surgery
Echocardiography, when required	Two Echocardiographic examinations.
Cardiac tissue that is removed or not used in the course of surgery is discarded/incinerated	Cardiac tissue that is removed or not used in the course of surgery is frozen and stored for laboratory-based analyses and measurements
Grafts removed from the leg, arm or from inside the chest are used for coronary bypass operation (if necessary)	Surplus tissue from these grafts will be obtained for the purposes of the study
Fat is dissected to allow access to either the heart or the grafts	Small biopsies of the dissected fat tissue from various sites (from the chest and from the leg

	if a vein from the leg is used for the routine procedure).
Blood samples, as part of the clinical management	Additional blood (approximately 50 mls in total) taken for measurements that are pertinent to this research project

What will I have to do?

1. Consent to taking part in this study by signing a form.
2. Undergo the study procedures, as described above.
3. Take your study medication and stop taking your usual statin, if applicable.
4. Avoid drinking large quantities of grapefruit juice (>1L per day) during the study period. This is because grapefruit juice may interfere with statins as well as with other drugs.

What is the drug that is being tested?

Atorvastatin belongs to a class of drugs called statins. Statins lower cholesterol which translates into a reduction in heart attacks and other complications in the long-term. Statins are widely used and very safe; very occasionally causing muscle pains or damage that requires stopping treatment (in less than 1 in 1000 patients).The dosage of atorvastatin in this study is 80 mg which is within safe limits and higher than the conventional dose used to lower cholesterol.

Are there any side effects of participating?

Most people do not have serious problems when taking Atorvastatin, but side effects can occur.

The following conditions cumulatively affect around 1% of patients (1 in 100) and are common to all statins: nausea, abdominal pain, constipation, wind, indigestion, headache, muscle or joint pain, weakness, diarrhea, insomnia, dizziness, chest pain, allergic reactions, skin rash, numbness, joint pain and back pain. It is unlikely that you would experience these side effects with atorvastatin if you have tolerated treatment with your usual statin well. These side effects resolve once the medication is stopped.

Are there any other possible risks from taking part?

Stopping your usual treatment with statins

There are no data to suggest that stopping statin treatment for a maximum of 12 days would be associated with an increased risk of heart attacks or other adverse events.

Electrical stimulation of the heart

Applying extra-stimuli to your heart's upper chambers (atria) with the external pacemaker may encourage the development of atrial fibrillation. The likelihood of this occurring is around 10%. However, as about 40% of patients develop atrial fibrillation spontaneously after cardiac surgery, the added risk of this procedure is relatively small. If you did develop atrial fibrillation in the course of this procedure, it would be managed in the usual way (i.e., as any post-operative atrial fibrillation episode).

What are the possible benefits?

There is no direct benefit to you in taking part in the trial. We hope that by studying inflammation following heart surgery and how statins may affect that, we will be able to understand problems such as post-operative atrial fibrillation and related heart rhythm abnormalities better. This may help to improve treatment of future patients like you.

What happens when the research study stops?

The investigators will write to you to tell you the results of the study and disclose the treatment group you were in (i.e. whether you received placebo or atorvastatin). Copies of any publications connected to this study will be available on request from Prof. Barbara Casadei (contact details below).

Will my taking part in the study be kept confidential?
Yes. We will follow ethical and legal practice and all information about you will be handled in confidence. All samples will be stored in an anonymous format (i.e. your name will not be directly attached to the samples) at the John Radcliffe Hospital or the Wellcome Trust Centre for Human Genetics, University of Oxford under the custodianship of the Department of Cardiovascular Medicine. The samples will be stored for up to 10 years and may be used in future research subject to separate ethical approval(which will be sought before end of trial), as our understanding of cardiovascular function grows. Alternatively samples may be transferred to a licensed tissue bank. Future research may include genetic research (see below).

If the information in Part 1 has interested you and you are considering participation, please read the additional information in Part 2 before making any decision.

PART 2

What if relevant new information becomes available?
Sometimes during the course of a research project, new information becomes available about the treatment/drug that is being studied. If this happens, your research doctor will tell you about it and discuss whether you want to continue in the study, or not depending on your position in the trial timeline. If you decided not to carry on, your research doctor would make arrangements for your care to continue. If you decided to continue in the study, you would be asked to sign an updated consent form. In addition, on receiving new information your research doctor might consider it to be in your best interests to withdraw you from the study. He/she will explain the reasons and arrange for your care to continue. If the study was stopped for any other reason, you would be told why and your continuing care would be arranged.

What will happen if I don't want to carry on with the study?
Since this research study is voluntary, you can withdraw from the study at any time. Any stored blood or tissue samples that can still be identified as yours and information we hold about you will be destroyed, if you wished. Alternatively, if you allowed us, we could make use of the information we had already collected about you. We can ensure that, if your samples and information were used for future research, this would be done entirely anonymously.

What if something goes wrong or I have a complaint?
Complaints
Any problems connected to the study would be dealt with initially by the researchers conducting the study (contact Dr Raja Jayaram on 01865 234914 or Professor Barbara Casadei or you can contact the University of Oxford Clinical Trials and Research Governance office on 01865 572245).

Harm
NHS indemnity operates in respect of the clinical treatment with which you are provided. In addition, the University of Oxford has appropriate insurance-related arrangements in place in respect of the University's role as Research Sponsor of this study.Non-negligent harm is not covered by the NHS indemnity scheme. Compensation

for harm arising from an accidental injury and occurring as a consequence of your participation in the study may be covered by the University of Oxford. If you are harmed and this is due to someone's negligence then you may have grounds for legal action for compensation against the University of Oxford (in respect of any harm arising out of the participation in the Clinical Trial) or the NHS (in respect of any harm which has resulted from the clinical procedure being undertaken).

Contacts
If the study researchers cannot answer your concerns, the Sponsor (University's Clinical trials and research governance office) may be contacted on 01865 572245.
If you wish to make a formal complaint please contact OUH Comments and Complaints on 01865 228913.

Participation in future research
We will ask if we can contact you about future studies. This is optional i.e., you can take part in this study but decline to be contacted again. If you consent, we will keep your contact details separately from the research data you have provided. Both your details and data will carry the same unique ID. This means your data is anonymised but that we can "link" details to data. In this way we can approach patients about studies relevant to their particular healthcare status. You can withdraw your consent for future contact at any time.

Involvement of the general practitioner
With your permission, the research doctors will write to your GP to inform them about your participation in the study. No extra visits to your GP will be required.

What will happen to any samples I give?
All samples will be retained in a secure environment for future analysis and will be stored in an anonymous format at the Department of Cardiovascular Medicine laboratory in the West Wing of John Radcliffe Hospital and/or in the Wellcome Trust Centre for Human Genetics of the University of Oxford under the custodianship of the Department of Cardiovascular Medicine. The samples will be stored for the entire duration of the clinical trial and may be used in future research as our understanding of cardiovascular disease grows for which separate ethical approval will be sought. Future research may include genetic research (see below). Alternatively samples may be transferred to a licensed tissue bank. Samples will be destroyed by the research team after the end of all ethical approvals.

Will any genetic tests be done?
It is possible that some samples will be used for genetic research. This research may be conducted by the study research team or collaborating research teams. The samples will be stored in an anonymous format but a record of who donated the samples will be kept so that we can relate any findings to your medical history. Keeping these records ensures that if you decide to withdraw your consent for us to keep your data, we will be able to destroy your samples. The genetic tests may involve looking at common variations in genes that affect the heart's response to inflammation and the anti-inflammatory effect of atorvastatin. We do not propose to test for inherited genetic diseases, or for conditions that will involve any other members of your family. There is no evidence to suggest that the results of these genetic studies are likely to have significant implications for you personally.

What will happen to the results of the research study?
We anticipate that the results will be published in a scientific journal for the benefit of the wider medical community. However, individual patients will not be identified in any

publication and your personal and clinical details will remain strictly confidential. Any scientific publications arising from the study will be available on request to all participants. The research will also form part of a thesis submitted by Dr Raja Jayaram towards an academic qualification. You would have no legal right to a share of any profits that may arise from the research.

Who is organizing and funding the research?
The investigators are Professor Barbara Casadei and Dr Raja Jayaram from the Dept of Cardiovascular Medicine with co-investigators from the Cardiothoracic Surgery Department at the John Radcliffe Hospital and the Clinical Trial Service Unit & Epidemiological Studies Unit (CTSU) of the University of Oxford. If you wish to know more about any aspect of the study, please contact Dr Jayaram on (01865) 234914 or Professor Barbara Casadei during office hours.The research is co-funded by the British Heart Foundation (http://www.bhf.org.uk/), Pfizer Pharmaceuticals and the Oxford Biomedical Research Centre (a partnership between the University of Oxford and Oxford University Hospitals, funded by the National Institute of Health Research http://www.oxfordbrc.org/)

Who has reviewed the study?
This study was given a favourable ethical opinion for conduct in the NHS by the Berkshire Research Ethics Committee. In addition, the study was approved by the Medicines and Healthcare Products Regulatory Agency (MHRA).

Where can I find independent information about taking part in research?
You can contact local branches of the NHS Patient Advisory Liaison Service (PALS). Here is their website: http://www.pals.nhs.uk or you can look up INVOLVE @ http://www.invo.org.uk/ which gives information about public involvement in clinical trials.

We thank you in advance for your co-operation.

Yours sincerely,

Dr. Raja Jayaram: (Raja.Jayaram@cardiov.ox.ac.uk) (Tel: 01865 234 914/ 07723354904)
Clinical research fellow in Cardio vascular Medicine

Prof. Barbara Casadei:
Professor of Cardiovascular Medicine & Honorary Consultant Cardiologist

Mr. Rana Sayeed, Mr. Ravi De Silva, Mr.Mario Petrou:
Consultant Cardiothoracic Surgeons
NHS Cardio-thoracic surgery, OUH Trust, Oxford.

2. Consent sheet

DEPARTMENT OF CARDIOVASCULAR MEDICINE
UNIVERSITY OF OXFORD
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OXFORD OX3 9DU

Oxford University Hospitals NHS
NHS Trust

UNIVERSITY OF OXFORD

CONSENT FORM (2.0/01.08.2012)

Study Full Title	Do statins prevent atrial rhythm disturbances in patients undergoing cardiac surgery?
Patient ID	
Researchers	Prof. Barbara Casadei, Dr. Raja Jayaram, Mr.Ravi De Silva, Mr.Rana Sayeed, Mr.Mario Petrou

Please Tick the box

1. I confirm that I have read and understand the information sheet (2.0/30.07.2012) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by authorized individuals from University of Oxford, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I permit these individuals access to my records. ☐
4. I understand why blood & tissue samples are being taken, how the samples will be collected, that giving samples for this research is voluntary and that I am free to withdraw my approval for use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. I understand that any data collected from the analyses of the samples will be retained for use in the results of the research study. ☐
5. I understand that my blood& tissue samples will be used in genetic research aimed at understanding the genetic influence on cardio vascular function, but that the results of these investigations are unlikely to have any implications for me personally. ☐
6. I understand why an electrophysiology study of my heart is done and why I am having a temporary pacing lead inserted. I give consent for this procedure. ☐
7. I agree to gift blood/tissue samples taken for the purpose of the research study to the University of Oxford. If a commercial product were developed as a result of this study, I will not profit financially from such a product ☐
8. I understand that my GP will (with my permission) be informed of the results of medical tests performed as part of the research, which are important for my health care. ☐
9. I agree to take part in the above study. ☐
10. (Optional). I agree to being contacted in the future to ask if I am interested in future related studies. ☐

Name of participant	Signature	Date
Name of person taking consent	Signature	Date

CHAPTER 5

STICS

STATIN **T**HERAPY **I**N **C**ARDIAC **S**URGERY

Background and rationale

Despite advances in surgical and perioperative care, postoperative complications after cardiac surgery remain frequent, leading to significant increases in mortality, morbidity and costs[552]. It has been suggested that starting statin therapy shortly before cardiac surgery may prevent such complications. In meta-analyses of non-randomised observational studies and of small randomised trials, short-term perioperative statin therapy (typically starting 2 to 7 days before surgery) has been associated with about a halving in the rate of postoperative atrial fibrillation (AF), along with lower rates of stroke and with shortened intensive care unit (ICU) and hospital stay [139,553,554]. In addition, it has been reported that perioperative statin therapy reduces the postoperative release of cardiac troponin I (cTnI), a marker of myocardial damage and an independent predictor of mortality after cardiac surgery[192] , and improves cardiac function[555,556].

