


# A randomised control trial of 5-Aminolevulinic Acid in combination with Sodium Ferrous Citrate to enhance Cardioprotection in Adults undergoing Cardiac Surgery (TALEN)

A Thesis Submitted for the Degree of Doctor of Philosophy

Trinity Term, 2024

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## Abstract

Haem oxygenase-1 (HO-1) is an evolutionarily highly conserved, ubiquitously expressed protein with critical antioxidant and pro-survival effects, primarily responsible for catalysing haem breakdown into equimolar quantities of carbon monoxide, biliverdin, and ferrous iron – each bioactive signalling molecules. Through its enzymatic role to catabolise haem, as well as through multiple non-enzymatic functions including favourable effects on immune cell regulation, immunometabolism and iron homeostasis, HO-1 represents an attractive therapeutic target for improving outcomes after cardiac surgery on cardiopulmonary bypass (CPB) which is associated with cardiac ischaemia-reperfusion injury, haem toxicity, sterile systemic inflammation with risk of organ injury. A prior Mendelian randomisation study in 2377 adult patients undergoing cardiac surgery on CPB linked genetically driven increased HO-1 function to lower acute kidney injury (AKI). Treatment with 5-aminolevulinic acid combined with sodium ferrous citrate (5-ALA-SFC) represents a pharmacological strategy to enhance HO-1 activity, mitigate haem-driven toxicity and reduce inflammation to potentially improve patient outcomes following cardiac surgery.

The TALEN trial was a phase II, single centre, randomised, double-blind, dose-finding adaptive translational trial evaluating the safety, tolerability and pharmacodynamic effects of oral 5-ALA-SFC administered pre-operatively for 3 days in 4 sequential dosing cohorts of patients (8 active:2 placebo) through evaluation of HO activity, immunophenotyping and biomarkers of organ injury to determine an optimal biological dose for future evaluation.

All dose level cohorts were successfully enrolled. A total of 48 patients were randomised with the resulting study population predominantly male with a median age of 69 years undergoing a mix of surgery (isolated coronary artery bypass grafting, aortic and mitral valve repair/replacement) on CPB. 5-ALA-SFC was well tolerated, with no adverse safety signals including no treatment-related serious adverse events or dose limiting toxicity. Drug compliance was excellent. A novel LC-MS/MS based HO activity assay developed for the trial in combination with monocyte immunoprofiling identified the highest dose of 5-ALA-SFC evaluated (300 mg/472 mg) as most robustly activating HO-1. Compared with placebo, treatment with 5-ALA-SFC was also associated with significantly reduced post-operative inflammation measured by CRP and IL-6. 5-ALA-SFC showed a significant renoprotective effect across all doses. No significant difference in peri-operative myocardial injury was identified using troponin I measurements although 5-ALA-SFC groups had numerically lower arrhythmia and inotrope requirements compared to placebo. Immunophenotyping by multiparameter flow cytometry revealed a 5-ALA-SFC treatment-associated monocyte phenotypic commitment to favour erythrophagocytosis and a ferrostatic, iron regulatory role.

The findings highlight the ability of pre-operative dosing with 5-ALA-SFC to induce HO-1, exert immunomodulatory effects including altered monocyte immunophenotype and induce renoprotection. These support the further evaluation of the potential of 5-ALA-SFC to improve clinical outcomes in patients undergoing cardiac surgery on CPB, particularly reducing risk of acute kidney injury, in appropriately powered larger trials.

**Acknowledgements**

None of the work presented in this thesis would have been possible without the generous participation of cardiac surgical patients, supported by their families, at the John Radcliffe Hospital in Oxford. Theirs is a contribution without personal reward and I thank them for their role in my own development, and in the success of the work presented in this thesis.

I would also like to acknowledge the support, teaching and patience shown to me by my supervision team. I would like to thank Dr Arash Yavari in particular. I could not have asked for better guide over the last few years. He has supported my development and has doggedly ensured this work is completed to a high standard. He has shaped my thinking more than he knows, and I am a better clinician for it. I sincerely hope I can continue to learn from him for many years to come.

As a clinician with no scientific background I have found working with career scientists enriching, not only for my own academic development but unexpectedly also in the way I now approach clinical problems. The support shown to me by members of Professor Hugh Watkins's group, specifically Dr James Brown, Dr Ying-jie Wang and Dr Kamayani Singh, was indispensable. I also received valuable support from members of Professor Keith Channon's group, and I have benefited from guidance in terms of experimental design and technique from Dr Marina Diotallevi and Dr Thomas Nicol - their willingness to teach me was instrumental to the successful completion of this DPhil. I was also able to work collaboratively with other groups within the university and would like to thank Professor Samira Lakhal-Littleton for a very instructive collaborative period spent with her group. I have learnt a great deal from her and Dr Mayra Vera-Aviles.

I would like to thank Professor Laurent Billot and Ms Severine Bompoint for supporting trial related statistical analyses – working with them both has represented a unique learning opportunity. I am also indebted to Mr Mark Soliano for guiding me through the intricacies of clinical trial conduct. Dr Rizwan Sarwar has generously supported me throughout, and this work would not have been completed without him.

I would like to acknowledge the driving force which my parents have always been. Their support and encouragement cannot be overstated. Finally, to my partner Richard, who I thank for getting this DPhil done with me.

**Declaration of work and attribution**

Except where specifically outlined, the work presented in this thesis is my own. It has not been submitted, either wholly or substantially, for another degree of this University, or for a degree at any other institution. I gratefully acknowledge contributions made by senior colleagues and fellow students within the broader university and externally.

Successful completion of the TALEN trial relied on several key collaborations. Dr Rizwan Sarwar was instrumental in the overall trial design and assisted with preparation of study related documents. Mr Samuel Paul was the trial coordinator for trial-setup and initial submission for ethical and regulatory approvals and ensured appropriate registration. The trial team was supported by the Department of Cardiovascular Medicine at the University of Oxford, in particular Dr Hanan Lamlum and Dr Mark Butler offered experienced advice throughout. Colleagues from the supporting contract research organisation, the George Clinical, specifically Dr Andrejs Faibusevics (Project Manager), Mr Mark Soliano (Clinical Research Associate) and Professor Laurent Billot and Ms Severine Bompoint (Trial Statisticians) as well as data managers, supported trial oversight, set up of the EDC, support with Safety Review Committee meetings, co-drafting of the Statistical Analysis Plan, undertaking the key statistical analyses and provision of data monitoring. Additional voting members of the Safety Review Committee – Dr Patrick Davey (Chair), Mr Rana Sayeed and Dr Matthew Rowland – were crucial in ensuring safety oversight for dose escalation decisions in this trial. Mr Rana Sayeed also offered invaluable senior surgical oversight in terms of trial design and ensured support from the wider Cardiac Surgical Departmental for the successful conduct of this trial.

A summary of specific scientific contributions and collaborations: Experimental Therapeutics, Oxford – Dr Rizwan Sarwar oversaw all Bioinformatics analyses (Chapter 4, Chapter 5); Cardiovascular Medicine, Oxford – Dr Kamayani Singh provided training in cell culture techniques and helped plan THP-1 experiments (Chapter 4); Dr Violetta Steeples carried out iPSC-CM experiments (Chapter 4); Dr Ying-jie Wang helped design and optimise the flow cytometry experiments and assisted with data analysis (Chapter 5); Dr Marina Diotallevi offered assistance with HO-1 quantification methods (Chapter 4); Dr Thomas Nicol offered training for mRNA experiments (Chapter 4 & 5); Department of Physiology, Anatomy and Genetics, Oxford – Dr Mayra Vera-Aviles oversaw all lipid peroxidation and hepcidin quantification experiments and provided training in these (Chapter 4); Genomic Medicine (Oxford) – Ms Parisa Yavari offered training in MDM culture techniques and oversaw these experiments (Chapter 4); Dr Mona Hashim advised on genotyping experiments (Chapter 4); External – Dr Shirin Akhter (Olink, Sweden) performed analyses for Olink-based proteomic readouts (Chapter 5); Professor Dorine Swinkels (Radboud, Netherlands) performed all NTBI assays (Chapter 4); Dr Michael Blackburn and Dr Janine Morsman (Quotient Sciences, Alnwick) co-developed (with input from the author and the Experimental Therapeutics team), validated and performed all HO activity assays (Chapter 4).

**Abbreviations and Acronyms**

| | |
|---|---|
| 5-ALA | 5-Aminolevulinic acid/δ-aminolevulinic acid |
| 5-ALA-SFC | 5-Aminolevulinic acid with Sodium ferrous citrate |
| $A_{2A}R$ | Adenosine receptor 2A |
| ACEi | Angiotensin converting enzyme inhibitor |
| ACT | Kaolin activated clotting time |
| ADAMTS13 | Disintegrin and metalloprotease with thrombospondin motifs 13 |
| ADP | Adenosine diphosphate |
| AE | Adverse event |
| AF | Atrial fibrillation |
| AKI | Acute kidney injury |
| AKT | Protein kinase B |
| ALAS | Aminolevulinic acid synthase |
| ALAS2 | Aminolevulinic acid synthase 2 |
| ALT | Alanine transaminase |
| AMP | Adenosine diphosphate |
| AMPK | AMP-activated protein kinase |
| ANCOVA | analysis of covariance |
| ANOVA | Analysis of variance |
| AP-1 | Activator protein-1 |
| ARB | Angiotensin receptor blocker |
| ARDS | Acute respiratory distress syndrome |
| ARE | Antioxidant response element |
| ASC | Apoptosis-associated speck like protein containing a caspase recruitment domain |
| AST | Aspartate transaminase |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |

| | |
|---|---|
| AVR | Aortic valve replacement |
| BACH1 | BTB domain and CNC homolog 1 |
| BMDM | Bone marrow derived macrophage |
| BMI | Body Mass Index |
| BMP | Bone morphogenetic protein |
| BP | Biological processes |
| BVR | Biliverdin reductase |
| CABG | Coronary artery bypass graft |
| CAC | Citric acid cycle |
| cAMP | Cyclic adenosine monophosphate |
| CCS | Canadian Cardiovascular Society |
| cf-Hb | Cell-free haemoglobin |
| cGMP | Cyclic guanosine monophosphate |
| CI | Confidence interval |
| CK-MB | Creatinine kinase M-B |
| CKD | Chronic kidney disease |
| CKD-EPI | Chronic Kidney Disease Epidemiology Collaboration |
| CL | Classical monocytes |
| CMR | Cardiac magnetic resonance |
| CNC | Cap'n'collar |
| CO | Carbon monoxide |
| CoE | Conservation of Expression |
| COPD | Chronic Obstructive Pulmonary Disease |
| CORMS | Carbon monoxide releasing molecules |
| CP | Ceruloplasmin |
| CPB | Cardiopulmonary bypass |
| CRP | C-reactive protein |

| | |
|---|---|
| CTCAE | Common Terminology Criteria for Adverse Events |
| cTnI | Cardiac troponin I |
| cTnI-AUC | Cardiac troponin area under the curve |
| DAMP | Danger associated molecular pattern |
| DC | Dendritic cells |
| Dcytb | Duodenal cytochrome b |
| DIC | Dicarboxylate carrier |
| DILI | Drug induced liver injury |
| DLT | Dose limiting toxicity |
| DMT1 | Divalent metal transporter 1 |
| ds | Double stranded |
| E. coli | Escherichia coli |
| ECG | Electrocardiogram |
| ECH | Erythroid cell-derived protein with cap-and-collar homology |
| eNOS | Endothelial nitric oxide synthase |
| EPO | Erythropoietin |
| EQ-5D | European Quality of Life-5 Dimensions |
| ERFE | Erythroferrone |
| ERK1/2 | Extracellular signal-regulated kinase 1/2 |
| FDR | False discovery rate |
| $Fe^{2+}$ | Ferrous iron |
| $Fe^{3+}$ | Ferric iron |
| FFA | Free fatty acids |
| Fig. | Figure |
| FiO2 | Fraction inspired oxygen |
| FLA | Fragment length analysis |
| FPN | Ferroportin |

| | |
|---|---|
| FTn | Ferritin |
| GDF15 | Growth differentiation factor 15 |
| GGT | Gamma-glutamyl transferase |
| GIK | Glucose-insulin-potassium |
| GPCR | G-protein coupled receptors |
| GSDMD | Gasdermin-D |
| GSK-3β | Glycogen synthase kinase-3β |
| $GT_n$ | guanine-thymine nucleotide |
| GTP | Guanosine Triphosphate |
| *HAMP* | Hepcidin antimicrobial peptide – hepcidin coding gene |
| Hb | Haemoglobin |
| HCP1 | Haem carrier protein 1 |
| HI | Haemolytic index |
| HIF-1α | Hypoxia-inducible factor 1 α |
| HIF-2a | Hypoxia inducible factor 2a |
| *HMOX1* | Haem oxygenase-1 gene |
| *HMOX2* | Haem oxygenase-2 gene |
| HnRNPK | Heterogenous nuclear ribonucleoprotein K |
| HO-1 | Haem oxygenase-1 |
| HO-2 | Haem oxygenase-2 |
| HR | Heart rate |
| HRG1 | Haem responsive gene protein 1 |
| hs-Trop I | High-sensitivity troponin I |
| HSF | Heat-shock factor |
| HUVEC | Human umbilical vein endothelial cell |
| ICU | Intensive care unit |
| IL | Interleukin |

| | |
|---|---|
| IMP | Investigational medicinal product |
| iNOS | Inducible nitric oxide synthase |
| IPC | Ischaemic preconditioning |
| iPSC-CM | Induced pluripotent stem cell derived cardiomyocytes |
| IQR | Interquartile range |
| IRI | Ischaemia-reperfusion injury |
| IRP | Iron regulatory protein |
| IRP1 | Iron regulatory protein 1 |
| ITM | Intermediate monocytes |
| JAK | Janus kinase |
| JAK/STAT | Janus kinase/signal transducer and activator of transcription |
| KDIGO | Kidney Disease: Improving Global Outcomes |
| KEAP1 | Kelch-like ECH-associated protein 1 |
| LCOS | Low cardiac output syndrome |
| LDH | Lactate dehydrogenase |
| $Lin^-$ | Lineage negative |
| LL | Homozygous long *HMOX1* $(GT)_n$ repeat polymorphism |
| LMM | Linear mixed model |
| LPS | Lipopolysaccharides |
| LV | Left ventricle |
| LVEF | Left ventricular ejection fraction |
| LVH | Left ventricular hypertrophy |
| MACE | Major adverse cardiovascular events |
| Max | Maximum |
| MDM | Monocyte derived macrophages |
| MHC | Major histocompatibility complex |
| MHRA | Medicines and Healthcare products Regulatory Agency |

| | |
|---|---|
| MI | Myocardial infarction |
| Min | Minimum |
| miRNA | microRNA |
| MPS | Mononuclear phagocyte system |
| mPTP | Mitochondrial permeability transition pore |
| n | Number of participants |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NC | Non-classical monocytes |
| Neh | NRF ECH homology |
| NET | Neutrophil extracellular trap |
| NF-kB | Nuclear factor kappa B |
| *NFE2L2* | Gene coding for nuclear factor E2-related factor 2 (NRF2) |
| NGAL | Neutrophil gelatinase–associated lipocalin |
| NK | Natural killer |
| NLRP3 | Nod like receptor protein with a PYRIN domain 3 |
| nNOS | Neuronal nitric oxide synthase |
| NO | Nitric oxide |
| NOS | Nitric oxide synthase |
| NRF2 | Nuclear factor erythroid 2-related factor 2 |
| NTBI | Non-transferrin-bound iron |
| NYHA | New York Heart Association |
| OPCAB | Off-pump coronary artery bypass graft |
| p:F | arterial oxygen partial pressure: fraction of inspiratory oxygen |
| PAMPs | Pathogen-associated molecular patters |
| PANTHER | Protein Analysis Through Evolutionary Relationships |
| $PaO_2$ | Arterial partial pressure of oxygen |
| PARP1 | Poly (ADP-ribose) polymerase-1 |

| | |
|---|---|
| PASP | Pulmonary artery systolic pressure |
| PBG | Porphobilinogen |
| PCA | Principal component analysis |
| PCD | Programmed cell death |
| PCR | Polymerase chain reaction |
| PD | Pharmacodynamic |
| PDE3 | Phosphodiesterase 3 |
| PDE5 | Phosphodiesterase 5 |
| PDH | Pyruvate dehydrogenase |
| PHD | Prolyl hydroxylase |
| PI3K | Phosphatidylinositol 3-kinase |
| PK | Pharmacokinetic |
| PKA | Protein kinase A |
| PKC | Protein Kinase C |
| PKCe | Protein Kinase C epsilon |
| PKG | Protein kinase G |
| PMI | Post-operative myocardial injury |
| PPIX | Protoporphyrin IX |
| PT | Prothrombin time |
| PVC | Polyvinyl chloride |
| Q1 Q3 | First and third quartile |
| QTc | Corrected QT interval |
| RAGE | Receptor of advanced glycation end products |
| RBC | Red blood cell |
| REC | Research and Ethics Committee |
| REML | Restricted maximum likelihood |
| RIPC | Remote ischaemic preconditioning |

| | |
|---|---|
| RISK | Reperfusion Ischaemia Salvage Kinase signalling |
| ROS | Reactive oxygen species |
| RRT | Renal replacement therapy |
| RT-qPCR | Real-time qunatitative polymerase chain reaction |
| SAE | Serious adverse event |
| SAFE | Survival activating factor enhancement |
| SBP | Systolic blood pressure |
| SD | Standard deviation |
| SDH | Succinate dehydrogenase |
| SE | Standard error |
| SFC | Sodium ferrous citrate |
| SL | Heterozygous *HMOX1* $(GT)_n$ repeat polymorphism |
| sMaf | Small musculoaponeurotic fibrosarcoma protein |
| SmPC | Summary of product characteristics |
| $SpO_2$ | Arterial oxygen saturation by spectroscopy |
| SR | Sarcoplasmic reticulum |
| SRC | Safety Review Committee |
| SS | Homozygous short *HMOX1* $(GT)_n$ repeat polymorphism |
| STAT3 | Signal transducer and activator of transcription 3 |
| STAT5 | Signal transducer and activator of transcription 5 |
| Succinyl-CoA | Succinyl-coenzyme A |
| SUSAR | Suspected unexpected serious adverse reaction |
| TBI | Transferrin bound iron |
| TCA | Tricarboxylic acid cycle |
| TfR1 | Transferrin receptor 1 |
| TIVA | Total intravenous anaesthesia |

| | |
|---|---|
| TLR | Toll-like receptor |
| TNF-a | Tumour necrosis factor alpha |
| TRAE | Treatment related adverse event |
| TRPV4 | Transient receptor potential vanilloid 4 |
| Tsat | Transferrin saturation |
| TWSG1 | Twisted gastrulation protein homologue 1 |
| ULN | Upper limit of normal |
| VIS | Vasopressor inotropic score |
| vWF | Von Willebrand Factor |
| WCC | White cell count |
| Yrs | Years |
| ZIP1 | ZRT/IRT-like protein 1 |
| ZnBG | Zinc deuteroporphyrin 2,4-bis glycol |

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# 1 Chapter 1. Introduction

## 1.1 Improving clinical outcomes after cardiac surgery - an important unmet need

Despite improvements in cardiac surgical techniques, including blood-based cardioplegia to limit myocardial ischaemia[1], a proportion of patients still develop prognostically important peri-operative myocardial injury (PMI)[2,3]. Post-operative cardiovascular complications such as heart failure and arrhythmias occur frequently and can be caused by PMI due to direct surgical trauma or the ischaemia-reperfusion injury (IRI) associated with cardiac surgery requiring cardiopulmonary bypass (CPB)[4].

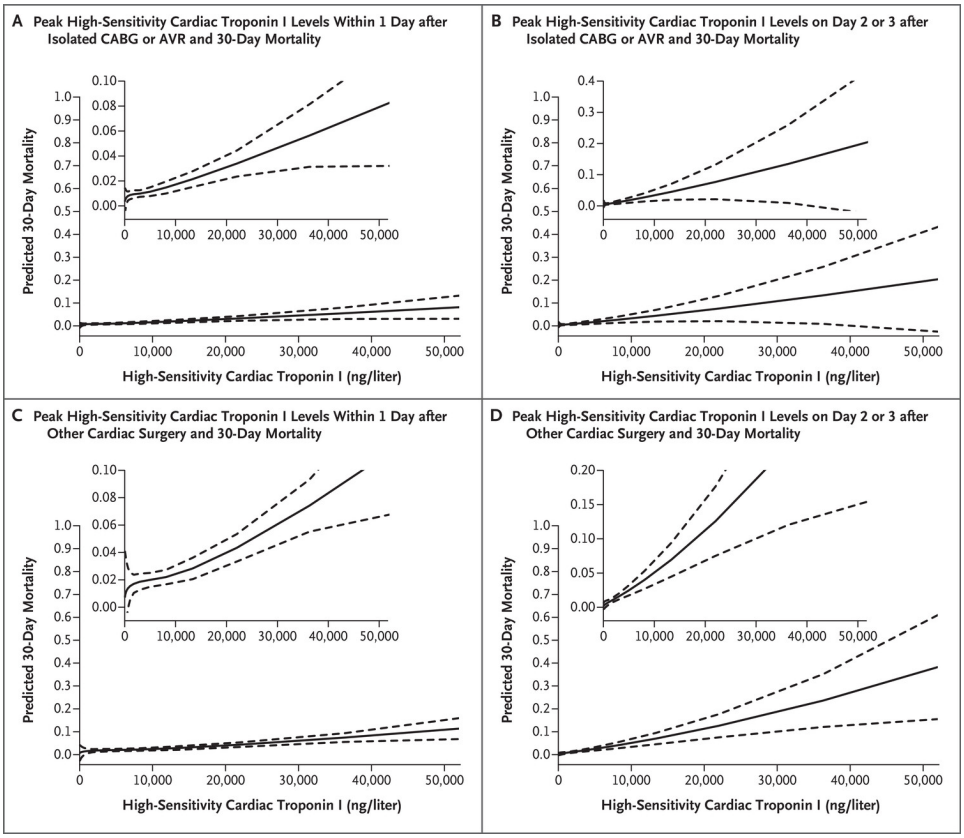



**Figure 1: The relationships between peak cardiac troponin I measurements and 30-day mortality across common cardiac surgical procedures in adults.**

The extent of peri-operative myocardial injury (PMI) (quantified by peak post-operative high sensitivity cardiac Troponin I) associates with an increased risk of death within 30 days. The relationship varies when comparing patients who underwent coronary artery bypass grafting (CABG) or aortic valve replacement (AVR) (panels A-B) to those patients undergoing other cardiac surgery (e.g., mitral valve surgery, combination procedures) (panels C-D), reflecting higher peak troponin values measured in these patients.[a]

[a] Reproduced from figure 1 Devereaux et al. *NEJM* 2022; 386(9): 827-836.

In addition to cardiovascular complications, CPB induces a profound acute haemolytic insult and systemic inflammatory response, which is associated with an increased risk of multi-organ dysfunction and death[5]. Non-cardiac complications (e.g., stroke, acute kidney injury [AKI]) impact long term survival and if occurring concurrently increase the risk of death [6]. Many of these (e.g., prolonged ventilation, AKI and stroke) also carry significant health economic costs[7,8]. With increasing age, co-morbidity burden and reduced organ reserve, patients undergoing cardiac surgery have become more complex and identifying novel approaches to improve patient outcomes remains an important clinical need and health economic challenge[9]. Addressing this need will, in part, rely on identifying novel therapeutic strategies aimed at carefully selected targets with sound biological rationale, ideally with human genetic validation. These would target both the cardiac ischaemia-reperfusion injury (IRI) and the adverse pro-inflammatory sequelae of CPB. Subsequent clinical translation will require thoughtful early phase studies, aimed at identifying the optimal biological dose and schedule of the intervention, in the correct patient population, coupled with effective trial execution. Appropriate selection of endpoints to confirm target engagement with evaluation of the dose-response relationship should allow for a preliminary evaluation of potential markers of efficacy including clinically relevant biomarkers.

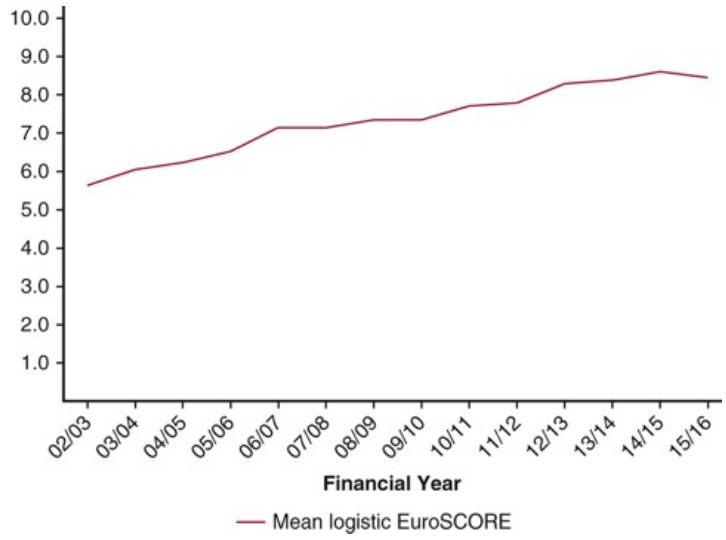



**Figure 2: The increasing risk profile of cardiac surgical patients in the United Kingdom.**

Surgical risk quantified using the logistic EuroSCORE (% risk of mortality). [b]

[b] Adapted from figure 7 Grant et al. *JCTCVS Open* 2021; 7: 259-269.

### 1.1.1 Cardiopulmonary bypass

The advent of CPB is regarded as one of the major advances of modern cardiac surgery – allowing a non-beating heart to be opened or operated upon while an extracorporeal circulation performs the functions of the heart and lungs[10]. Modern bypass circuits share the following basic characteristics: venous cannulation of the venae cava allows drainage of venous blood (by gravity) through polyvinyl chloride (PVC)/silicone tubing into a venous reservoir, which also receives blood from the intra-cardiac vent and surgical suction pump; from here blood is passed through a heat exchanger and gas exchanger by a systemic blood pump (centrifugal or roller); blood is then pumped back into the arterial system through an aortic cannula after passing through an arterial filter, with or without cardioplegia[11,12].

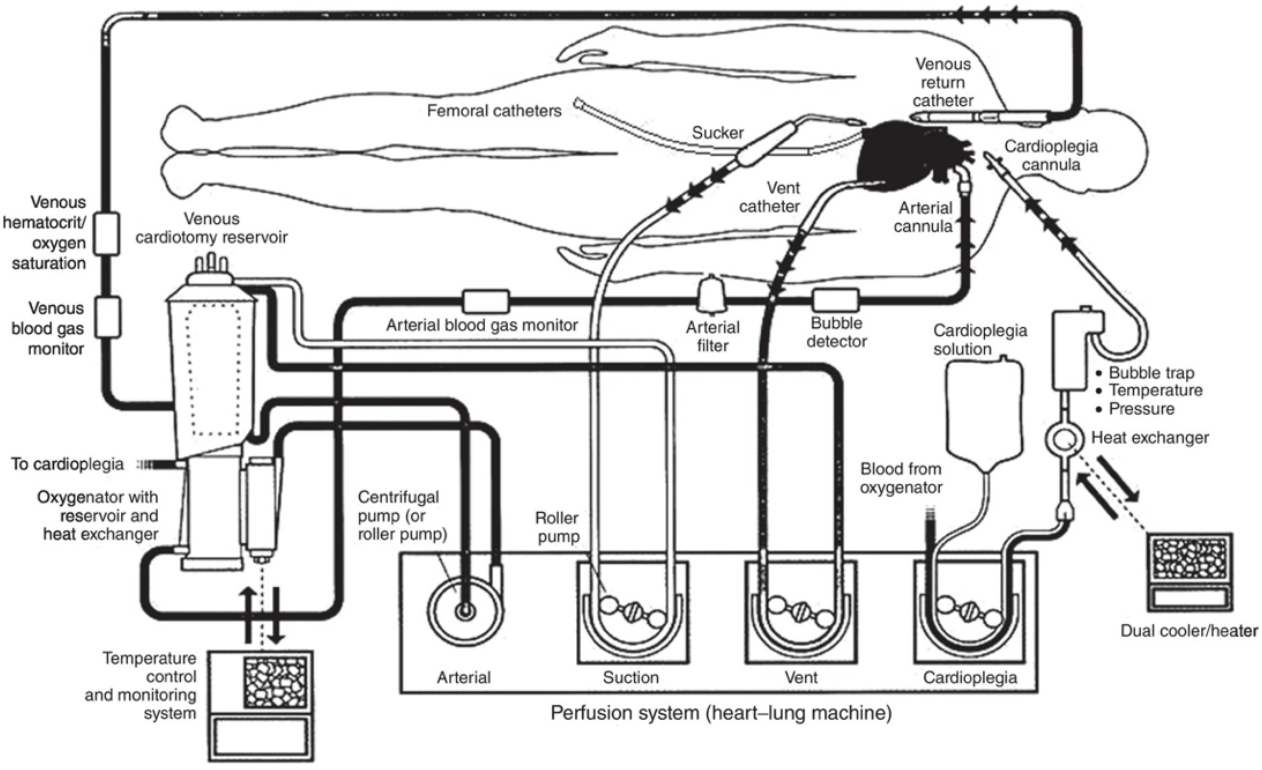



**Figure 3: A modern cardiopulmonary bypass circuit.**[c]

[c] Reproduced from figure 5 Coulson et al. Principles of Cardiopulmonary Bypass in *Core Topics in Cardiac Anaesthesia*. 2020: 30-44.

Although CPB has been instrumental in the development of cardiac surgery, it is associated with and a profound systemic inflammatory response capable of driving significant morbidity and mortality[13]. Contact of cellular (e.g., immune cells, platelets) and humoral (e.g., complement) elements with the extracorporeal circuit, along with significant haemolysis related to mechanical shear forces, operative trauma and IRI, cause immune activation which can be detrimental to the patient[13–15].

Our understanding of the systemic impact of CPB has led to ongoing improvements in perfusion practice (e.g., use of minimally invasive extracorporeal circuits, use of microporous oxygenators as first choice, use of heparin bonded circuits and centrifugal pumps for longer cases) with wide acceptance[16]. The independent contribution of CPB to worse patient outcomes has led to an interest in comparing outcomes between patients undergoing coronary artery bypass graft (CABG) surgery on- vs. off-CPB. Indeed, off-pump cardiac surgery has been linked to reduced rates of AKI[17]. Unfortunately, many cardiac surgical procedures can only be performed with CPB.

**Table 1: Post-operative complication risk associated with an increase of 30 minutes in CPB duration.[d]**

| Dependent variable | Odds ratio/β | 95% CI limits | *p* value |
|---|---|---|---|
| Post-operative blood loss (mL) | 78.1 (β) | 77.7, 78.5 | <0.0001 |
| ICU length of stay (d) | 0.245 (β) | 0.242, 0.246 | <0.0001 |
| Post-operative length of stay (d) | 0.42 (β) | 0.418, 0.423 | <0.0001 |
| Multiple RBC transfusions | 1.58 | 1.48, 1.68 | <0.0001 |
| Reoperation for bleeding | 1.10 | 1.02, 1.18 | 0.017 |
| Respiratory complications | 1.17 | 1.16, 1.17 | <0.0001 |
| Neurologic complications | 1.28 | 1.17, 1.39 | <0.0001 |
| Infective complications | 1.26 | 1.15, 1.38 | <0.0001 |
| Abdominal complications | 1.09 | 0.92, 1.30 | 0.32 |
| Renal complications | 1.31 | 1.22, 1.14 | <0.0001 |
| Multiorgan failure | 1.21 | 1.21, 1.21 | <0.0001 |
| Death | 1.57 | 1.43, 1.73 | <0.0001 |

Abbreviations: CPB – cardiopulmonary bypass; β - regression coefficient; CI - confidence interval; ICU – Intensive care unit; RBCs - red blood cells.

[d] Reproduced from table 9 Salis et al. *JCVA* 2008; 22(6): 814-822.

### 1.1.2 Myocardial injury ischaemia-reperfusion injury

Central to peri-operative myocardial dysfunction associated with cardiac surgery requiring CPB, is the inevitable IRI due to of cardioplegic arrest[18]. The latter constitutes a period of global cardiac ischaemia to enable surgery on a non-beating heart, followed by reperfusion of the organ with both events contributing cellular harm[19]. The pathophysiology of IRI is complex and involves several subcellular processes. In brief, ischaemia leads to myocardial energy failure, ATP-dependent transmembrane ion channel dysfunction and intracellular $Ca^{2+}$ accumulation – favouring mitochondrial permeability transition pore (mPTP) opening. A surge in reactive oxygen species (ROS) (e.g., superoxide anion, hydrogen peroxide) induced by reperfusion causes significant oxidative stress capable of overcoming cellular antioxidant mechanisms such as catalase, glutathione peroxidase and superoxide dismutase[20]. Unchecked, oxidative stress can ultimately lead to myocyte necrosis which, when associated with significant myocardial injury as quantified by biomarkers (e.g., creatinine kinase M-B [CK-MB], cardiac troponin I [cTnI]), correlates with post-operative mortality[3,21–23].

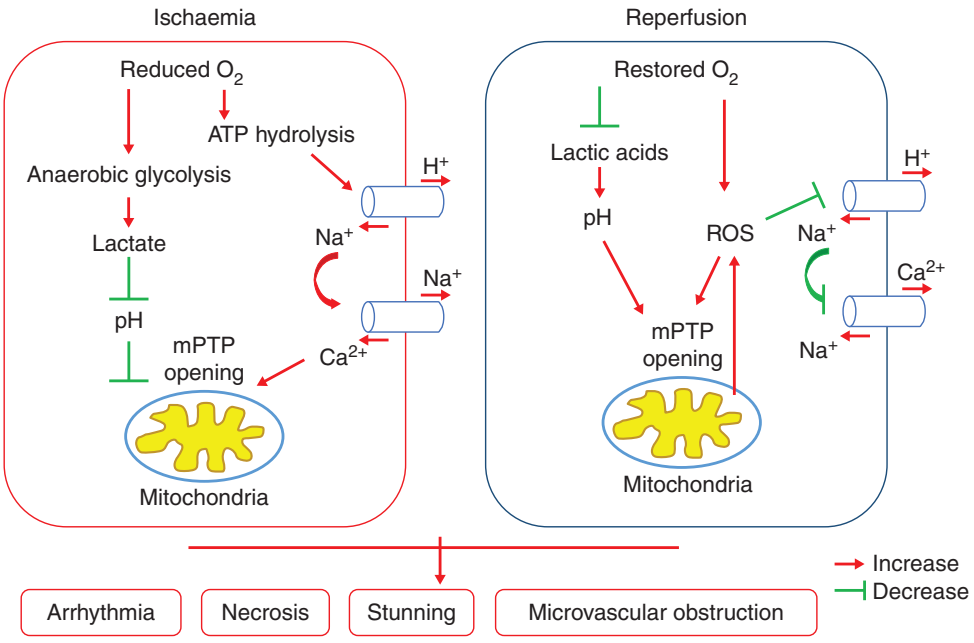



**Figure 4: Summary of the pathophysiology of myocardial ischaemia-reperfusion injury.**

Abbreviations: ATP – Adenosine triphosphate; mPTP - Mitochondrial permeability transition pore; ROS – Reactive oxygen species.[e]

[e] From figure 1 Xia et al. *BJA* 2016; 117 (Suppl): ii44-ii62.

Biomarker based diagnosis of clinically important PMI in the context of cardiac surgery is complicated due to the nature of the procedure itself, and previously suggested cTnI cut off values for the diagnosis of procedure related infarction have been challenged[3,24]. The gold standard for diagnosis and quantification of PMI is cardiac magnetic resonance imaging (CMR) with late gadolinium enhancement[25]. There is a strong correlation (r=0.83) between the amount of CMR quantified myocardial necrosis and cTnI release, as determined by the trapezoid area under the curve of serial cTnI measurements (cTnI-AUC)[26]. Unfortunately, this measure does not distinguish between infarcted myocardium and myocardium at risk[27]. Elevated post-operative cTnI levels predict poor short- and long-term post-operative outcomes[28], and remain useful clinical and research tools in quantifying myocardial injury in the post-operative period. As a manifestation of myocardial injury, between 4 and 30 % of cardiac surgical patients will develop a low cardiac output syndrome (LCOS), characterised by reduced cardiac pump function requiring inotropic agents (in up to 10% of patients) and/or mechanical circulatory support[29,30].

### 1.1.3 Cardioprotection

Cardioprotection in the context of cardiac surgery refers to peri-operative strategies aimed at reducing the negative sequelae of cardiac IRI[18]. Current standard cardioprotective practice includes measures such as the early treatment of intra-operative arrhythmias, maintaining normoglycaemia, use of mild hypothermia (31-33°C), continual removal of venous blood from the left ventricle (i.e, venting) and limiting the amount of time under CPB[31]. Cardioplegic arrest, aimed at minimising myocardial metabolic activity and oxygen demand, remains one of the most widely used cardioprotective measures. This is achieved through delivery of a hyperkalaemic blood-based solution into the aortic root (anterograde) or into the coronary sinus (retrograde), inducing diastolic arrest[32]. Several mechanistically attractive additional cardioprotective techniques (e.g., ischaemic- and pharmacological-preconditioning) have been investigated.

#### 1.1.3.1 Ischaemic preconditioning

Seminal work by Murry et al. demonstrated that repeated episodes of controlled, non-lethal cardiac ischaemia in mongrel dogs offered significant protection in the context of acute myocardial infarction (MI)[33]. Repeated episodes of non-lethal cardiac ischaemia were achieved by intermittent occlusion of the left circumflex coronary artery, followed by reperfusion. This was followed by a period of sustained occlusion to model an acute MI. Control animals were subject to sustained occlusion only. Infarct sizes were compared post-mortem and were greatly reduced in the intervention group (7.3% vs 29.4% in controls). With these findings, ischaemic preconditioning (IPC) became a favoured cardioprotection concept and was successfully trialled in 33 patients undergoing CABG with results suggesting significant clinical benefit[34,35]. Variations to the original paradigm have included delayed IPC and ischaemic postconditioning, the latter involving episodes of re-occlusion during revascularisation[36]. More recently remote ischaemic preconditioning (RIPC) has been proposed. RIPC relies on similar principles and involves controlled episodes of ischaemia being applied to a distant part of the body, typically a limb[37].

The precise cellular mechanisms underlying the putative benefits of IPC have not yet been fully elucidated but involve multiple overlapping signal transduction cascades outlined in **Fig. 5**. In response to various triggers which accumulate during ischaemia or surge during reperfusion (e.g., adenosine, bradykinin, ROS), three major pro-survival intracellular pathways are activated[38,39]. Membrane bound G-protein coupled receptors (GPCR) are stimulated by extracellular messengers and this activates protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG) and eNOS. This represents the PKC-eNOS-PKG pathway (highlighted in blue in Fig. 5). The Reperfusion Injury Salvage Kinase (RISK) pathway (highlighted in yellow in Fig. 5) is activated through stimulation of inhibitory GPCRs and eventual downstream inhibition of glycogen synthase kinase-3β (GSK-3β). Finally, the Survival Activating Factor Enhancement (SAFE) pathway (highlighted in green in Fig. 5) is activated by specific cytokine receptors with subsequent Janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction.

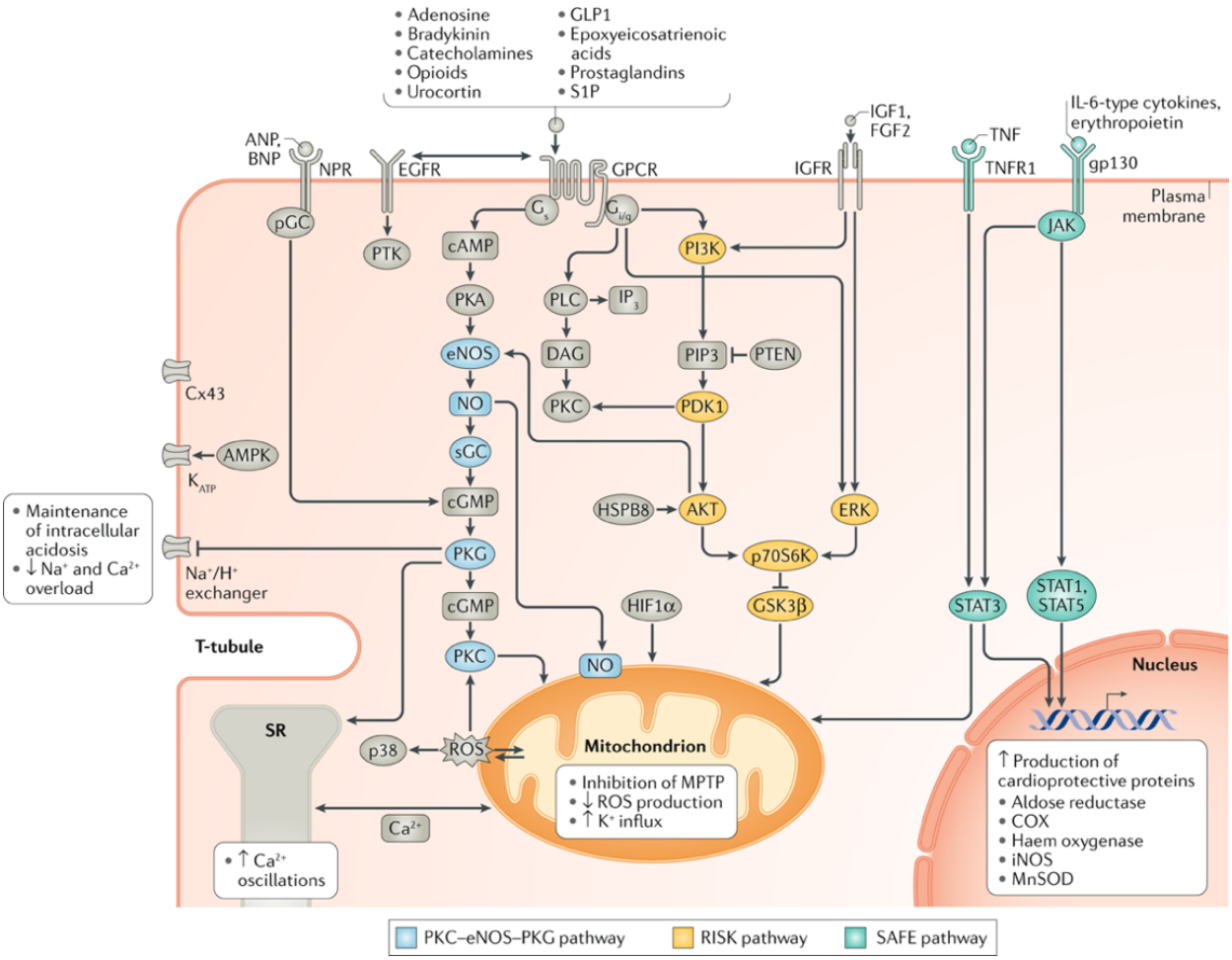



**Figure 5: Cardioprotective signal transduction cascades underpinning ischaemic preconditioning.**

Diverse extracellular triggers (e.g., bradykinin, ROS) activate interconnected intracellular pathways, communicating with effector organelles (mitochondrion, nucleus, sarcoplasmic reticulum [SR]) to protect cardiomyocytes.[f]

The most important final target of all three protective pathways, are the mitochondria[40]. Not only are mitochondria crucial in terms of ATP production, but they can also trigger cell death when they become dysfunctional during IRI (**Fig. 6**). JAK-STAT signalling also results in increased production of cardioprotective proteins (e.g., haem oxygenase) by the nucleus.

[f] Reproduced from figure 6 Heusch et al. *Nat. Rev. Cardiol.* 2020; 71: 773-789.

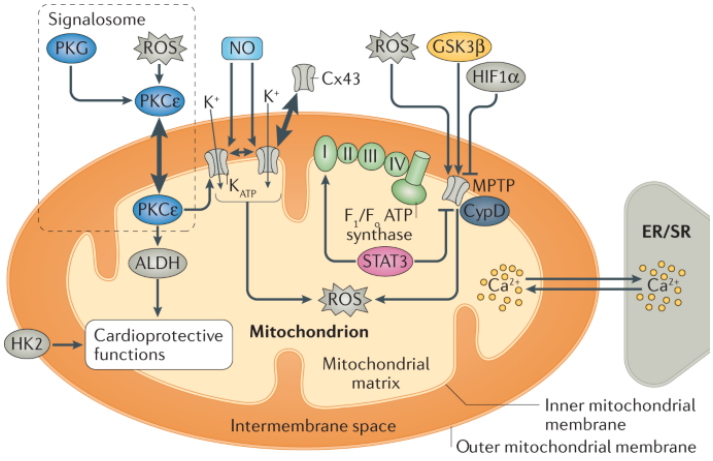



**Figure 6: The critical role of the mitochondria in cardioprotection.**

All three cardioprotective pathways converge on the mitochondria, causing $K^+$ influx, reduced reactive oxygen species (ROS) production and inhibition of the mitochondrial permeability transition pore (mPTP). This prevents matrix swelling and rupture of the outer mitochondrial membrane with release of cytochrome *c*, activation of caspases and subsequent apoptosis.[g]

### 1.1.3.2 Metabolic strategies for cardioprotection

Under normal aerobic conditions, the heart predominantly utilises free fatty acids (FFA) in the production of ATP[41]. Whilst FFAs represent highly energy dense substrates, aerobic glucose metabolism is more oxygen efficient, yielding more ATP per unit of oxygen utilized[42]. Therefore, metabolic therapies designed to augment aerobic glucose metabolism, such as the glucose-insulin-potassium (GIK) infusion[43] and oral perhexiline[43] have been advocated as oxygen sparing strategies, with the aim of improving myocardial metabolic efficiency in te context of cardiac surgery.

Several trials have examined the therapeutic potential of GIK in myocardial protection in cardiac surgical patients and are summarised in a recent meta-analysis [44]. Despite limitations, including changes in surgical and anaesthetic practice across the period the analysis covers, GIK was associated with a reduction in PMI, atrial fibrillation (AF) and AKI. One study, investigating the role of peri-operativeGIK infusions in patients with left ventricular hypertrophy (LVH) undergoing aortic valve replacement (AVR), demonstrated a significant reduction in the incidence of LCOS with improved post-operative haemodynamics (specifically: cardiac index, heart rate, central venous pressure and pulmonary artery

[g] Reproduced from figure 7 Heusch et al. *Nat. Rev. Cardiol.* 2020; 71: 773-789.

wedge pressure) and reduced inotrope requirements[45]. This improvement in reversible myocardial injury was associated with a significant increase in the activation of the RISK pathway, notably through increased AMP-activated protein kinase (AMPK) and protein kinase B (AKT) phosphorylation in myocardial samples. However, perhexiline treatment was not associated with improved outcomes after cardiac surgery[43].

Although the literature relating metabolism to cardioprotection has largely focused on attempts to modulate carbohydrate metabolism, there is evidence that other metabolites, including amino acids and citric acid cycle (CAC)/tricarboxylic acid cycle (TCA) intermediates, also contribute towards the myocardial response to ischaemia[46]. There is increasing recognition that metabolites such as succinate, in addition to their roles in generating energy through intermediate metabolism, have an important part to play in sensing and marshalling responses to cellular stress, including IRI (**Fig.7**) [47]. Under hypoxic/ischaemic conditions, myocardial succinate dehydrogenase acts in reverse to convert fuels such as aspartate, fumarate and glutamate to succinate via the CAC yielding both ATP and Guanosine Triphosphate (GTP)[48,49]. Succinate accumulates during ischaemia and is rapidly re-oxidised during reperfusion, driving ROS production[50]. This channelling of amino acids through the CAC has been proposed to maintain redox potentials and to yield high energy phosphates.

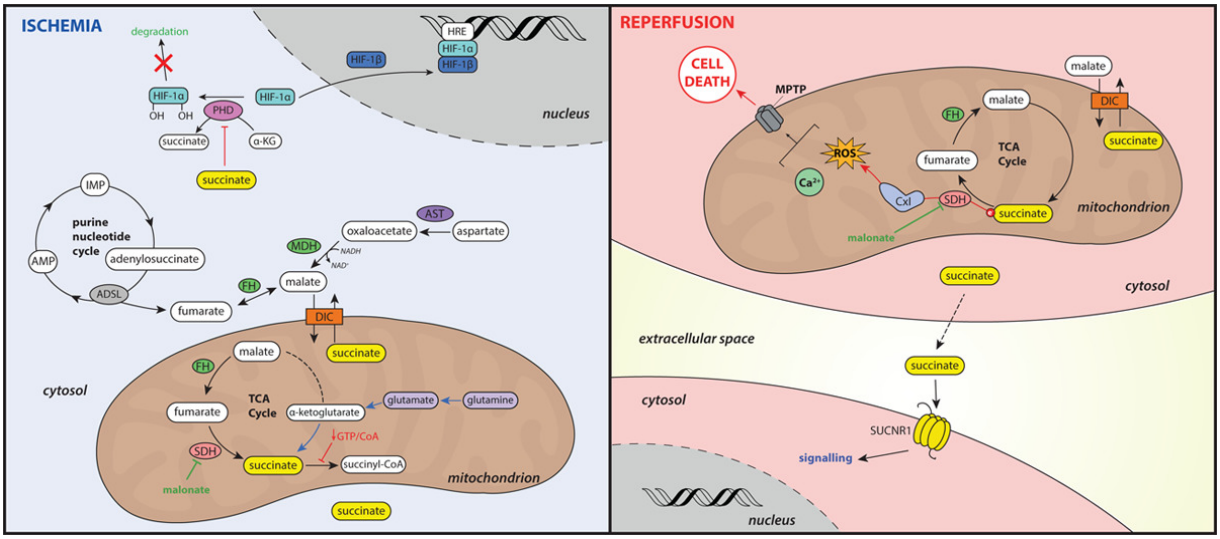



**Figure 7: The central role of succinate in myocardial ischaemia reperfusion injury.**

During ischaemia, mitochondrial succinate dehydrogenase (SDH) reversal facilitates conversion of fumarate to succinate. Under ischaemic conditions, guanosine triphosphate (GTP) depletion prevents conversion of succinate to succinyl coenzyme A, leading to mitochondrial succinate accumulation. Fumarate derives from the purine nucleotide cycle and upstream aspartate transamination, enters the mitochondrion via the dicarboxylate carrier (DIC) in exchange for succinate. Cytosolic succinate inhibits prolyl hydroxylases (PHD), preventing degradation of hypoxia-inducible factor 1 α (HIF-1α) enabling nuclear translocation where it regulates the transcription of genes involved in the response to ischaemia. At reperfusion, succinate is rapidly oxidised ultimately causing a surge in superoxide which can drive oxidative cell death. [h]

### 1.1.3.3 Pharmacological preconditioning

Mechanistically, both metabolic and ischaemic approaches to preconditioning share common signal transduction pathways and this has led to an effort to develop pharmacological strategies to target/intercept these nodes (i.e., receptor ligand, transduction and effector)[51]. Several clinical trials have investigated the protective effects of adenosine and, although there has been some evidence suggesting reduced infarct size in the context of acute MI, these findings have not been consistent[51,52]. Bradykinin, or acute angiotensin converting enzyme inhibitor (ACEi) induced increase in bradykinin, has also been studied. It is known that chronic ACEi or angiotensin receptor blocker (ARB) use is associated with a reduced incidence of acute MI[53]. In patients admitted with acute MI, early institution of an ACEi or ARB is associated with improved clinical outcomes[54,55]. Despite a plausible cardioprotective effect, due to the blood pressure lowering effects of these drugs, they have not been trialled as cardioprotective agents in

[h] Reproduced from figure 1 Kula-Alwar et al. *Circulation* 2019; 140: 1968-1970.

cardiac surgery. As commonly used drugs in cardiac anaesthesia, opioids are capable of activating multiple intracellular cardioprotective pathways[56–59]. Proving their efficacy as cardioprotective agents in cardiac surgery has proven challenging – they are always combined with other general anaesthetic agents (i.e., additional cardioprotective agents as described below) and no true placebo exists[60].

Nitric oxide (NO), as a trigger, mediator and effector in cardioprotection, remains an attractive target for pharmacological intervention[39]. Its effects on vascular smooth muscle tone, platelet aggregation and immune activation plausibly extend cellular protection beyond the classical intracellular cardioprotective pathways (e.g., RISK, SAFE)[61]. NO is synthesized by three NO synthase (NOS) isoforms – neuronal (nNOS), inducible (iNOS) and endothelial (eNOS) – and its role in cardiovascular health and disease remains a subject of considerable interest[62]. Phosphodiesterase 3 and 5 (PDE3, PDE5) inhibitors (e.g., milrinone, sildenafil), through their effects on NO-cyclic adenosine monophosphate (cAMP) or NO-cyclic guanosine monophosphate (cGMP) signalling and their effect on mPTP opening, have been shown to be cardioprotective in the setting of cardiac IRI[63–65]. PDE inhibitors also appear to act through JAK/STAT signalling, highlighting considerable cross talk between cardioprotective pathways[66].

Commonly used anaesthetic drugs have also been investigated as cardioprotective agents[56]. Volatile anaesthetics (e.g., sevoflurane, isoflurane, desflurane) are variably cardioprotective through pre- and post-conditioning[67]. Putative mechanisms differ between agents but have been shown to include activation of the Extracellular signal-regulated kinase 1/2 (ERK1/2), eNOS and JAK-STAT pathways with modulation of mPTP function[65,68,69]. Propofol, a commonly used induction and maintenance agent in anaesthesia, has been linked with cardioprotection but also the ability to counteract cardioprotective effects of approaches such as RIPC, possibly by acting as a ROS scavenger removing the pre-conditioning effects of the intervention[65]. Propofol is thought to exert its cardioprotective effects through its ability to prevent intracellular $Ca^{2+}$ overload (through inhibition of transient receptor potential vanilloid 4 (TRPV4) channels)[70], regulation of microRNA (miRNA) expression[71] and reduction of cardiac mast cell accumulation[72]. Noble gases (e.g., xenon, helium) partly exert their cardioprotective effects through their interaction with caveolins[73,74]. Caveolins, a family of membrane bound proteins

involved in receptor-independent endocytosis, represent an attractive therapeutic target due to their ability to interact with multiple intracellular protective pathways[75].

### 1.1.4 Strategies to improve translation of novel biological concepts into improved clinical outcomes after cardiac surgery

Improving patient outcomes after cardiac surgery remains an active area of research with several trials ongoing/recently concluded in the UK (e.g., Quality of Life After Cardiac Surgery – NCT04231461; Comparison Between Propofol and Inhalational Anaesthetic Agents on Cardiovascular Outcomes in Cardiac surgery – NCT04039854; The Pre-Op Energy Trial – NCT04015973)[76].

In the field of cardioprotection, there has been a disappointing failure to translate mechanistically attractive interventions, with either promising pre-clinical data and/or encouraging early phase trial findings, into clinically meaningful patient benefits in larger studies[77,78]. For example, RIPC, with compelling mechanistic and pre-clinical data to suggest efficacy, has not reliably improved patient outcomes such as reduced PMI[79,80]. This may partly be explained by the confounding effects of multiple co-morbidities and the choice of anaesthetic technique but a critical shortcoming in this field is a lack of robust phase II data. Establishing the correct dose, schedule, and timing of cardioprotection interventions as well as selecting the ideal patient population in which to investigate these, are crucial first steps in translation of a promising mechanism[77]. This, however, needs to be balanced with the need for pragmatism in trial design, ensuring interventions are tested in routine clinical settings and that findings are generalisable to the broader population[81]. The precise surgical intervention is also important. For example, valve surgery is known to be associated with additional (direct) traumatic injury which a cardioprotective agent would not be expected to impact upon. In addition, the induction of ischemia-reperfusion injury is inherent to the use of cardioplegic arrest, irrespective of surgical indication. Thus, patients undergoing cardiac valve replacement or repair on CPB also have the potential to experience benefit from cardioprotective strategies similar to those undergoing CABG[77,82], although the magnitude of this may differ.

Measuring the potential therapeutic impact of any intervention is a major consideration during trial design, and study endpoints in cardioprotection trials vary. As an alternative to CMR, many studies investigating cardioprotective interventions in cardiac surgery have relied on biomarkers, most commonly cTnI-AUC, to quantify the extent of myocardial damage secondary to IRI[9,83–86]. Direct surgical trauma to the myocardium, as well as aortic clamping, will cause biomarker release, which complicates the interpretation of these data[27]. Furthermore, peak levels of biomarkers measured peri-operatively and overall release kinetics will differ depending on the specific surgical intervention[3].

Transient peri-operative cardiovascular dysfunction, caused by direct myocardial injury and the systemic inflammatory response, is typically be treated using vasoactive and inotropic drugs (e.g., noradrenaline, dobutamine)[87]. Their use will mask haemodynamic dynamic compromise, and the utility of basic haemodynamic measurements (e.g., blood pressure) as markers of cardiovascular performance is diminished in this context. The degree of pharmacological support of the cardiovascular system required after surgery, is therefore an attractive alternative endpoint and is reflected by the inotropic/vasoactive-inotropic score (VIS)[88,89]. This score is calculated using a standardised equation incorporating dosages of commonly used drugs, and has been shown to predict morbidity and mortality in children and adults undergoing cardiac surgery.

Another frequently used endpoint in large, late stage confirmatory trials is a composite outcome of major adverse cardiovascular events (MACE)[9]. This includes death from a cardiac cause, non-fatal myocardial infarction, cardiac revascularisation and stroke. Other endpoints have included new onset atrial fibrillation (AF) and left ventricular ejection fraction (LVEF)[27]. Endpoints beyond myocardial injury alone, such as delirium screening[90], duration of ventilation or pneumonia[8] and incidence of acute kidney injury[91], are frequently measured. Beyond the initial post-operative phase, long-term morbidity may be significant and there is an increasing focus on health status and quality of life outcomes, such as the European Quality of Life-5 Dimensions (EQ-5D) score[9,92].

## 1.2 Cardiopulmonary bypass-induced haemolysis, inflammation, and iron mediated organ dysfunction

CPB, through mechanical damage to red blood cells (RBCs), contact of RBCs with air or non-endothelial surfaces, and sublethal RBC damage with premature RBC death, causes a predictable acute haemolytic insult[14]. Once RBCs are damaged, they release cell-free haemoglobin (cf-HB), haem and iron into the circulation[15,93]. In a study of 90 patients undergoing cardiac surgery, the extent of the haemolytic injury differed by surgical procedure, was associated with nitric oxide and haptoglobin consumption, and predicted AKI (**Fig. 8**) [14]. The body's innate ability to respond to haemolysis involves an efficient system of erythrophagocytosis (i.e., the clearance of damaged red cells from the circulation by immune cells), recycling of haem and iron to erythropoiesis and preventing cell-free haem and haemoglobin mediated cell damage by binding them to the scavenger proteins haemopexin and haptoglobin[94].

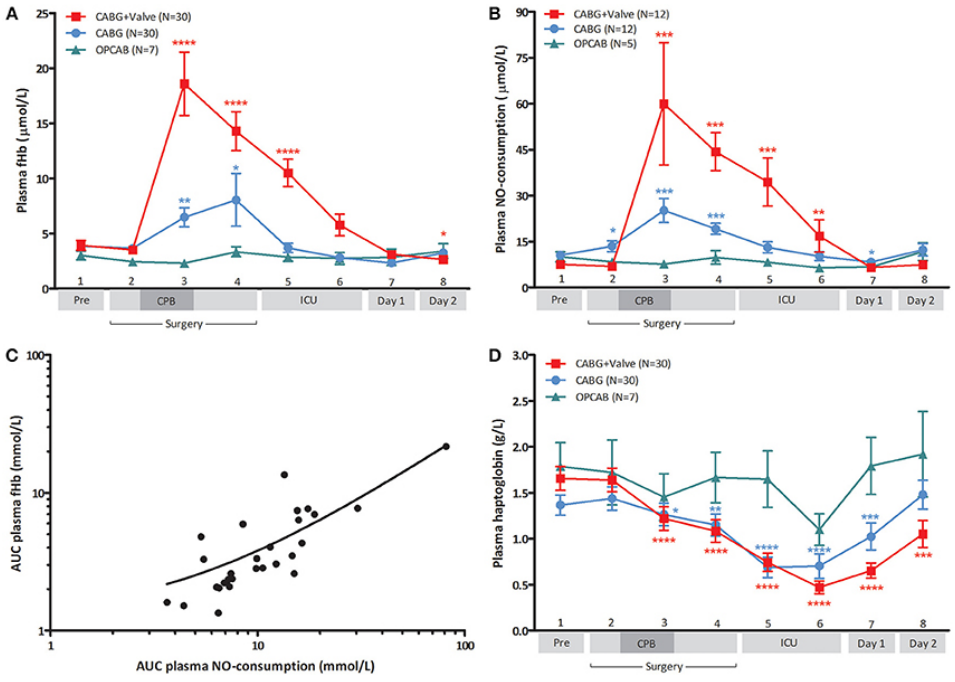



**Figure 8: The relationship between cardiopulmonary bypass and haemolysis.**

Cardiac surgery with cardiopulmonary bypass (CPB) is associated with an acute haemolytic insult quantified by plasma cell-free haemoglobin (cf-HB) which peaks in the initial hours after surgery (panel A). This correlates directly with plasma nitric oxide (NO) consumption (panels B-C) and indirectly with plasma haptoglobin levels (panel D).

Abbreviations: CABG, coronary artery bypass graft; OPCAB, off-pump coronary artery bypass graft.[i]

[i] Reproduced from figure 1 Windsant et al. *Front. Physiol.* 2014; 5: 1-9.

### 1.2.1 Cell-free haemoglobin and haem are toxic and cause immune activation

If severe enough, haemolysis can overcome the body's erythrophagocytic systems leading to an accumulation of cf-HB and haem[95]. cf-HB is known to be toxic through NO scavenging, causing vasoconstriction and endothelial, immune, and platelet activation[96–98]. The classic scavenging systems are Hb-haptoglobin complex clearance mediated by CD163, and haem-hemopexin complex clearance mediated by CD91. cf-HB has also been shown to reduce levels of circulating disintegrin and metalloprotease with thrombospondin motifs 13 (ADAMTS13), a protein essential in preventing uncontrolled clot formation through cleavage of large von Willebrand Factor (vWF) multimers[99]. Disordered microcirculatory function and widespread coagulation associated with haemolysis, can drive significant organ dysfunction in cardiac surgery, where it exacerbates injury during reperfusion and contributes to the so-called no-reflow phenomenon[14,32].

During haemolysis, cf-HB exists predominantly in αß-subunit heterodimers – these are small (32 kDa) particles capable of translocation into extravascular spaces (e.g., renal parenchyma) where they escape canonical intravascular scavenging proteins (e.g., haptoglobin)[94,100,101]. Hb can exist in different oxidation states, determined by the oxidation state of the haem iron moiety (i.e., ferrous [$Hb^{2+}$], ferric methaemoglobin [$Hb^{3+}$] or ferryl [$Hb^{4+}$]), with progressively toxic pro-oxidative capacity[102]. A more oxidative environment (e.g., IRI during cardiac surgery) drives the creation of more toxic Hb species and destabilises the haem-globin association resulting in cell-free haem[103].

Haem, formed from an iron atom and a tetrapyrrole protoporphyrin IX ring, is an essential component in many cells and is found as the prosthetic group of many haemoproteins including haemoglobin (oxygen transport), myoglobin (oxygen storage), oxidases (oxygen metabolism), cytochromes (electron transport) and antioxidant enzymes (e.g., catalases, haem peroxidase)[104]. Unbound haem is cytotoxic, sensitises cells to subsequent stressors (e.g., oxidative injury) and primes pro-inflammatory immune cells[105]. It is also lipophilic and can damage diverse intracellular organelles including the mitochondria (resulting in progressive impairment in mitochondrial respiration and ultrastructural damage), the lipid bilayer and nucleus[106]. It is also capable of injuring the proteosome and cytoskeleton[95]. Increased levels of unbound

haem in myocardial tissue, an organ very rich in intracellular haem compounds, have been associated with exaggerated tissue damage during ischaemia[107]. Increased levels of cytosolic haem have been observed in myocardial samples from patients with heart failure and, through its pro-oxidant effects, exogenous haem has been shown to cause cardiomyocyte dysfunction *in vitro*[108,109].

Systemically, haem induces inflammatory activation of the endothelium and sensitises it to pro-oxidant damage by granulocytes or toxic reactive oxygen species (ROS)[110]. Free haem is also a potent cytotoxic molecule sensed by the innate immune system, activating Toll-like receptors (TLRs)[111] and the Nod like receptor protein with a PYRIN domain 3 (NLRP3) inflammasome[112] to promote Nuclear factor kappa B (NF-κB) activation, tumour necrosis factor production and interleukin (IL)-1β and IL-18 processing. The ability of haem to activate multiple inflammatory pathways in the absence of an infectious stimulus has led to its categorisation as a Danger-Associated Molecular Pattern (DAMP), one of a group of molecules normally secured safely within the confines of the cell but which can be released because of sterile cellular injury after trauma (e.g., surgery), ischemia, or other mechanism of cell rupture[113]. Additionally, haem has been shown to induce long-lasting innate immune memory through cellular, transcriptional and chromatin accessibility changes[114].

Haem has also been shown to stimulate neutrophils to induce neutrophil extracellular trap (NET) formation[115]. NETs are decondensed chromatin strands decorated by granular enzymes, such as neutrophil elastase, to form extracellular fibres released by activated neutrophils which provide high concentrations of antimicrobial granules to kill bacteria[116]. In non-infectious settings, however, NETs are also associated with the promotion of hypercoagulability, inflammation and tissue damage. In patients undergoing cardiac surgery, there is a peri-operative increase in cell-free double-stranded (ds) DNA levels, a marker reflecting NET concentration, with higher levels of serum dsDNA correlating with and predicting renal dysfunction after CPB[117,118]. Haem also serves as both a substrate for and stimulant of haem oxygenase-1 (HO-1) transcription and very low levels can induce HO-1 expression, underlining this enzyme's critical regulatory and survival role[119]. The fates of haem are summarised in figure 9.

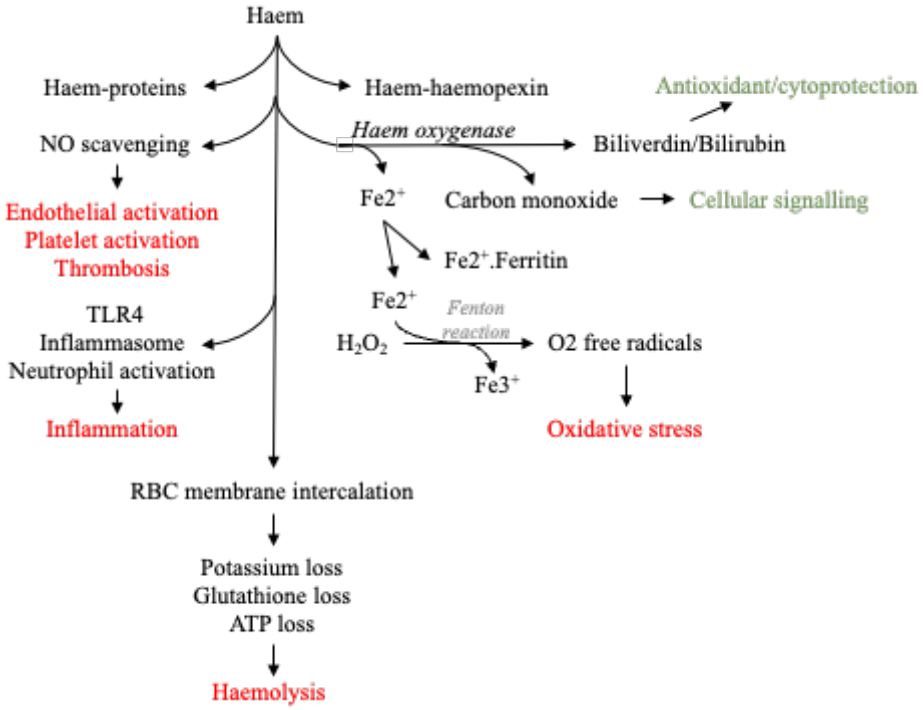



**Figure 9: Haem homeostasis and downstream signalling of unbound haem/degradation products.** Abbreviations: RBC – red blood cell; NO - nitric oxide; TLR4 - Toll-like receptor 4. [j]

### 1.2.2 Iron homeostasis and iron mediated cellular injury and death

Iron, an essential component of many cellular processes, is also capable of causing significant oxidative injury, and its levels are exquisitely regulated by complex homeostatic mechanisms summarised (**Fig. 6**) [120]. In brief, dietary ferric iron ($Fe^{3+}$) is reduced to its ferrous state ($Fe^{2+}$) by duodenal cytochrome b (Dcytb) and transported into the enterocyte via the divalent metal transporter 1 (DMT1). Dietary haem-bound iron enters the enterocyte via the haem carrier protein 1 (HCP1) or the haem responsive gene protein 1 (HRG1), liberating $Fe^{2+}$ after catabolism mediated by HO-1. Intracellular $Fe^{2+}$ is either exported via ferroportin (FPN), entering the bloodstream, or stored after oxidation to $Fe^{3+}$ and subsequent binding to ferritin. Exported $Fe^{2+}$ is immediately oxidised to $Fe^{3+}$ by hephaestin on the basolateral side of the enterocyte, enabling transferrin coupling. Absorbed iron is destined for erythropoiesis, under the control of erythropoietin (EPO), or recycling by macrophages. Uptake of transferrin bound iron (TBI) into macrophages is mediated by transferrin receptor 1 (TfR1). Macrophages also take up haem-bound iron

[j] Adapted from figure 2 Sawicki et al. *JAHA* 2015; 4(1): e001138.

through CD91-mediated uptake of haem-hemopexin complexes, and CD163-mediated uptake of Hb-haptoglobin complexes. From here, it is either oxidised and bound by ferritin, or exported by FPN. Exported macrophage $Fe^{2+}$ is oxidised by ceruloplasmin (CP), enabling ferritin coupling.

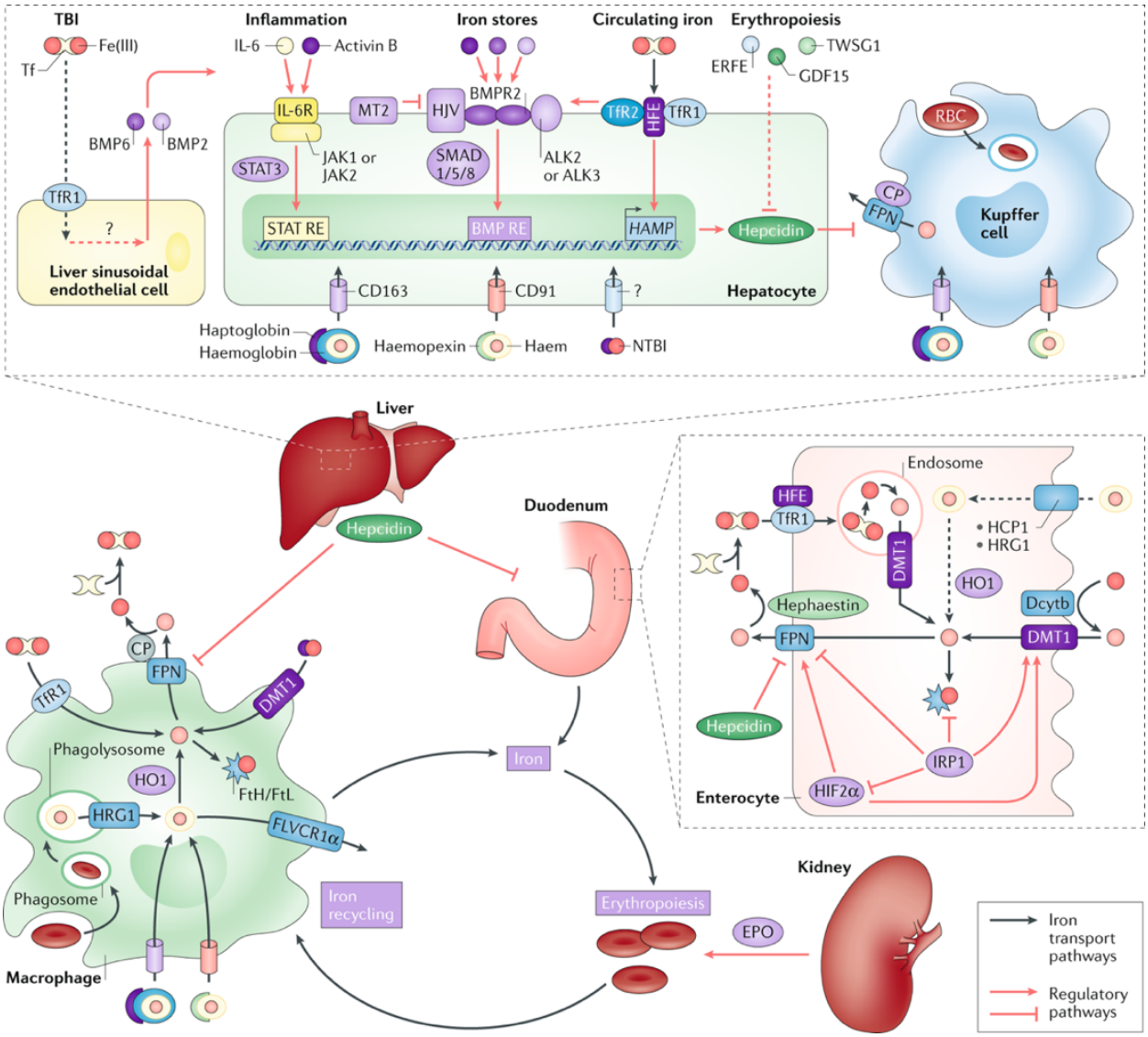



**Figure 10: Overview of iron homeostasis**.

Hepcidin is released from hepatocytes in response to transferrin bound iron (TBI), inflammation (mediated in part by IL-6) and increased iron stores signalled by bone morphogenetic protein (BMP) 2 and 6. Hepcidin release is negatively regulated by increased erythropoiesis which is signalled by the erythrokines erythroferrone (ERFE), growth differentiation factor 15 (GDF15) and twisted gastrulation protein homologue 1 (TWSG1). Hepcidin exquisitely controls iron homeostasis by balancing absorption with erythropoiesis and iron recycling.[k]

[k] Reproduced from figure 1 van Swelm et al. *Nat. Rev. Nephrol.* 2020; 16: 77-98.

Hepcidin (coded for by *HAMP*) and hypoxia-inducible factor 2α (HIF-2α) are critical regulators of iron homeostasis[120]. Hepcidin exercises exquisite control over the flow of iron into the bloodstream through its effect on FPN, reducing iron absorption from the duodenum as well as its export from macrophages and hepatocytes, thereby reducing circulating iron levels[121]. Transcription of *HAMP* (expressed primarily in hepatocytes) is under the control of various stimuli: inflammation, increased iron stores and circulating TBI increase hepcidin expression, while heightened erythropoietic drive decreases its expression, mediated via erythrokines. Once hepcidin is released, it binds to FPN leading to its internalisation and degradation[122]. Hypoxia and iron depletion (e.g., anaemia) causes heterodimerisation of hypoxia inducible factors and the resulting complex translocates to the nucleus, regulating the transcription of various iron regulatory genes. HIF-2α, transcriptionally activated by iron regulatory protein 1 (IRP1), plays a key role in regulating erythropoietin (EPO) expression, iron absorption and transport[123]. Disordered iron homeostasis, including elevated hepcidin levels, have been linked with post-operative AKI in patients undergoing surgery with CPB[124].

Circulating ferric iron is typically bound to transferrin and is regarded as inert, while unbound iron (or iron loosely bound to albumin or other low molecular weight compounds) represents a labile iron pool referred to as catalytic-, redox active- or non-transferrin-bound-iron (NTBI)[125]. NTBI, with its ability to freely take part in redox reactions (e.g., Fenton reaction), can produce ROS and represents a cytotoxic pool of iron[126]. NTBI is thought to form when there is iron overload (e.g., acute haemolysis) relative to transferrin binding capacity and has been mechanistically linked with significant cellular and sub-cellular toxicity, capable of initiating ferroptosis[127]. Ferroptosis is a distinct form of programmed cell death (PCD) initiated by the accumulation of intracellular free iron with subsequent lipid peroxidation and has been linked with multiple pro-inflammatory pathways[128]. Furthermore, circulating NTBI has been implicated as specifically cardiotoxic in the context of acute MI[129]. Cardiac surgery requiring CPB has been associated with higher transferrin saturation (Tsat) levels, favouring formation of NTBI[130]. In cardiac surgical patients with elevated catalytic iron, a 6.7-fold greater odds ratio of experiencing AKI requiring renal replacement therapy or in-hospital mortality, as a composite, in addition to greater risk of myocardial injury have been reported[93].

### 1.2.3 Haemolysis and ferroptosis contribute to acute kidney injury in cardiac surgery

AKI is a common complication after cardiac surgery, occurring in up to 20 – 30 % of patients, and is independently associated with a 5-year mortality risk as high as 30 %[131]. The pathophysiology of AKI following cardiac surgery is summarised in **Fig. 11**. Its pathogenesis involves a combination of factors including underlying patient co-morbidities, renal hypoperfusion (in part related to non-pulsatile blood flow during CPB), exposure to nephrotoxic drugs, thromboembolic events, oxidative stress, and the inflammatory response to surgery and CPB[132].

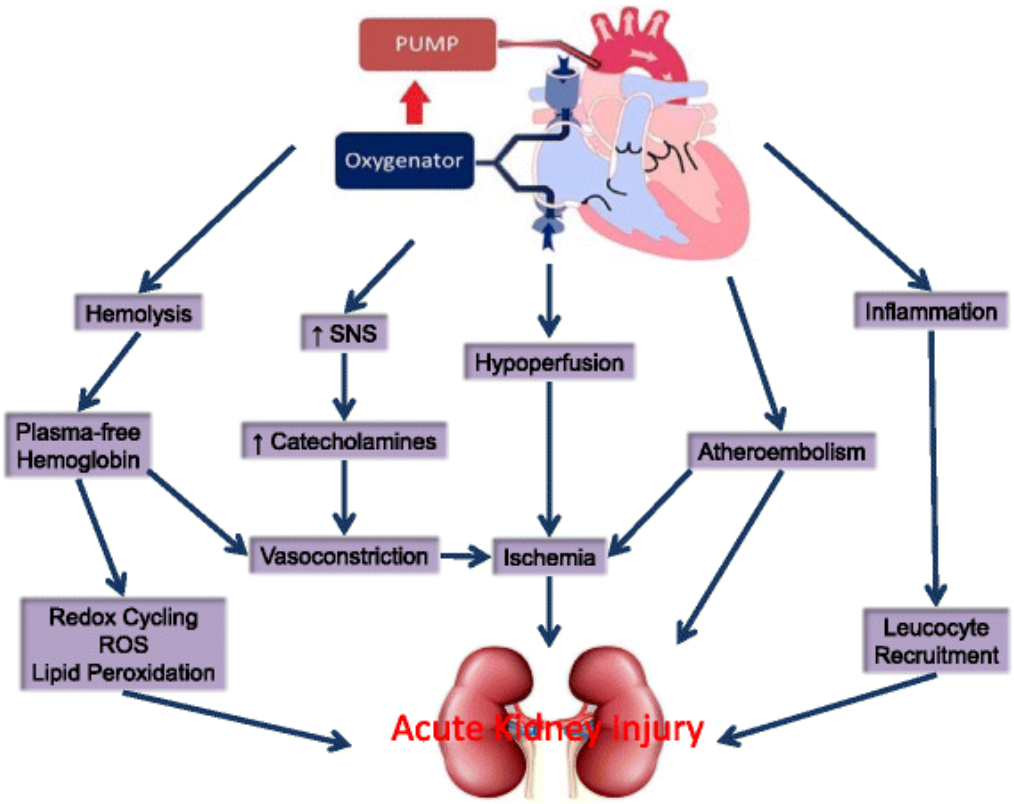



**Figure 11: The aetiology of acute kidney injury associated with cardiac surgery.**

The aetiology of acute kidney injury surgery is multifactorial, with several overlapping mechanisms (e.g., haemolysis, hypoperfusion) are directly attributable to cardiopulmonary bypass. [1]

Abbreviations: ROS – reactive oxygen species; SNS – sympathetic nervous system stimulation.

[1] Reproduced from figure 1 O'Neal et al. *Critical Care* 2016; 20: 187.

The link between the haemolytic insult associated with CPB and subsequent AKI has been investigated in a case control study[133]. Nested within a randomised, placebo-controlled trial investigating the effect of statin therapy to reduce AKI after cardiac surgery, this study demonstrated a link between increased levels of post-operative cf-HB, and AKI diagnosed using the Kidney Disease: Improving Global Outcomes (KDIGO) classification[134]. Haemolysis can cause kidney injury through multiple mechanisms including NO consumption with vasoconstriction and platelet aggregation[14]. Furthermore, cf-Hb is sequestered in the kidneys once it precipitates with Tamms Horsfall proteins in the tubular collecting systems and can also escape the vascular compartment, where haem-bound iron can mediate oxidative stress inducing so called pigment nephropathy[135]. Additionally, the kidneys appear to be particularly sensitive to toxicity mediated by circulating NTBI, as they can reabsorb iron even in conditions of excess[120]. Indeed, ferroptosis has been proposed as an early cellular mechanism for developing AKI in cardiac surgery[136,137]. Once triggered, ferroptosis can also lead to other forms of PCD (i.e., necroptosis, apoptosis) and inflammation, leading to established AKI and possible progression to chronic kidney disease (CKD). The presence of NTBI (or plasma catalytic iron as assayed) has also been associated with AKI and an increased mortality in a cohort of critically ill patients[138].

Addressing AKI in cardiac surgery remains an important clinical challenge and relies on identifying patients at risk of developing AKI, institution of prevention measures, and early diagnosis[132]. Various clinical prediction models (e.g., Cleveland Clinic Score) have been developed to identify patients at risk of developing AKI after cardiac surgery, and it is accepted that combining these with biomarkers aimed at early diagnosis may increase accuracy in identifying the correct time to intervene[139]. No specific therapies are known to reduce the incidence of AKI after cardiac surgery, and current guidance is to employ a ‘bundle’ of care encompassing primarily supportive measures to minimise renal injury[140]. Prompt diagnosis of established AKI is key to improving patient outcomes, and the current consensus recommendations are to use the KDIGO criteria[132].