On the basis of these findings, the American College of Cardiology/American Heart Association guideline for Coronary Artery Bypass Graft Surgery (CABG) [557] and the European Society of Cardiology guideline for AF management [558] currently recommend that statin therapy should be considered for the prevention of new onset AF and other postoperative complications after cardiac surgery. However, the evidence upon which these recommendations are based has some important limitations as it is derived either from non-randomised studies with their inherent potential for systematic biases in the assessment of treatment effects [559] or from randomised trials that involve only small numbers of highly selected patients in whom the allocation of statin treatment was not always blinded, postoperative outcomes were not always pre-specified or assessed systematically, and data analysis did not always involve “intention-to-treat” comparisons.

By contrast, the work presented in this chapter, the randomised placebo-controlled Statin in Cardiac Surgery Trial (STICS) involved 1922 patients scheduled to receive elective cardiac surgery and systematically evaluated the effects of perioperative rosuvastatin (20 mg daily) on, specifically, postoperative

AF assessed by continuous Holter ECG recordings and myocardial damage assessed by serial troponin I measurements, as well as on other in-hospital adverse outcomes recorded systematically.

Methods

Research ethics approval was obtained from the Institutional Review Board of the Fuwai Hospital and the Oxford Tropical Research Ethics Committee.

Eligibility

Patients were screened in the Department of Cardiac Surgery of the Fuwai Hospital in Beijing, China, either at the time of their outpatient appointment prior to planned surgery or after admission to hospital. Men and women aged over 18 years who were scheduled to receive elective coronary artery bypass grafting (CABG) and/or surgical aortic valve replacement (AVR) were eligible if they were in sinus rhythm, had no history of AF, and were not taking anti-arrhythmic medications (other than beta-blockers). Patients were considered ineligible if they had chronic liver disease, impaired renal function (creatinine >200 umol/L), untreated hypothyroidism, child-bearing potential, personal or family history of hereditary muscle disorders, inflammatory muscle disease or evidence of muscle problems, known intolerance to statins or history of muscle toxicity, ongoing treatment with fibrates, niacin, or inhibitors of cytochrome P450 or the P-glycoprotein (*e.g.*, cyclosporine, azole antifungal), or significant mitral valve disease (moderate or severe mitral regurgitation, *e.g.*, grade III or greater, mitral stenosis or mitral annular calcification).

Randomisation

Eligible patients were informed about the trial and asked for written informed consent. Consenting patients had a transthoracic echocardiography for the evaluation of left ventricular ejection fraction (LVEF) and left atrial size, and a blood sample taken for the determination of cTnI, NT-proBNP, and LDL cholesterol. Any statin therapy was stopped and patients were then randomly allocated to receive rosuvastatin 20 mg once daily or matching placebo for up to 8 days before surgery and 5 days thereafter. In patients receiving CABG, the choice of on- or off-pump surgery was made by the surgeon. Myocardial

protection in the on-pump procedure was achieved by cold blood cardioplegia and moderate systemic hypothermia. Postoperative management was in accordance with standard protocols.

Rosuvastatin (Crestor; AstraZeneca) tablets were purchased and matching placebo tablets were manufactured by Kaifeng Pharmaceutical Co. Ltd (China), who packed both the active and placebo tablets. The China-Oxford Center for International Health Research of the Fuwai Hospital labelled the packs with sequential numbers, according to a randomisation schedule generated at the Clinical Trial Service Unit (CTSU), University of Oxford, by personnel who had no further involvement in the trial. Subsequent retrieval of a random selection of 52 treatment packs found them all to be correctly labelled and only 4 packs were allocated out of sequence during the randomisation process.

Assessment of efficacy

The two pre-specified co-primary outcomes were postoperative AF detected by continuous Holter ECG monitoring for 5 days after surgery, and perioperative myocardial damage assessed by the area under the cTnI release curve derived from blood samples taken at 6, 24, 48 and 120 hours post-surgery. The secondary study outcomes were postoperative AF diagnosed clinically based on symptoms or routine ECG recordings, peak cTnI in the first 24 hours after surgery, the ratio between the maximum cTnI value during the first 120 hours after surgery and the cTnI value at randomisation, major in-hospital cardiac or cerebrovascular events (death, stroke, or myocardial infarction), duration of ICU and hospital stay, low cardiac output syndrome, pleural effusion, infections, LVEF at discharge, and blood biomarkers (NT-proBNP and LDL cholesterol). Postoperative myocardial infarction was diagnosed according to Thygesen *et al.*[560] LVEF and left atrial size were calculated using standard echocardiographic measurements.

Analysis of ECG, blood sample and right atrial appendages

Further details on the measurements below can be found in Chapter 2.
Holter ECG recordings were obtained using the Lifecard CF device (Space Labs Healthcare, Washington, USA) and downloaded to the Pathfinder Digital

software (Space Labs Healthcare). All ECG analyses were carried out in the Division of Cardiovascular Medicine, University of Oxford. AF was defined as non-sustained if it lasted between 10 beats and 10 minutes, and as sustained if it lasted longer than 10 minutes. All blood assays were carried out by the CTSU's Wolfson Laboratories, University of Oxford. Plasma and serum were separated by centrifugation at 1300g for 10 minutes at room temperature. Plasma was used for measuring LDL cholesterol (end point assay, N-geneous® reagents, calibrators and settings supplied by Genzyme Diagnostics, UK) using a Beckman Coulter AU680 and the N-terminal fragment of the prohormone brain natriuretic peptide (NT-Pro BNP) using a plate assay with electrochemiluminescence detection (Meso Scale Discovery). Serum was used for measuring cTnI (chemiluminescent immunoassay, reagents, calibrators and settings supplied by Beckman Coulter) using a Beckman Coulter ACCESS 2. At least two levels of quality control material were run for each assay: between-run precision was 2.5% for LDL cholesterol at 0.84 mmol/L, 8.9% for NT-proBNP at 275.5 pg/mL, and 6.1% for cTnI at 0.42 ng/mL. Samples of the right atrial appendages were collected prior to the commencement of CPB, were snap frozen in liquid nitrogen immediately and stored in -80^{o} until analysis. Atrial superoxide production was measured by 2 hydroxyethidium (2-OH- E+) detection by high-performance liquid chromatography and results are shown as the tiron-inhibitable fraction (see Chapter 2)

Statistical analysis

The effect of allocation to rosuvastatin on AF detected by Holter monitoring was calculated as the ratio of the odds of AF among rosuvastatin-allocated patients compared with the odds of AF among placebo-allocated patients. Assuming an AF rate of 35% in the placebo group, it was originally intended to randomise 1000 patients in order to have 84% power at P = 0.01 to detect a 30% proportional reduction in AF. However, as recruitment progressed, it became apparent that the blinded rate of AF (i.e., in both treatment groups combined) was about 20%. It was, therefore, decided to increase the sample size to at least 1900 patients in order still to have at least 80% power at P = 0.01 to detect a 30% proportional reduction.

Odds ratios were also used to test for differences in other dichotomous outcomes (e.g., in-hospital adverse events), while log-rank time-to-event methods were used for ICU stay and total hospital stay. For the co-primary endpoint, the area under each patient's cTnI release curve between 6 and 120 hours post-surgery was calculated, and analysis of covariance (ANCOVA) was used to compare the mean log area between those allocated rosuvastatin and those allocated placebo after adjustment for the baseline log troponin concentration. ANCOVA was also used to compare other blood biomarkers measured at 48 and 120 hours post-surgery, as well as postoperative transthoracic echocardiography measurements, after adjustment for baseline values. NT-proBNP was analysed on a log scale.

Pre-specified subgroup analyses of the co-primary outcomes of AF and cTnI release included subdivision by baseline age (≤60, >60 years), sex, prior statin use, baseline cTnI concentration, duration of randomized treatment before surgery (≤2, >2 days), type of surgery received (on-pump, off-pump; CABG only, AVR only), and postoperative use of non-steroidal anti-inflammatory drugs (NSAIDs) or steroids. Standard tests for heterogeneity were performed to check whether there was any good evidence that the effect in any particular subgroup differed significantly from the overall main effect.

All comparisons were made according to the "intention-to-treat" principle. All P-values are 2-sided and considered statistically significant, without allowance for multiple testing, at the 5% level. For comparisons of dichotomous outcomes, any patient with missing data was assumed not to have had the outcome, contributing to the denominator but not the numerator. Missing values for biomarkers, as well as for postoperative transthoracic echocardiography measures, were estimated by multiple imputation, with 10 replicate sets and combination across sets using Rubin's methods[561]. (These results were consistent with results using alternative "complete-case" analyses.)

Results

Patient characteristics and treatment

Between September 2011 and October 2013, 5429 patients scheduled for elective cardiac surgery were screened, 2721 did not meet the trial inclusion criteria (mostly because they had AF or needed mitral valve surgery), 340 refused to take part, and 446 were not recruited for other reasons **(Figure 5.1)**.