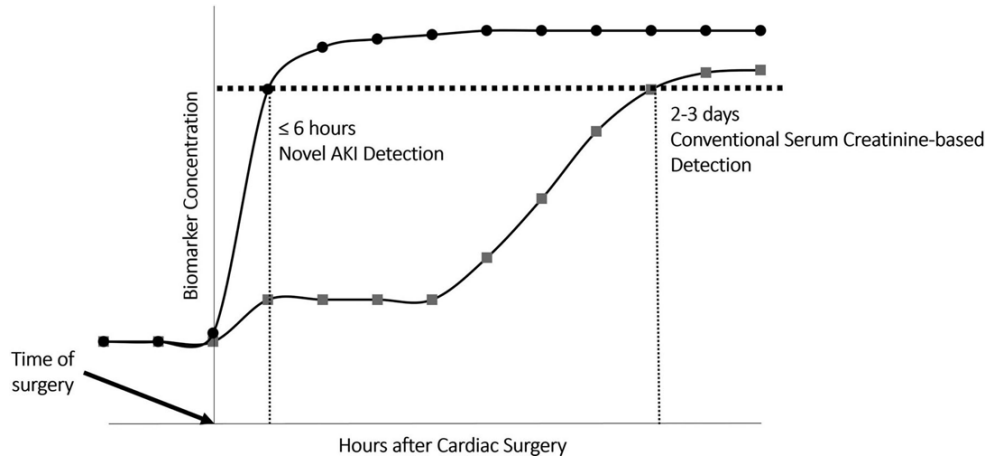



**Figure 12: Early detection of acute kidney injury using novel biomarkers.**

Using biomarkers such as Neutrophil gelatinase–associated lipocalin (NGAL) and Cystatin C may enable earlier detection of acute kidney injury (AKI) after cardiac surgery. These biomarkers have limitations in cardiac surgery (e.g., NGAL is less useful in patients with chronic kidney disease) and are not currently routinely used in clinical practice. [m]

### 1.2.4 Monocytes are capable of erythrophagocytosis and ferrostasis

Monocytes, together with macrophages and dendritic cells (DC), form part of the mononuclear phagocyte system (MPS)[141]. Monocytes are phenotypically diverse and are regarded as important innate and adaptive immune cells protecting the organism in times of stress, as well as representing important homeostatic cells[142]. Based on the relative surface expression of CD14 and CD16, monocytes have conventionally been defined through flow cytometry as either classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) or non-classical ($CD14^-CD16^+$)[143]. With the advent of new experimental approaches (e.g., single cell RNA sequencing), monocytes are now seen as so functionally and developmentally diverse that this simple classification approach is being revisited[144,145]. An alternative approach to the traditional taxonomy, focussing the functional role of monocytes in certain disease states, has been proposed[146].

In the context of damaged or senescent RBCs (e.g., during haemolysis), macrophages have long been held as the key effector MPS cells in erythrophagocytosis and ferrostasis[147,148]. Furthermore, macrophages in other tissues (e.g., heart, kidneys) have been shown to regulate harmful iron species in

[m] Reproduced from figure 2 Cheruku et al. *Anesthesiol.* 2023; 139: 880-898.

several disease states[149]. In muscle, for example, macrophages resident in injured tissue expressed higher levels of ferritin, CD163 and HO-1, suggesting a ferrostatic role via differentiation of cells to support the ability to control tissue levels of both bound (e.g., haem/myoglobin) and unbound, catalytically active iron[150]. Recent work has highlighted the ability of monocytes (classical > intermediate) to phagocytose damaged RBCs and scavenge NTBI, potentially limiting systemic toxicity induced by cf-HB, haem and iron[151]. This study evaluated gene and protein expression profiles in monocyte subsets comparing three groups of patients: an iron overload group from patients with disorders such as haemochromatosis; a blood transfusion group including patients with non-study related indications for RBC transfusion; and healthy controls. Experimentally, monocytes have been shown to represent rapidly recruitable immune cells capable of erythrophagocytosis, while also serving as progenitor cells for liver macrophages equipped specifically to phagocytose damaged RBCs and, crucially, store iron[152]. A switch in monocyte phenotype favouring an anti-inflammatory haemoglobin scavenging cell ($CD14^+$ $CD163^+$ HLA-$DR^-$) has been described following cardiac surgery, and has been proposed as an attractive therapeutic target in improving post-surgical outcomes[153,154].

## 1.3 Induction of haem oxygenase-1: a novel therapeutic strategy to improve outcomes after cardiac surgery requiring cardiopulmonary bypass

### 1.3.1 HO-1 - a master cytoprotective sentinel

Haem oxygenases (HO) are evolutionarily conserved, ubiquitously expressed enzymes which catalyse the rate-limiting step in the breakdown of haem into equimolar quantities of biliverdin (which is subsequently converted to bilirubin), carbon monoxide (CO) and ferrous iron ($Fe^{2+}$)[155]. HO exists in two major isoforms: a constitutively expressed haem oxygenase-2 (HO-2) and, haem oxygenase-1 (HO-1). HO-1 is highly inducible and responds to a variety of stimuli including haem, oxidative stress, hypoxia, ischaemia-reperfusion, lipopolysaccharide, heat shock, heavy metals and cytokines[156]. HO-1, a 32 kDa protein encoded for by a different gene (*HMOX1*) to HO-2 (*HMOX2*), can localise to the endoplasmic reticulum/microsomes where it may play a fundamental role as an inducible endogenous defence mechanism against cellular stress[155]. Activation of *HMOX1* transcription represents a unique intersection of four key cellular stress associated transcription factor pathways: heat-shock factor (HSF), Nuclear factor erythroid 2-related factor 2 (NRF2), nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) (**Fig. 13**) [157].

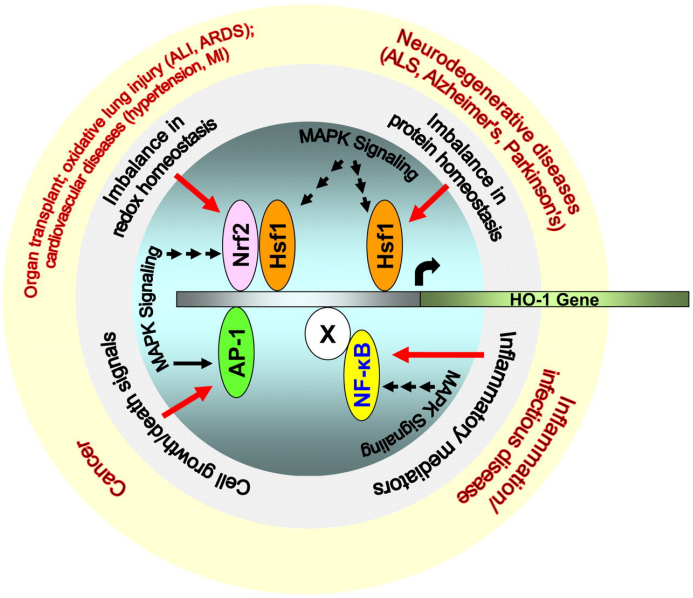



**Figure 13: Regulation of *HMOX1* transcription.**

*HMOX1* transcription is activated in response to diverse cellular stressors and represents a well-preserved cellular mechanism capable of responding to a variety of disorders. [n]

[n] Reproduced from figure 3 Alam et al. *Am J Respir Cell Mol Biol* 2007; 36 (2): 166-174.

Through its highly inducible catalytic action as well as potential non-enzymatic roles, such as regulation of mitochondrial quality[158] and promotion of mitochondrial biogenesis[159], HO-1 acts as a master cytoprotective sentinel capable of modulating oxidative stress[160]. Highlighting the critical survival role of HO-1 in humans, very few cases of complete HO-1 deficiency has been described[161–164]. Affected individuals have displayed exquisite sensitivity to oxidative injury with evidence of severe endothelial cell injury, widespread tissue iron deposition (likely reflecting disordered tissue iron homeostasis), renal tubular injury, interstitial lung disease, immune activation with haemophagocytosis, and premature death.

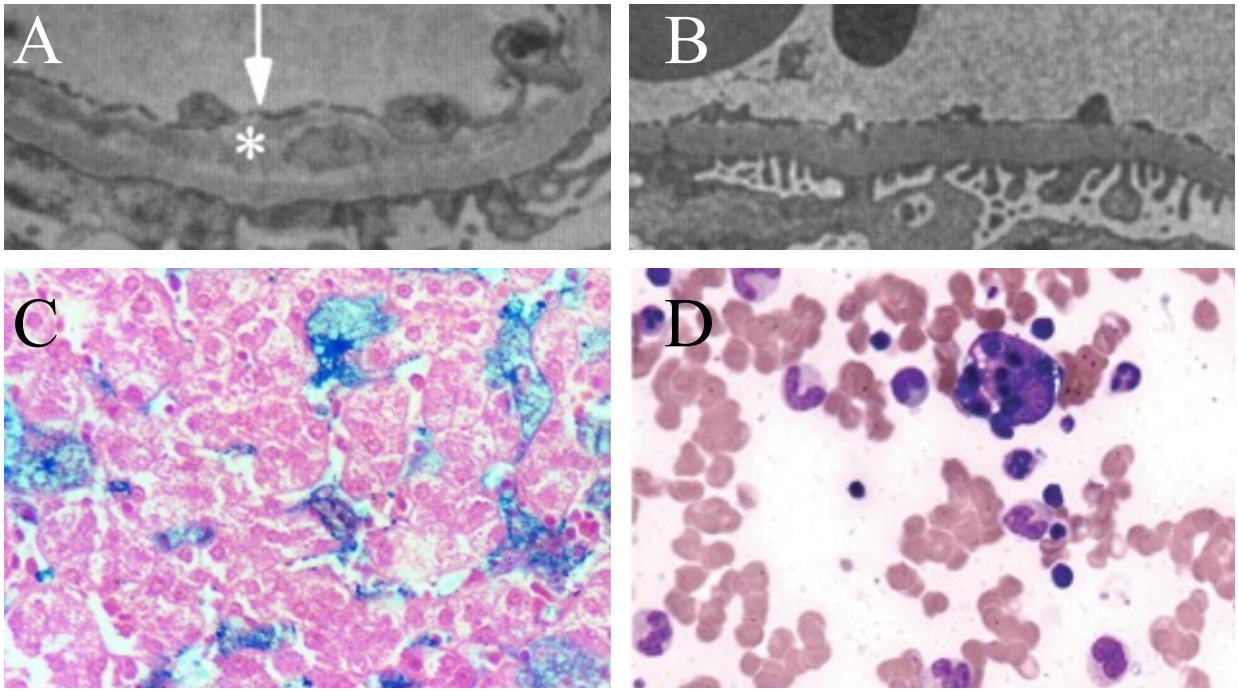



**Figure 14: Manifestations of human HO-1 deficiency.**

Electron microscopy showing glomerular endothelial detachment and subendothelial deposit in a 6-year old child with HO-1 deficiency (panel A) vs. healthy control renal glomerular endothelium (panel B); paraffin embedded liver sections showing widespread iron deposition (panel C); bone marrow aspiration sample showing haemophagocytosis (panel D).[o]

[o] Adapted from figure 2 Yachie et al. *JCI* 1999; 103(1): 129-135 and figure 2 Chau et al. *Pediatr Rheumatol* 2020; 18: 80.

### 1.3.2 HO-1 mediates haem breakdown and releases cytoprotective molecules

The pro-survival effects of HO-1 must be considered within the context of its canonical enzymatic function, summarised in **Fig. 15**. Principally, HO-1 plays a critical role in maintaining intracellular haem levels which, along with careful control of synthesis, incorporation into haemoproteins and trafficking, avoids haem mediated lipid peroxidation and oxidative stress[104,156]. The breakdown products of haem, specifically bilirubin and carbon monoxide, are bioactive signalling molecules in their own right and exert potent antioxidant, anti-inflammatory and anti-apoptotic functions to promote cell survival.

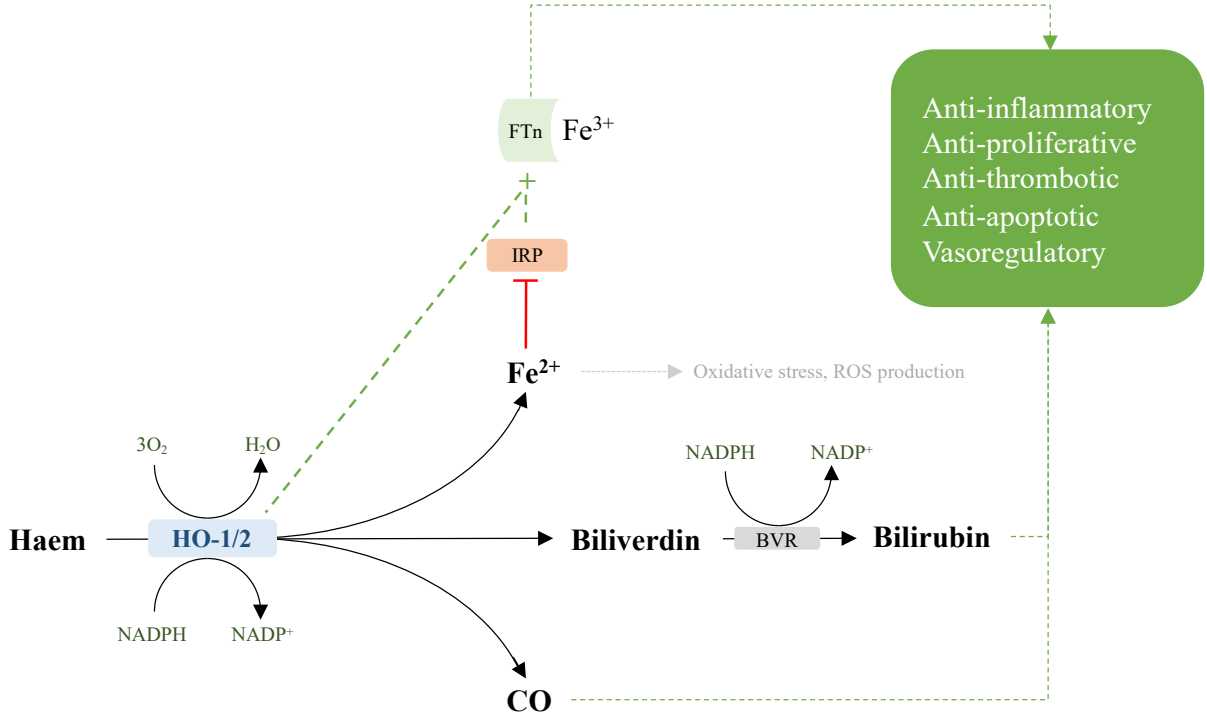



**Figure 15: The canonical function of the haem oxygenases.**

The HO enzymes catalyse the breakdown of haem into equimolar quantities of ferrous iron and biliverdin, which is converted into bilirubin by BVR, and carbon monoxide.

Abbreviations: BVR – biliverdin reductase; CO – carbon monoxide; FTn – ferritin; IRP - iron regulatory protein, HO-1/2 – Haem oxygenase-1/Haem oxygenase-2.[p]

CO had long been viewed as a toxin due to its ability to compete with $O_2$ for Hb binding[165]. The fact that HOs are ubiquitously expressed, implying that every cell in the body has the capacity to produce CO,

[p] Adapted from figure 1 Fredenburgh et al *Eur Heart J* 2015; 36(24): 1512-1518 and figure 1 Ryter et al *Mol Cell Biochem* 2002; 234-235(1-2): 249-263.

led to this dogma being questioned. CO has subsequently been shown to be cytoprotective in several disease states and interest in its therapeutic potential has led to the development of carbon monoxide releasing molecules (CORMS) as a distinct class of pharmacological agents[165–168]. CO, a colourless diatomic gas, is the most stable of three biologically important so-called gasotransmitters (the others being NO and $H_2S$), is capable of binding to transition metals (e.g., $Fe^{2+}$) and acts as an important intracellular signalling molecule[169,170]. In the cardiovascular system, CO has been shown to play an important regulatory role on vasomotor tone by binding guanylate cyclase and eNOS, favouring vasodilation and inhibition of platelet aggregation[171,172]. CO has also been shown to inhibit cardiac L-type $Ca^{2+}$ channels, inhibiting $Ca^{2+}$ influx into cardiomyocytes offering possible protection in cardiac IRI[173]. It's cardioprotective effect is further enhanced through its stabilising effect on the mPTP and maintenance of mitochondrial biogenesis[174,175]. CO also interacts with mitogen-activated protein kinases (MAPK) (e.g., p38) and caspase 3, with anti-inflammatory and anti-apoptotic effects[176]. In various pre-clinical models of disease with engineered loss of function of HO-1 (e.g., sepsis[177], allogenic aortic transplantation[178]) a phenotype associated with increased mortality was largely rescued by the subsequent introduction of CORMS to the experimental model, pointing to the central role of CO in the pro-survival effects of HO-1. HO-1 derived CO also appears to play an important role in the differentiation of myeloid progenitors into mature macrophages[179].

In mammals biliverdin is rapidly converted to bilirubin by biliverdin reductase (BVR), a nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction[180]. Bilirubin acts as a potent antioxidant by trapping hydroperoxyl radicals, cycling back to biliverdin as a consequence[181]. This rapid BVR-mediated cycling of biliverdin/bilirubin represents an important, scalable intracellular defence against ROS with various systemic benefits[182,183]. In human umbilical vein endothelial cells (HUVECs) exposed to tumour necrosis factor alpha (TNF-$\alpha$), bilirubin has been shown to be anti-inflammatory causing a reduction in the expression of cell surface adhesion molecules and subsequent polymorphonuclear cell adhesion[184]. The cardioprotective role of bilirubin has been tested in mice using a Langendorff model of cardiac IRI, where bilirubin pre-treatment was associated with a reduction in infarct size and preserved mitochondrial structure and function[185]. In humans, increased serum bilirubin levels have been associated

with reduced rates of acute MI[186,187], which was comparable to a favourable lipid profile in terms of risk stratification in one study[188]. The final product of enzymatic degradation of haem, ferrous iron, is potentially toxic through its ability to take part in oxidative reactions (e.g., Fenton reaction). However, $Fe^{2+}$ also promotes iron regulatory protein (IRP) mediated increased expression of ferritin, which has antioxidant and antiapoptotic functions[189,190].

### 1.3.3 HO-1 is a pleiotropic enzyme with non-canonical cellular protective functions

An important pleiotropic effect of HO-1 was first suggested after it was shown that a mutant form of the protein, devoid of enzymatic capabilities, still offered cytoprotection in vitro[191]. This can partly be explained by HO-1's ability to translocate within the cell, interact with pro-survival proteins and transcription factors, and undergo post-translational structural modifications[192].

Under conditions of cellular stress (e.g., hypoxia) HO-1, usually a cytosolic protein, is released from its C-terminus endoplasmic reticulum anchor and the truncated protein translocates/co-locates to the nucleus, suggesting an important transcriptional/regulatory function[193]. HO-1 can bind to heterogenous nuclear ribonucleoprotein K (HnRNPK), which then moves with HO-1 into the nucleus where it is an important modulator of DNA transcription[194]. HO-1 has also been identified in mitochondria where it has been associated with decreased lipid peroxidation[195]. Despite being devoid of the key features of a transcription factor (e.g., DNA binding motifs), HO-1 can regulate its own transcription through its effects on AP-1 and NF-κB as well as inhibiting pro-inflammatory signalling via inhibition of STAT3[193]. Interestingly, HO-1 has not demonstrated a similar inhibitory effect on Nrf2 signalling and it is thought that HO-1 may enhance Nrf2 signalling preferentially. HO-1 has also been shown to bind to nuclear DNA repair proteins (e.g., poly (ADP-ribose) polymerase-1 [PARP1] and DNA helicase II), potentially modulating the cellular response to oxidative stress[196]. In terms of post-translational modifications to HO-1, AKT has been shown to phosphorylate HO-1 at various protein residues without altering the enzymatic role, possibly pointing to a modification enabling a different, non-enzymatic, protein function[197]. Post-translational acetylation and ubiquitination of HO-1 has been demonstrated but the functional significance of these modifications is unknown[198].

The ability of HO-1 to interact with seemingly unrelated cellular processes has been highlighted. For example, there is significant cross talk between HO-1 and purine metabolism[199]. ATP, required as the primary energy source for most vital cellular processes, has been identified as an important extracellular alarmin, capable of mediating cellular responses in a paracrine and autocrine manner through P2X and P2Y receptors[200]. Through activation of the $P2X_7$ receptor, extracellular ATP has shown to be cytotoxic and proinflammatory[201,202]. Extracellular levels of ATP are controlled by 5'ectonucleoside triphosphate diphosphohydrolase (CD39) and ecto-5' nucleotidase (CD73) by stepwise degradation into adenosine diphosphate (ADP), adenosine diphosphate (AMP) and ultimately adenosine. Adenosine is regarded as an anti-inflammatory mediator so flow of this extracellular processing of ATP represents a distinct pro-survival pathway[199]. HO-1 derived bilirubin and CO favour increased expression of CD39 and the adenosine receptor 2A ($A_{2A}R$), with activation of $A_{2A}R$ leading to increased HO-1 expression representing an important pro-survival, feedforward mechanism. As further evidence of the interconnected nature of these two pathways, in a mouse model of renal IRI examining the therapeutic role of a CORM, the protective effect of the CORM was lost in CD39 knockout mice[203].

### 1.3.4 HO-1 is immunomodulatory and dampens activation of the sterile inflammasome

Not only is HO-1 activated in response to varied inflammatory stimuli as outlined, but it also functions as a key modulator of multiple immune cell types and of immunometabolism[204]. Specific favourable immunometabolic shifts associated with HO-1 expression have included promoted increased oxidative phosphorylation and AMPK production, with decreased glycolysis and generation of CAC cycle metabolites. HO-1, through its production of CO, has been shown to direct differentiation of myeloid progenitor cells into macrophages and promotes an anti-inflammatory, or M2-like, phenotype[205,206]. HO-1 also assumes central importance in the role of tissue resident macrophages in regulating iron homeostasis and the ability to protect tissues against oxidative stress[207]. HO-1 is constitutively expressed by immature dendritic cells (DC) and has been directly linked to their phagocytic and anti-inflammatory potential[204]. HO-1 induction maintains DCs in this immature state, promoting immune tolerance by reducing their production of pro-inflammatory cytokines, with a resultant reduction in T-cell effector responses[208]. Furthermore, HO-1 appears to prevent activation of the sterile inflammasome[209].

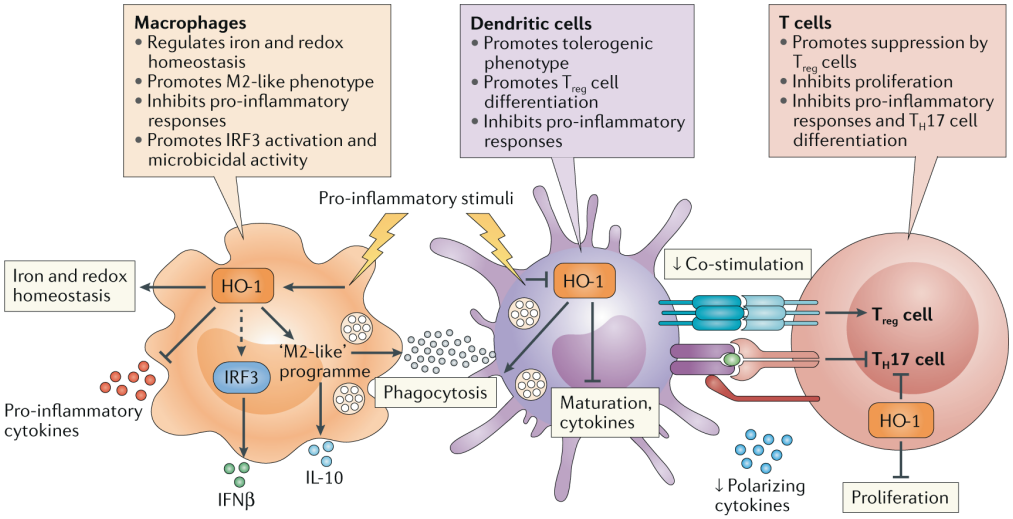



**Figure 16: The immunomodulatory role of HO-1 in immune cell subsets.**

HO-1 polarises immune cells to an anti-inflammatory phenotype, enabling phagocytosis, iron and redox homeostasis. [q]

Abbreviations: IFNß – interferon beta; IRF3 – interferon regulatory factor; Th17 – T Helper 17 cell; $T^{reg}$ – Regulatory T cell.

The NLRP3 inflammasome is an important intracellular complex able to sense, and respond to, multiple immunological stimuli including hypoxia[210]. It is made up of a sensor (NLRP3), an adapter (apoptosis-associated speck like protein containing a caspase recruitment domain (ASC)) and an effector (caspase-1). The system is primed in response to various extra-cellular stimuli (e.g., pathogen-associated molecular patters (PAMPs) or DAMPs, TNF) leading to NF-κB mediated NLRP3 transcription which can then be activated by multiple unrelated extra- and intra-cellular stimuli (e.g., infections, sterile inflammation mediated by DAMPs), culminating in the oligomerisation of NLRP3, ASC and caspase-1 [211]. The activated inflammasome activates caspase-1 which cleaves pro-interleukin 1β (pro-IL-1β) and pro-interleukin 18 (pro-IL-18) and gasdermin-D (GSDMD), enabling the release of the pro-inflammatory cytokines IL-1β and IL-18, and initiating pyroptosis, a highly inflammatory form of PCD. In humans, circulating monocytes possess constitutively activated caspase-1 and can release pro-inflammatory cytokines after a single stimulation of TLRs, escaping the requirement for classical two-step

[q] Reproduced from figure 3 Campbell et al *Nat. Rev. Immunol* 2021; 21(7): 411-425.

inflammasome activation[212,213]. A non-canonical pathway for inflammasome activation in human monocytes, involving caspase-4 and caspase-5, which does not require any NLRP3 inflammasome components, also exists[214].

Haem, as a prototypical DAMP, primes and activates the NLRP3 inflammasome through activation of TLRs[215], the Receptor of advanced glycation end products (RAGE)[216] as well as by the non-canonical pathway[217,218]. There is also experimental evidence that suggests haemolysis-associated cytotoxicity cannot only be explained solely by haem-induced inflammasome activation, suggesting other intracellular components (e.g., ATP, Hb) likely contribute significantly to this[219]. The ability of HO-1 to dampen the NLRP3 inflammasome response to classic priming/activation was demonstrated in bone marrow-derived macrophages (BMDMs) from HO-1 knockout vs. wild type mice[209]. The products of HO-1 mediated catabolism of haem (i.e., CO and bilirubin) have been suggested as the primary drivers of this beneficial relationship[220,221]. HO-1 upregulation by hemin, a commercially available oxidised form of haem, has subsequently been shown to reduce inflammasome activation in murine models of sepsis[222,223]. Targeting the inflammasome where unchecked activation may be present (e.g., acute haemolysis), represents an attractive therapeutic approach when the inflammatory fallout from the insult is liekly to be detrimental[224].

### 1.3.5 Evidence supporting HO-1 as a therapeutic node for cardio- and cyto-protection

HO-1 gain-of-function (e.g. transgenic or adenoviral-mediated overexpression or pharmacological induction) has been shown to be protective in multiple pre-clinical models of ischaemia-reperfusion injury involving the native or transplanted heart[225–228], liver[229,230], kidney[231] and ischaemic brain injury[232]. Possible mechanisms include an immunomodulatory mechanism with a reduction in the influx of post-ischaemic pro-inflammatory neutrophils and monocytes. This was validated in a porcine model of myocardial ischaemia[233].

In humans, a well described GT dinucleotide (GT)n repeat polymorphism in the *HMOX1* promoter region, located 250 base pairs (bp) upstream from the transcription start site, affects transcription,

conferring enhanced or reduced HO-1 activity, depending on the polymorphism status[234]. The size of this repeat length varies from 12 – 40 bp and exists in a bi-modal distribution of alleles between 22 and 31 bp in several populations studied. These alleles can be homozygous long (LL), homozygous short (SS) or heterozygous (SL). SS (variably described as a (GT)n repeat length of < 25 or < 27 in the literature) is associated with higher transcriptional activity and greater HO-1 expression and activity in vitro, with such cells showing greater resilience against oxidative stress and apoptosis[235,236].

Higher HO-1 activity conferred by the short (GT)n repeat polymorphism has been linked with a reduced incidence of cardiovascular disease/events[236–241] and improved outcomes after solid organ and hematopoietic stem cell transplants[242–244]. As an example of acute haemolysis, children with sickle cell disease had a reduced severity of acute crises and acute kidney AKI if they had the shorter (GT)n repeat polymorphism[245,246]. At odds with the apparent protective effect of genetically driven higher HO-1 activity at baseline, a shorter repeat has been linked with an increased risk of acute respiratory distress syndrome (ARDS)[247] and AKI[248] in critically unwell patients.

The link between the (GT)n dinucleotide repeat polymorphism status and AKI has also been studied in 2,377 adult patients undergoing CABG and/or valve surgery requiring CPB (**Fig 17**) [249]. This study employed a Mendelian randomisation approach and showed an association between HO-1 polymorphism status, determined by the number of (GT)n repeats, and the incidence of post-operative AKI. Consistent with a reno-protective role for HO-1 function in the setting of cardiac surgery, patients with longer *HMOX1* alleles – expected to have lower HO-1 expression/function – exhibited a 1.6-fold greater risk for developing AKI compared to those bearing the short *HMOX1* genotype. This relationship was stronger in those who received any packed red blood cell (pRBC) transfusions intra-operatively, notable since pRBC undergo progressive haemolysis to release free haemoglobin during storage[250]. Consistent with reduced capacity to catabolise toxic cell-free haem released during CPB, patients with longer *HMOX1* alleles exhibited higher levels of plasma free haemoglobin at the end of CPB[249].

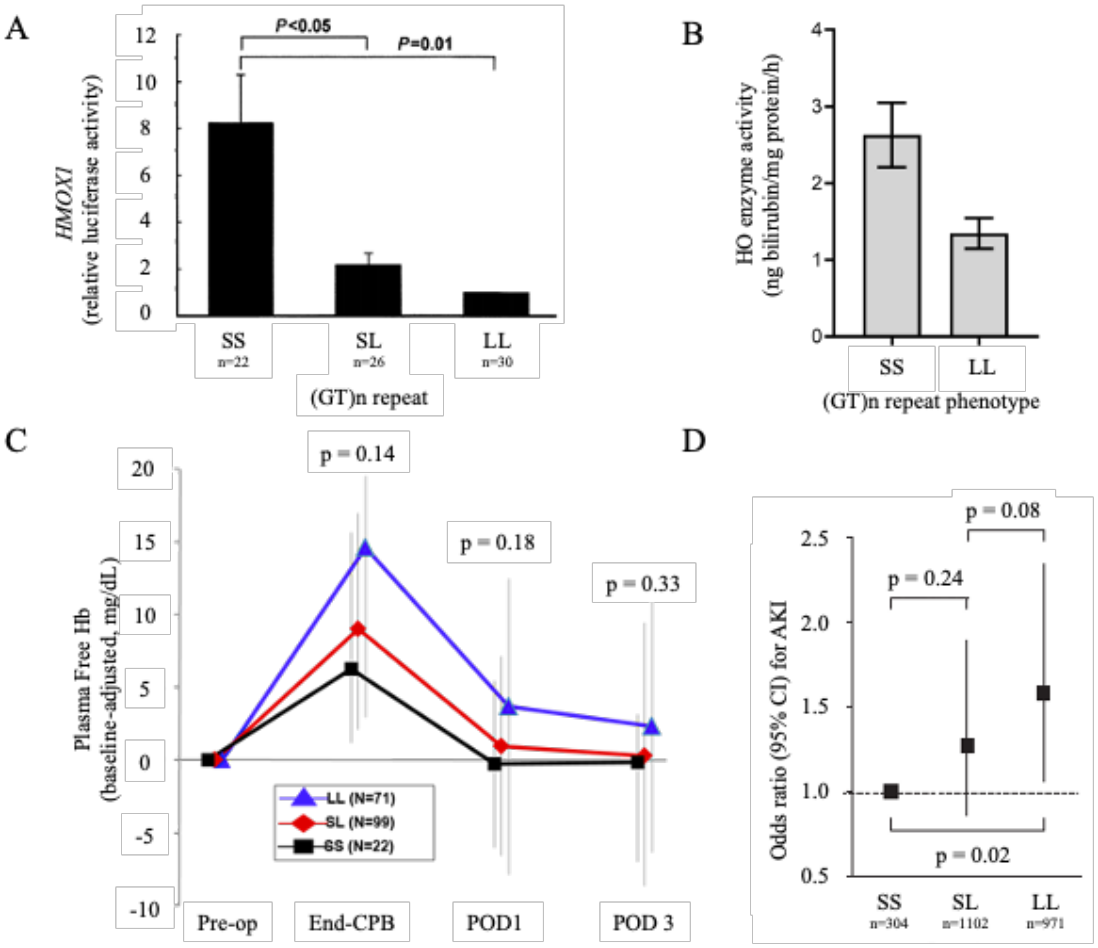



**Figure 17: Increased HO-1 activity reduces the incidence of acute kidney injury after cardiac surgery.**

A shorter (GT)n repeat polymorphism in the promoter region of *HMOX1* is associated with increased *HMOX1* transcription in human aortic smooth muscle cells (panel A) and increased HO activity in human lymphoblastoid cell lines, in response to $H_2O_2$ stress (panel B). Higher HO-1 activity, predicted by the repeat polymorphism status, was associated with a lower odds ratio of developing acute kidney injury (AKI) following cardiac surgery and this was potentially related to a less profound haemolytic injury quantified using cell-free haemoglobin (cf-HB) (panel C-D).

Abbreviations: SS – homozygous short; SL – heterozygous; LL – homozygous long; Hb – haemoglobin.[r]

A strategy of pharmacological HO-1 induction prior to elective IRI has been tested using a pre-operative infusion of haem arginate in the setting of renal transplantation[251] (NCT01430156: placebo-controlled study of 40 patients receiving deceased donor kidneys) and cardiac surgery[252] (NCT02314780: placebo-controlled study of 24 patients undergoing aortic valve replacement). In the cardiac surgical trial,

[r] Reproduced from figure 2 Chen et al. *Hum Genet* 2002; 111: 1-8; Hirai et al. *Blood* 2003; 102(5): 1619-1621; figure 1 Leaf et al. *J. Am Soc Nephrol* 2016; 27(11): 3291-3297.

investigating whether haem arginate would increase myocardial tissue expression of HO-1, haem arginate appeared to be safely tolerated by patients undergoing aortic valve replacement requiring CPB. HO-1 expression was increased in PBMCs as well as in cardiac tissue, at transcript and protein level. Interestingly, this study found that haem arginate increased HO-1 expression more profoundly in right atrial appendage tissue when compared to the left ventricle (LV), although the authors commented that this may be explained by the timing of tissue sampling (right atrial appendage samples were taken prior to institution of CPB, and LV biopsies were taken after).

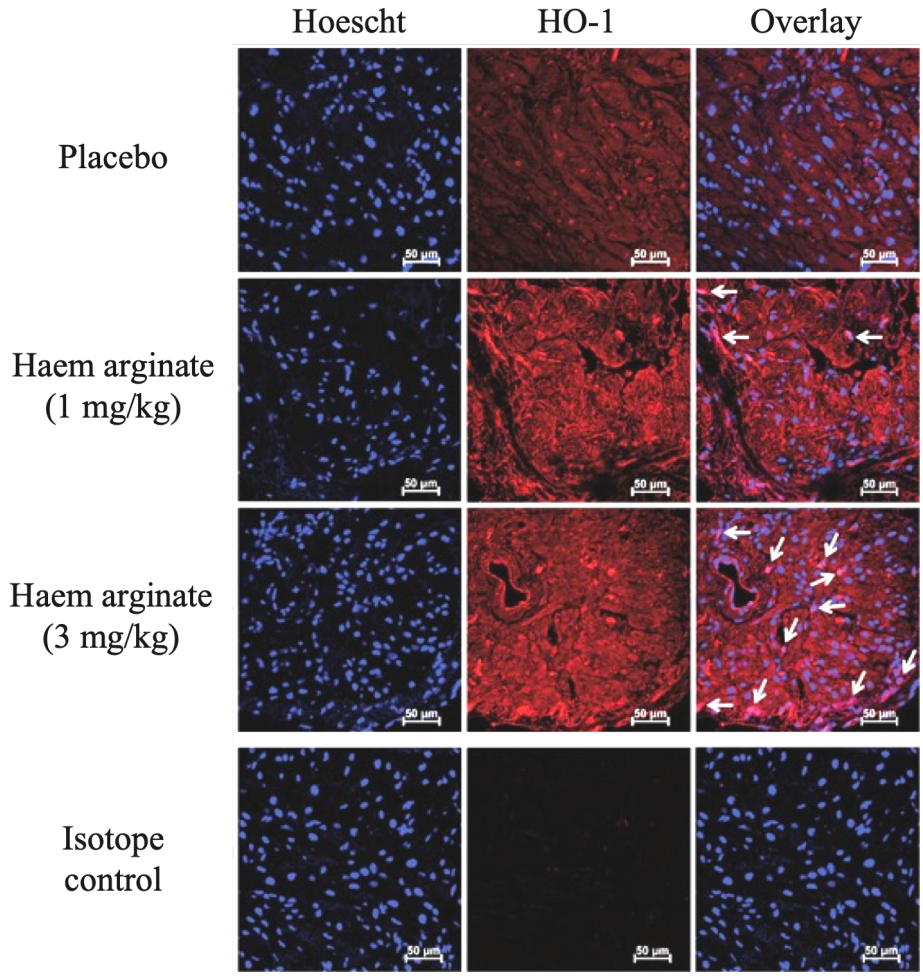



**Figure 18: Haem therapy increases HO-1 expression in human cardiac tissue.**

Immunofluorescence-based HO-1 detection demonstrating increased right atrial appendage protein expression in response to pre-operative intravenous haem arginate, in patients undergoing aortic valve replacement. Nuclear translocation/co-location is suggested in the higher dose group (indicated with white arrows). [s]

[s] Reproduced from figure 2 Andreas et al. *ATVB* 2018; 38: 2755-2762.

Nonetheless, both of these studies have shown that pre-operative dosing with haem arginate can increase HO-1 expression at mRNA and/or protein level in peripheral blood mononuclear cells (PBMCs) – highly enriched in HO-1 and containing key cellular effectors of its potential salutary effects – and organ tissue (kidney and tissue macrophages; cardiac atria and ventricle), with evidence of dose-responsivity haem across *HMOX1* (GT)n genotypes[253]. These studies illustrate that the substrate of HO-1, haem, can itself be used as a pharmacological HO-1 inducer.

### 1.4 5-ALA-SFC – Harnessing a physiological mechanism to upregulate HO-1

A distinct strategy to induce HO-1 expression is the use of 5-aminolevulinic acid (5-ALA), a metabolic precursor of haem, given orally together with sodium ferrous citrate (SFC) as an iron source, i.e. as 5-ALA-SFC[253]. 5-ALA (also known as δ-aminolevulinic acid) is a natural delta amino acid present in foods such as red wine, Asian ginseng, radish sprouts, green tea and vinegar[254]. 5-ALA is also synthesised within the mitochondria from the condensation of glycine and succinyl-coenzyme A (succinyl-CoA), the primary rate-limiting step in haem biosynthesis catalysed by ALA synthase (ALAS)[255]. This is then transferred to the cytosol where two ALA molecules are condensed by the enzyme ALA dehydratase to form the primary monopyrrole ring, porphobilinogen (PBG). Several subsequent synthetic steps lead to the formation of protoporphyrin IX (PPIX), into which ferrous iron is incorporated to form haem (**Fig. 19**). Haem regulates its own synthesis and breakdown through inhibition of ALAS and induction of HO-1 activity.

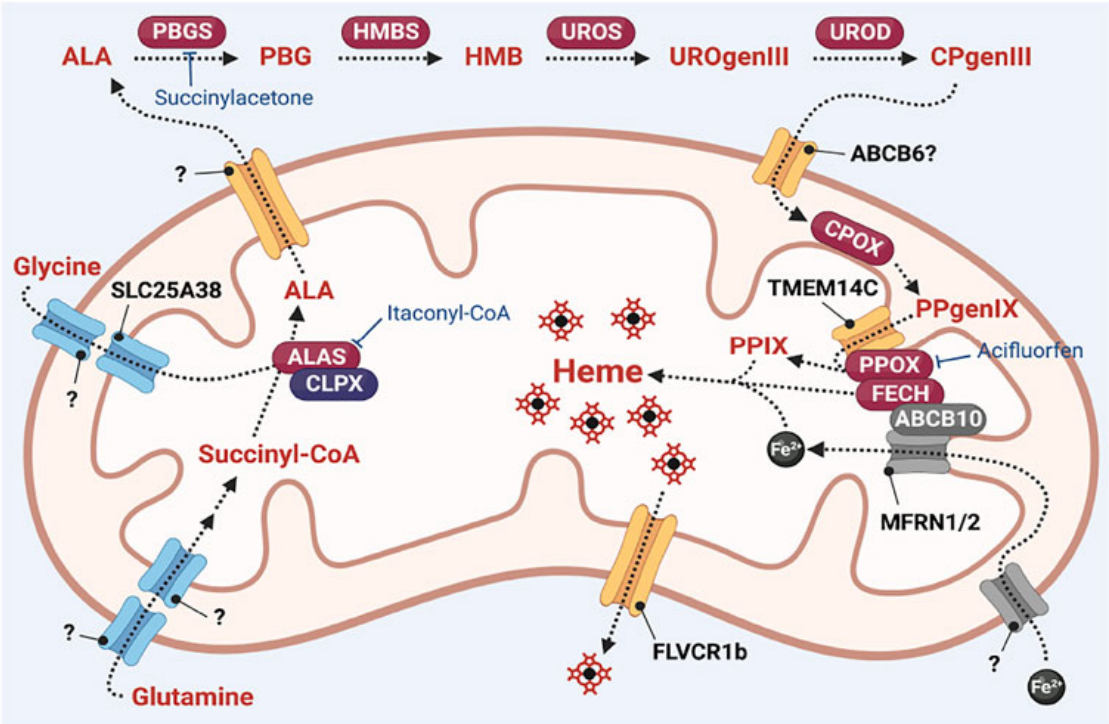



**Figure 19: The haem biosynthesis pathway.**

Haem biosynthesis involves several mitochondrial and cytosolic processes with the synthesis of aminolevulinic acid (ALA) and the incorporation of iron regarded as the rate limiting steps. [t]

[t] Reproduced from figure 2 Yien et al Front Cell Dev Biol 2022; 10: 895521.

### 1.4.1 5-ALA-SFC increases intracellular haem activating *HMOX1* transcription.

Intracellular haem activates *HMOX1* transcription by: i) binding of haem to the *HMOX1* repressor, BTB domain and CNC homolog 1 (BACH1); and ii) inducing phosphorylation of ERK1/2 and p38 MAPK, promoting HO-1 expression through an Nrf2 dependent pathway[207,256]. Accordingly, by increasing intracellular levels of haem, 5-ALA-SFC promotes increased HO-1 expression through this canonical pathway (**Fig. 20**) [257].

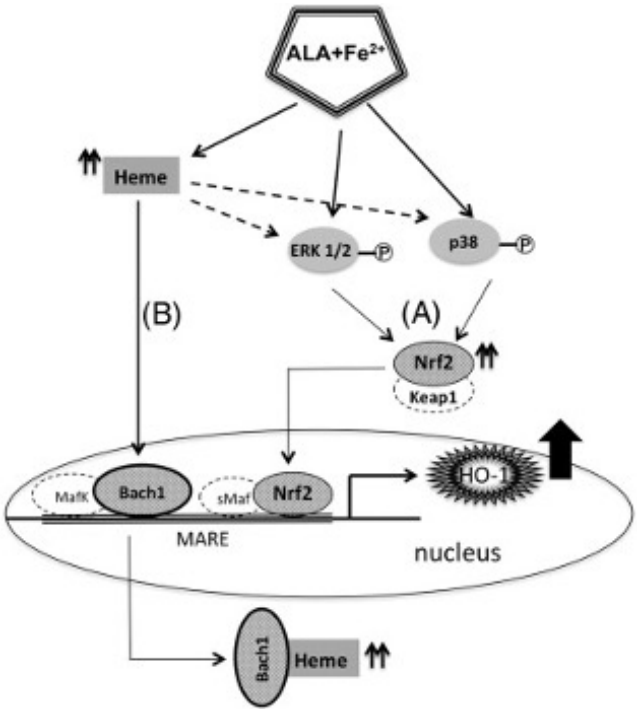



**Figure 20: 5-ALA-SFC mechanism of action.**

5-ALA and SFC are transported into cells where they are ultimately converted into haem, leading to dissociation of BACH1 from the antioxidant response element (ARE) in the nucleus. Nrf2 dissociates from Keap1 allowing nuclear translocation and activation of HO-1 transcription.[u]

Abbreviations: BACH1 - BTB domain and CNC homolog 1, Nrf2 - Nuclear factor erythroid 2-related factor 2, Keap1 - Kelch-like ECH-associated protein 1.

Nrf2 is a highly conserved transcriptional factor, encoded by the *NFE2L2* gene, that is at the centre of several cellular responses to oxidative stress and has been the focus of intense research as a target for cardioprotection[258]. In humans, the protein is highly regulated by seven distinct Nrf ECH (erythroid cell

[u] Reproduced from figure 6 Nishio et al. *Int Immunopharmacol* 2014; 19(2): 300-307.

derived protein with cap'n'collar (CNC) homology) homology (Neh) domains[259]. The Neh2 domain interacts with the negative regulator of Nrf2 activity, Kelch-like ECH-associated protein 1 (Keap1), ensuring homeostatic control of Nrf2 through ubiquitylation and subsequent proteasomal degradation[260]. Under conditions of oxidative stress, Keap1 is oxidised leading to stabilisation of Nrf2 and translocation into the nucleus[261]. In the nucleus, Nrf2 heterodimerises with small musculoaponeurotic fibrosarcoma proteins (sMaf), enabling transcription of cytoprotective target genes (Figure 10) after binding to the antioxidant response element (ARE) in the promoter region[262,263]. Understanding of the regulation of Nrf2 is evolving and is thought to include, amongst others, Keap1 independent interactions mediated at the Neh6 domain and possibly miRNAs[264,265]. Enhanced Nrf2 activity has proven central to reduced immune activation and the development of post-cardiac allograft vasculopathy in a mouse cardiac allotransplant model, underlining the crucial role the myriad Nrf2 target genes play in marshalling a pro-survival response to cardiac IRI[266]. Furthermore, in a mouse model of acute haemolysis, loss of Nrf2 function was associated with an increased risk of AKI[267]. Possible targets conferring protection in this model included HO-1, ferritin light chain (FTL), ferritin heavy chain (FTH) and glutathione.

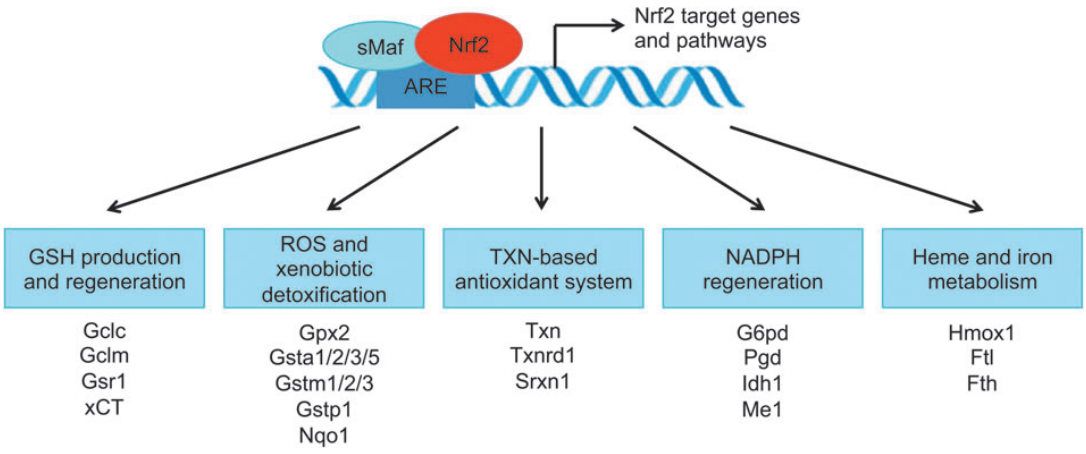



**Figure 21: Nrf2 target genes and putative roles.**

Nrf2 regulates the transcription of several genes involved in mammalian responses to toxic and oxidative insults. [v]

Abbreviations: ARE – antioxidant response element GSH – glutathione; NADPH Nicotinamide adenine dinucleotide phosphate; ROS – reactive oxygen species; sMaf – small Maf proteins; TXN – thioredoxin.

[v] Reproduced from Tonelli et al *Antioxid Redox Signal* 2018; 29(17): 1727-1745.

### 1.4.2 Pre-clinical studies demonstrating cyto- and cardio-protective effects of 5-ALA-SFC

5-ALA-SFC has shown a protective effect in a model of cyclosporin-induced nephrotoxicity, attenuating renal inflammation, apoptosis, fibrosis and tubular atrophy[268,269] and promoting expression of mitochondrial oxidative phosphorylation subunits in association with increased ATP levels[270]. These effects are potentially beneficial in the setting of elective ischaemic stress with its attendant cellular energetic challenge. Strikingly, administration of 5-ALA-SFC in a cardiac allotransplantation model was shown to promote permanent acceptance of the transplanted heart driven in part through effects on regulatory T cells and dendritic cells[271]. 5-ALA-SFC has also been demonstrated to exert significant protection against ROS through activation of the MAPK/Nrf2/HO-1 pathway in an immortalised mouse myocardial cell line[272].

In prior work by our own group, the potential for 5-ALA to induce cardioprotection has been investigated using a Langendorff model of global myocardial IRI injury in mice. In this model, hearts are *ex vivo* perfused for 20 minutes and then undergo 40 minutes of global normothermic ischaemia, followed by 90 mins of reperfusion. Mice who received enteral 5-ALA before IR injury had significantly reduced myocardial infarct size as a proportion of total left ventricular (LV) muscle volume compared to control mice (Fig. 22). This reduction in MI size was associated with functional protection, including amelioration of the reduction of coronary artery blood flow and myocardial contractility observed following global ischaemia. These findings are consistent with a direct cardioprotective effect of 5-ALA. To define the mechanism by which 5-ALA exerts this cardioprotective effect, the experiment was repeated with the addition of the HO-1 antagonist, zinc deuteroporphyrin 2,4-bis glycol (ZnBG). The cardioprotective effect of ALA was lost in mice co-treated with ZnBG (with ZnBG alone having no influence on MI size following global *ex vivo* IR injury). Taken together, these data are consistent with a cardioprotective effect of ALA mediated via an HO-1- dependent mechanism.

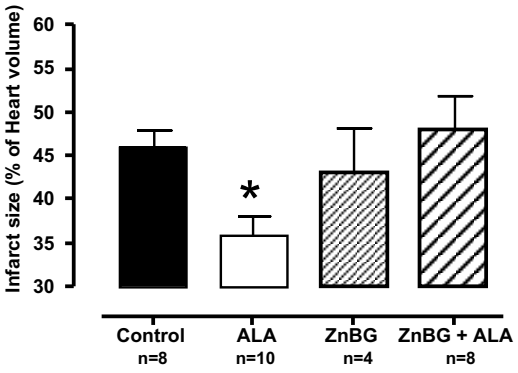



**Figure 22: 5-ALA reduces infarct size in a mouse Langendorff model of cardiac ischaemia.**

### 1.4.3 Current clinical use of 5-Aminolevulinic acid

A different formulation of 5-ALA, marketed as Gliolan, is authorised for use in adults to aid visualisation of malignant tissue during surgery for malignant glioma. 5-ALA-guided resection was recommended by NICE in the UK (NG99, published July 2018) to maximise the success rate of initial resection. It is used for this indication at a dose of 20 mg 5-ALA per kg body weight. Gliolan is dosed as an oral solution from bottles containing 1.5 g 5-ALA per bottle (i.e. suitable for a 75 kg patient). This is administered 3 hours before anaesthesia for neurosurgery. Gliolan's summary of product characteristics (SmPC)[273] highlights that there are no special instructions for the use of Gliolan in elderly patients with normal organ function. Gliolan's contra-indications are limited to acute/chronic porphyria, hypersensitivity, or pregnancy. Its substance-specific side effects are reported as hypotension, nausea, photosensitivity and photodermatosis. All of these are stated as uncommon side effects, defined as a frequency in patients of ≥ 1/1,000 to < 1/100.

A recent dose escalation study evaluated the safety and tolerability of higher doses of 5-ALA (> 20mg/kg) in patients undergoing elective neurosurgery[274]. Patients aged 27-88 years (mean age 60 years) undergoing resection of newly diagnosed or recurrent high-grade glioma, received up to 50 mg/kg body weight 5-ALA pre-operatively (i.e. 2.5-fold higher than the dose approved in Europe). This study enrolled 19 patients and reported no serious adverse events were reported. In the group receiving the highest dose, transient dermatological phenomena (redness and peeling) were reported. These were felt to be mild and should not necessarily prohibit the future use of a dose up to 50 mg/kg body weight of 5-

ALA, with the objective of achieving stronger tumour fluorescence. No adverse events (AE) of grade 2 or higher (by the Common Terminology Criteria for Adverse Events [CTCAE] grading) were reported. There were no instances of hypotension, anaemia, or nausea in any patient. In 3 cases, patients had transient elevations of liver enzymes. These elevations were mild, resolved spontaneously, and did not appear to be related to the dose of the study drug; accordingly, it was unclear whether this was related to the study drug, anaesthesia, or other unknown factors.

### 1.4.4 The safety profile of oral 5-ALA-SFC – pre-clinical and clinical experience to date

To date, oral 5-ALA-SFC has been safely administered to > 700 human subjects, including healthy volunteers and patients across several indications. Clinical trial exposure duration has ranged from 1 day to 12 weeks, and the doses administered have ranged from 5-ALA: 5 mg to 1500 mg, and SFC: 2.87 mg to 2351 mg. There have been no serious adverse reactions or deaths reported in any study.

5-ALA- SFC has been trialled in a phase 1 study of healthy Caucasian and Japanese volunteers[275]. Single oral doses of 5-ALA-SFC were found to be safe and well-tolerated at up to 1000 mg/1567 mg. Higher dose levels were less well-tolerated and associated with an increase in AEs. Gastrointestinal disorders (e.g., nausea, vomiting, dyspepsia, abdominal pain, abdominal distension and faecal discolouration) were most commonly encountered and were considered related or possibly related to 5-ALA-SFC. Nausea was the most commonly reported AE and typically occurred between 0.5- and 2.5-hours post-dose. Most events of nausea were mild. Nervous system disorders, including dizziness and headaches were reported in more than two subjects, and were considered related or possibly related to 5-ALA-SFC. All nervous system disorders were mild or moderate in severity. There were no treatment or dose-related trends in the mean or individual serum biochemistry, haematology, or urinalysis data during the single dosing phase of this study.

The same study also assessed multiple doses and longer courses of treatment with 5-ALA-SFC. 5-ALA-SFC given twice daily over 6.5 days was found to be safe and well tolerated when administered at dose levels of up to 300 mg /472 mg. The incidence of adverse events with doses of 150 mg/235 mg was

similar to placebo. Above 5-ALA-SFC 300 mg /472 mg the incidence of adverse events was higher, particularly in Caucasian subjects. The incidence of AEs rose sharply above dosages of 600 mg/944 mg. All adverse events were mild or moderate and resolved without treatment. Again, the most frequently reported adverse events were gastrointestinal and nervous system disorders.

After multiple doses, mild elevations in commonly used biochemical markers of liver function, namely alanine transaminase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (GGT), were identified in some subjects. Following the highest dose administered, 5-ALA-SFC 600 mg/940 mg, ALT was elevated beyond 3 times the upper limit of normal (ULN) in a single subject, constituting a drug induced liver injury (DILI). This was self-limiting and did not require medical intervention. There were no other apparent treatment or dose related trends in the mean or individual serum biochemistry, haematology, or urinalysis data during the multiple dosing phase of this study. Across the entire study, there were no treatment or dose-related trends in supine systolic and diastolic blood pressure, or pulse rate. There were no apparent treatment or dose-related trends in the 12-lead ECG parameters, including no evidence of prolongation of the QTc interval at any dose level of 5-ALA-SFC. There were no clinically important findings in the morphology of the 12-lead ECG for individual subjects at any dose level of 5-ALA-SFC.

In a phase II study studying the efficacy of 5-ALA-SFC to treat chemotherapy-induced anaemia concurrent with platinum-based chemotherapy, 17 patients were treated for approximately 8 weeks. There were two serious adverse events (appendicitis/ileocolitis and febrile neutropenia) neither of which were judged to be causally related to the investigational medicinal product (IMP)[276].

### 1.4.5 Pharmacokinetic (PK) characteristics of 5-ALA-SFC

5-ALA-SFC is rapidly absorbed after oral administration reaching peak plasma concentrations at 1-hour post-dose[275]. The timing of the dose, relative to the intake of food, slowed the time to peak concentration (reached 2 hours later), but area under the curve (AUC) analysis revealed no significant differences in systemic drug exposure. The drug readily redistributes to most tissues, with evidence of poor penetrance

across the blood brain barrier in mice. The majority of 5-ALA-SFC is incorporated into the haem biosynthetic pathway and does not undergo organ specific metabolism. Mean plasma half-life is approximately 1 hour, which appears to be independent of dose, and there is no evidence of drug accumulation after repeated dosing. After single and multiple doses, 8-20% of the drug is excreted renally in its unchanged form and there are no known active metabolites. The degree of renal elimination correlates with the dose and does not appear to be saturable (i.e., first order kinetics). Due to the absence of any significant effects on hepatic cytochromes, it is thought that use of 5-ALA-SFC will not result in significant drug-drug interactions. Small increases in plasma PPIX and plasma iron, in nano- and micro-molar ranges respectively, have been observed in healthy volunteers with a peak effect at 2-6 hours post-dose. Levels of both iron and haem returned to baseline within 12 hours. No cumulative effects on PPIX levels were observed with repeat dosing of 5-ALA-SFC.

### 1.4.6 The pharmacodynamic (PD) profile of 5-ALA-SFC

The PD effects of 5-ALA-SFC have principally been measured by assessing the drug's impact on HO-1 expression[277]. *HMOX1* mRNA expression levels measured using a quantitative polymerase chain reaction (qPCR) as well as HO-1 protein levels quantified by enzyme-linked immunosorbent assay (ELISA) in peripheral blood mononuclear cells (PBMCs) have been used to quantity drug response in healthy volunteers. HO-1 expression has also been assessed in specific immune cell subsets (plasmacytoid DCs and myeloid DCs) using flow cytometry. Significant increases in both PBMC *HMOX1* mRNA expression and HO-1 protein levels were demonstrated after repeated administration of 5-ALA-SFC (600 mg daily for 3 days). With repeated doses there was numerically greater *HMOX1* expression, which was not found to be statistically significant. Dose-related increases in blood protoporphyrin IX and most urinary porphyrins have been observed after administration of 5-ALA-SFC in phase 1 studies.

In summary, induction of HO-1 represents a highly attractive biological target to improve outcomes in patients undergoing cardiac surgery requiring CPB and pre-operative oral 5-ALA-SFC, with favourable safety, PK and PD profiles, may represent an accessible pharmacological agent to achieve this.

## 1.5 Hypothesis and objectives

The central hypothesis of this thesis is that oral administration of 5-ALA-SFC will cause dose-dependent induction of HO-1 in adult patients undergoing elective cardiac surgery on CPB. This will affect downstream signalling to mitigate excess peri-operative immune activation and haemolysis-driven organ injury, with a favourable tolerability and safety profile. Using a dose-escalation, randomised double-blind placebo-controlled design, the potential benefit of harnessing 5-ALA-SFC induced HO-1 activation as a node for cytoprotection in the context of cardiac surgery on CPB, will be assessed at the following levels:

1. Evaluate the safety and tolerability profile of 5-ALA-SFC in adult patients undergoing elective cardiac surgery on CPB:
    a. The tolerability profile will be assessed by measuring treatment compliance as well as the frequency of treatment interruptions. Adverse events attributed as potentially related to 5-ALA-SFC will be reported in full.
    b. The safety profile of 5-ALA-SFC in a contemporary patient population undergoing elective cardiac surgery on CPB will be characterised through assessment of adverse event data, vital signs and laboratory safety parameters. Attribution of causality will be reported, and any serious adverse events (SAEs) will be summarised in full.
    c. The feasibility of using serial cardiac troponin I measurements as a marker for PMI in early phase cardioprotection trials, will be assessed and preliminary kinetics will be examined, reporting any treatment-related differences.

2. Confirm target engagement by 5-ALA-SFC through assessment of HO-1 expression and activity using multiple, orthogonal approaches:
    a. Key circulating immune cells and tissues enriched for *HMOX1* will be identified through bioinformatics-based approaches, informing a strategy to evaluate HO-1 expression in discrete immune cell populations using flow cytometry. Using the results of this work, HO-1 induction by 5-ALA-SFC will also be assessed in key cell types *in vitro*.

b. *HMOX1* (GT)n promoter polymorphism status will be determined in patients undergoing cardiac surgery to investigate the correlation of genotype with readouts of baseline HO-1 function. Possible pharmacogenetic implications of the polymorphism status for 5-ALA-SFC will be explored by investigating for heterogeneity of responses to drug, by genotype.

c. The impact of 5-ALA-SFC on HO-1 biology will be investigated through quantification of HO-1 transcript and protein expression, as well as through quantification of enzyme function using a novel assay of HO enzymatic activity in PBMCs.

3. Establish the impact of HO-1 induction on CPB-associated haemolysis and iron-mediated cellular and organ injury, with a specific focus on the kidney:

   a. The degree of the haemolytic insult will be determined in patients undergoing cardiac surgery on CPB and potential differences related to 5-ALA-SFC will be investigated.

   b. The kinetics of circulating iron, systemic iron binding capacity and NTBI in response to cardiac surgery on CPB will be defined, and any treatment-related differences will be reported. Preliminary assessment of immune cell iron handling capacity will be investigated through assessment of the expression of selected genes encoding iron handling/homeostatic proteins.

   c. Levels of iron mediated lipid peroxidation markers and biomarkers of renal injury will be measured, and drug-related differences will be reported.

4. Evaluate the inflammatory response to cardiac surgery on CPB, and determine whether 5-ALA-SFC influences subsequent immune activation with a focus on cell types enriched for HO-1 as a potential mechanism to drive cyto-/organ-protection following cardiac surgery:

   a. Common biomarkers (e.g., C-reactive protein [CRP]) of immune activation/function will be assayed, and any treatment-related differences will be reported.

   b. Immunophenotyping by multiparameter flow cytometry will be used to evaluate whether 5-ALA-SFC differentially influences HO-1 expression in discrete monocyte subsets. Monocyte subsets will also be defined functionally, with specific reference to their ability to act as erythrophagocytic cells and their activation state.

c. Unbiased biological profiling of the inflammatory proteome will be undertaken to inform mechanistic understanding of the potential immunomodulatory properties of 5-ALA-SFC in cardiac surgical patients.

5. Integrate the safety, tolerability, pharmacodynamic and clinically relevant endpoint findings detailed above to identify an optimal biological dose of 5-ALA-SFC for further evaluation as a therapeutic strategy to improve patient outcomes following cardiac surgery on CPB.

# 2 Chapter 2. Methods

## 2.1 Pre-operative 5-Aminolevulinic acid to activate haem oxygenase to improve outcomes in cardiac surgery: A dose finding study – The "Treatment with 5-AminoLEvuliNic acid before cardiac surgery (TALEN)" trial protocol

### 2.1.1 Trial objectives and outcome measures

Trial objectives and outcomes are summarised in **Table 2**.

### 2.1.2 Trial design

The trial design followed a pragmatic approach with trial assessments and interventions mirroring usual clinical care as closely as possible[81]. The overall dose escalation trial design is summarised in **Fig. 23**.

#### 2.1.2.1 Dose escalation study design

To identify the OBD and evaluate the safety and tolerability profile of 5-ALA-SFC, participants were enrolled into sequential dose cohorts and randomised to receive either 5-ALA-SFC or placebo. Within each of the four planned dose cohorts, 8 participants were randomly allocated to receive 5-ALA-SFC, while 2 were allocated to receive placebo allowing 10 per cohort. The protocol allowed for the recruitment of additional participants within pre-defined boundaries (**appendix 8.1**).

#### 2.1.2.2 Cohort progression

Dose escalation and cohort progression was allowed after the SRC had reviewed and discussed blinded data for at least 8/10 (i.e., 80%) of evaluable participants per cohort, ensuring that at least six participants had taken the active drug. Per protocol, participants were considered evaluable if they had correctly taken ≥ 80% of study drug before cardiac surgery. They were required to have valid PD samples collected and key safety data available both at screening, prior to surgery and at least at one time point within 72 hours after surgery, allowing before and after treatment dose comparisons to be made. The protocol had defined stopping rules and the SRC were able to prohibit dose escalation if any safety concerns were raised with a potential causal relationship with 5-ALA-SFC.

Key safety data for dose escalation decision making included:

- Laboratory safety analyses (specifically liver and renal function tests)
- Adverse events (AE) and Serious Adverse Events (SAE) data, together with severity and investigator-attributed causality
- Dose-limiting toxicity (DLT) criteria were defined *a priori* in the protocol. If two or more participants at any 5-ALA-SFC dose level were found to have a DLT event, then dose-escalation would not proceed and the SRC could either recommend enrolment to a different (e.g. intermediate) dose or potentially close the trial. Dose limiting toxicity criteria were defined as:
- Unexpected AEs determined to be of severity grade 3 or higher (graded according to CTCAE Version 5.0) with reasonable possibility of causal relationship to 5-ALA-SFC
- Confirmed increase in the level of ALT (alanine aminotransferase) and/or AST (aspartate aminotransferase) to > 5 × ULN (upper limit of normal range) with reasonable possibility of causal relationship to 5-ALA-SFC
- Any Suspected Unexpected Serious Adverse Reactions (SUSAR).

### 2.1.3 Close of recruitment

TALEN closed to recruitment after the enrolment of ten evaluable participants to every cohort, as defined by the protocol. The SRC did not request any additional participants to be enrolled and the optional cohort was not utilised.

**Table 2: Summary of trial objectives with key outcome measures and timepoints of evaluation.**

| Objectives | Outcome Measures | Time point |
|---|---|---|
| **Primary Objective** | | |
| 1. To determine the recommended phase II dose (RP2D) of 5-ALA-SFC based on pharmacodynamic (PD) response and safety | • Changes in HO-1 expression and function in peripheral blood and immune cells<br>• Occurrence of adverse events / laboratory safety events defined as DLTs | HO-1: pre-dosing (baseline) and at end of dosing period immediately prior to cardiac surgery<br><br>During dosing and up to 72 hours post-operatively |
| **Secondary Objectives** | | |
| 1. To characterise the safety and tolerability profile of 5-ALA-SFC | • Incidence of treatment-related emergent AEs (including laboratory abnormalities) assessed using the Common Terminology Criteria for Adverse Events (CTCAE) v5.0<br>• Incidence of SAEs<br>• Worst grade of AE by CTCAE v5.0<br>• Frequency of treatment dose interruptions or discontinuations | From time of administration of placebo or IMP (i.e., treatment-emergent AEs, TEAEs) to 72 hours post-operatively<br><br>Safety assessed both up to time of surgery (i.e., pre-operative loading phase) and in the 72 hours immediately post-operatively |
| 2. Determine the PD response to 5-ALA-SFC in peripheral blood and immune cells, including peripheral blood mononuclear cells (PBMCs) | • HO enzyme activity in PBMCs<br>• HMOX1 mRNA expression in PBMCs<br>• HO-1 protein expression in PBMCs or serum/plasma<br>• HO-1 expression in circulating immune cell subpopulations by flow cytometry | Baseline (pre-dosing) and at end of dosing period immediately prior to surgery |
| 3. Preliminary evaluation of the kinetics of peri-operative myocardial injury | • Cardiac troponin I (cTnI) area under the curve for 72 hours | Measurements made at 0, 1, 6, 12, 24, 48 and 72 hours after release of aortic cross clamping |
| **Exploratory and Translational objectives** | | |

| | | |
|---|---|---|
| 1. Examine the PD influence of 5-ALA-SFC on HO-1 biology in myocardium | • Cardiac HO-1 expression (mRNA/protein)<br>• NRF2 target gene and haem transporter and other HO-/iron handling-related gene expression | Intraoperative (during surgery) where biopsy sample available |
| 2. Preliminary evaluation of the impact of 5-ALA-SFC on CPB-induced haemolysis | • Haemolysis, iron homeostasis markers (peripheral blood)<br>• Renal injury biomarkers (peripheral blood & urine) | Baseline and peri-operative period (blood/ urine sampling) |
| 3. Examine the impact of 5-ALA-SFC on the inflammatory, immune, metabolic, and oxidative profile, including stress response to CPB | • Immunophenotyping by multiparameter flow cytometry<br>• Biomarkers of inflammation, immune function, and metabolism<br>• Biomarkers of redox stress<br>• Unbiased biological profiling (e.g., RNA expression, metabolomics, proteomics)<br>• Primary cell culture experiments from patient-derived PBMCs and monocytes (e.g., differentiated in vitro to macrophages) to investigate cellular mechanisms of action and impact of 5-ALA-SFC on immune cell function | Baseline and peri-operative blood sampling<br>Intraoperative, where biopsy sample available<br>In vitro/tissue culture time points as appropriate |
| 4. Preliminary evaluation of the influence of *HMOX1* promoter polymorphism status on the PD response to 5-ALA-SFC | • Genotyping for *HMOX1* promoter polymorphism status | Single time point while on study |
| **Other key data captured** | | |
| | • Surgical risk scores | Pre-operative |

| | | |
|---|---|---|
| | • Echocardiography data including LV ejection fraction (where available) | Pre-operative |
| | • Anaesthetic and surgical operative data (including cardioplegia used) | At time of surgery |
| | • Details of Intensive Care Unit (ICU) stay (including number of ventilated days & length of stay) | Post-operative period on ICU |
| | • Incidence of Acute Kidney Injury (AKI) (including as per KDIGO definitions) within 72 hours of surgery[134] | At discharge from ICU or on cardiothoracic ward at 72 hours |
| Surgical risk stratification and clinically relevant markers of organ injury | • Incidence of Major Adverse Kidney Event (MAKE)[278]: death, persistent renal replacement therapy and/or more than 25 % reduction in eGFR | Until last study visit |
| | • Incidence of hypotensive/low-cardiac output state measured by vasoactive-inotropic score (VIS)[24,89] – maximum score over 24 hours, mean over 72 hours | At discharge from ICU |
| | • Incidence of type 5 MI (i.e., MI associated with CABG surgery) based on fourth universal definition of MI[24] (assessed only in those undergoing CABG surgery) | ≤48 hours after surgery |
| | • Incidence and severity of delirium (assessed using the confusion assessment method for the Intensive care Unit (CAM-ICU))[279] | During hospital admission |
| | • Post-operative complications | Operation until last study visit |

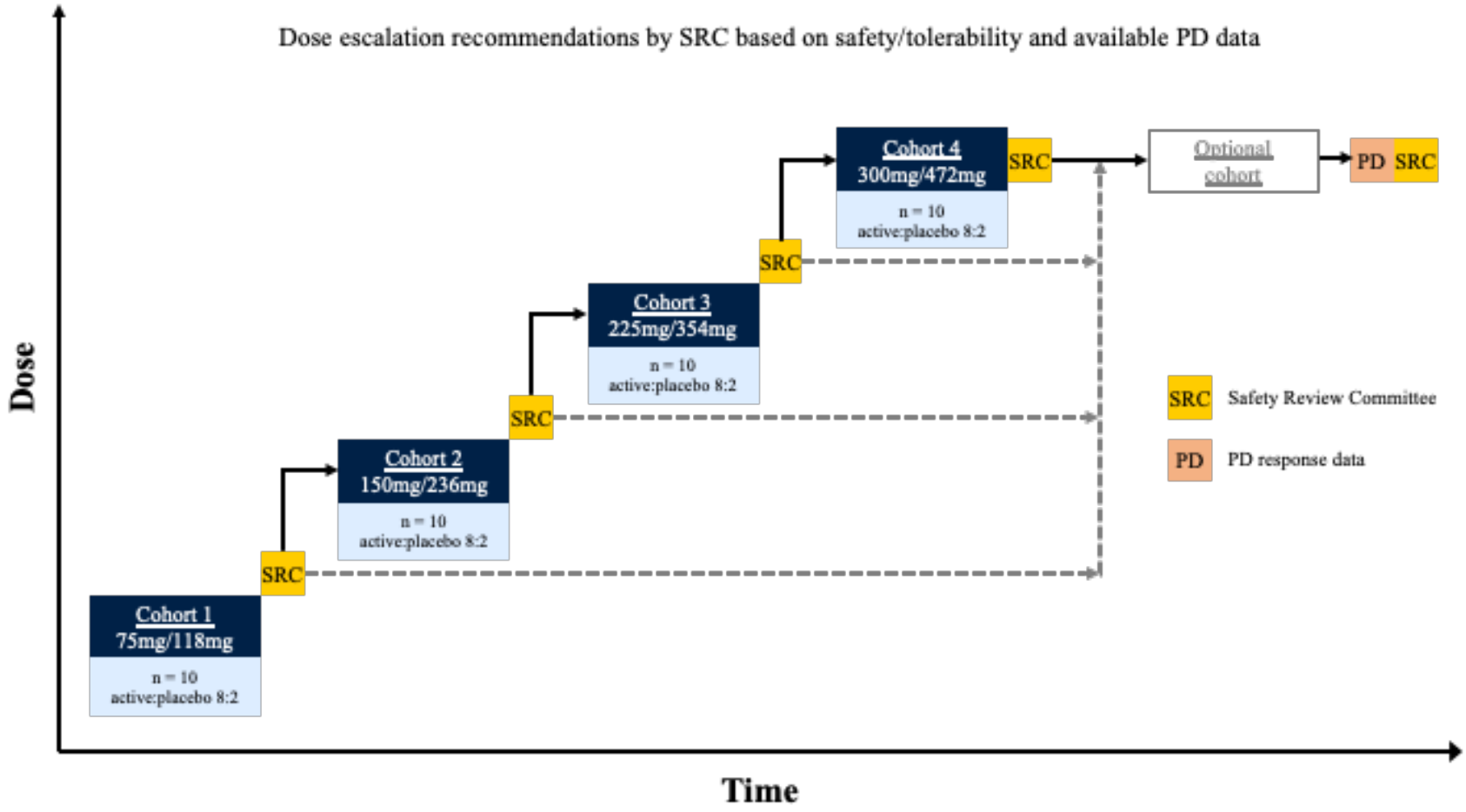



**Figure 23: TALEN dose escalation study design.**

Consecutive cohorts of 10 (8 active, 2 placebo) of increasing daily doses allowing comparison of 4 dose cohorts to placebo. Abbreviations: SRC – Safety Review Committee, PD – Pharmacodynamic. For drug dosing, dose of 5-ALA is listed first, then SFC.

### 2.1.4 Adaptive trial design features

Adaptive design elements were defined in the protocol to maximise scientific value, efficiency and flexibility of the trial while minimising any unnecessary exposure to participants and/or collection of unnecessary data[280]. Adaptations were envisaged to be made considering evolving trial data maintaining a focus on meaningful assessments, capturing essential and useful data while maintaining the validity and integrity of the trial[91]. Adaptations were designed to support selection of the optimal dose of 5-ALA-SFC to use in future studies, while avoiding unnecessary drug exposure, ensuring a pragmatic trial with applicability to a representative group of patients undergoing cardiac surgery on CPB[81].

### 2.1.5 Duration of subject participation

Patient participation in the trial started at the point of obtaining full written informed consent. Eligible participants were required to take a minimum of 3 days of 5-ALA-SFC or placebo immediately preceding their surgery and most study assessments continued post-operatively whilst the patient was in hospital, concluding with a final study visit which coincided with the final surgical follow-up appointment. This typically occurred between 6-10 weeks after surgery.

### 2.1.6 Study population

Adult patients (≥18 years) scheduled for non-emergent cardiac surgery requiring CPB (including CABG, valve replacement/repair or a combination thereof) who were willing and able to comply with study requirements and provided full written informed consent were eligible to be enrolled. Exclusion criteria were:

- Female participants who are of childbearing potential who are either unable or unwilling to use highly effective contraception, or who are pregnant or breast feeding.
- History of hypersensitivity to 5-ALA, SFC and/or porphyrins
- Acute or chronic types of porphyria
- Known genetic haemochromatosis or clinically significant iron overload
- History of clinically significant photosensitization

- Current long-term (> 3 months) use of amiodarone
- Concomitant use of hypericin extract (including St John's Wort) or concomitant therapeutic dose oral iron replacement
- Use of other investigational medical product(s) < 28 days prior to study or 5 half-lives, whichever is longer
- Cardiogenic shock/Low cardiac output syndrome requiring catecholamine infusion and/or mechanical circulatory support prior to induction of anaesthesia for cardiac surgery
- Recent acute myocardial infarction (within 2 weeks of surgery) – defined as elevated cardiac troponin > Upper Limit of Normal (ULN) together with symptoms of myocardial ischaemia and/or new ischaemic ECG changes
- Cardiac surgery without cardiopulmonary bypass or induced fibrillating heart surgery
- Inadequate renal or liver function defined as any of estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73m$^2$, ALT and/or AST > 2.5 x ULN, total bilirubin > 2 x ULN (unless documented Gilbert's syndrome), known cirrhosis or known active hepatitis infection
- Any other condition which, in the opinion of the Investigator, makes the patient unsuitable for or may compromise their participation in the study, e.g., due to safety concerns or compliance with clinical study procedures

This allowed for a representative study population for contemporary adult cardiac surgery to be recruited and, although more heterogenous than is usual for cardioprotection trial, followed a more pragmatic trial design allowing for future translation[81].

### 2.1.7 Trial assessments and procedures

Potential study participants were identified by clinical teams and offered a patient information sheet (PIS) during routine clinical care. The PIS included a key facts summary sheet to support potential participant decision making (see Appendix 6.2.1). Participants were requested to consider the information and were encouraged to feel free to raise questions to their surgical consultant/clinical team,

trial research team, their GP and/or any other independent parties to help them weigh their options and decide whether they wished to participate. If patients chose to participate, a member of the trial team obtained written informed consent, captured by means of an approved informed consent document (see Appendix 6.2.2). Participants were enrolled consecutively if they had provided consent and fulfilled trial eligibility criteria.

#### 2.1.7.1 Randomisation and prescription of the investigational medicinal product (IMP)

Once surgery had been scheduled, participants were randomised to a specific treatment allocation (i.e., 5-ALA-SFC or placebo). Randomisation followed a sequential randomisation list prepared by an independent unblinded trial statistician. The randomisation code corresponded with a treatment kit (active or placebo) which was prescribed and dispensed locally by pharmacy. Where patients required replacement treatment kits (in cases of surgical delays/cancellations) the unblinded trial statistician supplied a replacement kit number. Once randomised, participants received a patient diary to record details of study drug taken. In isolated instances, participants were re-allocated to a different dose cohort, with a replacement participant for the original cohort recruited and randomised. This typically occurred if a surgical date had been significantly postponed for logistical reasons by the patient's surgical care team. Contingency for this was pre-specified as one of the adaptive design features of the trial to minimise the impact of clinical delays on overall recruitment. For all cohorts, an active IMP (5-ALA-SFC) to placebo ratio of 8:2 per cohort was maintained.

#### 2.1.7.2 Trial data collection

TALEN followed a pragmatic trial design with most data capture and collection of biological samples occurring in tandem with usual clinical care as summarised in **Fig. 24**. Recruitment and follow-up visits were timed to coincide with planned surgical clinical visits as far as possible. Peri-operative data capture and sample collection took place in operating theatres, the intensive care unit and surgical ward. Source documents for TALEN included electronic hospital clinical, laboratory and pharmacy records, and patient diaries. Data was captured using an electronic case report form (eCRF) in the form of a fully integrated electronic database designed in collaboration with software managers at a contract research

organisation (George Clinical). Database servers were in the United States and all application traffic was encrypted in transit across all public networks using secure sockets layer (SSL) (2048 bit key). Certain data sets (e.g., molecular pharmacodynamic readouts) were recorded and analysed separately to the eCRF, ensuring no patient identifiable data was shared. The database platform has been widely used and is General Data Protection Regulation (GDPR) and 21 Code of Federal Regulation (CFR) Part 11 compliant. Access was password controlled and all data handling was GDPR compliant. Where source documents in paper form were used (e.g., patient diaries), these were stored in a locked office.

#### 2.1.7.3 Sample collection and handling

Collection, handling, and analysis of samples which formed part of the standard of care pathway (e.g., full blood count, renal profile and electrolytes and liver enzymes) was in accordance with local National Health Service (NHS) standard laboratory practice. Trial specific research samples were collected, prepared, stored and/or shipped in accordance with instructions provided in the sample handling manual which is summarised in **Table 3**. An up-to-date log of patient samples (including exact location of samples) was always available in the Electronic Master File (eTMF). Blood and tissue samples were stored in a linked anonymised fashion – identifiable only through a trial participant identification number, linked with patient details through the subject identification code list held in the trial master file, compliant with the 1998 Data Protection Act. Where samples were shared with external collaborating laboratories, only the subject identifier was supplied. Storage of human tissue for research purposes met the requirements as set out in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act.

Plasma separation from whole blood: whole blood centrifuged at 1500 x G (swinging bucket, 4°C) for 10 minutes. Clear plasma above buffy coat was pipetted out carefully and aliquoted into 1.8 mL cryovials than immediately stored at -80°C.