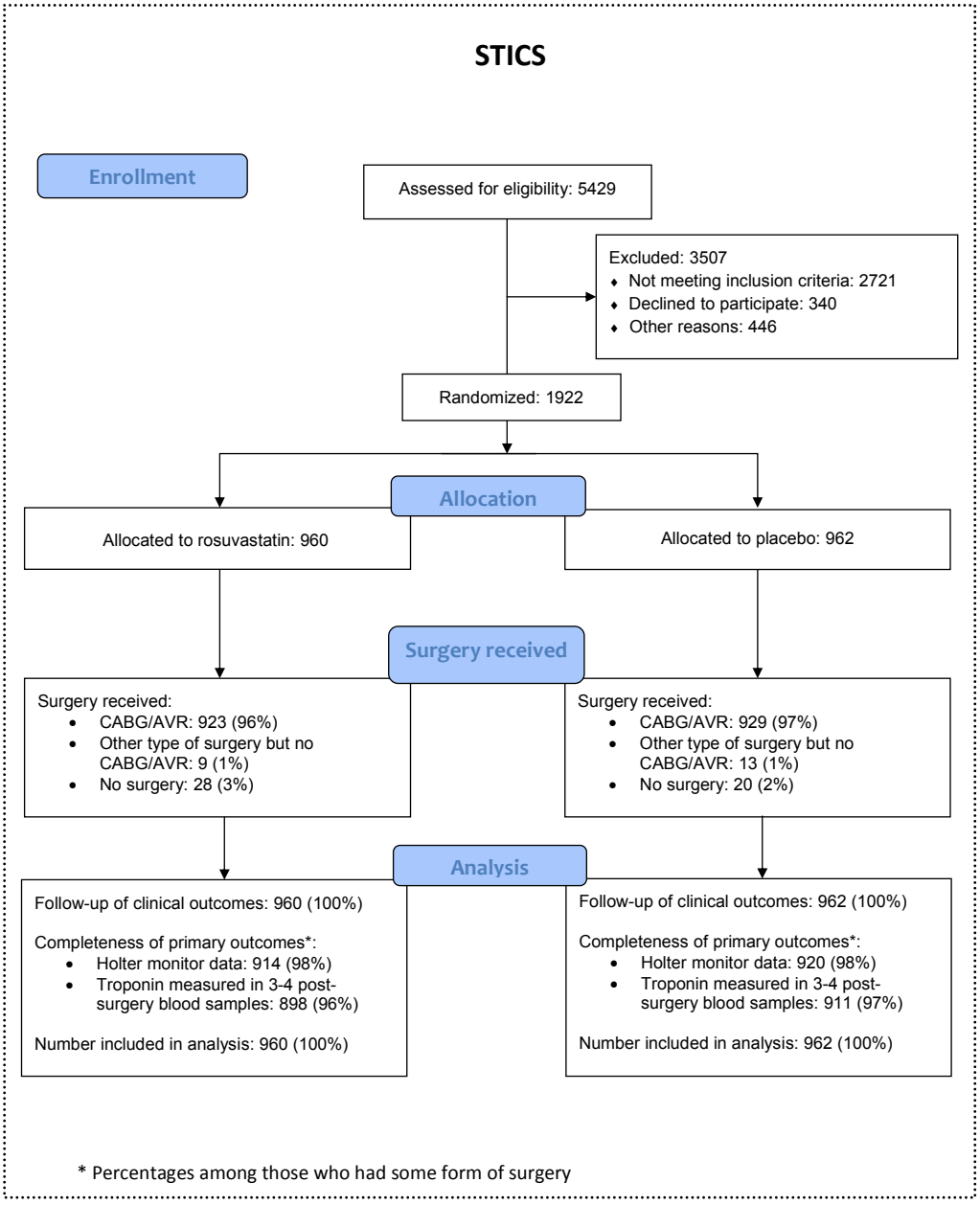

Figure 5.1: Consolidated standards of reporting trials flow diagram
STICS – Statin Therapy in Cardiac Surgery

The mean age of the 1922 randomised patients (960 allocated rosuvastatin and 962 allocated placebo) was 59.4 (SD 9.4) years, 21% were female, 31% had a diagnosis of diabetes, 64% of hypertension and 29% of prior myocardial infarction, and prior statin use was recorded in 34%, with good balance of the baseline characteristics between the randomised groups (**Table 5.1**).

	Rosuvastatin	Placebo
Number randomised	960	962
Age, years	59.3 (9.4)	59.5 (9.5)
≤60	516 (54%)	504 (52%)
>60	444 (46%)	458 (48%)
Women	194 (20%)	205 (21%)
Body mass index, kg/m^2	25.7 (3.2)	25.7 (3.1)
Current smoker	229 (24%)	245 (25%)
Past medical history		
Hypertension	621 (65%)	614 (64%)
Myocardial infarction	282 (29%)	274 (28%)
Stroke/transient ischaemic attacks	115 (12%)	119 (12%)
Peripheral arterial disease	23 (2%)	19 (2%)
Heart failure	46 (5%)	37 (4%)
Chronic obstructive pulmonary disease	5 (1%)	14 (1%)
Diabetes mellitus	310 (32%)	291 (30%)
Chronic kidney disease	10 (1%)	8 (1%)
Current/recent medication		
Beta blockers	813 (85%)	804 (84%)
NSAIDs/Steroids	16 (2%)	7 (1%)
Insulin	140 (15%)	158 (16%)
Contrast agents (last 2 weeks)	383 (40%)	379 (39%)
Antiplatelets/anticoagulants	792 (83%)	779 (81%)
Calcium channel blockers	445 (46%)	424 (44%)
ACEi/ARB	385 (40%)	384 (40%)
Nitrates	798 (83%)	800 (83%)
Diuretics	197 (21%)	213 (22%)
Statins	321 (33%)	332 (35%)
Details of scheduled surgery		
On-pump procedure	508 (53%)	515 (54%)
Off-pump procedure	422 (44%)	429 (45%)
CABG	832 (87%)	838 (87%)
AVR	117 (12%)	119 (12%)

Mean (SD) or n (%) shown.
ACEi, inhibitors of the angiotensin converting enzyme; ARB, angiotensin receptor blockers

Table 5.1 – Baseline characteristics by randomised treatment allocation

The randomised study treatment was started at a median of 1 day before surgery. Among randomised patients who had surgery, the average compliance to the scheduled treatment between randomisation pre-operatively and 5 days

postoperatively was 92% in both treatment groups. Allocation to rosuvastatin was associated with significantly lower LDL cholesterol by 48 hours and 5 days after surgery (**Table 5.2**).

	Rosuvastatin	Placebo	p-value
Number randomised	960	962	
LDL cholesterol (mmol/L)			
Baseline	2.14 (0.02)	2.07 (0.02)	-
48 hours*	0.96 (0.01)	1.28 (0.01)	<0.0001
120 hours*	1.29 (0.02)	2.01 (0.01)	<0.0001
Post-surgery troponin			
Ratio of peak post-surgery cTnI to baseline cTnI	237 (12)	230 (12)	0.66
Peak cTnI within first 24 hours, ng/mL	2.6 (0.1)	2.6 (0.1)	0.89
NT-proBNP (pg/mL)			
Baseline	944 (42)	951 (42)	-
48 hours*	7545 (161)	7416 (164)	0.58
Left atrial size (cms)			
Baseline	3.6 (0.01)	3.6 (0.01)	-
Discharge*	3.4 (0.01)	3.4 (0.01)	0.21
LVEF (%)			
Baseline	60.5 (0.3)	61.0 (0.3)	-
Discharge*	59.3 (0.2)	59.2 (0.2)	0.65
Major in-hospital SAEs			
Atrial fibrillation[#]	149 (16%)	117 (12%)	0.03
Arrhythmias other than AF	427 (44%)	431 (45%)	0.89
Low cardiac output syndrome	28 (3%)	28 (3%)	0.99
Pleural effusion	84 (9%)	95 (10%)	0.40
Stroke	5 (1%)	5 (1%)	0.99
Myocardial Infarction	37 (4%)	41 (4%)	0.65
Heart failure	75 (8%)	72 (7%)	0.79
Infection	80 (8%)	90 (9%)	0.43
Death	3 (0%)	1 (0%)	0.34
Time from surgery to discharge from			
Intensive care (hours)	42 (21 - 71)	41 (21 - 69)	0.17
Hospital (days)	7 (7 - 9)	7 (7 - 9)	0.18

Values are arithmetic means (SE) for LDL cholesterol, left atrial size and LVEF, geometric means (approximate SE) for the peak post-surgery:baseline cTnI ratio, the peak cTnI within the first 24 hours and NT-proBNP, n (%) for major in-hospital SAEs and product-limit estimates of the median (IQR) for time to intensive care/hospital discharge.
* Estimated by analysis of covariance after adjustment for the baseline value (missing data imputed using multiple imputation).
[#] Identified by routine ECG or symptoms, rather than by Holter monitoring.

Table 5.2: Effects on blood biomarkers, left atrial size and left ventricular ejection fraction, major in-hospital SAEs and duration of ICU/hospital stay.

Following randomisation, surgery was cancelled in 48 patients (3% assigned rosuvastatin and 2% assigned placebo). 87% of randomised patients had CABG

surgery (3% combined with AVR), 10% had AVR alone and 1% had some other form of surgery; 55% of the surgery was on-pump (**Figure 5.1**). Following surgery, 92% of the patients received beta-blockers and 60% received NSAIDs or dexamethasone (5-10 mg od for 1-2 days).

Primary outcomes

Postoperative AF was detected by continuous Holter ECG recordings for 5 days after surgery in 400 randomised patients and was sustained in 92% of cases. There were 203 patients with postoperative AF (21%) in the rosuvastatin group and 197 patients (20%) in the placebo group (OR 1.04, 95% CI 0.84 to 1.30, P = 0.72, **Figure 5.2**). As expected, the incidence of postoperative AF was higher in older patients and those undergoing on-pump surgery, but it was not affected by sex, prior use of statin therapy or postoperative use of anti-inflammatory drugs (including dexamethasone). Pre-specified subgroup analyses did not find that age, sex, prior statin use, baseline cTnI concentration, type of cardiac surgery, or postoperative use of anti-inflammatory drugs had a significant impact on the effect of allocation to rosuvastatin on the incidence of postoperative AF.

The co-primary outcome of perioperative myocardial damage was defined as the area under the curve of serial cTnI measurements in the postoperative period (6-120 hours). As shown in **Figure 5.3**, allocation to rosuvastatin had no effect on cTnI release after surgery (+1%, 95% CI from -9% to +13%, P = 0.80). As was the case for postoperative AF, the effect of the study treatment on postoperative cTnI did not differ in any of the pre-specified subgroups (**Figure 5.4**), nor were there significant differences between the treatment groups for the secondary outcomes of the ratio of peak postoperative to baseline cTnI or of the peak level of cTnI in the first 24 hours after surgery (**Table 5.2**).

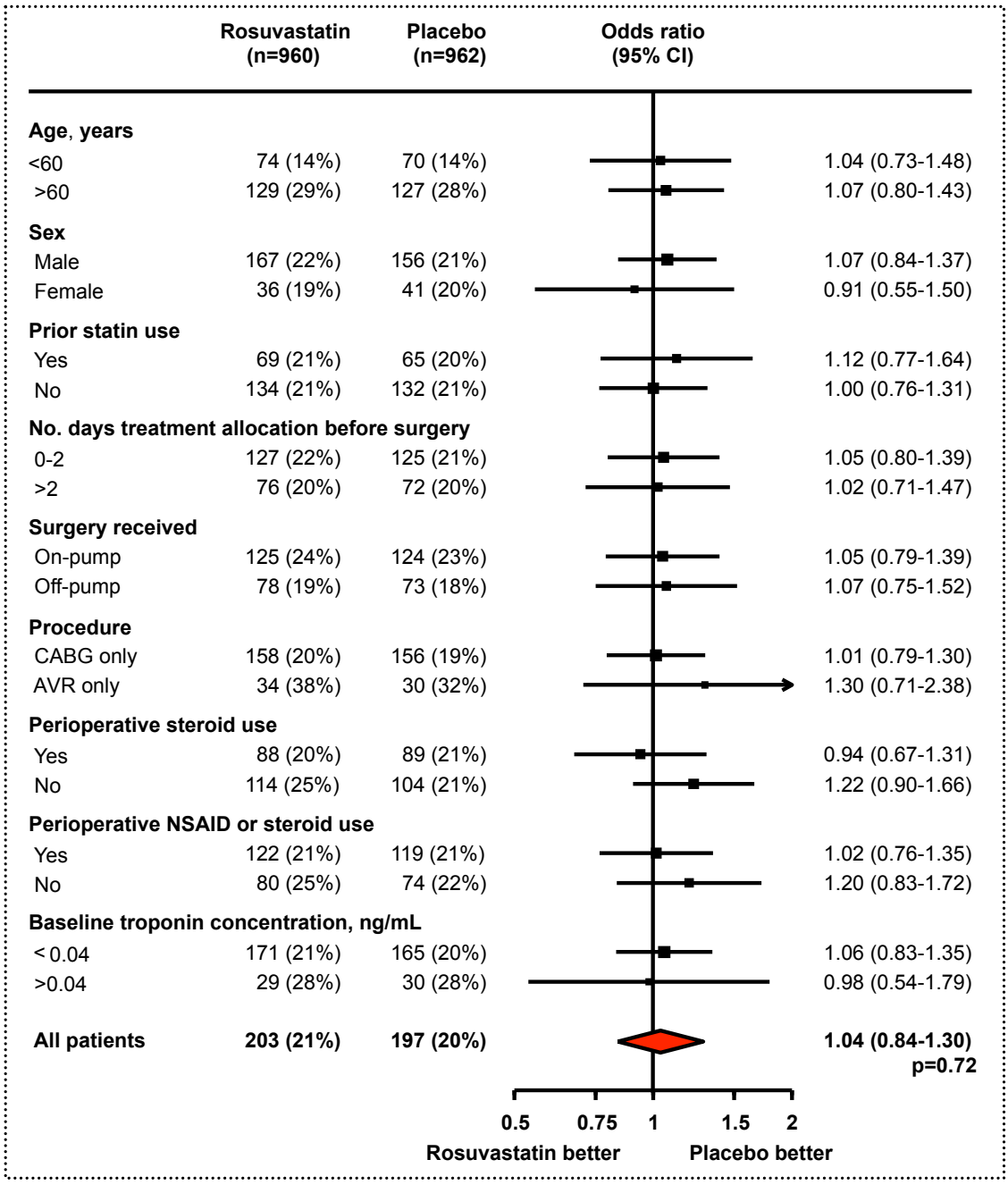

Figure 5.2 - Effect of rosuvastatin on postoperative Holter monitor AF, overall and in predefined subgroups

Number of patients with postoperative AF and the corresponding percentage for each group shown as n (%). Odd ratios comparing the outcome among participants assigned to rosuvastatin with the outcome among those assigned to placebo are plotted. For subcategories, odd ratios are plotted as squares and horizontal lines represent 95% confidence intervals. Overall odd ratio represented by the red diamond and statistical significance test shown on the right side. Squares or diamond to the left of the solid vertical

line indicate benefit with rosuvastatin, but the benefit is significant (P<0.05) only if the horizontal lines or diamond does not overlap the solid vertical line.

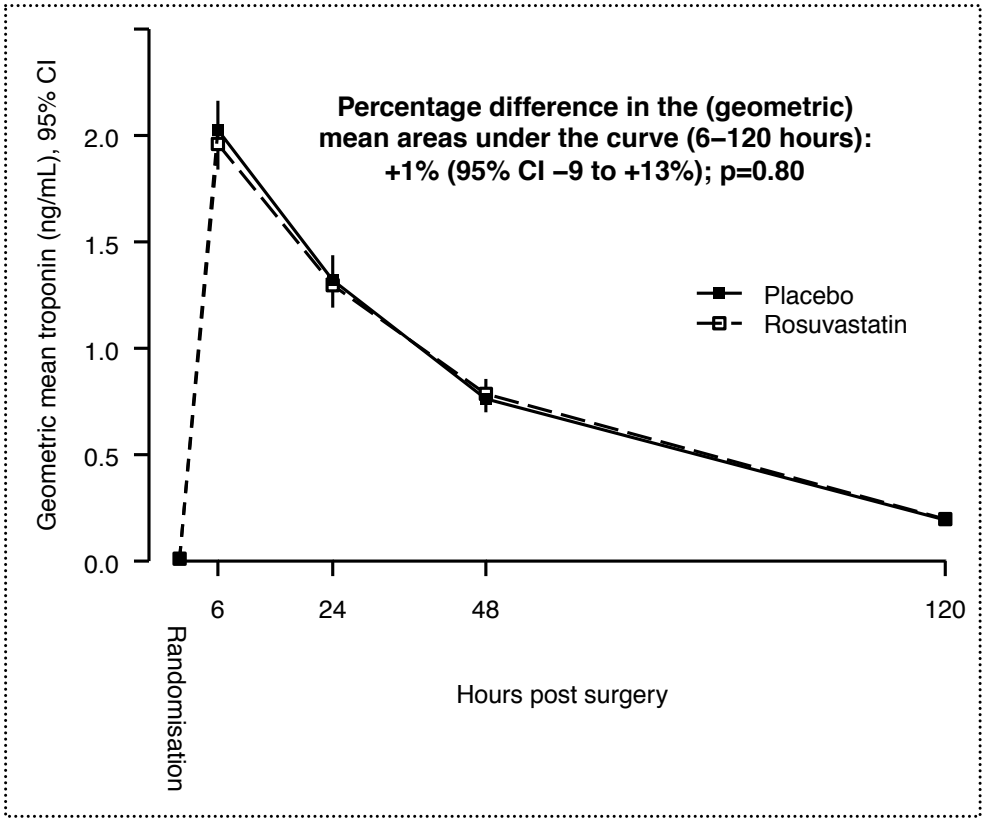

Figure 5.3 - Effect of Rosuvastatin on myocardial injury

Other adverse outcomes

Postoperative adverse outcomes were monitored for 5 days after surgery. Atrial fibrillation was identified by routine ECG or symptoms in 16% of patients who were assigned to rosuvastatin and 12% of those assigned placebo (P=0.03, **Table 5.2**). Otherwise, there were no significant differences between the treatment groups in the rates of postoperative serious adverse events, including any other heart rhythm disturbance, stroke, myocardial infarction, heart failure, infection, or death. Nor did the length of ICU or hospital stay differ significantly between the treatment groups (**Table 5.2**). Echocardiographic parameters, such as left atrial size and LVEF, did not differ between the treatment groups. NT-proBNP increased significantly 48 hours after surgery but was not altered by treatment allocation (**Table 5.2**).

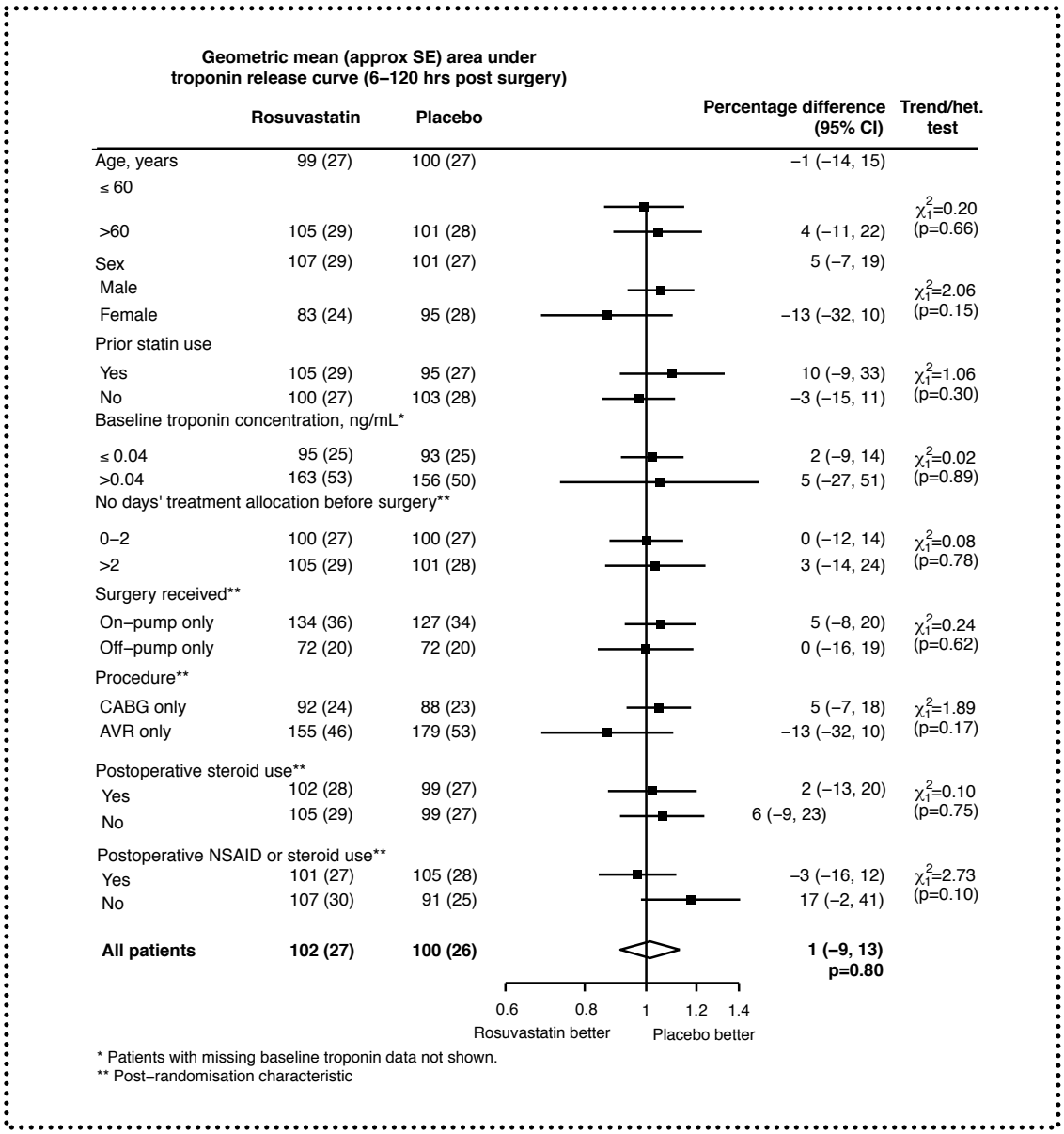

Figure 5.4 - Effect of rosuvastatin on myocardial injury, overall and in predefined subgroups

Effect of rosuvastatin on atrial superoxide production before CPB

To assess, whether treatment with rosuvastatin was associated with antioxidant effects, superoxide release was measured in atrial samples obtained before CPB by detecting 2 - OH - (E+) using HPLC. **Figure 5.5** show that, there was no difference between the randomised groups in myocardial superoxide production before CPB. Additional work on atrial samples to investigate this finding further is ongoing.