Serum separation from whole blood: whole blood was collected into BD Vacutainer™ SST™ II tubes and centrifuged at 1500 x G (swinging bucket, 4°C) for 10 minutes. Clear serum above the separation gel was pipetted out carefully and aliquoted into 1.8 mL cryovials then immediately stored at -80°C.

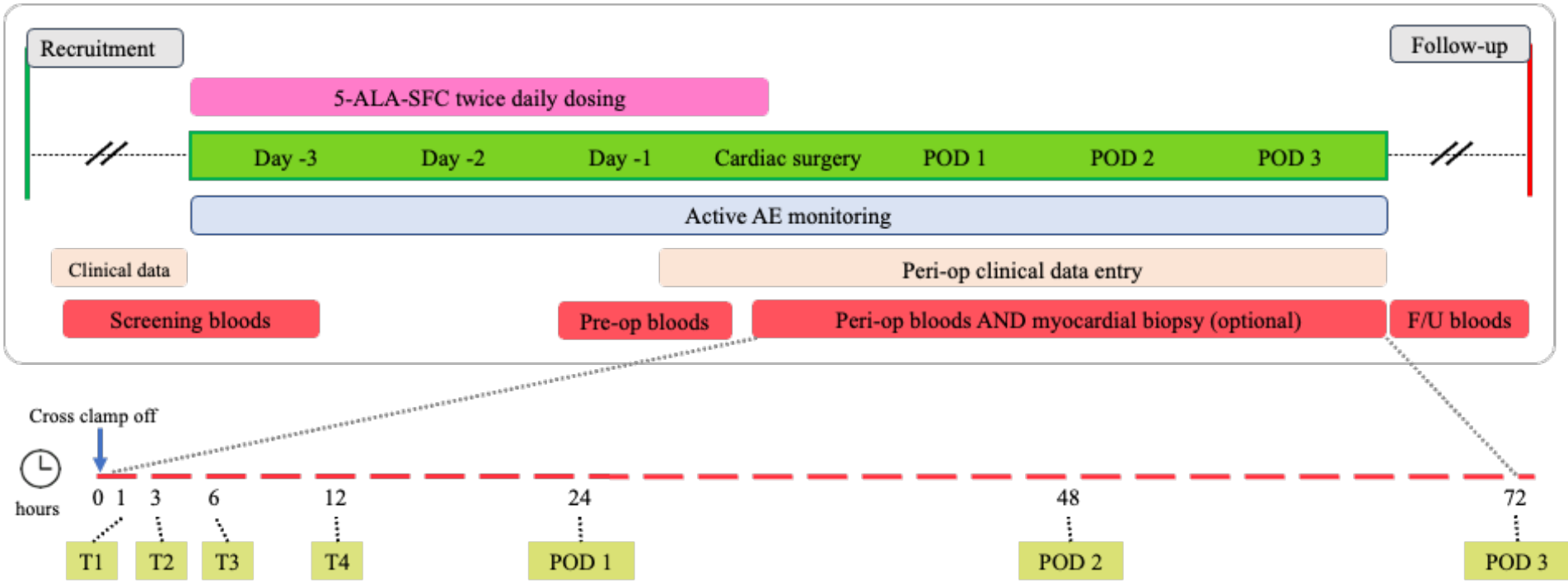



**Figure 24: Summary of participant journey, trial activities and sampling strategy.**

Abbreviations: AE – adverse events; F/U – follow-up; POD - post-operative day; Peri-op – peri-operative, T1 - one hour after removal of the aortic cross clamp; T2 - three hours after/admission to ICU; T3 - six hours after; T4 - 12 hours after cross clamp removal.

**Table 3: Sample handling for trial specific samples analysed locally or by external collaborators.**

| Time-point | Sample | Handling |
|---|---|---|
| Screening – peripheral venepuncture | 1. 5 mL EDTA anticoagulated whole blood for DNA extraction and plasma separation | Stored at 4-8°C, proceed to DNA extraction and plasma separation within 2 hours of collection |
| | 2. 5 mL whole blood in Tempus™ Blood RNA Tube for RNA extraction | Stored at 4-8°C, transferred to -20°C within 2 hours of collection |
| | 3. 16 mL whole blood in BD Vacutainer® CPT™ tubes for peripheral blood mononuclear cell (PBMC) isolation and cryopreservation | Stored at room temperature, proceed to cellular isolation and cryopreservation within 2 hours of collection |
| Pre-operative – blood typically taken from central venous catheter placed by anaesthetist after induction of anaesthesia | 1. 5 mL EDTA anticoagulated whole blood for plasma separation | As above |
| | 2. 5 mL whole blood into BD Vacutainer™ SST™ II tubes for serum separation | Stored at 4-8°C, serum separation and cryopreservation within 2 hours of collection |
| | 3. 5 mL whole blood in Tempus™ Blood RNA Tube for RNA extraction | As above |
| | 4. 16 mL whole blood in BD Vacutainer® CPT™ tubes for peripheral blood mononuclear cell (PBMC) isolation and cryopreservation | As above |
| | 5. 5-10 mL urine from urinary catheter bag, in sterile specimen jar | Collected in sterile specimen jar, stored at -20°C within 2 hours of collection. |
| Intra-operative: after cardioplegic arrest and institution of CPB | 1. Cardiac tissue collection (with specific consent from patients in agreement with consultant surgeon). Biopsies taken by surgeons using a standardised approach. | Samples from one, or a combination, of: right atrial appendage, left atrial appendage, left ventricle (needle core biopsy) divided as possible. One sample placed in cryovial and snap frozen in liquid nitrogen and stored at -80°C. The second sample immediately fixed in 10% formalin and transferred to 70% ethanol in deionised water, stored at 4-8°C. |
| | 1. 5 mL EDTA anticoagulated whole blood for plasma separation (only T1) | As above |

| | | |
|---|---|---|
| Peri-operative blood sampling: T1, T2, T3, T4* | 2. 5 mL whole blood into BD Vacutainer™ SST™ II tubes for serum separation | As above. Samples stored overnight at 4-8°C, processed within 24 hours as above and stored at -80°C. |
| Post-operative: POD 1, POD 2, POD 3 | 1. 5 mL EDTA anticoagulated whole blood for plasma separation | As above |
| | 2. 5 mL whole blood into BD Vacutainer™ SST™ II tubes for serum separation | As above, processed and stored at -80°C within 2 hours of collection. |
| | 3. 5 mL whole blood in Tempus™ Blood RNA Tube for RNA extraction | As above |
| | 4. 16 mL whole blood in BD Vacutainer® CPT™ tubes for peripheral blood mononuclear cell (PBMC) isolation and cryopreservation | As above |
| | 5. 5-10 mL urine from urinary catheter bag, in sterile specimen jar | As above |

* T1 – one hour after removal of the aortic cross clamp, T2 – three hours after removal of the aortic cross clamp/admission to ICU, T3 – six hours after removal of the aortic cross clamp, T4 – 12 hours after removal of the aortic cross clamp, POD 1-3 – post-operative day 1, 2 and 3.

Isolation and cryopreservation of peripheral blood mononuclear cells: PBMCs were isolated (observing strict asepsis in a tissue cabinet thoroughly cleaned with ethanol) according to product guidance for BD Vacutainer™ CPT™ Tube with Sodium Citrate and density solution, with minimal alterations.

In brief:

- Cells were resuspended by gently inverting the tubes 8 to 10 times. Cellular isolation during initial centrifugation step (swinging bucket) was at 1500xg, room temperature for 30 minutes (the longer initial centrifugation step has been shown to be associated with reduced red blood cell contamination). Typical separation was visible as in **Fig. 25** below.

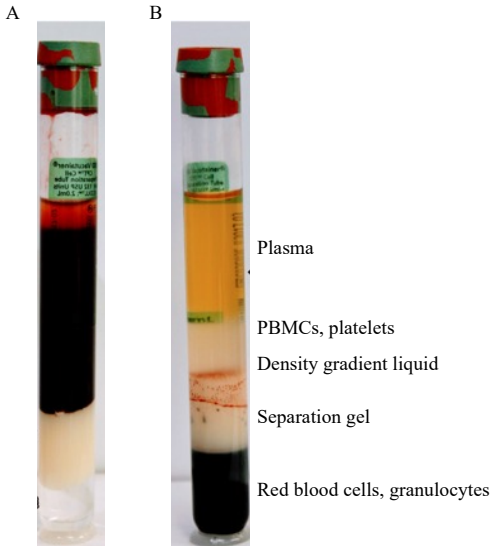



**Figure 25: PBMC isolation.**

BD Vacutainer™ CPT™ Tubes before (A) and after (B) initial centrifugation, showing typical separation.

- PBMCs were re-suspended in plasma by gently inverting the tube 5 to 10 times. The entire contents of each tube above the gel were transferred with a sterile pipette into a 50 mL Falcon tube, taking care that the separation gel was not disturbed in any way. Each tube was rinsed gently with 5 mL PBS to dislodge any adherent cells. PBS was added to the Falcon tube to ensure final volume of 35 mL.

- The second centrifugation step followed immediately: room temperature, 300 x g for 10 minutes. Visual inspection confirmed the presence of a cellular pellet and supernatant was discarded. The pellet was loosened and resuspended in 3 mL ACK lysing buffer (red cell lysis) for three minutes. Lysis was quenched with 35 mL RPMI + 5% FCS (fetal calf serum). Centrifugation repeated as above, supernatant discarded. Cells were resuspended in 10 mL RPMI + 5% FCS and centrifugation repeated. Cells were counted with a Cellometer automated cell counter and viability was assessed using the Trypan blue method.
- Resuspension volume was calculated to allow for a final cellular concentration of 2.5 x $10^6$/mL. The cellular pellet was again loosened and resuspended in 100% FBS (using half the calculated resuspension volume). An equal volume of freeze media (20% DMSO in FBS) was added, in drop-wise fashion while swirling the sample gently, to the cellular suspension. 2 mL aliquots of the final solution were added to cryovials rapidly (minimising time that cells were exposed to DMSO at room temperature).
- Cryovials were immediately transferred to a Mr Frosty freezing container (pre-cooled to 4-8°C, 100% isopropyl alcohol to the fill line) and then to the -80°C freezer. Long term storage was at -80°C after at least 24 hours in the freezing container to allow for controlled cryopreservation.

#### 2.1.7.4 Urgent unblinding and participant safety

There was guidance in the protocol for investigators on urgent unblinding procedures in the event that knowledge of treatment allocation was judged necessary for a participant's clinical care by their treating clinician. No instances of urgent unblinding occurred during the study.

#### 2.1.7.5 Participant withdrawal and end of trial

Participants who did not start dosing but proceeded to surgery were withdrawn from the trial. In some instances, participants were withdrawn after randomisation (and rarely, after taking a single/multiple doses of IMP). This occurred due to patients, for e.g., experiencing side effects or whose management was changed to a non-surgical intervention by their treating clinical team. Data relating to this were

captured and reported. In all cases, data and samples collected up to the point of withdrawal were used in line with the initial informed consent obtained.

### 2.1.8 Investigational medicinal product (IMP)

#### 2.1.8.1 IMP description, labelling, packaging and handling

The Active Pharmaceutical Ingredients (APIs) were manufactured according to the principles of Good Manufacturing Practice (GMP) in Japan on behalf of SBI Pharmaceuticals, and supplied to Quotient Sciences, Alnwick, Northumberland. Quotient Sciences manufactured and packaged the final IMP and was responsible for Qualified Person (QP) release and shipment to the central trial pharmacy at the John Radcliffe Hospital in Oxford.

The IMP was supplied in two bottles of capsules per treatment kit as follows:

5-ALA HCl capsule: Supplied for oral administration as a dark green, opaque, size 0, hypromellose (HPMC) capsule containing drug alone at a dosage strength of 75mg (58.7mg as 5-ALA). There were no excipients. These were packaged in bottle 1 or 2 of the treatment kit.

- SFC capsule: Supplied for oral administration as a dark green, opaque, size 0, HPMC capsule containing drug alone at a dosage strength 118mg (12.5mg as Fe). There were no excipients. These were packaged in bottle 1 or 2 of the treatment kit.
- Placebo capsule: Supplied for oral administration as a dark green, opaque, size 0, HPMC capsule containing 125mg lactose. The capsule shells were of non-animal origin, and were comprised of HPMC, titanium dioxide, and a copper complex of chlorophyllins. These were packaged in bottle 1 and 2 of the treatment kit.

All IMPs were labelled according to European Union GMP Annexure 13 requirements. IMPs were packaged in coded treatment kits – the code corresponding with the randomisation code, supplied directly to Quotient Sciences by the independent unblinded trial statistician.

#### 2.1.8.2 Dose and exposure duration justification

TALEN was a dose finding trial where sequential cohorts of subjects received ascending doses of 5-ALA-SFC contingent on favourable safety and tolerability data reviewed by the safety review committee (SRC). The starting dose of 5-ALA-SFC was 75mg/118mg, increased twofold between cohorts to a maximum dose of 300mg/472mg dosed orally twice daily for three days pre-operatively with an additional dose on the day of surgery. All the dose levels had previously been used safely[275], including the maximum dose to be used, 5-ALA-SFC at 300 mg/472 mg twice daily (cohort 4). Higher doses (e.g., 5-ALA-SFC at 600 mg/940 mg twice daily for 6.5 days) had been tested in previous trials but were associated with elevations in liver function tests in some individuals and judged clinically significant in 1 individual with ALT > 3 x ULN.

5-ALA itself is licenced at a dose of 20 mg/kg (i.e. 1200 mg, 1500 mg or 2000 mg for a 65 kg, 75 kg or 100 kg individual, respectively) 3 hours before anaesthesia for major neurosurgery in the context of malignant glioma[273]. This equates, as a single dose, to between 20-fold or 5-fold higher levels of 5-ALA than used in TALEN (cohort 1, 75 mg 5-ALA, and cohort 4, 300 mg 5-ALA, respectively).

Co-administration of sodium ferrous citrate served as a concurrent source of iron designed to maximise the HO-1 PD response to 5-ALA. The iron content of SFC was 12.5 mg per 118 mg capsule. This compares to licenced oral formulations of iron: 68 mg for ferrous fumarate (210 mg capsules) dosed 1-2 times daily, 65 mg for ferrous sulphate (200 mg capsules) dosed 2-3 times daily and 35 mg for ferrous gluconate (300 mg capsules) dosed at 1.2-1.8 g daily for the treatment of iron deficiency anaemia in adults. This equates to iron administration of 136-210 mg daily, typically for several months. In TALEN, cohort 1 (118 mg SFC bd) received 25 mg of iron daily, while cohort 4 received 100 mg iron daily up to a maximum of 3.5 days.

Key clinical trials for dose- nd duration-justifcaiton are summarised below.

**Summary of key clinical trials using 5-ALA-SFC:**

| Trial | n | Population & objective | Dose and duration |
|---|---|---|---|
| Brooks, A. (2013) [75] | 120 | Phase 1, double blind, placebo-controlled, single and multiple oral doses of 5-ALA-SFC safety, tolerability and pharmacokinetic study in healthy volunteers | Group 1 (n = 80): 10 groups, 6 active:2 placebo, receiving single doses of 5-ALA-SFC 50–1500mg /78.4–2351 mg, respectively<br>Group 2 (n = 40): 5 groups, 6 active:2 placebo, receiving twice daily doses of 5-ALA-SFC from 150 – 600mg/235 – 940mg for 6 days followed by a single dose on day 7 |
| Saitama, I. (2016)[76] | 17 | Phase II, single arm, open label study of 5-ALA-SFC in patients with platinum chemotherapy- induced anaemia investigating the influence on haemoglobin levels | 5-ALA-SFC 150mg/235mg twice daily for 8 weeks |
| Ito, H., et al. (2018)[68] | 40 | Open label, non-placebo-controlled study evaluating PBMC HO-1 expression response to 5-ALA-SFC in healthy volunteers | Group 1 (n = 5): 5-ALA-SFC 600mg/940mg given once<br>Group 2 (n = 40): 5-ALA-SFC 150-600mg/235-942mg once<br>Group 3 (n = 5): 5-ALA-SFC 600mg/942mg given once daily for 3 days |

#### 2.1.8.3 Supply, administration and returns of IMP

IMP was prescribed at the point of randomisation and dispensed by the trial pharmacy. The pharmacy was also responsible for inventory and drug accountability tasks. Patients were instructed to take capsules from each bottle in the morning and evening with the dosing cohort and stage of the trial defining overall dose (i.e., total capsule number) for three days before their surgery. On the day of surgery patients were asked to take the morning dose only. No known adverse drug interactions with the IMP are known and no limits were placed on the use of concomitant medication. As the IMP is known to be a photosensitising agent, caution was advised when other photosensitising drugs (e.g., tetracyclines, sulphonamides, fluoroquinolones) were to be used. As a precaution, patients with current long-term use of amiodarone (> 6 months) were excluded from enrolment.

Compliance with study drug treatment was assessed using a patient diary. Patients were asked to return all remaining IMP when they were admitted for surgery, along with their patient diary. The number of capsules returned vs. number of capsules expected to have been used was assessed as a further measure of compliance. Where discrepancies existed, these were explored directly with participants and documented in the patient diary. Returned IMP was taken to the central trial pharmacy and documented on the accountability log.

#### 2.1.8.4 Storage of IMP

Drugs were stored in their coded packaging in the Oxford University Hospitals NHS Foundation Trust pharmacy under refrigerated conditions (4-8°C). IMP was supplied to patients in a temperature-controlled package with instructions to refrigerate the kit at home.

#### 2.1.8.5 IMP destruction

Returned or unused IMP was destroyed by the local trial pharmacy in accordance with the Medicines for Human Use (Clinical Trials) Regulations 2004 (2006), as hazardous waste. Records of the destruction of any returned IMPs were filed in the Pharmacy Site File, and / or written confirmation provided to the Sponsor at the end of the trial to confirm destruction of surplus IMP, in accordance with local policies and procedures.

### 2.1.9 Pharmacovigilance and safety reporting

#### 2.1.9.1 Adverse event reporting

The definitions of adverse events that were used for TALEN are summarised in the **Table 4** below.

**Table 4: Trial definitions of adverse events.**

| | |
|---|---|
| Adverse Event (AE) | Any untoward medical occurrence in a participant to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product. |

| | |
|---|---|
| Adverse Reaction (AR) | An untoward and unintended response in a participant to an investigational medicinal product which is related to any dose administered to that participant.<br><br>The phrase "response to an investigational medicinal product" means that a causal relationship between a trial medication and an AE is at least a reasonable possibility, i.e., the relationship cannot be ruled out.<br><br>All cases judged by either the reporting medically qualified professional or the Sponsor as having a reasonable suspected causal relationship to the trial medication qualify as adverse reactions. |
| Serious Adverse Event (SAE) | A serious adverse event is any untoward medical occurrence that:<br>• results in death<br>• is life-threatening<br>• requires inpatient hospitalisation or prolongation of existing hospitalisation<br>• results in persistent or significant disability/incapacity<br>• consists of a congenital anomaly or birth defect.<br><br>Other 'important medical events' may also be considered serious if they jeopardise the participant or require an intervention to prevent one of the above consequences.<br><br>NOTE: The term "life-threatening" in the definition of "serious" refers to an event in which the participant was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.<br><br>An official hospital admission will be defined as inpatient hospital stay equal to or greater than 24 hours.<br><br>Hospitalisation or prolongation of a hospitalisation is a criterion for considering an AE to be serious. In the absence of an AE, the participating investigator should not report hospitalisation or prolongation of hospitalisation (e.g. for social/other discharge reasons). A hospitalisation planned before the start of the study for a pre-existing condition that has not worsened does not constitute an SAE (e.g., elective hospitalization for a total knee replacement due to a pre-existing condition of osteoarthritis of the knee that has not worsened during the study).<br><br>Disability is defined as a substantial disruption in a person's ability to conduct normal life functions.<br><br>If there is any doubt whether the information constitutes an AE or SAE, please report the event. |
| Serious Adverse Reaction (SAR) | An adverse event that is both serious and, in the opinion of the reporting Investigator, believed with reasonable probability to be due to one of the trial treatments, based on the information provided.<br><br>A "reasonable probability" of a relationship conveys that there are facts, evidence, and/or arguments to suggest a causal relationship, rather than a relationship cannot be ruled out. |
| Suspected Unexpected Serious Adverse Reaction (SUSAR) | A serious adverse reaction, the nature and severity of which is not consistent with the information about the medicinal product in question set out:<br>• in the case of a product with a marketing authorisation, in the summary of product characteristics (SmPC) for that product. |

- in the case of any other investigational medicinal product, in the investigator's brochure (IB) relating to the trial in question.

A SUSAR is therefore an AE which is serious (defined under SAE), causally related to study treatment and unexpected (i.e., not consistent with the applicable reference safety information).

Active reporting of AEs was in effect from the point of consent through to the final study visit. Outside this period of surveillance, there was a requirement to report any SARs or SUSARs which may have been identified through participant self-reporting and/or review of participant's medical notes. Where AEs were encountered these were evaluated immediately and relevant data were captured in the trial database.

In brief:

- Details of all AEs were captured in the trial database and these data were presented to the Safety Review Committee (SRC) regularly.
- SAEs, unless exempt in the protocol on the grounds of expected sequelae of cardiac surgery, and SUSARs were to be reported to the independent CRO medical monitor for the trial within 24 hours of the event occurring.
- All SUSARs were expected to be evaluated and reported to the Research Ethics Committee (REC) and Medicines and Healthcare products Regulatory Agency (MHRA), within statutory timelines.
- SAEs exempt from expedited reporting would not be reported to the medical monitor for the trial unless initiated at the discretion of the principal investigator (PI). All SAEs were reported to the PI within 24 hours of the event occurring.
- If a participant withdrew consent for further processing of data, this did not preclude the reporting of SARs and SUSARs.
- AEs considered related to IMP were followed up until resolution, or the event was considered stable.

#### 2.1.9.2 Anticipated SAEs in the context of cardiac surgery – justification for risk-adapted safety surveillance and reporting exemptions

TALEN adopted a tailored approach to AE monitoring and reporting. Patients undergoing cardiac surgery encounter frequent adverse events related to co-morbidities and the surgical intervention itself. Accordingly, with regulatory approval the protocol specified adverse events which occur in the context of cardiac surgery on CPB and whose occurrence would not routinely trigger an expedited safety report. In instances where the PI/Investigator felt the event was of a more serious magnitude than expected (i.e. potentially related to IMP) these will be reported as serious adverse events/reactions following the usual processes. All reported adverse event data (whether expected or unexpected in the context of cardiac surgery) were captured in the eCRF.

The following SAEs were exempt from routine expedited reporting:

- Foreseeable AEs associated with cardiac surgery – the list was specified in the trial protocol and is summarised in Table 8.
- Hospitalisation for a pre-existing condition or deterioration thereof (including ischaemic heart disease).
- Treatment on an emergency, outpatient basis for an event not fulfilling any of the definitions of serious and not resulting in hospital admission.
- SAEs listed in the trial protocol as outcome measures, e.g., hypotension, acute kidney injury, cardiogenic shock, renal failure, peri-operative MI.

**Table 5: Foreseeable AEs associated with cardiac surgery which would not routinely trigger expedited safety reporting.**

| **Cardiovascular events** |
|---|
| • Atrial, ventricular or nodal arrhythmia |
| • Hypertensive states |
| • Air and other macro/micro-embolism |
| • Aortic dissection or haematoma |
| • Intra-cardiac fistula or pseudoaneurysm |
| • Cardiac failure with or without pulmonary oedema |
| • Peri-operative MI or graft occlusion or coronary artery occlusion or spasm |
| • Prosthetic or native valvular/paravalvular regurgitation or other valve dysfunction or damage |
| • Low cardiac output states and vasodilatory shock |

- Systolic anterior motion of mitral valve &/or left ventricular outflow tract obstruction
- Pulmonary hypertension
- Haemodynamic support (including inotropes, vasopressors, vasodilators, pulmonary artery catheter, IABP or LVAD)
- Cardiac arrest requiring resuscitation, DC cardioversion/defibrillation, chest re-opening, or external and/or internal cardiac massage
- Pericardial effusion/ tamponade or pericarditis

**Neurological events**

- Stroke or TIA or intracranial/subarachnoid haemorrhage
- Seizure, stupor or coma
- Delirium and acute confusional states; cognitive dysfunction, including memory deficits
- Peripheral neuropathy

**Pulmonary events**

- Endotracheal tube-related complications
- Reintubation/prolonged requirement for mechanical ventilation or difficulty weaning from respiratory support
- Initiation of non-invasive ventilatory support after extubation (e.g., continuous positive airway pressure, high flow nasal oxygen)
- Acute lung injury or ARDS
- Pneumothorax, pleural effusion, haemothorax, diaphragmatic dysfunction or atelectasis

**Gastro-intestinal events**

- Diarrhoea or constipation
- Nausea and/or vomiting
- GI ulceration with or without GI bleed or perforation
- Bowel ischaemia/obstruction with or without laparotomy
- Hyperglycaemia requiring insulin therapy; Pancreatitis

**Renal events**

- Acute renal failure
- New requirement for haemofiltration or dialysis

**Haematological and bleeding events**

- Excess bleeding including requiring re-operation or surgical exploration or that related to anticoagulant/antiplatelet use; haematoma
- Platelet or clotting factor deficiency and/or replacement
- Anaemia; blood/other blood-derived product transfusion
- Transfusion reaction
- Haemolysis
- Arterial or venous thromboembolic events
- Valve thrombosis or intra-cardiac thrombus formation
- Heparin-induced thrombocytopenia

**Other surgical complications**

- Adverse reaction to anaesthesia/analgesia including pain
- Infective: line-related infections, mediastinitis, respiratory infection, systemic sepsis or bacteraemia, sternal/other wound infection or cellulitis, ulcer, lymphocele, endocarditis, osteomyelitis, sternal dehiscence, colitis, infective diarrhoea, UTI

#### 2.1.9.3 Causality assessment

The relationship of each AE to the trial medication was determined by a suitable medically qualified individual at the study site and, where appropriate, the trial medical monitor. Where the investigators

and medical monitor differed in their causality assessments, it was expected that both reports would be shared with the sponsor for review. This was not required for this trial. AEs were judged to be related (the adverse event follows a reasonable temporal sequence from trial medication administration and/or a relationship between the trial medication and AE cannot reasonably be ruled out) or unrelated (the adverse event is probably produced by the participant's clinical state or by other modes of therapy administered to the participant).

#### 2.1.9.4 Development safety update reports (DSUR)

These were developed in collaboration with SBI Pharmaceuticals for every year that the trial was active and was submitted to the MHRA, REC and local NHS trust within 60 days of the data lock point (last day of the month prior to the month of the Development International Birth Date of ALA-SFC – 1st November 2012, by convention).

### 2.1.10 Trial oversight – the Safety Review Committee (SRC)

The SRC was responsible for the overall safety oversight of the trial through periodic review of safety data. The full roles and responsibilities of the SRC, timing and purpose of meetings were described in the SRC charter, which was finalised before the trial began recruiting.

In brief:

- The SRC consisted of an independent chair (a senior clinician with appropriate cardiovascular and/or critical care clinical expertise), the PI, a suitably qualified co-investigator and the trial statistician.
- The SRC conducted ongoing review of all blinded safety, tolerability, and available PD data to make decisions on dose escalation. Provision was made to allow the SRC to view un-blinded data if any safety concerns were identified, however this was not required and the SRC remained blinded to treatment allocation throughout.
- The specific aims of the SRC included: identifying any specific safety concerns, requesting further information from investigators as required, evaluating the risk of continuing the trial, deciding

whether it was appropriate to recommend dose escalation and finally recommend a dose of 5-ALA-SFC for future trial evaluation.

### 2.1.11 Quality assurance procedures

TALEN was conducted in accordance with an approved, up-to-date version of the trial protocol and governed by the principles of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Guideline for Good Clinical Practice (GCP), the Research Governance Framework and all trial specific Standard Operating Procedures (SOPs). The trial was sponsored by the University of Oxford.

#### 2.1.11.1 Monitoring

A trial specific monitoring plan was developed which outlined the procedures for monitoring in detail. Regular study-related monitoring was performed by a senior clinical research associate (CRA) from the George Clinical. Data were evaluated for compliance with the protocol and accuracy in relation to source documents. TALEN accepted 100% source document verification for all data entered. At the end of each monitoring visit (in person or remotely via a video call), the CRA prepared monitoring reports outlining follow-up actions, these were shared with the CI and filed in the eTMF.

## 2.2 Confirmation of target engagement and dose response in key human cell types

Unless otherwise specified, all laboratory work was undertaken at the Wellcome Centre for Human Genetics in Oxford. All required safety training was completed before experimental work began. Processing and storage of human biological samples was carried out in line with local safety guidance and provisions in the 2004 Human Tissue Act and the 2006 Human Tissue (Scotland) Act were observed.

### 2.2.1 *In silico* identification of key human circulating cells and tissues enriched for *HMOX1*

Using publicly available transcriptomic datasets, tissues and cells enriching for *HMOX1* were identified using *HMOX2* as a control. Tissues across the entire body, across haematological cell development lines

and across circulating blood cells were considered. Transcriptomic data were downloaded from online repositories. For RNA sequencing (RNAseq) datasets, transcripts had already been mapped to a recent genome assembly. However, microarray identifiers had been mapped to older genome assemblies. Therefore, probesets were mapped to their genomic locations using BioMart in the latest release of Ensembl[281].

For expression analyses in tissues across the body, human and murine datasets were interrogated, and these are summarised in **Table 6** below.

**Table 6: Transcriptomic datasets used to evaluate tissues enriching for HMOX1**.

| **Organism** | **Human** | **Mouse** |
|---|---|---|
| Dataset | Genotype-Tissue Expression (GTEx)[282] | GeneAtlas[283] |
| Sampling | 751 donors within 24 hours of death | Pools of 4-6 healthy male C57/BL6 mice (unless female organs) |
| Samples | 11,688 | 2 biological replicates |
| Source | 53 tissues/cells | 91 tissues/cells |
| Expression profiling | RNAseq quantified with Illumina TrueSeq | Affymetrix GeneChip Mouse Genome 430 2.0 Array (2008) which had >45,000 probesets from >39,000 transcripts from >34,0000 genes, designed from the UniGene database (Build 107, June 2002) and refined with the draft mouse genome (April 2002) |
| Download | GTEx download portal<br>Analysis v7 (15/1/16) was mapped to Genome Reference Consortium Human Build 37, Transcripts per million (TPM) by tissue normalisation | GCRMA normalised data from NCBI GEO GSE10246 |

For expression analyses across haematological cells, atlases of systematic transcriptomic profiling from multiple states of haematological cell development in in human and mouse were interrogated. Details of these datasets are shown in **Table 7** below. Conservation of the transcriptional signature of gene expression across species was compared with the 'Comparative' tool on the ImmGen website, which uses the Pearson correlation coefficient to calculate Conservation of Expression (CoE) across the cells,

ranging from +1 (indicating highly conserved) to -1 (indicating highly divergent)[284]. This metric was developed by mapping orthologous genes in human and mouse and comparing the expression in cell types.

**Table 7: Transcriptomic datasets used to evaluate haematological cells enriching for HMOX1**.

| **Organism** | **Human** | **Mouse** |
|---|---|---|
| Dataset | Differentiation Map (DMap)[285] | Immunological Genome Project Phase 1 (ImmGen)[286] |
| Sampling | 4-7 donors/sample | 5-week-old C57/BL6 male mice (some pools 10-30) |
| Samples | 38 states of haematopoiesis from umbilical cord blood and adult peripheral blood or flow cytometry selection | 178 states of haematopoiesis by blunt dissection or flow cytometry selection |
| Expression profiling | Affymetrix GeneChip HT-HG U133A Early Access Array<br>22,944 probesets<br>dbEST 28/4/01, WUSTL Feb 01, GenBank 25/4/01, RefSeq 30/4/01, UniGene 20/4/01<br>HT arrays released in 2011 | Affymetrix GeneChip Mouse Gene 1.0 ST Array<br>28,853 genes (25 probes/gene)<br>Genome assembly Feb 06, RefSeq 3/4/07, GenBank 13/11/06<br>ST arrays released in 2007 |
| Download | NCBI GEO GSE26014<br>Raw microarray data downloaded and normalised with RMAExpress v 1.1.0 | Immgen website RMA normalised data |

For the analysis of expression of *HMOX1* in circulating blood cells, three independent human RNAseq datasets were reviewed. Details of these datasets are shown in **Table 8** below. The Human Protein Atlas has combined all three datasets to in a consensus dataset of normalised expression, which was downloaded from the Human Protein Atlas website[287].

**Table 8: Transcriptomic datasets used for the evaluation of circulating human blood cells.**

| Dataset | Database of Immune Cell eQTLs, expression and epigenomics (DICE)[288] | Absolute Immune Signature (ABIS)[289] | Human Blood Atlas[290] |
|---|---|---|---|
| Sampling | 91 healthy donors (San Diego Blood Bank) | 13 healthy Singaporean donors | 6 healthy Swedish donors (3 male, 3 female) |

| | | | |
|---|---|---|---|
| | | (9 male, 4 female) | |
| Samples | 13 cell types | 27 cell types and PBMCs | 17 cell types and PBMCs |
| Expression profiling | RNAseq | RNAseq | RNAseq |

Expression data were downloaded as transcripts per million (TPM) and converted to a log10 scale using the formula log10(1+TPM) and plotted in a box and whisker plot showing the median and interquartile range. For the microarray data, normalised gcRMA or RMA data were expressed on a log2 scale and plotted as mean ± standard error. In the one case where the data repository used another method of normalisation, the raw microarray data were downloaded and the normalised with RMAExpress v 1.1.0[291]. All of these analyses were performed using Microsoft Excel. Transcript expression from circulating human blood cells was graphed in heatmaps drawn in Java TreeView 3.0 [292].

### 2.2.2 *In vitro* evaluation of target engagement – 5-ALA-SFC mediated activation of HO-1

#### 2.2.2.1 *In vitro* evaluation of HO-1 activation with 5-ALA with SFC in human monocytes

Human THP-1 cells are widely used in in vitro models examining monocyte biology[293]. They are an immortalised monocytic leukemic cell line and were sourced from Public Health England (Cat no. 8808120103, Lot no. 20A042 ECSH03.T) and used to test the hypothesis that 5-ALA with SFC would activate HO-1 in vitro.

The cells were revived as outlined below:

- Cells were rapidly thawed in a water bath (37°C) until 1-2 ice crystals were visible and carefully pipetted into a 15 mL Falcon tube, adding 4 mL revival growth media (20% Foetal Bovine Serum (FBS, Cat no. F7524-500ML) in RPMI 1640 with L. glutamine (Cat no. 21875034).
- Resuspended cells were centrifuged at 100 x g for 5 minutes at room temperature, discarding the supernatant. Cells were resuspended in 1 mL revival media, counted and assessed for viability using the Trypan Blue protocol – a 1:1 solution of the cell suspension and 0.2 % Trypan blue was counted using an automated cell counter (Nexcelom Cellometer Auto 1000). Following revival 10.32 X $10^6$ viable cells/mL with 91.8% overall viability was found.

- After an additional centrifugation step as above, the visible pellet was resuspended in 20 mL revival growth media (final cell concentration = 5.16 X $10^5$ cells/mL), transferred into a 100 mL Sarstedt culture flask (kept upright) and placed in a sterile incubator – set at 37°C with 5 % $CO_2$.
- The culture flask was reviewed daily for colour change and maintenance of culture conditions to establish that revival had been successful and to identify when the cell culture reached the exponential growth phase.
- Once the cells had entered the exponential growth phase, serial passages were performed by decanting the contents of the culture flasks into a 50 mL Falcon tube, centrifuging at 300 x g at room temperature and resuspending the visible pellet in 5 mL growth media (10% Foetal Bovine Serum (FBS, Cat no. F7524-500ML) in RPMI 1640 with L. glutamine (Cat no. 21875034). At each passage cells were counted and viability was assessed as above and resuspended in an appropriate volume aiming for a final concentration of approximately 5 X $10^5$ cells/mL in each flask.
- Cells were kept in culture and prepared for in vitro experiments with 5-ALA with SFC.

THP-1 cells in culture were plated into non-adherent tissue culture plates aiming for a concentration of approximately 1 x $10^6$ cells/mL. 5-ALA and SFC were supplied as dry powder by Arcinova (ARC Trinova limited). A 1 M dilution of 5-ALA (1.68 G in 10 mL de-ionised water) was prepared and diluted in RPMI 1640 with L. glutamine (Cat no. 21875034) to a final concentration of 100 mM. A 100 mM concentration of SFC (0.527 mg in 10 mL de-ionised water) was prepared.

Treatment conditions were:

- Positive control: Hemin (Sigma Aldrich, Cat no. 51280) at final concentrations 10 μM, 25 μM and 50 μM
- Positive control: LPS (Sigma Aldrich, Cat no. L5293) at final concentrations of 1 μM and $H_2O_2$ 400 μM
- 5-ALA final concentrations 30 μM, 300 μM and 1000 μM, AND SFC final concentrations 15 μM, 150 μM and 500 μM
- Negative control – vehicle only

At the end of the experiment the contents of each well were transferred into a 2 mL Eppendorf tube, centrifuged at 500 x g for 5 minutes at room temperature, discarding the supernatant. Cell pellets were snap frozen in liquid nitrogen and stored at – 80°C for subsequent protein extraction.

For lysis and protein extraction cell pellets were thawed on wet ice and resuspended in 500 µL chilled PBS, centrifuged at 450 x g for 5 minutes at 4°C, discarding the supernatant. Cells were lysed by adding a lysis buffer (10 mL Cell lytic reagent (Sigma, C2978), 1 tablet protease inhibitor (Roche, 693159001) and 1 tablet PhosphoSTOP (Roche, 4906837001)) and incubating at room temperature for 15 minutes on a shaker. Lysis buffer volumes were calculated to ensure at least 125 µL for each 1 x $10^6$ cells, typically 500 – 1000 µL. Lysed cells were centrifuged at 12,000 x g for 10 minutes to pellet the cellular debris. Protein containing supernatant was then transferred to a 2 mL Eppendorf tube on wet ice. Protein was quantified using the BCA protein assay kit (Thermofisher Scientific, 232528 and 1859078) according to the manufacturer's guidance without modification.

Samples made up with 4 x LaemmLi buffer (Nu-Page 4X, Novex Life Technologies, NP0008), dithiothreitol (DTT) 10 % (Thermofisher Scientific, R0861) and sterile water aiming for a protein concentration of 5 µg per sample, as per **Table 9** below. These were incubated at 90°C for five minutes and allowed to cool to room temperature.

**Table 9: Sample preparation for Western Blot quantification of HO-1 and NLRP3.**

| Sample | Protein* (µg/µL) | Sample (µL) | DTT (µL) | $H_2O$ (µL) | Loading Buffer (µL) |
|---|---|---|---|---|---|
| 1 | 1.186 | 21.1 | 7.5 | 27.7 | 18.75 |
| 2 | 1.019 | 24.5 | 7.5 | 24.2 | 18.75 |
| 3 | 1.468 | 17.0 | 7.5 | 31.7 | 18.75 |
| 4 | 1.989 | 12.6 | 7.5 | 36.2 | 18.75 |
| 5 | 1.765 | 14.2 | 7.5 | 34.6 | 18.75 |
| 6 | 1.188 | 21.0 | 7.5 | 27.7 | 18.75 |

| Sample | Protein* (µg/µL) | Sample (µL) | DTT (µL) | $H_2O$ (µL) | Loading Buffer (µL) |
|---|---|---|---|---|---|
| 7 | 1.273 | 19.6 | 7.5 | 29.1 | 18.75 |
| 8 | 1.09 | 22.9 | 7.5 | 25.8 | 18.75 |
| 9 | 0.765 | 32.7 | 7.5 | 16.1 | 18.75 |
| 10 | 0.731 | 34.2 | 7.5 | 14.6 | 18.75 |
| 11 | 0.609 | 41.1 | 7.5 | 7.7 | 18.75 |
| 12 | 1.162 | 21.5 | 7.5 | 27.2 | 18.75 |

*Protein concentration by BCA assay

Pre-prepared 4 – 12 % bis-tris gels (Invitrogen, NP0335BOX) were prepared by rinsing with distilled water and loaded into the tank as per manufacturer's guidance. The tank was filled carefully with 1X SDS running buffer (Invitrogen, B000102-5L). After loading the molecular ladder (100 kDa) and 30 µL sample to wells, electrophoresis (150 V for 60 minutes) was performed.

The nitrocellulose membrane was prepared by cutting this to a similar size of the gel and placing it in the transfer buffer (15g of glycine + 3.03g of Tris Base - add 800mL of distilled water and then 200 mL of methanol). The blotting chamber was prepared by layering sponge, 2 x filter paper, gel, nitrocellulose membrane and 2 x filter paper in order and loading this into the transfer tank. Transfer was run in a cold room (4°C) at 300 mA for 90 minutes. The membrane was carefully removed after transfer and washed 5 x with cold 0.05 % Tween® (Sigma Aldrich, P9416) in PBS and then blocked in 5 % milk in PBS for one hour at room temperature.

The primary antibodies – HO-1 Rabbit mAb (Cell Signalling, #5853) and NLRP3 Rabbit mAb (Cell Signalling #15101) – were added to 5% milk in PBS to a final concentration of 1:1000. The membrane was added to this and left overnight on the rocker at 4°C, protected from light. This was washed three times for five minutes with cold 0.05 % Tween® (Sigma Aldrich, P9416) in PBS. The secondary antibodies (IRDye® 800CW Goat anti-Mouse IgG (Li-Cor, 926-32210) and IRDye® 680RD Goat anti-Rabbit IgG (Li-Cor, 926-68071)) were added to 5 % milk with PBS to a final concentration of 1:25,000 and added to the membrane, incubating at room temperature for one hour (protected from light). The

membrane was removed and washed as above. Image processing was with the Li-Cor Odyssey® CLx Infrared Imaging System and Acquisition software. Analysis was performed in Image Studio™, Li-Cor Biosciences and presented as optical density relative to ß-tubulin.

#### 2.2.2.2 Assessment of HO-1 activation with 5-ALA-SFC in primary human monocyte-derived macrophages (MDM)

This experiment formed part of a collaboration with Prof. Julian Knight's group (Nuffield Department of Medicine, University of Oxford). Cells were obtained from National Health Service Blood and Transplant (NHSBT) under an internal agreement with the collaborating group under Ethical approval.

The methodology used for monocyte separation and macrophage differentiation was:

- PBMCs were isolated from a leukocyte cone provided by NHSBT, using the Ficol density gradient method. All cells were extracted from the cone with the cone being flushed gently with HBSS (sterile filtered) until the contents ran clear, into a T75 Sarstedt tissue culture flask. 15 mL of these cells in suspension were layered onto 12 mL of Ficoll® in Falcon tubes and centrifuged at 400 x g for 30 minutes at room temperature. Separation typically allowed for delineation of the PBMC monolayer (**Fig. 26**). Using a sterile Pasteur pipette, the PBMC monolayer and plasma were aspirated carefully and transferred into 50 mL Falcon tubes on ice. From here cells were handled on wet ice/at 4°C. Cells were centrifuged at 300 x g for 7 minutes at 4°C, repeating the wash step with 20 mL HBSS thereafter.

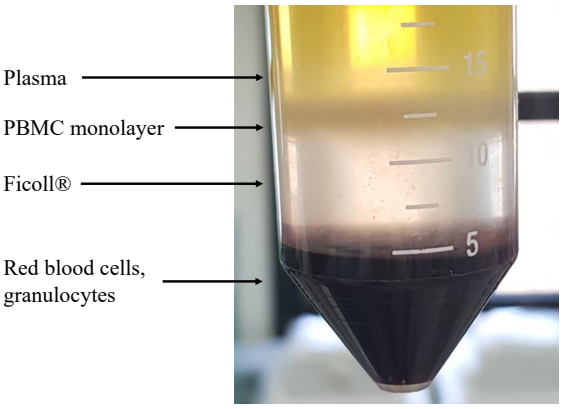



**Figure 26: PBMC isolation by the Ficoll® denisty gradient method.**

- Cells were resuspended in 20 mL MACS® buffer (Miltenyi Biotec, Cat. No. 130-091-221) and centrifuged as above while performing a cell count manually. Resuspension volume was calculated to allow 80 µL MACS® buffer and 15 µL CD14 microbeads (Miltenyi Biotec, Cat. No. 130-050-201) for each $10^7$ cells. Cells were resuspended and incubated at 4°C for 15 minutes. Volume was topped up to 50 mL with MACS® buffer and centrifuged for seven minutes as above. Cellular pellets were resuspended in 2 mL MACS® buffer. The LS columns (Miltenyi Biotec, Cat. No. 130-042-401) were prepared by washing them with 2 mL MACS® buffer with the cell suspension being loaded after. Separation was with the MidiMACS™ separator. Columns were washed three times using 2 mL MACS® buffer, collecting the flow through. 5 mL buffer added with elution of the CD14+ cells after adding 5 mL MACS® buffer using the column plunger. 10 mL PBS added to the cell suspension and centrifuged for seven minutes as above, repeating once.
- Cells were resuspended in MDM media (RPMI 1640 (ThermoFisher Scientific) supplemented with 1% L-glutamine, 1% penicillin-streptomycin, 10% FBS, 1% non-essential amino acids, 1% sodium pyruvate, 0.1% M-CSF). The resuspension volume was calculated to allow for a final cell concentration of 2 x $10^6$ cells/mL of media. 2 x $10^6$ cells were plated per well in an adherent, flat bottomed, 6-well sterile tissue culture plate and cultured in a sterile incubator – set at 37°C with 5 % $CO_2$.
- On day 3 the cells were checked to be adherent to the bottom of each well under the microscope. 1 mL of fresh MDM media was added to each well and re-incubated. This was a modification of the standard procedure (which requires careful aspiration of surface layer of MDM media in culture to be replaced with fresh media).
- On day 6 cells were checked to be adherent to the bottom of each well under the microscope. All media was carefully aspirated, ensuring not to disturb the adherent cells. 2 mL fresh MDM media was added, and cells were incubated for 24 hours as above. Cells were then ready for treatment with 5-ALA and SFC. Cells were not polarised prior to exposure for this experiment.

Treatment conditions were:

- Positive control: LPS stimulation only, LPS followed by haemin co-stimulation after 3 hours.
- 5-ALA:SFC at final concentrations of 30 μM:15 μM: , 300 μM:150 μM and 1000 μM:500 μM
- Negative control – vehicle only

At the end of exposure, all media was removed and 2 mL PBS on wet ice was added to wash. This was then aspirated carefully and 1 mL PBS + 5 mM EDTA was added to each well. Using a scraper, cells were detached from the bottom of each well. The contents from each well were carefully aspirated and transferred to an Eppendorf tube, on wet ice. 10 μL of each sample was taken for counting while the samples were centrifuged at 400 x g for 5 minutes at 4°C. For RNA extraction a minimum of 50,000 cells were required for each condition. RNA extraction proceeded immediately using the RNeasy® Mini kit (Qiagen, 74104) in accordance with the manufacturer's protocol without modifications. RNA quantity and quality was assessed using spectrophotometry. Relative quantification of *HMOX1* was determined by RT-qPCR using Taqman™ chemistry using the ΔΔCt method. B-actin (*ACTB*) was selected as the housekeeping gene.

#### 2.2.2.3 Assessment of HO-1 activation with 5-ALA-SFC in human induced pluripotent stem cell derived cardiomyocytes (iPSC-CMs)

For this experiment differentiated cells were kindly provided by Dr Violetta Steeples (Department of Cardiovascular Medicine, University of Oxford).

The methods for this experiment in brief:

- KOLF2-C1 induced pluripotent stem cells (iPSC) were maintained in Essential 8 Medium (A1517001, ThermoFisher Scientific) on tissue culture-treated plates coated with a 1:100 dilution of Matrigel Growth Factor Reduced Basement Membrane Matrix, Phenol Red-free, LDEV-free (356231, Corning) in DMEM/F12 (11330032, ThermoFisher Scientific).
- iPSC were differentiated into cardiomyocytes (CM) by modulation of the Wnt signalling pathway. On day 0, mesoderm induction was performed by treating iPSC at about 90% confluence with 8 μM

CHIR-99021 (S2924, Selleck) diluted in differentiation media (RPMI 1640 (11875093, ThermoFisher Scientific) supplemented with B27 minus insulin supplement (A1895601, ThermoFisher Scientific)). On day 1 CHIR-99021 was diluted to 4 μM by adding 1 volume of differentiation media. On day 2 CHIR-99021 was replaced with fresh differentiation media. On day 3 cardiomyocyte specification was initiated by replacing media with 5 μM IWR1 (S7086, Selleck) diluted in differentiation media. On day 5 media was changed to fresh differentiation media.

- From day 7 onwards media was changed every other day to fresh cardiomyocyte maintenance media (RPMI 1640 with B27 supplement (17504044, ThermoFisher Scientific)). Cells were visually inspected and wells with more than 80% cells beating were frozen in Cryostor CS10 (07930, StemCell). Cells were defrosted into replating media (RPMI 1640 with B27 supplement, 10 % knockout serum replacement (ThermoFisher Scientific, 10828028), 2 μM thiazovivin (S1459, Selleck)) and 250,000 cells per well plated. Media was changed after 24 hours to cardiomyocyte maintenance media. Media was changed every other day to fresh cardiomyocyte maintenance media.
- On day 29 cells were treated with either 1000 μM 5-ALA and 500 μM SFC, 300 μM 5-ALA and 150 μM SFC, 30 μM 5-ALA and 15 μM SFC or vehicle diluted in maintenance media at incubated for 24 hours.

RNA was extracted by lysis by scraping in RLT buffer and RNA extracted using RNeasy mini kit (Qiagen, 74104) in accordance with the manufacturer's protocol without modifications. Relative quantification of *HMOX1* determined by RT-qPCR using TaqMan™ chemistry using the ΔΔCt method. B-actin (*ACTB*) was selected as the housekeeping gene.

## 2.3 Genotyping the HMOX1 promoter (GT)n repeat copy number variant (CNV) polymorphism

DNA was extracted from whole blood using the DNeasy® Blood and Tissue kit from Qiagen, following the protocol for nucleated whole blood without modifications.

In brief:

- EDTA tube inverted 5-10 times gently to resuspend cells and then 10 µL was transferred to an Eppendorf tube containing 20 µL proteinase K and 190 µL PBS. 200 µL Buffer AL (ethanol added) was added and mixed thoroughly by vortexing and incubated at 56°C for ten minutes.
- 200 µL pure ethanol was added and mixed thoroughly by vortexing. The mixture pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube. Centrifuged at > 6000 x g for 1 min, discarding the flow-through and collection tube.
- Multiple sequential wash steps followed by adding wash buffers (Buffer AW1 and AW2), centrifuging as above and discarding flow- through and collection tube each time.
- DNA was eluted by adding 50 µL Buffer AE onto membrane and incubating the sample at room temperature for five minutes. Centrifugation for 1 min at ≥ 6000 x g followed, collecting the flow-through which contained DNA. Sample quality and DNA quantity measured immediately with spectrophotometry (NanoDrop® ND-1000 UV-Vis Spectrophotometer) and stored at 4-8°C.

For the design and optimisation of the Touchdown polymerase chain reaction (PCR) genotyping experiment, transcript HMOX1-201 (ID: ENST00000216117.9) was selected from Ensembl[294]. Key transcript considerations are summarised in **Fig. 27**. Commonly used primers were selected from the literature[236–238,241,248,249,295–301] and tested during optimisation. These are summarised in **Table 10**.

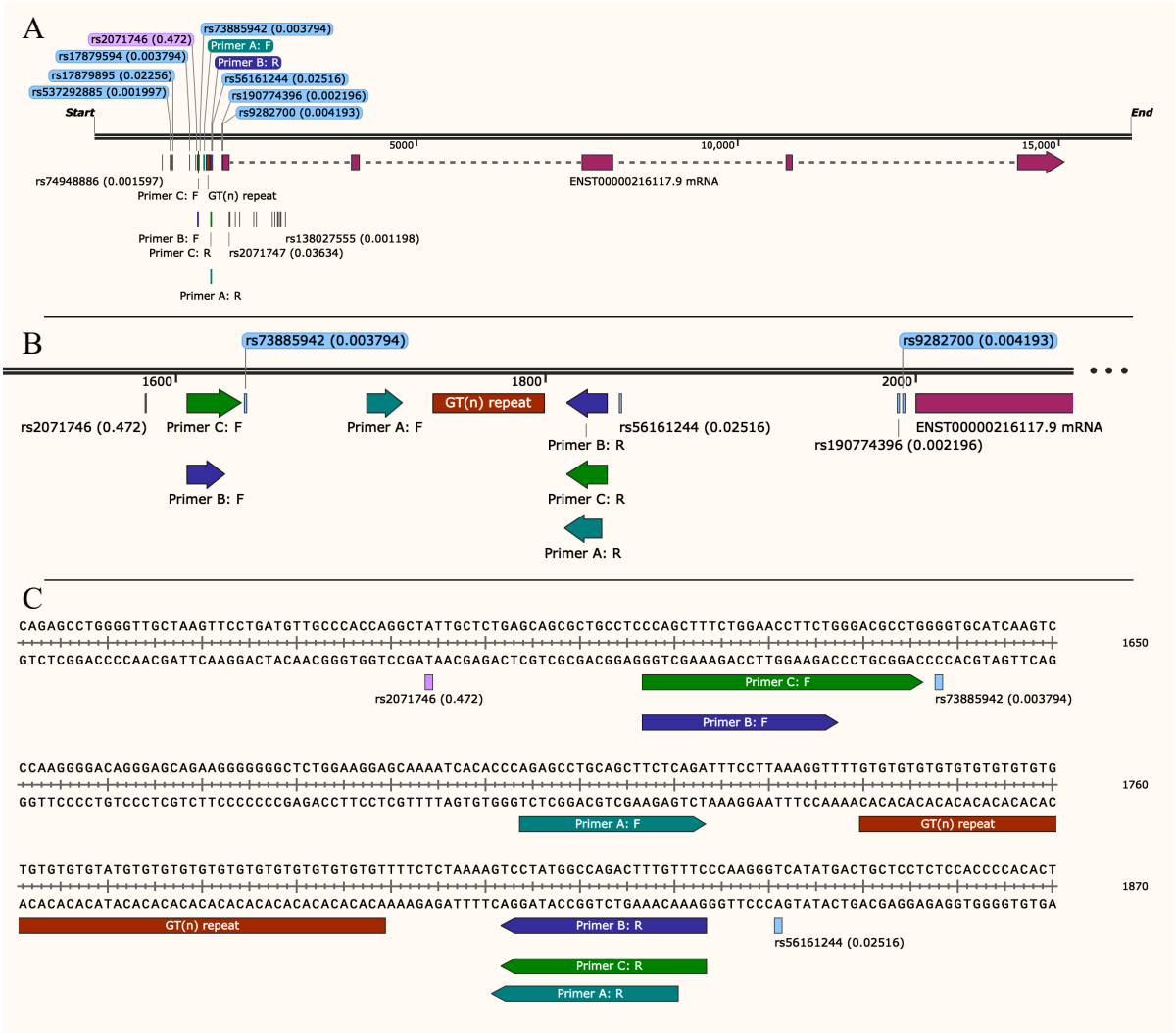



**Figure 27: Annotation of HMOX 1.**

Transcript ENST00000216117.9 with frequently used primers and single nucleotide polymorphisms (SNPs) with a population Minor Allele Frequency (MAF) > 0.1 % in the same region. A – entire gene, B- promoter region and C – area of (GT)n repeat.

**Table 10: PCR primer sets from literature review.**

| | **Notes** |
|---|---|
| Primer set A<br>`Forward: AGAGCCTGCAGCTTCTCAGA`<br>`Reverse: ACAAAGTCTGGCCATAGGAC` | • F and R primers: 100% ID<br>• E-val = 0.11<br>• SNP flanks 3' end of R-primer |
| Primer set B<br>`Forward: CCAGCTTTCTGGAACCTTCTG`<br>`Reverse: GAAACAAAGTCTGGCCATAGGA` | • F-primer: 100 % ID, E-val 0.028. 10 other regions with 100% ID but much higher E-val<br>• R-primer: 21-mer, 100 % ID, E-val 0.007 |
| Primer Set C<br>`Forward: CCAGCTTTCTGGAACCTTCTGGGACGCCTG`<br>`Reverse: GAAACAAAGTCTGGCCATAGGA` | • F-primer: 100% ID, E-val 6 x 10-7. 8 other near 100 % hits but with higher E-val (>2).<br>• R-primer: 100% ID, E-val 0.007. |

Primer set B performed best using the touchdown PCR protocol used by Leaf et al[249]. Using these conditions, the primer set used by these authors was also tested and found that this set did not perform well. A master mix was prepared using the Qiagen Taq™ PCR core kit using the following quantities of components per reaction: 10 X PCR buffer (2.5 µL), dNTP mix (0.5 µL), Taq™-polymerase (0.25 ™), nuclease free water (up to 18.75 µL), primer pair (10 µM, 1 µL per primer) and template DNA (volume to approximate 50 ng DNA).

The PCR was performed using the following conditions:

| | | | |
|---|---|---|---|
| Step 1 | 95°C | 15 min | Denaturation |
| Step 2 | 95°C | 30 sec | Denaturation |
| Step 3 | 65°C (-0.5°C/cycle) | 30 sec | Annealing |
| Step 4 | 72°C | 60 sec | Extension |
| Step 5 | Go to step 2 | 24 x | |
| Step 6 | 95°C | 30 sec | Denaturation |
| Step 7 | 54°C | 30 sec | Annealing |
| Step 8 | 72°C | 60 sec | Extension |
| Step 9 | Go to step 6 | 24 x | |
| Step 10 | 72°C | 10 min | Holding |
| Step 11 | 25°C | 30 sec | |
| Step 12 | 4°C | infinity | |

PCR products were checked by electrophoresis using a 1.8% Agarose gel. 20 µL PCR product was mixed thoroughly with 5 µL loading buffer (New England Biolabs, B7024S) and loaded into each well. A 100-base pair (bp) molecular ladder (New England Biolabs, N3231S) was used as the size standard. PCR products were purified using the ExoSAP-IT™ (ThermoFisher Scientific, 78201) reagent according to the manufacturer's guidance. 2 µL purified PCR products were prepared for analysis by adding 10 µL Hi-Di Formamide loading buffer (Applied Biosystems, 4440753) and 0.5 µL internal size standard. In this instance GeneScan™ 500 ROX™ dye (Applied Biosystems, 401734) was used. Samples were submitted to Eurofins Genomics for size calling through Fragment Length Analysis. Expected fragment length 227 bp with the template transcript containing 31 (GT)n repeats. Allelic determination was defined as either homozygous short (SS) (< 27 bp), homozygous long (LL) (≥ 27 bp) or heterozygous (SL).

## 2.4 RNA extraction and Real time Quantitative Polymerase Chain Reaction for relative gene expression

RNA extraction was performed in a sterile vertical laminar flow tissue culture cabinet. All surfaces and equipment were cleaned with 70 % ethanol and RNase AWAY™ Surface Decontaminant (ThermoFisher Scientific, 7000TS1) prior to starting. Unless otherwise stated, all RNA extraction was with the RNEasy® Mini Kit (Qiagen, 74104) according to the manufacturer's instructions.

Modifications to the manufacturer's protocol were:

- For elution 50 μL nuclease free water was placed onto the column and left at room temperature for 5 minutes before centrifugation.

RNA quality and integrity were immediately assessed using spectrophotometry (NanoDrop® ND-1000 UV-Vis Spectrophotometer) and stored at -20°C. Acceptable samples were samples with a 260/280 ratio of > 1.8 and a 260/230 ratio of > 2. All samples with ratios outside these ranges have been reported. cDNA synthesis from RNA was with the Quantitect® Reverse Transcriptase kit (Qiagen, 20531) according to the manufacturer's protocol without modifications. cDNA was stored at -20°C. RT-qPCR using TaqMan™ chemistry followed according to manufacturer's guidance without modification. (β)-actin (*ACTB*) was selected as the housekeeping gene given its stable expression in PBMCs and its apparently stable expression in immune cells before and after immune activation[302]. Genes of interest are summarised in **Table 11** below which also reflects TaqMan™ probes selected (ThermoFisher Scientific). The housekeeping gene was VIC labelled and all other genes were FAM labelled to allow for multiplexing. Unless otherwise stated all probes spanned exons and would not detect gDNA. cDNA, Fast Advanced Master Mix (Applied Biosystems, 4444965), probes and nuclease free $H_2O$ was plated according to the manufacturer's guidance using a 384-well plate setup. PCR was performed after plating according to the Fast set-up using the QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems, 4485701) and data were quality checked and analysed using the QuantStudio™ Design and

Analysis software package. Gene expression relative to the housekeeping gene was derived using the ΔΔCt method.

**Table 11: TaqMan™ probes for RT-qPCR.**

| Gene | Associated protein | Catalogue number |
|---|---|---|
| **Housekeeping gene** | | |
| *ACTB* | (β)-actin | Hs99999903_m1 |
| **Haem oxygenase enzyme isoforms** | | |
| *HMOX1* | HO-1 | Hs01110250_m1 |
| *HMOX2* | HO-2 | Hs01558390_m1 |
| **Nrf2 pathway activation - mechanism of action of drug** | | |
| *NFE2L2** | Nuclear factor erythroid 2-related factor 2 | Hs00975961_g1 |
| *TXN** | Thioredoxin | Hs01555214_g1 |
| *ME1* | Malic enzyme 1 | Hs00159110_m1 |
| *NQO1* | NAD(P)H dehydrogenase (quinone) | Hs00168547_m1 |
| *GSR* | Glutathione-disulfide reductase | Hs00167317_m1 |
| **Haemolysis, erythrophagocytosis and iron handling** | | |
| *CD163* | Scavenger receptor cysteine-rich type 1 protein M130 | Hs00174705_m1 |
| *LRP1* | Low-density lipoprotein receptor-related protein 1 | Hs00233856_m1 |
| *FTL** | Ferritin light chain | Hs00902546_g1 |
| *FTH** | Ferritin heavy chain | Hs01000477_g1 |
| *TFRC* | Transferrin receptor protein 1 | Hs00951083_m1 |
| *SLC40A1* | Ferroportin | Hs00205888_m1 |
| *SLC11A2* | Divalent metal transporter 1 | Hs00167206_m1 |
| *SLC39A14* | Metal cation symporter ZIP14 | Hs00299262_m1 |
| *HAMP* | Hepcidin | Hs00221783_m1 |
| **Inflammation and cytoprotection** | | |
| *NFKB1* | Nuclear factor kappa-light-chain-enhancer of activated B cells | Hs00765730_m1 |
| *CASP1* | Caspase-1 | Hs00354836_m1 |
| *NLRP3* | NACHT, LRR and PYD domains-containing protein 3 | Hs00918082_m1 |
| *ENTPD1* | Ectonucleoside triphosphate diphosphohydrolase-1 OR CD39 | Hs00969558_m1 |
| *IL-1B* | Pro-interleukin-1 beta | Hs01555410_m1 |
| *IL-6* | Interleukin-6 | Hs00174131_m1 |
| *IL-10* | Interleukin-10 | Hs00961622_m1 |

* Detects gDNA

## 2.5 Circulating HO-1 levels by enzyme-linked immunosorbent assay (ELISA)

Determination of plasma protein concentration was performed using the HO-1 (human) ELISA kit (Enzo, ADI-EKS-800) without modifications.

Cryopreserved plasma from TALEN trial participants was thawed on ice and mixed by pipetting prior to sample analysis. During optimisation of this experiment, undiluted plasma performed best. During analysis of trial samples, 50 µL standard (reducing concentration, prepared according to manufacturer's guidance) and 50 µL undiluted plasma were plated in duplicate. The ELISA proceeded as per manufacturer's guidance. Absorbance measured using the CLARIOstar® Plus (BMG Labtech) at 450 nm wavelength. Data were analysed using the MARS Data Analysis software package (BMG Biotech). Concentrations of HO-1 in plasma samples were interpolated from the four-parameter logistic regression standard curve for HO-1 (**Fig. 28**).

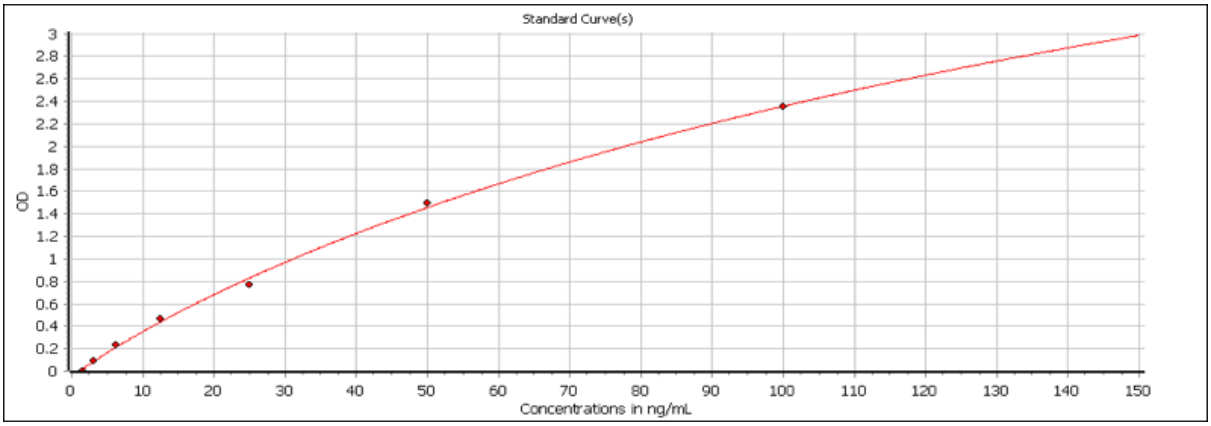



**Figure 28: Representative standard curve for HO-1 ELISA using a four-parameter logistic regression analysis.**

## 2.6 HO-1 enzyme activity in PBMCs through biliverdin quantitation by Ultra-high Performance Liquid Chromatography-tandem Mass Spectrometry (UPLC-MS/MS)

The proposed assay relied on the canonical biochemical function of HO-1: the catalyst driving the breakdown of haem into equimolar quantities of $Fe^{2+}$, CO and biliverdin (**Fig. 29**) [155]. The rate of production of these molecules can serve as clinically useful biomarkers of HO enzymatic function. However, CO production can occur through both HO-dependant and HO-independent mechanisms, decreasing the specificity of CO as a marker of enzyme function[167]. Biliverdin, a more specific marker of enzyme activity, is rapidly converted to bilirubin by biliverdin reductase. Bilirubin is highly unstable in vitro, limiting its usefulness as a biomarker[303]. Biliverdin, on the other hand, is highly stable in vitro and an attractive alternative. By creating conditions favouring the conversion of bilirubin to biliverdin in vitro, a stable biomarker for enzyme activity exists (**Fig. 30**). As the inducible isoform of the enzyme, it is assumed that any increase over baseline function represents HO-1, rather than HO-2, activity.

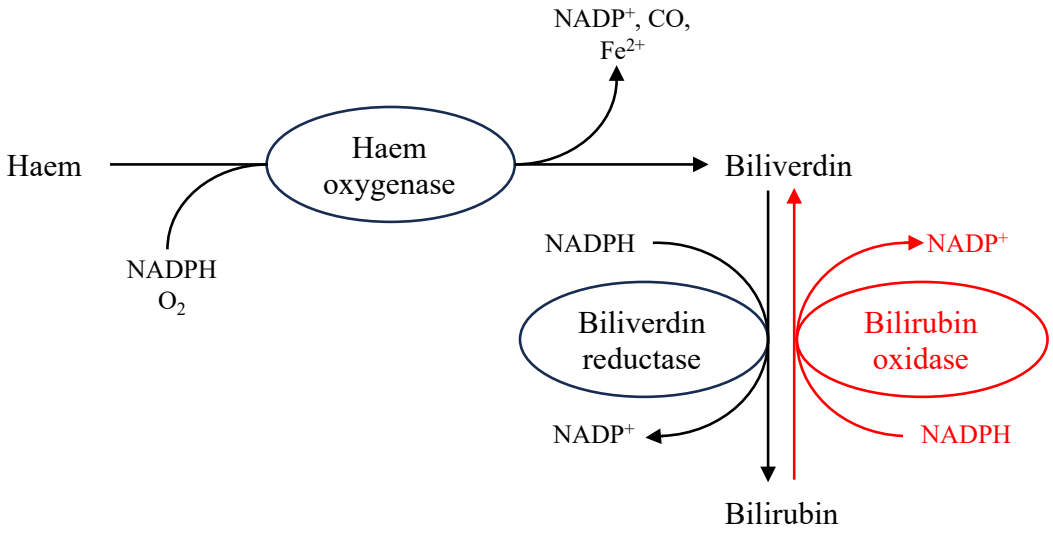



**Figure 29: Schematic of the biliverdin/bilirubin cycle. By supplementing bilirubin oxidase and NADPH in vitro, oxidation of bilirubin to biliverdin is favoured.**[w]

Iwamori et al.[303] described an assay to determine HO activity using the rate of biliverdin production in murine renal biopsies. I proposed developing and validating a similar substrate-driven enzymatic

[w] Figure adapted from Iwamori et al.[303]

function assay in human PBMCs which was developed as part of a scientific collaboration with senior method development scientists (Dr Michael Blackburn and Dr Janine Morsman) at Quotient Sciences. All method development experiments, and subsequent trial sample analyses, were carried out in their laboratory in Alnwick, Northumberland. All method development experiments, and subsequent trial sample analyses, were carried out in their laboratory in Alnwick, Northumberland. The laboratory is fully compliant with the principles of Good Laboratory Practice (GLP) and subject to regular regulatory (e.g., MHRA) inspection.

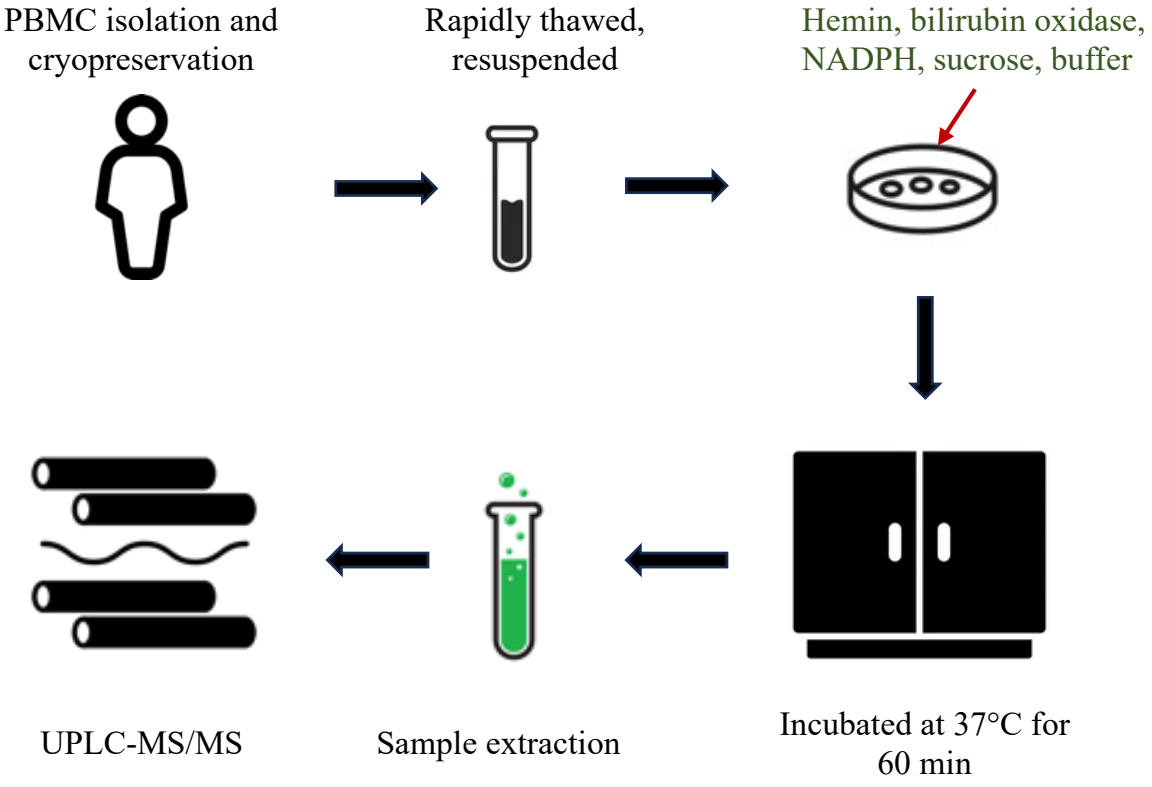



**Figure 30: UPLC-MS/MS quantification of biliverdin as a biomarker of HO activity in human PBMCs.**

Methods in brief:

- Calibration solutions using reagent grade biliverdin (Sigma, BCCC7472) were prepared to cover a concentration range of 25 – 500 ng/mL for each run of the assay. This represented the range of linearity i.e., biliverdin mass could accurately be interpolated between these values (**Fig. 31**). Outside these values the linear formula would be applied to ntify biliverdin.

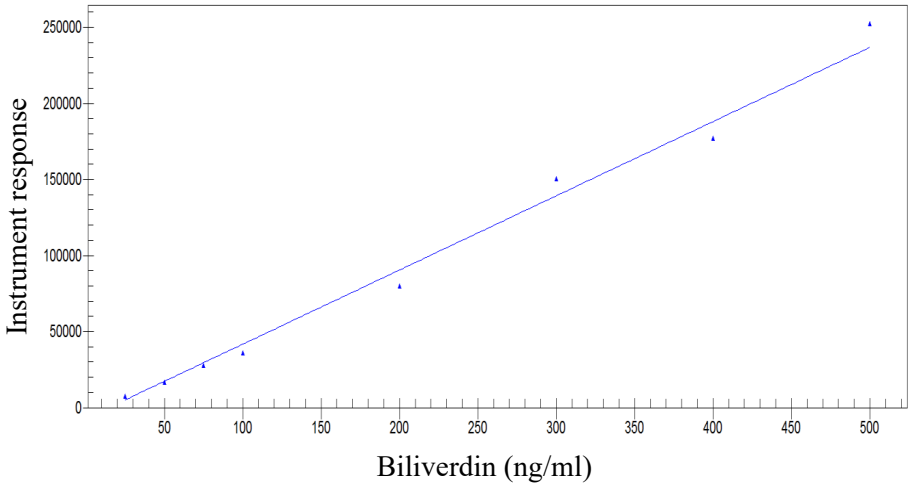



**Figure 31: Representative calibration curve for biliverdin in human PBMCs.**

Regression method: linear, weighting factor 1/X. Response = Slope x Concentration + Intercept. Slope = 487.88, Intercept = -7089.3, R2 = 0.9901

- 50 µL of rapidly thawed human PBMCs (standardised to 2.5 x $10^6$ cells/mL) were combined with 20 mM sodium phosphate buffer pH 7.4/1% Triton (Invitrogen™, HFH10), 1 mM NADPH (Sigma Aldrich, 2646-71-1), 25 mM sucrose, 5 µM hemin (Sigma Aldrich, H9039), 0.025 U bilirubin oxidase (Sigma Aldrich, B0390) and water in a final volume of 200 µL. Incubation was carried out at 37°C for 60 min at 750 rpm on a Thermomixer.
- Samples were extracted with 400 µL of 1% formic acid in acetonitrile and vortex mixed for 20 s. After a spin at 16,100 x g for 5 min, 150 µL of supernatant was mixed with 100 µL of water. Extracts were transferred to a 2 mL 96-well plate and covered with a pre-slit sealing mat. An aliquot (5 µL) of each processed sample was then analysed by UPLC-MS/MS on a Waters Xevo TQ-XS mass spectrometer. Biliverdin was detected by tracking the Electrospray Ion Source (ESI) positive ion for the precursor and daughter ion (mass to charge ratio (m/z) 583.3 – 297.1).
- Data were acquired using MassLynx (version 4.2) and integrated using TargetLynx (version 4.2). Peak area results were exported into the Watson Laboratory Information System (LIMS). Watson LIMS was used to calculate the standard curve parameters and subsequent concentration of biliverdin (linear model using a weighting of 1/concentration).
- The method was validated both in male and female fresh and cryopreserved PBMCs prior to trial sample analysis.

## 2.7 High-dimensional flow cytometry

This experiment was designed and run in collaboration with Dr Ying-Jie Wang (Cardiovascular Medicine, University of Oxford). Through unbiased analyses of publicly available transcriptomic datasets (discussed in chapter 4) monocytes were identified as important circulating immune cells for the study of HO-1 biology. Classification and immunophenotyping of monocyte populations was designed to enable standard monocyte subset evaluation as well as novel aspects with a focus on cellular function[146]. HO-1 expression in these cells was an important potential PD readout of efficacy of 5-ALA-SFC. A multi-parameter design incorporating these considerations resulted in a selection of antibodies as listed in **Table 12**.

Experimental protocol:

- Cryopreserved PBMCs were rapidly thawed in a water bath at 37°C until one or two ice crystals remained. This was immediately transferred to a 15 mL Falcon tube containing 10 mL RPMI 1640 with L. glutamine (Cat no. 21875034) and centrifuged at 300 x g for 10 minutes at 4°C, discarding the supernatant. Samples kept on wet ice and all reagents kept at 4°C/on wet ice.
- The pellet was resuspended in 10 mL PEB buffer (200 uL EDTA in 50 mL PBS), repeating the centrifugation step. The pellet was resuspended in blocking buffer (5 mL Rabbit serum (Gibco, Cat no. 16120-099 100ML), 5 mL Mouse serum (Invitrogen, Cat no. 10410), 5 mL Goat serum (ThermoFisher Scientific, Cat no. 31872), 35 mL Flow Cytometry Stain Buffer (FSB) (BD Bioscience, Cat no. 554656) and 500 µL Human Fc receptor block (Biolegend, Cat no. 422301)). Resuspension volume was calculated to allow for 50 µL cell suspension per well for the optimisation plate setup. Resuspended cells were set aside for 45 mins at 4°C.
- Single-colour sample cocktail: 49 µL Blocking buffer and 1 µL specific surface antibody OR 50 µL Blocking buffer only – for intracellular single colour samples (final antibody dilution 1:100).
- All-colour antibody cocktail: 35 µL Blocking buffer and 1 µL all surface antibodies (final antibody dilution 1:100).

- Fluorescence minus one (FMO)[x] antibody (surface) cocktails were prepared as follows:
  - ⇒ Where no surface antibodies are omitted: 35 µL Blocking buffer and 1 µL all surface antibodies (final antibody dilution 1:100).
  - ⇒ Where only one surface antibody was omitted: 36 µL Blocking buffer and 1 µL remaining surface antibodies (final antibody dilution 1:100).
  - ⇒ Where three surface antibodies were omitted: 38 µL Blocking buffer and 1 µL all remaining surface antibodies (final antibody dilution 1:100). Three antibodies were omitted to cover the negative population channel (see Table 12) (final antibody dilution 1:100).
- 50 µL cell suspension added to each cocktail and left for 1 hour at 4°C (protecting samples from light with aluminium foil). Samples were then centrifuged at 300 x g at 4°C for 10 minutes, discarding the supernatant. Cell pellets resuspended in 100 µL PEB buffer and centrifuged as above. Pellets were resuspended in 100 µL Fixation/Permeabilization buffer (Invitrogen, Cat no. 00-5123-543) and left at room temperature for 30 minutes, protecting the samples from light. 100 µL Permeabilization buffer (Invitrogen, Cat no. 00-85333-56) was added, and samples were centrifuged at 300 x g for 5 minutes at room temperature, discarding the supernatant.
- Intracellular staining as follows:
  - ⇒ For single colour samples, if no intracellular antibodies were to be added, samples were resuspended in 100 µL 1:1 Permeabilization buffer with Blocking buffer. Where intracellular antibodies were being added, samples were resuspended in 99 µL 1:1 Permeabilization buffer with Blocking buffer.
  - ⇒ For multicolour samples, cells were resuspended in 97 µL 1:1 Permeabilization buffer with Blocking buffer.
  - ⇒ For FMO samples, where a single intracellular antibody was to be omitted, samples were resuspended in 99 µL 1:1 Permeabilization buffer with Blocking buffer. All other samples were resuspended in 100 µL 1:1 Permeabilization buffer with Blocking buffer.

[x] FMO samples are control samples including all antibodies minus one. They are used to set the upper limit of acceptable background signal on the omitted antibody/channel.

⇒ Samples were left for 15 minutes at room temperature before adding 1 µL intracellular antibody and left again for 30 mins at room temperature. Samples protected from light throughout (final antibody concentration 1:100).

- Samples were centrifuged at 300 x g for 5 minutes at room temperature, discarding the supernatant and resuspended in 200 µL Permeabilization buffer repeating the centrifugation step. Before analysis samples were resuspended in 650 µL FSB.
- Compensation controls were prepared using 2 drops of the appropriate beads (see Table 12) and 2 µL of each antibody. No stain controls were prepared by omitting all antibodies above but treating the samples in an identical manner in other respects.
- All sample analysis performed on the BD LSRFortessa™ X20 Analyzer using BD FACSDiva™ Software for setup and automatic compensation matrix calculation. All data analysis was performed using FlowJo™ version 10.9.

The proposed gating strategy is outlined in **Fig. 32**.

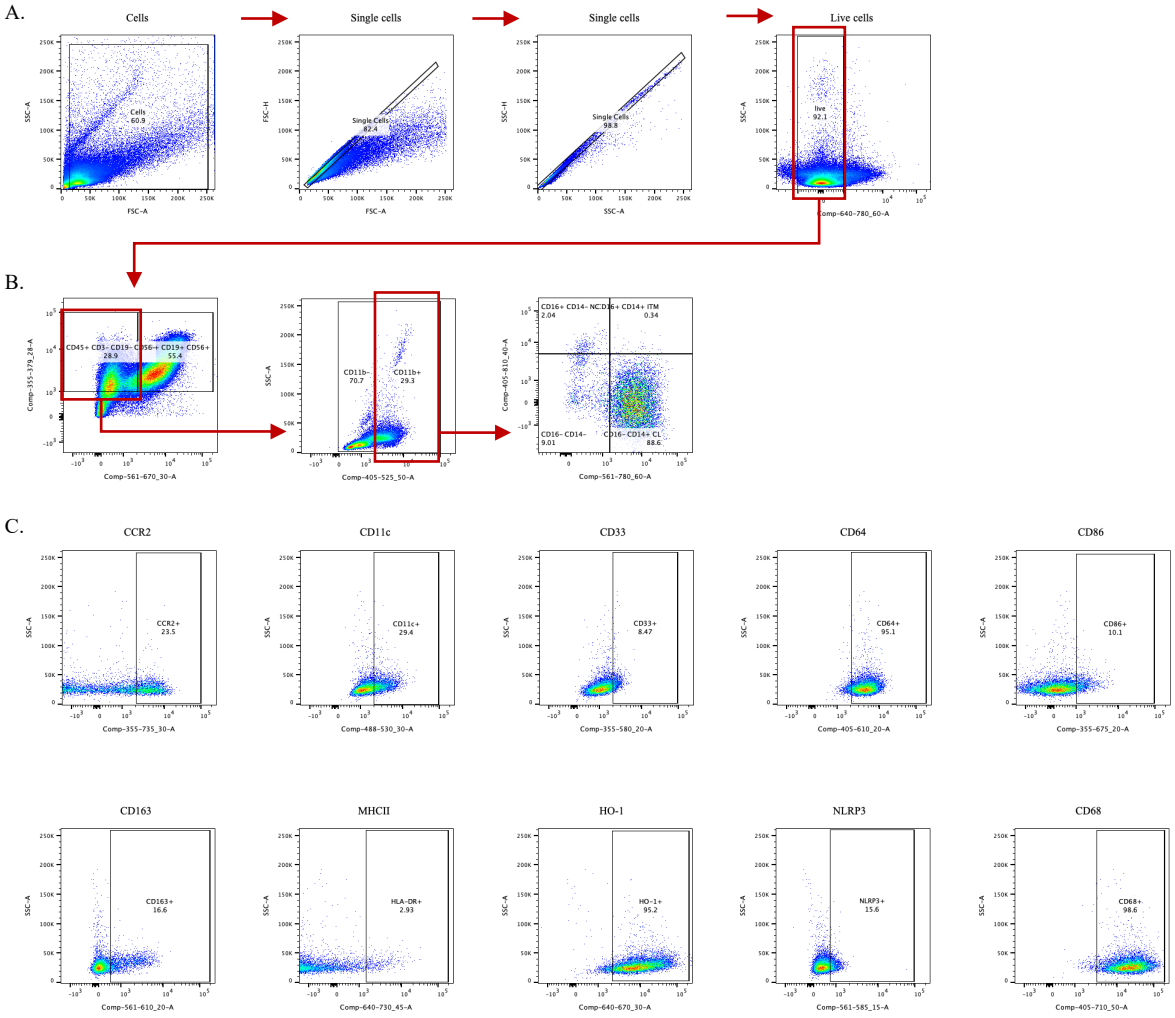



**Figure 32: Proposed monocyte gating strategy.**

A - exclusion of debris, inclusion of singlets and live cells, B - inclusion of $CD45^{+}$ $Lin^{-}$($CD3^{-}CD19^{-}$ $CD56^{-}$), CD11b+ to identify monocytes, C – immunophenotyping by surface and intracellular markers.

**Table 12: Antibody selection for high dimensional flow cytometry experiment.**

| Antibody | Cell/function | Location | Fluorophore | Clone | Isotype | Cat. Number |
|---|---|---|---|---|---|---|
| CD11c | Dendritic cell | Surface | **BB515** | B-ly6 | Mouse BALB/c IgG1, κ | 564490 |
| HO-1 | Haem oxygenase-1 | Intracellular | **APC** | 23 | Mouse IgG1 | 566391 |
| HLA-DR | Antigen presenting | Surface | **AF700** | G46-6 | Mouse IgG2a, κ | 560743 |
| FVS780 | Viability | Amine dye | **FVS780** | n/a | n/a | 565388 |
| CD11b | Myeloid cell | Surface | **BV480** | ICRF44 | Mouse IgG1, κ | 746704 |
| CD64 | Monocyte/macrophage | Surface | **BV605** | 10.1 | Mouse BALB/c IgG1, κ | 740406 |
| CD68 | Macrophage/neutrophil | Intracellular | **BV711** | Y1/82A | Mouse BALB/c IgG2b, κ | 565594 |
| CD16 | Monocyte | Surface | **BV786** | 3GB | Mouse BALB/c x DBA/2 | 563690 |
| NLRP3[#] | Inflammasome | Intracellular | **PE** | 768319 | Rat IgG2A Clone # 768319 | IC7578P[+] |
| CD163 | Monocyte/macrophage | Surface | **PE-Dazzle** | GHI/61 | Mouse BALB/c IgG1, κ | 562670 |
| CD3* | T cell (lymphocytes) | Surface | **PE-Cy5** | UCHT1 | Mouse BALB/c IgG1, κ | 555334 |
| CD19* | B cell (lymphocytes) | Surface | **PE-Cy5** | HIB19 | Mouse IgG1, κ | 555414 |
| CD56* | Natural Killer cell | Surface | **PE-Cy5** | MSK39 | Mouse IgG1, κ | 555517 |
| CD14 | Monocyte | Surface | **PE-Cy7** | M**φ**P9 | Mouse BALB/c IgG2b, κ | 562698 |
| CD45 | Pan-immune | Surface | **BUV395** | HI30 | Mouse IgG1, κ | 563792 |
| CD33 | Monocyte | Surface | **BUV563** | WM53 | Mouse BALB/c IgG1, κ | 741369 |
| CD86 | Monocyte/macrophage | Surface | **BUV661** | 331 (FUN-1) | Mouse BALB/c IgG1, κ | 741629 |
| CCR2 | Monocyte/macrophage | Surface | **BUV737** | LS132.1D9 | Mouse BALB/c IgG2a, κ | 749075 |

Abbreviations: CD – cluster of differentiation, HLA-DR – Human Leukocyte Antigen – DR isotype, FVS – fixable viability stain, NLRP3 – NLR Family Pyrin Domain Containing 3, CCR2 – C-C Motif Chemokine Receptor 2

[#] Compensation beads used during experimental optimisation – BD Biosciences Rat, all other antibodies – BD Biosciences Mouse

* Lymphocytes and NK cells shared a common fluorophore as they were grouped together as a negative channel for exclusion.

[+] Supplied by R+D systems, all other antibodies supplied by BD Biosciences

Fluorophores are denoted in colour indicating the specific laser employed to excite them. Detection was at pre-specified voltage (determined during experimental optimisation) settings to avoid spectral overlap.

## 2.8 Serum and plasma markers of haemolysis, iron homeostasis and lipid peroxidation

### 2.8.1 Haemolytic index as a marker of cell-free haemoglobin (cf-Hb)

All serum samples were submitted to the Clinical Biochemistry laboratory at the John Radcliffe Hospital (Oxford University Hospitals NHS Foundation Trust) for biochemical analysis undergo an automated determination of the haemolytic index (HI). This automated absorbance assay is required to determine the risk of interference of cf-Hb on standard biochemical readouts (e.g., creatinine) and reliably predicts the absolute quantity of cf-Hb present in serum[304].

### 2.8.2 Non-transferrin bound iron (NTBI)

NTBI was determined using an assay based on the chelation-ultrafiltration-detection approach. In short, this assay comprises mobilization of serum NTBI by the weak iron-mobilizing chelator nitrilotriacetate (NTA), after which the chelated NTBI is separated from transferrin-bound iron by ultrafiltration and measured spectrophotometrically by the addition of thioglycolic acid (TGA) and bapthophenanthroline disulfonic acid (BPT)[305]. The lower limit of detection (LLOD) of the NTBI assay is 0.607 μmol/L. This work was carried out as part of a collaboration with Professor Dorine Swinkels (Laboratory Medicine, Radboud University, The Netherlands). Cryopreserved serum samples were shipped to the Netherlands on dry ice and processed in line with their internal standard operating procedures. Data were supplied as final concentrations in .pdf format.

### 2.8.3 Plasma hepcidin determination

To determine concentrations of plasma hepcidin the Simple Plex Human Hepcidin Cartridge (Bio-Techne, Cat no. SPCKB-PS-000984) was used. This bespoke hepcidin cartridge enables rapid, automated ELISA quantification of protein using the Ella Automated Immunoassay System (Bio-Techne, Cat no. 600-100). The assay was performed as per manufacturer's guidance without modification. Hepcidin determination experiments were performed in Professor Samira Lakhal-Littleton's laboratory (Department of Physiology, Anatomy & Genetics, University of Oxford, United Kingdom).

In brief:

- EDTA plasma was thawed on wet ice diluted to a final dilution of 1:100 by adding the sample diluent.
- 35 µL dilute plasma aliquoted into microcentrifuge tube and centrifuged at 8,000-10,000 g for 4 minutes after which 30 µL of the supernatant was carefully aspirated and transferred to another microcentrifuge tube.
- 30 µL of the appropriate sample diluent was added to the supernatant and mixed thoroughly. 50 µL of this mixture was transferred into the appropriate cartridge inlet, after adding the loading buffer into the appropriate inlet.
- ELISA performed as per product guidance and data exported in Excel format.

### 2.8.4 Lipid peroxidation by determination of plasma malondialdehyde (MDA) by colorimetry

Plasma MDA levels were determined using the Lipid peroxidation (MDA) Assay (Colorimetric) (Abcam, Cat no. ab233471) according to manufacturer's guidance without modification. During optimisation it was found that the optimal dilution of plasma samples was 1:50. MDA determination experiments were performed in Professor Samira Lakhal-Littleton's laboratory (Department of Physiology, Anatomy & Genetics, University of Oxford, United Kingdom).

Method in brief:

- Cryopreserved plasma thawed on wet ice and handled on wet ice throughout.
- 50 µL of diluted samples (diluted in dilution buffer) and MDA standards (prepared as per product guidance) plated in duplicated using a 96-well, clear bottomed microplate, including blank controls.
- 10 µL of the MDA Colour Reagent Solution added to each well, left at room temperature for 30 minutes. 40 µL of the Reaction Solution and incubated for 60 minutes.
- Absorbance measured using an absorbance plate reader with path-check correction at an optical density (OD) wavelength of 695 – 700 nm.
- Concentrations of MDA (µMol) were interpolated from the linear regression standard curve for MDA (see Figure 16). Data were exported in Excel format.

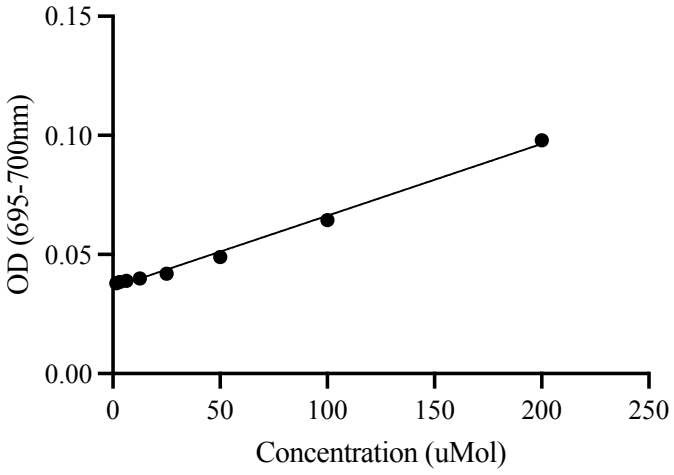



**Figure 33: Representative linear regression standard curve for MDA.**

## 2.9 Inflammatory proteomic profiling using the Olink® Explore 384: Inflammation platform.

This assay was performed in collaboration with Dr Paolo Piazza (Oxford Genomics Centre, University of Oxford). This is an Olink® accredited facility. All data generated underwent quality control checks in Uppsala, Sweden, before release. Olink® uses novel Proximity Extension Assay (PEA) technology coupled with next generation Sequencing (NGS) which allows it to reliably perform high-multiplex and high throughput biomarker analyses[306]. The assay procedure is summarised in **Fig. 34**.

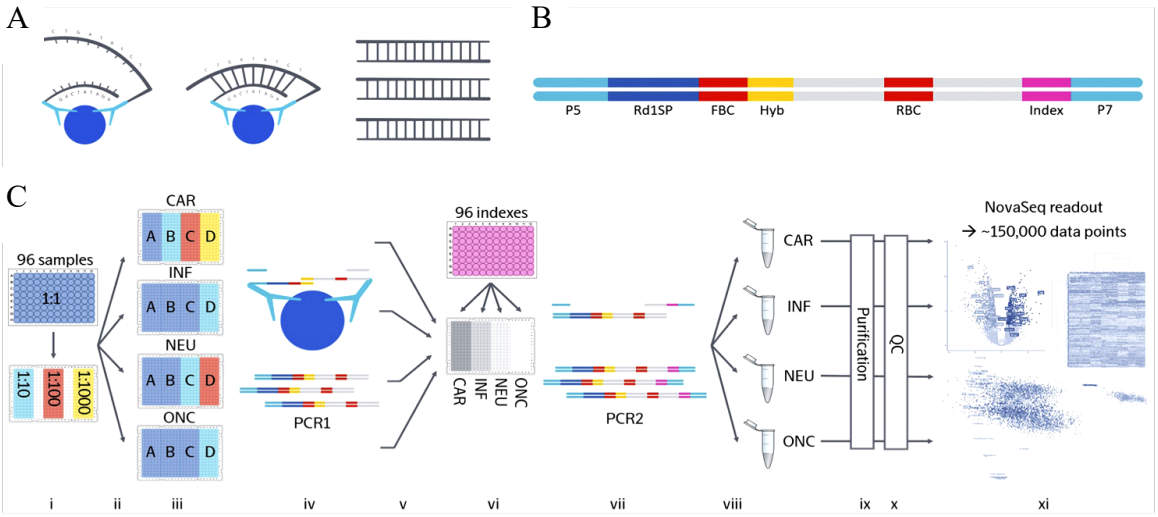



**Figure 34: Summary of Olink Explore platform.**

A - Matched PEA probes, with unique oligonucleotides, bind to their specific proteins in the sample, allowing pairwise DNA hybridization generating detectable amplicons. B – PEA probe design includes Illumina adaptor sequences (P5 and P7), barcodes for protein identification (FBC and RBC), indices for sample identification and a sequence for pairwise hybridisation of DNA sequences when in proximity (Hyb). C – Hybridised DNA sequences are amplified by PCR. During PCR 1, universal primers allow for extension of the PEA probeset (which includes Illumina's P5 adapter sequence). During PCR 2 sample specific indices and Illumina's adapter sequence (P7) are added. The resulting library DNA sequences allows for downstream identification of high multiplex proteins.[y]

The assay is controlled at each stage (incubation, extension and amplification) by the addition of internal controls, included in each incubation reaction. Each plate will also include external controls (biological sample control, negative control and a plate control made up of pooled healthy plasma samples). The extension and plate controls are used for normalisation of data. Data are summarised as Normalised Protein eXpression (NPX). NPX is a unique quantification on the log2 scale. Values are calculated from

[y] Reproduced from figure 1 Wik et al. *Mol Cell Proteomics* 2021; 20: 100168.

the number of matched counts from the sequencing data. NPX generation is, broadly, by normalisation of data to the extension control, followed by log2 transformation then level adjustment using the plate control.

Statistical analysis was kindly performed by Dr Shirin Akhtar (Olink Proteomics AB, Uppsala, Sweden). A linear mixed model (LMM) was used to identify proteins that differed significantly between groups, defined as a p-value < 0.05 with Benjamini-Hochberg false discovery rate (FDR) correction for multiple comparisons. Comparisons were made to assess the impact of the drug (i.e., active vs. placebo), impact of the timepoint (i.e., effect of the surgical insult), impact of the dose vs. placebo or a comparison of these.

Using lists of proteins with significant dynamic changes from the Olink analyses, Gene Ontology (GO) analyses were be performed by assessing for changes in the biological processes (BP) using the PANTHER (Protein Analysis Through Evolutionary Relationships) classification[307]. UniProt accession numbers were uploaded and compared to the reference of the 368 proteins that were assayed on the Olink® Explore Inflammation 384 panel to assess for enrichment of BPs. Comparisons were assessed for significance using Fisher's Exact test, with no correction for multiple comparisons. A minimum requirement of two significantly differentially expressed proteins in each top-level BP was required.

### 2.10 Statistics

Unless otherwise specified, all statistical analyses were in accordance with the TALEN Statistical Analysis Plan (SAP) Version 1.0 (Final, 04 October 2023), which superseded the trial protocol with regards to statistical analysis. The SAP was jointly developed with Professor Laurent Billot (Lead trial statistician, The George Institute for Global Health, Sydney, Australia) and approved prior to investigator unblinding. All statistical analysis performed by the SAS Enterprise Guide (version 8.3 or above) and R (version 4.0.0 or above). Internal analyses were performed using a combination of Microsoft® Excel for Mac (Version 16.78) and GraphPad Prism for macOS (Version 10.1.0).

#### 2.10.1 General approach to statistical analysis

All trial data were summarised chronologically by protocol defined timepoints. All safety, exploratory efficacy, demographic, and other baseline data were summarized by treatment group (dose level of 5-ALA-SFC or placebo) and total, as applicable. Statistical analysis tests were conducted at a two-sided alpha = 0.05 unless otherwise stated. Continuous data were presented descriptively, using number of observations, as arithmetic mean, standard deviation (SD), geometric mean and geometric SD (if applicable), median, interquartile range (IQR), minimum and maximum. Categorical data were summarized descriptively using frequencies and percentages. Unless otherwise stated, denominators for percentages were the number of patients in the analysis population with non-missing variables of interest for the column of interest. Laboratory assessment values that were < Lower Limit of Quantification (LLOQ) were converted to numeric values by replacing with ½ of LLOQ unless otherwise specified. Where they were > Upper Limit of Quantification (ULOQ) the upper value of quantification was used.

The main between-group comparisons focused on the differential effect of the respective 5-ALA-SFC dose level vs pooled placebo. Analyses of the impact of drug dosing pre-operatively was made to support the trial's primary objective. This was to understand the impact of 5-ALA-SFC on PD endpoints free of the confounding effect of cardiac surgery. Analyses of the impact of different dose levels on the trajectory of PD response during the peri-operative period were also made. It was accepted, *a priori*, that data transformation and/or alternative non-parametric approaches for the same hypotheses could be

considered and used, should the model/hypothesis assumptions fail. Unless otherwise specified, no imputations for missing data were applied and the main analysis was based on all available data. Outliers were included in summary tables and listings but where clinically justified (e.g., an unanticipated iatrogenic intra-operative/on-table severe surgical complication) analyses were performed with and without the respective datapoints. Given the nature of the study as a translational, proof-of-concept, phase II dose-finding trial, hypothesis testing was regarded as exploratory. Any statistically significant findings were reviewed and interpreted for potential biological and clinical relevance whilst considering the totality, consistency, and directionality of the data (i.e., considering whether any orthogonal outcome measures supported the finding).

### 2.10.2 Definition of analysis sets and proposed comparisons

The study population was condensed into analysis sets as described in **Table 13** below.

**Table 13: TALEN trial analysis sets.**

| Definition | Use of analysis set |
|---|---|
| **Full analysis set (FAS)**<br>All randomised patients analysed according to randomised treatment assignment who have:<br>• taken at least 1 dose of trial medication; and<br>• not withdrawn. | • Safety and tolerability<br>• Efficacy and PD response |
| **Primary analysis set (PAS)**<br>All patients in the FAS population who:<br>• underwent the planned on-pump cardiac surgery without unanticipated iatrogenic, intra-operative /on-table severe surgical injury* | • Safety and tolerability<br>• Efficacy and PD response |

* Examples include iatrogenic coronary injury arising during cardiac surgery. Such iatrogenic on-table surgical injuries, while uncommon, can jeopardise a substantial region of myocardium and be associated with significant morbidity and/or potentially mortality.

### 2.10.3 Primary analysis

Treatment-related changes in HO-1-related PD measures between pre-dosing (screening) and end of dosing period (pre-operatively) were analysed using analysis of covariance (ANCOVA). For this, the independent variables were the baseline value (explanatory baseline covariate) and the treatment group,

and the dependent variable was the value at the end of the dosing period immediately prior to cardiac surgery. The mean, standard error of the mean (SEM), mean differences between each active dose group and the (pooled) placebo (the reference or control cohort) were calculated from the ANCOVA and presented with 95% confidence intervals (CI) for the differences. P-values for the difference in effect related to each dose level of 5-ALA-SFC vs. placebo were derived and presented from the ANCOVA model. The same model was re-run after adding *HMOX1* (GT)n repeat promoter polymorphism status as a continuous covariate.

### 2.10.4 Secondary analyses

Unless otherwise specified, all secondary analyses were performed using either a linear mixed model (LMM) using time as a categorical variable, with no correction for multiple comparisons ; OR a LMM using Restricted Maximum Likelihood (REML), correcting for multiple comparisons by controlling the False Discovery Rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli was used to determine whether any differences observed between treatment allocation groups was significant. The choice of LMM by dataset will be annotated in the results secton of this thesis.

Cardiac troponin I analysis will calculate the arithmetic mean of the area under the curve (AUC) for the natural logarithm of serial troponin values between 0 and 72. Analysis of the troponin I AUCs will be performed on the log-scale; that is after natural logarithm transformation of individual AUCs (AUC72). Group means of troponin l (AUC72) will be compared using an analysis of variance (ANOVA). The effect of the intervention against placebo will be estimated using the ratio of geometric means and its 95% confidence interval, obtained by back-transforming (exponentiating) the log-transformed results to the original scale.

Where statistical approaches deviate from these, this will be explained in relevant sections of this thesis.

### 2.10.5 Interim analyses

The SRC conducted review of blinded safety, tolerability, and available PD data at the end of each dosing cohort to decide whether dose escalation should proceed. The optional dose cohort was ultimately not utilised (after discussion with the industry provider of IMP for the trial in consideration of availability of IMP). No formal interim analyses were conducted.

# 3 Chapter 3 – Pre-operative 5-ALA-SFC as a strategy to improve patient outcomes after cardiac surgery on cardiopulmonary bypass

## 3.1 Introduction

The "Treatment with 5-AminoLEvuliNic acid before cardiac surgery (TALEN)" trial was a prospective, randomised, double-blind, placebo-controlled phase II study of pre-operative 5-ALA-SFC in adult patients undergoing non-emergent cardiac surgery on CPB. The study examined the potential of 5-ALA-SFC to induce HO-1, as a novel cardio- and cyto-protective strategy with the potential to improve post-operative patient outcomes. Using a placebo-controlled, dose-escalation study design to evaluate the pharmacodynamic response to 4 different dose levels of 5-ALA-SFC, alongside assessments of safety and tolerability, TALEN aimed to determine the optimum biological dose (OBD) of 5-ALA-SFC for future clinical testing. This would represent a dose that was well tolerated without adverse safety signals in this relatively high-risk patient population under cardiac surgery and which resulted in meaningful induction of HO-1 activity and, ideally, was associated with preliminary signals suggestive of efficacy (e.g. using clinically relevant biomarkers of organ injury/dysfunction). Further exploratory work focussed on better understanding the drug's mechanism of action and evidence for a putative immunomodulatory role in mitigating the cytotoxic insult associated with CPB-induced haemolysis. Serial high-sensitivity cardiac troponin I measurements were used to quantify peri-operative myocardial damage. Other data captured included in-depth clinical (e.g., incidence of delirium, requirement for cardiovascular support), haematological and biochemical assessment preliminary signals suggestive of efficacy/activity and to potential drug-related (direct/indirect) toxicities.

The specific objectives of the work presented in this chapter are as follows:

1. Report all TALEN trial initiation, set-up, conduct and recruitment activities, in line with CONSORT[308] principles.
2. Summarise the baseline clinical characteristics of the enrolled study population in TALEN.

3. Detail the observed safety profile and tolerability of pre-operative 5-ALA-SFC across a range of doses in adult patients undergoing elective cardiac surgery on CPB, including treatment compliance, AEs, clinical safety data, ECG findings and biochemical laboratory readouts.
4. Investigate the kinetics of cardiac troponin I release as a surrogate for PMI across a range of different cardiac surgical procedures and the potential impact of 5-ALA-SFC on these profiles.

## 3.2 Results

### 3.2.1 TALEN trial logistics and the impact of the 2019 SARS-CoV-2 (COVID) pandemic

TALEN was a single centre study undertaken in the Oxford Heart Centre at the John Radcliffe Hospital in Oxford, United Kingdom. The trial received ethical approval from the Oxford A Research and Ethics Committee (REC ID number: 20/SC/0272) and was registered on EudraCT (2020-001135-27). Regulatory approval from the Medicines and Healthcare products Regulatory Agency (MHRA) was obtained on the 9th of September 2020. After a delay in trial initiation in the context of the global SARS-CoV-2 (COVID) pandemic and a prolonged period of cessation of all non-COVID-19 research studies at the hospital, the first TALEN participant was recruited on the 21st of February 2021. Initial progress on recruitment was impacted by a gradual staged resumption of non-urgent cardiac surgery, focussing initially on highly symptomatic patients in need of cardiac valve surgery rather than patients undergoing elective CABG which the trial enrolment population was initially exclusively focused on. To address this impact on recruitment, a substantial protocol amendment was submitted and approved by the MHRA on the 4th August 2021. This protocol amendment allowed for the recruitment of patients scheduled for valve repair/replacement surgery, in addition to CABG surgery, as was proposed in the original approved trial protocol. The broader eligibility criteria allowed for more effective recruitment and the last patient was recruited on the 24th of March 2022. The final follow-up study visit took place on the 30th of June 2022, concluding patient involvement in the trial. The study was funded by an industry grant from the manufacturers of 5-ALA-SFC, SBI Pharmaceuticals Co., Ltd., and sponsored by the University of Oxford.

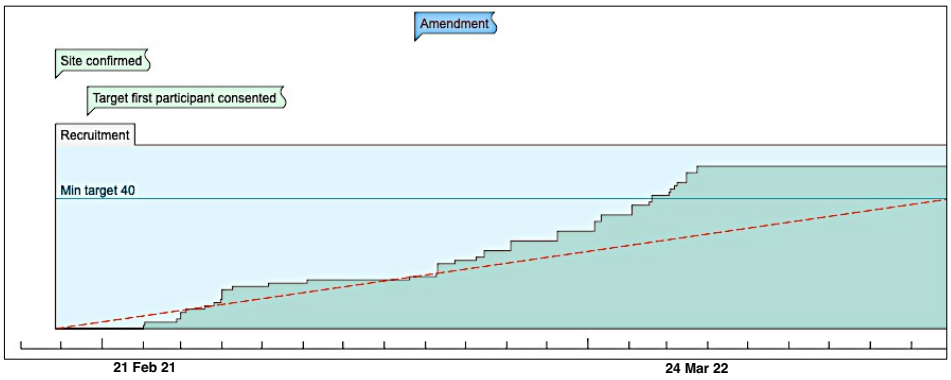



**Figure 35: TALEN recruitment tracker.**

### 3.2.2 TALEN trial participants

From December 2020 until May 2022, 64 adult male and female patients who were scheduled for non-emergent cardiac surgery on CPB were informed of the TALEN trial by their primary clinical care team. A total of 11 patients declined to participate. Patients who indicated interest in participating in the trial to their treating clinicians were invited to attend a screening visit. 3 patients did not meet protocol eligibility criteria and there were 2 withdrawals for surgical logistical reasons: surgery was cancelled in 1 patient; 1 patient's surgical date was moved, thereby preceding randomisation. 48 patients were randomised after providing full written, informed consent. A total of 6 patients were withdrawn from the trial before final analysis: 1 patient moved their care to a different hospital prior to surgery; 1 patient had their surgery moved to an earlier date which did not leave sufficient time to complete dosing; 1 patient had their surgery cancelled indefinitely by their clinical care team; 1 patient withdrew due to a headache judged to be related to the IMP; 1 patient had their surgical procedure cancelled on the table; and in 1 case the research study team was not informed of a late change to the patient's date for surgery such that key trial assessments were not completed. Full details of subject disposition, treatment allocation and reasons for withdrawal from the trial are set out as a CONSORT diagram in **Fig. 36**.

The final, evaluable trial population included 42 patients, constituting the full analysis set (FAS) defined as all randomised patients analysed according to the randomised treatment who had taken at least 1 dose of trial medication and not withdrawn. In addition to this overall population, additional analysis of the population of randomised patients after excluding any patients who had experienced unanticipated iatrogenic, on-table severe surgical injury unrelated to the study treatment or any who had ultimately not undergone cardiac surgery on CPB was undertaken. The latter constituted the primary analysis set (PAS). Two patients experienced intra-operative myocardial infarction, both related to mechanical placement of valve prostheses and judged by their treating clinician not to be related to the study drug. A further patient underwent off-pump CABG. These 3 patients were excluded from the PAS before unblinding of the randomization code and knowledge of treatment group (5-ALA-SFC or placebo) allocation.

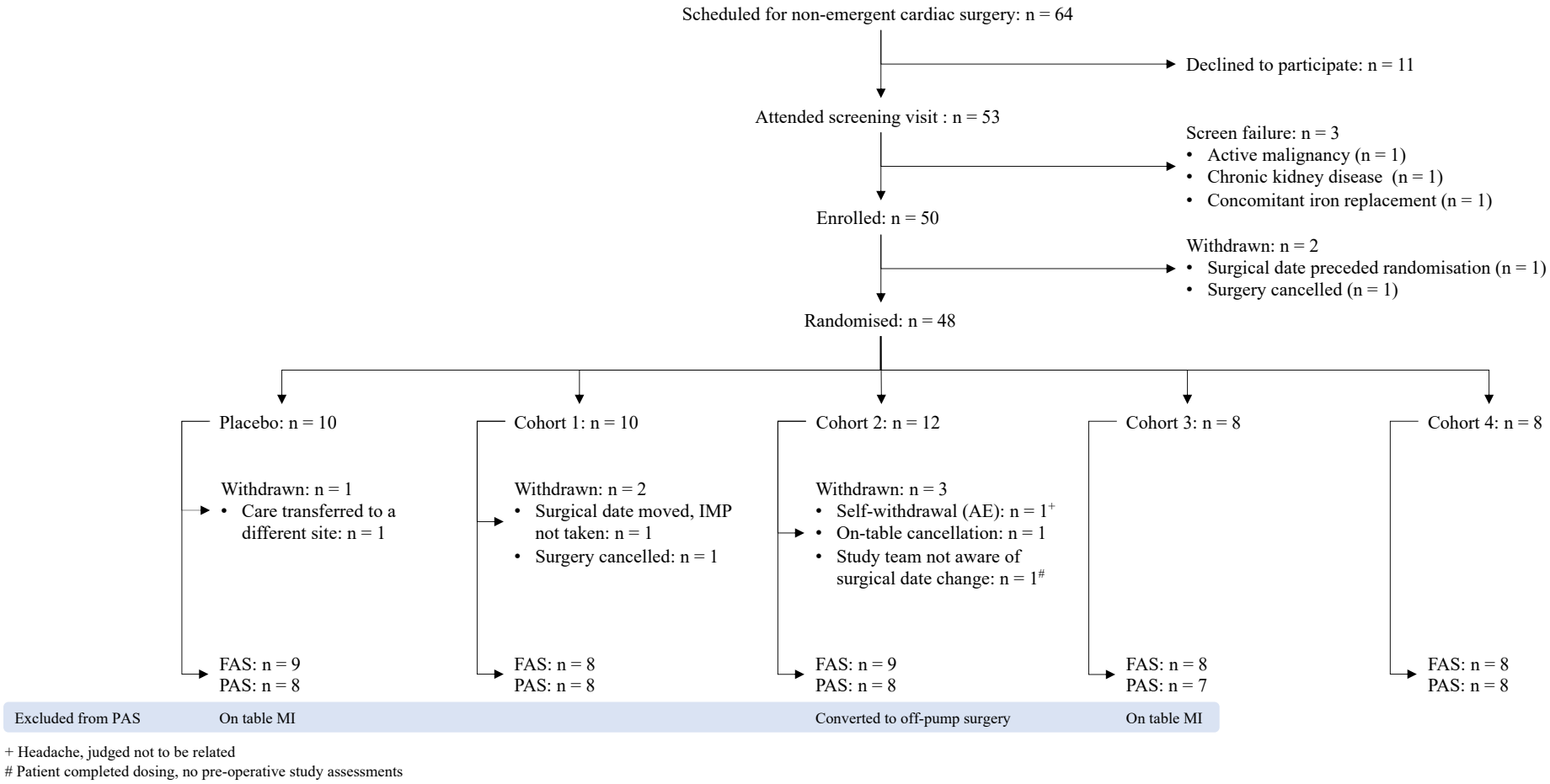



**Figure 36: TALEN CONSORT diagram.**

Abbreviations: IMP – investigational medicinal product; AE – adverse event; FAS – full analysis set; PAS – primary analysis set; MI – myocardial infarction.

Patient baseline clinical characteristics are summarised in **Table 14**. Across the trial the median age (IQR) of patients was 69 (60; 73) years. Patients were predominantly male (88%) and Caucasian (95%). Patients recruited to the highest two 5-ALA-SFC treatment allocation groups were slightly younger (median age 64 and 63 years, respectively) and there was also a slightly higher proportion of female participants in these groups when taken together, compared to other allocation groups. 78% of patients in the placebo group had a moderate or heavy alcohol intake history. This was higher than in patients randomised to the active 5-ALA-SFC treatment allocation groups. Overall, 45% of patients were never smokers.

Across the trial 67% of patients had hypertension, 50% had hyperlipidaemia and 17% had diabetes. A minority of patients (7%) had experienced a previous MI. Most had a degree of functional limitation due to cardiovascular symptoms. Dyspnoea was assessed using the New York Heart Association (NYHA) classification, with 64% of patients overall in NYHA Class II and 17% in NYHA Class III. No participants in the 5-ALA-SFC 75 mg/118 mg or 300 mg/472 mg dose allocation groups were NYHA Class III. Most participants experienced a degree of exertional angina, with 48% experiencing Canadian Cardiovascular Society (CCS) Grade I angina, 29% Grade II and 7% each experiencing Grade III and IV angina.

In the 5-ALA-SFC 75 mg/118 mg treatment allocation group (lowest dose evaluated), 100% of patients underwent isolated CABG surgery, in line with the original protocol defined inclusion criteria. Within the 5-ALA-SFC 300 mg/472 mg treatment allocation group (highest dose evaluated), by comparison, only 1 patient (13%) underwent an isolated CABG procedure. Overall, the majority of procedures were elective as opposed to urgent. Patients randomly allocated to the placebo group underwent a range of surgical procedures (CABG, combination CABG with valve, and isolated valve procedures), allowing for a balanced control group when evaluating for drug-related changes.

**Table 14: Baseline clinical characteristics.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| **Age (yrs)** | | | | | | |
| Mean (SD) | 68 (9) | 67 (8) | 70 (5) | 64 (9) | 63 (11) | 67 (8) |
| Median (IQR) | 70 (64; 73) | 68 (62; 73) | 72 (68; 73) | 64 (57; 71) | 63 (55; 73) | 69 (60; 73) |
| min max | 51 79 | 52 74 | 60 75 | 52 75 | 46 77 | 46 79 |
| **Ethnicity (%)** | | | | | | |
| Caucasian | 8/9 (89%) | 8/8 (100%) | 8/9 (89%) | 8/8 (100%) | 8/8 (100%) | 40/42 (95%) |
| Asian | 0/9 (0%) | 0/8 (0%) | 1/9 (11%) | 0/8 (0%) | 0/8 (0%) | 1/42 (2%) |
| Other | 1/9 (11%) | 0/8 (0%) | 0/9 (0%) | 0/8 (0%) | 0/8 (0%) | 1/42 (2%) |
| **Male sex (%)** | 9/9 (100%) | 7/8 (88%) | 8/9 (89%) | 6/8 (75%) | 7/8 (88%) | 37/42 (88%) |
| **BMI ($kg/m^2$)** | | | | | | |
| Mean (SD) | 30.8 (4.7) | 28.4 (2.6) | 28.1 (2.9) | 28.0 (3.9) | 28.8 (9.4) | 28.9 (5.1) |
| Median (IQR) | 31.8 (28.6; 34.3) | 28.3 (27.2; 29.3) | 27.2 (26.1; 30.0) | 29.9 (24.4; 30.3) | 26.8 (23.7; 29.6) | 28.7 (25.0; 30.4) |
| min max | 22.9 36.6 | 24.3 33.2 | 24.7 33.5 | 22.1 33.0 | 19.9 50.7 | 19.9 50.7 |
| **Alcohol use (%)** | | | | | | |
| None | 0/9 (0%) | 1/8 (13%) | 3/9 (33%) | 0/8 (0%) | 0/8 (0%) | 4/42 (10%) |
| Moderate | 3/9 (22%) | 2/8 (13%) | 5/9 (22%) | 4/8 (13%) | 5/8 (13%) | 19/42 (45%) |
| Heavy | 5/9 (56%) | 2/8 (25%) | 0/9 (0%) | 3/8 (38%) | 3/8 (38%) | 13/42 (31%) |
| Unknown | 1/9 (11%) | 3/8 (38%) | 1/9 (11%) | 1/8 (13%) | 0/8 (0%) | 6/42 (14%) |
| **Tobacco use (%)** | | | | | | |
| Never smoker | 4/9 (44%) | 2/8 (25%) | 6/9 (67%) | 3/8 (38%) | 4/8 (50%) | 19/42 (45%) |
| Current smoker | 1/9 (11%) | 0/8 (0%) | 0/9 (0%) | 1/8 (13%) | 0/8 (0%) | 2/42 (5%) |
| Former smoker | 3/9 (33%) | 5/8 (63%) | 3/9 (33%) | 3/8 (38%) | 4/8 (50%) | 18/42 (43%) |
| Unknown | 1/9 (11%) | 1/8 (13%) | 0/9 (0%) | 1/8 (13%) | 0/8 (0%) | 3/42 (7%) |
| **Medical history (%)** | | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| Hypertension | 7/9 (78%) | 7/8 (88%) | 5/9 (56%) | 3/8 (38%) | 6/8 (75%) | 28/42 (67%) |
| Diabetes | 0/9 (0%) | 2/8 (25%) | 4/9 (44%) | 1/8 (13%) | 0/8 (0%) | 7/42 (17%) |
| Hyperlipidaemia | 3/9 (33%) | 7/8 (88%) | 3/9 (33%) | 4/8 (50%) | 4/8 (50%) | 21/42 (50%) |
| Vasculopathy | 2/9 (22%) | 0/8 (0%) | 0/9 (0%) | 1/8 (13%) | 0/8 (0%) | 3/42 (7%) |
| Documented MI | 0/9 (0%) | 1/8 (13%) | 0/9 (0%) | 1/8 (13%) | 1/8 (13%) | 3/42 (7%) |
| Documented AF | 1/9 (11%) | 0/8 (0%) | 2/9 (22%) | 0/8 (0%) | 2/8 (25%) | 5/42 (12%) |
| COPD | 0/9 (0%) | 0/8 (0%) | 2/9 (22%) | 0/8 (0%) | 0/8 (0%) | 2/42 (5%) |
| **Medications history (%)** | | | | | | |
| Aspirin | 4 (40%) | 10 (100%) | 2 (17%) | 3 (38%) | 3 (38%) | 22 (46%) |
| Clopidogrel | 1 (10%) | 2 (20%) | 2 (17%) | 0 (0%) | 1 (13%) | 6 (13%) |
| Beta blocker | 8 (80%) | 6 (60%) | 7 (58%) | 3 (38%) | 3 (38%) | 27 (56%) |
| Regular nitrate | 0 (0%) | 4 (40%) | 0 (0%) | 1 (13%) | 0 (0%) | 5 (10%) |
| $Ca^{2+}$ channel blocker | 3 (30%) | 6 (60%) | 1 (8%) | 1 (13%) | 2 (25%) | 13 (27%) |
| ACEi or ARB | 6 (60%) | 7 (70%) | 3 (25%) | 2 (25%) | 4 (50%) | 22 (46%) |
| Statin | 6 (60%) | 10 (100%) | 9 (75%) | 6 (75%) | 6 (75%) | 37 (77%) |
| Ezetimibe | 0 (0%) | 1 (10%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (2%) |
| Warfarin | 1 (10%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (13%) | 2 (4%) |
| Other anticoagulant | 2 (20%) | 0 (0%) | 6 (50%) | 0 (0%) | 1 (13%) | 9 (19%) |
| Glyceryl trinitrate | 10 (100%) | 10 (100%) | 12 (100%) | 8 (100%) | 8 (100%) | 48 (100%) |
| **Cardiovascular symptomatic status** | | | | | | |
| **NYHA class (%)** | | | | | | |
| Class I | 2/9 (22%) | 2/8 (25%) | 1/9 (11%) | 1/8 (13%) | 2/8 (25%) | 8/42 (19%) |
| Class II | 3/9 (33%) | 6/8 (75%) | 6/9 (67%) | 6/8 (75%) | 6/8 (75%) | 27/42 (64%) |
| Class III | 4/9 (44%) | 0/8 (0%) | 2/9 (22%) | 1/8 (13%) | 0/8 (0%) | 7/42 (17%) |
| **CCS grade (%)** | | | | | | |
| Grade I | 5/9 (56%) | 2/8 (25%) | 4/9 (44%) | 3/8 (38%) | 6/8 (75%) | 20/42 (48%) |
| Grade II | 1/9 (11%) | 5/8 (63%) | 2/9 (22%) | 2/8 (25%) | 2/8 (25%) | 12/42 (29%) |
| Grade III | 2/9 (22%) | 0/8 (0%) | 0/9 (0%) | 1/8 (13%) | 0/8 (0%) | 3/42 (7%) |
| Grade IV | 0/9 (0%) | 1/8 (13%) | 0/9 (0%) | 2/8 (25%) | 0/8 (0%) | 3/42 (7%) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| Not documented | 1/9 (11%) | 0/8 (0%) | 3/9 (33%) | 0/8 (0%) | 0/8 (0%) | 4/42 (10%) |
| **LV impairment (%) *** | | | | | | |
| Mildly impaired | 0/8 (0%) | 2/8 (25%) | 1/8 (13%) | 0/8 (0%) | 1/8 (13%) | 4/40 (10%) |
| Moderately impaired | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/40 (0%) |
| Severely impaired | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/8 (0%) | 0/40 (0%) |
| **Pulmonary hypertension (%)** [+] | | | | | | |
| Moderate | 1/8 (13%) | 0/8 (0%) | 1/9 (11%) | 0/8 (0%) | 0/8 (0%) | 2/41 (5%) |
| **Planned procedure (%)** | | | | | | |
| Isolated AVR | 0/8 (0%) | 0/8 (0%) | 1/6 (17%) | 5/8 (63%) | 3/8 (38%) | 9/38 (24%) |
| Isolated CABG | 5/8 (63%) | 8/8 (100%) | 3/6 (50%) | 2/8 (25%) | 1/8 (13%) | 19/38 (50%) |
| Isolated MVR | 1/8 (13%) | 0/8 (0%) | 0/6 (0%) | 1/8 (13%) | 3/8 (38%) | 5/38 (13%) |
| MV Repair | 2/8 (25%) | 0/8 (0%) | 2/6 (33%) | 0/8 (0%) | 0/8 (0%) | 4/38 (11%) |
| MV Repair + CAB | 0/8 (0%) | 0/8 (0%) | 0/6 (0%) | 0/8 (0%) | 1/8 (13%) | 1/38 (3%) |
| **Weighting of procedure (%)** | | | | | | |
| Elective | 8/9 (89%) | 6/8 (75%) | 9/9 (100%) | 6/8 (75%) | 8/8 (100%) | 37/42 (88%) |
| Urgent | 1/9 (11%) | 2/8 (25%) | 0/9 (0%) | 2/8 (25%) | 0/8 (0%) | 5/42 (12%) |
| **EuroSCORE II[7] – Predicted mortality** | | | | | | |
| Mean (SD) | 1.7 (0.9) | 1.3 (0.7) | 1.3 (0.4) | 1.1 (0.7) | 1.6 (1.9) | 1.4 (1.0) |
| Median (IQR) | 1.6 (1.0; 2.0) | 1.2 (0.8; 1.6) | 1.4 (1.1; 1.6) | 1.0 (0.7; 1.4) | 0.8 (0.6; 1.6) | 1.2 (0.8; 1.6) |
| min max | 0.8 3.7 | 0.6 2.7 | 0.7 2.0 | 0.5 2.7 | 0.5 6.1 | 0.5 6.1 |
| **The Society of Thoracic Surgeons (STS) Cardiac Surgery Risk Models[309] – STS score** | | | | | | |
| **Risk of operative mortality (%)** | | | | | | |
| Mean (SD) | 1.04 (0.63) | 0.55 (0.22) | 1.40 (1.11) | 1.06 (0.68) | 1.28 (1.37) | 1.07 (0.90) |
| Median (IQR) | 0.88 (0.52; 1.57) | 0.56 (0.37; 0.66) | 0.93 (0.54; 2.51) | 0.87 (0.57; 1.34) | 0.82 (0.66; 1.31) | 0.80 (0.52; 1.20) |
| min max | 0.41 2.02 | 0.27 0.92 | 0.32 3.20 | 0.39 2.49 | 0.16 4.51 | 0.16 4.51 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| **Risk of operative morbidity or mortality (%)** | | | | | | |
| Mean (SD) | 7.4 (3.4) | 13.3 (23.3) | 11.0 (9.7) | 7.8 (3.1) | 8.0 (4.7) | 9.5 (11.1) |
| Median (IQR) | 7.9 (5.5; 9.9) | 5.0 (4.2; 6.9) | 7.8 (7.2; 11.0) | 7.6 (5.5; 9.7) | 7.1 (6.1; 9.1) | 7.3 (5.1; 9.9) |
| min max | 2.1 12.6 | 3.3 70.9 | 2.8 35.6 | 4.0 12.8 | 1.7 18.2 | 1.7 70.9 |
| **Risk of renal failure (%)** | | | | | | |
| Mean (SD) | 1.51 (1.61) | 0.73 (0.60) | 1.41 (0.91) | 0.89 (0.57) | 1.10 (0.68) | 1.14 (0.98) |
| Median (IQR) | 1.12 (0.64; 1.54) | 0.54 (0.42; 0.74) | 1.10 (0.82; 1.95) | 0.80 (0.38; 1.29) | 1.08 (0.64; 1.41) | 0.89 (0.51; 1.54) |
| min max | 0.33 5.62 | 0.27 2.15 | 0.30 3.16 | 0.28 1.87 | 0.17 2.40 | 0.17 5.62 |
| **Risk of permanent stroke (%)** | | | | | | |
| Mean (SD) | 0.85 (0.26) | 0.67 (0.39) | 0.91 (0.39) | 0.95 (0.37) | 0.86 (0.43) | 0.85 (0.37) |
| Median (IQR) | 0.83 (0.62; 0.91) | 0.53 (0.44; 0.78) | 0.92 (0.74; 1.04) | 0.80 (0.74; 1.23) | 0.88 (0.55; 1.12) | 0.81 (0.56; 1.04) |
| min max | 0.56 1.43 | 0.32 1.54 | 0.33 1.50 | 0.44 1.59 | 0.20 1.53 | 0.20 1.59 |
| **Risk of prolonged ventilation (%)** | | | | | | |
| Mean (SD) | 5.4 (2.2) | 2.9 (0.8) | 7.3 (9.0) | 4.4 (1.9) | 5.1 (4.0) | 5.1 (4.7) |
| Median (IQR) | 6.0 (3.4; 6.8) | 2.9 (2.3; 3.7) | 4.4 (3.4; 6.6) | 4.3 (2.8; 5.7) | 4.0 (3.0; 5.8) | 4.0 (3.0; 6.3) |
| min max | 1.8 8.3 | 1.8 4.0 | 1.5 30.9 | 2.1 7.5 | 0.8 14.0 | 0.8 30.9 |
| **Probability of long length of hospital stay (>14d, %)** | | | | | | |
| Mean (SD) | 3.6 (2.0) | 2.1 (1.0) | 4.4 (3.5) | 2.4 (1.3) | 2.9 (2.2) | 3.1 (2.3) |
| Median (IQR) | 3.6 (2.4; 4.0) | 1.9 (1.5; 2.6) | 3.2 (2.3; 5.3) | 2.1 (1.5; 3.2) | 2.6 (1.6; 3.4) | 2.5 (1.6; 3.6) |
| min max | 1.4 8.0 | 0.8 3.9 | 1.2 11.8 | 1.1 4.9 | 0.4 7.7 | 0.4 11.8 |

* Left ventricular functional impairment: mildly impaired – LVEF 31%-50%; moderately impaired - LVEF 21%-30%; severely impaired - LVEF 20% or less

[+] Moderate pulmonary hypertension - PASP 31-55 mmHg

Abbreviations: ACEi – angiotensin converting enzyme inhibitor; AF – atrial fibrillation; ARB – angiotensin receptor blocker; BMI – body mass index; CCS – Canadian Cardiovascular Society Angina Grade; COPD – chronic obstructive pulmonary disease; LVEF – left ventricular ejection fraction; MI – myocardial infarction; NYHA – New York Heart Association functional class; PASP – pulmonary artery systolic pressure; yrs – years.

n – number of participants; SD – standard deviation; IQR – interquartile range; min – minimum; max – maximum.

### 3.2.3 The tolerability profile of 5-ALA-SFC in patients undergoing cardiac surgery on CPB

Overall compliance with the study drug was excellent. Most patients (90%) took every required dose correctly (i.e., 100% compliance) and all took ≥80% of the required doses correctly, a protocol requirement for inclusion in the primary analysis. A single participant in the placebo allocation group took too many capsules at individual dosing timepoints, accounting for the maximum value within this group's compliance dataset (170%). In several patients who had begun taking their study treatment, their surgery was postponed by their clinical care team for logistic reasons unrelated to participation in the trial, resulting in either a prolonged period of dosing (if surgery proceeded within four days), or a period of redosing ahead of a future date for surgery (with the trial protocol providing contingency and guidance for dosing of study drug for both scenarios). These patients would have taken more capsules compared to others in their treatment allocation group, explaining the broad standard deviation (SD) and interquartile range (IQR) for data relating to the number of capsules taken. There was one AE-related treatment interruption during the trial when 1 patient withdrew due to a headache. This patient had a history of regular migraine headaches, and it was felt by their treating clinician at the time the episode represented one of the patient's usual headaches. The headache was self-limiting and did not require medical intervention. As this event led to a study drug interruption and ultimate withdrawal of the participant from the trial, this treatment interruption was judged to be related to the AE.

**Table 15: Study drug compliance.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| **Study drug compliance (%)** | | | | | | |
| 100 % required doses taken | 8/9 (89%) | 8/8 (100%) | 7/9 (78%) | 7/8 (88%) | 8/8 (100%) | 39/42 (93%) |
| ≥ 80% required doses taken | 9/9 (100%) | 8/8 (100%) | 9/9 (100%) | 8/8 (100%) | 8/8 (100%) | 42/42 (100%) |
| ≥1 required dose(s) taken | 9 /9 (100%) | 8/8 (100%) | 9/9 (100%) | 8/8 (100%) | 8/8 (100%) | 42/42 (100%) |
| **Compliance (% required capsules taken)** | | | | | | |
| Mean (SD) | 108 (24) | 100 (0) | 97 (6) | 98 (5) | 100 (0) | 101 (12) |
| Median (IQR) | 100 (100; 100) | 100 (100; 100) | 100 (100; 100) | 100 (100; 100) | 100 (100; 100) | 100 (100; 100) |
| min max | 100 171 | 100 100 | 86 100 | 86 100 | 100 100 | 86 171 |
| **Number of capsules taken** | | | | | | |
| Mean (SD) | 46 (25) | 18 (6) | 46 (23) | 53 (17) | 63 (18) | 45 (24) |
| Median (IQR) | 46 (26; 57) | 14 (14; 22) | 32 (28; 56) | 48 (42; 63) | 56 (56; 64) | 44 (28; 56) |
| min max | 14 94 | 14 28 | 24 89 | 36 84 | 48 104 | 14 104 |

Abbreviations: SD – standard deviation; IQR – interquartile range.

### 3.2.4 The safety profile of 5-ALA-SFC in adult patients undergoing elective cardiac surgery

Across the trial 64% of all participants encountered at least one adverse event (AE). The most common AEs, experienced by more than 5% of participants in the study, were peri-operative hypotension (21%) and AKI (12%). Both hypotension and AKI are anticipated AEs in the context of cardiac surgery (i.e. risks irrespective of interventional clinical trial participation). The majority of AEs (83%) were not attributed to the study treatment by the treating clinician. Seven participants (17%) experienced AEs, graded as mild, that were considered by the treating investigators to be related to the study treatment. Treatment-related AEs (TRAEs) most affected the gastrointestinal system and are a recognised adverse effect of 5-ALA-SFC described in the Investigator Brochure for the drug. These were dyspepsia (5%), nausea (5%), abdominal discomfort (2%) and abdominal pain (2%). One participant (2%) reported having experienced a “fish oil after-taste” following dosing, which was felt to represent a sensory, rather than gastrointestinal, disturbance. This was the only TRAE encountered which had not previously been linked with 5-ALA-SFC. It was judged to be mild and resolved spontaneously.

Nine SAEs in four participants (10%) were reported during the trial. All SAEs were anticipated potential adverse sequelae in the context of cardiac surgery (and had been pre-defined in the trial protocol) and causality was not attributed to the study treatment by treating investigators. In summary, these four participants represented either particularly high-risk surgical candidates who required a prolonged period of CPB (e.g., patients 005 and 031) or participants who suffered severe unexpected intra-operative complications (e.g., 036 and 039). All SAEs observed were defined in the protocol as potentially anticipated in patients undergoing cardiac surgery on CPB and were, therefore, not automatically subject to expedited reporting to regulatory authorities. No SUSARs or instances of DLTs with respect to renal and/or liver function were reported and there were no deaths for the entirety of participant involvement in this trial.

There were no signals for differences in reported safety findings between treatment allocation groups and no dose-associated rise in the frequency of AEs was observed. SAEs occurred in the placebo as well as in active 5-ALA-SFC treatment allocation groups.

AEs are summarised in **table 16**, and individual listings are captured in **table 17**.

**Table 16: Summary of adverse events: grading and causality attribution.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) | Total (n=42) |
|---|---|---|---|---|---|---|
| **Any AE (event count; patient count (%))** | 12; 6 (66.7%) | 9; 3 (37.5%) | 18; 8 + 1* (88.9%) | 12; 5 (62.5%) | 6; 5 (62.5%) | 57; 27 + 1* (64%) |
| **AE severity* (% of event)** | | | | | | |
| Mild | 8 (67%) | 6 (67%) | 13 (72%) | 6 (50%) | 4 (67%) | 37 (64%) |
| Moderate | 3 (25%) | 1 (11%) | 3 (17%) | 2 (17%) | 2 (33%) | 11 (19%) |
| Severe | 1 (8%) | 2 (22%) | 2 (11%) | 4 (33%) | 0 (0%) | 9 (16%) |
| **Any SAE (event count; patient count (%))** | 1; 1 (11. %) | 2; 1 (13%) | 2; 1 (11%) | 4; 1 (13%) | 0 (0 %) | 9; 4 (10%) |
| **Any SUSAR (event count; patient count (%))** | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) |
| **IMP related AEs (event count; patient count (%))** | 1; 1 (11%) | 1; 1 (13%) | 3; 3 (33%) | 1; 1 (13%) | 1; 1 (13%) | 7; 7 (17%) |
| **IMP related SAEs (event count; patient count (%))** | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) | 0; 0 (0%) |
| **IMP interruption (event count; patient count (%))** | 0; 0 (0%) | 0; 0 (0%) | 1; 0 + 1* (0%) | 0; 0 (0 %) | 0; 0 (0 %) | 1; 0 +1* (0%) |

Abbreviations: AE – Adverse event; SAE – Serious adverse event; SUSAR – Suspected unexpected serious adverse reaction; IMP – investigational medicinal product.

* Subject 023 withdrawn from trial, not included in full analysis set (FAS) or primary analysis set (PAS).

## Table 17: Adverse event listings.

| Allocation, ID | AE | Relatedness | Grade | Resolution |
|---|---|---|---|---|
| **Placebo** | | | | |
| 019 | Hypertension | Unrelated | Mild | Resolved |
| | Peralgia paraesthetica | Unrelated | Mild | Unresolved |
| 025 | Hypotension, intra-op | Unrelated | Moderate | Resolved |
| | Acute kidney injury | Unrelated | Mild | Resolved |
| | Hypocalcaemia | Unrelated | Mild | Resolved |
| | Tachyarrhythmia | Unrelated | Mild | Resolved |
| 028 | Hypotension, intra-op | Unrelated | Moderate | Resolved |
| 034 | ***Abdominal pain*** | ***Related*** | ***Mild*** | ***Resolved*** |
| 039 | **Myocardial infarction*** | **Unrelated** | **Severe** | **Resolved⁺** |
| | Acute kidney injury | Unrelated | Moderate | Resolved |
| 041 | Sternal pain | Unrelated | Mild | Resolved |
| | Sternal wound infection | Unrelated | Mild | Resolved |
| **Active: 75 mg/118 mg** | | | | |
| 002 | Haemorrhage | Unrelated | Moderate | Resolved |
| 005 | **Ischaemic stroke*** | **Unrelated** | **Severe** | **Resolved⁺** |
| | **Acute kidney injury*** | **Unrelated** | **Severe** | **Resolved** |
| 013 | ***Fish oil taste*** | ***Related*** | ***Mild*** | ***Resolved*** |
| | Hypotension, intra-op | Unrelated | Mild | Resolved |
| | Hypokalaemia | Unrelated | Mild | Resolved |
| | Hyperglycaemia | Unrelated | Mild | Resolved |
| | Hypotension, post-op | Unrelated | Mild | Resolved |
| | Constipation | Unrelated | Mild | Resolved |
| **Active: 150 mg/236 mg** | | | | |
| 020 | ***Nausea, post-dose*** | ***Related*** | ***Mild*** | ***Resolved*** |
| | Hypotension, intra-op | Unrelated | Mild | Resolved |
| | Nausea, post-op | Unrelated | Mild | Resolved |
| | Hypotension, post-op | Unrelated | Mild | Resolved |
| | Hypertension, post-op | Unrelated | Mild | Resolved |
| | Blurry vision, post-op | Unrelated | Mild | Unresolved |
| 021 | Hypotension, intra-op | Unrelated | Mild | Resolved |
| | Hypotension, post-op | Unrelated | Mild | Resolved |
| | Urinary retention | Unrelated | Moderate | Resolved |
| 022 | Hypotension, intra-op | Unrelated | Mild | Resolved |
| | Transient ischaemic attack | Unrelated | Mild | Resolved |
| 023[#] | Headache | Unrelated | Mild | Resolved |
| 024 | ***Nausea*** | ***Related*** | ***Mild*** | ***Resolved*** |
| 029 | Ileus, post-op | Unrelated | Moderate | Resolved |
| 030 | Hypotension, intra-op | Unrelated | Moderate | Resolved |
| 031 | **Hypotension, post-op*** | **Unrelated** | **Severe** | **Resolved** |
| | **Atrial fibrillation, post-op*** | **Unrelated** | **Severe** | **Resolved** |
| 035 | ***Abdominal discomfort*** | ***Related*** | ***Mild*** | ***Resolved*** |
| **Active: 225 mg /354 mg** | | | | |
| 033 | Hypotension, post-op | Unrelated | Moderate | Resolved |
| | Pleural effusion, post-op | Unrelated | Mild | Unresolved |
| 036 | **Myocardial infarction*** | **Unrelated** | **Severe** | **Resolved⁺** |
| | **Ventricular fibrillation*** | **Unrelated** | **Severe** | **Resolved** |
| | **Hypotension, intra-op*** | **Unrelated** | **Severe** | **Resolved** |

| Allocation, ID | AE | Relatedness | Grade | Resolution |
|---|---|---|---|---|
| | **Acute kidney injury*** | **Unrelated** | **Severe** | **Resolved** |
| | Upper limb weakness | Unrelated | Mild | Unresolved |
| 040 | Acute kidney injury | Unrelated | Mild | Resolved |
| | Hypotension, post-op | Unrelated | Mild | Resolved |
| | Dyspnoea | Unrelated | Moderate | Unresolved |
| 042 | ***Dyspepsia, post-dose*** | ***Related*** | ***Mild*** | ***Resolved*** |
| 043 | Chest numbness, post-op | Unrelated | Mild | Unresolved |
| **Active: 300 mg/472 mg** | | | | |
| 045 | Pericardial effusion, post-op | Unrelated | Mild | Resolved |
| | Transient ischaemic attack | Unrelated | Mild | Resolved |
| 046 | Atrial fibrillation | Unrelated | Moderate | Resolved |
| 047 | Headache | Unrelated | Mild | Unresolved |
| 048 | ***Dyspepsia, post-dose*** | ***Related*** | ***Mild*** | ***Resolved*** |
| 053 | Pericardial effusion, post-op | Unrelated | Moderate | Resolved |

* Serious adverse event. Treatment-related events are italicised.

Abbreviations: AE – adverse event; ID – patient identifier; Intra-op – intra-operative; post-op - post-operative.

All AE data, together with key safety laboratory data and clinical findings for each cohort were reviewed by the SRC prior to further dose escalation. No safety concerns were raised by the SRC at any point, and the trial proceeded without pause to recruitment or dosing. Between dosing cohorts, decisions to dose escalate were unanimously agreed upon by the SRC. All SAEs and relevant clinical data were individually reviewed by the SRC.

### 3.2.5 Peri-operative haemodynamic findings in TALEN trial participants

Intensive monitoring of haemodynamic and respiratory parameters in the peri-operative period was carried out in all TALEN trial participants. Small increases in heart rate (HR) and systolic blood pressure (SBP) were seen consistently between the screening and immediate pre-operative timepoints across all treatment allocation groups (**Fig. 37 A-B**). In the post-operative period, a downward trend in particularly SBP readings was noted as expected in the context of major cardiac surgery and general anaesthesia but the profile of this was indistinguishable between placebo and active treatment groups. Changes in respiratory observations (e.g., oxygen saturation [$SpO_2$], ratio of arterial oxygen partial pressure [PaO2] and fraction of inspiratory oxygen [FiO2] [p:F]) were also consistent between treatment groups (**Fig. 37 C- D**). No significant dose-dependent changes in cardiorespiratory function assessed using standard physiological parameters were observed. Descriptive statistics of haemodynamic data are summarised in Appendix 8.5.

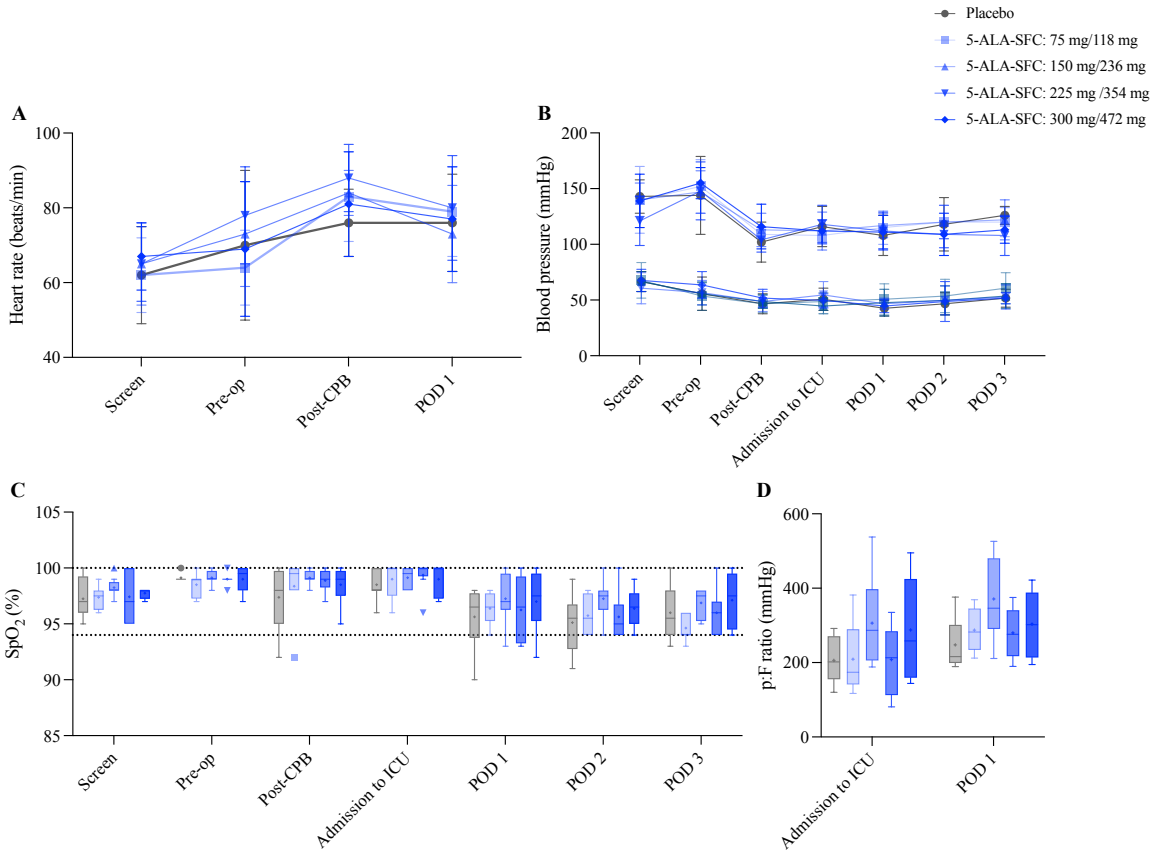



**Figure 37: Effect of 5-ALA-SFC on peri-operative observations.**

Data presented as mean, ± SD (panel A and B); median, IQR, Tukey's upper and lower adjacent values with outside values (panel C and D).

### 3.2.6 The impact of 5-ALA-SFC on cardiac conduction intervals

To examine the potential effects of 5-ALA-SFC dosing on cardiac conduction, 12-lead electrocardiograms (ECGs) performed at screening and on the first post-operative day were compared (**Fig. 38)**. On the first post-operative day, similar prolongation of QTc was seen across all treatment allocation groups. When compared to baseline values, these changes were not clinically significant (i.e., < 1.5 x baseline), and in most patients (86%) the interval remained within normal limits. There were no instances of Torsade du Pointes, a life-threatening manifestation of prolonged $QT_c$, and clinicians did not report any concerning trends in post operative ECG findings. No dose dependant changes in cardiac conduction times, including PR interval, QRS duration and QTc, were observed.

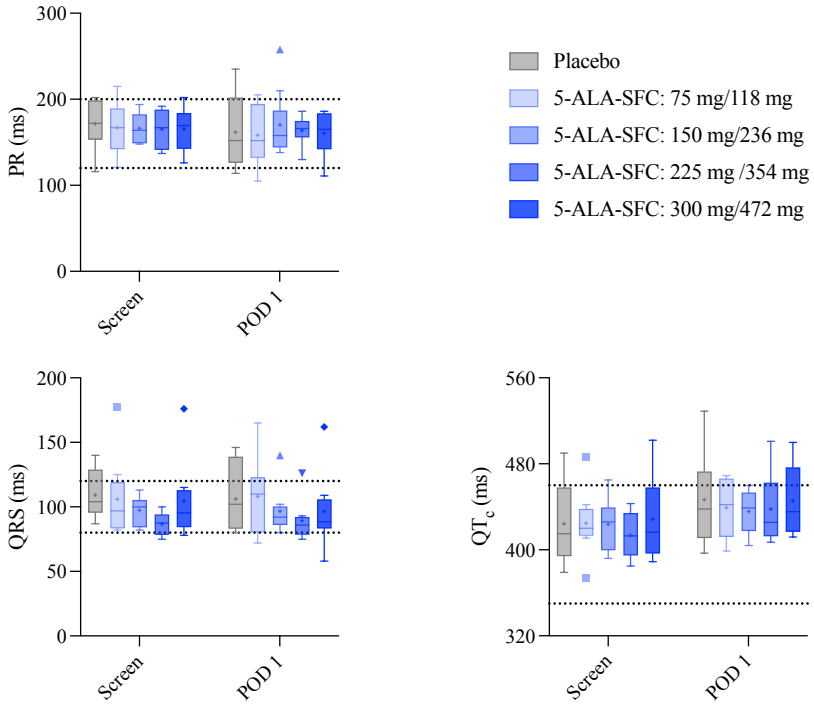



**Figure 38: Cardiac conduction intervals by ECG.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Abbreviations: POD – post-operative day; ms – milliseconds.

**Table 18: Cardiac conduction intervals – descriptive statistics.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Screening** | | | | | |
| **PR (ms)** | | | | | |
| Mean (SD) | 172 (29.4) | 167 (30.9) | 163 (17.8) | 165 (23.1) | 165 (26.1) |
| Median (IQR) | 172 (153; 199) | 167 (142; 190) | 164 (148; 183) | 167 (141; 188) | 170 (143; 184) |
| Min Max | 116 202 | 121 215 | 148 194 | 137 192 | 126 202 |
| **QRS (ms)** | | | | | |
| Mean (SD) | 109 (19.3) | 106 (32.1) | 97 (11.3) | 87 (9.0) | 104 (31.6) |
| Median (IQR) | 104 (96; 129) | 97 (83; 119) | 100 (84; 106) | 88 (78; 94) | 96 (84; 113) |
| Min Max | 87 140 | 82 177 | 82 113 | 75 100 | 78 176 |
| **$QT_c$ (ms)** | | | | | |
| Mean (SD) | 424 (37.4) | 425 (31.4) | 424 (35.7) | 414 (20.9) | 429 (38.8) |
| Median (IQR) | 415 (394; 458) | 420 (413; 438) | 426 (400; 440) | 413 (395; 434) | 417 (397; 458) |
| Min Max | 378 490 | 374 486 | 392 465 | 385 443 | 389 502 |
| **POD 1** | | | | | |
| **PR (ms)** | | | | | |
| Mean (SD) | 161 (54.7) | 158 (35.7) | 170 (13.0) | 164 (17.4) | 160 (27.3) |
| Median (IQR) | 152 (126; 202) | 152 (132; 195) | 158 (144; 187) | 166 (156; 175) | 165 (142; 184) |
| Min Max | 114 235 | 105 205 | 138 258 | 130 186 | 111 186 |
| **QRS (ms)** | | | | | |
| Mean (SD) | 106 (26.5) | 108 (30.4) | 87 (17.7) | 89 (16.0) | 97 (30.1) |
| Median (IQR) | 102 (83; 109) | 110 (80; 123) | 88 (78; 94) | 86 (78; 92) | 89 (83; 106) |
| Min Max | 80 146 | 72 165 | 75 100 | 75 126 | 58 162 |
| **$QT_c$ (ms)** | | | | | |
| Mean (SD) | 447 (45.8) | 439 (27.2) | 436 (19.3) | 438 (32.6) | 446 (32.5) |
| Median (IQR) | 438 (411; 473) | 442 (412; 467) | 439 (418; 454) | 426 (413; 463) | 436 (417; 477) |
| Min Max | 397 529 | 399 469 | 404 460 | 407 501 | 412 500 |

Abbreviations: ms – milliseconds; SEM – standard error of the mean; IQR – interquartile range; Min – minimum; Max – maximum

### 3.2.7 The impact of 5-ALA-SFC and cardiac surgery on haematological indices

Haemoglobin (Hb), white cell count (WCC) and platelets were assayed regularly in all trial participants as part of the safety evaluation and data are summarised in **Fig. 39** and **table 19**. A consistent minor decrease in all indices is noted between the screening and pre-operative time points, across all treatment allocation groups (**Fig. 39 A-C**). The most prominent changes observed were for the Hb and platelet count. The change from baseline and impact of cardiac surgery was comparable across treatment allocation groups (**Fig 39. D-F**) and no statistically significant differences vs. placebo were observed at any dose. No 5-ALA-SFC dose-dependent changes in Hb, WCC or platelet count over time, were observed.

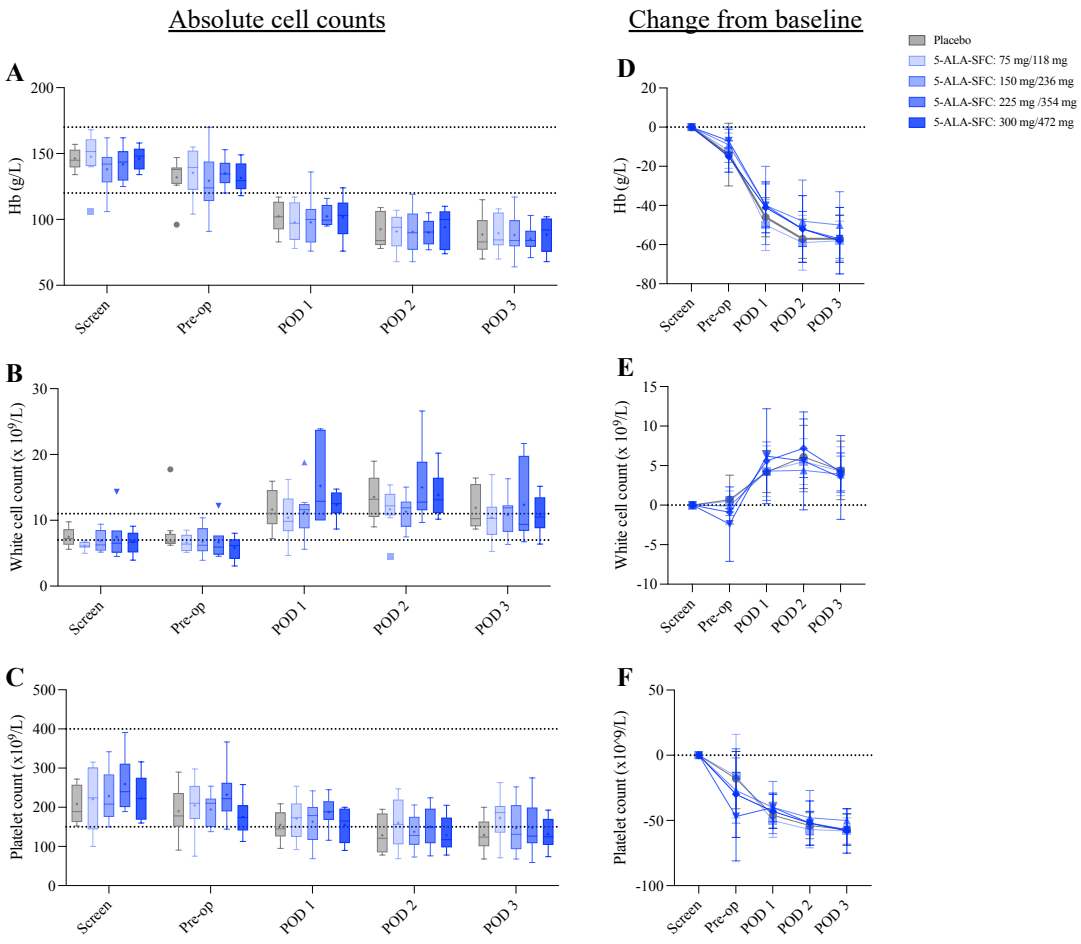



**Figure 39: The impact of 5-ALA-SFC on routine haematological measurements.**

**(Tukey style box and whisker plots) and change from baseline (mean ± SD) in the peri-operative period.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Within the 225 mg/354 mg treatment allocation group there was a more pronounced increase in the WCC in the post-operative period (**Fig. 39 C**). When the PAS (excluding patients who experienced major surgical complications, associated with prolonged CPB time and surgical trauma and subsequent immune activation) was analysed, this difference was not as pronounced. The mean (SD) was 13.78 (5.15) x $10^9$/L cells in the PAS, vs. 20.8 (16.9) x $10^9$/L cells in the FAS. Change from baseline data were comparable across treatment allocation groups. Coagulation was not formally assessed in all patients at all time points and this is not standard practice at the surgical centre, which relies primarily on point of care coagulation assessments (e.g., kaolin activated clotting time [ACT]). Where data were available, these were captured and are included in Appendix 8.6.

Changes observed in haematological laboratory parameters were temporally related to cardiac surgery and across a linear mixed model (LMM) using restricted maximum likelihood (REML), with timepoint (i.e., relationship to cardiac surgery) identified as a significant fixed effect factor (p-value < 0.0001 for all indices).

**Table 19: Descriptive statistics and analyses of haematological indices.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Hb (g/dL)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 146 (8) | 148 (19) | 138 (16) | 142 (13) | 146 (9) |
| Median (IQR) | 145 (143; 152) | 152 (141; 161) | 142 (129; 147) | 144 (130; 151) | 148 (138; 153) |
| Min Max | 134 157 | 106 168 | 106 162 | 125 162 | 134 158 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 132 (15) | 135 (18) | 129 (23) | 135 (10) | 131 (11) |
| Median (IQR) | 138 (128; 139) | 140 (123; 150) | 124 (115; 143) | 135 (128; 141) | 129 (124; 140) |
| Min Max | 96 147 | 104 155 | 91 170 | 120 153 | 118 149 |
| **POD 1** | | | | | |
| Mean (SD) | 100 (15) | 98 (14) | 98 (19) | 102 (8) | 103 (13) |
| Median (IQR) | 102 (92; 113) | 97 (86; 111) | 100 (84; 101) | 100 (97; 108) | 100 (95; 113) |
| Min Max | 76 117 | 78 117 | 76 136 | 95 116 | 86 124 |
| **POD 2** | | | | | |
| Mean (SD) | 92 (14) | 91 (13) | 91 (17) | 90 (10) | 94 (14) |
| Median (IQR) | 84 (82; 106) | 94 (81; 101) | 90 (79; 100) | 91 (82; 97) | 99 (82; 105) |
| Min Max | 74 109 | 68 107 | 68 119 | 77 105 | 74 110 |
| **POD 3** | | | | | |
| Mean (SD) | 92 (14) | 91 (13) | 91 (17) | 90 (10) | 94 (14) |
| Median (IQR) | 84 (82; 106) | 94 (81; 101) | 90 (79; 100) | 91 (82; 97) | 99 (82; 105) |
| Min Max | 74 109 | 68 107 | 68 119 | 77 105 | 74 110 |

**White blood cell count (x $10^9$/L)**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Screening** | | | | | |
| Mean (SD) | 7.5 (1.4) | 6.1 (0.6) | 6.9 (1.6) | 14.6 (20.4) | 6.7 (1.7) |
| Median (IQR) | 7.2 (6.6; 8.1) | 6.1 (5.9; 6.7) | 6.3 (5.5; 7.8) | 7.1 (5.4; 11.4) | 6.7 (5.6; 8.1) |
| Min Max | 5.6 9.8 | 5.0 6.9 | 5.2 9.4 | 4.5 64.5 | 4.0 9.1 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 8.1 (3.7) | 6.6 (1.3) | 6.8 (2.1) | 12.2 (15.7) | 5.8 (1.7) |
| Median (IQR) | 6.7 (6.5; 7.5) | 6.4 (5.5; 7.8) | 6.2 (5.9; 8.7) | 6.2 (4.9; 10.0) | 6.2 (4.7; 6.9) |
| Min Max | 6.2 17.7 | 5.1 8.5 | 3.9 10.4 | 4.5 50.6 | 3.1 8.1 |
| **POD 1** | | | | | |
| Mean (SD) | 11.7 (3.0) | 10.4 (3.6) | 11.2 (3.6) | 20.8 (16.9) | 12.3 (2.0) |
| Median (IQR) | 11.1 (9.8; 13.9) | 9.9 (8.5; 12.9) | 11.6 (9.4; 12.2) | 13.5 (11.0; 23.8) | 12.5 (11.3; 13.8) |
| Min Max | 7.2 15.9 | 4.7 16.2 | 5.6 18.8 | 10.0 60.2 | 8.7 14.8 |
| **POD 2** | | | | | |
| Mean (SD) | 13.5 (3.4) | 11.7 (3.3) | 11.3 (2.4) | 20.2 (15.6) | 13.9 (3.4) |
| Median (IQR) | 13.2 (11.1; 16.4) | 12.2 (11.1; 13.5) | 11.9 (9.2; 12.5) | 13.2 (11.7; 22.8) | 13.1 (11.5; 15.6) |
| Min Max | 9.0 19.0 | 4.4 15.4 | 7.5 15.1 | 9.7 56.4 | 10.2 20.2 |
| **POD 3** | | | | | |
| Mean (SD) | 11.9 (3.3) | 10.4 (3.5) | 10.8 (3.1) | 18.1 (17.2) | 10.9 (2.9) |
| Median (IQR) | 10.3 (9.1; 15.1) | 10.3 (8.3; 12.0) | 11.9 (9.5; 12.1) | 10.5 (8.6; 20.7) | 10.5 (9.1; 13.2) |
| Min Max | 8.7 16.4 | 5.3 17.0 | 6.3 16.3 | 6.7 58.5 | 6.4 15.2 |
| **Platelet count (x $10^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 208 (48) | 221 (79) | 232 (69) | 260 (69) | 223 (57) |
| Median (IQR) | 189 (170; 248) | 224 (162; 290) | 222 (176; 285) | 240 (209; 299) | 222 (169; 263) |
| Min Max | 152 272 | 100 315 | 150 342 | 189 391 | 160 316 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 190 (61) | 205 (67) | 194 (41) | 232 (67) | 176 (46) |
| Median (IQR) | 178 (154; 210) | 210 (178; 244) | 210 (158; 219) | 222 (191; 261) | 174 (142; 203) |
| Min Max | 91 290 | 75 298 | 138 254 | 144 367 | 113 258 |
| **POD 1** | | | | | |
| Mean (SD) | 155 (38) | 171 (53) | 163 (54) | 188 (38) | 155 (43) |
| Median (IQR) | 145 (141; 182) | 174 (135; 204) | 179 (120; 193) | 189 (172; 210) | 165 (119; 193) |
| Min Max | 95 209 | 92 254 | 69 242 | 116 245 | 90 200 |
| **POD 2** | | | | | |
| Mean (SD) | 129 (47) | 160 (64) | 138 (44) | 150 (50) | 130 (45) |
| Median (IQR) | 121 (88; 181) | 157 (112; 215) | 128 (106; 165) | 149 (115; 187) | 118 (99; 161) |
| Min Max | 78 195 | 69 247 | 73 206 | 76 224 | 78 205 |
| **POD 3** | | | | | |
| Mean (SD) | 129 (40) | 173 (58) | 147 (66) | 148 (68) | 132 (40) |
| Median (IQR) | 124 (105; 162) | 187 (136; 203) | 131 (111; 164) | 127 (109; 190) | 125 (108; 160) |
| Min Max | 68 200 | 71 263 | 68 252 | 59 275 | 74 193 |
| **Change in haematological indices, active vs placebo (LMM using REML)** | | | | | |
| **Hb (g/dL)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 132.0 | 135.4 | 129.3 | 135.0 | 131.5 |
| Mean difference vs placebo (SE) | | 3.4 (7.9) | -2.7 (9.1) | 3.0 (6.2) | -0.5 (6.3) |
| p-value | | 0.6793 | 0.7737 | 0.6347 | 0.9378 |
| **POD 1** | | | | | |
| Observed mean | 102.3 | 97.9 | 97.8 | 102.4 | 101.8 |
| Mean difference vs placebo (SE) | | -4.4 (6.5) | -4.5 (7.5) | 0.1 (5.0) | -0.5 (6.8) |
| p-value | | 0.5058 | 0.5551 | 0.9935 | 0.9332 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 2** | | | | | |
| Observed mean | 92.7 | 90.9 | 90.6 | 90.1 | 94.0 |
| Mean difference vs placebo (SE) | | -1.8 (6.5) | -2.1 (7.1) | -2.6 (5.7) | 1.3 (6.8) |
| p-value | | 0.7872 | 0.7714 | 0.6619 | 0.8475 |
| **POD 3** | | | | | |
| Observed mean | 88.6 | 89.6 | 88.1 | 85.1 | 88.4 |
| Mean difference vs placebo (SE) | | 1.0 (7.0) | -0.5 (7.2) | -3.5 (6.0) | -0.2 (6.8) |
| p-value | | 0.8810 | 0.9518 | 0.5761 | 0.9792 |
| **White blood cell count (x $10^9$/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 8.12 | 6.61 | 6.77 | 6.67 | 5.84 |
| Mean difference vs placebo (SE) | | -1.51 (1.13) | -1.35 (1.43) | -1.45 (0.92) | -2.28 (1.45) |
| p-value | | 0.2229 | 0.3705 | 0.1654 | 0.1590 |
| **POD 1** | | | | | |
| Observed mean | 11.65 | 10.41 | 11.23 | 15.23 | 12.34 |
| Mean difference vs placebo (SE) | | -1.24 (1.66) | -0.42 (1.74) | 3.58 (1.54) | 0.69 (1.07) |
| p-value | | 0.4788 | 0.8134 | 0.0597 | 0.5435 |
| **POD 2** | | | | | |
| Observed mean | 13.55 | 11.67 | 11.33 | 14.99 | 13.87 |
| Mean difference vs placebo (SE) | | -1.88 (1.79) | -2.22 (1.56) | 1.44 (2.69) | 0.32 (1.14) |
| p-value | | 0.3291 | 0.1909 | 0.6115 | 0.7872 |
| **POD 3** | | | | | |
| Observed mean | 11.90 | 10.43 | 10.83 | 12.36 | 10.91 |
| Mean difference vs placebo (SE) | | -1.47 (1.87) | -1.07 (1.73) | 0.46 (2.90) | 0.99 (0.99) |
| p-value | | 0.4577 | 0.5534 | 0.8786 | 0.3515 |
| **Platelet count (x $10^9$/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 190.2 | 204.6 | 194.4 | 232.3 | 175.9 |
| Mean difference vs placebo (SE) | | 14.4 (31.2) | 4.2 (24.4) | 42.1 (31.1) | -14.3 (25.9) |
| p-value | | 0.6509 | 0.8651 | 0.1974 | 0.5885 |
| **POD 1** | | | | | |
| Observed mean | 155.2 | 171.3 | 163.3 | 187.8 | 155.4 |
| Mean difference vs placebo (SE) | | 16.1 (22.7) | 8.1 (22.0) | 32.6 (18.7) | 0.2 (19.9) |
| p-value | | 0.4923 | 0.7179 | 0.1019 | 0.9940 |
| **POD 2** | | | | | |
| Observed mean | 128.7 | 160.3 | 137.6 | 150.1 | 129.5 |
| Mean difference vs placebo (SE) | | 31.6 (27.7) | 8.9 (21.7) | 21.4 (23.7) | 0.8 (22.32) |
| p-value | | 0.2746 | 0.6871 | 0.3801 | 0.9707 |
| **POD 3** | | | | | |
| Observed mean | 129.3 | 173.1 | 146.9 | 147.9 | 131.5 |
| Mean difference vs placebo (SE) | | 43.8 (24.6) | 17.6 (25.9) | 18.6 (27.6) | 2.2 (19.4) |
| p-value | | 0.0998 | 0.5088 | 0.5150 | 0.9126 |

Abbreviations: SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – Standard error of the mean.