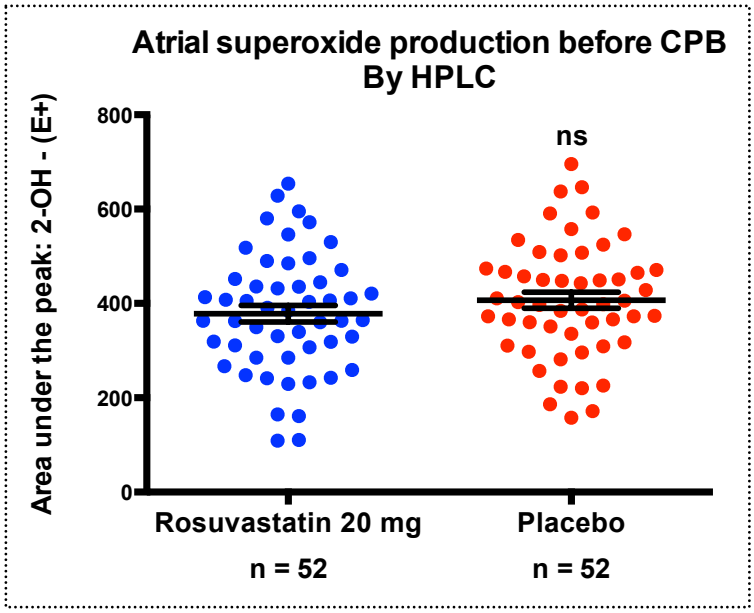

Figure 5.5: Average superoxide production in homogenates of the right atrial appendages taken before CPB, detected by HPLC. ns = not significant as p = 0.25 by unpaired t test. Data were expressed as mean ± SEM.

Discussion

It is generally accepted that the reduction in coronary events and stroke observed in patients taking long-term statin therapy is largely, if not wholly, mediated by sustained LDL cholesterol lowering [562]. However, it has been proposed that statins also produce clinical benefits that are independent of their effects on lipoproteins (*i.e.*, "pleiotropism")[563]. Beneficial effects of statin therapy in the postoperative setting[139,553,554] are plausible since inflammation and oxidative stress have been implicated in the pathogenesis of postoperative complications, and anti-inflammatory, antioxidant and cardioprotective effects

are amongst the pleiotropic actions of statins[518]. In particular, the incidence of postoperative AF coincides with the peak of the systemic inflammatory response after cardiac surgery[564], is independently associated with atrial markers of oxidative stress[98,99], and has been reported to be partially prevented by anti-inflammatory drugs (such as corticosteroids[565] or colchicine[566]).

The first randomised trial (ARMYDA-3) of perioperative atorvastatin (40 mg od) that was both placebo-controlled and had postoperative AF as its primary end-point reported a proportional reduction of 61% (95% CI 15-81%, P=0.017) in the odds of AF among 200 statin naïve patients[186]. Overall, in an updated meta-analysis of the published data from randomised trials in patients undergoing elective cardiac surgery, allocation to perioperative statin therapy (started between 48 hours and three weeks before surgery) was associated with an approximate halving in the incidence of postoperative AF (**Figure 5.6**). However, the trials that support the results of ARMYDA-3 (**Table 5.3**) appear to have a number of limitations, including: involving even smaller numbers of patients and (of most relevance) AF cases, not systematically excluding patients who already had AF (but excluding those who were taking a statin, limiting their relevance to current practice), not having postoperative AF as a pre-specified outcome, not systematically assessing AF by continuous ECG monitoring or blind to treatment allocation, and not analyzing the results by "intention-to-treat" (Figure 5.7). Indeed, of the 4 randomised trials that had postoperative AF as a pre-defined outcome[186,567-569], only ARMYDA-3 involved systematic and blinded assessment of AF and analysis according to "intention-to-treat" [186]. By contrast, the STICS trial is larger than all of these trials combined, only excluded patients who had pre-existing AF, mitral valve disease, or specific contraindications to statin therapy, recorded high levels of compliance to the allocated study treatment (as also indicated by the rapid reduction in LDL cholesterol in the rosuvastatin group), evaluated the pre-specified and pre-defined outcomes systematically in a blinded manner, and compared outcomes between the randomised treatment groups on an "intention-to-treat" basis. Given its design and large size, STICS is able to rule out more than a 15-20% proportional reduction in AF with reasonable certainty. Since STICS was specifically designed to provide an independent "hypothesis-testing" assessment of the effects of perioperative

statin therapy on AF (as well as on myocardial damage) that had been reported by “hypothesis-generating” meta-analyses of previous small trials, the results were considered separately in the updated meta-analysis. Moreover, given the highly significant interaction between the observed effect on AF in STICS and the previous trials, it is not statistically appropriate to combine them.

Age is the strongest risk factor for developing postoperative AF [570] and off-pump cardiac surgery has been associated with a lower incidence of postoperative AF[571]. In STICS, the incidence of postoperative AF was 14% in patients younger than 60 years versus 28% in those older than 60 years, and 24% with on-pump versus 18% with off-pump surgery. Nevertheless, there was no suggestion that the effect of rosuvastatin on postoperative AF differed with age or with surgical modality. The discrepancy between clinical diagnosis of AF and the events detected by continuous holter monitoring which included both sustained and non-sustained AF (Based on the criteria defined earlier in the chapter) suggests that many AF episodes in the postoperative period remain clinically asymptomatic. Characterisation of the relationship between variables generated by the “PATHFINDER”, the holter analytical platform (but not presented in this thesis) such as AF burden or number as well as duration of AF episodes and clinical use of antiarrhythmic drugs may further elucidate the underlying reasons. As ambulatory arrhythmia monitoring is not widely available, the results from this analysis may aid in development of practice guidelines and refine diagnostic accuracy of postoperative AF, risk stratification as well as management.

The average duration of randomised treatment before surgery in STICS was shorter than reported by most of the previous trials; however, even among patients in whom study treatment was started more than 2 days before surgery, allocation to rosuvastatin therapy did not reduce the incidence of postoperative AF. Prior statin use among the patients entered in STICS was 34% (in keeping with previously published data in similar populations in China [572]), but the effect of allocation to rosuvastatin on AF did not differ between patients who had been on statin therapy before randomisation and those who (as in all of the previous trials) had not. In the previous trials, perioperative statin therapy appeared to be

equally beneficial in patients of Caucasian or South-East Asian ethnicity and, although atorvastatin was tested in most of those trials, benefits had been reported with pravastatin, simvastatin and Rosuvastatin (Figure **5.6**). The previous trials however only included patients who had not been taking statin therapy prior to cardiac surgery; among the 1269 such patients in STICS, there was no beneficial effect of starting statin therapy on postoperative AF or myocardial injury. Studies evaluating pharmacokinetics of rosuvastatin in Chinese population has shown that, the relationship between dosage and efficacy is linear and there is little accumulation of the drug following repeated administration[573,574]. STICS was a hypothesis generating trial testing whether biological effects of HMG Co reductase inhibition independent of LDL lowering referred to as "pleiotropic effects" are beneficial in patients undergoing cardiac surgery. These effects have been shown to set in very rapidly following initiation of statin therapy and equally disappear after cessation of treatment [518]. This is of particular relevance in STICS as trial medications were initiated close to the day of surgery following cessation of previous statin treatment. Among such 653 patients in STICS, the finding that there was no benefit from continuing statin therapy versus stopping it prior to surgery suggests that previous statin therapy has no measurable impact on study outcome measures and that it is not necessary to continue statin therapy during the perioperative period to prevent postoperative complications.

The suggestion that statin therapy might reduce perioperative myocardial damage derives from one randomised placebo-controlled trial of rosuvastatin (20 mg od) in 200 patients having CABG surgery [555] and a non-blinded randomised trial of perioperative simvastatin (20 mg od) in 151 patients having non-coronary cardiac surgery[556]. In both trials, allocation to a statin was associated with a significant reduction in cTnI release during 5-8 days after surgery and, in the open trial, with better LV function[556]. By contrast, in the much larger randomised and blinded STICS trial, allocation to rosuvastatin had no effect on postoperative cTnI release, NT-proBNP 48 hours after cardiac surgery or LVEF at discharge. Nor were there any effects of the randomly allocated statin therapy on any of these outcomes in any of the pre-specified subgroups.

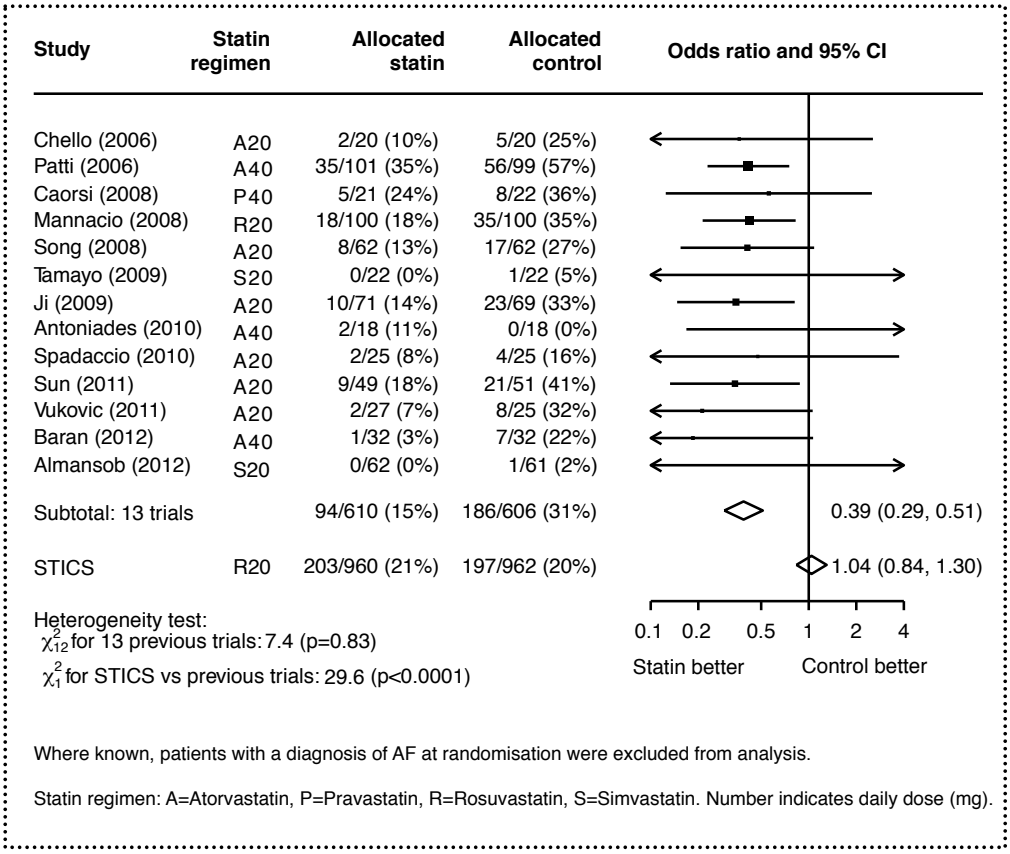

Figure 5.6 - Updated meta-analysis of effect of statin therapy on atrial fibrillation in cardiac surgery trials. AF (%)/Randomised

First author (year)	Number of randomised patients	Duration of randomised treatment before surgery	Patients with AF excluded	AF pre-specified outcome	AF assessed by continuous ECG monitoring	Blinded assessment of AF	Intention-to-treat analysis
Chello (2006)[553]	40	3 weeks	No	No	Yes	Yes	Yes
Patti (2007)[138]	200	6 days	Yes	Yes	Yes	Yes	Yes
Caorsi (2008)[554]	43	2 days	No	No	?	No	Yes
Mannacio (2008)[533]	200	7 days	No	No	No	Yes	Yes
Song (2008)[545]	124	3 days	Yes	Yes	Yes	No	Yes
Tamayo (2008)[555]	44	3 weeks	No	No	?	No	Yes
Ji (2009)[546]	140	7 days	Yes	Yes	Yes	Yes	No
Antoniades (2012)[99]	42	3 days	No	No	No	Yes	Yes
Spadaccio (2010)[556]	50	3 weeks	No	No	Yes	Yes	Yes
Sun (2011)[547]	100	7 days	Yes	Yes	Yes	Yes	No
Vukovic (2011)[557]	57	3 weeks	No	No	?	No	Yes
Baran (2011)[558]	64	14 days	Yes	No	?	Yes	No
Almansob (2012)[534]	151	5-7 days	No	No	?	No	No

Table 5.3 - Characteristics of previous trials of perioperative statin therapy in cardiac surgery (as shown in figure 5.6).

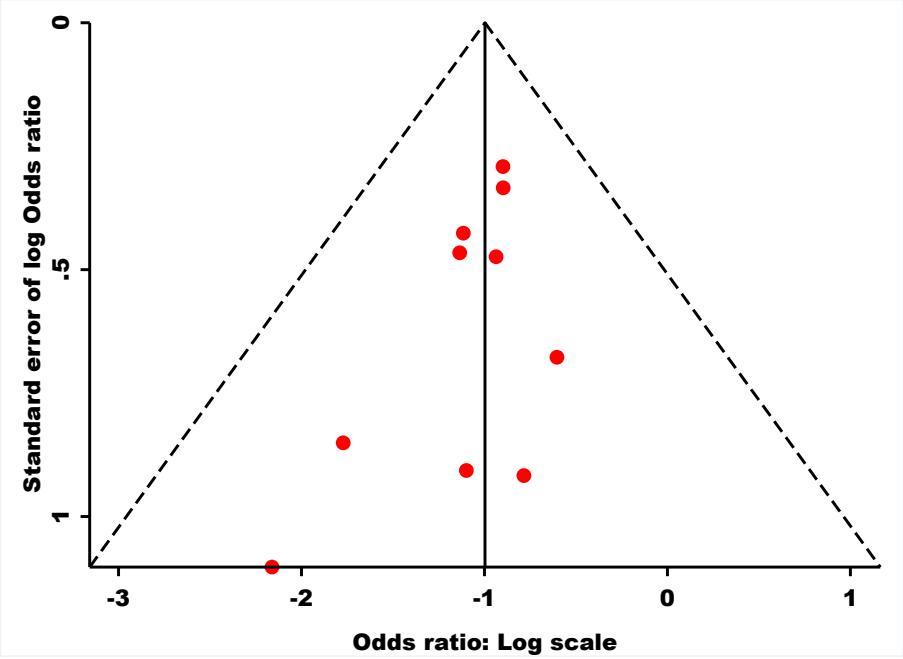

Figure 5.7: Funnel plot of the effect of perioperative statin therapy on atrial fibrillation in 13 cardiac surgery trials showing asymmetry by Egger's test (p = 0.007). The solid vertical line represents the summary estimate of the treatment effect, derived using fixed-effect meta-analysis.

Conclusions

Perioperative statin therapy does not prevent postoperative AF or perioperative myocardial damage in patients undergoing elective cardiac surgery, and nor does it affect hospital stay, postoperative LV function, or the incidence of major cardiovascular adverse events. Although the beneficial effects of long-term statin therapy in patients requiring myocardial revascularization are well established, the findings presented in this chapter do not support routine use of short-term perioperative rosuvastatin for the prevention of postoperative complications of elective cardiac surgery.

Supplement

1: Patient Information leaflet

You are being invited to take part in a research study where, in addition to being given all of the treatments that are standard, patients who are about to have heart surgery are randomly allocated to receive either 20 mg rosuvastatin daily or matching dummy "placebo" tablets for up to 14 days.

Please take the time to read the following information and consider carefully your participation in this study. If anything is not clear or you would like more information, please ask the research staff.

You are entirely free to decide whether or not to take part in this study. If you choose not to take part, the standard of care given by your doctors will not be affected. Moreover, if you decide to take part, you would be free to withdraw at any time without giving a reason.

What is the purpose of the trial?

Patients with heart disease are often prescribed "statin" drugs because it has been shown that their long-term use reduces the risks of having a heart attack, stroke or other problems and they are one of the most widely used treatments in modern medical practice, with very few side-effects.

During coronary artery bypass surgery (CABG), including when diseased heart valves are repaired or replaced, there is an increased risk of injury to the heart muscle. Following such heart surgery, up to half of all patients develop an irregular heartbeat ("arrhythmia") called "atrial fibrillation" (AF). These arrhythmias do not usually last a long time, but they can lead to complications requiring a longer hospital stay.

In addition to lowering cholesterol, experimental studies have suggested that statins have rapid anti-inflammatory, anti-oxidant and anti-arrhythmic effects during and after heart surgery. But it is not known whether these effects are of any real benefit to patients and no statin regimen, including the 20 mg rosuvastatin daily dose being studied in this trial, is known to be indicated in this setting. As a consequence, some doctors routinely stop statin therapy before heart surgery, some continue it during surgery and if you are not on statin therapy routinely like the 50-70% of patients scheduled for open heart surgery in Fuwai hospital, your physician is unlikely to prescribe statins either.
The objective of this study is to assess whether perioperative statin treatment translates into better post-operative outcomes for patients undergoing cardiac surgery.

More precisely, this study aims to find out whether a short course of a commonly used statin (20 mg rosuvastatin once a day) reduces damage to heart muscle and the likelihood of irregular heartbeats developing in patients shortly after heart surgery. In order to address these questions without any bias, half of the patients who join the study will receive rosuvastatin tablets and half will receive matching dummy "placebo" tablets (which look like rosuvastatin but do not contain active statin).

The decision about which participants receive rosuvastatin and which participants receive placebo in the study will be decided according to a computer-generated random order. The treatment that is allocated to a particular patient will be unknown by both the participant and their doctors (although members of the health-care team can find out if this is needed for the care of a patient).

Why have I been invited?

You have been invited because you are about to have coronary artery surgery with/without valve replacement or an isolated valve surgery at the Fuwai Hospital. It is intended that about 1000 patients having such surgery will join the study.

What would taking part involve?

If, after considering this information leaflet and asking any questions that you have about the study, you agree to take part then you would be invited to sign a written consent form. The procedures would then involve the following steps, with most of them being part of the routine clinical procedures:

Data collection: Information about your past medical history (for example, whether you have high blood pressure, kidney or liver disease, diabetes or have had a heart attack) and the treatments that you take would be recorded on a study form along with pertinent details of the ultrasound scan of your heart (echocardiogram) performed as a part of your care before surgery. Before your discharge from hospital, information about your operation, your recovery after surgery and your treatment would also be recorded on a study form including the echocardiography report after surgery. This information would be held confidentially and used only for this research study.

Blood sampling: The equivalent of two teaspoon (one tea spoon = approximately 5 mls) of blood would be taken from a blood vessel in your arm immediately after you join the study. Subsequently, a similar amount of blood would be taken from a sterile tube which is inserted into one of your neck veins as a part of the standard care for heart surgery (or taken from your arm if the sterile tube is not available) on the day of your surgery, on each of the 3 days after surgery and on 5th day after the surgery.

These samples would be used to measure your blood levels of cholesterol and some inflammatory and other biomarkers. We may also use the samples to analyse variation in your genes. The samples would be held in frozen storage in order to allow them to be used for future blood analyses.

Urine sampling: The equivalent of two teaspoon (one tea spoon = approximately 5 mls) of urine will be collected twice (6 hrs and 24hrs) after the surgery. Normally you will be having a urinary catheter inserted as a part of your routine care after surgery and the sample will be collected from the bag attached to the catheter. In the event of you not having the catheter, you will be requested to provide the sample in a bottle. This sample will be used to measure some of the biomarkers reflective of whether the kidneys have sustained any injury after the surgery.

Study treatment: You would be asked to take the first study tablet immediately after joining the study and then to take one study tablet a day for 5 days after your surgery. The study tablets that you receive would either all contain 20 mg rosuvastatin or all contain placebo.

If you currently take a statin (atorvastatin, lovastatin, pravastatin, rosuvastatin, simvastatin) then you would be asked to stop it. When study treatment is completed i.e. after post-operative day 5, you would be able to start taking your usual statin treatment again.

You would also have to avoid drinking more than 1 litre per day of grapefruit juice or using herbal medicines while taking the study treatment. This is because large quantities of grapefruit juice may interfere with statins, and so may some kinds of herbal medicine.

Collecting routinely-removed tissue samples: During heart surgery, small amounts of heart tissue are routinely removed and discarded. If you agree to join the study then we would collect some of these samples. This would allow us to test whether short-term treatment with rosuvastatin reduces inflammation and improves markers of function in heart tissue. These samples would be stored securely and used for research purposes including genetic research.

***Recording electrical activity of your heart continuously*:** Following surgery, your heart rhythm would be recorded for up to 6 days using a special device called a Holter monitor. This is a small battery-powered electronic device that is linked via cables to sticky pads attached to the skin on your chest. It is a non-invasive, painless process that would not interfere with your routine care.

Are there any possible adverse effects of taking part in the study?

Side-effects of rosuvastatin: Rarely, statins can cause muscle pain and/or weakness with abnormal muscle enzyme blood tests, which is called “myopathy”. Typically, this occurs in about 1 in 10,000 people per year of statin therapy, although it may occur more commonly in Chinese people. In the very large JUPITER trial, there were very few problems with 20 mg rosuvastatin daily given for about 3 years. In the present study, 20 mg rosuvastatin daily is only to be given for up to 14 days, so the likelihood of myopathy occurring is extremely low. If evidence of myopathy emerges during the course of your study treatment (clinical symptoms supported by lab evidence), the study treatment would be stopped immediately.

Stopping current statin therapy: Half of the people who join the study – including those who are already taking statin therapy beforehand – will be allocated placebo tablets which do not contain rosuvastatin. In normal practice, some doctors routinely stop statin therapy before heart surgery whereas other doctors continue it during surgery. It is not known whether stopping statin therapy for a short period (up to 14 days in the present study) before and after heart surgery has any effect. The beneficial effects on heart attacks and strokes due to lowering blood cholesterol levels with statins typically emerge only after several months of treatment. The present study will assess whether there are other effects of statin therapy that occur more rapidly around the time of surgery.

What will happen if I don’t want to carry on with the study?

Participation in this study is voluntary and you can withdraw from it at any time without any change in the standard of care you will receive. If you decided that you did not want to continue taking the study treatment then you could allow us to follow your progress so that information about you still contributed to the research. Alternatively, if you wanted to withdraw from the study entirely then we would destroy all of the information and blood/tissue samples that can be identified as relating to you.

What if relevant new information becomes available?

If relevant new information about the benefits or safety of using statin therapy during and after heart surgery emerges while you are taking the study treatment then we would tell you about it and discuss whether you want to continue in the study. Your own doctors might also consider it to be in your best interests to withdraw you from the study.