Linear mixed model with Restricted Maximum Likelihood. Correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 3.2.8 The impact of 5-ALA-SFC and cardiac surgery on markers of liver injury

Monitoring for potential liver injury during the trial included regularly assaying ALT, ALP, albumin and bilirubin in all trial participants. Across the trial, no 5-ALA-SFC dose-dependent changes in ALT, ALP or albumin were observed. Increases in bilirubin at the pre-operative timepoint were significantly different vs. placebo with a dose-dependent effect consistent with a pharmacodynamic effect of HO-1 activation - this finding is discussed in a subsequent section of this thesis. Importantly, the mean increase in bilirubin observed at this timepoint in the group receiving even the highest dose of 5-ALA-SFC (300 mg/472 mg) did not exceed 1.5 x ULN and did not constitute a protocol-defined DLT.

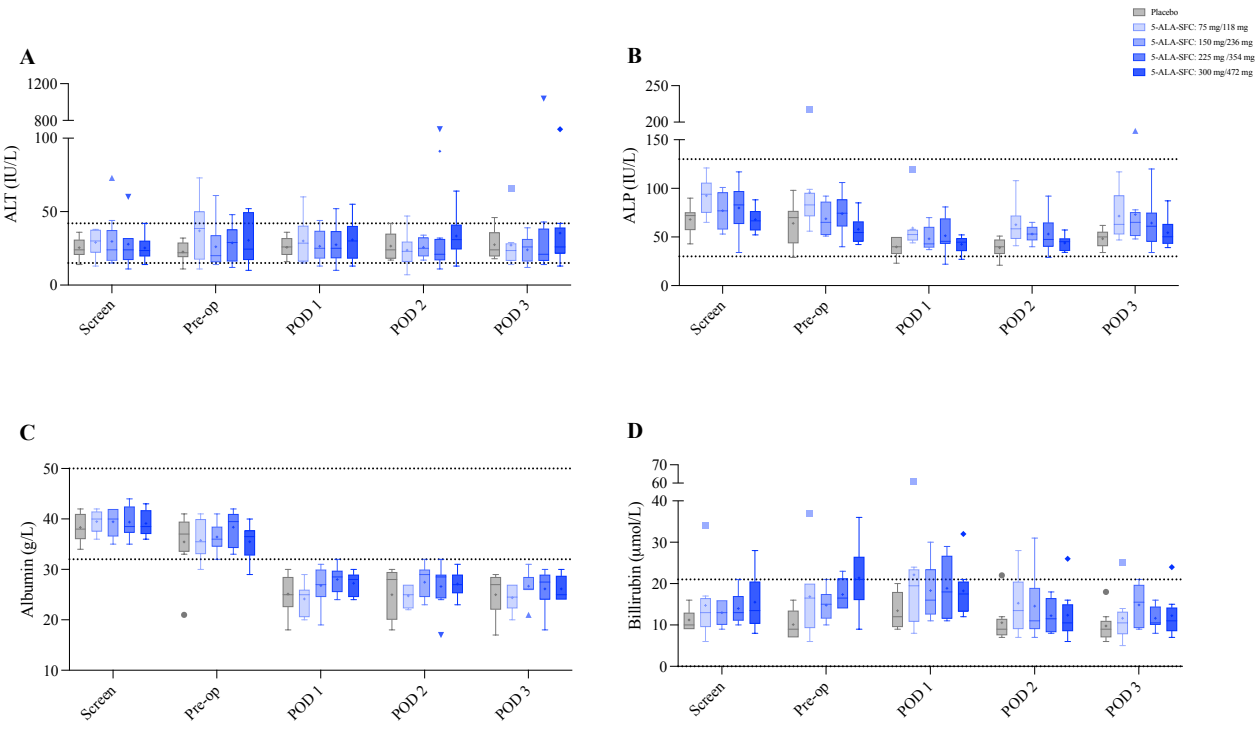



**Figure 40: The impact of 5-ALA-SFC on circulating biochemical parameters of liver injury and function.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Abbreviations: ALT - alanine transaminase; ALP – alkaline phosphatase; Pre-op – pre-operative; POD – post-operative day.

There were significant individual increases in ALT, particularly in the post-operative period, and individual patient level data are summarised in **Fig. 41**. In the 5-ALA-SFC 75 mg/118 mg treatment allocation group a single patient had a pre-operative rise in ALT which did not exceed 1.5 x ULN, was transient and returned to baseline levels in the post-operative period. In the 5-ALA-SFC 225 mg/354 mg treatment allocation group, a single patient had significantly elevated ALT values on the second and third post-operative days. This accounts for the higher mean and outlier datapoints in this group. This individual had not displayed a significant pre-operative rise from baseline (i.e., the period assumed to be most reflective of an isolated drug effect without the confounder of changes due to cardiac surgery). The rise was likely to reflect the occurrence of an intra-operative MI with an associated period of profound cardiovascular instability in this patient rather than DILI. A further participant, within the 5-ALA-SFC 300 mg/472 mg treatment allocation group, had an isolated ALT rise on the third post-operative day, which returned to baseline during their hospital admission. Due to the temporal relationship with the final dose of the study treatment (> 72 hours) and the drug's short half-life, this was judged to be unrelated to 5-ALA-SFC.

ALP data remained within normal limits and were comparable between all allocation groups with no significant differences identified between them. Albumin levels decreased in the post-operative period in all treatment groups as expected in the context of major surgical stress but there were no statistically significant differences when considering patients treated with 5-ALA-SFC vs. placebo. The temporal relationship to surgery was the most important factor influencing albumin levels, and within a LMM using REML, timepoint was the most significant fixed factor influencing the model (p-value < 0.0001). There were no significant differences between treatment groups.

Finally, a single patient had an elevated bilirubin level > 1.5 x ULN which remained elevated peri-operatively. This individual had a diagnosis of Gilbert's syndrome at baseline, and the elevation was not ascribed to 5-ALA-SFC.

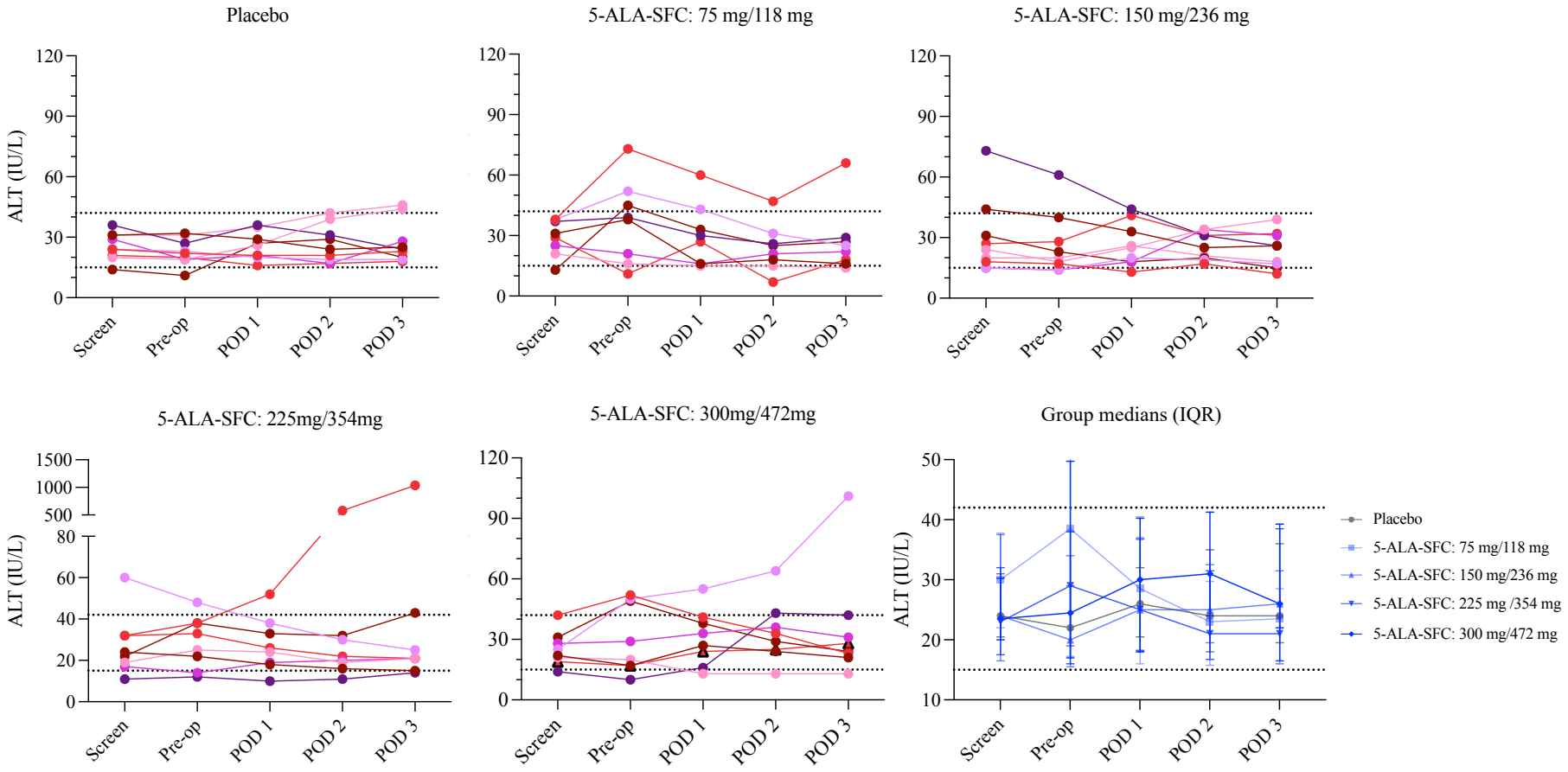



**Figure 41: Alanine transaminase -individual participant data.**

**Table 20: Biochemical parameters of hepatoxicity and function.**

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **ALT (IU/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 25 (7) | 29 (9) | 30 (19) | 30 (17) | 25 (9) |
| Median (IQR) | 24 (21; 30) | 30 (23; 38) | 24 (18; 31) | 28 (19; 32) | 24 (20; 30) |
| Min Max | 14 36 | 13 38 | 15 73 | 11 60 | 14 42 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 23 (7) | 37 (21) | 26 (15) | 29 (13) | 31 (17) |
| Median (IQR) | 22 (19; 27) | 39 (19; 49) | 20 (17; 28) | 29 (18; 38) | 25 (17; 50) |
| Min Max | 11 32 | 11 73 | 14 61 | 12 48 | 10 52 |
| **POD 1** | | | | | |
| Mean (SD) | 26 (7) | 30 (16) | 26 (11) | 28 (13) | 31 (14) |
| Median (IQR) | 26 (21; 29) | 29 (16; 38) | 25 (18; 33) | 25 (19; 36) | 30 (20; 40) |
| Min Max | 16 36 | 15 60 | 13 44 | 10 52 | 13 55 |
| **POD 2** | | | | | |
| Mean (SD) | 27 (9) | 24 (12) | 26 (7) | 101 (211) | 33 (15) |
| Median (IQR) | 24 (19; 31) | 23 (17; 29) | 25 (20; 31) | 20 (16; 32) | 31 (25; 40) |
| Min Max | 17 42 | 7 47 | 17 34 | 11 579 | 13 64 |
| **POD 3** | | | | | |
| Mean (SD) | 27 (10) | 27 (17) | 24 (9) | 150 (359) | 35 (28) |
| Median (IQR) | 24 (20; 28) | 24 (17; 28) | 26 (17; 31) | 21 (18; 34) | 26 (22; 37) |
| Min Max | 18 46 | 14 66 | 12 39 | 14 1038 | 13 101 |
| **ALP (IU/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 68 (14) | 92 (19) | 77 (19) | 80 (26) | 68 (12) |
| Median (IQR) | 72 (64; 75) | 94 (78; 105) | 77 (60; 96) | 83 (66; 96) | 67 (59; 76) |
| Min Max | 43 90 | 65 121 | 53 101 | 34 117 | 52 88 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 64 (21) | 96 (51) | 69 (18) | 74 (20) | 58 (14) |
| Median (IQR) | 70 (46; 77) | 83 (73; 92) | 65 (53; 83) | 75 (62; 87) | 55 (47; 66) |
| Min Max | 29 98 | 56 218 | 51 92 | 40 106 | 42 85 |
| **POD 1** | | | | | |
| Mean (SD) | 40 (10) | 59 (25) | 48 (12) | 51 (19) | 42 (9) |
| Median (IQR) | 40 (34; 50) | 53 (47; 57) | 43 (40; 60) | 46 (43; 66) | 45 (37; 49) |
| Min Max | 23 50 | 44 119 | 37 70 | 22 81 | 27 52 |
| **POD 2** | | | | | |
| Mean (SD) | 39 (10) | 63 (21) | 53 (8) | 53 (20) | 44 (8) |
| Median (IQR) | 40 (34; 46) | 59 (48; 70) | 53 (49; 58) | 48 (42; 62) | 45 (36; 48) |
| Min Max | 21 51 | 41 108 | 40 65 | 29 92 | 34 57 |
| **POD 3** | | | | | |
| Mean (SD) | 48 (9) | 72 (25) | 73 (33) | 64 (26) | 54 (16) |
| Median (IQR) | 50 (42; 53) | 63 (53; 88) | 65 (54; 74) | 61 (47; 73) | 50 (45; 60) |
| Min Max | 34 62 | 47 117 | 48 151 | 34 120 | 39 87 |
| **Albumin (g/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 38 (3) | 40 (2) | 39 (3) | 39 (3) | 39 (3) |
| Median (IQR) | 38 (37; 40) | 40 (38; 41) | 40 (37; 42) | 39 (38; 42) | 39 (37; 42) |
| Min Max | 34 42 | 36 42 | 35 42 | 35 44 | 36 43 |
| **Pre-operatively** | | | | | |

| Readout Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 35 (6) | 36 (4) | 36 (3) | 38 (4) | 36 (4) |
| Median (IQR) | 37 (34; 39) | 36 (33; 39) | 36 (35; 38) | 40 (36; 41) | 37 (34; 38) |
| Min Max | 21 41 | 30 41 | 32 41 | 33 42 | 29 40 |
| **POD 1** | | | | | |
| Mean (SD) | 25 (4) | 24 (3) | 27 (4) | 28 (3) | 27 (2) |
| Median (IQR) | 25 (23; 28) | 25 (21; 26) | 27 (25; 30) | 29 (26; 30) | 28 (25; 29) |
| Min Max | 18 30 | 20 29 | 19 31 | 24 32 | 24 30 |
| **POD 2** | | | | | |
| Mean (SD) | 25 (5) | 25 (2) | 27 (3) | 27 (5) | 27 (3) |
| Median (IQR) | 28 (21; 29) | 25 (23; 27) | 29 (25; 30) | 29 (25; 29) | 27 (26; 29) |
| Min Max | 18 30 | 22 27 | 23 32 | 17 32 | 23 31 |
| **POD 3** | | | | | |
| Mean (SD) | 25 (4) | 24 (3) | 27 (3) | 26 (4) | 26 (3) |
| Median (IQR) | 27 (22; 28) | 25 (23; 27) | 26 (26; 28) | 28 (24; 29) | 25 (24; 29) |
| Min Max | 17 29 | 20 27 | 21 31 | 18 30 | 24 30 |
| **Bilirubin (µmol/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 11 (2) | 15 (8) | 13 (3) | 14 (4) | 16 (7) |
| Median (IQR) | 10 (9; 13) | 13 (10; 16) | 13 (10; 16) | 14 (11; 17) | 14 (11; 20) |
| Min Max | 9 16 | 6 34 | 9 16 | 10 21 | 8 28 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 10 (3) | 17 (10) | 15 (4) | 17 (4) | 21 (8) |
| Median (IQR) | 9 (7; 13) | 17 (10; 20) | 15 (12; 16) | 17 (14; 21) | 21 (16; 26) |
| Min Max | 7 16 | 6 37 | 10 21 | 14 23 | 9 36 |
| **POD 1** | | | | | |
| Mean (SD) | 13 (4) | 22 (17) | 18 (7) | 19 (7) | 18 (6) |
| Median (IQR) | 12 (10; 17) | 20 (12; 23) | 16 (13; 20) | 18 (12; 26) | 18 (14; 20) |
| Min Max | 9 20 | 8 61 | 11 30 | 11 29 | 12 32 |
| **POD 2** | | | | | |
| Mean (SD) | 11 (5) | 15 (7) | 15 (8) | 12 (4) | 12 (6) |
| Median (IQR) | 9 (8; 11) | 14 (10; 20) | 11 (10; 17) | 11 (8; 17) | 11 (9; 14) |
| Min Max | 7 22 | 7 28 | 7 31 | 8 18 | 6 26 |
| **POD 3** | | | | | |
| Mean (SD) | 10 (4) | 12 (6) | 15 (5) | 12 (3) | 12 (5) |
| Median (IQR) | 9 (8; 10) | 11 (9; 13) | 16 (10; 20) | 11 (10; 14) | 11 (9; 14) |
| Min Max | 6 18 | 5 25 | 9 21 | 8 16 | 7 24 |
| **Change in hepatic biochemical indices, active vs placebo (LMM using REML)** | | | | | |
| **ALT (IU/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 22.67 | 36.88 | 26.11 | 28.75 | 30.50 |
| Mean difference vs placebo (SE) | | 14.21 (7.60) | 3.44 (5.59) | 6.08 (4.98) | 7.83 (6.48) |
| p-value | | 0.0972 | 0.5504 | 0.2489 | 0.2582 |
| **POD 1** | | | | | |
| Observed mean | 25.67 | 30.00 | 26.44 | 27.50 | 30.88 |
| Mean difference vs placebo (SE) | | 4.33 (5.97) | 0.77 (4.26) | 1.83 (5.20) | 5.21 (5.40) |
| p-value | | 0.4857 | 0.8578 | 0.7318 | 0.3579 |
| **POD 2** | | | | | |
| Observed mean | 26.56 | 23.75 | 25.75 | 91.13 | 33.38 |
| Mean difference vs placebo (SE) | | -2.81 (5.24) | -0.77 (3.85) | 64.57 (69.81) | 6.82 (6.23) |
| p-value | | 0.6014 | 0.8426 | 0.3856 | 0.2962 |

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 3** | | | | | |
| Observed mean | 27.44 | 27.13 | 24.00 | 149.80 | 35.38 |
| Mean difference vs placebo (SE) | | -0.31 (6.82) | -3.44 (4.61) | 122.36 (127.00) | 7.94 (10.43) |
| p-value | | 0.9634 | 0.4657 | 0.3675 | 0.4671 |
| **ALP (IU/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 64.11 | 96.13 | 68.78 | 73.88 | 57.75 |
| Mean difference vs placebo (SE) | | 32.02 (19.33) | 4.67 (9.25) | 9.77 (10.18) | -6.36 (8.74) |
| p-value | | 0.3461 | 0.9630 | 0.7482 | 0.8799 |
| **POD 1** | | | | | |
| Observed mean | 39.78 | 59.38 | 48.22 | 51.38 | 42.25 |
| Mean difference vs placebo (SE) | | 19.60 (9.28) | 8.44 (5.17) | 11.60 (7.38) | 2.47 (4.42) |
| p-value | | 0.1828 | 0.3356 | 0.3822 | 0.9476 |
| **POD 2** | | | | | |
| Observed mean | 38.89 | 62.63 | 53.00 | 53.00 | 43.63 |
| Mean difference vs placebo (SE) | | 23.74 (8.19) | 14.11 (4.24) | 14.11 (7.62) | 4.74 (4.28) |
| p-value | | 0.0520 | **0.0150*** | 0.2597 | 0.6517 |
| **POD 3** | | | | | |
| Observed mean | 48.22 | 71.50 | 73.00 | 64.38 | 54.25 |
| Mean difference vs placebo (SE) | | 23.28 (9.22) | 24.78 (12.08) | 16.16 (9.82) | 6.03 (6.304) |
| p-value | | 0.1004 | 0.2092 | 0.3573 | 0.7532 |
| **Albumin (g/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 35.44 | 35.75 | 36.44 | 38.38 | 35.50 |
| Mean difference vs placebo (SE) | | 0.31 (2.45) | 1.00 (2.22) | 2.94 (2.38) | 0.06 (2.38) |
| p-value | | 0.9025 | 0.6609 | 0.2406 | 0.9817 |
| **POD 1** | | | | | |
| Observed mean | 25.11 | 24.13 | 26.78 | 28.00 | 27.25 |
| Mean difference vs placebo (SE) | | -0.98 (1.71) | 1.67 (1.78) | 2.89 (1.58) | 2.14 (1.53) |
| p-value | | 0.5728 | 0.3634 | 0.0877 | 0.1835 |
| **POD 2** | | | | | |
| Observed mean | 25.00 | 24.75 | 27.44 | 26.63 | 27.13 |
| Mean difference vs placebo (SE) | | -0.25 (1.85) | 2.44 (1.96) | 1.63 (2.32) | 2.13 (1.87) |
| p-value | | 0.8949 | 0.2329 | 0.4940 | 0.2775 |
| **POD 3** | | | | | |
| Observed mean | 25.00 | 24.38 | 26.67 | 26.13 | 26.13 |
| Mean difference vs placebo (SE) | | -0.62 (1.67) | 1.67 (1.67) | 1.13 (1.98) | 1.13 (1.66) |
| p-value | | 0.7145 | 0.3345 | 0.5786 | 0.5088 |

Abbreviations: SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – Standard error of the mean.

Linear mixed model with Restricted Maximum Likelihood. Correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 3.2.9 The impact of 5-ALA-SFC and cardiac surgery on electrolytes and acid-base balance

Across the study, a slight decrease in sodium levels in all treatment allocation groups was observed on post-operative days two and three (**Fig. 42 A**). Two patients developed moderate hyponatraemia ($Na^+$ = 125 – 129 mmol/L). These instances occurred in the 225 mg/354 mg and 300 mg/472 mg groups on the second post-operative (129 mmol/L) and third post-operative day (127 mmol/L), respectively. All other episodes of hyponatraemia were mild ($Na^+$ = 130 – 135 mmol/L). 7 patients (17%) developed instances of clinically important hyperkalaemia (i.e., K+ > 5.3 mmol/L) (**Fig. 42 B**). Post-operatively, slightly lower K+ levels were observed in patients who had been allocated to receive 5-ALA-SFC, but this was not statistically significant. Fluctuations in sodium and potassium measurements were transient, consistent with changes expected post cardiac surgery on CPB, required no specific medical intervention and were judged by treating clinicians to be unrelated to 5-ALA-SFC.

Hyperchloremic metabolic acidosis was the most common acid-base disturbance observed in this study (**Fig. 42 C-E**). This is characterised by a pH < 7.35 (or normal if compensated), standard bicarbonate < 23 mmol/L and chloride > 105 mmol/L. There was a temporal relationship between surgery and the most profound increase in chloride and within the statistical model applied for analysis, time was identified as the most significant fixed effect ($p < 0.0001$). The data suggested that patients allocated to receive 5-ALA-SFC tended to higher pH readings on the first post-operative day, tending towards less acidosis. In the 300 mg/472 mg treatment allocation group, for example, a mean difference (SE) of 0.04 (0.02) with a p-value of 0.0314 was observed vs. placebo.

Most patients developed hyperglycaemia intra-operatively consistent with a stress response to surgery, without clear differences between treatment groups (**Fig. 42 F**). There was a marked increase in lactate at the end of CPB in all patients (**Fig. 42 G**). Data suggest a potential impact of 5-ALA-SFC to decrease lactate/impact anaerobic metabolism post-operatively. At the post-CPB timepoint, this was statistically significant for the 5-ALA-SFC 300 mg/472 mg treatment allocation group (mean difference (SE) vs. placebo = -1.24 (0.52) mmol/L, p-value = 0.0383).

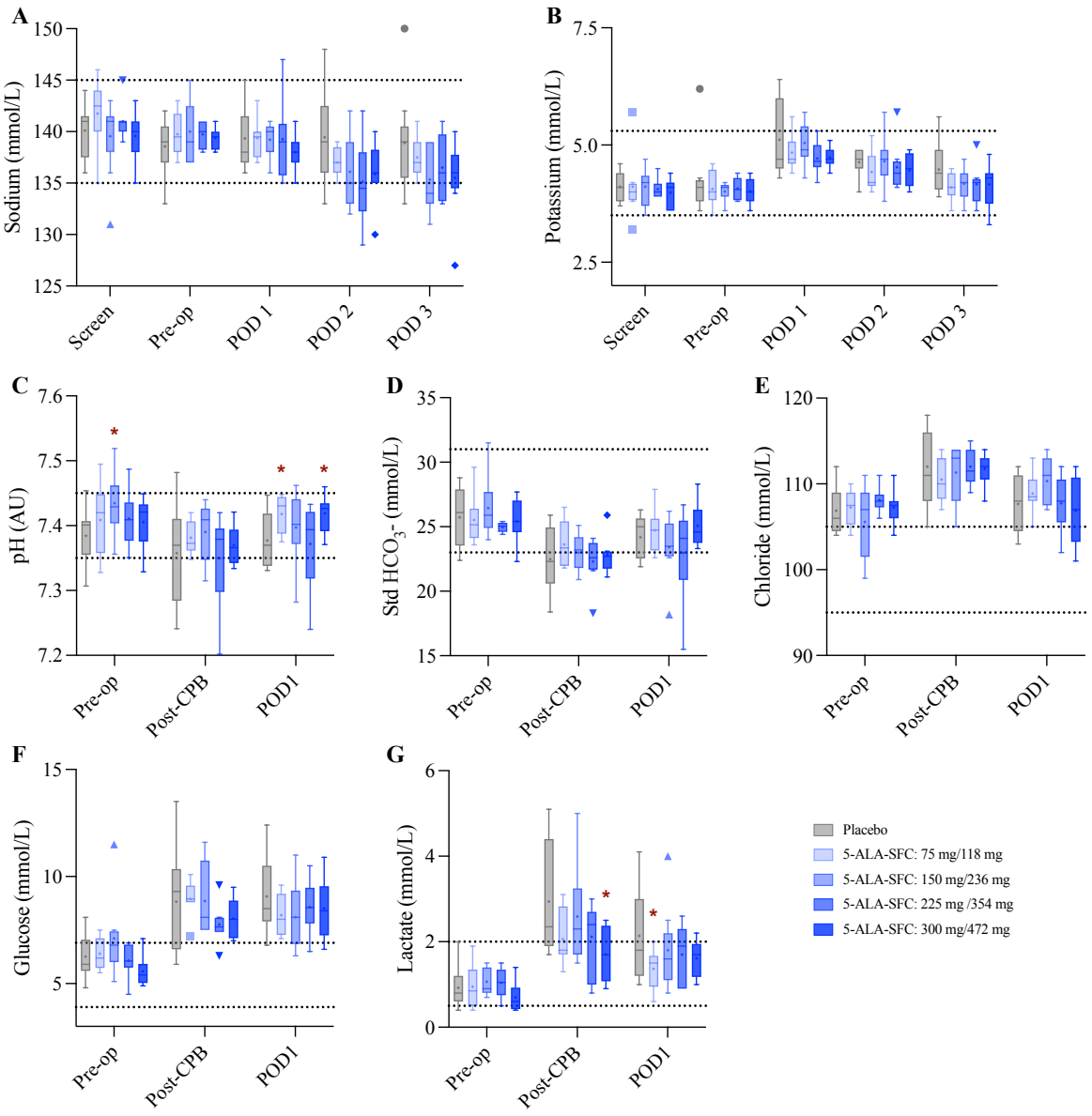



**Figure 42: Electrolyte and acid-base indices.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values. * p-value < 0.05.

Abbreviations: POD – post-operative day; Std $HCO_3$- – standard bicarbonate.

## Table 21: Effects of 5-ALA-SFC on peri-operative electrolyte and acid-base balance.

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Sodium (mmol/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 140 (0.9) | 141 (1.2) | 140 (1.2) | 141 (0.6) | 140 (1.0) |
| Median (IQR) | 141 (138; 142) | 143 (140; 144) | 141 (138; 142) | 141 (140; 141) | 140 (138; 141) |
| Min Max | 136 144 | 135 146 | 131 143 | 139 145 | 135 143 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 139 (0.9) | 140 (0.8) | 140 (1.0) | 140 (0.5) | 140 (0.4) |
| Median (IQR) | 139 (137; 141) | 140 (138; 142) | 139 (137; 143) | 140 (138: 141) | 140 (138; 140) |
| Min Max | 133 142 | 137 143 | 137 145 | 138 141 | 138 141 |
| **POD 1** | | | | | |
| Mean (SD) | 139 (1.0) | 140 (0.7) | 139 (0.6) | 139 (1.4) | 138 (0.6) |
| Median (IQR) | 138 (137; 142) | 140 (138; 140) | 140 (138; 141) | 139 (136; 141) | 138 (137; 139) |
| Min Max | 133 150 | 137 143 | 136 141 | 135 147 | 135 141 |
| **POD 2** | | | | | |
| Mean (SD) | 139 (1.5) | 137 (0.5) | 136 (1.1) | 135 (1.4) | 136 (1.1) |
| Median (IQR) | 139 (136; 143) | 137 (136; 139) | 135 (133; 139) | 135 (132; 138) | 136 (135; 138) |
| Min Max | 133 150 | 135 139 | 132 142 | 129 142 | 130 140 |
| **POD 3** | | | | | |
| Mean (SD) | 138 (4.9) | 138 (0.7) | 135 (1.0) | 137 (1.1) | 136 (1.4) |
| Median (IQR) | 139 (136; 141) | 137 (136; 139) | 134 (133; 139) | 136 (133; 140) | 136 (135; 138) |
| Min Max | 133 150 | 135 141 | 131 139 | 133 141 | 127 140 |
| **Potassium (mmol/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 4.1 (0.1) | 4.1 (0.3) | 4.1 (0.1) | 4.1 (0.1) | 4.0 (0.1) |
| Median (IQR) | 4.1 (3.8; 4.4) | 4.0 (3.8; 4.2) | 4.2 (3.7; 4.4) | 4.0 (3.9; 4.2) | 4.1 (3.6; 4.2) |
| Min Max | 3.7 4.6 | 3.2 5.7 | 3.5 4.7 | 3.9 4.5 | 3.6 4.4 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 4.2 (0.3) | 4.1 (0.1) | 4.0 (0.1) | 4.1 (0.1) | 4.0 (0.1) |
| Median (IQR) | 4.1 (3.8 (4.3) | 4.0 (3.8; 4.5) | 4.1 (3.9; 4.2) | 4.1 (3.8; 4.3) | 4.0 (3.8; 4.3) |
| Min Max | 3.6 6.2 | 3.5 4.6 | 3.6 4.2 | 3.8 4.4 | 3.6 4.4 |
| **POD 1** | | | | | |
| Mean (SD) | 5.1 (0.3) | 4.8 (0.1) | 5.0 (0.1) | 4.7 (0.1) | 4.7 (0.1) |
| Median (IQR) | 4.7 (4.5; 6.0) | 4.7 (4.6; 5.1) | 4.9 (4.8; 5.4) | 4.7 (4.5; 5.0) | 4.7 (4.6; 4.9) |
| Min Max | 4.3 6.4 | 4.4 5.6 | 4.3 5.7 | 4.2 5.3 | 4.4 5.1 |
| **POD 2** | | | | | |
| Mean (SD) | 4.6 (0.1) | 4.4 (0.1) | 4.7 (0.2) | 4.5 (0.2) | 4.5 (0.1) |
| Median (IQR) | 4.7 (4.5; 4.9) | 4.2 (4.1; 4.8) | 4.7 (4.4; 4.9) | 4.4 (4.2; 4.7) | 4.5 (4.1; 4.8) |
| Min Max | 4.0 4.9 | 4.0 5.2 | 3.8 5.7 | 4.1 5.7 | 4.0 4.9 |
| **POD 3** | | | | | |
| Mean (SD) | 4.5 (0.2) | 4.1 (0.1) | 4.2 (0.1) | 4.2 (0.1) | 4.2 (0.2) |
| Median (IQR) | 4.4 (4.1; 4.9) | 4.1 (3.9; 4.4) | 4.2 (3.9; 4.4) | 4.2 (3.8; 4.3) | 4.3 (3.8; 4.4) |
| Min Max | 3.9 5.6 | 3.6 4.5 | 3.6 4.7 | 3.6 5.0 | 3.3 4.8 |
| **pH** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 7.39 (0.04) | 7.41 (0.05) | 7.44 (0.05) | 7.41 (0.04) | 7.41 (0.04) |
| Median (IQR) | 7.40 (7.36; 7.41) | 7.42 (7.36; 7.45) | 7.43 (7.40; 7.46) | 7.41 (7.38; 7.44) | 7.42 (7.38; 7.43) |
| Min Max | 7.31 7.45 | 7.33 7.50 | 7.36 7.52 | 7.35 7.49 | 7.33 7.45 |
| **Post-CPB** | | | | | |
| Mean (SD) | 7.36 (0.08) | 7.38 (0.03) | 7.39 (0.04) | 7.35 (0.07) | 7.37 (0.013 |
| Median (IQR) | 7.37 (7.28; 7.41) | 7.37 (7.36; 7.41) | 7.41 (7.35; 7.43) | 7.38 (7.30; 7.40) | 7.3 (7.34; 7.39) |

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Min Max | 7.24 7.48 | 7.35 7.42 | 7.32 7.44 | 7.20 7.42 | 7.33 7.42 |
| **POD 1** | | | | | |
| Mean (SD) | 1.38 (0.04) | 7.42 (0.03) | 7.40 (0.06) | 7.37 (0.07) | 7.42 (0.03) |
| Median (IQR) | 7.37 (7.34; 7.42) | 7.43 (7.39; 7.44) | 7.40 (7.37; 7.44) | 7.39 (7.32; 7.42) | 7.43 (7.39; 7.44) |
| Min Max | 7.33 7.45 | 7.38 7.45 | 7.28 7.46 | 7.24 7.43 | 7.37 7.46 |
| **Chloride (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 106.9 (2.3) | 107.3 (2.1) | 105.6 (4.1) | 108.1 (1.5) | 107.3 (2.1) |
| Median (IQR) | 106 (105; 109) | 108 (105; 109) | 107 (102; 109) | 108 (107; 109) | 108 (106; 108) |
| Min Max | 104 112 | 104 110 | 99 111 | 109 115 | 104 111 |
| **Post-CPB** | | | | | |
| Mean (SD) | 112.0 (4.5) | 110.5 (2.6) | 111.3 (3.7) | 112.0 (2.1) | 111.8 (1.9) |
| Median (IQR) | 111 (108; 116) | 110 (108; 113) | 113 (108; 114) | 112 (110; 114) | 112 (111; 113) |
| Min Max | 104 118 | 107 114 | 105 114 | 109 115 | 108 114 |
| **POD 1** | | | | | |
| Mean (SD) | 107.7 (3.4) | 108.9 (2.6) | 110.3 (2.7) | 107.8 (3.2) | 106.9 (4.0) |
| Median (IQR) | 108 (105; 111) | 109 (108; 111) | 111 (108; 113) | 108 (106; 111) | 107 (103; 111) |
| Min Max | 103 112 | 105 113 | 107 114 | 102 112 | 101 112 |
| **Standard bicarbonate (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 25.7 (2.3) | 25.5 (1.9) | 26.4 (2.3) | 25.0 (0.4) | 25.4 (1.7) |
| Median (IQR) | 26.1 (23.6; 27.9) | 25.2 (24.1; 26.4) | 25.9 (24.9; 27.7) | 25.1 (24.6; 25.3) | 25.4 (24.6; 27.0) |
| Min Max | 22.4 28.8 | 23.6 29.6 | 24.0 31.5 | 24.4 25.4 | 22.3 27.7 |
| **Post-CPB** | | | | | |
| Mean (SD) | 22.5 (2.5) | 23.6 (1.8) | 23.0 (1.4) | 22.3 (1.9) | 22.8 (1.4) |
| Median (IQR) | 22.3 (20.6; 25.0) | 23.4 (22.0; 25.4) | 23.2 (21.8; 24.2) | 22.6 (21.7; 23.87) | 22.7 (21.8; 23.1) |
| Min Max | 18.4 25.9 | 21.8 26.5 | 20.9 25.1 | 18.3 24.1 | 21.1 25.9 |
| **POD 1** | | | | | |
| Mean (SD) | 24.2 (1.7) | 24.7 (1.7) | 23.4 (2.3) | 23.0 (3.6) | 25.1 (1.7) |
| Median (IQR) | 25.0 (22.6; 25.7) | 24.8 (23.2; 25.6) | 23.5 (22.7; 25.3) | 24.1 (20.9; 25.5) | 24.6 (23.8; 26.3) |
| Min Max | 22.4 28.8 | 22.6 27.9 | 18.2 26.2 | 15.5 26.7 | 23.3 28.3 |
| **Lactate (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.9 (0.5) | 1.0 (0.5) | 1.1 (0.3) | 1.0 (0.3) | 0.7 (0.3) |
| Median (IQR) | 0.8 (0.6; 1.2) | 0.9 (0.5; 1.4) | 0.9 (0.8; 1.4) | 1.1 (0.8; 1.4) | 0.6 (0.4; 0.9) |
| Min Max | 0.4 2.0 | 0.4 1.9 | 0.7 1.5 | 0.7 1.5 | 0.4 1.4 |
| **Post-CPB** | | | | | |
| Mean (SD) | 2.9 (1.3) | 2.1 (0.7) | 2.6 (1.1) | 2.1 (0.9) | 1.7 (0.6) |
| Median (IQR) | 2.4 (2.0; 4.0) | 1.8 (1.7; 2.8) | 2.3 (1.7; 3.3) | 2.6 (1.3; 2.9) | 1.7 (1.1; 2.4) |
| Min Max | 1.7 5.1 | 1.3 3.1 | 1.5 5.0 | 0.8 6.4 | 0.9 2.5 |
| **POD 1** | | | | | |
| Mean (SD) | 2.7 (1.1) | 1.4 (0.5) | 1.8 (1.0) | 1.7 (0.7) | 1.6 (0.4) |
| Median (IQR) | 1.8 (1.4; 3.7) | 1.5 (1.0; 1.7) | 1.6 (1.1; 2.2) | 2.0 (1.0; 2.5) | 1.7 (1.2; 2.0) |
| Min Max | 1.0 6.8 | 0.6 2.0 | 0.8 4.0 | 0.9 9.8 | 1.0 2.2 |
| **Glucose (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.3 (1.1) | 6.4 (0.7) | 7.1 (1.8) | 6.1 (0.8) | 5.6 (0.7) |
| Median (IQR) | 5.9 (5.6; 7.1) | 6.2 (5.7; 7.1) | 6.8 (6.0; 7.5) | 6.1 (5.8; 6.8) | 5.4 (5.0; 5.9) |
| Min Max | 4.8 8.1 | 5.5 7.5 | 5.1 11.5 | 4.5 6.9 | 4.9 7.1 |
| **Post-CPB** | | | | | |
| Mean (SD) | 8.8 (2.5) | 9.0 (0.9) | 8.9 (1.8) | 7.8 (0.9) | 8.0 (0.9) |
| Median (IQR) | 9.3 (6.6; 10.4) | 9.0 (8.8; 9.6) | 8.1 (7.5; 10.8) | 7.7 (7.4; 8.1) | 8.0 (7.1; 8.9) |

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Min Max | 5.9 13.5 | 7.2 10.1 | 7.5 11.6 | 6.3 10.5 | 7.0 9.5 |
| **POD 1** | | | | | |
| Mean (SD) | 9.1 (1.8) | 8.2 (1.0) | 8.1 (1.6) | 8.6 (1.2) | 8.5 (1.4) |
| Median (IQR) | 8.5 (7.9; 10.5) | 8.0 (7.3; 9.2) | 8.1 (6.9; 9.4) | 8.6 (7.8; 9.5) | 8.4 (7.3; 9.6) |
| Min Max | 6.8 12.4 | 7.1 9.6 | 6.3 11.0 | 6.5 10.5 | 6.6 10.9 |
| **Change in electrolyte and acid-base parameters, active vs placebo (LMM using REML)** | | | | | |
| **Sodium (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 138.6 | 139.8 | 140.0 | 139.8 | 139.4 |
| Mean difference vs placebo (SE) | | 1.2 (1.2) | 1.4 (1.3) | 1.2 (1.0) | 0.8 (1.0) |
| p-value | | 0.3241 | 0.2916 | 0.2591 | 0.4188 |
| **POD 1** | | | | | |
| Observed mean | 139.3 | 139.4 | 139.2 | 139.3 | 138.0 |
| Mean difference vs placebo (SE) | | 0.1 (1.2) | -0.1 (1.2) | 0.0 (1.7) | -1.3 (1.2) |
| p-value | | 0.9733 | 0.9255 | 0.9615 | 0.2834 |
| **POD 2** | | | | | |
| Observed mean | 139.4 | 137.0 | 136.1 | 135.1 | 135.9 |
| Mean difference vs placebo (SE) | | -2.4 (1.6) | -3.3 (1.9) | -4.3 (2.1) | -3.5 (1.8) |
| p-value | | 0.1521 | 0.0958 | 0.0551 | 0.0714 |
| **POD 3** | | | | | |
| Observed mean | 138.9 | 137.5 | 135.3 | 136.5 | 135.5 |
| Mean difference vs placebo (SE) | | -1.4 (1.8) | -3.6 (1.9) | -2.4 (2.0) | -3.4 (2.1) |
| p-value | | 0.4546 | 0.0882 | 0.2520 | 0.1338 |
| **Potassium (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 4.24 | 4.06 | 4.01 | 4.08 | 4.01 |
| Mean difference vs placebo (SE) | | -0.18 (0.29) | -0.23 (0.27) | -0.16 (0.27) | -0.23 (0.27) |
| p-value | | 0.5413 | 0.4012 | 0.5449 | 0.4173 |
| **POD 1** | | | | | |
| Observed mean | 5.11 | 4.84 | 5.04 | 4.71 | 4.74 |
| Mean difference vs placebo (SE) | | -0.27 (0.30) | -0.07 (0.31) | -0.40 (0.29) | -0.37 (0.28) |
| p-value | | 0.3801 | 0.8307 | 0.2025 | 0.2122 |
| **POD 2** | | | | | |
| Observed mean | 4.63 | 4.43 | 4.66 | 4.53 | 4.46 |
| Mean difference vs placebo (SE) | | -0.20 (0.18) | 0.03 (0.20) | -0.10 (0.21) | -0.17 (0.15) |
| p-value | | 0.2636 | 0.9133 | 0.6091 | 0.2870 |
| **POD 3** | | | | | |
| Observed mean | 4.48 | 4.10 | 4.18 | 4.16 | 4.16 |
| Mean difference vs placebo (SE) | | -0.38 (0.22) | -0.30 (0.27) | -0.32 (0.25) | -0.32 (0.26) |
| p-value | | 0.1164 | 0.2086 | 0.2230 | 0.2446 |
| **pH (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 7.39 | 7.41 | 7.44 | 7.41 | 7.41 |
| Mean difference vs placebo (SE) | | 0.02 (0.02) | 0.05 (0.02) | 0.02 (0.02) | 0.02 (0.02) |
| p-value | | 0.3087 | **0.0318 *** | 0.2184 | 0.3139 |

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Post-CPB** | | | | | |
| Observed mean | 7.36 | 7.38 | 7.39 | 7.35 | 7.37 |
| Mean difference vs placebo (SE) | | 0.02 (0.03) | 0.03 (0.03) | -0.01 (0.04) | 0.01 (0.03) |
| p-value | | 0.4055 | 0.2955 | 0.8394 | 0.6749 |
| **POD 1** | | | | | |
| Observed mean | 7.38 | 7.42 | 7.40 | 7.37 | 7.42 |
| Mean difference vs placebo (SE) | | 0.04 (0.02) | 0.02 (0.02) | -0.01 (0.03) | 0.04 (0.02) |
| p-value | | **0.0363 *** | 0.4058 | 0.84 | **0.0314 *** |
| **Chloride (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 106.9 | 107.3 | 105.6 | 108.1 | 107.3 |
| Mean difference vs placebo (SE) | | 0.4 (1.2) | -1.3 (1.7) | 1.2 (1.1) | 0.4 (1.2) |
| p-value | | 0.7652 | 0.4338 | 0.2630 | 0.7623 |
| **Post-CPB** | | | | | |
| Observed mean | 112.0 | 110.5 | 111.3 | 112.0 | 111.8 |
| Mean difference vs placebo (SE) | | -1.5 (1.7) | -0.7 (1.9) | 0.0 (1.7) | -0.2 (1.6) |
| p-value | | 0.4055 | 0.7353 | > 0.9999 | 0.8813 |
| **POD 1** | | | | | |
| Observed mean | 107.7 | 108.9 | 110.3 | 107.8 | 106.9 |
| Mean difference vs placebo (SE) | | 1.2 (1.4) | 2.6 (1.4) | 0.1 (1.6) | -0.8 (1.8) |
| p-value | | 0.4005 | 0.0822 | 0.9592 | 0.6681 |
| **Standard bicarbonate (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 25.72 | 25.53 | 26.44 | 24.96 | 25.43 |
| Mean difference vs placebo (SE) | | -0.19 (1.03) | 0.72 (1.09) | -0.76 (0.79) | -0.329 (0.98) |
| p-value | | 0.8505 | 0.5150 | 0.3617 | 0.7669 |
| **Post-CPB** | | | | | |
| Observed mean | 22.49 | 23.63 | 23.00 | 22.31 | 22.81 |
| Mean difference vs placebo (SE) | | 1.14 (1.04) | 0.51 (0.96) | -0.18 (1.06) | 0.32 (0.97) |
| p-value | | 0.2913 | 0.6015 | 0.8698 | 0.7437 |
| **POD 1** | | | | | |
| Observed mean | 24.19 | 24.74 | 23.40 | 22.98 | 25.06 |
| Mean difference vs placebo (SE) | | 0.55 (0.82) | -0.79 (0.95) | -1.21 (1.39) | 0.87 (0.81) |
| p-value | | 0.5160 | 0.4219 | 0.4038 | 0.3004 |
| **Lactate (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.92 | 0.95 | 1.07 | 1.04 | 0.70 |
| Mean difference vs placebo (SE) | | 0.03 (0.25) | 0.15 (0.20) | 0.12 (0.21) | -0.22 (0.21) |
| p-value | | 0.9148 | 0.4824 | 0.5887 | 0.3060 |
| **Post-CPB** | | | | | |
| Observed mean | 2.94 | 2.06 | 2.59 | 2.11 | 1.70 |
| Mean difference vs placebo (SE) | | -0.88 (0.52) | -0.35 (0.60) | -0.83 (0.57) | -1.24 (0.52) |
| p-value | | 0.1253 | 0.5709 | 0.1753 | **0.0383 *** |

| Readout<br>Time point | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Observed mean | 2.14 | 1.36 | 1.80 | 1.70 | 1.61 |
| Mean difference vs placebo (SE) | | -0.78 (0.41) | -0.34 (0.50) | -0.44 (0.46) | -0.53 (0.41) |
| p-value | | 0.0902 | 0.5099 | 0.3561 | 0.2273 |
| **Glucose (mmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 6.26 | 6.40 | 7.11 | 6.08 | 5.58 |
| Mean difference vs placebo (SE) | | 0.14 (0.44) | 0.85 (0.70) | -0.18 (0.45) | -0.68 (0.44) |
| p-value | | 0.7465 | 0.2458 | 0.6942 | 0.1405 |
| **Post-CPB** | | | | | |
| Observed mean | 8.82 | 8.96 | 8.86 | 7.76 | 8.04 |
| Mean difference vs placebo (SE) | | 0.14 (0.87) | 0.04 (1.00) | -1.06 (0.88) | -0.78 (0.88) |
| p-value | | 0.8758 | 0.9786 | 0.2572 | 0.3946 |
| **POD 1** | | | | | |
| Observed mean | 9.07 | 8.20 | 8.10 | 8.58 | 8.51 |
| Mean difference vs placebo (SE) | | -0.87 (0.69) | -0.97 (0.80) | -0.49 (0.74) | -0.56 (0.78) |
| p-value | | 0.2322 | 0.2441 | 0.5166 | 0.4885 |

Abbreviations: SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – Standard error of the mean.

Linear mixed model with Restricted Maximum Likelihood. Correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 3.2.10 Anaesthetic and surgical procedural data

Participants in this study were admitted to the cardiothoracic ICU immediately post-operatively as per routine standard of care for cardiac surgery. Most patients were discharged to the ward on the second post-operative day (73%) and subsequently discharged from hospital within one week (78%). A single patient in the 5-ALA-SFC 225 mg/354 mg treatment allocation group had a prolonged ICU stay (30 days) due to a major intra-operative complication. They were ultimately transferred to a different hospital and spent a total of 60 days in hospital as an inpatient. Clinical data reflecting care while patients were admitted to the Heart Centre are summarised in **table 22**.

Levels of organ dysfunction and support required were approximately comparable between groups. Although similar numbers of patients required some form of pharmacological cardiovascular support at the first post-operative day timepoint, there was a suggestion that the overall dosage of drugs required was slightly lower in the highest two active treatment allocation groups. Patients in these two groups also had a lower frequency of arrhythmias (including AF), and fewer required post-operative cardiac pacing at post-operative day 1, most apparent for the highest dose 5-ALA-SFC group. No clear treatment-related differences were identified in terms of post-operative blood loss or volumes of crystalloid infused. It is notable that no patients in the 5-ALA-SFC 300 mg/472 mg treatment allocation group received post-operative packed red blood cell transfusion on the intensive care unit. The overall frequency of acute kidney injury, defined using the KDIGO classification[134], was 14 % for this study. Most cases were identified in the placebo and the 5-ALA-SFC 225 mg/354 mg treatment allocation groups. When corrected for patients who had experienced major intra-operative complications, the highest-grade cases of AKI (KDIGO > 2) were excluded.

At the final follow up visit, it was found that one patient had positive data for the composite renal endpoint (major kidney event [MAKE]) and one patient for the composite cardiovascular endpoint (major cardiovascular event [MACE]). There had been no deaths. There was no evidence for a dose-dependent treatment-related increase in the requirement for organ support or in the incidence of post-operative clinical complications.

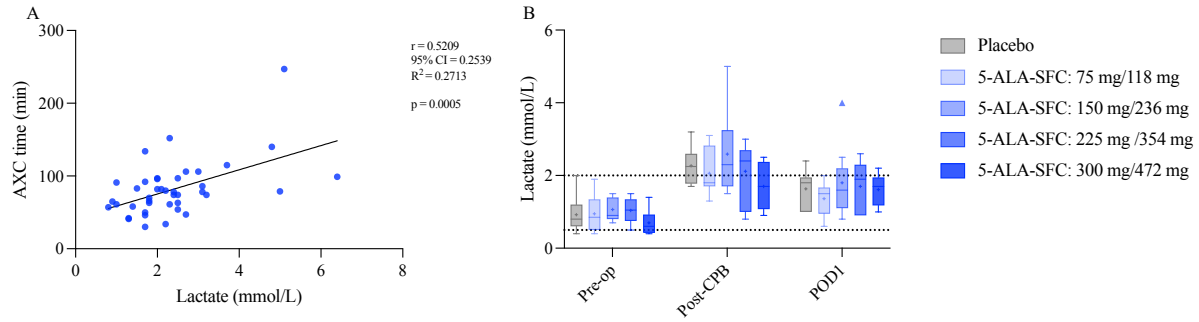



**Figure 43: The correlation between aortic cross clamp time and serum lactate.**

Panel B data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Abbreviations: AXC – aortic cross clamp; Pre-op – pre-operative; CPB – cardiopulmonary bypass; POD – post-operative day.

**Table 22: Key peri-operative anaesthetic and surgical data.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Anaesthetic induction** | | | | | |
| **Propofol (mg)** | | | | | |
| N (%) | 9 (100%) | 7 (88%) | 7 (78%) | 7 (78%) | 7 (88%) |
| Mean (SD) | 101.7 (43) | 102.9 (57) | 72.86 (29) | 84.29 (15) | 71.43 (24) |
| Median (IQR) | 90 (72.50; 120) | 120 (40; 120) | 80 (50; 100) | 80 (70; 100) | 80 (50; 100) |
| Min Max | 50 200 | 30 200 | 30 100 | 70 100 | 40 100 |
| **Other induction agent:** | | | | | |
| Etomidate (n) | 0 | 1 | 2 | 0 | 0 |
| Midazolam (n) | 0 | 0 | 0 | 0 | 1 |
| Fentanyl (n) | 0 | 0 | 0 | 1 | 0 |
| **Anaesthetic maintenance** | | | | | |
| **Volatile: Isoflurane (ET%)** | | | | | |
| N | 9 (100%) | 7 (88%) | 7 (78%) | 8 (78%) | 8 (100%) |
| Mean (SD) | 0.88 (0.23) | 0.9 (0.20) | 0.7 (0.24) | 0.8 (0.23) | 1.0 (0.17) |
| Median (IQR) | 0.9 (0.7; 1.0) | 1.1 (0.8; 1.1) | 0.5 (0.5; 0.9) | 0.9 (0.6; 1.0) | 1.0 (0.9; 1.0) |
| Min Max | 0.5 1.3 | 0.6 1.1 | 0.45 1.0 | 0.5 1.2 | 0.6 1.2 |
| **Intravenous agent: Propofol (mg/hr)** | | | | | |
| N | 6 (67%) | 1 (13%) | 6 (67%) | 3 (40%) | 7 (88%) |
| Mean (SD) | 247 (137) | 100 (NA) | 192 (141) | 167 (185) | 307 (197) |
| Median (IQR) | 300 (88; 356) | 100 (NA) | 210 (45; 313) | 80 (40; 380) | 300 (80; 540) |
| Min Max | 50 380 | NA | 45 350 | 40 380 | 40 540 |
| **Maintenance technique** | | | | | |
| Single agent: volatile (N) | 2 (22%) | 7 (88%) | 3 (33%) | 5 (63%) | 1 (13%) |
| Single agent: TIVA (N) | 1 (11%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Combination (N) | 5 (56%) | 1 (13%) | 5 (56%) | 3 (38%) | 7 (88%) |
| **Intravenous fluid therapy in theatre in addition to CPB pump priming fluid** | | | | | |
| **Crystalloid: Hartmann's solution (L)** | | | | | |
| Mean (SD) | 1.2 (0.3) | 1.6 (1.1) | 1 (1) | 1.1 (0.6) | 1.3 (1.0) |
| Median (IQR) | 1.0 (1.0; 1.0) | 1.5 (1.0; 2.0) | 1.0 (0.0; 1.5) | 1.0 (1.0; 1.5) | 1.0 (0.4; 2.0) |
| Min Max | 1.0 2.5 | 1.0 4.0 | 0.0 3.0 | 1.0 2.0 | 0.0 3.0 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Total aortic cross clamp time (min) FAS** | | | | | |
| Mean (SD) | 101.6 (69) | 66.5 (20) | 87.5 (26) | 79.3 (23) | 71.3 (18) |
| Median (IQR) | 74.1 (54.0; 146.0) | 66.5 (47; 84) | 82.5 (65.5; 109.3) | 79.0 (58; 104.3) | 69.5 (59.3; 88.8) |
| Min Max | 30 247 | 42 97 | 51 134 | 47 106 | 41 96 |
| **Total aortic cross clamp time (min) PAS** | | | | | |
| Mean (SD) | 102.1 (73) | 66.5 (20) | 87.5 (26) | 76.4 (23) | 71.3 (18) |
| Median (IQR) | 74 (42; 149) | 66.5 (47; 84) | 82.5 (65.5; 109.3) | 78 (57; 106) | 69.5 (59.3; 88.8) |
| Min Max | 30 247 | 42 97 | 54 134 | 47 106 | 41 96 |

Abbreviations: TIVA – Total intravenous anaesthesia; CPB – Cardiopulmonary bypass; SD – standard deviation; IQR – interquartile range; FAS – Full analysis set; PAS – Primary analysis set

### 3.2.11 Peri-operative high-sensitivity cardiac Troponin I measurements

Consistent with published data, cardiac surgery was associated with a marked increase in circulating hs-TropI reflecting peri-operative myocardial injury. The increase was most noticeable in the placebo group and peaked in the initial 12 hours after CPB in both the FAS (**Fig. 44 A**) and PAS (**Fig. 44 C**), the latter pre-specified analysis population excluding two patients who had experienced substantial intraoperative MIs. In contrast there was a tendency to lower hs-TropI elevation in all 5-ALA-SFC treated groups in the initial post-operative period (to ~6-12 hours). In patients allocated to receive 5-ALA-SFC at the highest dose level of 300 mg/472 mg the data suggested a delayed rise to peak shifting the release profile rightward. When considering only the PAS, excluding 1 patient each from the placebo and 225 mg/364 mg treatment allocation groups outlined above, the overall pattern of lower troponin release at the earliest timepoints post-surgery and delayed more persistent rise in hs-TropI in the highest 5-ALA-SFC dose allocation group was still noticeable.

Geometric area under the curve (AUC) analysis was the pre-specified primary analysis for quantifying extent of PMI, evaluating the overall increase in hs-TropI over 72 hours (Fig. 45 B). There was no statistically significant difference between treatment groups in hs-TropI. Specifically patients allocated to the 5-ALA-SFC 300 mg/472 mg treatment group had a mean difference versus placebo (95% CI) of 18.5 (-76.6; 113.7) with a p-value of 0.6842.

Next, the impact of different surgical interventions on the kinetics of hs-TropI was considered (**Fig. 44 D-E**), particularly considering the baseline differences in surgical procedures across treatment arms. Participants undergoing CABG, AVR, or a combination of both procedures, had markedly lower peak post-operative troponin values and overall lower release profiles, compared to those undergoing mitral valve surgery. After separating patients by surgical intervention, and pooling all patients allocated to receive 5-ALA-SFC together, patients receiving the active therapy tended to have lower hs-TropI values at almost every timepoint for both classes of procedure. Across the trial, there were only 15 missing data points for hs-Trop I, representing a missing data frequency of 5 %.

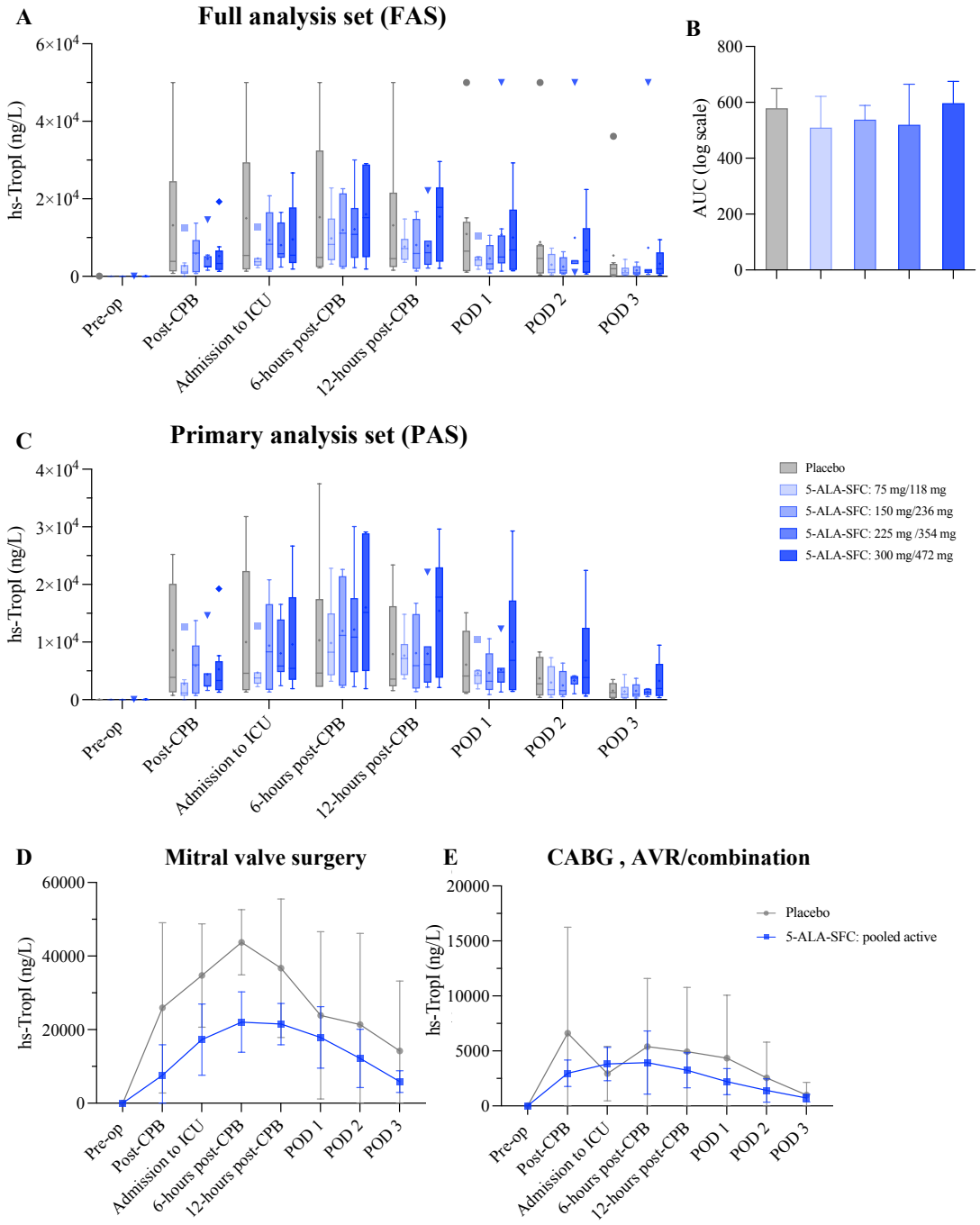



**Figure 44: The impact of 5-ALA-SFC on peri-operative troponin I**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A and C), mean, ± SD (panel B, D-E).

Abbreviations – CABG – coronary artery bypass grafting; AVR – aortic valve replacement; AUC – area under the curve.

**Table 23: Peri-operative troponin I descriptive statistics and AUC analysis.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Cardiac troponin I (ng/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 15 (19) | 13 (15) | 7 (5) | 12 (21) | 37 (44) |
| Median (IQR) | 7 (6; 10) | 9 (3; 17) | 6 (4; 10) | 6 (3; 8) | 13 (4; 70) |
| Min Max | 3 58 | 2 46 | 2 15 | 2 64 | 2 117 |
| **Post-CPB** | | | | | |
| Mean (SD) | 13198 (16766) | 2693 (4129) | 5920 (4727) | 4535 (4278) | 5276 (6003) |
| Median (IQR) | 3909 (1276; 23945) | 1156 (789; 2523) | 6045 (1241; 9156) | 2639 (2338; 5059) | 3343 (1718; 5764) |
| Min Max | 772 50000 | 9 12601 | 742 13733 | 1596 14611 | 1291 19264 |
| **Admission to ICU (2-3 hrs post CPB)** | | | | | |
| Mean (SD) | 15017 (18027) | 4716 (3659) | 9387 (7743) | 8027 (5226) | 9586 (9173) |
| Median (IQR) | 5417 (1912; 27065) | 3769 (2766; 4777) | 8300 (1790; 16386) | 5855 (4797; 13936) | 5441 (3440; 17809) |
| Min Max | 1349 50000 | 2241 12789 | 1340 20803 | 2416 16559 | 1897 26676 |
| **3 – 6 hours post-CPB** | | | | | |
| Mean (SD) | 15263 (18566) | 9834 (6742) | 11954 (8863) | 12173 (9418) | 16012 (11386) |
| Median (IQR) | 4882 (2581; 27477) | 8244 (4725; 13365) | 11150 (2395; 21447) | 10848 (4767; 17641) | 15148 (5968; 28798) |
| Min Max | 2220 50000 | 3189 22819 | 2104 22608 | 2244 30053 | 1908 29102 |
| **4 – 12 hours post CPB** | | | | | |
| Mean (SD) | 13164 (16831) | 7682 (3707) | 8091 (6584) | 7964 (6769) | 15413 (10404) |
| Median (IQR) | 4575 (2479; 19814) | 7166 (4758; 9599) | 5927 (1920; 14880) | 6103 (2949; 9296) | 17783 (4370; 20783) |
| Min Max | 1575 50000 | 3609 14802 | 1407 16744 | 2168 22135 | 2124 29635 |
| **POD 1** | | | | | |
| Mean (SD) | 10972 (15570) | 4573 (2633) | 4661 (3723) | 10814 (16151) | 10039 (10029) |
| Median (IQR) | 6509 (1558; 13118) | 4218 (2919; 5029) | 3213 (1701; 7995) | 5016 (3658; 8916) | 6828 (1714; 16259) |
| Min Max | 1086 50000 | 1866 10388 | 887 10584 | 1333 50000 | 1444 29268 |
| **POD 2** | | | | | |
| Mean (SD) | 8860 (15754) | 3003 (2733) | 2504 (2312) | 9988 (17677) | 6785 (7784) |
| Median (IQR) | 4629 (773; 7799) | 1749 (779; 5609) | 1559 (786; 4241) | 4077 (3170; 4143) | 3853 (1031; 10724) |
| Min Max | 384 50000 | 434 7315 | 511 6353 | 1041 50000 | 615 22452 |
| **POD 3** | | | | | |
| Mean (SD) | 5408 (11600) | 1454 (1430) | 1530 (1303) | 7388 (17225) | 3281 (3362) |
| Median (IQR) | 2022 (308; 2919) | 936 (351; 2239) | 959 (499; 2662) | 1426 (958; 1904) | 1958 (664; 5582) |
| Min Max | 195 36142 | 209 4373 | 300 3699 | 533 50000 | 359 9480 |
| **Cardiac troponin I geometric AUC (log scale)** | | | | | |
| Mean (SD) | 579 (91) | 510 (133) | 538 (61) | 520 (173) | 597 (93) |
| Mean 95% CI | 509, 648 | 399, 621 | 487, 588 | 375, 664 | 519, 675 |
| Median (Q1; Q3) | 581 (488; 667) | 542 (476; 579) | 546 (486; 586) | 556 (489; 625) | 589 (508; 676) |
| min max | 468 675 | 220 664 | 447 620 | 128 689 | 495 736 |
| Mean difference vs placebo (95% CI) | | -68.7 (-185.0; 47.6) | -40.7 (-121.7; 40.2) | -58.9 (-199.3; 81.5) | 18.5 ( -76.6; 113.7) |
| p-value | | 0.2271 | 0.3003 | 0.3853 | 0.6842 |

### 3.2.12 Relevant intensive care and follow-up data

Participants in this study were routinely admitted to the cardiothoracic ICU immediately post-operatively. Most patients were discharged to the ward on the second post-operative day (73%) and subsequently discharged from hospital within one week (78%). A single patient in the 5-ALA-SFC 225 mg/354 mg treatment allocation group had a prolonged ICU stay (30 days) due to a major intra-operative complication. They were ultimately transferred to a different hospital and spent a total of 60 days in hospital as an inpatient. Clinical data reflecting care while patients were admitted to hospital are summarised in **table 24**.

Levels of organ dysfunction and support required were approximately comparable between groups. Although similar numbers of patients required some form of pharmacological cardiovascular support at the first post-operative day timepoint, there was a suggestion that the overall dosage of drugs required was slightly lower in the highest two active treatment allocation groups. Patients in these two groups also had a lower frequency of arrhythmias, and fewer required post-operative cardiac pacing at post-operative day 1. No clear treatment-related differences were identified in terms of post-operative blood loss or volumes of crystalloid infused. It is notable that no patients in the 5-ALA-SFC 300 mg/472 mg treatment allocation group received post-operative packed red blood cell transfusion on the intensive care unit. The frequency of acute kidney injury, defined using the KDIGO classification[134], was 14 % for this study. Most cases were identified in the placebo and the 5-ALA-SFC 225 mg/354 mg treatment allocation groups. When corrected for patients who had suffered major intra-operative complications, the highest-grade cases of AKI (KDIGO > 2) were excluded.

At the final follow up visit, it was found that one patient had positive data for the composite renal endpoint (major kidney event [MAKE]) and one patient for the composite cardiovascular endpoint (major cardiovascular event [MACE]). There had been no deaths. There was not a dose-dependent treatment-related increase in the requirement for organ support or the incidence of post-operative clinical complications in this study.

## Table 24: Clinical data: ICU to hospital discharge.

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Duration of stay** | | | | | |
| **ICU (days)** | | | | | |
| Mean (SD) | 3.2 (2.2) | 2.5 (1.6) | 1.6 (1.1) | 4.3 (9.6) | 2.3 (0.9) |
| Median (IQR) | 5 (1.5; 5.0) | 2 (1.0; 4.0) | 1 (1.0; 2.0) | 1 (1.0; 1.5) | 2 (2.0; 2.8) |
| Min Max | 1 7 | 1 5 | 1 4 | 1 30 | 1 4 |
| **Hospital (days)** | | | | | |
| Mean (SD) | 7.0 (2.5) | 6.2 (3.4) | 8.2 (5.7) | 12.6 (19.2) | 6.4 (0.7) |
| Median (IQR) | 6 (4.5; 9.5) | 5 (4.0; 6.8) | 6 (5.5; 8.5) | 6 (5.3; 7.5) | 7 (6.0; 7.0) |
| Min Max | 4 10 | 4 14 | 4 23 | 4 60 | 5 7 |
| **Organ function** | | | | | |
| **SOFA score at POD 1 (AU)** | | | | | |
| Mean (SD) | 5 (2.7) | 4 (2.0) | 3 (2.4) | 4 (2.6) | 3 (1.6) |
| Median (IQR) | 7 (2.5; 7.0) | 4 (3.0; 4.8) | 2 (1.5; 5.5) | 3 (2.3; 5.3) | 4 (2.0; 4.0) |
| Min Max | 1 8 | 1 8 | 0 7 | 1 9 | 1 6 |
| **Delirium during hospital stay** | | | | | |
| Incidence (n, %) | 1 (11%) | 0 (0%) | 1 (11%) | 0 (0%) | 0 (0%) |
| **Cardiovascular support** | | | | | |
| **Patients requiring pharmacological support at POD 1** | | | | | |
| N (%) | 5 (56%) | 3 (38%) | 4 (44%) | 4 (50%) | 5 (63%) |
| **VIS at POD 1 (AU)** | | | | | |
| Mean (SD) | 9.7 (12.0) | 4.0 (7.3) | 8.0 (12.1) | 7.5 (15.8) | 3.0 (3.4) |
| Median (IQR) | 3 (0.0; 22.0) | 0 (0.0; 6.3) | 0 (0.0; 19, 0) | 2 (0.0; 6.3) | 2 (0.0; 8.0) |
| Min Max | 0 30 | 0 21 | 0 19 | 0 46 | 0 8 |
| **Patients requiring internal cardiac pacing at POD 1** | | | | | |
| N (%) | 3 (33%) | 3 (38%) | 5 (56%) | 1 (13%) | 1 (13%) |
| **Documented atrial fibrillation during the first 24 post-operative hours** | | | | | |
| N (%) | 3 (67%) | 1 (13%) | 1 (11%) | 0 (0%) | 0 (0%) |
| **Other arrythmia during the first 24 post-operative hours** | | | | | |
| N (%) | 2 (22%) | 1 (13%) | 1 (11%) | 1 (13%) | 0 (0%) |
| **Respiratory support** | | | | | |
| **p:F ratio (AU) POD 1** | | | | | |
| Mean (SD) | 264 (78) | 325 (90) | 402 (139) | 280 (66) | 304 (90) |
| Median (IQR) | 225 (205; 311) | 318 (254; 377) | 373 (301; 486) | 276 (228; 335) | 302 (217; 387) |
| Min Max | 189 390 | 212 491 | 211 648 | 190 375 | 195 422 |
| **Post-operative blood loss (L)** | | | | | |
| **POD 1** | | | | | |
| Mean (SD) | 0.72 (0.44) | 0.71 (0.23) | 0.49 (0.24) | 0.51 (0.27) | 0.76 (0.53) |
| Median (IQR) | 0.54 (0.42; 0.95) | 0.67 (0.51; 0.93) | 0.39 (0.25; 0.72) | 0.41 (0.29; 0.78) | 0.67 (0.33; 0.87) |
| Min Max | 0.39 1.74 | 0.44 1.11 | 0.24 0.92 | 0.21 0.92 | 0.23 1.93 |
| **POD 2** | | | | | |
| Mean (SD) | 0.75 (0.51) | 1.09 (0.61) | 0.55 (0.32) | 0.58 (0.68) | 0.18 (0.21) |
| Median (IQR) | 0.66 (0.50; 0.90) | 0.85 (0.63; 1.73) | 0.39 (0.30; 0.72) | 0.40 (0.07; 0.78) | 0.12 (0.01; 0.30) |
| Min Max | 0.00 1.89 | 0.44 2.05 | 0.24 1.25 | 0.00 2.09 | 0.00 0.6 |
| **POD 3** | | | | | |
| Mean (SD) | 0.21 (0.56) | 0.29 (0.50) | 0.05 (0.12) | 0.09 (0.21) | 0.06 (0.14) |
| Median (IQR) | 0.00 (0.00; 0.07) | 0.13 (0.00; 0.41) | 0.00 (0.00; 0.05) | 0.00 (0.00; 0.11) | 0.00 (0.00; 0.04) |
| Min Max | 0.00 1.70 | 0.00 1.46 | 0.00 0.37 | 0.00 0.61 | 0.00 0.40 |
| **Crystalloid: Hartmann's solution (L)** | | | | | |
| **POD 1** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 3.05 (0.9) | 2.89 (0.81) | 2.51 (0.67) | 2.11 (0.94) | 3.76 (0.80) |
| Median (IQR) | 3.08 (1.99; 4.00) | 3.03 (1.2; 3.67) | 2.45 (1.84; 3.13) | 2.1 (1.3; 2.89) | 3.71 (3.28; 4.28) |
| Min Max | 1.90 4.03 | 1.86 3.81 | 1.74 3.56 | 0.85 3.59 | 2.52 5.09 |
| **POD 2** | | | | | |
| Mean (SD) | 0.20 (0.43) | 0.2 (0.24) | 0.42 (0.51) | 0.34 (0.71) | 0.38 (0.80) |
| Median (IQR) | 0.0 (0.0; 0.24) | 0.13 (0.0; 0.44) | 0.4 (0.0; 0.66) | 0.01 (0.0; 0.51) | 0.3 (0.15; 0.61) |
| Min Max | 0.0 1.31 | 0.0 0.61 | 0.0 1.55 | 0.0 2.08 | 0.07 0.89 |
| **POD 3** | | | | | |
| Mean (SD) | 0.06 (0.1) | 0.05 (0.01) | 0.14 (0.33) | 0.00 (0.00) | 0.16 (0.24) |
| Median (IQR) | 0.0 (0.0; 0.14) | 0.0 (0.0; 0.13) | 0.0 (0.0; 0.13) | 0.0 (0.0; 0.0) | 0.08 (0.0; 0.23) |
| Min Max | 0.0 0.25 | 0.0 0.25 | 0.0 1.0 | 0.0 0.0 | 0.0 0.72 |
| **Post-operative transfusion: packed red cells (L)** | | | | | |
| N (%) | 4 (44%) | 2 (25%) | 2 (22%) | 3 (38%) | 0 (0%) |
| Total volume (L) | 2.1 | 2.0 | 0.56 | 2.9 | 0 |
| **Acute kidney injury (n, %) FAS** | | | | | |
| KDIGO: I | 2 (22%) | 1 (13%) | 0 (0%) | 1 (13%) | 0 (0%) |
| KDIGO: II | 1 (11%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| KDIGO: III | 0 (0%) | 0 (0%) | 0 (0%) | 1 (13%) | 0 (0%) |
| **Acute kidney injury (n, %) PAS** | | | | | |
| KDIGO: I | 2 (25%) | 1 (13%) | 0 (0%) | 1 (15%) | 0 (0%) |
| KDIGO: II | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| KDIGO: III | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |

Abbreviations: TIVA – Total intravenous anaesthesia; CPB – Cardiopulmonary bypass; SEM – standard error of the mean; IQR – interquartile range; FAS – Full analysis set; PAS – Primary analysis set

### 3.3 Discussion

The TALEN trial represents the first investigation of 5-ALA-SFC in an adult cardiac surgical population. Trial participants were allocated to either placebo, or one of four active 5-ALA-SFC dosage treatment allocation groups. The groups were balanced in terms of cardiovascular symptomatic status and surgical risk but, despite randomisation, with some differences in breakdown of individual surgical procedures. Taken together, the high treatment compliance, low incidence and severity of treatment-related AEs and very low frequency of treatment interruptions in this study suggest that pre-operative dosing with oral 5-ALA-SFC, up to a dose of 300 mg/472 mg for three days prior to surgery, is well tolerated in patients undergoing cardiac surgery requiring CPB. The majority of patients were advanced in age, with significant co-morbidities and on multiple concomitant medications, reflecting a typical population of patients undergoing elective cardiac surgery, adding significantly to our understanding of the safe use of 5-ALA-SFC. Although the overall incidence of AEs was relatively high, this was not an unexpected finding given the complexity of a cardiac surgical population and the invasive nature of the surgery itself. All SAEs encountered were those anticipated and not infrequent in the context of major cardiac surgery and no deaths occurred.

With regard to haemodynamic assessments, the rise in SBP and HR observed at the pre-operative timepoint in all groups is likely attributable to the anxiety experienced by patients ahead of major surgery. Overall, no evidence was found for a drug associated hypotensive effect at the doses evaluated and which had previously been described at higher doses of 5-ALA[310]; this neutral haemodynamic effect is an important consideration for any novel therapeutic in the context of cardiac surgery. A degree of haemodynamic suppression in the post-operative period was observed in all treatment allocation groups, with a similar requirement for cardiovascular pharmacological support between them. Due to underlying comorbidities and the procedure itself, a significant proportion of patients undergoing cardiac surgery will develop cardiovascular compromise in the peri-operative period[30]. Predictable fluctuations in cardiorespiratory physiology, induced by anaesthesia and surgery are commonly encountered[311]. This adds complexity when attributing causality in cardiac surgical clinical trials if cardiovascular compromise is encountered. In the absence of differences between treatment groups and placebo,

together with the lack of 5-ALA-SFC dose-dependent changes in post-operative SBP and HR, it is most likely that these changes are attributable to the surgery. Similarly, changes to the QTc interval on the first post-operative day are likely to be explained by residual anaesthetic effects, the influence of concomitant medications, and the post-operative state[312].

Although treatment with 5-ALA-SFC has not previously been linked with bone marrow suppression[275], understanding the impact of this drug on haematological function in the context of cardiac surgery, is important. A consistent mild decrease in Hb, WCC and platelet count was observed at the pre-operative time point across groups. This could be explained by the dilutional effect of crystalloid infusion immediately after induction of anaesthesia (prior to sampling), and subsequent autologous priming of the CPB pump before instituting bypass, would exaggerate this[313]. Post-operative fluctuations were consistent across groups and most likely related to surgery. Cardiac surgery requiring cardiopulmonary bypass is associated with surgical and non-surgical blood loss, activation of the coagulation cascade, immune activation, and predictable changes in haematological indices (e.g., Hb, WCC, platelet count) and measures of coagulation (e.g., prothrombin time [PT])[314].

Specific assessment for potential treatment-related hepatotoxicity in this population was important given that 5-ALA-SFC at high doses has been associated with transient elevations in liver enzymes in healthy volunteers[275]. 5-ALA-SFC was not associated with dose-dependent increases in ALT or ALP in this study, suggesting a lack of specific hepatotoxic effects in this population at the investigated doses. The drop in albumin levels observed in this study has been well described in the literature and is thought to be a result of several factors including dilution, immune activation, and blood loss[315]. Also observed were fluid, electrolyte and acid-base balance disturbances that are commonly encountered in patients undergoing cardiac surgery[316]. Hyperchloremic metabolic acidosis, for example, is well described and is thought to be due, in part, to fluid therapy before and during CPB[317].

Cardiac surgery induces a predictable hyperglycaemic neuroendocrine stress response, accounting for the higher plasma glucose readings encountered in trial particpants[318]. Early onset hyperlactataemia (i.e.,

high blood lactate which is present at the end of CPB which can persist for some hours) occurs commonly in patients undergoing cardiac surgery and its aetiology includes factors such as tissue hypoperfusion and high levels of circulating endogenous catecholamines[319]. The degree of hyperglycaemia and hyperlactataemia corresponded with the duration of CPB and more profound surgical neuroendocrine stress. The electrolyte and acid-base data from this study likely reflect usual peri-operative fluctuations in physiology, rather than a primary drug effect, and do not identify any safety concerns for the use of 5-ALA-SFC in patients undergoing cardiac surgery. The tendency to lower systemic lactate levels, observed in patients allocated to the higher dose 5-ALA-SFC groups, is intriguing in suggesting a possible favorable metabolic effect and reduced reliance on anaerobic glycolysis for energy generation but is hypothesis generating at this stage and requires further exploration in a future adequately powered study.

Collection of hs-TropI AUC as a surrogate marker of PMI was confirmed to be feasible in this trial. The study design employed electronic prescription to define specific timepoints for blood sampling by critical care/cardiothoracic nursing staff, an intervention which less than 5% samples were missed – a common challenge when using this study endpoint, where high levels of missing troponin data effects the validity of trial findings [9,84]. Therefore, the ongoing use of regular biomarker assessment, as a surrogate for imaging-based quantification of PMI, can be recommended as a tool in cardioprotection trials in cardiac surgery. The findings of this study demonstrate that of others that the surgical intervention performed (i.e., CABG, AVR or combination of both vs. mitral valve procedures) has important differential effects on the peak levels and overall release of hs-TropI, specifically that mitral valve procedures (which require incision of cardiac tissue itself to access the valve) are associated with much higher degree of PMI. This observation has been made before[3], and consideration should be given to either focusing on patients undergoing a specific type of surgery or stratifying patients at randomisation based on surgical intervention, when designing larger cardioprotection trials. In this study, data pooled by surgical procedure (see Fig. 44 D-E) provide better discrimination between active and placebo when assessing for a potential drug effect. Importantly, this study was not powered to identify any cardioprotective effects of 5-ALA-SFC and no statistically significant dose-dependent changes were observed. The

suggestion that patients allocated to the highest dose treatment group experienced a delayed and sustained increase in troponin I, warrants further investigation.

TALEN was designed as a translational, proof of mechanism, dose-ranging, single-centre study and not designed or powered to identify statistically significant differences in clinical endpoints and no account was made for multiple comparison testing. Accordingly, findings with respect to clinical outcomes in the post-operative period should be cautiously interpreted and considered as hypothesis generating for future assessment and validation in adequately powered studies. With these caveats in mind, the mean and median SOFA organ dysfunction scores as well as the vasoactive-inotropic (VIS) score reflecting the degree of pharmacological support required of the patient's cardiovascular system (and a predictor of mortality) were lower in 5-ALA-SFC treated groups compared to placebo. Similarly, the proportions of patients in KDIGO I and II and those with documented arrhythmia were highest in the placebo group. In conjunction with the lower troponin release profiles compared to placebo when examining the same types of surgery, these findings may point to a potential multi-organ (including cardio-) protective effect of 5-ALA-SFC in the context of surgery on CPB.

In summary, pre-operative dosing with 5-ALA-SFC was well-tolerated with a good safety profile across all doses examined in adult patients undergoing cardiac surgery on CPB. Patients treated with 5-ALA-SFC tended to have numerically lower SOFA organ dysfunction scores, less arrhythmia and lower vasoactive-inotropic pharmacological support requirements than those treated with placebo. These findings support the further evaluation of 5-ALA-SFC in adequately powered trials focused on clinical endpoints.

# 4 Chapter 4. Oral 5-ALA-SFC to induce HO-1 function - a strategy to reduce the effects of CPB associated haemolysis and iron mediated organ injury

## 4.1 Introduction

Clinical trials investigating interventions aimed at improving outcomes in cardiac surgery have frequently failed to translate promising biological mechanisms into patient benefit[77]. Recently, there has been a focus on reducing both the impact of cardiac IRI associated with surgery requiring CPB and, separately, reducing the extent and impact of CPB-associated haemolysis[320]. 5-ALA-SFC, through its potential to induce HO-1, mitigate haem toxicity and subsequent inflammatory response[204], represents an attractive therapeutic option capable of addressing both these insults. Confidence in optimal dose selection of 5-ALA-SFC for evaluation in large outcome-driven trials in cardiac surgery will be informed by evaluation of its safety profile combined with proximate pharmacodynamic measures of HO-1 activity and biomarkers supporting its potential ability to reducing the adverse impact of CPB-associated haemolysis and subsequent organ injury.

Accordingly, the specific objectives of the work presented in this chapter are:

1. Identify key human cells and tissues enriched for *HMOX1* by interrogating publicly available datasets, allowing for accurate selection of physiologically relevant *in vitro* models investigating drug effect, and focus on key circulating (immune) cells in which to evaluate drug dose-pharmacodynamic response in patients.
2. Evaluate for evidence of target engagement and a dose response relationship to 5-ALA-SFC in key immune and tissue cell lines, *in vitro*.
3. Within the TALEN patient population:
    a. Define the study population's *HMOX1* (GT)n- repeat promoter polymorphism status.
    b. Determine the presence of target engagement by 5-ALA-SFC (i.e., evidence for increased HO-1 expression and/or enzymatic activity, particularly focused on the change from screening to immediate pre-operative timepoint) using multiple orthogonal approaches, to maximise confidence in selection of the OBD for HO-1 activation for future clinical trial evaluation.

c. Examine the impact of 5-ALA-SFC on NRF2-dependent cellular signalling through gene expression analyses of circulating immune cells.
d. Examine the impact of 5-ALA-SFC on the extent of CPB-associated haemolysis, iron homeostasis, and iron mediated cellular injury.
e. Investigate the potential for pre-operative dosing with 5-ALA-SFC to mitigate renal injury in response to cardiac surgery on CPB through evaluation of a range of blood and urine-based peri-operative biomarkers (including those in routine clinical use).

## 4.2 Results

### 4.2.1 *In silico* identification of human cells and tissues enriching for *HMOX1*

The expression of *HMOX1* was evaluated across multiple tissues in human (GTEx[282]) and mouse (GeneAtlas[283]). In both species, tissues/cells of the reticuloendothelial system (e.g., spleen, bone marrow macrophages) were found to enrich for *HMOX1*. In human tissues, this expression was highest in the spleen and in mouse, six out of the top ten cells/tissues were of the mononuclear phagocyte system (MPS), bone marrow tenth- and the spleen fourteenth highest (**Figs. 45-46**). Relative expression varied widely across tissues, whereas *HMOX2* expression was relatively stable (Appendix 8.3). Circulating immune cells exhibited a dynamic range of *HMOX1* expression in human (DMap[285]) and mouse (ImmGen[286]), in contrast with reasonably stable *HMOX2* expression. From these analyses, cells of the MPS were found to enrich for *HMOX1* (**Figs. 47-48**) and this expression profile was highly conserved across species. This was formally assessed by determining the Conservation of Expression (CoE)[284] metric which was found to be 0.89. This metric ranges from -1 (poorly conserved) to + 1 (highly conserved). Finally, interrogation of three independent human RNAseq datasets[288–290] confirmed that monocytes enriched for *HMOX1* most profoundly (**Fig. 49**). These data were normalised and combined in a consensus dataset[290] which found that non-classical, then classical and intermediate monocytes enriched most significantly for *HMOX1.*

This work also explored differential gene expression in cell types enriched for *HMOX1,* with a specific focus on ontological classification by scavenger and haem biosynthesis function. Strikingly, immune cells enriching for *HMOX1* also enriched for various genes encoding for proteins with important scavenging/innate immune functions (e.g., *MSR1*, *MARCO*, *CD36*, *CD68*, *CLEC7A* and *CXCL16*) (Fig. 50). Data summarised by heatmap (exported from the Human Protein Atlas[287]) suggested that particularly high levels of *CD36* and *CD68* were co-expressed with *HMOX1*. As expected, *HMOX1* was co-expressed with various genes involved in haem biosynthesis, transport, and breakdown pathways (e.g., *ALAS*, *UROS*, *UROD*, *PPOX, BLVRA*).

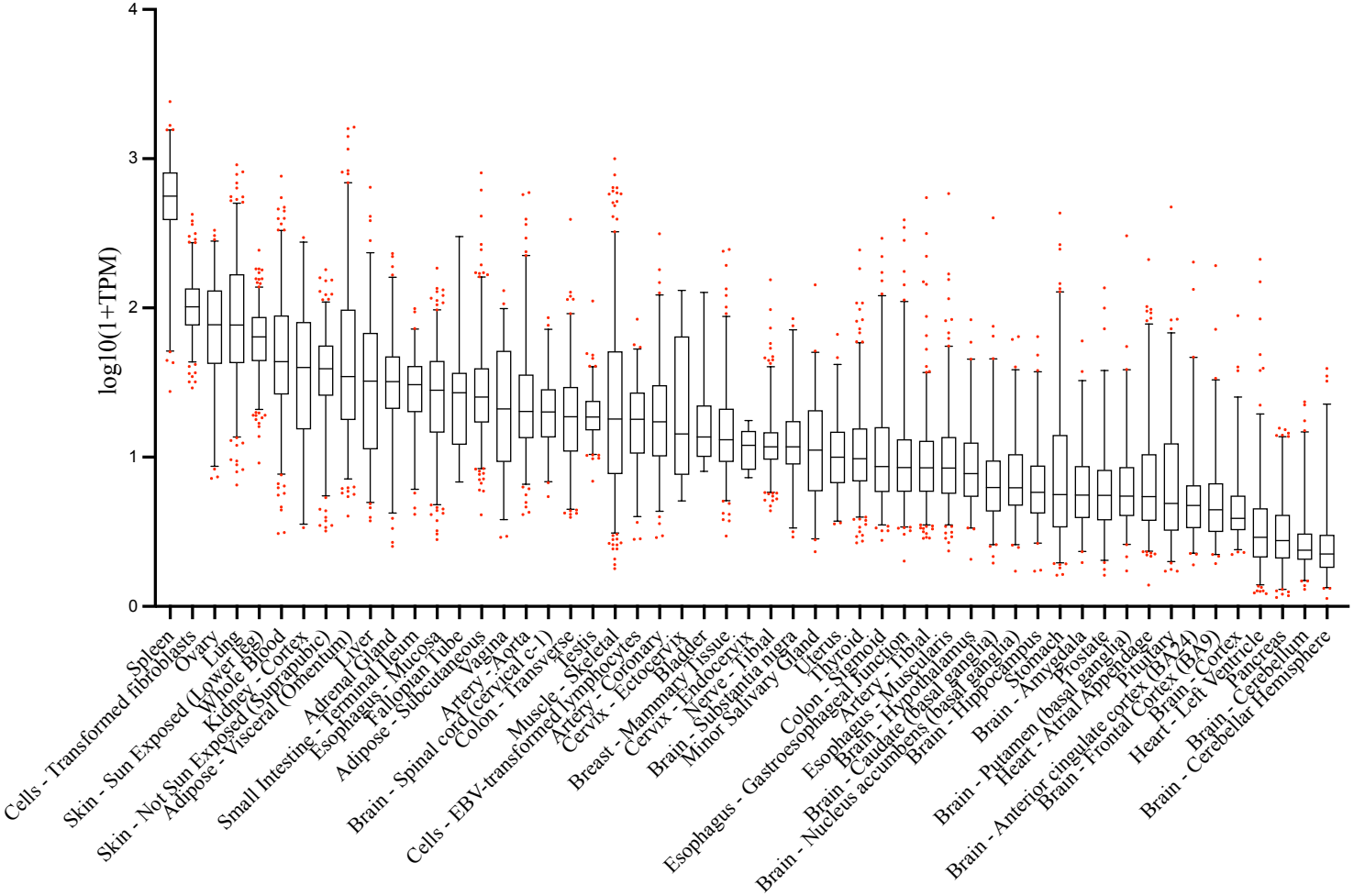



**Figure 45: *HMOX1* expression in human tissues (GTEx).**

Data presented as: median, IQR and 95% CI. Abbreviations: TPM – transcript/million, IQR – interquartile range, CI – confidence interval.

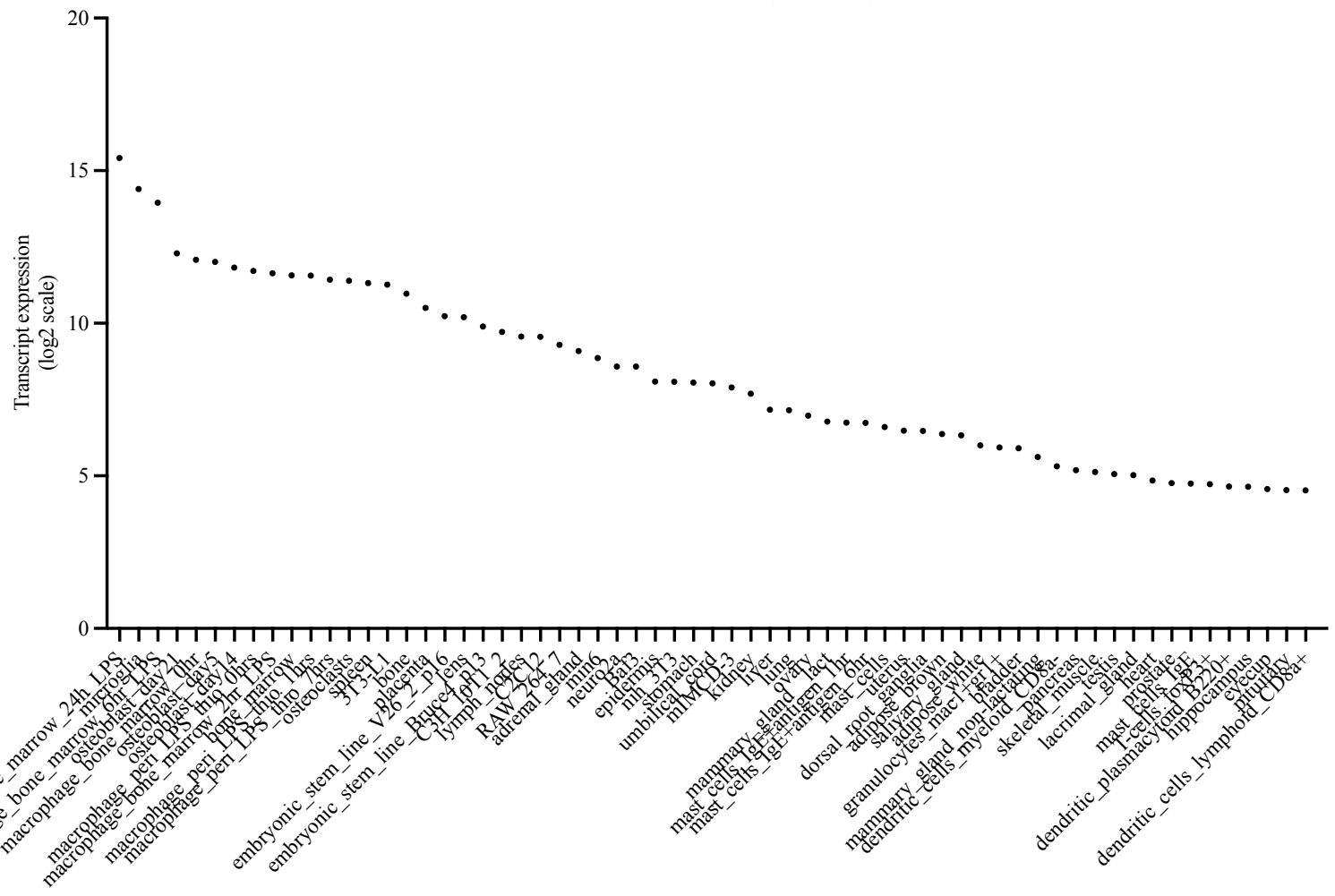



**Figure 46: *HMOX1* expression in mouse tissues (GeneAtlas).**

Data presented as: mean.

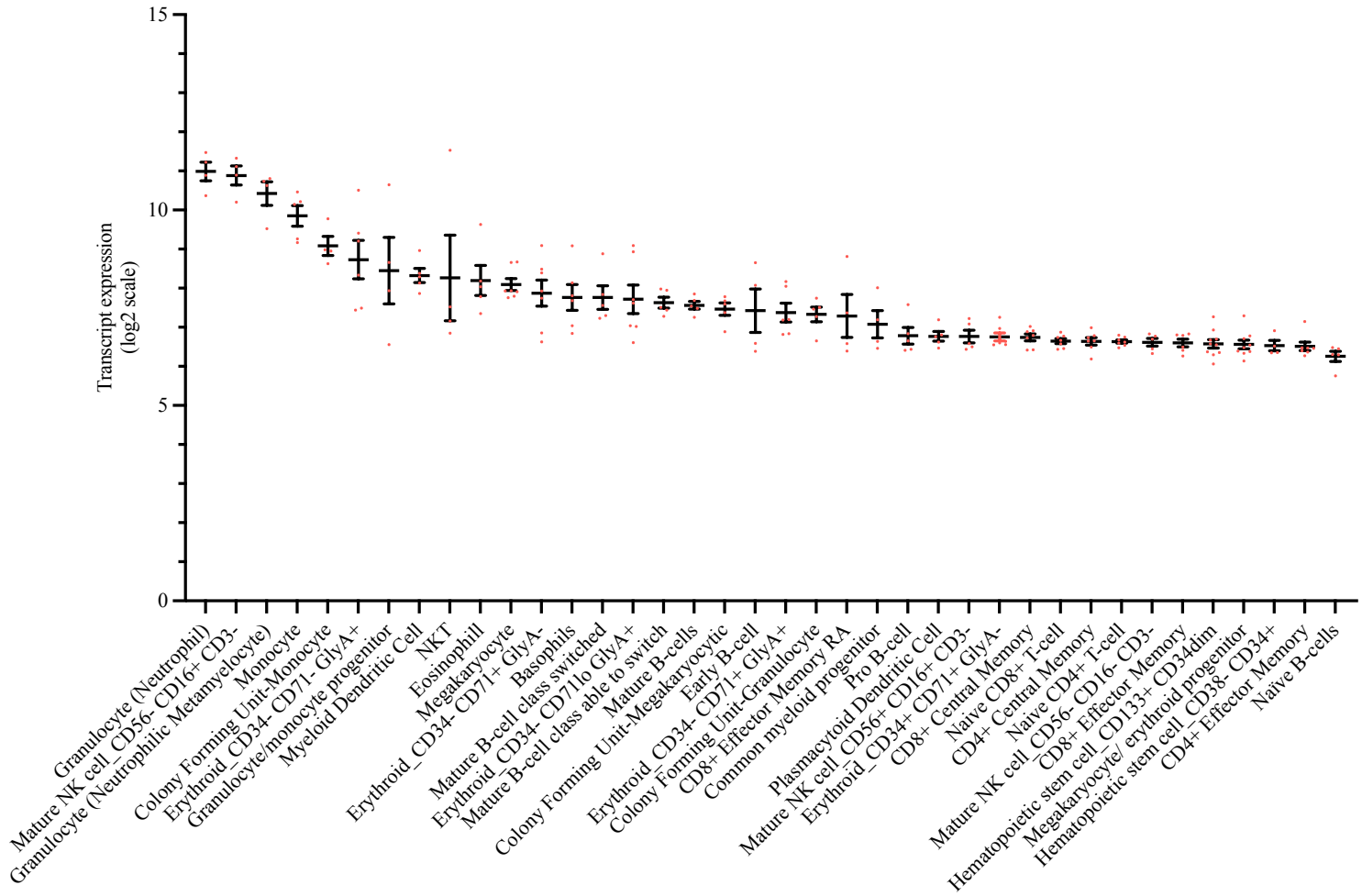



**Figure 47: Human HMOX1 expression in circulating immune cells (DMap).**

Data presented as: mean, standard error of the mean (SE).

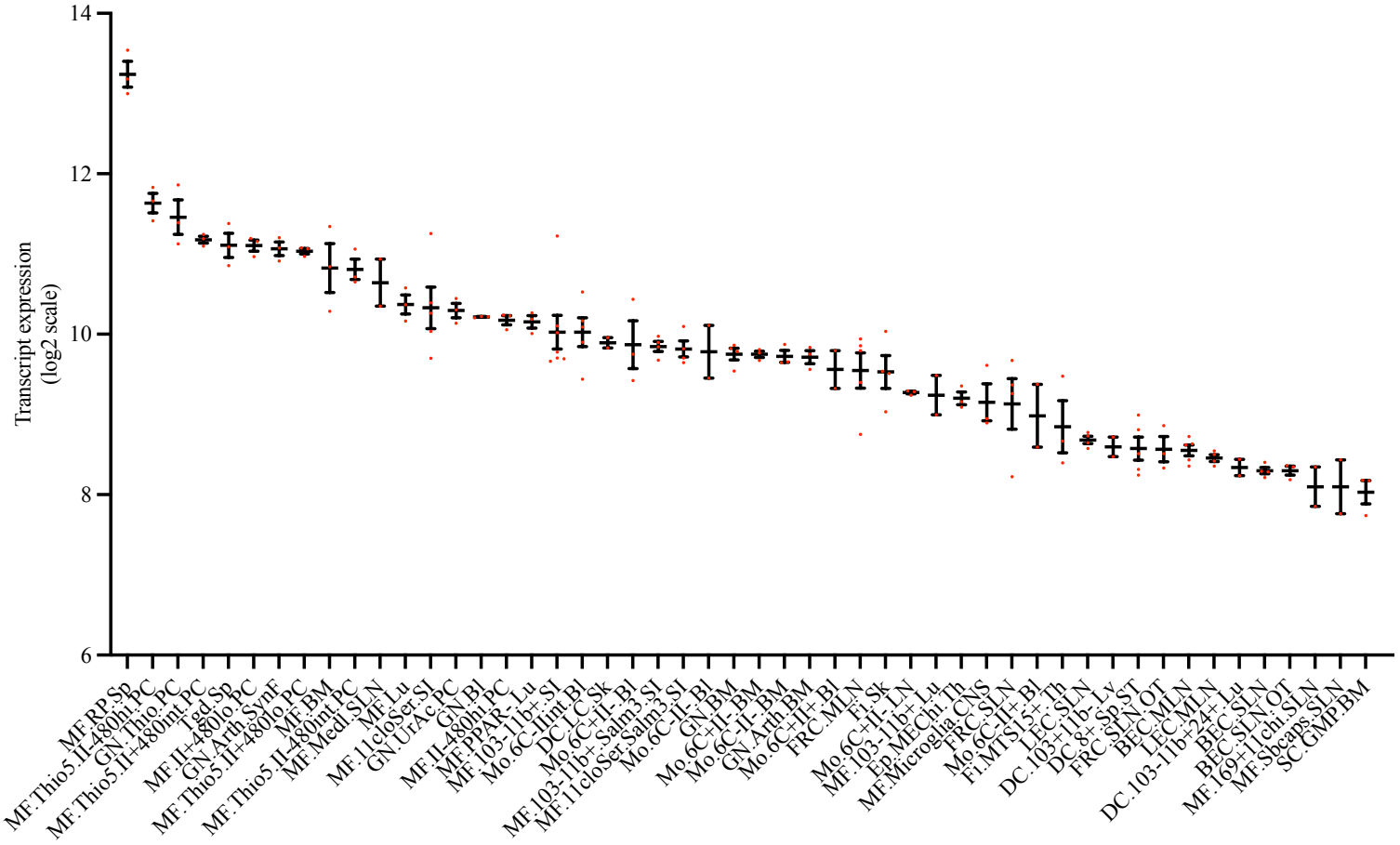



**Figure 48: Murine HMOX1 expression in circulating immune cells (Immgen).**

Data presented as: Mean, standard error of the mean (SE).

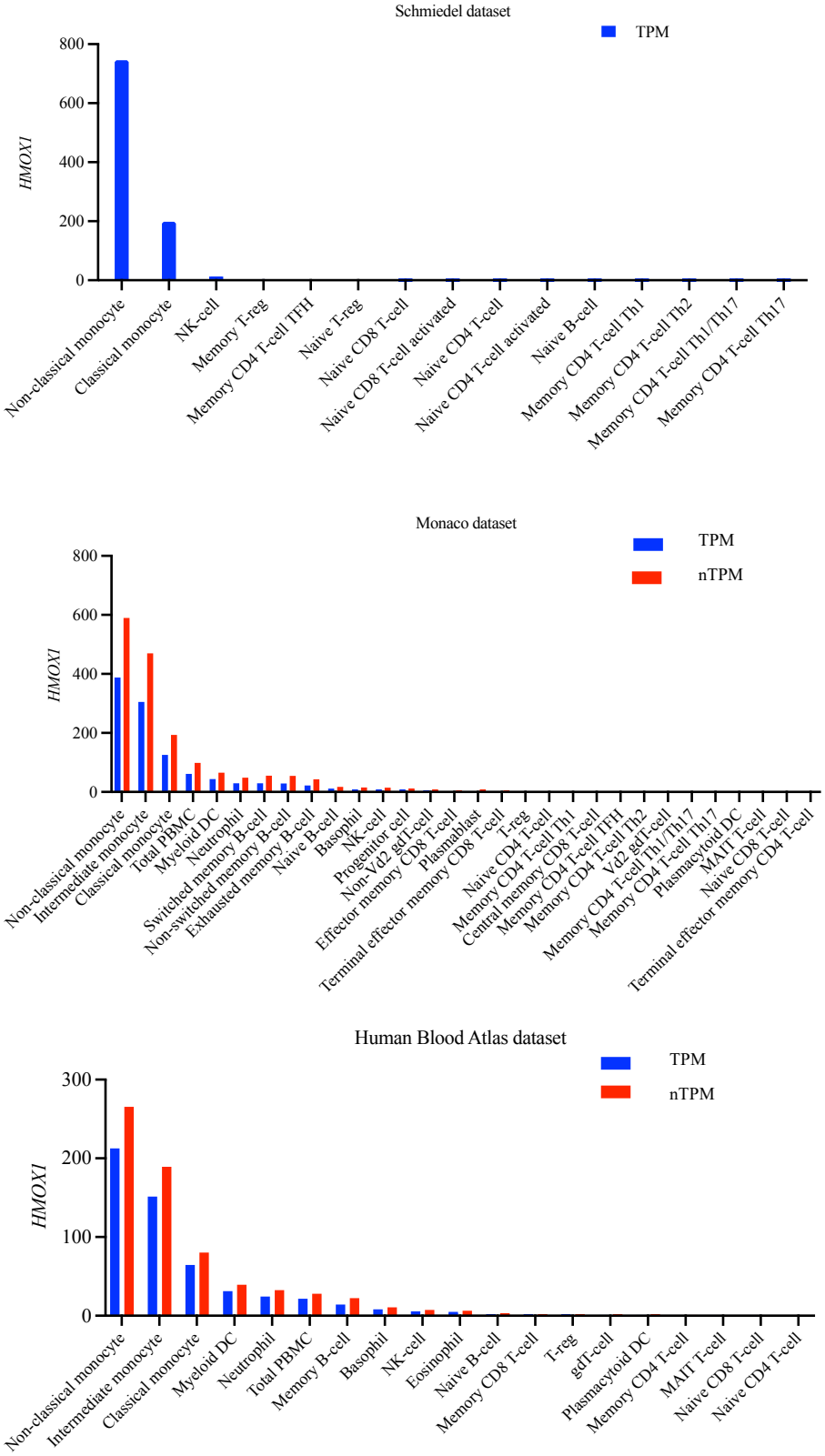



**Figure 49: HMOX1 expression in human circulating immune cells (Human Protein atlas).**

Abbreviations: TPM – transcripts per million; nTPM – normalised.

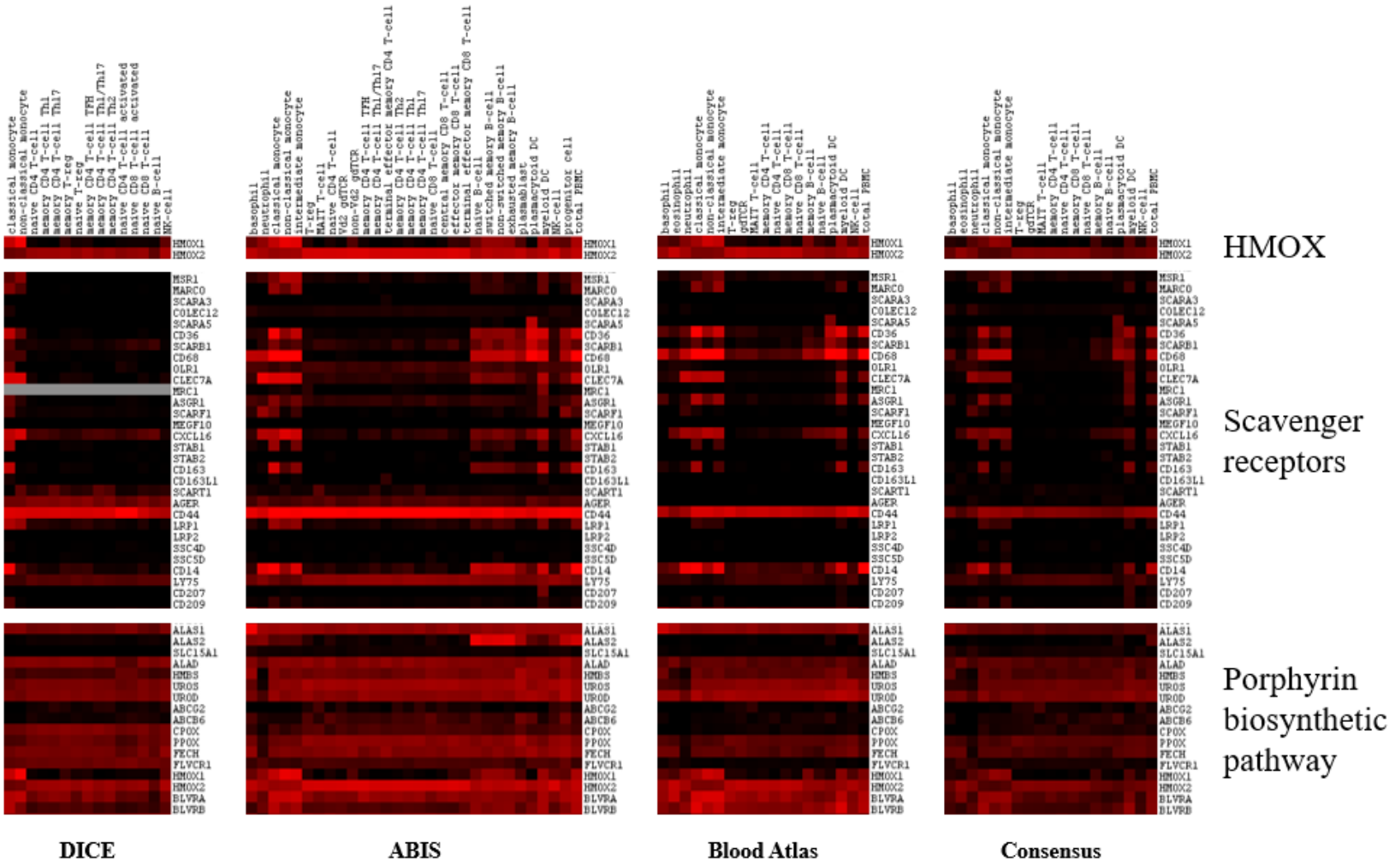



**Figure 50: Expression profile of genes involved in haem synthesis and scavenging in human immune cells and their relationship to *HMOX1* expression.**

### 4.2.2 In vitro confirmation of 5-ALA-SFC target engagement in key cell types

Using an immortalised monocytic leukemic cell line (THP-1 cells) the response to increasing doses of 5-ALA-SFC was investigated *in vitro*. When compared to negative treatment conditions (exposure to vehicle only), 5-ALA-SFC caused a dose-dependent increase in HO-1 expression quantified using Western blot (**Fig. 51**). This was more pronounced than the dose response seen when cells were exposed to increasing concentrations of hemin, an agent known to induce HO-1 expression. The experiment was repeated with the same treatment conditions and cells were stained for HO-1 and analysed using flow cytometry (**Fig. 52**). These data suggested not only an increased protein expression in the 5-ALA-SFC treated group (quantified as mean fluorescent intensity), but also that a higher proportion of cells expressed the protein when treated with increasing concentrations of 5-ALA-SFC (low concentration 8.13 % vs. high concentration 75.2%). Again, no increase in expression of HO-1 was seen in the vehicle-treated negative control, and a less pronounced response was observed in cells exposed to hemin. For the latter experiment lipopolysaccharides (LPS) from *Escherichia coli* (*E. coli)* were used as a further positive control.

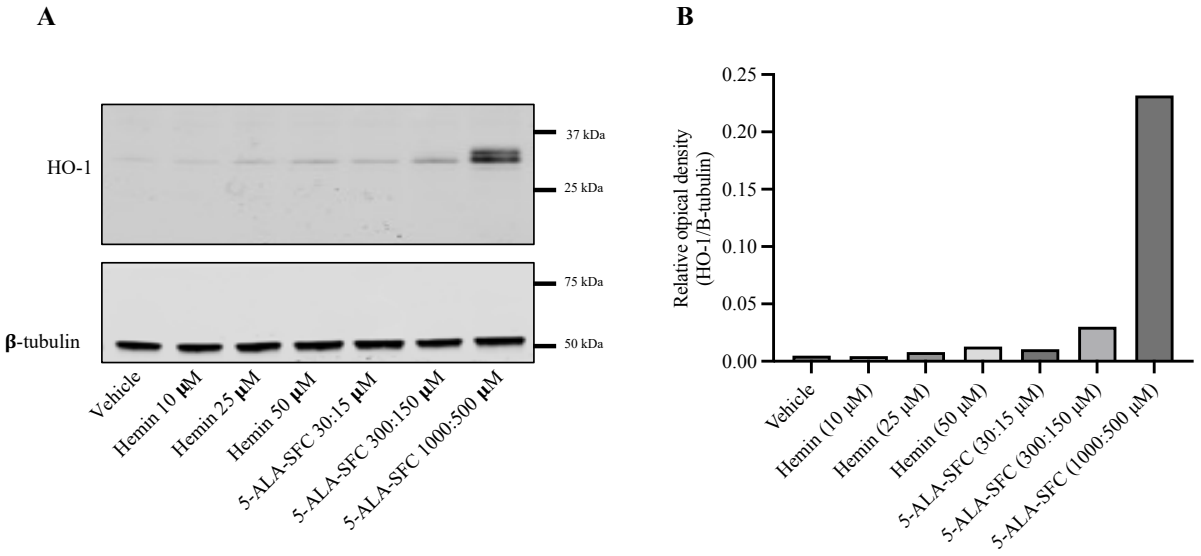



**Figure 51: 5-ALA-SFC induces HO-1 expression in THP-1 cells.**

Western Blot with HO-1 and β-Tubulin (panel A); relative optical density of HO-1 to β-Tubulin (panel B).

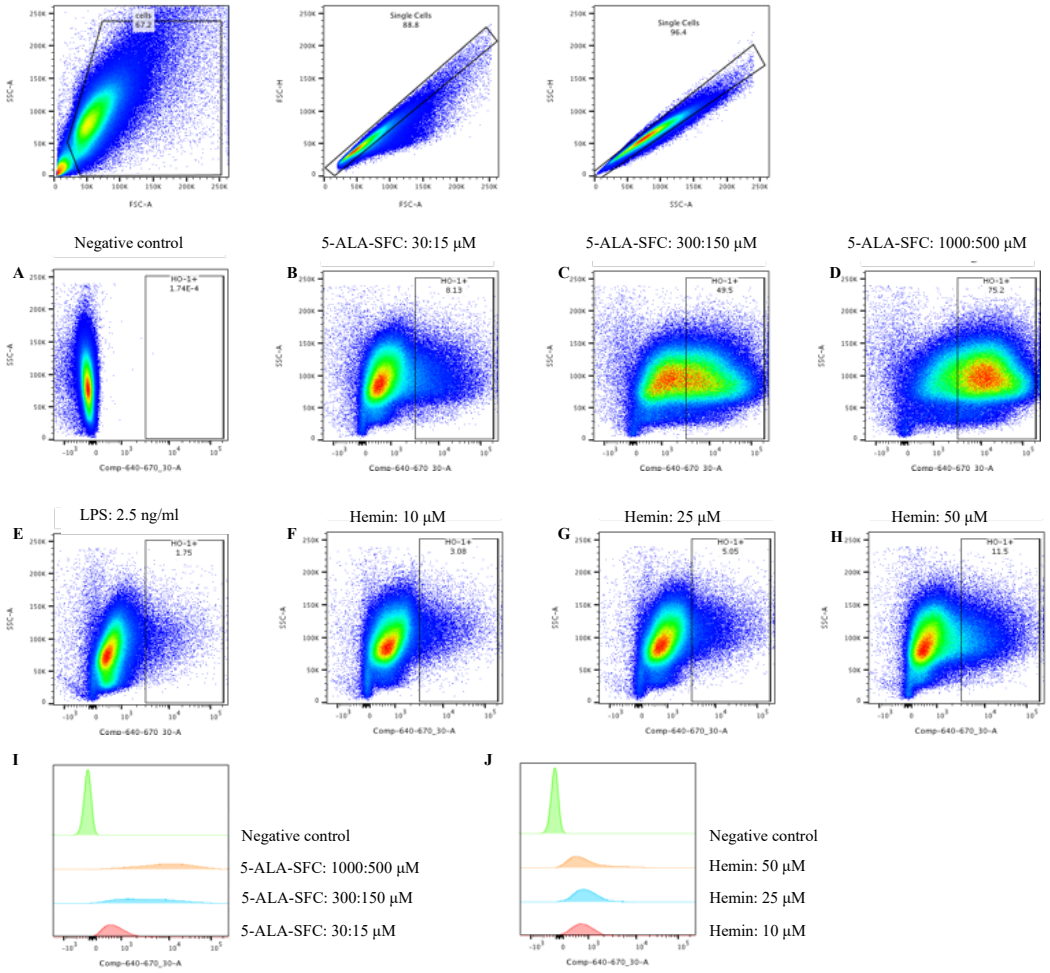



**Figure 52: HO-1$^{+}$ THP-1 cells in response to 5-ALA-SFC exposure.**

Representative flow cytometry traces showing dose-dependent induction of HO-1 expression in THP-1 cells in response to 5-ALA-SFC (panel B-D) compared to LPS (panel E) and hemin (panel F-H) as positive controls. Histograms (panel I-J) quantify the response with a rightward shift representing the scale of change.

The ability of 5-ALA-SFC to induce HO-1 mRNA expression in human immune cells was further explored using primary human monocyte-derived macrophages (MDM) that were cultured in identical conditions and exposed to either vehicle or increasing concentrations of 5-ALA-SFC. This showed a significant dose-dependent increase in *HMOX1* gene expression normalised to *ACTB* (**Fig. 53 A**) with s no statistically significant difference in *HMOX2* expression across treatment groups relative to *ACTB* **(Fig 53 B)**, and a significant increase in the *HMOX1*:*HMOX2* ratio (**Fig. 53 C**).

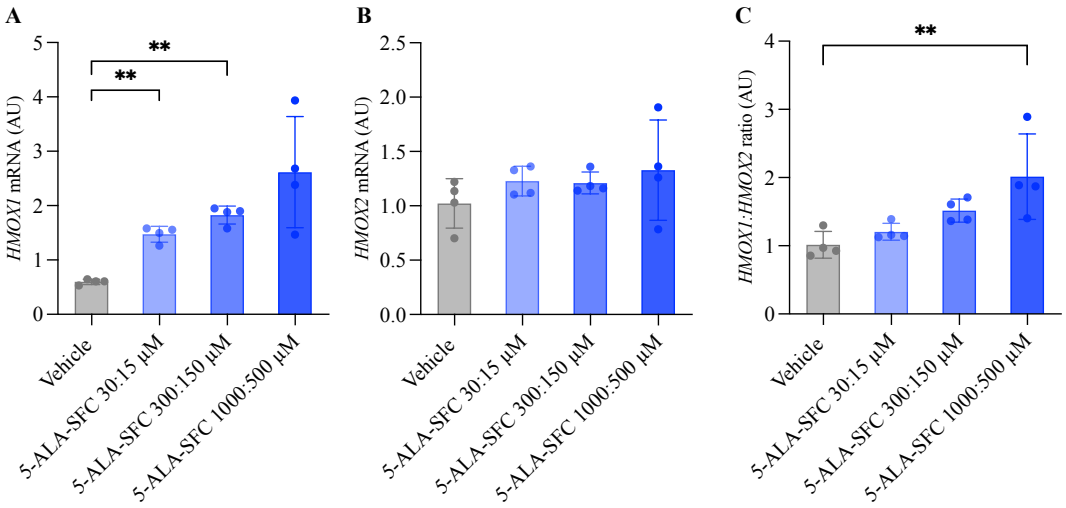



**Figure 53: The impact of 5-ALA-SFC on *HMOX1* expression in primary human MDMs.**

A dose-dependent increase in *HMOX1* expression is demonstrated in MDMs treated with 5-ALA-SFC (panel A). The significance of the treatment effect at the highest dose is further appreciated by normalising *HMOX1* to *HMOX2* data (panel C).

** = $p < 0.05$, one-way ANOVA vs. vehicle, Dunnett's multiple comparisons.

Finally, *in vitro* evidence of target engagement in human cardiomyocytes was investigated by treating induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) with 5-ALA-SFC at increasing concentrations, compared to a vehicle-only treated negative control (**Fig. 54**). Statistically significant increases in relative *HMOX1* expression were identified in iPSC-CMs exposed to the two higher concentrations of 5-ALA-SFC. Data from these experiments also suggested a potential biphasic response to the drug, with the highest increase in relative gene expression observed in cells exposed to the intermediate 5-ALA-SFC concentration selected, and a less profound effect in the highest concentration tested.

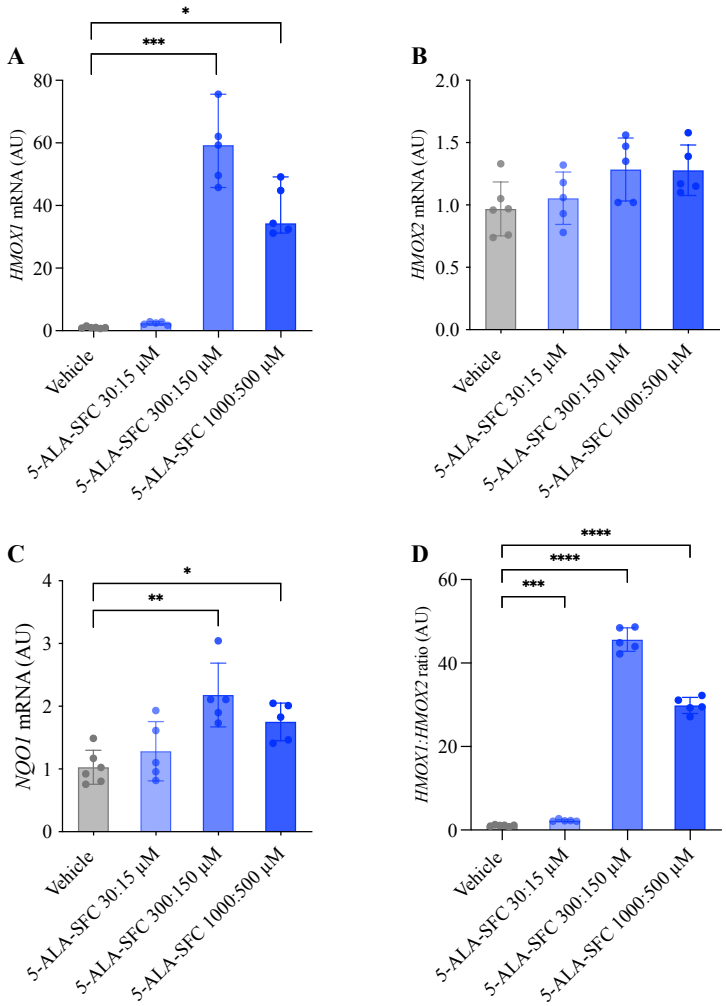



**Figure 54: 5-ALA-SFC increases *HMOX1* expression in iPSC-CMs.**

*HMOX1* mRNA expression increases most significantly under intermediate 5-ALA-SFC concentration conditions and the data suggest a U-shaped iPSC-CM response to the drug (panel A). The significance of this finding is further enhanced by normalising data to HMOX2 (panel D). *NQO1* expression, a canonical NRF2 target gene, is increased in response to 5-ALA-SFC (panel C).

* = $p < 0.05$, ** = $p < 0.05$, *** = $p < 0.01$, **** = $p < 0.001$ one-way ANOVA vs. vehicle, Dunnett's multiple comparisons.

### 4.2.3 Frequency distribution of the *HMOX1* promoter (GT)n repeat polymorphism within the TALEN trial study population

Following informed consent, PBMC DNA from all TALEN trial participants was extracted, quantified and assessed for quality (spectrophotometry data are summarised in appendix 8.7) prior to performing a PCR aimed at amplifying a defined region within the promoter of *HMOX1*, containing a well described (GT)n repeat polymorphism. This was successfully performed in all 48 adult patients scheduled to undergo non-urgent cardiac surgery. PCR products were assessed for quality by 1.8% Agarose gel

electrophoresis and amplicons were visually estimated to be of the expected molecular size. Overall, the quality of PCR products was sufficient to proceed with fragment length analysis (FLA) by capillary electrophoresis (**Fig. 55 A-C**). Representative FLA data illustrate allele homozygosity (**Fig. 55 B**) and heterozygosity (**Fig. 55 C**). Assigning amplicon lengths for all submitted samples was possible and the quality of the data was acceptable. The length of the (GT)n repeat was calculated and defined as short (S) (≤ 27 GT repeats) or long (L) (> 27 repeats).

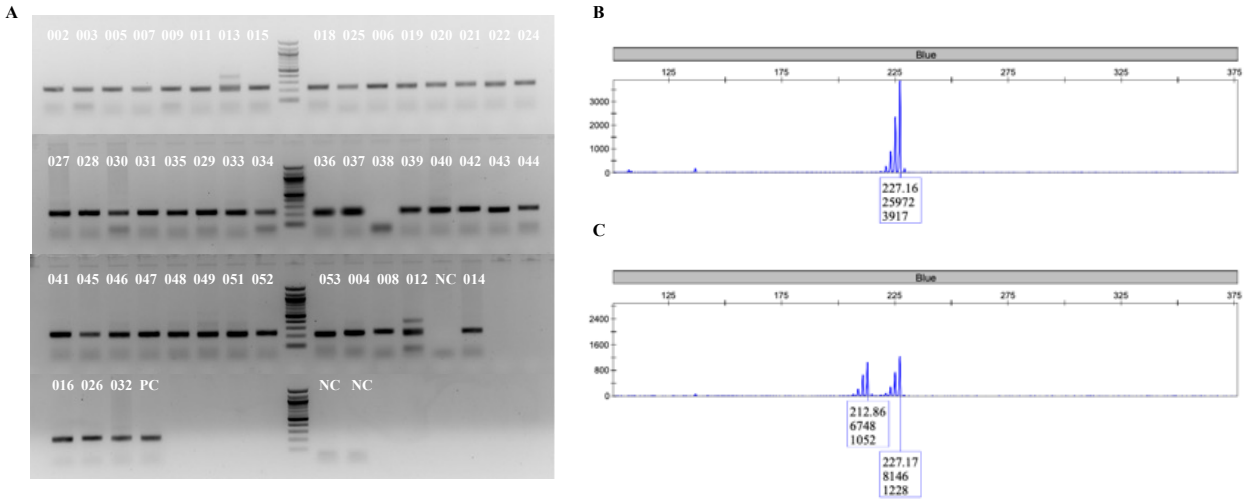



**Figure 55: Electrophoresis of PCR products amplifying the *HMOX1* promoter region of interest with fragment length analysis by capillary electrophoresis.**

Abbreviations: NC – negative control; PC – positive control.

Through considering both alleles within one patient sample, patients were assigned a genotype of either homozygous short (SS), heterozygous short (SL) or homozygous long (LL). Findings from TALEN trial patients showed a biphasic population distribution in repeat lengths, with peak frequencies seen at a (GT)n repeat of 18 and 32 (**Fig. 56 A**). Reflecting the relatively small sample size per treatment group, *HMOX1* (GT)n length polymorphisms were not equally distributed across treatment allocation groups within the trial (Fig. 56 B). The 5-ALA-SFC 225 mg/354 mg allocation group was most balanced (SS 38 %, SL 25 %, and LL 38 %) with no SS individuals in either the placebo or lowest two dose 5-ALA-SFC treatment allocation groups. Across the trial there were 4 SS (10 %), 23 SL (55%) and 15 LL (35%) individuals. Notably, the highest dose group within the active arm had only 1 LL and only one SS patient.

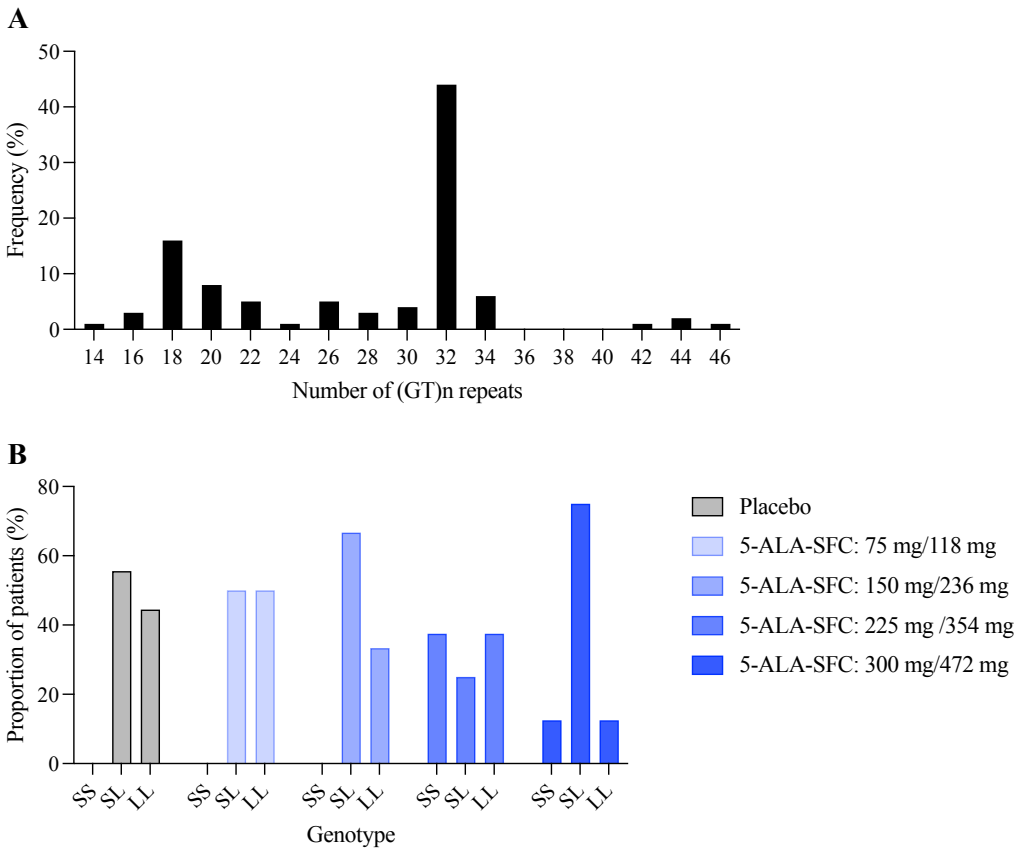



**Figure 56: TALEN trial participant microsatellite *HMOX1* promoter (GT)n repeat polymorphism status.**

Allelic length population frequency (panel A) and distribution of genotypes across treatment allocation groups within the TALEN trial (panel B).

### 4.2.4 HO-1 activation as evidence of 5-ALA-SFC target engagement in patients undergoing cardiac surgery on CPB

#### 4.2.4.1 Enzyme activity in human PBMCs

The rate of biliverdin production, as a measure of HO enzyme activity, was measured in cryopreserved PBMCs from all TALEN trial participants using a novel LC-MS/MS-based assay designed specifically for the trial (**Fig. 57 A-B**). The assay performed well and accurate quantification of biliverdin was achieved in most samples. As the assay cannot distinguish between biliverdin production from HO-1 vs. HO-2, findings are reported as HO activity. It is assumed that any increase in enzyme activity over baseline represents inducible HO-1, rather than constitutive HO-2, activity. Higher baseline values for bilirubin concentration and its derivative, HO activity, were seen in the 5-ALA-SFC 300 mg/472 mg

treatment allocation group, compared to all other patients. This could be explained by inter-group differences in the *HMOX1* promoter (GT)n repeat polymorphism status of patients, as described above, which revealed that patients in the 300 mg/472 mg treatment allocation group had a single patient with the LL genotype (i.e., most patients had a genotype associated with higher baseline enzyme activity). Enzyme activity was predicted by the polymorphism status (**Fig. 57 C**) with higher baseline (i.e. pre-treatment) enzyme function seen in the SL and particularly SS genotype groups compared to those bearing the LL genotype. Serum bilirubin, quantified independently by the hospital NHS laboratory laboratory, was also numerically highest at screening baseline in this treatment allocation group (**Fig. 57 D**).

The specified primary statistical analysis using analysis of covariance (ANCOVA) which takes into account baseline values revealed a significant increase in biliverdin concentration at the end of dosing timepoint (i.e. Pre-op) over baseline in the highest dose treatment group (mean difference [95 % CI] vs. placebo = 253.0 ng/mL [17.6; 488.3], p-value = 0.0363). This treatment effect was further enhanced when the analysis was corrected for *HMOX1* promoter genotype (mean difference [95 % CI] vs. placebo = 283.5 ng/mL [31.3, 535.7]; p-value = 0.0293), suggesting some degree of differential response to the drug depending on patient HO-1 promoter polymorphism genotype. A pre-specified supportive secondary analysis using a LMM with time as a categorical variable confirmed these findings. Within this model, the most significant change in HO activity was seen at the pre-operative timepoint (biliverdin [ng/mL]: mean difference [95 % CI] vs. placebo = 246.8 [100; 393], p-value = 0.0015; HO activity [pmol biliverdin/million cells/hour]: mean difference [95 % CI] vs. placebo = 514.7 [119, 910], p-value = 0.0119).

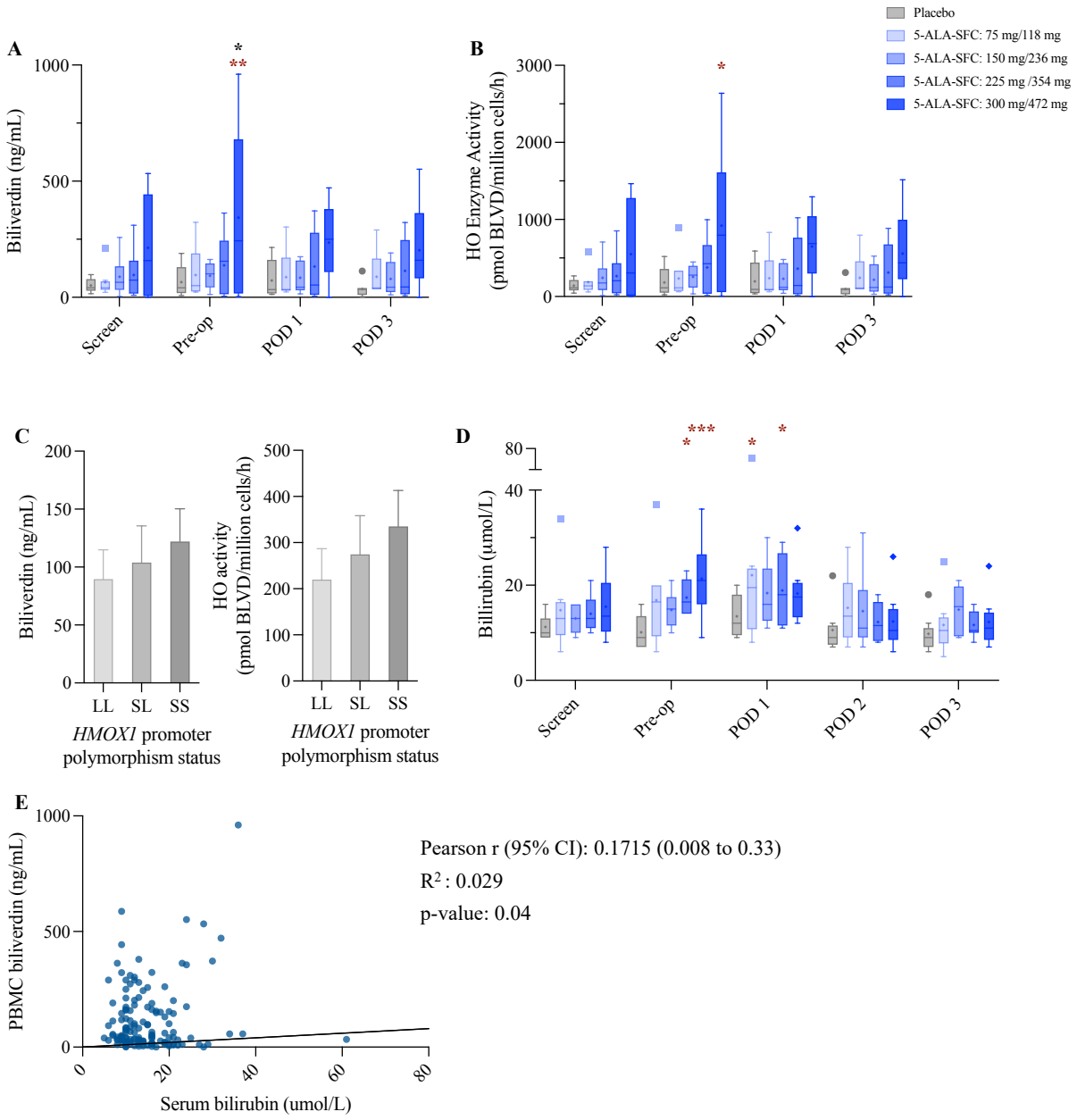



**Figure 57: The impact of 5-ALA-SFC on haem oxygenase activity.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (A, B, D) and mean with SE (C).

Significance from ANCOVA in black, from the LMM in red: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Abbreviations: IQR – interquartile range; SE – standard error of the mean.

5-ALA-SFC was also associated with a striking dose-dependent increase in serum bilirubin amongst cardiac surgical patients, particularly at the pre-operative timepoint (**Fig. 57 D**). Using a LMM approach, the most significant increase was seen pre-operatively in the 5-ALA-SFC 300 mg/472 mg treatment

allocation group (mean difference [95 % CI] vs. placebo = 7.3 [3.1; 11.5] mmol/L, p-value = 0.0008). Although serum bilirubin did not strongly correlate with PBMC LC-MS/MS measured biliverdin concentration ($R^2$ = 0.0029) the correlation was statistically significant (p-value = 0.04) (**Fig. 57 E**), validating the assay and the potential for routinely measured serum bilirubin to serve as an indirect marker of HO-1 activation in response to 5-ALA-SFC in the appropriate clinical context.

**Table 25: HO activity in human PBMCs in response to 5-ALA-SFC – descrptive statistics and inferential analysis**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **PBMC HO activity (biliverdin (ng/mL))** | | | | | |
| **Screening** | | | | | |
| n (%) | 8 (89%) | 7 (88%) | 8 (89%) | 8 (100%) | 7 (88%) |
| Mean (SD) | 52.7 (30.3) | 68.2 (65.8) | 94.8 (81.7) | 96.4 (102.8) | 213.3 (214.1) |
| Median (IQR) | 47.1 (28.7; 78.3) | 50.5 (31.3; 72.9) | 80.3 (34.5; 133.5) | 74.2 (18.8; 133.3) | 158.0 (5.2; 443.0) |
| Min Max | 15.9 97.8 | 21.4 212.0 | 4.0 258.0 | 8.7 310.0 | 0.1 533.0 |
| **Pre-op** | | | | | |
| n (%) | 7 (78%) | 6 (75%) | 6 (67%) | 7 (88%) | 7 (88%) |
| Mean (SD) | 70.9 (71.7) | 85.1 (117.3) | 105.9 (47.6) | 137.4 (135.4) | 407.5 (381.3) |
| Median (IQR) | 50.6 (17.5; 154.0) | 41.4 (26.5; 55.6) | 112.0 (60.6; 146.0) | 155.0 (13.0; 244.0) | 356.0 (131.0; 587.0) |
| Min Max | 8.3 189.0 | 22.8 323.0 | 41.8 163.0 | 5.0 363.0 | 2.7 961.0 |
| **POD 1** | | | | | |
| n (%) | 7 (78%) | 5 (63%) | 8 (89%) | 8 (100%) | 8 (100%) |
| Mean (SD) | 78.5 (89.2) | 86.6 (121.1) | 92.6 (64.1) | 132.2 (151.0) | 209.0 (168.4) |
| Median (IQR) | 36.1 (13.3; 201.0) | 33.8 (32.9; 40.0) | 72.4 (38.2; 159.0) | 52.6 (11.3; 276.5) | 200.5 (65.0; 335.0) |
| Min Max | 12.7 215.0 | 23.1 303.0 | 27.0 175.0 | 4.9 372.0 | 0.1 471.0 |
| **POD 3** | | | | | |
| n (%) | 7 (78%) | 5 (63%) | 4 (44%) | 8 (100%) | 7 (88%) |
| Mean (SD) | 36.7 (37.2) | 88.5 (112.7) | 96.2 (72.7) | 113.7 (128.2) | 203.2 (190.7) |
| Median (IQR) | 39.2 (10.8; 40.3) | 40.3 (34.1; 44.0) | 79.2 (39.3; 153.0) | 44.8 (13.4; 232.0) | 160.0 (81.9; 363.0) |
| Min Max | 3.0 113.0 | 34.0 290.0 | 35.3 191.0 | 7.4 322.0 | 0.3 552.0 |
| **PBMC HO activity (pmol biliverdin/million cells/hour)** | | | | | |
| **Screening** | | | | | |
| n (%) | 8 (89%) | 7 (88%) | 8 (89%) | 8 (100%) | 6 (75%) |
| Mean (SD) | 145 (83) | 187 (181) | 260 (225) | 265 (282) | 551 (636) |
| Median (IQR) | 129 (79; 215) | 139 (86; 200) | 220 (95; 367) | 204 (52; 366) | 305 (14; 1217) |
| Min Max | 44 269 | 59 582 | 11 709 | 24 851 | 0 1464 |
| **Pre-op** | | | | | |
| n (%) | 7 (78%) | 6 (75%) | 6 (67%) | 7 (88%) | 7 (88%) |
| Mean (SD) | 195 (197) | 234 (322) | 291 (131) | 377 (372) | 921 (944) |
| Median (IQR) | 139 (48; 423) | 114 (73; 153) | 308 (166; 401) | 426 (36; 670) | 796 (58; 1612) |
| Min Max | 23 519 | 63 887 | 115 448 | 14 997 | 7 2639 |
| **POD 1** | | | | | |
| n (%) | 7 (78%) | 5 (63%) | 8 (89%) | 8 (100%) | 7 (88%) |
| Mean (SD) | 216 (245) | 238 (333) | 254 (176) | 363 (415) | 648 (446) |
| Median (IQR) | 99 (37; 552) | 93 (90; 110) | 199 (105; 437) | 144 (31; 759) | 687 (299; 1044) |
| Min Max | 35 590 | 63 832 | 74 481 | 13 1022 | 0 1294 |
| **POD 3** | | | | | |
| n (%) | 7 (78%) | 5 (63%) | 4 (44%) | 8 (100%) | 7 (88%) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 101 (102) | 243 (310) | 264 (200) | 312 (352) | 558 (524) |
| Median (IQR) | 108 (30; 111) | 111 (94; 121) | 217 (108; 420) | 123 (37; 637) | 439 (225; 997) |
| Min Max | 8 310 | 93 796 | 97 525 | 20 884 | 1 1516 |
| **Change in PBMC HO activity screening vs pre-operatively (ANCOVA)** | | | | | |
| **PBMC HO activity (biliverdin (ng/mL)) - Unadjusted** | | | | | |
| Observed mean (SE) | 100.2 (64.9) | 108.3 (75.4) | 117.2 (68.9) | 127.8 (63.7) | 353.2 (89.2) |
| Difference vs placebo (95% CI) | | 8.1 (-196.3, 212.5) | 17.0 (-177.3, 211.4) | 27.7 (-161.7, 217.1) | 253.0 (17.6, 488.3) |
| p-value | | 0.9353 | 0.8576 | 0.7653 | **0.0363 *** |
| **PBMC HO activity (biliverdin (ng/mL)) – Adjusted for *HMOX1* promoter polymorphism status** | | | | | |
| Observed mean (SE) | 90.4 (66.7) | 95.2 (78.1) | 117.6 (69.5) | 134.8 (65.0) | 373.9 (94.0) |
| Difference vs placebo (95% CI) | | 4.8 (-202.1, 211.8) | 27.3 (-171.3, 225.9) | 44.5 (-152.5, 241.5) | 283.5 (31.3, 535.7) |
| p-value | | 0.9619 | 0.7783 | 0.6441 | **0.0293 *** |
| **PBMC HO activity (pmol biliverdin/million cells/hour) - Unadjusted** | | | | | |
| Observed mean (SE) | 282.5 (180.5) | 296.5 (210.6) | 320.5 (192.2) | 339.5 (178.2) | 796.0 (216.3) |
| Difference vs placebo (95% CI) | | 14.0 (-556, 584) | 38.0 (-504, 580) | 57.0 (-470, 584) | 513.6 ( -80, 1107) |
| p-value | | 0.9600 | 0.8860 | 0.8253 | 0.0866 |
| **PBMC HO activity (pmol biliverdin/million cells/hour) – Adjusted for *HMOX1* promoter polymorphism status** | | | | | |
| Observed mean (SE) | 255.7 (186.3) | 261.0 (218.6) | 320.2 (194.2) | 356.2 (181.6) | 845.8 (229.5) |
| Difference vs placebo (95% CI) | | 5.3 (-572.1, 583) | 64.5 (-489.4, 619) | 100.5 (-448, 649) | 590.2 ( -51, 1231) |
| p-value | | 0.9850 | 0.8116 | 0.7082 | 0.0694 |
| **Change in PBMC HO activity, active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **PBMC HO activity (biliverdin (ng/mL))** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 100.2 (33.1) | 105.5 (37.8) | 103.8 (34.3) | 131.1 (31.7) | 171.1 (39.2) |
| Difference vs placebo (95% CI) | | 5.3 ( -95.6, 106.2) | 3.6 ( -93.0, 100.2) | 30.9 ( -62.0, 123.8) | 70.9 ( -38.4, 180.3) |
| p-value | | 0.9150 | 0.9401 | 0.5031 | 0.1959 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 105.4 (43.3) | 111.0 (50.2) | 122.4 (45.6) | 132.3 (42.6) | 352.2 (56.4) |
| Difference vs placebo (95% CI) | | 5.6 (-127, 138) | 17.0 (-109, 143) | 26.9 (-95, 149) | 246.8 (100, 393) |
| p-value | | 0.9329 | 0.7870 | 0.6597 | **0.0015 **** |
| **POD 1** | | | | | |
| Observed mean (SE) | 110.4 (43.3) | 103.8 (55.2) | 101.2 (40.5) | 139.7 (40.5) | 112.8 (45.8) |
| Difference vs placebo (95% CI) | | -6.5 (-147, 134) | -9.2 (-128, 110) | 29.3 (-90, 148.3) | 2.4 (-128, 133) |
| p-value | | 0.9255 | 0.8770 | 0.6223 | 0.9703 |
| **POD 3** | | | | | |
| Observed mean (SE) | 84.7 (43.4) | 101.7 (55.1) | 87.7 (54.1) | 121.2 (40.5) | 48.3 (49.5) |
| Difference vs placebo (95% CI) | | 17.0 (-123, 157) | 3.0 (-137, 143) | 36.5 ( -83, 156) | -36.5 (-174, 101) |
| p-value | | 0.8086 | 0.9661 | 0.5404 | 0.5946 |
| **PBMC HO activity (pmol biliverdin/million cells/hour)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 276.2 (96.1) | 286.0 (109.3) | 278.2 (99.5) | 353.1 (92.2) | 360.1 (118.9) |
| Difference vs placebo (95% CI) | | 9.8 (-284, 303) | 1.9 (-279, 283) | 76.9 (-194, 348) | 83.9 (-240, 408) |
| p-value | | 0.9462 | 0.9889 | 0.5668 | 0.6016 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 290.2 (124.1) | 302.1 (143.6) | 330.6 (130.6) | 355.6 (122.3) | 804.8 (147.3) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | 11.9 (-368, 392) | 40.4 (-322, 402) | 65.4 (-286, 417) | 514.7 (119, 910) |
| p-value | | 0.9498 | 0.8229 | 0.7088 | **0.0119 *** |
| **POD 1** | | | | | |
| Observed mean (SE) | 303.5 (124.0) | 281.4 (157.9) | 271.8 (116.3) | 377.3 (116.3) | 215.6 (153.5) |
| Difference vs placebo (95% CI) | | -22.1 (-424, 380) | -31.7 (-374, 310) | 73.8 (-268, 416) | -87.9 (-496, 321) |
| p-value | | 0.9123 | 0.8527 | 0.6658 | 0.6667 |
| **POD 3** | | | | | |
| Observed mean (SE) | 235.0 (124.3) | 274.6 (157.4) | 232.1 (154.3) | 326.6 (116.3) | 59.8 (153.5) |
| Difference vs placebo (95% CI) | | 39.5 (-363, 442) | -3.0 (-403, 397) | 91.5 (-251, 434) | -175.2 (-585, 234) |
| p-value | | 0.8438 | 0.9881 | 0.5928 | 0.3931 |
| **Change in serum bilirubin (µmol/L), active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **PBMC HO activity (biliverdin (ng/mL))** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 13.1 (0.7) | 15.0 (0.7) | 15.5 (0.7) | 15.2 (0.8) | 14.2 (0.7) |
| Difference vs placebo (95% CI) | | 1.9 (-0.1,3.9) | 2.4 (0.5,4.3) | 2.0 (-0.1,4.2) | 1.1 (-1.0,3.1) |
| p-value | | 0.0590 | **0.0161 *** | 0.0657 | 0.2939 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 12.3 (1.4) | 15.8 (1.6) | 15.3 (1.5) | 17.8 (1.8) | 19.6 (1.6) |
| Difference vs placebo (95% CI) | | 3.5 (-0.7,7.7) | 3.0 (-1.1,7.0) | 5.5 (0.9,10.1) | 7.3 (3.1,11.5) |
| p-value | | 0.1020 | 0.1487 | **0.0186 *** | **0.0008 ***** |
| **POD 1** | | | | | |
| Observed mean (SE) | 15.5 (1.5) | 21.0 (1.6) | 18.8 (1.5) | 20.5 (1.8) | 16.5 (1.6) |
| Difference vs placebo (95% CI) | | 5.5 (1.2,9.8) | 3.3 (-0.9,7.4) | 4.9 (0.3,9.6) | 1.0 (-3.4,5.3) |
| p-value | | **0.0125 *** | 0.1200 | **0.0385 *** | 0.6631 |
| **POD 2** | | | | | |
| Observed mean (SE) | 12.7 (1.5) | 14.2 (1.6) | 15.1 (1.5) | 13.0 (2.0) | 10.6 (1.6) |
| Difference vs placebo (95% CI) | | 1.5 (-2.8,5.8) | 2.4 (-1.8,6.6) | 0.3 (-4.6,5.2) | -2.0 (-6.3,2.3) |
| p-value | | 0.4868 | 0.2557 | 0.8973 | 0.3520 |
| **POD 3** | | | | | |
| Observed mean (SE) | 11.9 (1.5) | 10.5 (1.6) | 14.9 (1.6) | 11.0 (1.8) | 10.5 (1.6) |
| Difference vs placebo (95% CI) | | -1.3 (-5.6,3.0) | 3.0 (-1.2,7.3) | -0.9 (-5.6,3.7) | -1.4 (-5.7,2.9) |
| p-value | | 0.5413 | 0.1623 | 0.6983 | 0.5270 |

Abbreviations: SD – standard deviation; IQR – inter-quartile range; SE – standard error of the mean; CI – confidence interval; ANCOVA – analysis of covariance; LMM – linear mixed model; PBMC – peripheral blood mononuclear cells; HO – haem oxygenase

#### 4.2.4.2 The impact of 5-ALA-SFC on PBMC *HMOX1* expression

RT-qPCR was performed using RNA extracted from cryopreserved PBMCs. Between the screening timepoint and immediately before surgery (i.e., reflecting drug effect free of the confounder of impact of cardiac surgery on gene expression), quantification of gene expression by $2^{(-\Delta\Delta Ct)}$ did not identify a statistically significant increase in relative *HMOX1* expression by 5-ALA-SFC (**Fig. 58 A**). However, an important temporal relationship between cardiac surgery with a transient upregulation of *HMOX1* expression across all treatment allocation groups over baseline was seen on the first post-operative day. Within a LMM using REML, timepoint was identified as a significant fixed effect factor in predicting relative *HMOX1* expression (p-value = < 0.0001) consistent with the ability of surgical stress to induce *HMOX1* expression.

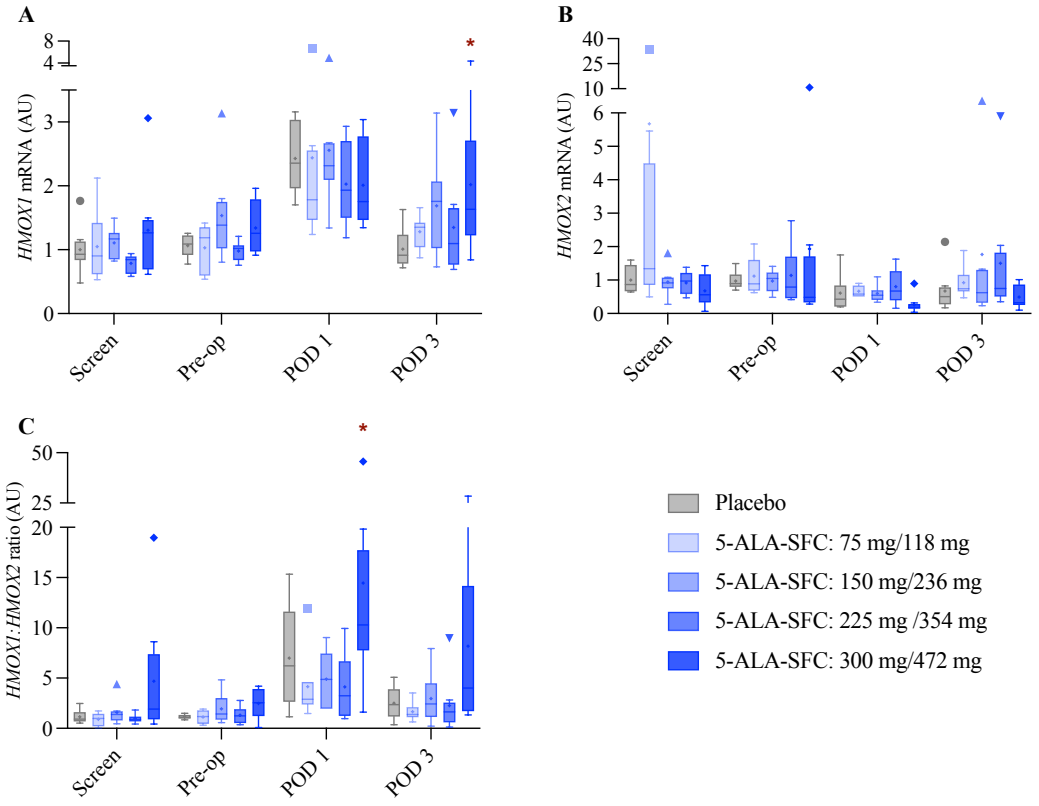



**Figure 58: Effect of cardiac surgery on relative *HMOX1* expression in cardiac surgical patients.** Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values. Significance from the LMM in red: * = $p < 0.05$. Abbreviations: AU – arbitrary units.

In the post-operative period, patients who had been treated with 5-ALA-SFC at the highest dose displayed a sustained increase in relative *HMOX1* expression compared to placebo-treated patients. Using a LMM, the 5-ALA-SFC 300 mg/472 mg treatment allocation group had the most significant remaining increase in expression at post-operative day 3 (mean difference [95 % CI] vs. placebo = 1.01 [0.22; 1.80] AU, p-value = 0.0127). When *HMOX1* expression was expressed as a ratio to *HMOX2*, there was also a significant difference at post-operative day 1 (mean difference [95 % CI] vs. placebo = 6.49 [1.53, 11.46] AU, p-value = 0.0112) (**Fig. 58 C**). Notably, when considering the entirety of the LMM model, the overall fixed effect of 5-ALA-SFC at a dose of 300 mg/472 mg on *HMOX1:HMOX2* was significant (mean difference [95 % CI] vs. placebo = 3.85 [0.80, 6.90], p-value = 0.0150) suggesting the potential for the highest dose evaluated to impact HO-1 beyond the day of surgery into the early post-operative period.