What will happen to the results of the study?

It is intended that the results of the study will be published in a scientific journal for the benefit of the wider medical community and future patients. Individual participants in the study would not be identified in any publication, and their details would remain confidential.

Will information about me be kept confidential?

Information collected about you on the study forms would be entered onto computers in the China Oxford Centre for International Health Research at the Fuwai Hospital in Beijing. These data will be stored securely, with personal identifiers (such as patients' names) held separately from all other information to which they will be linked by a unique number. Blood,urine and tissue samples and Holter monitor recordings will also be stored separately from personal identifiers. Final analyses will only involve "anonymised" data from all participants rather than any specific individual.

Access to all study information will be restricted to authorized study personnel on a need-to-know basis, and will be controlled by usernames and passwords. Authorized people from regulatory agencies, drug company etc. may look at the information to ensure that the study is being carried out correctly, but will be bound by rules of confidentiality.

Who is organizing and funding the research?

This study has been designed and coordinated by the Department of Cardiac Surgery/ China-Oxford Centre for International Health Research at the Fuwai Hospital and the Department of Cardiovascular Medicine/Clinical Trial Service Unit at Oxford University/UK. The study has been reviewed and approved by an independent research ethics committee at the Fuwai Hospital.

Packaged study treatment (rosuvastatin and placebo) has been purchased by the study organizers. The rosuvastatin is marketed drug produced by Astra Zeneca, and the placebo tablets have been made for the study by a qualified pharmaceutical factory holding a certificate of good manufacturing practices for pharmaceutical products from the China State Food & Drug Administration (SFDA).

What if you have a concern with the study?

If you have a concern about any aspect of the study at any time, you should speak with a member of the research team who will do their best to answer your questions. If you still have any concern and/or wish to complain, please contact the study coordinator at the address below. In the unlikely event of you being harmed as a result of taking part in the trial, compensation would be paid if the injury probably resulted from taking the study treatment or from any study-specific procedure. You would also be entitled to free treatment at the Fuwai Hospital for such complications.

Contact details of the coordinating centre:

STICS trial co-ordinator
China Oxford Centre for International Health Research
Fuwai Hospital
167 Beilishi Road,
Beijing, China, 100037

Telephone: 10-88365200

Fax: 10-88365201

Email: stics@fwoxford.org

2. Consent sheet

Version 3 – September 1 , 2011

DEPARTMENT OF CARDIO-THORACIC SURGERY &
CHINA-OXFORD CENTRE FOR INTERNATIONAL HEALTH RESEARCH
FUWAI HOPSITAL
167 Beilishi Road, Xicheng District, Beijing, China

Study title	**STICS: Statin Therapy In Cardiac Surgery**
Patient ID	

Agreement to participate:	**Yes**	**No**
• I confirm that I have read and understood the information sheet for the above study. I have had the opportunity to consider the information, ask questions and had these answered satisfactorily.	☐	☐
• I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	☐	☐
• I understand free treatment and compensation will be provided if any injury/adverse events arise as a result of study procedures.	☐	☐
• I understand that relevant information from my medical notes may be looked at by authorized study staff. I permit these individuals to access my records and understand that all the data relating to the study will be kept confidential.	☐	☐
• I understand why blood and urine samples are being taken, how the samples will be collected, that giving samples for this research is voluntary and that it will be used for research purposes, including genetic research.	☐	☐
• I understand why tissue samples from my heart are being collected, how the samples are collected, that giving samples for this research is voluntary, and that it will be used for research purposes.	☐	☐
• I agree to take part in the above study.	☐	☐
• I give permission to store my blood, urine and tissue samples for use in future research	☐	☐

Name of the participant	**Date**	**Signature**

Name of the Doctor	**Date**	**Signature**

CHAPTER 6

GENERAL DISCUSSION

Key findings

The work presented in this thesis elucidates the molecular mechanisms underlying myocardial nitroso-redox balance during elective cardiac surgery on CPB, analyses the effects of perioperative HMG CoA inhibition with Atorvastatin 80 mg on myocardial nitroso-redox balance as well as changes in postoperative right atrial effective refractory period (STARR) and investigates the impact of short-term intensive therapy with Rosuvastatin 20 mg on common postoperative outcomes after elective cardiac surgery (STICS). The key findings are; I) Elective cardiac surgery on CPB is associated with decreased bioavailability of nitric oxide due to *S*-glutathionylation of eNOS II) Perioperative therapy with Atorvastatin 80mg for an average of two days before and continued for five days after the surgery redressed increase in right atrial superoxide production, maintained functionally coupled status of NOS and bioavailability of NO during CPB in the absence of a measurable effect on changes in postoperative right atrial effective refractory period III) Intensive perioperative treatment with Rosuvastatin 20 mg in patients undergoing elective on- or off-pump CABG, AVR or combined CABG and AVR did not prevent postoperative atrial fibrillation detected by continuous holter monitoring, perioperative myocardial injury detected by cTnI release or other common in-hospital complications.

Taken together, the work presented in this thesis does not support short-term intensive therapy with HMG Co-A inhibitors for the primary indication of prevention of in-hospital peri- and postoperative complications in patients undergoing elective cardiac surgery.

General discussion

Redox signaling achieves a degree of functional specificity by confining the production or limiting the spread of ROS to discrete intracellular micro domains where redox-sensitive targets are in abundance[492]. In the presence of oxidative stress associated with cardiac surgery, dysregulation of these signaling pathways can result in perioperative myocardial dysfunction and postoperative arrythmias[87]. Given the failure of systemic antioxidant therapy to confer any

benefit[487], inhibition of specific oxidase systems (e.g., of NOX2 oxidases[89,98]) by statin therapy might mitigate oxidative stress in relevant subcellular compartments and decrease the incidence of perioperative myocardial dysfunction and postoperative atrial fibrillation in patients undergoing cardiac surgery. However, from the findings reported in STICS, it is clear that perioperative short-term intensive therapy with statins does not translate into beneficial outcomes after elective cardiac surgery raising important questions on the reasons behind the failure of translation.

Reactive oxygen and nitrogen species mediated post-translational modifications

Covalent post-translational modification of proteins[575], usually targets cysteine residues owing to the strongly nucleophilic sulfhydryl side chain[466] and is a powerful mechanism that modulates protein function and stability. Redox signaling mediated by oxidation-reduction reactions, primarily involves the reactive cysteine residues in the target protein complex[433,576,577]. By spatially restricting the generation of ROS and post-translational modifications, redox signaling exerts diverse effects on protein stability, localization and interaction. NO-dependent modification of cysteine residues may also modulate protein function in a similar manner[434,578].

Configuration of electrons in the D-orbital of the sulphur atom located at the centre of the thiol group in the terminal side chain of cysteine residues allows multiple oxidation states[579]. This inherent diversity[580] can result in alteration of protein function by a range of potentially reversible modifications such as *S*-nitrosylation[581,582], sulfhydration[583], *S*-glutathionylation[584,585], formation of disulfide bonds[586-588], sulfenylation[589,590] or irreversible oxidative processes such as carbonylation[591] and reactions mediated by sulphonic acids[592].The formation of an individual oxidative post translational modification depend primarily on the reactivity of individual cysteine residues determined by its location on the surface of the protein, pKa of thiols or composition of neighboring amino acid residues[593-596]. In addition, local redox environment, proximity of neighbouring SH groups, amides or sulfenic acid residues also can impact the reactivity of cysteine residues[466,597]. Accordingly, susceptibility of individual proteins to

various oxidative post-translational modifications and hence their function may vary, thereby allowing the cardiovascular system to mount an appropriate response depending on the magnitude and duration of oxidative stimuli. Moreover, it has been shown that, these mechanisms may impact other post-translational modifications such as phosphorylation, acetylation, ubiquitination, and the cross talk between the two processes may ultimately determine the biological outcomes[585,593,598-605].

Among cysteines' post-translational modifications, *S*-glutathionylation and *S*-nitrosylation have attracted much attention, partly due to better availability of methods that can identify these modifications in biological samples [606-611]. It has been suggested that a close relationship exists among the mechanistic as well as functional aspects of these modifications [612-614]. Supporting this notion further, it has been demonstrated that the function of number of proteins involved in cardiac metabolism, calcium handling, ion channel function and inotropy (such as the mitochondrial complexes [615-618], GAPDH[619,620], HIF-1alpha[621], Na, K- ATPase[622-624], calcium-calmodulin kinase II[625], calcium channels[626,627], ryanodine receptor-2 [103,628-630], SERCA[631-633], actin[634], titin[635], troponin-I[636], phospholamban[637], and protein kinase A and G[638,639]) can be modified by these mechanisms. For instance, *S*-glutathionylation as well as *S*-nitrosylation, protects Ryanodine receptor-2 against irreversible oxidation of redox-sensitive cysteine residues located on its surface [104,640,641], decreases NO synthesis by eNOS[431,512] and activates SERCA[631,642]. Functionally, both reversible protein *S* - glutathionylation and *S* - nitrosylation have been linked to cardioprotection[643] as well as induction of adaptive physiological responses during ischemia/reperfusion[626], whereas dysregulation of protein *S* - nitrosylation and irreversible protein oxidation have been associated with contractile dysfunction and arrythmogenesis[644-649]. Similarly, both redox and NO signaling are involved in the regulation of myocardial excitation-contraction coupling, excitability, and adaptation to hypoxia [650,651]. The apparent lack of benefit of intensive therapy with statins in STICS or, in other words, similar incidence of postoperative AF and other major in hospital outcomes in the randomised groups, may have been due to this convergence of ROS and NO mediated signaling mechanisms as well as corresponding post-translational modification

of proteins on functional outcomes. This is supported by the findings reported in chapter three and four (STARR) where it was shown that signaling mechanisms in the myocardium during elective cardiac surgery is primarily mediated by ROS whereas perioperative HMG CoA inhibition shifted the balance towards reactive nitrogen species.

Future directions

The paradigm of lack of efficacy[652] or even increased mortality[653] associated with systemic antioxidant therapy favored a more targeted approach focusing on subcellular enzymatic sources of oxidant species. NOX2-containing NADPH oxidases are an attractive target for therapeutic intervention as they are an important source of ROS production in the human myocardium, function primarily to generate ROS in a highly regulated and spatially confined manner, are stimulated by inflammation, and their activity has been associated with new onset AF after cardiac surgery[98,654-657]. In the work presented in this thesis, inhibition of NOX2-NADPH oxidases by statin therapy did not translate into improvement in postoperative outcomes after elective cardiac surgery, necessitating further research for other therapeutic targets.