**Table 26: Relative expression of *HMOX1* and *HMOX2* in response to 5-ALA-SFC.**

| | **Placebo (n=9)** | **75 mg/118 mg (n=8)** | **150 mg/236 mg (n=9)** | **225 mg/354 mg (n=8)** | **300 mg/472 mg (n=8)** |
|---|---|---|---|---|---|
| ***HMOX1* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.37) | 1.05 (0.55) | 1.11 (0.24) | 0.79 (0.14) | 1.31 (0.79) |
| Media (IQR) | 0.93 (0.84; 1.13) | 0.90 (0.62; 1.42) | 1.17 (0.85; 1.27) | 0.84 (0.62; 0.9) | 1.26 (0.69; 1.46) |
| Min Max | 0.48 1.77 | 0.53 2.12 | 0.82 1.5 | 0.58 0.94 | 0.61 3.06 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.06 (0.17) | 1.03 (0.37) | 1.53 (0.73) | 0.98 (0.15) | 1.34 (0.41) |
| Median (IQR) | 1.09 (0.92; 1.22) | 1.19 (0.59; 1.35) | 1.38 (1.02; 1.75) | 1.02 (0.84; 1.07) | 1.26 (0.97; 1.79) |
| Min Max | 0.78 0.13 | 0.54 1.42 | 0.81 3.14 | 0.76 1.2 | 0.92 1.96 |
| **POD 1** | | | | | |
| Mean (SD) | 2.43 (0.57) | 2.44 (1.78) | 2.56 (1.06) | 2.03 (0.65) | 2.01 (0.67) |
| Median (IQR) | 2.36 (1.96; 3.04) | 1.78 (1.47; 2.56) | 2.32 (2.09; 2.67) | 1.93 (1.5; 2.7) | 1.75 (1.47; 2.78) |
| Min Max | 1.7 3.16 | 1.24 6.69 | 1.34 4.96 | 1.19 2.93 | 1.34 3.04 |
| **POD 3** | | | | | |
| Mean (SD) | 1.0 (0.31) | 1.28 (0.25) | 1.69 (0.76) | 1.35 (0.81) | 2.02 (1.14) |
| Median (IQR) | 0.92 (0.78; 1.24) | 1.35 (1.04; 1.43) | 1.76 (1.02; 2.07) | 1.1 (0.76; 1.65) | 1.63 (1.23; 2.71) |
| Min Max | 0.72 1.63 | 0.87 1.66 | 0.73 3.34 | 0.69 3.14 | 0.84 4.36 |
| ***HMOX2* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.39) | 5.68 (11.28) | 0.95 (0.43) | 0.92 (0.33) | 0.68 (0.48) |
| Median (IQR) | 0.86 (0.67; 1.45) | 0.13 (0.85; 4.50) | 0.91 (0.75; 1.08) | 0.97 (0.58; 1.22) | 0.56 (0.33; 1.18) |
| Min Max | 0.44 1.60 | 0.50 33.32 | 0.28 1.81 | 0.47 1.38 | 0.07 1.43 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.97 (0.27) | 1.21 (0.56) | 0.97 (0.26) | 1.14 (0.84) | 1.93 (3.60) |
| Median (IQR) | 0.90 (0.80; 1.45) | 0.89 (0.68; 1.61) | 1.05 (0.67; 1.23) | 0.79 (0.46; 1.78) | 0.49 (0.33; 1.71) |
| Min Max | 0.70 1.50 | 0.62 2.08 | 0.49 1.41 | 0.42 2.78 | 0.28 10.73 |
| **POD 1** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 0.61 (0.53) | 0.66 (0.17) | 0.6 (0.25) | 0.80 (0.52) | 0.26 (0.27) |
| Median (IQR) | 0.43 (0.21; 0.84) | 0.58 (0.51; 0.84) | 0.54 (0.41; 0.70) | 0.67 (0.39; 1.27) | 0.18 (0.14; 0.29) |
| Min Max | 0.20 1.75 | 0.51 0.90 | 0.34 1.10 | 0.15 1.63 | 0.04 0.89 |
| **POD 3** | | | | | |
| Mean (SD) | 0.67 (0.63) | 0.93 (0.45) | 1.77 (3.13) | 1.51 (1.85) | 0.49 (0.34) |
| Median (IQR) | 0.51 (0.28; 0.79) | 0.74 (0.66; 1.19) | 0.63 (0.32; 1.30) | 0.75 (0.51; 1.82) | 0.33 (0.25; 0.87) |
| Min Max | 0.17 2.14 | 0.47 1.88 | 0.23 9.45 | 0.35 5.90 | 0.10 1.01 |
| **Change in PBMC *HMOX1* expression relative to *ACTB*, active vs placebo (LMM, time as a categorical variable)** | | | | | |
| ***HMOX1* mRNA (AU)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 1.50(0.19) | 1.58 (0.19) | 1.93 (0.19) | 1.45 (0.19) | 1.79 (0.19) |
| Difference vs placebo (95% CI) | | 0.08 ( -0.45, 0.62) | 0.43 ( -0.11, 0.96) | -0.05 ( -0.59, 0.49) | 0.29 ( -0.26, 0.84) |
| p-value | | 0.7525 | 0.1170 | 0.8561 | 0.2899 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 1.06 (0.28) | 1.03 (0.28) | 1.53 (0.28) | 0.98 (0.28) | 1.34 (0.28) |
| Difference vs placebo (95% CI) | | -0.03 (-0.81, 0.75) | 0.47 (-0.31, 1.25) | -0.08 (-0.87, 0.70) | 0.28 (-0.51, 1.07) |
| p-value | | 0.9361 | 0.2329 | 0.8306 | 0.4827 |
| **POD 1** | | | | | |
| Observed mean (SE) | 2.43 (0.28) | 2.44 (0.28) | 2.56 (0.28) | 2.03 (0.28) | 2.01 (0.28) |
| Difference vs placebo (95% CI) | | 0.01 (-0.77, 0.79) | 0.13 (-0.65, 0.91) | -0.40 (-1.19, 0.38) | -0.42 (-1.21, 0.37) |
| p-value | | 0.9769 | 0.7428 | 0.3100 | 0.2938 |
| **POD 3** | | | | | |
| Observed mean (SE) | 1.01 (0.28) | 1.28 (0.28) | 1.69 (0.28) | 1.35 (0.28) | 2.02 (0.28) |
| Difference vs placebo (95% CI) | | 0.27 (-0.51, 1.05) | 0.68 (-0.10, 1.46) | 0.34 (-0.44, 1.12) | 1.01 (0.22, 1.80) |
| p-value | | 0.4887 | 0.0874 | 0.3901 | **0.0127 *** |
| ***HMOX2* mRNA (AU)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 0.74 (0.29) | 0.93 (0.31) | 1.11 (0.29) | 1.14 (0.29) | 0.88 (0.29) |
| Difference vs placebo (95% CI) | | 0.19 (-0.69, 1.07) | 0.36 (-0.47, 1.20) | 0.40 (-0.43, 1.22) | 0.14 (-0.68, 0.97) |
| p-value | | 0.6612 | 0.3851 | 0.3352 | 0.7307 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 0.96 (0.50) | 1.15 (0.51) | 0.96 (0.50) | 1.13 (0.50) | 1.92 (0.50) |
| Difference vs placebo (95% CI) | | 0.19 (-1.24, 1.61) | -0.00 (-1.40, 1.40) | 0.17 (-1.24, 1.57) | 0.96 (-0.44, 2.36) |
| p-value | | 0.7948 | 0.9977 | 0.8144 | 0.1774 |
| **POD 1** | | | | | |
| Observed mean (SE) | 0.60 (0.50) | 0.70 (0.54) | 0.59 (0.53) | 0.79 (0.50) | 0.25 (0.50) |
| Difference vs placebo (95% CI) | | 0.10 (-1.38, 1.58) | -0.01 (-1.46, 1.44) | 0.19 (-1.21, 1.60) | -0.35 (-1.75, 1.05) |
| p-value | | 0.8970 | 0.9908 | 0.7841 | 0.6222 |
| **POD 3** | | | | | |
| Observed mean (SE) | 0.67 (0.50) | 0.96 (0.51) | 1.76 (0.50) | 1.50 (0.50) | 0.48 (0.50) |
| Difference vs placebo (95% CI) | | 0.29 (-1.14, 1.71) | 1.09 (-0.31, 2.50) | 0.83 (-0.57, 2.23) | -0.19 (-1.59, 1.22) |
| p-value | | 0.6865 | 0.1241 | 0.2411 | 0.7906 |
| ***HMOX1:HMOX2* (AU)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 3.74 (0.99) | 2.58 (1.02) | 3.35 (1.01) | 2.82 (0.99) | 7.59 (1.08) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | -1.16 (-4.02, 1.70) | -0.39 (-3.25, 2.47) | -0.92 (-3.75, 1.90) | 3.85 (0.80, 6.90) |
| p-value | | 0.4145 | 0.7826 | 0.5117 | **0.0150 *** |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 1.33 (1.72) | 1.40 (1.72) | 2.02 (1.71) | 1.56 (1.72) | 1.70 (1.77) |
| Difference vs placebo (95% CI) | | 0.07 (-4.77, 4.90) | 0.69 (-4.15, 5.52) | 0.22 (-4.61, 5.06) | 0.36 (-4.60, 5.33) |
| p-value | | 0.9776 | 0.7781 | 0.9264 | 0.8846 |
| **POD 1** | | | | | |
| Observed mean (SE) | 7.18 (1.72) | 4.40 (1.84) | 4.97 (1.83) | 4.37 (1.72) | 13.67 (1.77) |
| Difference vs placebo (95% CI) | | -2.78 (-7.78, 2.22) | -2.21 (-7.22, 2.79) | -2.81 (-7.64, 2.03) | 6.49 (1.53, 11.46) |
| p-value | | 0.2714 | 0.3809 | 0.2505 | **0.0112 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 2.70 (1.72) | 1.93 (1.72) | 3.06 (1.71) | 2.52 (1.72) | 7.40 (1.77) |
| Difference vs placebo (95% CI) | | -0.77 (-5.61, 4.06) | 0.35 (-4.48, 5.19) | -0.18 (-5.02, 4.65) | 4.69 (-0.27, 9.66) |
| p-value | | 0.7501 | 0.8851 | 0.9403 | 0.0635 |

Abbreviations: AU – arbitrary units; LMM – linear mixed model; PBMC – peripheral blood mononuclear cells; SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – standard error of the mean; CI – confidence interval.

#### 4.2.4.3 The effect of 5-ALA-SFC on PBMC HO-1 expression and plasma HO-1 levels

Cryopreserved PBMCs from all TALEN trial participants were thawed and analysed using high dimensional flow cytometry. Negative selection of natural killer (NK) cells and lymphocytes ($Lin^-$: $CD3^-$ $CD19^-$ $CD56^-$) allowed for interrogation of all other $CD45^+$ immune cells within the PBMC compartment (e.g., monocytes and dendritic cells [DC]). Flow cytometry data suggested that 5-ALA-SFC increased the proportion of non-NK/lymphocyte immune cells that express HO-1 in a dose-dependent fashion (**Fig. 59 A**). By fitting a LMM using REML, this was found to be most significant in the highest dose treatment allocation group at the pre-operative time point, suggesting a primary drug effect (mean difference [SE] vs. placebo = 12.08 [4.47] %, p-value = 0.0197). Although not statistically significant, this pattern persisted into the post-operative period, implying a fixed monocyte/DC phenotypic commitment towards increased HO-1 expression. Plasma HO-1, as determined by ELISA, was not similarly discernibly increased pre-operatively and no statistically significant drug effects were found. Increases after cardiac surgery were seen across all treatment allocation groups and here, numerically lower levels of HO-1 were seen in patients who had been treated with 5-ALA-SFC (**Fig. 59 B**).

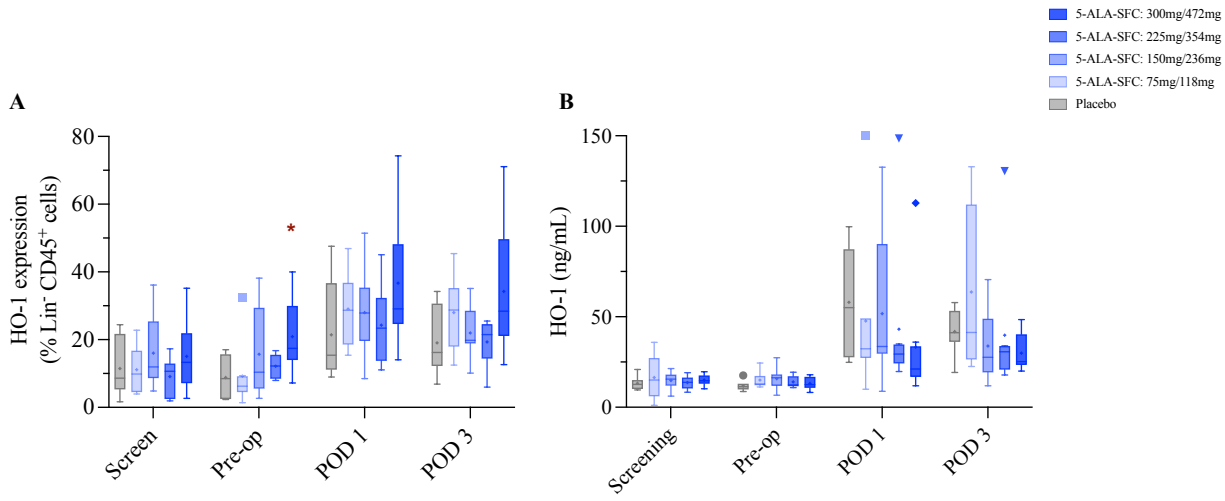



**Figure 59: Impact of 5-ALA-SFC on immune cell expression of HO-1 (A) and circulating plasma HO-1 (B).**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Significance from the LMM in red: * = $p < 0.05$.

**Table 27: Immune cell HO-1 expression profile and plasma HO-1 in response to 5-ALA-SFC.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **HO-1$^+$/Lin$^-$CD45$^+$ (%)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 11.49 (8.59) | 11.12 (6.89) | 16.04 (10.79) | 9.12 (5.85) | 15.10 (10.50) |
| Median (IQR) | 8.63 (5.34; 21.72) | 9.84 (4.49; 16.8) | 11.96 (8.66; 25.44) | 10.65 (2.48; 12.98) | 13.33 (7.12; 21.89) |
| Min Max | 1.66 24.41 | 3.94 22.76 | 4.83 36.11 | 1.92 17.28 | 2.64 35.18 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 8.82 (6.43) | 9.24 (9.74) | 15.69 (13.23) | 12.16 (3.54) | 20.91 (10.62) |
| Median (IQR) | 8.51 (2.49; 15.65) | 6.28 (4.49; 9.27) | 10.4 (5.51; 29.42) | 12.25 (8.40; 15.56) | 17.44 (13.99; 29.98) |
| Min Max | 2.33 17.07 | 1.38 32.44 | 2.7 38.18 | 7.98 16.78 | 7.25 40.00 |
| **POD 1** | | | | | |
| Mean (SD) | 21.43 (14.70) | 29.03 (10.63) | 28.00 (12.77) | 24.27 (12.03) | 36.68 (20.13) |
| Median (IQR) | 15.43 (11.15; 36.71) | 28.66 (18.56; 36.83) | 27.89 (19.64; 35.36) | 23.41 (13.69; 32.33) | 29.08 (24.63; 48.21) |
| Min Max | 8.99 47.59 | 15.38 46.92 | 8.48 50.46 | 11.07 45.06 | 14.03 74.29 |
| **POD 3** | | | | | |
| Mean (SD) | 19.10 (9.94) | 28.01 (10.78) | 21.96 (7.92) | 19.34 (7.13) | 34.27 (19.40) |
| Median (IQR) | 16.2 (12.25; 30.67) | 28.73 (17.92; 35.23) | 19.82 (18.85; 28.54) | 21.52 (14.41; 24.57) | 28.4 (21.12; 49.66) |
| Min Max | 6.89 34.25 | 12.5 45.39 | 10.15 35.15 | 6.01 25.49 | 12.61 71.09 |
| **Circulating HO-1 (ng/mL)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 13.7 (3.7) | 16.5 (12.4) | 14.7 (4.7) | 13.2 (3.6) | 15.0 (3.1) |
| Median (IQR) | 13.1 (10.3; 15.2) | 15.0 (7.6; 24.4) | 15.6 (11.9; 17.7) | 12.7 (9.7; 15.0) | 14.7 (13.1; 17.2) |
| Min Max | 10.0 20.9 | 1.1 35.9 | 6.2 21.4 | 8.4 19.1 | 10.2 19.7 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 12.1 (2.9) | 15.1 (4.6) | 15.8 (6.0) | 13.2 (2.5) | 13.1 (3.4) |
| Median (IQR) | 12.1 (9.8; 13.0) | 12.9 (12.2; 17.3) | 16.4 (12.2; 17.7) | 12.2 (11.5; 15.9) | 12.5 (10.9; 15.9) |
| Min Max | 8.7 17.6 | 11.3 24.4 | 6.7 27.3 | 10.9 17.6 | 8.2 18.0 |
| **POD 1** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 62.7 (28.6) | 47.7 (46.6) | 51.8 (43.7) | 28.0 (5.6) | 33.2 (33.0) |
| Median (IQR) | 65.8 (34.9; 90.2) | 32.3 (27.2; 49.2) | 33.6 (30.4; 72.4) | 29.1 (24.0; 34.1) | 21.2 (17.6; 31.6) |
| Min Max | 25.3 99.8 | 10.0 150.0 | 8.8 132.7 | 19.7 35.0 | 11.8 112.8 |
| **POD 3** | | | | | |
| Mean (SD) | 45.1 (8.9) | 63.6 (47.0) | 33.8 (19.4) | 26.9 (6.8) | 30.0 (10.4) |
| Median (IQR) | 44.8 (37.0; 55.3) | 41.4 (30.3; 91.2) | 27.6 (21.4; 44.9) | 29.4 (20.6; 33.2) | 25.3 (23.6; 36.8) |
| Min Max | 35.8 57.9 | 22.5 132.9 | 11.9 70.6 | 17.8 34.0 | 20.0 48.4 |
| **Change in proportion of HO-1$^+$ Lin$^-$CD45$^+$ cells, active vs placebo (Mixed effects analysis, REML).** | | | | | |
| **HO-1$^+$/Lin$^-$CD45$^+$ (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 8.824 | 9.244 | 15.69 | 12.16 | 20.91 |
| Mean difference vs placebo (SE) | | 0.42 (4.21) | 6.87 (5.27) | 3.34 (2.83) | 12.08 (4.47) |
| p-value | | 0.9222 | 0.2206 | 0.2662 | **0.0197 *** |
| **POD 1** | | | | | |
| Observed mean | 21.43 | 29.03 | 28.00 | 24.27 | 36.23 |
| Mean difference vs placebo (SE) | | 7.60 (6.70) | 6.57 (7.16) | 2.84 (7.41) | 15.25 (9.42) |
| p-value | | 0.2817 | 0.3768 | 0.7091 | 0.1388 |
| **POD 3** | | | | | |
| Observed mean | 19.10 | 28.01 | 22.09 | 19.34 | 34.27 |
| Mean difference vs placebo (SE) | | 8.92 (5.35) | 2.86 (4.80) | 0.24 (4.75) | 15.17 (7.82) |
| p-value | | 0.1196 | 0.5629 | 0.9610 | 0.0792 |
| **Change in circulating HO-1 (ELISA), active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **HO-1 (ng/mL)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 38.2 (7.5) | 45.0 (9.0) | 33.7 (7.5) | 33.0 (7.5) | 25.2 (7.5) |
| Difference vs placebo (95% CI) | | 6.9 (-17.2, 31.0) | -4.5 (-26.1, 17.2) | -5.2 (-26.7, 16.3) | -13.0 (-34.7, 8.6) |
| p-value | | 0.5653 | 0.6777 | 0.6258 | 0.2300 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 12.8 (9.9) | 14.5 (11.4) | 15.7 (9.8) | 14.7 (9.8) | 12.9 (9.8) |
| Difference vs placebo (95% CI) | | 1.7 (-28.6, 31.9) | 3.0 (-24.8, 30.7) | 1.9 (-25.9, 29.6) | 0.1 (-27.7, 27.9) |
| p-value | | 0.9124 | 0.8327 | 0.8936 | 0.9962 |
| **POD 1** | | | | | |
| Observed mean (SE) | 58.9 (9.9) | 61.0 (12.3) | 51.7 (9.8) | 43.7 (9.8) | 32.9 (9.8) |
| Difference vs placebo (95% CI) | | 2.1 (-29.5, 33.6) | -7.2 (-35.0, 20.6) | -15.2 (-42.9, 12.6) | -26.0 (-53.8, 1.8) |
| p-value | | 0.8957 | 0.6052 | 0.2783 | 0.0663 |
| **POD 3** | | | | | |
| Observed mean (SE) | 42.8 (9.9) | 59.7 (12.4) | 33.7 (9.8) | 40.5 (9.8) | 29.7 (9.8) |
| Difference vs placebo (95% CI) | | 16.9 (-15.0, 48.7) | -9.1 (-36.9, 18.7) | -2.3 (-30.0, 25.4) | -13.1 (-40.9, 14.7) |
| p-value | | 0.2935 | 0.5161 | 0.8685 | 0.3511 |

Abbreviations: LMM – linear mixed model; REML – restricted maximum likelihood; SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – standard error of the mean; CI – confidence interval

Linear mixed model with Restricted Maximum Likelihood. Correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 4.2.4.4 The impact of peri-operative 5-ALA-SFC on NRF2 dependent gene expression

As 5-ALA-SFC causes *HMOX1* transcription via a NRF2-dependent mechanism[269], establishing whether the drug influences transcription of other NRF2-dependent genes was investigated as a downstream measure of HO-1 activation. Using RNA extracted from cryopreserved PBMCs, the relative expression of key NRF2-related genes was determined by RT-qPCR with TaqMan™ chemistry (**Fig. 60**).

Using a LMM with REML, expression of *NFE2L2*, coding for NRF2, was found to be significantly increased after dosing with 5-ALA-SFC 225 mg/354 mg (mean difference [SE] vs. placebo = 0.33 [0.09] AU, p-value = 0.0152) (Fig. 60 A). Pre-operatively, relative expression of *NQO1*, coding for NAD(P)H Quinone Dehydrogenase 1, increased most in the 5-ALA-SFC 300 mg/472 mg treatment allocation group although this did not reach formal statistical significance (mean difference [SE] vs. placebo = 1.98 [0.99] AU, p-value = 0.2439) (**Fig. 60 B**). Relative expression of *ME1*, coding for malic enzyme 1, showed a tendency to greater expression in all groups that had received 5-ALA-SFC, vs. placebo. Surgery appeared to affect the relative expression of this gene, with the most substantial increase seen at post-operative day 1. Notably, within the 5-ALA-SFC 300 mg/472 mg treatment allocation group a less pronounced increase in relative expression of *ME1* (median [IQR] = 1.85 [1.39; 3.09]) vs. placebo (median [IQR] = 4.30 [2.07; 5.30]) was observed. A similar relationship with the surgical insult is appreciated in the relative expression of *TXN*, coding for thioredoxin. (**Fig. 60 C-D**).

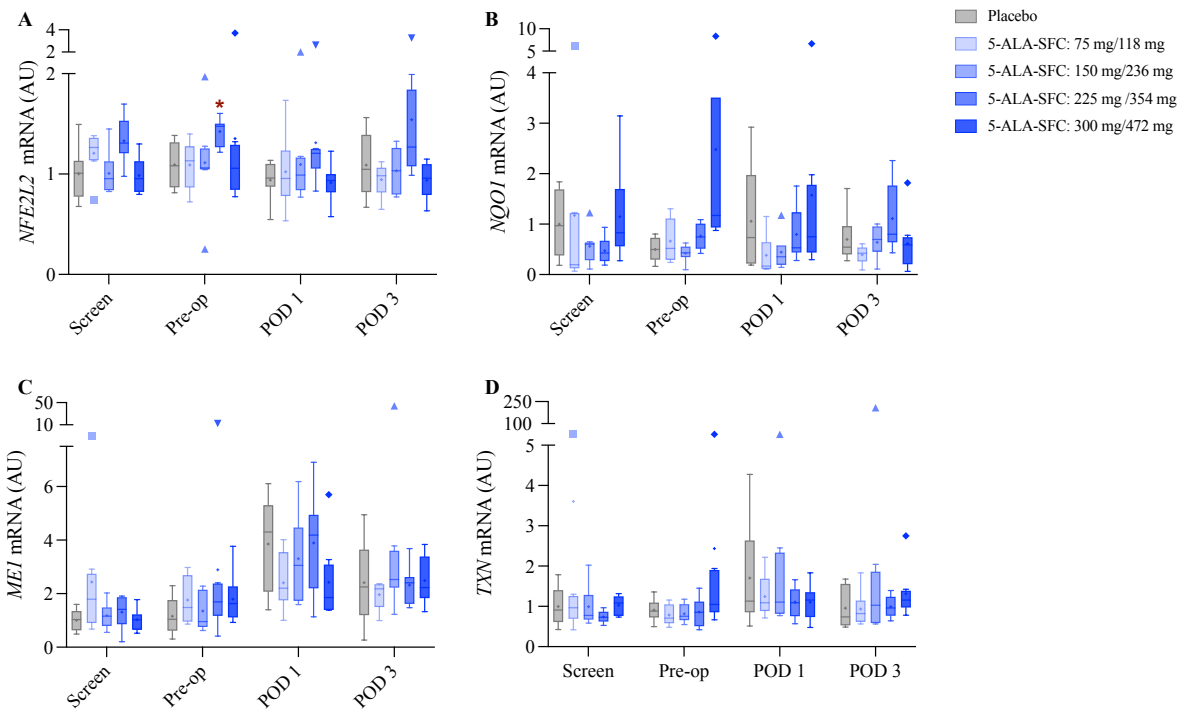



**Figure 60: The impact of 5-ALA-SFC on NRF2 dependent gene expression in human PBMCs.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Significance from the LMM using REML mixed effects analysis shown in red: * = $p < 0.05$.

Abbreviations: AU – arbitrary units; *NFE2L2* – gene for nuclear factor erythroid 2–related factor (NRF2); *NQO1* – gene for NAD(P)H Quinone Dehydrogenase 1; *ME1* – gene for malic enzyme 1; *TXN* – gene for thioredoxin.

**Table 28: Impact of 5-ALA-SFC on NRF2 dependent gene expression.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| ***NFE2L2* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.26) | 1.21 (0.21) | 1.0 (0.21) | 1.33 (0.23) | 0.99 (0.19) |
| Median (IQR) | 1.0 (0.77; 1.13) | 1.27 (1.14; 1.36) | 0.95 (0.84; 1.13) | 1.31 (1.21; 1.53) | 0.95 (0.82; 1.13) |
| Min Max | 0.67 1.49 | 0.74 1.38 | 0.83 1.45 | 0.98 1.70 | 0.80 1.30 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.09 (0.23) | 1.09 (0.23) | 1.11 (0.47) | 1.42 (0.14) | 1.35 (0.97) |
| Median (IQR) | 1.08 (0.87; 1.31) | 1.13 (0.87; 1.28) | 1.06 (1.05; 1.26) | 1.48 (1.27; 1.5) | 1.06 (0.84; 1.29) |
| Min Max | 0.81 1.38 | 0.72 1.40 | 0.25 1.97 | 1.22 1.60 | 0.77 3.71 |
| **POD 1** | | | | | |
| Mean (SD) | 0.94 (0.19) | 1.02 (0.37) | 1.10 (0.40) | 1.31 (0.55) | 0.92 (0.19) |
| Median (IQR) | 0.96 (0.87; 1.10) | 0.96 (0.78; 1.24) | 0.10 (0.84; 1.17) | 1.21 (1.05; 1.25) | 0.94 (0.82; 0.10) |
| Min Max | 0.54 1.13 | 0.53 1.73 | 0.77 2.01 | 0.83 2.62 | 0.58 1.23 |
| **POD 3** | | | | | |
| Mean (SD) | 1.087 (0.317) | 0.94 (0.16) | 1.03 (0.23) | 1.54 (0.55) | 0.94 (0.18) |
| Median (IQR) | 1.05 (0.82; 1.39) | 0.98 (0.82; 1.07) | 1.03 (0.79; 1.26) | 1.27 (1.08; 1.84) | 0.96 (0.79; 1.10) |
| Min Max | 0.67 1.56 | 0.65 1.12 | 0.77 1.33 | 0.99 3.36 | 0.63 1.15 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| ***NQO1* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.66) | 1.18 (2.10) | 0.56 (0.33) | 0.47 (0.25) | 1.15 (0.94) |
| Median (IQR) | 0.97 (0.38; 1.69) | 0.20 (0.12; 1.22) | 0.62 (0.28; 0.64) | 0.43 (0.27; 0.67) | 0.83 (0.56; 1.70) |
| Min Max | 0.19 1.84 | 0.07 6.23 | 0.11 1.23 | 0.19 0.94 | 0.27 3.15 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.50 (0.23) | 0.66 (0.42) | 0.42 (0.18) | 0.76 (0.25) | 2.48 (2.74) |
| Median (IQR) | 0.50 (0.30; 0.73) | 0.52 (0.29; 1.12) | 0.43 (0.35; 0.56) | 0.75 (0.51; 1.01) | 1.17 (0.93; 3.51) |
| Min Max | 0.16 0.81 | 0.24 1.31 | 0.10 0.63 | 0.42 1.09 | 0.87 8.33 |
| **POD 1** | | | | | |
| Mean (SD) | 1.06 (1.03) | 0.38 (0.39) | 0.45 (0.35) | 0.79 (0.52) | 1.57 (2.13) |
| Median (IQR) | 0.74 (0.22; 1.98) | 0.17 (0.11; 0.65) | 0.35 (0.20; 0.58) | 0.53 (0.44; 1.23) | 0.75 (0.44; 1.78) |
| Min Max | 0.19 2.92 | 0.10 1.15 | 0.15 1.18 | 0.28 1.76 | 0.30 6.67 |
| **POD 3** | | | | | |
| Mean (SD) | 0.70 (0.47) | 0.39 (0.18) | 0.64 (0.31) | 1.11 (0.67) | 0.61 (0.55) |
| Median (IQR) | 0.54 (0.40; 0.96) | 0.42 (0.26; 0.54) | 0.70 (0.45; 0.96) | 0.80 (0.64; 1.77) | 0.59 (0.20; 0.74) |
| Min Max | 0.27 1.71 | 0.09 0.61 | 0.11 1.01 | 0.43 2.26 | 0.06 1.82 |
| ***ME1* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.39) | 2.44 (2.44) | 1.18 (0.47) | 1.31 (0.61) | 1.03 (0.40) |
| Median (IQR) | 1.04 (0.63; 1.35) | 1.80 (0.90; 2.74) | 1.16 (0.80; 1.49) | 1.42 (0.86; 1.87) | 1.02 (0.65; 1.23) |
| Min Max | 0.49 1.60 | 0.68 8.26 | 0.55 2.03 | 0.20 1.91 | 0.53 1.78 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.16 (0.67) | 1.77 (0.87) | 1.35 (0.69) | 2.90 (3.84) | 1.80 (0.98) |
| Median (IQR) | 1.04 (0.62; 1.76) | 1.48 (0.95; 2.69) | 0.96 (0.75; 2.15) | 1.70 (1.18; 2.39) | 1.63 (1.11; 2.28) |
| Min Max | 0.30 2.29 | 0.87 2.98 | 0.62 2.29 | 0.41 12.25 | 0.93 3.77 |
| **POD 1** | | | | | |
| Mean (SD) | 3.85 (1.78) | 2.41 (1.05) | 3.30 (1.6) | 3.90 (1.83) | 2.42 (1.48) |
| Median (IQR) | 4.30 (2.07; 5.30) | 2.20 (1.75; 3.55) | 3.06 (1.74; 4.47) | 4.19 (2.20; 4.94) | 1.85 (1.39; 3.09) |
| Min Max | 1.40 6.11 | 1.01 4.02 | 1.60 6.18 | 1.14 6.91 | 1.38 5.70 |
| **POD 3** | | | | | |
| Mean (SD) | 2.41 (1.52) | 1.97 (0.52) | 7.74 (14.77) | 2.32 (0.71) | 2.49 (0.91) |
| Median (IQR) | 2.25 (1.19; 3.65) | 2.79 (1.50; 2.36) | 2.53 (2.22; 3.60) | 2.40 (1.62; 2.62) | 2.22 (1.83; 3.39) |
| Min Max | 0.27 4.94 | 0.99 2.38 | 1.23 44.26 | 1.48 3.68 | 1.33 3.84 |
| ***TXN* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.47) | 3.61 (7.69) | 0.99 (0.49) | 0.74 (0.15) | 1.03 (0.23) |
| Median (IQR) | 0.91 (0.61; 1.40) | 0.97 (0.69; 1.26) | 0.75 (0.67; 1.29) | 0.73 (0.62; 0.87) | 1.08 (0.77; 1.25) |
| Min Max | 0.42 1.79 | 0.42 22.62 | 0.59 2.02 | 0.53 0.97 | 0.74 1.32 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.91 (0.26) | 0.78 (0.25) | 0.81 (0.23) | 0.86 (0.36) | 2.44 (3.59) |
| Median (IQR) | 0.89 (0.73; 1.09) | 0.71 (0.58; 1.05) | 0.76 (0.65; 1.06) | 0.85 (0.51; 1.12) | 1.05 (0.85; 1.90) |
| Min Max | 0.50 1.36 | 0.48 1.16 | 0.55 1.76 | 0.42 1.45 | 0.67 11.24 |
| **POD 1** | | | | | |
| Mean (SD) | 1.70 (1.28) | 1.25 (0.52) | 7.59 (17.82) | 1.10 (0.38) | 1.10 (0.42) |
| Median (IQR) | 1.13 (0.85; 2.64) | 1.09 (0.88; 1.69) | 1.10 (0.82; 2.34) | 1.09 (0.75; 1.42) | 1.16 (0.73; 1.36) |
| Min Max | 0.51 4.27 | 0.72 2.22 | 0.77 51.66 | 0.57 1.66 | 0.48 1.83 |
| **POD 3** | | | | | |
| Mean (SD) | 0.96 (0.50) | 0.94 (0.42) | 26.94 (73.29) | 0.99 (0.26) | 1.32 (1.16) |
| Median (IQR) | 0.74 (0.53; 1.56) | 0.82 (0.62; 1.15) | 1.03 (0.58; 1.86) | 0.96 (0.77; 1.24) | 1.16 (0.97; 1.39) |
| Min Max | 0.49 1.68 | 0.57 1.83 | 0.56 208.3 | 0.64 1.40 | 0.78 2.75 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Change in relative gene expression, active vs placebo (Mixed effects analysis, REML).** | | | | | |
| ***NEF2L2*** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.09 | 1.09 | 1.11 | 1.42 | 1.35 |
| Mean difference vs placebo (SE) | | 0.00 (0.12) | 0.02 (0.18) | 0.33 (0.09) | 0.26 (0.35) |
| p-value | | > 0.9999 | 0.9998 | **0.0152 *** | 0.8669 |
| **POD 1** | | | | | |
| Observed mean | 0.94 | 1.02 | 1.10 | 1.54 | 0.94 |
| Mean difference vs placebo (SE) | | 0.08 (0.15) | 0.16 (0.16) | 0.37 (0.20) | 0.02 (0.09) |
| p-value | | 0.9408 | 0.7157 | 0.2731 | 0.9973 |
| **POD 3** | | | | | |
| Observed mean | 1.03 | 1.07 | 1.06 | 1.40 | 1.05 |
| Mean difference vs placebo (SE) | | 0.04 (0.13) | 0.03 (0.14) | 0.37 (0.29) | 0.02 (0.13) |
| p-value | | 0.6285 | 0.9836 | 0.3901 | 0.6206 |
| ***NQO1*** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.50 | 0.66 | 0.43 | 0.76 | 2.48 |
| Mean difference vs placebo (SE) | | 0.16 (0.17) | -0.08 (0.10) | 0.26 (0.12) | 1.98 (0.99) |
| p-value | | 0.7705 | 0.8700 | 0.1865 | 0.2439 |
| **POD 1** | | | | | |
| Observed mean | 1.06 | 0.38 | 0.44 | 0.79 | 1.57 |
| Mean difference vs placebo (SE) | | -0.69 (0.46) | -0.62 (0.39) | -0.27 (0.38) | 0.51 (0.59) |
| p-value | | 0.4652 | 0.4059 | 0.8908 | 0.8025 |
| **POD 3** | | | | | |
| Observed mean | 0.81 | 0.65 | 0.52 | 0.79 | 1.45 |
| Mean difference vs placebo (SE) | | -0.16 (0.18) | -0.27 (0.14) | -0.02 (0.26) | 0.64 (0.23) |
| p-value | | 0.3646 | 0.9811 | 0.3846 | 0.9870 |
| ***ME1*** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.16 | 1.76 | 1.34 | 2.90 | 1.80 |
| Mean difference vs placebo (SE) | | 0.60 (0.39) | 0.19 (0.35) | 1.74 (1.38) | 0.64 (0.44) |
| p-value | | 0.3799 | 0.9500 | 0.5667 | 0.4471 |
| **POD 1** | | | | | |
| Observed mean | 3.85 | 2.40 | 3.29 | 3.90 | 2.42 |
| Mean difference vs placebo (SE) | | -1.45 (0.74) | -0.56 (0.88) | 0.04 (0.90) | -1.43 (0.82) |
| p-value | | 0.2169 | 0.9239 | > 0.9999 | 0.2841 |
| **POD 3** | | | | | |
| Observed mean | 2.41 | 1.97 | 7.74 | 2.32 | 2.49 |
| Mean difference vs placebo (SE) | | -0.44 (0.57) | 5.33 (5.25) | -0.09 (0.59) | 0.08 (0.62) |
| p-value | | 0.8473 | 0.7127 | 0.9996 | 0.9998 |
| ***TXN*** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.91 | 0.78 | 0.81 | 0.86 | 2.44 |
| Mean difference vs placebo (SE) | | -0.13 (0.13) | -0.10 (0.12) | -0.05 (0.16) | 1.53 (1.27) |
| p-value | | 0.7256 | 0.8518 | 0.9909 | 0.5998 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Observed mean | 1.70 | 1.25 | 7.59 | 1.10 | 1.10 |
| Mean difference vs placebo (SE) | | -0.55 (0.49) | 5.89 (6.32) | -0.60 (0.47) | -0.60 (0.48) |
| p-value | | 0.7584 | 0.7623 | 0.5542 | 0.5581 |
| **POD 3** | | | | | |
| Observed mean | 0.96 | 0.93 | 26.94 | 0.99 | 1.32 |
| Mean difference vs placebo (SE) | | -0.03 (0.24) | 25.98 (25.91) | 0.03 (0.20) | 0.36 (0.28) |
| p-value | | > 0.9999 | 0.7202 | 0.9995 | 0.5370 |

Relative expression by 2(-ΔΔCt). Linear mixed model with Restricted Maximum Likelihood. Correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

Abbreviations: SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum; SE – standard error of the mean

### 4.2.5 The impact of 5-ALA-SFC on CPB-associated haemolysis and iron homeostasis

#### 4.2.5.1 Quantifying the extent of the haemolytic injury associated with CPB

The degree of CPB-associated haemolysis was determined using the haemolytic index (HI), an automated absorbance assay which determines the presence of cf-Hb in serum. The HI was measured in all serum samples from TALEN trial participants submitted for biochemical analysis (**Fig. 61 A-B**). Across all treatment allocation groups, the presence of cf-Hb peaked immediately after CPB and returned to near baseline levels by the 12-hour post-CPB timepoint (**Fig. 61 A**). The data suggested less profound haemolysis as measured by the HI in the 5-ALA-SFC 300 mg/472 mg treatment allocation group at this timepoint, although within a LMM using REML, this finding was not statistically significant (mean difference [SE] vs. placebo: -0.30 [0.20] AU, p-value = 0.1669). Overall, a treatment specific effect on the degree of the haemolytic insult was not observed and within the statistical model, timepoint (i.e., relation to cardiac surgery) was found to be the most important factor impacting the degree of haemolysis (p-value < 0.0001) (**Fig. 61 B**).

Haptoglobin, a plasma protein which binds cf-Hb and allows for its clearance from the circulation, was assayed in all TALEN trial patients (**Fig. 61 C**). No statistically significant treatment-related effects on haptoglobin during the perioperative period were found. Serum lactate dehydrogenase (LDH) was increased in all treatment allocation groups during the post-operative period (**Fig. 61 D**). It is noted that

data from all active treatment groups showed a non-significant tendency to lower serum LDH in the early post-operative period. The 5-ALA-SFC 75 mg/118 mg treatment allocation group, for example, showed a mean difference (95 % CI) vs. placebo of -90 (-297; 117) IU/L with a p-value of 0.3910.

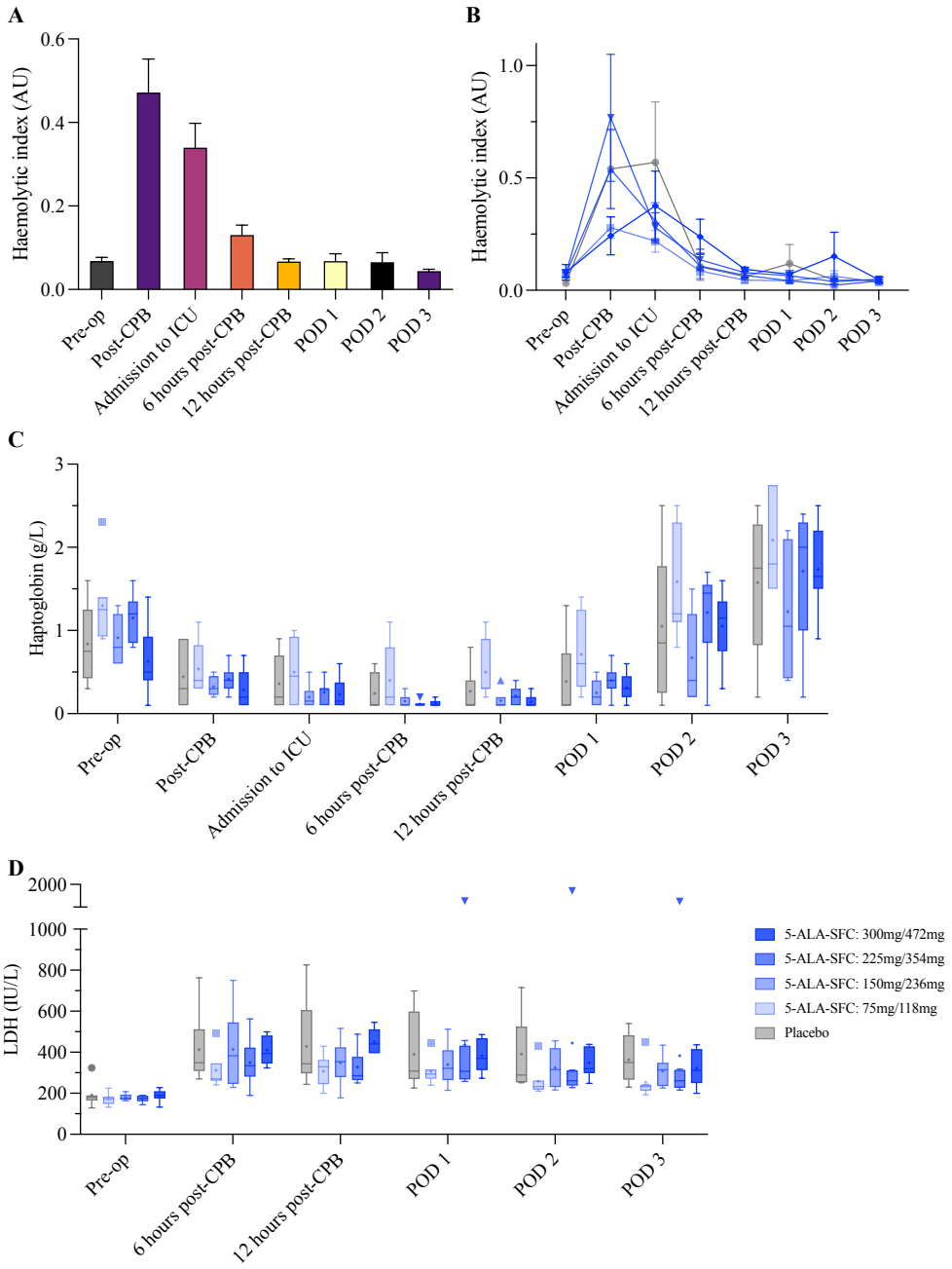



**Figure 61: The impact of 5-ALA-SFC on CPB-associated haemolysis.**

Data presented as mean (SE) (panel A-B); median, IQR, Tukey's upper and lower adjacent values with outside values (panel C-D).

**Table 29: Descriptive statistics and inferential statistics of markers of haemolysis.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Haemolytic index (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.03 (0.02) | 0.08 (0.04) | 0.07 (0.05) | 0.08 (0.05) | 0.08 (0.10) |
| Median (IQR) | 0.02 (0.02; 0.05) | 0.07 (0.05; 0.08) | 0.07 (0.03; 0.09) | 0.06 (0.04; 0.11) | 0.04 (0.03; 0.09) |
| Min Max | 0.01 0.07 | 0.05 0.17 | 0.03 0.18 | 0.01 0.17 | 0.01 0.31 |
| **Post-CPB** | | | | | |
| Mean (SD) | 0.54 (0.47) | 0.28 (0.13) | 0.54 (0.49) | 0.77 (0.80) | 0.24 (0.24) |
| Median (IQR) | 0.36 (0.29; 0.58) | 0.30 (0.16; 0.40) | 0.37 (0.11; 0.79) | 0.53 (0.32; 0.79) | 0.13 (0.04; 0.50) |
| Min Max | 0.26 1.58 | 0.05 0.42 | 0.11 1.60 | 0.22 2.67 | 0.03 0.59 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 0.57 (0.65) | 0.22 (0.14) | 0.31 (0.23) | 0.28 (0.18) | 0.38 (0.41) |
| Median (IQR) | 0.25 (0.07; 1.29) | 0.20 (0.10; 0.37) | 0.25 (0.13; 0.47) | 0.23 (0.10; 0.48) | 0.26 (0.06; 0.53) |
| Min Max | 0.05 1.61 | 0.06 0.42 | 0.10 0.75 | 0.10 0.54 | 0.05 1.21 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 0.11 (0.15) | 0.09 (0.06) | 0.11 (0.16) | 0.14 (0.13) | 0.24 (0.19) |
| Median (IQR) | 0.05 (0.04; 0.15) | 0.07 (0.05; 0.14) | 0.05 (0.02; 0.10) | 0.10 (0.11; 0.48) | 0.22 (0.06; 0.40) |
| Min Max | 0.03 0.41 | 0.02 0.18 | 0.01 0.49 | 0.03 0.40 | 0.04 0.54 |
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 0.06 (0.04) | 0.05 (0.03) | 0.07 (0.03) | 0.08 (0.05) | 0.09 (0.02) |
| Median (IQR) | 0.05 (0.02; 0.10) | 0.04 (0.02; 0.08) | 0.07 (0.03; 0.10) | 0.09 (0.05; 0.18) | 0.10 (0.08; 0.11) |
| Min Max | 0.02 0.10 | 0.01 0.09 | 0.02 0.10 | 0.02 0.17 | 0.07 0.12 |
| **POD 1** | | | | | |
| Mean (SD) | 0.12 (0.24) | 0.04 (0.03) | 0.04 (0.02) | 0.07 (0.05) | 0.07 (0.03) |
| Median (IQR) | 0.03 (0.02; 0.08) | 0.04 (0.02; 0.0.06) | 0.04 (0.02; 0.07) | 0.05 (0.01; 0.10) | 0.08 (0.05; 0.10) |
| Min Max | 0.02 0.71 | 0.01 0.10 | 0.01 0.08 | 0.01 0.20 | 0.02 0.10 |
| **POD 2** | | | | | |
| Mean (SD) | 0.05 (0.02) | 0.06 (0.07) | 0.02 (0.01) | 0.04 (0.03) | 0.15 (0.30) |
| Median (IQR) | 0.05 (0.02; 0.07) | 0.04 (0.01; 0.10) | 0.02 (0.01; 0.04) | 0.04 (0.02; 0.09) | 0.04 (0.02; 0.09) |
| Min Max | 0.01 0.08 | 0.01 0.21 | 0.01 0.04 | 0.02 0.09 | 0.02 0.90 |
| **POD 3** | | | | | |
| Mean (SD) | 0.05 (0.03) | 0.04 (0.03) | 0.04 (0.03) | 0.05 (0.03) | 0.05 (0.04) |
| Median (IQR) | 0.05 (0.01; 0.08) | 0.04 (0.01; 0.05) | 0.04 (0.02; 0.06) | 0.04 (0.02; 0.09) | 0.04 (0.02; 0.09) |
| Min Max | 0.01 0.10 | 0.01 0.09 | 0.01 0.10 | 0.02 0.10 | 0.01 0.10 |
| **Haptoglobin (g/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.89 (0.46) | 1.30 (0.46) | 0.89 (0.33) | 1.15 (0.28) | 0.63 (0.41) |
| Median (IQR) | 0.80 (0.50; 1.30) | 1.25 (0.95; 1.40) | 0.80 (0.60; 1.20) | 1.20 (0.90; 1.30) | 0.50 (0.40; 0.85) |
| Min Max | 0.30 1.60 | 0.90 2.30 | 0.40 1.30 | 0.80 1.60 | 0.10 1.40 |
| **Post-CPB** | | | | | |
| Mean (SD) | 0.48 (0.34) | 0.54 (0.31) | 0.31 (0.14) | 0.41 (0.16) | 0.25 (0.24) |
| Median (IQR) | 0.45 (0.15; 0.80) | 0.40 (0.30; 0.75) | 0.30 (0.25; 0.40) | 0.40 (0.30; 0.50) | 0.15 (0.10; 0.40) |
| Min Max | 0.10 0.90 | 0.30 1.10 | 0.10 0.50 | 0.20 0.70 | 0.00 0.70 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 0.40 (0.32) | 0.50 (0.39) | 0.20 (0.14) | 0.26 (0.14) | 0.23 (0.20) |
| Median (IQR) | 0.35 (0.10; 0.60) | 0.45 (0.10; 0.90) | 0.15 (0.10; 0.25) | 0.30 (0.10; 0.30) | 0.15 (0.10; 0.30) |
| Min Max | 0.10 0.90 | 0.10 1.00 | 0.10 0.50 | 0.10 0.50 | 0.10 0.60 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 0.26 (0.21) | 0.40 (0.39) | 0.15 (0.08) | 0.12 (0.04) | 0.10 (0.06) |
| Median (IQR) | 0.15 (0.10; 0.45) | 0.20 (0.10; 0.80) | 0.10 (0.10; 0.20) | 0.10 (0.10; 0.10) | 0.10 (0.10; 0.10) |
| Min Max | 0.10 0.60 | 0.10 1.10 | 0.10 0.30 | 0.10 0.20 | 0.00 0.20 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 0.29 (0.25) | 0.50 (0.35) | 0.16 (0.11) | 0.21 (0.11) | 0.14 (0.09) |
| Median (IQR) | 0.20 (0.10; 0.40) | 0.30 (0.30; 0.90) | 0.10 (0.10; 0.20) | 0.20 (0.10; 0.30) | 0.10 (0.10; 0.10) |
| Min Max | 0.10 0.80 | 0.20 1.10 | 0.10 0.40 | 0.10 0.40 | 0.10 0.30 |
| **POD 1** | | | | | |
| Mean (SD) | 0.38 (0.42) | 0.71 (0.46) | 0.25 (0.17) | 0.40 (0.19) | 0.31 (0.16) |
| Median (IQR) | 0.10 (0.10; 0.50) | 0.60 (0.35; 1.10) | 0.20 (0.10; 0.40) | 0.40 (0.30; 0.50) | 0.30 (0.20; 0.40) |
| Min Max | 0.10 1.30 | 0.20 1.40 | 0.10 0.50 | 0.10 0.70 | 0.10 0.60 |
| **POD 2** | | | | | |
| Mean (SD) | 1.07 (0.82) | 1.59 (0.67) | 0.76 (0.49) | 1.22 (0.58) | 1.05 (0.41) |
| Median (IQR) | 1.10 (0.40; 1.70) | 1.20 (1.10; 2.30) | 0.80 (0.30; 1.20) | 1.45 (1.10; 1.50) | 1.15 (0.80; 1.30) |
| Min Max | 0.10 2.50 | 0.80 2.50 | 0.20 1.50 | 0.10 1.70 | 0.30 1.60 |
| **POD 3** | | | | | |
| Mean (SD) | 1.66 (0.81) | 2.09 (0.56) | 1.39 (0.76) | 1.71 (0.81) | 1.74 (0.50) |
| Median (IQR) | 2.00 (1.20; 2.30) | 1.80 (1.50; 2.75) | 1.55 (0.60; 2.10) | 2.00 (1.00; 2.30) | 1.65 (1.50; 2.10) |
| Min Max | 0.20 2.50 | 1.50 2.75 | 0.40 2.20 | 0.20 2.40 | 0.90 2.50 |
| **Lactate dehydrogenase (LDH) (IU/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 169 (23) | 171 (28) | 181 (16) | 173 (16) | 189 (29) |
| Median (IQR) | 178 (153; 186) | 174 (149; 184) | 176 (169; 194) | 176 (161; 188) | 191 (177; 209) |
| Min Max | 129 193 | 133 225 | 163 208 | 144 191 | 133 228 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 398 (169) | 312 (87) | 413 (181) | 350 (120) | 409 (72) |
| Median (IQR) | 331 (314; 428) | 270 (260; 347) | 383 (259; 523) | 334 (280; 423) | 394 (365; 464) |
| Min Max | 260 763 | 241 492 | 229 750 | 190 563 | 324 499 |
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 411 (200) | 308 (79) | 348 (112) | 328 (85) | 451 (63) |
| Median (IQR) | 337 (284; 478) | 329 (244; 364) | 354 (278; 425) | 286 (264; 379) | 441 (396; 478) |
| Min Max | 263 826 | 200 429 | 177 516 | 251 488 | 395 546 |
| **POD 1** | | | | | |
| Mean (SD) | 380 (180) | 306 (61) | 339 (99) | 436 (340) | 383 (81) |
| Median (IQR) | 298 (286; 333) | 294 (273; 316) | 322 (265; 406) | 308 (270; 408) | 370 (319; 462) |
| Min Max | 243 699 | 239 443 | 214 513 | 258 1262 | 273 486 |
| **POD 2** | | | | | |
| Mean (SD) | 376 (169) | 259 (73) | 325 (91) | 444 (513) | 348 (72) |
| Median (IQR) | 296 (265; 474) | 233 (220; 263) | 316 (230; 421) | 261 (238; 309) | 320 (303; 427) |
| Min Max | 226 715 | 210 432 | 216 456 | 228 1712 | 248 439 |
| **POD 3** | | | | | |
| Mean (SD) | 352 (104) | 253 (81) | 309 (71) | 383 (350) | 322 (86) |
| Median (IQR) | 351 (266; 377) | 233 (217; 243) | 315 (242; 348) | 261 (228; 312) | 311 (260; 399) |
| Min Max | 230 540 | 193 448 | 226 434 | 215 1244 | 200 436 |
| **Change in haemolytic index (AU), active vs placebo (Mixed effects analysis, REML).** | | | | | |
| **Haemolytic index (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.03 | 0.08 | 0.07 | 0.08 | 0.08 |
| Mean difference vs placebo (SE) | | 0.04 (0.02) | 0.04 (0.02) | 0.04 (0.04) | 0.05 (0.04) |
| p-value | | 0.0187 | 0.0626 | 0.0498 | 0.2073 |
| **Post-CPB** | | | | | |
| Observed mean | 0.54 | 0.28 | 0.54 | 0.77 | 0.24 |
| Mean difference vs placebo (SE) | | -0.26 (0.18) | 0.00 (0.25) | 0.23 (0.33) | -0.30 (0.20) |
| p-value | | 0.1999 | 0.9961 | 0.5091 | 0.1669 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Admission to ICU** | | | | | |
| Observed mean | 0.57 | 0.22 | 0.31 | 0.28 | 0.38 |
| Mean difference vs placebo (95% CI) | | -0.35 (0.27) | -0.26 (0.28) | -0.29 (0.28) | -0.19 (0.31) |
| p-value | | 0.2521 | 0.3891 | 0.3364 | 0.5484 |
| **POD 1** | | | | | |
| Observed mean | 0.12 | 0.04 | 0.04 | 0.07 | 0.07 |
| Mean difference vs placebo (95% CI) | | -0.08 (0.09) | -0.08 (0.09) | -0.05 (0.09) | -0.05 (0.09) |
| p-value | | 0.4021 | 0.4013 | 0.5583 | 0.5960 |
| **POD 2** | | | | | |
| Observed mean | 0.05 | 0.06 | 0.02 | 0.04 | 0.15 |
| Mean difference vs placebo (95% CI) | | 0.02 (0.03) | -0.02 (0.01) | -0.01 (0.01) | 0.11 (0.11) |
| p-value | | 0.5210 | 0.0504 | 0.6641 | 0.3567 |
| **POD 3** | | | | | |
| Observed mean | 0.05 | 0.04 | 0.04 | 0.05 | 0.05 |
| Mean difference vs placebo (95% CI) | | -0.01 (0.02) | -0.01 (0.01) | 0.0 (0.02) | 0.0 (0.01) |
| p-value | | 0.5343 | 0.7555 | 0.8833 | > 0.9999 |
| **Change in circulating markers of haemolysis, active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **Haptoglobin (g/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 0.72 (0.04) | 0.73 (0.05) | 0.61 (0.04) | 0.57 (0.05) | 0.78 (0.05) |
| Difference vs placebo (95% CI) | | 0.01 (-0.12,0.14) | -0.11 (-0.23,0.01) | -0.15 (-0.27, -0.02) | 0.07 (-0.06,0.19) |
| p-value | | 0.8550 | 0.0824 | 0.0233* | 0.2855 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 0.95 (0.11) | 1.10 (0.12) | 0.95 (0.12) | 1.05 (0.12) | 0.86 (0.12) |
| Difference vs placebo (95% CI) | | 0.14 (-0.17,0.46) | -0.00 (-0.33,0.32) | 0.09 (-0.22,0.41) | -0.09 (-0.41,0.22) |
| p-value | | 0.3683 | 0.9946 | 0.5655 | 0.5614 |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 0.49 (0.12) | 0.34 (0.12) | 0.38 (0.12) | 0.31 (0.12) | 0.49 (0.12) |
| Difference vs placebo (95% CI) | | -0.16 (-0.48,0.17) | -0.11 (-0.45,0.22) | -0.18 (-0.51,0.16) | -0.01 (-0.33,0.32) |
| p-value | | 0.3447 | 0.5138 | 0.2972 | 0.9690 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 0.38 (0.13) | 0.23 (0.14) | 0.28 (0.12) | 0.16 (0.12) | 0.39 (0.13) |
| Difference vs placebo (95% CI) | | -0.15 (-0.52,0.23) | -0.10 (-0.46,0.26) | -0.22 (-0.58,0.14) | 0.01 (-0.37,0.38) |
| p-value | | 0.4367 | 0.5809 | 0.2197 | 0.9746 |
| **POD 1** | | | | | |
| Observed mean (SE) | 0.44 (0.11) | 0.51 (0.12) | 0.34 (0.12) | 0.26 (0.12) | 0.55 (0.12) |
| Difference vs placebo (95% CI) | | 0.07 (-0.25,0.39) | -0.10 (-0.43,0.22) | -0.18 (-0.51,0.15) | 0.11 (-0.21,0.42) |
| p-value | | 0.6704 | 0.5285 | 0.2813 | 0.5094 |
| **POD 2** | | | | | |
| Observed mean (SE) | 1.13 (0.11) | 1.36 (0.13) | 0.87 (0.13) | 1.08 (0.13) | 1.29 (0.12) |
| Difference vs placebo (95% CI) | | 0.23 (-0.10,0.55) | -0.26 (-0.60,0.08) | -0.05 (-0.39,0.29) | 0.15 (-0.16,0.47) |
| p-value | | 0.1788 | 0.1380 | 0.7669 | 0.3357 |
| **POD 3** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Observed mean (SE) | 1.72 (0.11) | 1.86 (0.13) | 1.58 (0.12) | 1.58 (0.12) | 1.97 (0.12) |
| Difference vs placebo (95% CI) | | 0.14 (-0.19,0.46) | -0.14 (-0.46,0.19) | -0.14 (-0.47,0.18) | 0.25 (-0.06,0.57) |
| p-value | | 0.4154 | 0.4003 | 0.3906 | 0.1152 |
| **LDH (IU/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 328 (23) | 265 (22) | 306 (22) | 333 (24) | 363 (27) |
| Difference vs placebo (95% CI) | | -62 (-128,3) | -22 (-88,44) | 6 (-62,73) | 35 (-38,108) |
| p-value | | 0.0617 | 0.5078 | 0.8630 | 0.3379 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 169 (55) | 171 (55) | 181 (55) | 173 (55) | 190 (55) |
| Difference vs placebo (95% CI) | | 2 (-152,156) | 12 (-143,166) | 4 (-150,158) | 21 (-134,176) |
| p-value | | 0.9801 | 0.8798 | 0.9615 | 0.7925 |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 293 (90) | 223 (90) | 234 (90) | 245 (110) | 397 (91) |
| Difference vs placebo (95% CI) | | -69 (-321,183) | -59 (-311,193) | -47 (-329,234) | 105 (-149,358) |
| p-value | | 0.5875 | 0.6457 | 0.7414 | 0.4173 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 382 (78) | 291 (70) | 297 (70) | 309 (70) | 400 (111) |
| Difference vs placebo (95% CI) | | -90 (-297,117) | -85 (-291,122) | -72 (-279,135) | 18 (-250,287) |
| p-value | | 0.3910 | 0.4207 | 0.4920 | 0.8921 |
| **POD 1** | | | | | |
| Observed mean (SE) | 351 (59) | 308 (59) | 349 (59) | 327 (59) | 451 (70) |
| Difference vs placebo (95% CI) | | -43 (-208,122) | -2 (-167,163) | -23 (-188,141) | 101 (-80,281) |
| p-value | | 0.6059 | 0.9787 | 0.7788 | 0.2743 |
| **POD 2** | | | | | |
| Observed mean (SE) | 355 (59) | 259 (55) | 325 (59) | 444 (55) | 349 (55) |
| Difference vs placebo (95% CI) | | -96 (-255,64) | -30 (-195,136) | 90 (-70,249) | -6 (-167,155) |
| p-value | | 0.2379 | 0.7234 | 0.2693 | 0.9416 |
| **POD 3** | | | | | |
| Observed mean (SE) | 336 (55) | 253 (55) | 309 (55) | 382 (55) | 322 (55) |
| Difference vs placebo (95% CI) | | -83 (-237,71) | -27 (-181,127) | 47 (-108,201) | -14 (-169,142) |
| p-value | | 0.2897 | 0.7314 | 0.5521 | 0.8634 |

Abbreviations: AU – arbitrary units; SD – standard deviation; IQR – interquartile range; SE – standard error; CI – confidence interval, LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 4.2.5.2 The effect of 5-ALA-SFC on peri-operative iron homeostasis and NTBI

Serum iron was found to be increased pre-operatively in all patients who had received active 5-ALA-SFC treatment with an apparent dose-response relationship consistent with increasing administered elemental iron content and indirectly supporting the treatment compliance data (**Fig. 62 A**). The most

marked increase was seen in the 300 mg/472 mg treatment allocation group (mean difference [95 % CI] vs. placebo = 10.9 [0.5; 21.2] µmol/L, p-value = 0.0397). In this treatment group, the mean pre-operative serum iron reading of 30 µmol/L approached the reference value for the upper limit of normal (31 µmol/L). No instances of pre-operative iron deficiency were observed. Post-operatively, a consistent drop in serum iron levels was seen in all trial patients with no statistically significant treatment-related differences between groups. Post-operative serum iron levels were comparable across groups, and no significant 5-ALA-SFC dose-dependent changes were observed (**Fig. 62 B**).

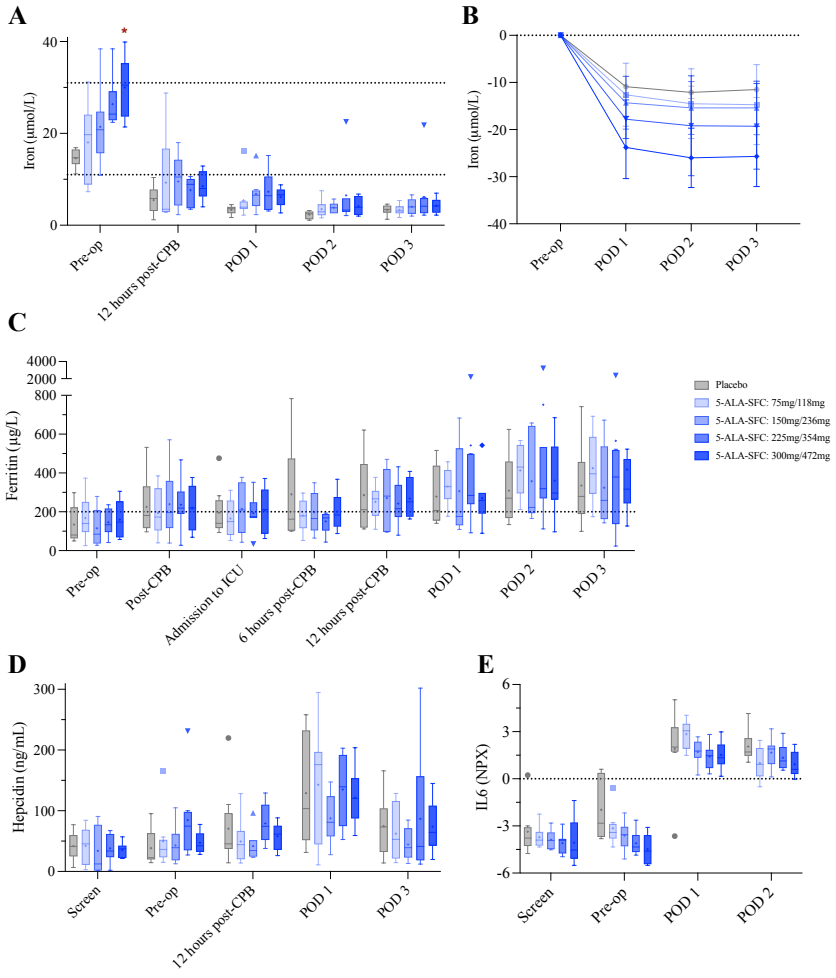



**Figure 62: 5-ALA-SFC and serum iron homeostasis.**

Data presented as mean (SE) (panel B); median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, C – E).

Abbreviations: NPX – normalised protein expression; SE – standard error of the mean.

Post-operative increases in serum ferritin (**Fig. 62 C**) and hepcidin (**Fig. 62 D**), the latter with kinetics broadly similar to that of serum iron reduction, were seen in all treatment allocation groups and this was accompanied by an increase in the expression of IL-6 (**Fig. 62 E**). Although not statistically significant, patients receiving active treatment appeared to have a tendency to lower post-operative serum hepcidin. For example, in the 5-ALA-SFC 150 mg/236 mg treatment allocation group hepcidin was lower vs. placebo (mean difference [SE] = -30.4 [19.4] ng/mL, p-value = 0.3783) on the third post-operative day. Taken together, these data suggest an acute post-operative functional iron deficient state across all groups in response to surgical stress, likely driven by increased hepcidin secretion. Similarly, 5-ALA-SFC treated groups tended to have lower IL-6 levels at all timepoints compared to placebo, a difference most marked for the end of dosing/pre-operative timepoint.

Cardiac surgery was associated with an acute decrease in serum transferrin levels in all patients, and no significant treatment-related differences were observed between groups (**Fig. 63 A**). It is notable that within the 5-ALA-SFC 300 mg/472 mg treatment allocation group there was a significant increase in transferrin saturation (Tsat) ratios immediately before surgery (mean difference [95 % CI] vs. placebo = 19 [3; 25] %, p-value = 0.0288) (**Fig. 63 B**). A 5-ALA-SFC dose related change in pre-operative Tsat readings, was apparent and this corresponds with higher serum iron levels described above. This was followed by a marked reduction in post-operative Tsat levels across all treatment groups below the lower reference range to levels typically associated with iron deficiency.

As a measure of catalytically active iron, peri-operative serum non-transferrin bound iron (NTBI) was quantified in all TALEN trial participants. This showed that Tsat and NTBI correlated significantly ($R^2$ = 0.2203, p-value = 0.0067) (**Fig. 63 C**). Linear regression analysis placed the y-intercept at 49.56%, suggesting that at approximately Tsat > 50%, a pool of non-transferrin bound iron began to appear in the serum. In this study, NTBI was only detected in patients who had been allocated to the active treatment arm, and all patients who had detectable pre-operative NTBI had been allocated to the highest two dosage 5-ALA-SFC allocation groups. NTBI peaked in the immediate hours after CPB and was undetectable by the first post-operative day across all treatment allocation groups (**Fig. 63 D**). As a marker potentially

reflecting NTBI-related lipid peroxidation, plasma malondialdehyde (MDA) was measured. When considering the post-CPB timepoint, the data suggested a dose-related reduction in oxidative stress in 5-ALA-SFC treated patients. For example, in the 5-ALA-SFC 300 mg/472 mg allocation group a mean difference (SE) of -22.06 (19.02) µMol vs. placebo was found, although this was not statistically significant (p-value = 0.6017).

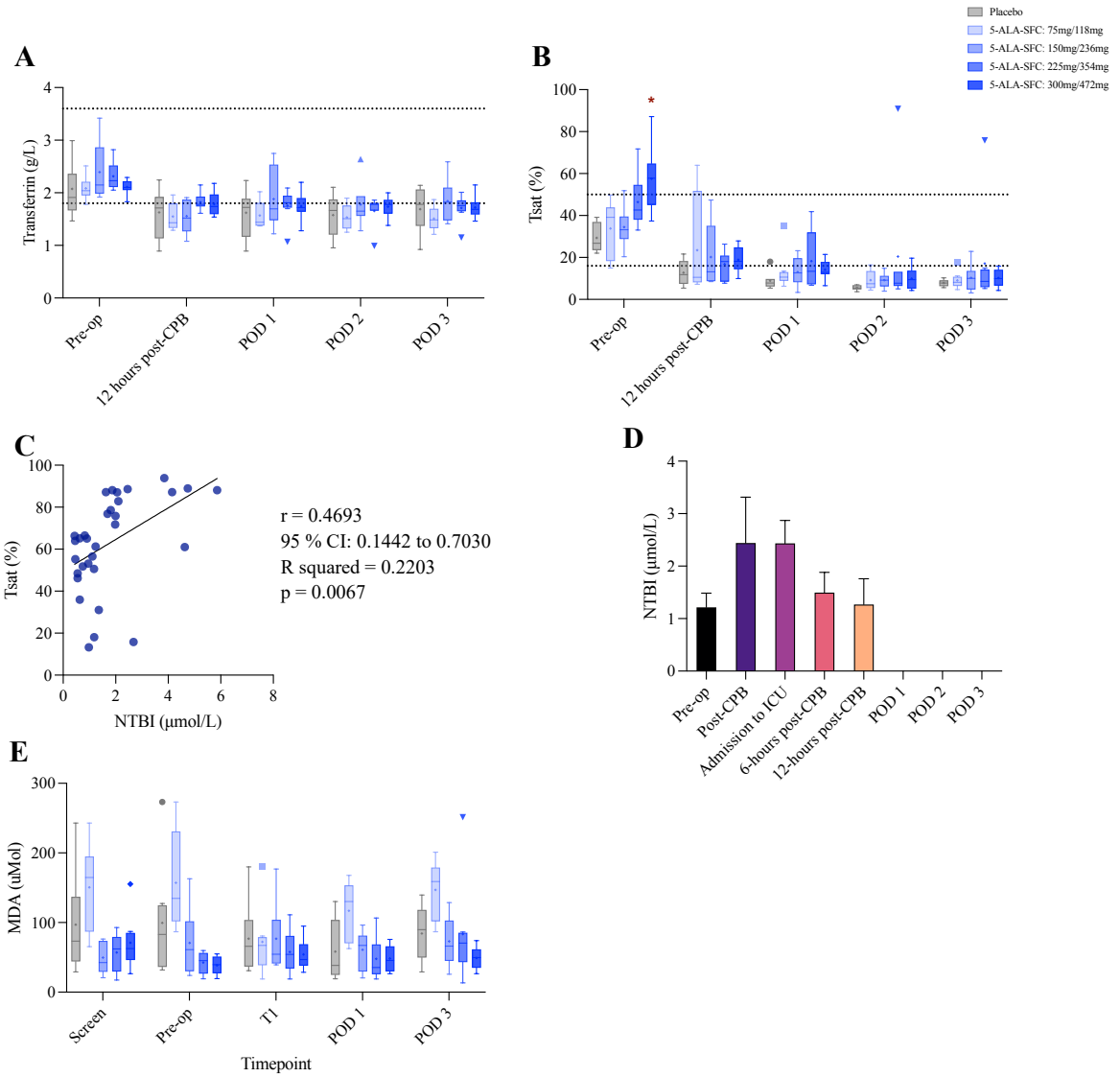



**Figure 63: The effect of 5-ALA-SFC and non-transferrin bound iron associated lipid peroxidation in cardiac surgery.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, B, E) mean (SE) (panel D).

Abbreviations: Tsat – transferrin saturation; NTBI – non-transferrin bound iron; MDA – malondialdehyde.