As demonstrated in this thesis as well as in many previous reports, increasing age is the strongest risk factor for the development of postoperative AF[658,659]. Mitochondrial dysfunction characterized by increased ROS production has been associated with ageing[660-662] as well as postoperative AF[663]. In addition, mitochondria have been characterized as the primary effectors of I/R injury in ageing hearts[447-449]. Notwithstanding these findings and implication in permanent AF[89], it is unclear whether mitochondria derived ROS have a direct role in the pathogenesis of new onset AF and myocardial dysfunction following I/R. For instance, a lot needs to be discerned regarding the molecular mechanisms of mitochondrial ROS production under physiological and pathological conditions. In this respect, micro RNAs [664] and redox post-translational modifications of mitochondrial electron transport chain[665-667] are an expanding field of research. It has been suggested that NOX2-derived ROS may stimulate mitochondrial ROS production following many pathological processes and in the same token, mitochondrial ROS induce superoxide production from

NADPH Oxidase during hypoxia[457,458] though the related molecular mechanisms remain unclear[455]. Since NOX2 activity has been associated with arrythmogenesis[88,98,119,668,669] and I/R injury[333,452], the cross talk between the two oxidase systems might be of functional significance in the context of cardiac surgery. Unraveling the mechanisms by which redox posttranslational modifications modulate proteins involved in E-C coupling and metabolism will be critical. In this regard, identifying the cysteine residues targeted by post-translational modification and their impact on protein function and subcellular localization will further advance this area of research. Together, the insights gleaned from these investigations may result in the development of more successful cardioprotective strategies.

Concluding remarks

The challenges which lie ahead in development of interventions to prevent perioperative myocardial dysfunction and arrhythmias in cardiac surgery are underscored by the need for a clearer understanding of redox sensing, signaling, and targets. An ideal intervention should preserve ROS mediated physiological adaptive responses during cellular stress while preventing the deleterious effects of ROS on myocardial excitability and metabolism.
The work presented in this thesis indicates that short-term statin therapy, at the doses conventionally use to lower LDL-cholesterol, has a significant impact on the myocardial redox state of patients who had cardiac surgery on CPB by both inhibiting NOX2 and mitochondrial oxidases and preserving NOS activity mostly through the prevention of NOS *S*-glutathionylation. However, these presumably favorable actions are not associated with cardioprotection, improved post-operative left ventricular function, or prevention of AF after cardiac surgery. Taken together, these findings cast some doubt on the pathogenic role of myocardial nitroso-redox imbalance on in-hospital postoperative complications in patients undergoing elective cardiac surgery and support the prevailing view that most, if not all, of the beneficial effect of statin therapy in patients is due to LDL cholesterol lowering.

APPENDICES

1: Timing and venue of research

Work	Venue	Period
Chapter 3	1,2,3	2010 -12
Chapter 4	1,2,3	2012 -14
Chapter 5	1,2,4,5,6	2012 -14

Number	Description
1	Prof Casadei lab, RDM division of Cardiovascular Medicine, University of Oxford
2	British Heart Foundation Molecular Cardiology lab, Wellcome Trust Gene Centre, University of Oxford
3	Division of Cardiothoracic Surgery, Oxford Heart centre, Oxford University Hospitals NHS Trust
4	National Clinical Research Center of Cardiovascular Diseases, National Center for Cardiovascular Diseases*
5	Department of Cardiovascular Surgery, Fuwai Hospital*
6	Clinical Trial Services Unit, University of Oxford
*	Affiliated to Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China

2: Sources of research funding

I was funded by the Oxford Biomedical Research Centre (NIHR) project grant and British Heart Foundation programme grant awarded to Prof. Casadei. The project specific costs were met from the grants awarded to Prof Casadei as below.

- Chapter 3: The British Heart Foundation.
- Chapter 4: An unrestricted Grant from Pfizer pharmaceuticals, British Heart Foundation, Oxford Biomedical Research Centre (NIHR).
- Chapter 5: British Heart Foundation, FP7 European Network for Translational Research in Atrial Fibrillation (EUTRAF), Oxford Biomedical Research Centre (NIHR), Clinical Trial Service Unit (Oxford University), UK Medical Research Council, and a small unrestricted grant from AstraZeneca.

None of the funders had any role in the design, conduct, analysis or interpretation of the studies.

3: Attributions

I am extremely grateful for the contributions made to this research by colleagues and collaborators as noted in Chapter 2 and also in the next section. Apart from the exceptions described below, I am responsible for setting up the studies, the recruitment of the patients, collection of biological samples, conduct of experiments, data analysis and preparation of the thesis.

STICS: Practical conduct of the study was undertaken by staff at National Clinical Research Center of Cardiovascular Diseases, Fuwai Hospital, Beijing, China. The accredited staff at Wolfson's laboratories, CTSU, UK, performed the biomarker assays. Dr. Jonathan Emberson, the University senior statistician, did the statistical analysis.

4: Acknowledgements

I am very grateful to my supervisor Prof Casadei for giving me this opportunity, nurturing and guiding me throughout the DPhil period. The programme, I was blessed with, was unique in so many ways. It suited my career track as an academic intensivist as well as laid foundation for a future clinician scientist path with the acquisition of a diverse set of basic scientific skills and training in large-scale clinical studies. The weekly lab meetings were excellent learning opportunity with direct supervision where I was also able to discuss issues if any, receive helpful comments and constructive feedbacks. Outside the lab meeting, Prof Casadei was always accessible despite a demanding busy schedule round the year and offered valuable advice and guidance promptly as and when I needed them. I extend my sincere gratitude to Prof Casadei for providing me this high-quality training programme and expert supervision.

I thank my joint supervisor Dr Michael Hill for being a friend, philosopher and guide during this journey. I have learned some qualities by this relationship, not

the least of which is efficient management of multiple complex projects, related professional life while being a great family man.

I also thank Prof Zhengming Chen for his role as joint supervisor and also appreciate his support during my time in Fuwai Hospital, Beijing, China.

A research programme of this dimension would not have become possible without the help and support from many colleagues and collaborators.

I thank the following individuals as detailed below.

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- Keshav Nahar – For the friendship and support in the lab.
- Silvia Guarguagli (SG), Azhar Hussein (AH), Mei Hua Zhang (MZ), Nicky Goodfellow (NG), Svetlana Reilly (SR); SG and AH – Analysis of Holter recordings, MZ – Technical inputs for *S*-Glutathionylation IP/IB, SR-Training for ROS measurements, qPCR, NG –qPCR.
- Eunice Berry – For the excellent help in the capacity as Prof Casadei's secretary.

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- Dr Nikhil Pal: For the friendship and the collaboration for the Echocardiography measurements in STARR.
- Dr Mark Crabtree and Ashley Hale: For the training and technical input for working with HPLC platform.
- Mr Phil Townsend and Dr James Brown: For the help in the capacity as lab managers.
- Mrs Lynn Clee, Heidi Crook and Emma Heaton - Administration.
- Prof Hugh Watkins: For the help in the capacity as the head of the department.

Oxford University Hospitals NHS Trust:

- Dr Michael Jones: For the friendship and the collaboration for the atrial effective refractory period measurements (Technical input and duplicate analysis of ECG recordings).
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- Prof P. Foex: For being there in the background.
- Dr Oliver Dyar, Dr Vivian Addy – For supporting out of programme leave application.

Health Education, Thames Valley (Formerly Oxford PGMDE):

- Dr Michael Bannon – Postgraduate Dean for approving out of programme leave application.

Principal Investigators and Research Staff at the China-Oxford Center for International Health Research of the Fuwai Hospital:

I would like to say big thanks to everyone I had the pleasure to meet and work with in the context of STICS, especially to Pang Xin, Dai Hao, Yan Zhao, Xiaoshuai and Libo Hou.

Clinical Trial Services Unit, University of Oxford:

I would like to acknowledge the support given by Dr Jonathan Emberson for the statistical analysis. Also, I would also like to thank Dr Aishwarya Kumar and other colleagues in the Wolfson Laboratories for their work on biomarker assays.

Industry:
I had interacted with many personnel affiliated with many industries and had obtained technical as well as logistical support for setting up the projects, analytical platforms and related processes. In particular, I would like to thank Dr Iqbal Minhas of Pfizer Limited, UK, Mr Stephen Allen of Medtronic UK and Mr Ryan Hill of Spacelabs Healthcare.

All patients:
I am most grateful to all of the patients who agreed to take part in the studies, kindly donated biological samples, and patiently cooperated for the measurements during one of the most difficult stages of their lives while recovering from major surgery. Many of the professional interactions expanded into long lasting friendships, in particular, Mr. David Nye for his pastoral support and warmth.

Family:
Finally, I would like to express my love and gratitude to my wife Srividhya for being there all the time, helping and shouldering the responsibilities of bringing up our two lovely sons, Adi and Aghi who missed their dad when it mattered on many occasions. Without their assistance and understanding, none of this would have been realistic.

5: Publications arising from the work in this thesis

1: R Jayaram, N Goodfellow, K Nahar, MH Zhang, S Reilly, MJ Crabtree, R De Silva, R Sayeed, B Casadei. On-Pump Cardiac Surgery in Humans Induces Myocardial Nitric Oxide Synthase Dysfunction via S-Glutathionylation of eNOS. Circulation. 2014; 130: A13082.

2: R Jayaram, N Goodfellow, K Nahar, MH Zhang, S Reilly, MJ Crabtree, R De Silva, R Sayeed, B Casadei.Mechanisms of myocardial nitroso redox imbalance following elective cardiac surgery on cardiopulmonary bypass Cardiovasc Res (2014) 103 (suppl 1): S117.

3: Zhe Zheng, Raja Jayaram, Lixin Jiang, Jonathan Emberson, Yang Zhao, Qi Li, Silvia Guarguagli, Michael Hill, Zhengming Chen, Rory Collins , Barbara Casadei. Perioperative rosuvastatin treatment in cardiac surgery for the prevention of postoperative atrial fibrillation and myocardial damage (At the time of submission of the thesis, under peer review in NEJM)

6: Other publications during the DPhil training period

1: Idigo WO, Reilly S, Zhang MH, Zhang YH, **Jayaram R**, Carnicer R, Crabtree MJ, Balligand JL, Casadei B. Regulation of endothelial nitric-oxide synthase (NOS) S-glutathionylation by neuronal NOS: evidence of a functional interaction between myocardial constitutive NOS isoforms. J Biol Chem. 2012 Dec 21; 287(52): 43665-73. PubMed PMID: 23091050.

2: Antoniades C, Demosthenous M, Reilly S, Margaritis M, Zhang MH, Antonopoulos A, Marinou K, Nahar K, **Jayaram R**, Tousoulis D, Bakogiannis C, Sayeed R, Triantafyllou C, Koumallos N, Psarros C, Miliou A, Stefanadis C, Channon KM, Casadei B. Myocardial redox state predicts in-hospital clinical outcome after cardiac surgery effects of short-term pre-operative statin treatment. J Am Coll Cardiol. 2012 Jan 3; 59(1): 60-70. PMID: 22192670.

3: Reilly SN, **Jayaram R**, Nahar K, Antoniades C, Verheule S, Channon KM, Alp NJ, Schotten U, Casadei B. Atrial sources of reactive oxygen species vary with the duration and substrate of atrial fibrillation: implications for the antiarrhythmic effect of statins. Circulation. 2011 Sep 6; 124(10): 1107-17. PubMed PMID: 21844076.

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7: Awards and Prizes

1: **Poster prize** distributed by Cardiovascular Research: Frontiers in Cardiovascular Biology, European Society of Cardiology - 2014

2: **International Travel Grant:** Council of Basic Cardiovascular Sciences (BCVS): American Heart association – 2014.

3: **Abstract Travel Grant:** Council of Basic Cardiovascular Sciences (BCVS): American Heart association – 2014.

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