**Table 30: Descriptive and inferential statistics of markers of haemolysis.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Serum iron (µmol/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 14.5 (1.8) | 18.0 (8.4) | 19.8 (5.2) | 26.4 (6.0) | 30.0 (6.4) |
| Median (IQR) | 14.5 (13.7; 15.9) | 19.8 (9.9; 23.3) | 20.8 (15.9; 24.4) | 24.2 (23.0; 26.1) | 30.5 (24.6; 34.2) |
| Min Max | 11.2 16.9 | 7.3 31.2 | 10.9 25.1 | 22.4 38.4 | 21.4 39.9 |
| **Post-CPB** | | | | | |
| Mean (SD) | 20.1 (8.8) | 18.5 (5.8) | 70.0 (82.0) | 18.4 (8.3) | 30.0 (9.4) |
| Median (IQR) | 17.5 (12.9; 30.0) | 17.2 (13.9; 23.0) | 28.8 (16.8; 164.4) | 18.4 (12.5; 24.2) | 30.0 (23.3; 36.6) |
| Min Max | 12.9 30.0 | 13.4 26.0 | 16.8 164.4 | 12.5 24.2 | 23.3 36.6 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 20.1 (11.4) | 17.4 (7.8) | 22.1 (8.0) | 25.0 (6.9) | 29.7 (7.3) |
| Median (IQR) | 22.5 (12.3; 27.9) | 19.4 (13.6; 21.5) | 18.6 (16.9; 22.3) | 24.1 (20.9; 24.7) | 29.7 (23.4; 35.9) |
| Min Max | 4.3 31.0 | 6.0 26.3 | 16.7 35.9 | 18.9 36.6 | 22.3 37.0 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 17.5 (11.4) | 17.3 (11.0) | 17.0 (9.9) | 18.0 (4.3) | 23.4 (7.9) |
| Median (IQR) | 19.5 (3.2; 29.1) | 16.3 (6.7; 27.0) | 15.9 (10.6; 23.3) | 19.6 (12.1; 20.1) | 22.9 (16.7; 28.4) |
| Min Max | 2.5 29.8 | 4.2 35.0 | 3.1 33.8 | 11.9 23.5 | 15.1 33.8 |
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 5.7 (3.0) | 9.3 (10.0) | 8.1 (6.4) | 7.7 (3.0) | 8.5 (3.2) |
| Median (IQR) | 6.2 (3.2; 7.5) | 3.5 (2.9; 16.7) | 6.3 (3.2; 10.5) | 8.9 (3.7; 10.1) | 8.0 (7.0; 11.3) |
| Min Max | 1.2 10.4 | 2.8 28.8 | 2.3 18.0 | 3.5 10.6 | 4.0 12.9 |
| **POD 1** | | | | | |
| Mean (SD) | 3.6 (1.0) | 5.4 (4.4) | 5.5 (1.9) | 7.3 (4.4) | 6.2 (2.1) |
| Median (IQR) | 3.9 (3.0; 4.0) | 4.0 (3.6; 5.1) | 5.8 (4.1; 7.0) | 6.5 (3.5; 10.1) | 6.7 (4.7; 7.7) |
| Min Max | 1.7 5.1 | 2.2 16.2 | 2.3 7.8 | 3.1 15.2 | 2.7 8.8 |
| **POD 2** | | | | | |
| Mean (SD) | 2.3 (0.8) | 3.5 (1.9) | 3.9 (1.2) | 6.5 (7.2) | 4.2 (2.0) |
| Median (IQR) | 2.5 (1.7; 2.8) | 3.0 (2.3; 4.4) | 3.8 (2.6; 4.7) | 3.3 (2.9; 5.9) | 3.9 (2.4; 6.2) |
| Min Max | 1.1 3.2 | 1.6 7.5 | 2.6 5.8 | 2.1 22.5 | 2.0 6.8 |
| **POD 3** | | | | | |
| Mean (SD) | 3.2 (1.1) | 3.3 (1.1) | 4.4 (2.3) | 6.1 (6.5) | 4.3 (1.6) |
| Median (IQR) | 3.4 (2.6; 3.6) | 3.1 (2.7; 3.9) | 4.0 (2.6; 5.5) | 4.2 (2.7; 5.8) | 4.2 (3.2; 5.3) |
| Min Max | 1.3 4.6 | 1.7 5.4 | 2.0 8.7 | 2.2 21.8 | 2.2 7.0 |
| **Ferritin (µg/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 134 (96) | 168 (112) | 115 (96) | 146 (68) | 160 (97) |
| Median (IQR) | 81 (66; 165) | 140 (96; 236) | 84 (39; 183) | 133 (103; 209) | 148 (74; 237) |
| Min Max | 50 298 | 26 373 | 28 279 | 42 237 | 57 306 |
| **Post-CPB** | | | | | |
| Mean (SD) | 225 (148) | 194 (123) | 239 (185) | 236 (125) | 219 (119) |
| Median (IQR) | 181 (125; 289) | 173 (104; 288) | 197 (142; 290) | 219 (194; 285) | 220 (114; 318) |
| Min Max | 97 532 | 40 385 | 40 571 | 28 468 | 69 377 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 196 (128) | 166 (99) | 215 (134) | 192 (96) | 208 (122) |
| Median (IQR) | 141 (120; 236) | 150 (108; 206) | 209 (107; 345) | 175 (168; 250) | 211 (95; 296) |
| Min Max | 94 476 | 53 311 | 44 378 | 35 352 | 62 372 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 290 (251) | 181 (88) | 198 (110) | 150 (61) | 201 (99) |
| Median (IQR) | 162 (104; 453) | 179 (136; 246) | 166 (104; 297) | 169 (161; 185) | 184 (136; 246) |
| Min Max | 100 783 | 53 297 | 65 350 | 44 193 | 88 368 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 286 (193) | 252 (90) | 271 (171) | 243 (118) | 269 (108) |
| Median (IQR) | 211 (122; 444) | 267 (203; 286) | 281 (96; 404) | 216 (204; 308) | 252 (186; 351) |
| Min Max | 112 622 | 110 378 | 95 471 | 80 432 | 163 408 |
| **POD 1** | | | | | |
| Mean (SD) | 279 (149) | 331 (94) | 307 (227) | 542 (684) | 270 (139) |
| Median (IQR) | 206 (162; 388) | 330 (266; 410) | 176 (139; 515) | 284 (241; 497) | 259 (191; 298) |
| Min Max | 141 516 | 177 458 | 109 683 | 92 2202 | 90 543 |
| **POD 2** | | | | | |
| Mean (SD) | 309 (181) | 413 (134) | 358 (216) | 752 (1094) | 361 (190) |
| Median (IQR) | 270 (184; 322) | 430 (300; 533) | 222 (193; 643) | 319 (269; 534) | 297 (271; 484) |
| Min Max | 134 624 | 212 568 | 165 658 | 112 3212 | 97 685 |
| **POD 3** | | | | | |
| Mean (SD) | 335 (198) | 425 (176) | 324 (206) | 566 (746) | 418 (349) |
| Median (IQR) | 279 (202; 414) | 396 (304; 566) | 259 (171; 459) | 380 (178; 513) | 317 (253; 421) |
| Min Max | 100 741 | 174 692 | 143 672 | 23 2358 | 127 1238 |
| **Hepcidin (ng/mL)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 41.9 (22.5) | 42.3 (33.0) | 33.9 (40.9) | 38.2 (22.7) | 35.3 (12.8) |
| Median (IQR) | 41.2 (24.7; 59.9) | 45.5 (10.9; 69.0) | 12.5 (1.4; 77.1) | 33.6 (23.5; 61.7) | 37.5 (22.2; 42.7) |
| Min Max | 6.7 76.9 | 3.0 84.3 | 1.1 90.8 | 2.0 67.3 | 21.3 57.2 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 38.4 (29.2) | 49.8 (48.2) | 43.0 (30.8) | 84.7 (65.6) | 75.5 (18.0) |
| Median (IQR) | 22.8 (19.9; 63.0) | 36.3 (23.3; 52.3) | 39.3 (18.1; 62.2) | 74.8 (34.8; 98.2) | 42.3 (32.5; 62.8) |
| Min Max | 14.6 92.3 | 15.0 164.5 | 12.7 104.8 | 21.3 231.1 | 28.3 77.5 |
| **Post-CPB** | | | | | |
| Mean (SD) | 70.6 (66.4) | 49.5 (37.2) | 41.8 (26.1) | 79.1 (33.1) | 58.1 (21.7) |
| Median (IQR) | 45.5 (37.1; 96.3) | 44.2 (20.0; 66.7) | 34.6 (24.3; 51.6) | 74.3 (51.9; 110.0) | 61.5 (35.6; 75.5) |
| Min Max | 14.2 22.0 | 14.0 128.1 | 23.2 96.6 | 37.9 129.3 | 26.2 88.3 |
| **POD 1** | | | | | |
| Mean (SD) | 128.9 (90.3) | 143.0 (96.0) | 87.5 (41.1) | 135.1 (58.8) | 121.8 (45.4) |
| Median (IQR) | 103.6 (51.7; 232.5) | 175.9 (44.5; 197.1) | 81.0 (57.5; 123.9) | 139.6 (74.8; 192.1) | 120.3 (87.2; 153.7) |
| Min Max | 31.5 258.2 | 10.9 295.0 | 27.8 147.5 | 52.6 203.0 | 59.3 203.9 |
| **POD 3** | | | | | |
| Mean (SD) | 74.9 (48.5) | 62.4 (46.5) | 44.5 (26.0) | 86.8 (103.5) | 73.8 (40.9) |
| Median (IQR) | 73.4 (32.2; 104.0) | 52.8 (21.1; 116.3) | 39.3 (22.7; 71.3) | 140.0 (74.8; 192.1) | 41.2 (18.2; 156.9) |
| Min Max | 14.0 165.9 | 14.6 128.4 | 13.3 84.7 | 52.7 203.0 | 12.2 302.0 |
| **Transferrin (g/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.1 (0.5) | 2.1 (0.2) | 2.4 (0.5) | 2.3 (0.3) | 2.1 (0.1) |
| Median (IQR) | 1.9 (1.7; 2.3) | 2.0 (2.0; 2.2) | 2.2 (2.0; 2.7) | 2.2 (2.1; 2.4) | 2.1 (2.1; 2.2) |
| Min Max | 1.5 3.0 | 1.8 2.5 | 1.9 3.4 | 2.1 2.8 | 1.8 2.3 |
| **Post-CPB** | | | | | |
| Mean (SD) | 1.6 (0.6) | 1.3 (0.3) | 1.8 (0.6) | 1.7 (0.5) | 1.8 (0.3) |
| Median (IQR) | 1.5 (1.2; 2.3) | 1.2 (1.1; 1.5) | 1.5 (1.4; 2.5) | 1.7 (1.4; 2.1) | 1.8 (1.6; 2.0) |
| Min Max | 1.2 2.3 | 0.9 1.6 | 1.4 2.5 | 1.4 2.1 | 1.6 2.0 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 1.7 (0.5) | 1.5 (0.3) | 1.9 (0.7) | 1.8 (0.2) | 1.7 (0.2) |
| Median (IQR) | 1.5 (1.3; 2.0) | 1.4 (1.3; 1.6) | 1.7 (1.6; 2.5) | 1.7 (1.6; 1.9) | 1.6 (1.6; 1.8) |
| Min Max | 1.2 2.3 | 1.2 2.0 | 1.0 2.7 | 1.6 2.0 | 1.6 2.0 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 1.7 (0.4) | 1.5 (0.3) | 1.8 (0.7) | 1.7 (0.2) | 1.6 (0.2) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Median (IQR) | 1.7 (1.2; 1.8) | 1.3 (1.2; 1.7) | 1.7 (1.3; 2.3) | 1.7 (1.6; 1.9) | 1.7 (1.5; 1.7) |
| Min Max | 1.1 2.3 | 1.2 2.0 | 1.1 3.0 | 1.5 2.0 | 1.4 1.8 |
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 1.6 (0.4) | 1.6 (0.3) | 1.9 (0.6) | 1.8 (0.3) | 1.7 (0.3) |
| Median (IQR) | 1.7 (1.6; 1.8) | 1.4 (1.4; 1.8) | 1.7 (1.5; 2.3) | 1.8 (1.7; 1.9) | 1.7 (1.6; 1.9) |
| Min Max | 0.9 2.2 | 1.4 2.0 | 1.2 2.8 | 1.1 2.1 | 1.3 2.2 |
| **POD 1** | | | | | |
| Mean (SD) | 1.6 (0.4) | 1.6 (0.3) | 1.9 (0.6) | 1.8 (0.3) | 1.7 (0.3) |
| Median (IQR) | 1.7 (1.6; 1.8) | 1.4 (1.4; 1.8) | 1.7 (1.5; 2.3) | 1.8 (1.7; 1.9) | 1.7 (1.6; 1.9) |
| Min Max | 0.9 2.2 | 1.4 2.0 | 1.2 2.8 | 1.1 2.1 | 1.3 2.2 |
| **POD 2** | | | | | |
| Mean (SD) | 1.6 (0.4) | 1.5 (0.2) | 1.8 (0.4) | 1.7 (0.3) | 1.7 (0.2) |
| Median (IQR) | 1.6 (1.3; 1.8) | 1.5 (1.3; 1.7) | 1.7 (1.6; 1.9) | 1.8 (1.7; 1.8) | 1.8 (1.6; 1.9) |
| Min Max | 1.0 2.1 | 1.3 1.9 | 1.3 2.6 | 1.0 1.9 | 1.4 2.0 |
| **POD 3** | | | | | |
| Mean (SD) | 1.7 (0.4) | 1.5 (0.2) | 1.8 (0.4) | 1.7 (0.3) | 1.7 (0.2) |
| Median (IQR) | 1.8 (1.4; 2.0) | 1.5 (1.4; 1.7) | 1.8 (1.5; 2.1) | 1.8 (1.7; 1.9) | 1.7 (1.6; 1.8) |
| Min Max | 0.9 2.1 | 1.2 1.9 | 1.4 2.6 | 1.2 2.0 | 1.5 2.2 |
| **Transferrin saturation (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 30 (7) | 38 (15) | 36 (10) | 47 (14) | 57 (15) |
| Median (IQR) | 30 (24; 36) | 44 (24; 49) | 36 (30; 42) | 43 (40; 49) | 58 (47; 63) |
| Min Max | 22 42 | 17 57 | 20 52 | 33 72 | 37 87 |
| **Post-CPB** | | | | | |
| Mean (SD) | 50 (5) | 68 (23) | 50 (25) | 42 (8) | 70 (34) |
| Median (IQR) | 52 (44; 53) | 63 (52; 85) | 45 (27; 77) | 42 (36; 47) | 70 (46; 94) |
| Min Max | 44 53 | 47 100 | 27 77 | 36 47 | 46 94 |
| **Admission to ICU** | | | | | |
| Mean (SD) | 51 (25) | 53 (25) | 55 (26) | 58 (18) | 71 (20) |
| Median (IQR) | 60 (34; 68) | 61 (38; 74) | 60 (31; 68) | 48 (48; 61) | 70 (54; 88) |
| Min Max | 14 69 | 17 75 | 27 88 | 44 87 | 50 92 |
| **Six hours post-CPB** | | | | | |
| Mean (SD) | 41 (25) | 54 (35) | 47 (21) | 42 (11) | 59 (21) |
| Median (IQR) | 52 (11; 65) | 46 (18; 99) | 43 (38; 56) | 41 (31; 55) | 51 (44; 79) |
| Min Max | 8 68 | 15 101 | 18 89 | 26 55 | 37 82 |
| **Twelve-hours post CPB** | | | | | |
| Mean (SD) | 14 (6) | 27 (27) | 21 (16) | 17 (7) | 19 (6) |
| Median (IQR) | 14 (9; 19) | 12 (9; 59) | 13 (9; 26) | 18 (8; 21) | 18 (16; 24) |
| Min Max | 5 22 | 8 73 | 8 47 | 8 26 | 10 28 |
| **POD 1** | | | | | |
| Mean (SD) | 10 (4) | 15 (10) | 13 (7) | 18 (14) | 14 (5) |
| Median (IQR) | 8 (7; 10) | 12 (11; 14) | 13 (9; 19) | 13 (7; 28) | 13 (13; 18) |
| Min Max | 5 18 | 7 40 | 3 23 | 7 42 | 6 21 |
| **POD 2** | | | | | |
| Mean (SD) | 10 (12) | 11 (5) | 9 (4) | 20 (31) | 10 (6) |
| Median (IQR) | 7 (6; 7) | 9 (7; 15) | 9 (6; 11) | 8 (6; 13) | 10 (5; 14) |
| Min Max | 5 40 | 5 19 | 4 15 | 5 91 | 4 20 |
| **POD 3** | | | | | |
| Mean (SD) | 8 (2) | 10 (5) | 10 (6) | 17 (24) | 10 (4) |
| Median (IQR) | 8 (6; 9) | 9 (7; 12) | 10 (5; 14) | 9 (6; 14) | 10 (8; 13) |
| Min Max | 5 10 | 5 20 | 3 23 | 5 76 | 4 16 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **MDA (µmol)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 97.0 (70.8) | 150.6 (62.7) | 49.7 (23.5) | 56.9 (27.4) | 70.9 (39.5) |
| Median (IQR) | 73.3 (44.0; 136.9) | 164.9 (86.7; 194.9) | 42.7 (29.2; 73.8) | 62.1 (29.6; 19.3) | 62.6 (46.2; 85.2) |
| Min Max | 29.17 243.1 | 65.3 243.1 | 20.8 76.3 | 17.4 92.9 | 26.5 155.4 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 99.5 (79.1) | 157.0 (70.32) | 70.7 (49.2) | 42.6 (15.3) | 38.4 (12.9) |
| Median (IQR) | 83.0 (36.3; 124.9) | 134.8 (101.5; 231.0) | 61.2 (30.0; 102.1) | 45.2 (26.5; 56.5) | 39.6 (27.0; 51.2) |
| Min Max | 31.96 273.1 | 86.7 273.1 | 24.1 163.0 | 19.1 59.8 | 19.3 55.0 |
| **Post-CPB** | | | | | |
| Mean (SD) | 76.9 (49.3) | 72.2 (48.5) | 76.9 (49.5) | 57.7 (29.8) | 54.3 (21.5) |
| Median (IQR) | 65.99 (36.6; 103.8) | 67.1 (38.5; 79.1) | 54.4 (41.0; 104.1) | 54.3 (33.8; 80.9) | 46.9 (38.1; 69.0) |
| Min Max | 30.9 180.0 | 18.8 179.9 | 39.3 176.9 | 19.0 111.0 | 28.5 94.8 |
| **POD 1** | | | | | |
| Mean (SD) | 58.3 (43.1) | 117.1 (42.4) | 60.9 (27.7) | 48.0 (29.8) | 48.4 (19.4) |
| Median (IQR) | 38.4 (24.8; 103.7) | 430.3 (70.0; 153.6) | 67.3 (29.6; 81.4) | 35.4 (25.8; 68.7) | 45.5 (29.8; 65.9) |
| Min Max | 19.1 130.2 | 62.6 167.9 | 20.4 96.3 | 19.0 106.6 | 26.6 75.9 |
| **POD 3** | | | | | |
| Mean (SD) | 94.3 (38.4) | 147.0 (42.1) | 73.1 (34.7) | 83.4 (72.2) | 49.1 (15.6) |
| Median (IQR) | 89.9 (49.8; 118.3) | 158.8 (101.7; 179.1) | 66.2 (44.7; 102.9) | 70.5 (43.1; 85.4) | 49.7 (34.9; 61.7) |
| Min Max | 29.2 139.7 | 86.7 200.9 | 25.8 128.6 | 13.3 251.3 | 26.5 74.0 |
| **Change in circulating markers of haemolysis, active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **Serum iron (µmol/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 12.8 (1.7) | 12.5 (1.4) | 19.5 (1.5) | N/A | 14.4 (1.9) |
| Difference vs placebo (95% CI) | | -0.3 (-4.5,3.9) | 6.7 (2.3,11.0) | N/A | 1.6 (-4.1,7.2) |
| p-value | | 0.8791 | **0.0036 **** | N/A | 0.5690 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 16.5 (3.6) | 19.0 (3.5) | 20.2 (3.5) | 24.9 (4.1) | 27.4 (3.6) |
| Difference vs placebo (95% CI) | | 2.5 (-7.3,12.2) | 3.7 (-6.1,13.5) | 8.3 (-2.5,19.2) | 10.9 (0.5,21.2) |
| p-value | | 0.6204 | 0.4616 | 0.1317 | **0.0397 *** |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 22.5 (5.8) | 19.8 (5.0) | 71.3 (5.7) | N/A | 27.7 (7.0) |
| Difference vs placebo (95% CI) | | -2.7 (-17.6,12.3) | 48.8 (32.8,64.7) | N/A | 5.2 (-13.0,23.3) |
| p-value | | 0.7260 | **<.0001 ***** | N/A | 0.5741 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 22.0 (5.0) | 18.1 (4.4) | 22.5 (4.4) | 25.4 (5.8) | 27.3 (5.0) |
| Difference vs placebo (95% CI) | | -3.9 (-17.0,9.2) | 0.5 (-12.6,13.6) | 3.4 (-11.8,18.5) | 5.3 (-8.8,19.5) |
| p-value | | 0.5592 | 0.9380 | 0.6624 | 0.4591 |
| **POD 1** | | | | | |
| Observed mean (SE) | 5.6 (3.6) | 6.4 (3.5) | 5.9 (3.5) | 7.0 (4.1) | 3.6 (3.6) |
| Difference vs placebo (95% CI) | | 0.8 (-9.0,10.6) | 0.3 (-9.5,10.1) | 1.4 (-9.4,12.3) | -2.0 (-12.3,8.3) |
| p-value | | 0.8706 | 0.9468 | 0.7924 | 0.7031 |
| **POD 2** | | | | | |
| Observed mean (SE) | 4.2 (3.8) | 4.5 (3.5) | 4.4 (3.7) | 6.1 (4.5) | 1.6 (3.6) |
| Difference vs placebo (95% CI) | | 0.2 (-9.9,10.4) | 0.2 (-10.3,10.7) | 1.9 (-9.9,13.6) | -2.6 (-13.3,8.0) |
| p-value | | 0.9624 | 0.9693 | 0.7546 | 0.6262 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 3** | | | | | |
| Observed mean (SE) | 5.0 (3.6) | 4.3 (3.5) | 4.8 (3.5) | 5.6 (4.1) | 1.7 (3.6) |
| Difference vs placebo (95% CI) | | -0.7 (-10.5,9.0) | -0.2 (-10.0,9.6) | 0.6 (-10.3,11.4) | -3.3 (-13.6,7.0) |
| p-value | | 0.8811 | 0.9651 | 0.9193 | 0.5279 |
| **Ferritin (µg/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 270 (29) | 242 (32) | 280 (32) | 363 (32) | 236 (33) |
| Difference vs placebo (95% CI) | | -28 (-116,60) | 10 (-77,98) | 93 (6,180) | -34 (-123,56) |
| p-value | | 0.5254 | 0.8118 | **0.0375** * | 0.4487 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 150 (80) | 129 (84) | 162 (84) | 143 (84) | 134 (84) |
| Difference vs placebo (95% CI) | | -21 (-249,208) | 12 (-217,240) | -7 (-236,221) | -16 (-244,213) |
| p-value | | 0.8583 | 0.9197 | 0.9500 | 0.8922 |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 241 (80) | 155 (84) | 245 (97) | 233 (84) | 193 (84) |
| Difference vs placebo (95% CI) | | -85 (-314,144) | 5 (-243,253) | -7 (-236,221) | -47 (-276,181) |
| p-value | | 0.4642 | 0.9698 | 0.9493 | 0.6837 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 218 (84) | 170 (107) | 221 (97) | 210 (90) | 202 (97) |
| Difference vs placebo (95% CI) | | -48 (-316,220) | 4 (-250,258) | -8 (-251,236) | -16 (-270,238) |
| p-value | | 0.7267 | 0.9781 | 0.9508 | 0.9020 |
| **POD 1** | | | | | |
| Observed mean (SE) | 294 (80) | 292 (84) | 353 (84) | 539 (84) | 279 (90) |
| Difference vs placebo (95% CI) | | -2 (-231,227) | 59 (-170,287) | 244 (16,473) | -16 (-253,221) |
| p-value | | 0.9847 | 0.6120 | **0.0362** * | 0.8954 |
| **POD 2** | | | | | |
| Observed mean (SE) | 325 (80) | 374 (84) | 386 (90) | 742 (90) | 335 (84) |
| Difference vs placebo (95% CI) | | 49 (-179,278) | 61 (-176,298) | 416 (179,653) | 10 (-219,239) |
| p-value | | 0.6713 | 0.6142 | **0.0006** *** | 0.9320 |
| **POD 3** | | | | | |
| Observed mean (SE) | 351 (80) | 386 (84) | 371 (84) | 562 (84) | 393 (84) |
| Difference vs placebo (95% CI) | | 35 (-194,263) | 19 (-209,248) | 211 (-18,439) | 41 (-187,270) |
| p-value | | 0.7664 | 0.8677 | 0.0703 | 0.7228 |
| **Transferrin (g/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 1.8 (0.0) | 1.6 (0.0) | 1.7 (0.0) | N/A | 1.8 (0.0) |
| Difference vs placebo (95% CI) | | -0.2 (-0.3,-0.1) | -0.1 (-0.2,0.0) | N/A | 0.0 (-0.1,0.1) |
| p-value | | **0.0020** ** | 0.2093 | N/A | 0.7722 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 2.1 (0.1) | 2.1 (0.1) | 2.2 (0.1) | 2.2 (0.1) | 2.2 (0.1) |
| Difference vs placebo (95% CI) | | 0.0 (-0.2,0.3) | 0.1 (-0.2,0.3) | 0.1 (-0.2,0.3) | 0.0 (-0.2,0.3) |
| p-value | | 0.9587 | 0.5334 | 0.6586 | 0.9269 |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 1.7 (0.1) | 1.3 (0.1) | 1.5 (0.2) | N/A | 1.7 (0.2) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | -0.4 (-0.8,0.0) | -0.2 (-0.6,0.2) | N/A | 0.0 (-0.4,0.5) |
| p-value | | 0.0658 | 0.4262 | N/A | 0.8883 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 1.8 (0.1) | 1.6 (0.1) | 1.6 (0.1) | 1.6 (0.1) | 1.7 (0.1) |
| Difference vs placebo (95% CI) | | -0.2 (-0.6,0.1) | -0.2 (-0.5,0.2) | -0.2 (-0.6,0.2) | -0.1 (-0.5,0.2) |
| p-value | | 0.1747 | 0.3370 | 0.2620 | 0.4826 |
| **POD 1** | | | | | |
| Observed mean (SE) | 1.7 (0.1) | 1.6 (0.1) | 1.7 (0.1) | 1.6 (0.1) | 1.8 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.3,0.2) | 0.0 (-0.2,0.3) | -0.1 (-0.4,0.2) | 0.1 (-0.1,0.4) |
| p-value | | 0.7021 | 0.8249 | 0.4880 | 0.4042 |
| **POD 2** | | | | | |
| Observed mean (SE) | 1.7 (0.1) | 1.6 (0.1) | 1.7 (0.1) | 1.6 (0.1) | 1.8 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4,0.1) | -0.1 (-0.3,0.2) | -0.1 (-0.4,0.2) | 0.1 (-0.2,0.3) |
| p-value | | 0.3332 | 0.7140 | 0.3780 | 0.6550 |
| **POD 3** | | | | | |
| Observed mean (SE) | 1.7 (0.1) | 1.6 (0.1) | 1.7 (0.1) | 1.6 (0.1) | 1.8 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4,0.1) | -0.0 (-0.3,0.3) | -0.1 (-0.4,0.2) | 0.1 (-0.2,0.3) |
| p-value | | 0.3927 | 0.9627 | 0.4787 | 0.5087 |
| **Transferrin saturation (%)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 30 (3) | 35 (2) | 32 (2) | N/A | 34 (3) |
| Difference vs placebo (95% CI) | | 6 (-1,12) | 2 (-4,9) | N/A | 4 (-4,13) |
| p-value | | 0.1003 | 0.4785 | N/A | 0.3064 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 34 (6) | 39 (6) | 37 (6) | 45 (7) | 53 (6) |
| Difference vs placebo (95% CI) | | 6 (-10,21) | 4 (-12,20) | 11 (-6,29) | 19 (3,35) |
| p-value | | 0.4754 | 0.6281 | 0.1913 | **0.0228 *** |
| **Post-CPB** | | | | | |
| Observed mean (SE) | 54 (9) | 70 (8) | 54 (9) | N/A | 67 (11) |
| Difference vs placebo (95% CI) | | 16 (-8,40) | -0 (-26,25) | N/A | 13 (-16,42) |
| p-value | | 0.1934 | 0.9756 | N/A | 0.3790 |
| **Admission to ICU** | | | | | |
| Observed mean (SE) | 53 (8) | 53 (7) | 56 (7) | 62 (9) | 67 (8) |
| Difference vs placebo (95% CI) | | 0 (-21,21) | 3 (-18,24) | 9 (-15,33) | 14 (-8,36) |
| p-value | | 0.9835 | 0.7508 | 0.4794 | 0.2185 |
| **POD 1** | | | | | |
| Observed mean (SE) | 13 (6) | 16 (6) | 15 (6) | 20 (7) | 9 (6) |
| Difference vs placebo (95% CI) | | 3 (-13,18) | 2 (-14,17) | 7 (-10,24) | -4 (-20,13) |
| p-value | | 0.7350 | 0.8276 | 0.4143 | 0.6444 |
| **POD 2** | | | | | |
| Observed mean (SE) | 14 (6) | 11 (6) | 11 (6) | 23 (7) | 5 (6) |
| Difference vs placebo (95% CI) | | -2 (-19,14) | -3 (-19,14) | 9 (-9,28) | -9 (-25,8) |
| p-value | | 0.7688 | 0.7516 | 0.3282 | 0.3153 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 3** | | | | | |
| Observed mean (SE) | 11 (6) | 11 (6) | 12 (6) | 19 (7) | 5 (6) |
| Difference vs placebo (95% CI) | | 0 (-16,16) | 1 (-14,17) | 8 (-9,25) | -5 (-22,11) |
| p-value | | 0.9796 | 0.8767 | 0.3495 | 0.5112 |
| **Change in hepcidin (ng/mL), active vs placebo (Mixed effects analysis, REML).** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 38.4 | 49.8 | 43.0 | 84.7 | 47.5 |
| Mean difference vs placebo (SE) | | 11.4 (19.9) | 4.5 (15.0) | 46.3 (25.4) | 9.1 (12.1) |
| p-value | | 0.9410 | 0.9939 | 0.2682 | 0.8652 |
| **Post-CPB** | | | | | |
| Observed mean | 70.6 | 49.5 | 41.8 | 79.1 | 58.1 |
| Mean difference vs placebo (SE) | | -21.1 (26.9) | -28.8 (25.5) | 8.5 (26.2) | -12.5 (24.7) |
| p-value | | 0.8486 | 0.6378 | 0.9921 | 0.9600 |
| **POD 1** | | | | | |
| Observed mean | 128.9 | 143.0 | 87.5 | 135.1 | 121.8 |
| Mean difference vs placebo (SE) | | 14.1 (46.6) | -41.4 (35.1) | 6.2 (38.1) | -7.1 (35.7) |
| p-value | | 0.9939 | 0.6051 | 0.9994 | 0.9987 |
| **POD 3** | | | | | |
| Observed mean | 74.9 | 62.4 | 44.5 | 86.8 | 73.8 |
| Mean difference vs placebo (SE) | | -12.5 (23.7) | -30.4 (19.4) | 11.9 (40.4) | -1.2 (22.4) |
| p-value | | 0.9548 | 0.3783 | 0.9942 | > 0.9999 |
| **Change in MDA (µmol), active vs placebo (Mixed effects analysis, REML).** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 99.51 | 157.0 | 70.66 | 42.55 | 38.39 |
| Mean difference vs placebo (SE) | | 57.49 (37.43) | -28.85 (32.93) | -56.96 (28.49) | -61.12 (28.34) |
| p-value | | 0.3829 | 0.7946 | 0.2244 | 0.1822 |
| **Post-CPB** | | | | | |
| Observed mean | 76.92 | 72.15 | 76.87 | 57.67 | 54.32 |
| Mean difference vs placebo (SE) | | -4.77 (24.44) | -0.05 (25.58) | -19.25 (20.36) | -22.60 (19.02) |
| p-value | | 0.9989 | > 0.9999 | 0.7545 | 0.6017 |
| **POD 1** | | | | | |
| Observed mean | 58.27 | 117.1 | 60.93 | 47.98 | 48.36 |
| Mean difference vs placebo (SE) | | 58.83 (21.38) | 2.66 (18.12) | -10.29 (18.53) | -9.91 (16.72) |
| p-value | | **0.0499 *** | 0.9996 | 0.9463 | 0.9326 |
| **POD 3** | | | | | |
| Observed mean | 84.27 | 147.0 | 73.13 | 83.40 | 49.14 |
| Mean difference vs placebo (SE) | | 62.73 (20.12) | -11.14 (18.29) | -0.87 (28.91) | -35.13 (14.64) |
| p-value | | **0.0250 *** | 0.9280 | > 0.9999 | 0.1149 |

| **NTBI (µmol/L): All patients** | | | | | |
|---|---|---|---|---|---|
| | **Pre-op** | **Post-CPB** | **Admission to ICU** | **6 hours post-CPB** | **12 hours post-CPB** |
| Mean (SEM) | 1.21 (0.27) | 2.44 (0.88) | 2.43 (0.44) | 1.50 (0.39) | 1.27 (0.49) |
| Median (IQR) | 1.17 (0.65; 1.81) | 1.59 (0.91; 3.35) | 1.99 (0.86; 3.83) | 1.11 (0.55; 2.06) | 0.97 (0.53; 2.31) |
| Min Max | 0.47 1.98 | 0.43 8.03 | 0.63 5.86 | 0.43 4.75 | 0.46 2.68 |

Abbreviations: SD – standard deviation; IQR – interquartile range; SE – standard error; CI – confidence interval.

#### 4.2.5.3 The effect of 5-ALA-SFC on iron handling genes Impact of 5-ALA-SFC on PBMC iron handling

To investigate whether 5-ALA-SFC had any effect on immune cell iron handling related gene expression, RT-qPCR using Taqman™ chemistry was performed on RNA extracted from PBMC (**Fig. 64**).

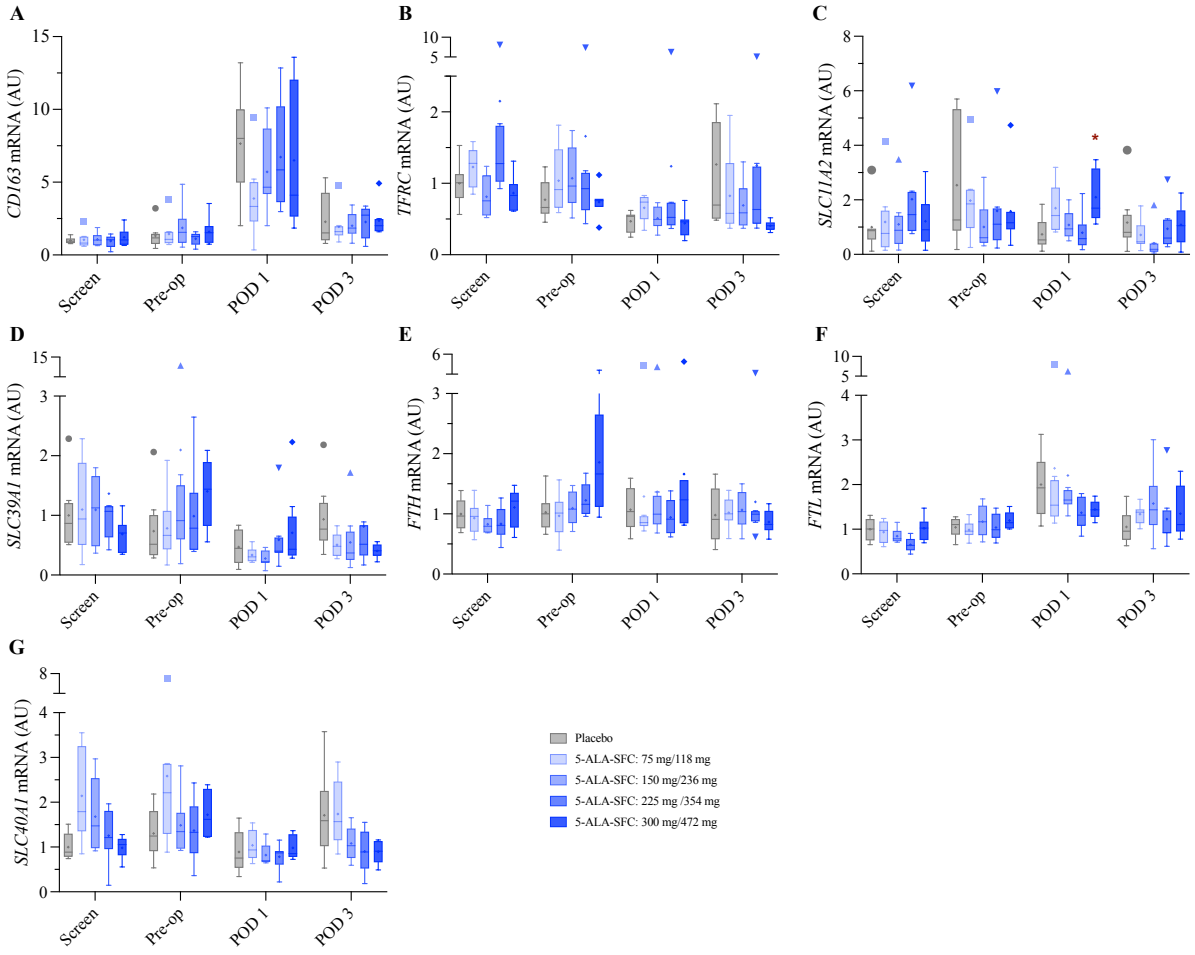



**Figure 64: Relative expression of key iron handling genes in human PBMCs.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Abbreviations: AU – arbitrary units.

There was a clear temporal relationship between surgery and an increased relative expression of PBMC *CD163* and within a LMM using REML time was identified as the most significant fixed effect factor (p-value = < 0.0001) (**Fig. 64 A**). No 5-ALA-SFC dose-dependent changes to differential expression of *CD163* were found. *TFRC* expression (coding for the membrane bound transferrin receptor) was reduced

across all treatment allocation groups on the first post-operative day, with a slightly higher decrement observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group (observed mean [SE] vs. placebo = -0.03 [0.08] AU, p-value = 0.9870) (**Fig.64 B**). By the third post-operative day, the highest dose treatment allocation group diverges further (mean difference [SE)] vs. placebo = -0.80 [0.44] AU, p-value = 0.2390).

A further mechanism for cellular iron uptake is via the divalent metal transporter 1 (DMT1), coded for by *SLC11A2*. A potential drug-related increase in differential expression of *SLC11A2* was seen on the first post-operative day in the 5-ALA-SFC 300 mg/472 mg treatment allocation group (mean difference [SE] vs. placebo = 1.35 [0.39] AU, p-value = 0.0153) (**Fig. 64 C**). At the pre-operative timepoint a non-significant increase in the relative expression of *SLC39A1* (coding for ZRT/IRT-like protein 1 [ZIP1]) in response to 5-ALA-SFC treatment was observed, and this was most marked in the highest dose treatment allocation (mean difference [SE] vs. placebo = 0.67 [0.31]AU, p-value = 0.1460) (**Fig. 64 D**). Relative expression of this gene was noticeably reduced during the post-operative period across all treatment allocation groups.

At the pre-operative time point there was a suggestion of 5-ALA-SFC dose-dependent increase in the relative expression of *FTH* (coding for ferritin heavy chain) (**Fig. 64 E**) although this finding was not statistically significant. No significant post-operative treatment-related differences were identified. The effect on relative expression of *FTL* (coding for ferritin light chain) was very subtle and lacked the suggestion of dose dependency displayed by *FTH* expression (**Fig. 64 F**). For example, relative expression in the 300 mg/472 mg treatment allocation group appeared only marginally different to placebo at the pre-operative timepoint (mean difference [SE] vs. placebo = 0.15 [0.10] AU, p-value = 0.4258).

Finally, the relative expression of *SLC40A1* (encoding ferroportin) was considered. Baseline relative expression varied widely between groups (**Fig. 64 G**). There was no suggestion of a dose-dependent difference vs. placebo. Conversely, on the third post-operative day, relative expression of *SLC40A1*

tended to lower values in patients treated with the active drug. Here there was an apparent dose-dependent relationship between gene expression and 5-ALA-SFC with the most marked decrease vs. placebo observed in the highest dose allocation group (mean [SE] vs. placebo = -0.82 [0.35] AU, p-value = 0.1378). Although no statistically significant treatment-related differences were identified at each timepoint, across the LMM using REML statistical model, data did suggest a treatment-related effect (p-value = 0.0196).

**Table 31: Descriptive and inferential statistics of relative expression of iron handling genes.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| ***CD163* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.0 (0.21) | 1.05 (0.58) | 1.08 (0.46) | 0.94 (0.42) | 1.22 (0.61) |
| Median (IQR) | 0.95 (0.82; 1.14) | 0.82 (0.64; 1.26) | 1.02 (0.67; 1.37) | 1.03 (0.54; 1.26) | 1.04 (0.70; 1.61) |
| Min Max | 0.78 1.40 | 0.59 2.32 | 0.64 1.89 | 0.21 1.43 | 0.65 2.41 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.32 (0.84) | 1.44 (1.02) | 1.87 (1.39) | 1.10 (0.43) | 1.60 (0.92) |
| Median (IQR) | 1.18 (0.76; 1.46) | 1.07 (0.80; 1.59) | 1.56 (0.84; 2.50) | 1.26 (0.70; 1.44) | 1.53 (0.83; 2.00) |
| Min Max | 0.45 3.20 | 0.72 3.83 | 0.54 4.85 | 0.38 1.58 | 0.74 3.54 |
| **POD 1** | | | | | |
| Mean (SD) | 7.66 (3.57) | 3.88 (2.68) | 5.72 (2.78) | 6.72 (3.69) | 6.50 (4.81) |
| Median (IQR) | 8.00 (4.97; 10.00) | 3.33 (2.29; 5.01) | 4.66 (4.18; 8.69) | 5.84 (3.61; 10.21) | 4.11 (2.64; 12.05) |
| Min Max | 2.01 13.21 | 0.35 9.45 | 2.01 10.11 | 2.97 12.84 | 1.85 13.60 |
| **POD 3** | | | | | |
| Mean (SD) | 2.28 (1.83) | 1.94 (1.21) | 2.01 (0.88) | 2.26 (1.08) | 2.35 (1.10) |
| Median (IQR) | 1.52 (1.01; 4.28) | 1.61 (1.34; 1.97) | 1.85 (1.46; 2.82) | 2.73 (1.13; 3.72) | 2.02 (1.64; 2.48) |
| Min Max | 0.79 5.30 | 0.89 4.80 | 0.81 3.44 | 0.58 3.34 | 1.59 4.92 |
| ***TFRC* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.28) | 1.23 (0.28) | 0.81 (0.28) | 2.15 (2.42) | 0.86 (0.25) |
| Median (IQR) | 1.01 (0.79; 1.13) | 1.28 (0.94; 1.47) | 0.76 (0.55; 1.11) | 1.28 (1.02; 1.81) | 0.63 (0.62; 0.99) |
| Min Max | 0.56 1.53 | 0.85 1.59 | 0.52 1.24 | 0.93 8.08 | 0.61 1.31 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.77 (0.27) | 1.04 (0.45) | 1.07 (0.43) | 1.66 (2.32) | 0.74 (0.22) |
| Median (IQR) | 0.66 (0.58; 1.02) | 0.91 (0.66; 1.48) | 0.96 (0.73; 1.50) | 0.93 (0.62; 1.15) | 0.76 (0.67; 0.78) |
| Min Max | 0.46 1.23 | 0.59 1.81 | 0.52 1.74 | 0.44 7.36 | 0.38 1.12 |
| **POD 1** | | | | | |
| Mean (SD) | 0.47 (0.14) | 0.66 (0.18) | 0.51 (0.16) | 1.24 (2.02) | 0.44 (0.18) |
| Median (IQR) | 0.54 (0.31; 0.56) | 0.74 (0.50; 0.81) | 0.49 (0.40; 0.68) | 0.52 (0.41; 0.73) | 0.46 (0.27; 0.49) |
| Min Max | 0.25 0.62 | 0.34 0.83 | 0.28 0.73 | 0.37 6.22 | 0.20 0.76 |
| **POD 3** | | | | | |
| Mean (SD) | 1.27 (1.23) | 0.82 (0.57) | 0.69 (0.32) | 1.25 (1.59) | 0.41 (0.07) |
| Median (IQR) | 0.70 (0.50; 1.86) | 0.58 (0.43; 1.28) | 0.59 (0.41; 0.93) | 0.63 (0.44; 1.24) | 0.41 (0.35; 0.45) |
| Min Max | 0.48 4.00 | 0.37 1.95 | 0.37 1.30 | 0.37 5.09 | 0.31 0.52 |
| ***SLC11A2* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.89) | 1.19 (0.31) | 1.10 (1.11) | 2.02 (1.80) | 1.21 (0.97) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Median (IQR) | 0.87 (0.55; 0.95) | 0.77 (0.26; 1.61) | 0.88 (0.39; 1.42) | 1.46 (0.86; 2.29) | 0.90 (0.47; 1.85) |
| Min Max | 0.12 3.09 | 0.15 4.16 | 0.16 3.49 | 0.76 6.18 | 0.15 3.04 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.54 (2.32) | 1.97 (1.40) | 1.01 (0.92) | 1.60 (1.85) | 1.58 (1.44) |
| Median (IQR) | 1.26 (0.87; 5.33) | 1.85 (0.97; 2.37) | 0.61 (0.42; 1.66) | 1.11 (0.52; 1.70) | 1.16 (0.91; 1.56) |
| Min Max | 0.18 5.70 | 0.25 4.95 | 0.32 2.83 | 0.23 5.98 | 0.34 4.74 |
| **POD 1** | | | | | |
| Mean (SD) | 0.74 (0.57) | 1.70 (0.91) | 1.08 (0.52) | 0.80 (0.66) | 2.09 (0.94) |
| Median (IQR) | 0.53 (0.37; 1.19) | 1.43 (0.90; 2.37) | 0.94 (0.66; 1.52) | 0.58 (0.34; 1.10) | 1.70 (1.32; 3.15) |
| Min Max | 0.12 1.84 | 0.83 3.20 | 0.50 2.00 | 0.18 2.23 | 1.12 3.47 |
| **POD 3** | | | | | |
| Mean (SD) | 1.17 (1.15) | 0.72 (0.54) | 0.41 (0.58) | 0.94 (0.82) | 1.09 (0.71) |
| Median (IQR) | 0.91 (0.62; 1.45) | 0.47 (0.38; 1.08) | 0.19 (0.10; 0.40) | 0.60 (0.38; 1.28) | 1.06 (0.45; 1.62) |
| Min Max | 0.11 3.82 | 0.14 1.78 | 0.05 1.82 | 0.28 2.74 | 0.09 2.25 |
| ***SLC39A1* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.58) | 1.10 (0.76) | 1.10 (0.58) | 1.37 (0.38) | 0.69 (0.29) |
| Median (IQR) | 0.87 (0.54; 1.21) | 0.94 (0.50; 1.88) | 1.13 (0.48; 1.66) | 1.07 (0.63; 1.15) | 0.71 (0.37; 0.84) |
| Min Max | 0.51 2.29 | 0.17 2.29 | 0.37 1.80 | 0.42 4.71 | 0.35 1.16 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.73 (0.60) | 0.78 (0.55) | 2.10 (3.52) | 0.99 (0.77) | 1.40 (0.59) |
| Median (IQR) | 0.52 (0.33; 1.00) | 0.67 (0.43; 1.08) | 0.91 (0.60; 1.51) | 0.78 (0.42; 1.38) | 1.44 (0.82; 1.89) |
| Min Max | 0.29 2.06 | 0.17 1.92 | 0.19 10.74 | 0.40 2.65 | 0.56 2.09 |
| **POD 1** | | | | | |
| Mean (SD) | 0.47 (0.29) | 0.34 (0.12) | 0.28 (0.14) | 0.59 (0.51) | 0.71 (0.67) |
| Median (IQR) | 0.45 (0.20; 0.76) | 0.31 (0.23; 0.43) | 0.23 (0.20; 0.41) | 0.40 (0.37; 0.63) | 0.43 (0.33; 0.98) |
| Min Max | 0.09 0.83 | 0.21 0.56 | 0.07 0.46 | 0.15 1.80 | 0.28 2.23 |
| **POD 3** | | | | | |
| Mean (SD) | 0.93 (0.58) | 0.51 (0.20) | 0.55 (0.52) | 0.81 (0.95) | 0.40 (0.11) |
| Median (IQR) | 0.77 (0.58; 1.2) | 0.48 (0.32; 0.68) | 0.37 (0.25; 0.73) | 0.52 (0.33; 0.83) | 0.41 (0.32; 0.51) |
| Min Max | 0.35; 2.20 | 0.27 0.82 | 0.12 1.72 | 0.17 3.10 | 0.22 0.56 |
| ***FTH* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.25) | 0.93 (0.26) | 0.83 (0.16) | 0.83 (0.27) | 1.11 (0.32) |
| Median (IQR) | 0.96 (0.76; 1.23) | 0.96 (0.70; 1.10) | 0.79 (0.69; 0.93) | 0.81 (0.65; 1.08) | 1.21 (0.77; 1.35) |
| Min Max | 0.67 1.39 | 0.57 1.39 | 0.68 1.14 | 0.44 1.26 | 0.41 1.47 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.03 (0.30) | 0.97 (0.36) | 1.10 (0.28) | 1.22 (0.27) | 1.86 (0.94) |
| Median (IQR) | 1.00 (0.78; 1.18) | 1.01 (0.69; 1.20) | 1.08 (0.85; 1.37) | 1.16 (1.00; 1.49) | 1.66 (1.12; 2.65) |
| Min Max | 0.66 1.63 | 0.40 1.57 | 0.68 4.12 | 0.62 1.38 | 0.94 3.59 |
| **POD 1** | | | | | |
| Mean (SD) | 1.07 (0.36) | 1.29 (1.25) | 1.37 (1.13) | 0.94 (0.29) | 1.66 (1.45) |
| Median (IQR) | 1.04 (0.78; 0.43) | 0.85 (0.80; 0.97) | 0.99 (0.83; 1.30) | 0.91 (0.68; 1.23) | 1.24 (0.84; 1.56) |
| Min Max | 0.59 1.59 | 0.72 4.37 | 0.68 4.12 | 0.62 1.38 | 0.81 4.88 |
| **POD 3** | | | | | |
| Mean (SD) | 0.98 (0.45) | 1.02 (0.25) | 1.07 (0.32) | 1.20 (0.80) | 0.87 (0.19) |
| Median (IQR) | 0.91 (0.58; 1.42) | 1.00 (0.89; 1.24) | 1.04 (0.82; 1.36) | 1.00 (0.87; 1.05) | 0.83 (0.73; 1.05) |
| Min Max | 0.41 1.66 | 0.59 1.39 | 0.59 1.50 | 0.62 3.15 | 0.58 1.17 |
| ***FTL* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.24) | 0.94 (0.24) | 0.84 (0.16) | 0.65 (0.15) | 1.02 (0.26) |
| Median (IQR) | 1.01 (0.75; 1.23) | 0.97 (0.69; 1.18) | 0.79 (0.73; 0.96) | 0.64 (0.53; 0.78) | 1.03 (0.76; 1.17) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Min Max | 0.65 1.31 | 0.61 1.24 | 0.71 1.16 | 0.44 0.91 | 0.70 1.47 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.05 (0.22) | 0.99 (0.19) | 1.17 (0.35) | 1.04 (0.29) | 1.20 (0.19) |
| Median (IQR) | 1.11 (0.88; 1.23) | 0.96 (0.87; 1.13) | 1.17 (0.86; 1.68) | 0.99 (0.81; 1.35) | 1.15 (1.02; 1.38) |
| Min Max | 0.66 1.28 | 0.69 1.33 | 0.72 1.68 | 0.69 1.48 | 1.01 1.51 |
| **POD 1** | | | | | |
| Mean (SD) | 2.00 (0.70) | 2.37 (2.32) | 2.21 (1.64) | 1.37 (0.35) | 1.44 (0.19) |
| Median (IQR) | 1.93 (1.34; 2.51) | 1.54 (1.28; 2.10) | 1.66 (1.55; 1.88) | 1.32 (1.08; 1.73) | 1.44 (1.28; 1.62) |
| Min Max | 1.07 3.12 | 1.14 8.05 | 1.30 6.23 | 0.85 1.80 | 1.15 1.74 |
| **POD 3** | | | | | |
| Mean (SD) | 1.05 (0.37) | 1.34 (0.21) | 1.58 (0.73) | 1.23 (0.67) | 1.35 (0.58) |
| Median (IQR) | 0.96 (0.76; 1.32) | 1.38 (1.13; 1.45) | 1.44 (1.10; 1.98) | 0.93 (0.91; 1.43) | 1.10 (0.95; 1.98) |
| Min Max | 0.63 1.74 | 1.01 1.67 | 0.67 3.01 | 0.62 2.77 | 0.78 2.30 |
| ***SLC40A1* mRNA (AU)** | | | | | |
| **Screen** | | | | | |
| Mean (SD) | 1.00 (0.30) | 2.14 (1.03) | 1.68 (0.81) | 1.25 (0.60) | 0.98 (0.25) |
| Median (IQR) | 0.88 (0.78; 1.30) | 1.79 (1.35; 3.25) | 1.47 (0.97; 2.54) | 1.22 (0.95; 1.81) | 1.06 (0.82; 1.18) |
| Min Max | 0.74 1.51 | 0.85 3.55 | 0.92 2.97 | 0.15 1.97 | 0.56 1.28 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.30 (0.54) | 2.59 (1.90) | 1.49 (0.67) | 1.37 (0.67) | 1.72 (0.49) |
| Median (IQR) | 1.24 (0.91; 1.80) | 2.21 (1.29; 2.86) | 1.34 (0.96; 1.77) | 1.33 (0.86; 1.91) | 1.62 (1.22; 2.30) |
| Min Max | 0.54 2.18 | 0.89 6.93 | 0.92 2.81 | 0.36 2.43 | 1.22 2.39 |
| **POD 1** | | | | | |
| Mean (SD) | 0.89 (0.46) | 1.04 (0.34) | 0.82 (0.25) | 0.78 (0.28) | 0.98 (0.26) |
| Median (IQR) | 0.76 (0.54; 1.33) | 0.94 (0.75; 1.38) | 0.70 (0.66; 1.04) | 0.87 (0.61; 0.91) | 0.85 (0.78; 1.29) |
| Min Max | 0.34 1.65 | 0.63 1.54 | 0.65 1.29 | 0.22 1.15 | 0.72 1.37 |
| **POD 3** | | | | | |
| Mean (SD) | 1.71 (0.96) | 1.74 (0.74) | 1.08 (0.37) | 0.90 (0.47) | 0.89 (0.25) |
| Median (IQR) | 1.59 (1.02; 2.26) | 1.57 (1.15; 2.46) | 1.03 (0.75; 1.41) | 0.89 (0.52; 1.35) | 0.91 (0.66; 1.14) |
| Min Max | 0.53 3.58 | 0.84 2.90 | 0.59 1.66 | 0.18 1.55 | 0.49 1.16 |
| **Change in relative expression of key iron handling genes, active vs placebo (LMM, REML)** | | | | | |
| ***CD163* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.32 | 1.44 | 1.87 | 1.10 | 1.60 |
| Difference vs placebo (SE) | | 0.12 (0.47) | 0.55 (0.57) | -0.22 (0.33) | 0.28 (0.44) |
| p-value | | 0.9966 | 0.7437 | 0.9122 | 0.9150 |
| **POD 1** | | | | | |
| Observed mean | 7.66 | 3.88 | 5.72 | 6.72 | 6.50 |
| Difference vs placebo (SE) | | -3.77 (1.58) | -1.94 (1.60) | -0.94 (1.82) | -1.16 (2.12) |
| p-value | | 0.0999 | 0.7437 | 0.9122 | 0.9150 |
| **POD 3** | | | | | |
| Observed mean | 2.28 | 1.94 | 2.01 | 2.26 | 2.35 |
| Difference vs placebo (SE) | | -0.36 (0.77) | -0.27 (0.72) | -0.02 (2.12) | 0.07 (0.75) |
| p-value | | 0.9759 | 0.9859 | > 0.9999 | > 0.9999 |
| ***TRFC* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.77 | 1.04 | 1.07 | 1.66 | 0.74 |
| Difference vs placebo (SE) | | 0.27 (0.19) | 0.30 (0.18) | 0.89 (0.82) | -0.03 (0.13) |
| p-value | | 0.4538 | 0.3254 | 0.6758 | 0.9965 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Observed mean | 0.47 | 0.66 | 0.51 | 1.24 | 0.44 |
| Difference vs placebo (SE) | | 0.19 (0.08) | 0.04 (0.07) | 0.77 (0.72) | -0.03 (0.08) |
| p-value | | 0.1117 | 0.9485 | 0.6755 | 0.9870 |
| **POD 3** | | | | | |
| Observed mean | 1.27 | 0.82 | 0.69 | 1.25 | 0.47 |
| Difference vs placebo (SE) | | -0.45 (0.48) | -0.57 (0.45) | -0.02 (0.71) | -0.80 (0.44) |
| p-value | | 0.7697 | 0.5487 | > 0.9999 | 0.2390 |
| ***SLC11A2* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 2.54 | 1.97 | 1.02 | 1.60 | 1.60 |
| Difference vs placebo (SE) | | -0.57 (0.96) | -1.52 (0.89) | -0.94 (1.05) | -0.94 (0.98) |
| p-value | | 0.9374 | 0.3136 | 0.7865 | 0.7412 |
| **POD 1** | | | | | |
| Observed mean | 0.74 | 1.70 | 1.08 | 0.80 | 2.09 |
| Difference vs placebo (SE) | | 0.96 (0.38) | 0.34 (0.27) | 0.06 (0.31) | 1.35 (0.39) |
| p-value | | 0.0818 | 0.5400 | 0.9990 | **0.0153 *** |
| **POD 3** | | | | | |
| Observed mean | 1.17 | 0.72 | 0.41 | 0.94 | 1.09 |
| Difference vs placebo (SE) | | -0.45 (0.45) | -0.76 (0.46) | -0.23 (0.50) | -0.08 (0.48) |
| p-value | | 0.7106 | 0.3265 | 0.9698 | 0.9993 |
| ***SLC39A1* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.74 | 0.78 | 2.10 | 0.99 | 1.41 |
| Difference vs placebo (SE) | | 0.04 (0.28) | 1.36 (1.26) | 0.25 (0.35) | 0.67 (0.31) |
| p-value | | 0.9994 | 0.6755 | 0.8777 | 0.1460 |
| **POD 1** | | | | | |
| Observed mean | 0.47 | 0.34 | 0.28 | 0.59 | 0.71 |
| Difference vs placebo (SE) | | -0.13 (0.11) | -0.19 (0.11) | 0.12 (0.21) | 0.24 (0.26) |
| p-value | | 0.5880 | 0.3191 | 0.9359 | 0.7649 |
| **POD 3** | | | | | |
| Observed mean | 0.93 | 0.51 | 0.55 | 0.81 | 0.40 |
| Difference vs placebo (SE) | | -0.42 (0.22) | -0.38 (0.28) | -0.12 (0.39) | -0.53 (0.21) |
| p-value | | 0.2205 | 0.4549 | 0.9932 | 0.1048 |
| ***FTH* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.03 | 0.97 | 1.10 | 1.22 | 1.11 |
| Difference vs placebo (SE) | | -0.06 (0.17) | 0.07 (0.15) | 0.19 (0.14) | 0.08 (0.34) |
| p-value | | 0.9894 | 0.9709 | 0.4767 | 0.1235 |
| **POD 1** | | | | | |
| Observed mean | 1.07 | 1.29 | 1.34 | 0.94 | 1.65 |
| Difference vs placebo (SE) | | 0.22 (0.46) | 0.27 (0.42) | -0.13 (0.16) | 0.59 (0.56) |
| p-value | | 0.9663 | 0.8836 | 0.8534 | 0.6926 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 3** | | | | | |
| Observed mean | 0.98 | 1.02 | 1.07 | 1.20 | 0.87 |
| Difference vs placebo (SE) | | 0.04 (0.19) | 0.09 (0.20) | 0.22 (0.32) | -0.11 (0.17) |
| p-value | | 0.9977 | 0.9742 | 0.8955 | 0.9077 |
| ***FTL* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.05 | 0.99 | 1.17 | 1.04 | 1.20 |
| Difference vs placebo (SE) | | -0.06 (0.10) | 0.12 (0.15) | -0.01 (0.13) | 0.15 (0.10 |
| p-value | | 0.9404 | 0.7976 | > 0.9999 | 0.4258 |
| **POD 1** | | | | | |
| Observed mean | 2.00 | 2.37 | 2.21 | 1.37 | 1.44 |
| Difference vs placebo (SE) | | 0.37 (0.86) | 0.21 (0.63) | -0.63 (0.28) | -0.56 (0.26) |
| p-value | | 0.9773 | 0.9916 | 0.1323 | 0.1725 |
| **POD 3** | | | | | |
| Observed mean | 1.05 | 1.34 | 1.58 | 1.23 | 1.35 |
| Difference vs placebo (SE) | | 0.29 (0.15) | 0.53 (0.29) | 0.18 (0.27) | 0.30 (0.24) |
| p-value | | 0.2257 | 0.2710 | 0.9114 | 0.5686 |
| ***SLC40A1* mRNA (AU)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.30 | 2.59 | 1.47 | 1.37 | 1.71 |
| Difference vs placebo (SE) | | 1.29 (0.70) | 0.17 (0.32) | 0.07 (0.31) | 0.41 (0.27) |
| p-value | | 0.2768 | 0.9419 | 0.9984 | 0.3739 |
| **POD 1** | | | | | |
| Observed mean | 0.89 | 1.04 | 0.82 | 0.78 | 0.98 |
| Difference vs placebo (SE) | | 0.15 (0.20) | -0.07 (0.18) | -0.11 (0.19) | 0.09 (0.19) |
| p-value | | 0.8706 | 0.9852 | 0.9453 | 0.9627 |
| **POD 3** | | | | | |
| Observed mean | 1.71 | 1.74 | 1.08 | 0.90 | 0.89 |
| Difference vs placebo (SE) | | 0.03 (0.43) | -0.63 (0.37) | -0.81 (0.37) | -0.82 (0.35) |
| p-value | | > 0.9999 | 0.3081 | 0.1656 | 0.1378 |

Abbreviations: AU – arbitrary units; SD – standard deviation; IQR – interquartile range; SE – standard error; CI – confidence interval.

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 4.2.6 The impact of 5-ALA-SFC on peri-operative markers of renal injury

Considering serum urea, creatinine and estimated glomerular filtration rate (eGFR), reflecting routine markers of renal function used clinically, a strikingly congruent signal for improved or better preserved post-operative renal function was observed across all treatment allocation groups compared with placebo (**Fig. 65 A-F**). A possible 5-ALA-SFC dose-dependent relationship with improved renal function on the second and third post-operative days is seen, and this relationship becomes clearer when evaluating only the PAS, i.e. excluding patients who had experienced significant on-table surgical complications unrelated to the drug (**Fig. 65 G**). Across a LMM with time as a categorical variable, highly statistically significant reductions in urea (mean difference [95 % CI] vs. placebo = -1.4 [-2.3; 0.6] mmol/L, p-value = 0.0013) and creatinine (mean difference [95 % CI] vs. placebo = -12 [-221; -3] μmol/L, p-value = 0.0116) as well as improvements in eGFR (mean difference [95 % CI] vs. placebo = 8 [3; 13] mL/min/1.73 $m^2$, p-value = 0.0035) were observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group overall, showing a treatment-related fixed effect at this dose. Statistically significant differences vs. placebo were observed particularly apparent from the second post-operative day onwards.

The hospital NHS biochemistry laboratory had applied the 2009 Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI [2009]) formula to creatinine values when reporting eGFR which had an upper limit for detection of 90 mL/min/1.73 $m^2$ (i.e., where patients had an eGFR > 90 mL/min/1.73 $m^2$, this would be reported as 90 mL/min/1.73 $m^2$). Using creatinine data from the NHS laboratory, eGFR was also calculated using the latest 2021 equation (CKD-EPI [2021]) and data re-analysed (**Fig. 66 A**). Across the LMM, a significant treatment-related fixed effect was again observed in the highest 5-ALA-SFC dose allocation group (mean [95% CI] vs. placebo = 9 [3; 15] mL/min/1.73 $m^2$, p-value = 0.0025). There was a treatment-related dose-dependent increase in eGFR (i.e. improvement in renal function) in the 5-ALA-SFC-treated groups, with the most profound difference vs. placebo observed in the 300 mg/472 mg treatment allocation group on the second post-operative day (mean difference [95 % CI] vs. placebo = 18 [5; 30] mL/min/1.73 $m^2$, p-value = 0.0061) consistent with a renoprotective effect most marked at the highest dose evaluated.

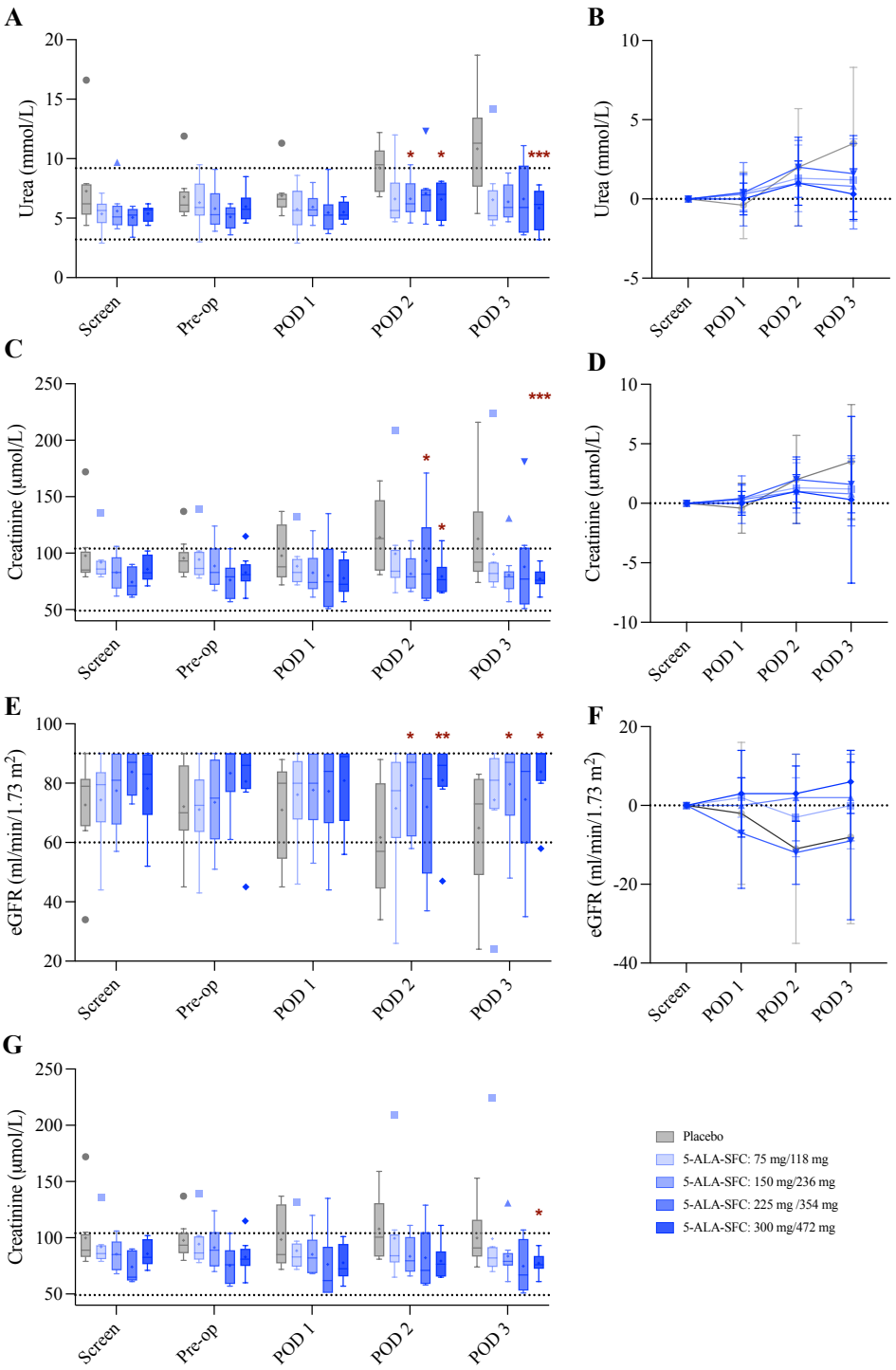



**Figure 65: Impact of 5-ALA-SFC on routine renal function indices in patients undergoing cardiac surgery.**

Summary data presented in panel A, C, E, G presented as median, IQR, Tukey's upper and lower adjacent values with outside values; change from baseline data presented in panel B, D, F presented as mean, SE. Panel E – CKD-EPI (2009); Panel G – PAS data only.

Significance from LMM: in red. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Abbreviations: eGFR – estimated glomerular filtration rate, PAS – primary analysis set.

To further explore for a renoprotective role of HO-1 induction through 5-ALA-SFC, novel biomarkers of renal injury were also assayed. There was a dose-dependent decrease in post-operative serum Cystatin C levels with 5-ALA-SFC treatment, consistent with reduced renal injury in patients treated with the study drug (**Fig. 66 B**), which was most significant in the 300 mg/472 treatment allocation group on the third post-operative day (mean [95% CI] vs. placebo = -0.3 [-0.5; -0.1], p-value = 0.0054). Across the model there was a significant treatment-related fixed effect at this dose (p-value = 0.0100). After recalculation of eGFR by combining serum creatinine with Cystatin C data with the 2021 CKD-EPI (Creatinine-Cystatin C) equation, the LMM demonstrated a significant treatment-related fixed effect evident at the highest dose (mean difference [95% CI] vs. placebo = 9 [2; 16] mL/min/1.73 $m^2$, p-value = 0.0095 (**Fig. 66 C**).

The final biomarker used to assess renal function was the early marker of injury, urinary NGAL. There was a treatment-related reduction in NGAL that was the most significant on the first post-operative day (**Fig. 66 D**) which a clearer signal for dose-response when urinary NGAL was normalised to urinary creatinine, showing statistically significant differences vs. placebo in patients allocated to each of the highest three doses of 5-ALA-SFC (**Fig. 66 E**). This was most marked in the 300 mg/472 mg group (mean [95% CI] vs. placebo = -3.30 [-5.89; -0.11] mg/mmol, p-value = 0.0261).

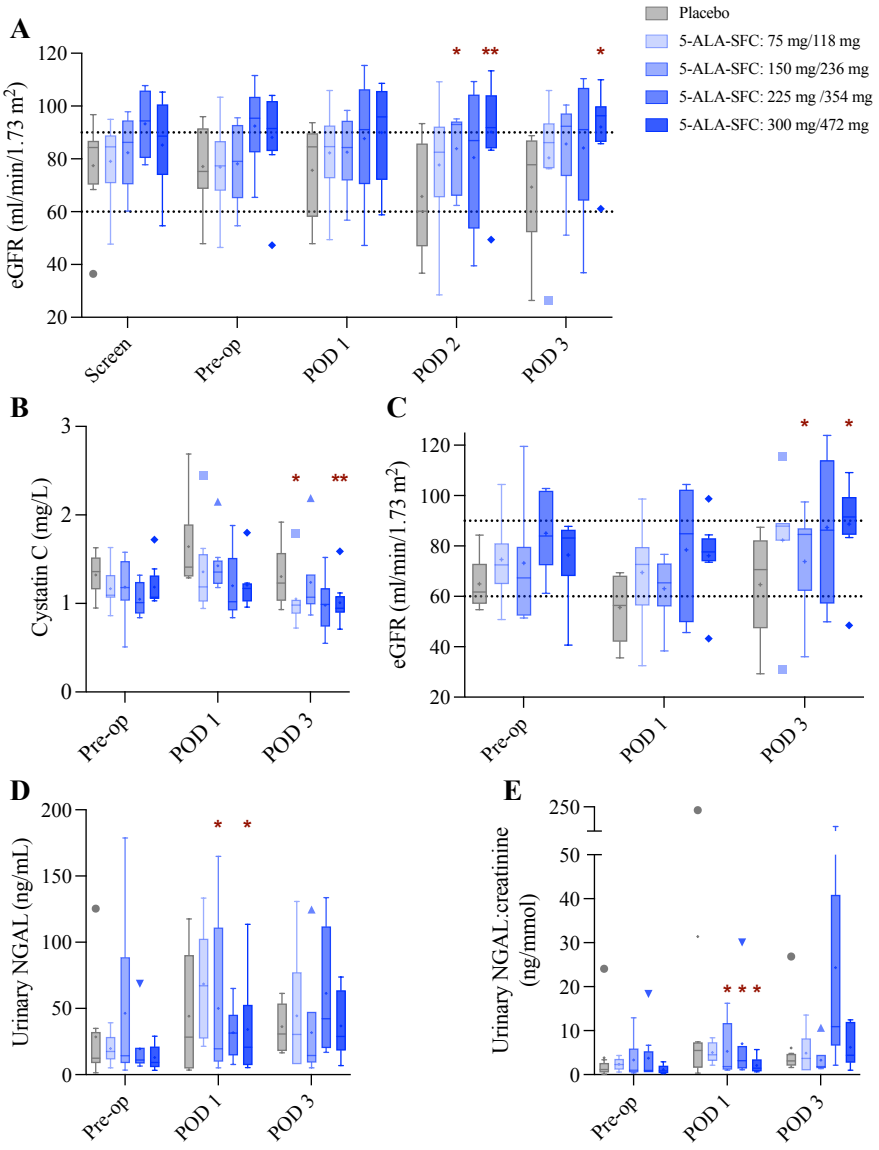



**Figure 66: Impact of 5-ALA-SFC on markers of kidney injury in patients undergoing cardiac surgery.**

Summary data presented in panel A, C, E, G (presented as median, IQR, Tukey's upper and lower adjacent values with outside values), change from baseline data presented in panel B, D, F. (presented as mean, SE). Panel E – CKD-EPI (2009). Panel G – PAS data only.

Significance from LMM in red: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001.

Abbreviations: eGFR – estimated glomerular filtration rate, PAS – primary analysis set.

**Table 32: Descriptive and inferential statistics of biomarkers of renal function and injury.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Serum urea (mmol/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 7.3 (3.7) | 5.4 (1.3) | 5.6 (1.7) | 5.1 (0.9) | 5.4 (0.7) |
| Median (IQR) | 6.2 (5.4; 7.8) | 5.7 (4.7; 6.2) | 5.1 (4.5; 5.9) | 5.3 (4.6; 5.7) | 5.7 (4.7; 5.9) |
| Min Max | 4.4 16.6 | 2.9 7.1 | 4.1 9.7 | 3.4 6.0 | 4.4 6.2 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.8 (2.1) | 6.3 (2.0) | 5.8 (1.7) | 5.1 (1.0) | 6.0 (1.3) |
| Median (IQR) | 6.1 (5.8; 7.0) | 5.9 (5.3; 7.8) | 5.3 (4.8; 6.5) | 5.4 (4.5; 5.8) | 5.8 (5.0; 6.6) |
| Min Max | 5.2 11.9 | 3.0 9.5 | 3.9 9.1 | 3.6 6.2 | 4.6 8.5 |
| **POD 1** | | | | | |
| Mean (SD) | 6.9 (1.8) | 5.8 (1.8) | 5.9 (1.1) | 5.5 (1.7) | 5.5 (0.8) |
| Median (IQR) | 6.6 (6.0; 7.0) | 5.7 (4.5; 7.1) | 5.7 (5.3; 6.1) | 5.3 (4.3; 6.0) | 5.3 (5.0; 6.2) |
| Min Max | 5.2 11.3 | 2.9 8.6 | 4.4 8.0 | 3.7 9.1 | 4.5 6.8 |
| **POD 2** | | | | | |
| Mean (SD) | 9.2 (1.9) | 6.6 (2.5) | 6.6 (1.6) | 7.1 (2.3) | 6.6 (1.6) |
| Median (IQR) | 9.5 (7.5; 10.6) | 5.7 (5.2; 7.3) | 6.2 (5.7; 7.4) | 7.0 (5.6; 7.4) | 7.0 (5.1; 8.0) |
| Min Max | 6.8 12.2 | 4.7 12.0 | 4.6 9.5 | 4.5 12.3 | 4.4 8.1 |
| **POD 3** | | | | | |
| Mean (SD) | 10.8 (4.1) | 6.6 (3.3) | 6.4 (1.5) | 6.6 (3.0) | 5.9 (1.7) |
| Median (IQR) | 11.3 (8.0; 12.7) | 5.2 (4.9; 6.8) | 5.9 (5.1; 7.2) | 5.9 (3.9; 9.4) | 6.2 (4.5; 7.3) |
| Min Max | 5.4 18.7 | 4.4 14.2 | 4.7 8.8 | 3.6 11.1 | 3.2 7.8 |
| **Serum creatinine (µmol/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 98 (29) | 92 (19) | 83 (15) | 74 (13) | 86 (11) |
| Median (IQR) | 85 (83; 98) | 86 (81; 93) | 83 (69; 96) | 71 (63; 88) | 83 (78; 97) |
| Min Max | 79 172 | 79 136 | 62 106 | 61 90 | 71 102 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 96 (18) | 94 (20) | 89 (19) | 76 (17) | 83 (16) |
| Median (IQR) | 93 (85; 94) | 87 (82; 101) | 83 (74; 103) | 79 (60; 86) | 81 (76; 88) |
| Min Max | 79 137 | 78 139 | 67 124 | 57 104 | 60 115 |
| **POD 1** | | | | | |
| Mean (SD) | 98 (25) | 88 (19) | 83 (19) | 80 (31) | 78 (16) |
| Median (IQR) | 88 (81; 117) | 83 (76; 93) | 74 (68; 91) | 75 (54; 100) | 73 (67; 93) |
| Min Max | 72 137 | 72 132 | 61 120 | 51 135 | 57 101 |
| **POD 2** | | | | | |
| Mean (SD) | 114 (33) | 100 (46) | 82 (16) | 93 (40) | 79 (16) |
| Median (IQR) | 113 (87; 135) | 84 (78; 99) | 79 (69; 85) | 82 (60; 117) | 77 (66; 87) |
| Min Max | 81 164 | 65 209 | 66 111 | 58 171 | 65 111 |
| **POD 3** | | | | | |
| Mean (SD) | 113 (46) | 99 (51) | 81 (21) | 88 (43) | 77 (10) |
| Median (IQR) | 92 (84; 121) | 82 (76; 92) | 79 (75; 79) | 77 (56; 103) | 76 (73; 84) |
| Min Max | 74 216 | 70 224 | 57 131 | 51 181 | 61 93 |
| **Serum cystatin (mg/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.3 (0.2) | 1.2 (0.2) | 1.2 (0.4) | 1.0 (0.2) | 1.2 (0.2) |
| Median (IQR) | 1.4 (1.2; 1.5) | 1.1 (1.1; 1.3) | 1.2 (1.0; 1.5) | 1.0 (0.9; 1.2) | 1.1 (1.1; 1.2) |
| Min Max | 1.0 1.6 | 0.9 1.6 | 0.5 1.6 | 0.8 1.3 | 1.0 1.7 |
| **POD 1** | | | | | |
| Mean (SD) | 1.3 (0.4) | 1.1 (0.4) | 1.2 (0.4) | 1.0 (0.3) | 1.0 (0.3) |
| Median (IQR) | 1.2 (1.0; 1.6) | 1.0 (0.9; 1.3) | 1.1 (1.0; 1.3) | 1.0 (0.8; 1.1) | 0.9 (0.9; 1.0) |
| Min Max | 0.9 1.9 | 0.7 1.8 | 0.9 2.2 | 0.6 1.5 | 0.7 1.6 |
| **POD 3** | | | | | |
| Mean (SD) | 1.6 (0.5) | 1.3 (0.5) | 1.4 (0.3) | 1.2 (0.4) | 1.2 (0.3) |
| Median (IQR) | 1.4 (1.3; 1.8) | 1.2 (1.0; 1.4) | 1.4 (1.2; 1.5) | 1.0 (0.9; 1.5) | 1.2 (1.0; 1.2) |
| Min Max | 1.3 2.7 | 0.9 2.4 | 1.2 2.2 | 0.8 1.9 | 1.0 1.8 |
| **Urinary NGAL (ng/mL)** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Pre-operatively** | | | | | |
| Mean (SD) | 25.5 (39.0) | 19.7 (11.6) | 51.0 (67.8) | 22.8 (23.6) | 12.1 (9.7) |
| Median (IQR) | 9.6 (8.6; 24.0) | 17.6 (13.6; 25.0) | 14.3 (9.8; 88.8) | 10.3 (8.4; 42.2) | 7.4 (5.6; 21.9) |
| Min Max | 0.6 125.4 | 5.1 39.2 | 3.4 178.8 | 6.6 68.6 | 3.3 29.0 |
| **POD 1** | | | | | |
| Mean (SD) | 352.7 (873.5) | 68.5 (43.0) | 56.0 (66.1) | 69.1 (112.0) | 32.4 (35.9) |
| Median (IQR) | 37.0 (12.0; 104.0) | 67.2 (29.1; 92.6) | 23.1 (13.8; 137.8) | 25.5 (11.1; 65.1) | 20.2 (11.0; 38.9) |
| Min Max | 3.4 2512.0 | 21.5 133.3 | 5.2 164.9 | 7.7 319.1 | 5.4 113.5 |
| **POD 3** | | | | | |
| Mean (SD) | 62.0 (74.3) | 41.5 (52.9) | 34.8 (50.6) | 245.1 (454.7) | 36.8 (25.2) |
| Median (IQR) | 38.3 (23.3; 57.6) | 11.7 (8.0; 49.0) | 12.1 (10.3; 21.8) | 63.9 (23.4; 133.7) | 29.0 (22.1; 60.4) |
| Min Max | 16.5 241.5 | 7.8 130.9 | 5.2 124.7 | 16.9 1168.7 | 6.8 73.8 |
| **Urinary NGAL:urinary creatine ratio (ng/mmol)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.23 (0.29) | 0.21 (0.14) | 0.55 (0.74) | 0.28 (0.23) | 0.14 (0.11) |
| Median (IQR) | 0.10 (0.09; 0.30) | 0.18 (0.12; 0.28) | 0.18 (0.11; 1.01) | 0.17 (0.12; 0.56) | 0.08 (0.06; 0.27) |
| Min Max | 0.01 0.92 | 0.06 0.46 | 0.05 1.80 | 0.08 0.66 | 0.04 0.31 |
| **POD 1** | | | | | |
| Mean (SD) | 3.72 (9.42) | 0.79 (0.51) | 0.60 (0.69) | 0.73 (1.00) | 0.39 (0.38) |
| Median (IQR) | 0.36 (0.15; 0.84) | 0.86 (0.26; 1.19) | 0.26 (0.18; 1.36) | 0.30 (0.18; 0.71) | 0.31 (0.16; 0.45) |
| Min Max | 0.04 27.01 | 0.22 1.37 | 0.08 1.81 | 0.15 2.95 | 0.06 1.26 |
| **POD 3** | | | | | |
| Mean (SD) | 0.47 (0.33) | 0.29 (0.25) | 0.41 (0.56) | 1.78 (2.37) | 0.48 (0.33) |
| Median (IQR) | 0.36 (0.21; 0.66) | 0.13 (0.11; 0.54) | 0.16 (0.14; 0.28) | 0.95 (0.40; 1.77) | 0.38 (0.31; 0.73) |
| Min Max | 0.16 1.12 | 0.10 0.58 | 0.09 1.40 | 0.16 6.46 | 0.07 1.00 |
| **eGFR* (mL/min/1.73 m$^2$)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 73 (16) | 74 (15) | 77 (13) | 84 (7) | 78 (14) |
| Median (IQR) | 79 (67; 80) | 80 (68; 84) | 81 (70; 90) | 87 (77; 90) | 83 (70; 90) |
| Min Max | 34 90 | 44 90 | 57 90 | 73 90 | 52 90 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 72 (14) | 71 (14) | 74 (15) | 83 (11) | 81 (15) |
| Median (IQR) | 70 (66; 82) | 73 (65; 81) | 75 (62; 87) | 90 (78; 90) | 86 (79; 90) |
| Min Max | 45 90 | 43 90 | 51 90 | 61 90 | 45 90 |
| **POD 1** | | | | | |
| Mean (SD) | 71 (16) | 76 (15) | 78 (13) | 77 (17) | 81 (14) |
| Median (IQR) | 80 (57; 84) | 80 (70; 87) | 80 (69; 90) | 84 (68; 90) | 89 (72; 90) |
| Min Max | 45 88 | 46 90 | 53 90 | 44 90 | 56 90 |
| **POD 2** | | | | | |
| Mean (SD) | 62 (19) | 72 (21) | 79 (14) | 72 (22) | 81 (15) |
| Median (IQR) | 57 (46; 77) | 78 (64; 87) | 87 (64; 90) | 82 (53; 90) | 86 (80; 90) |
| Min Max | 34 88 | 26 90 | 58 90 | 37 90 | 47 90 |
| **POD 3** | | | | | |
| Mean (SD) | 65 (21) | 74 (22) | 80 (15) | 75 (20) | 84 (11) |
| Median (IQR) | 73 (59; 81) | 81 (72; 88) | 87 (74; 90) | 84 (62; 90) | 90 (82; 90) |
| Min Max | 24 83 | 24 90 | 48 90 | 35 90 | 58 90 |
| **eGFR# (mL/min/1.73 m$^2$)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 77 (18) | 79 (15) | 82 (13) | 93 (13) | 85 (17) |
| Median (IQR) | 84 (72; 86) | 85 (72; 89) | 86 (75; 94) | 94 (81; 105) | 89 (74; 98) |
| Min Max | 36 97 | 48 95 | 60 98 | 78 108 | 55 105 |
| **Pre-operatively** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 77 (15) | 77 (17) | 78 (16) | 92 (15) | 88 (18) |
| Median (IQR) | 75 (71; 88) | 77 (69; 86) | 79 (66; 92) | 95 (84; 102) | 92 (84; 101) |
| Min Max | 48 96 | 46 103 | 55 96 | 65 112 | 47 104 |
| **POD 1** | | | | | |
| Mean (SD) | 76 (17) | 82 (17) | 83 (14) | 88 (23) | 90 (18) |
| Median (IQR) | 85 (61; 90) | 85 (75; 92) | 84 (73; 94) | 91 (72; 106) | 96 (76; 104) |
| Min Max | 48 94 | 49 106 | 57 98 | 47 115 | 59 109 |
| **POD 2** | | | | | |
| Mean (SD) | 66 (20) | 78 (24) | 84 (14) | 81 (27) | 90 (19) |
| Median (IQR) | 60 (49; 83) | 83 (68; 92) | 93 (68; 94) | 87 (57; 104) | 92 (85; 103) |
| Min Max | 37 93 | 28 109 | 62 95 | 40 109 | 49 113 |
| **POD 3** | | | | | |
| Mean (SD) | 69 (22) | 80 (24) | 86 (17) | 84 (26) | 92 (15) |
| Median (IQR) | 78 (63; 86) | 86 (77; 93) | 92 (79; 97) | 91 (66; 106) | 96 (87; 99) |
| Min Max | 26 89 | 26 106 | 51 100 | 37 110 | 61 110 |
| **eGFR$^{+}$ (mL/min/1.73 m$^2$)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 65 (10) | 75 (16) | 73 (23) | 85 (16) | 76 (16) |
| Median (IQR) | 62 (57; 66) | 72 (65; 81) | 67 (52; 80) | 84 (73; 101) | 83 (72; 86) |
| Min Max | 55 84 | 51 104 | 51 120 | 61 103 | 41 88 |
| **POD 1** | | | | | |
| Mean (SD) | 56 (13) | 69 (20) | 63 (12) | 78 (25) | 76 (15) |
| Median (IQR) | 56 (45; 68) | 73 (60; 79) | 65 (57; 73) | 85 (53; 101) | 78 (74; 82) |
| Min Max | 36 69 | 32 99 | 38 77 | 46 104 | 43 99 |
| **POD 3** | | | | | |
| Mean (SD) | 65 (20) | 82 (25) | 74 (21) | 87 (29) | 89 (18) |
| Median (IQR) | 71 (48; 78) | 88 (82; 89) | 85 (62; 87) | 86 (62; 114) | 92 (85; 99) |
| Min Max | 29 87 | 31 116 | 36 97 | 50 124 | 48 109 |
| **Change in biochemical indices of renal function, active vs placebo (LMM, time as a categorical variable)** | | | | | |
| **Serum urea (µmol/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 7.4 (0.3) | 6.3 (0.3) | 6.2 (0.3) | 6.2 (0.3) | 5.9 (0.3) |
| Difference vs placebo (95% CI) | | -1.0 (-1.9; -0.2) | -1.2 (-2.0; -0.4) | -1.1 (-2.0; -0.3) | -1.4 (-2.3; -0.6) |
| p-value | | **0.0128 *** | **0.0033 **** | **0.0091 **** | **0.0013 **** |
| **Screening** | | | | | |
| Observed mean (SE) | 6.4 (0.6) | 5.5 (0.6) | 5.7 (0.6) | 5.4 (0.6) | 5.6 (0.7) |
| Difference vs placebo (95% CI) | | -0.8 (-2.5; 0.8) | -0.7 (-2.3; 0.9) | -0.9 (-2.6; 0.8) | -0.8 (-2.5; 1.0) |
| p-value | | 0.3243 | 0.4139 | 0.2787 | 0.3794 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 5.9 (0.6) | 6.5 (0.6) | 5.9 (0.6) | 5.5 (0.6) | 6.0 (0.7) |
| Difference vs placebo (95% CI) | | 0.6 (-1.1; 2.3) | -0.0 (-1.7; 1.6) | -0.4 (-2.1; 1.3) | 0.1 (-1.6; 1.9) |
| p-value | | 0.4900 | 0.9731 | 0.6166 | 0.8952 |
| **POD 1** | | | | | |
| Observed mean (SE) | 6.1 (0.6) | 6.0 (0.6) | 6.0 (0.6) | 5.8 (0.6) | 5.6 (0.7) |
| Difference vs placebo (95% CI) | | -0.1 (-1.9; 1.6) | -0.1 (-1.7; 1.6) | -0.2 (-2.0; 1.5) | -0.5 (-2.3; 1.3) |
| p-value | | 0.8860 | 0.9416 | 0.7785 | 0.5669 |
| **POD 2** | | | | | |
| Observed mean (SE) | 8.4 (0.6) | 6.8 (0.6) | 6.7 (0.6) | 7.5 (0.6) | 6.6 (0.7) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | -1.6 (-3.4; 0.1) | -1.7 (-3.4; -0.0) | -1.0 (-2.7; 0.8) | -1.9 (-3.7; -0.1) |
| p-value | | 0.0672 | **0.0438 *** | 0.2683 | **0.0408 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 10.0 (0.6) | 6.8 (0.6) | 6.5 (0.6) | 7.0 (0.6) | 5.8 (0.7) |
| Difference vs placebo (95% CI) | | -3.3 (-5.0; -1.5) | -3.6 (-5.2; -1.9) | -3.0 (-4.8; -1.3) | -4.2 (-6.0; -2.4) |
| p-value | | **0.0003 *** ** | **<.0001 *** ** | **0.0008 *** ** | **<.0001 *** ** |
| **Serum creatinine (PAS) (µmol/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 93 (3) | 90 (3) | 87 (3) | 93 (3) | 81 (3) |
| Difference vs placebo (95% CI) | | -4 (-13; 5) | -7 (-16; 2) | 0 (-10; 10) | -12 (-21; -3) |
| p-value | | 0.4267 | 0.1382 | 0.9959 | **0.0116 *** |
| **Screening** | | | | | |
| Observed mean (SE) | 87 (7) | 87 (7) | 86 (7) | 85 (7) | 86 (7) |
| Difference vs placebo (95% CI) | | -1 (-20; 18) | -1 (-20; 17) | -2 (-22; 18) | -1 (-21; 19) |
| p-value | | 0.9509 | 0.8960 | 0.8402 | 0.9260 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 85 (7) | 90 (7) | 92 (7) | 87 (7) | 84 (7) |
| Difference vs placebo (95% CI) | | 4 (-15; 24) | 7 (-12; 26) | 2 (-18; 22) | -2 (-21; 18) |
| p-value | | 0.6750 | 0.4959 | 0.8636 | 0.8563 |
| **POD 1** | | | | | |
| Observed mean (SE) | 88 (7) | 84 (7) | 86 (7) | 91 (7) | 78 (7) |
| Difference vs placebo (95% CI) | | -4 (-24; 16) | -2 (-21; 18) | 4 (-17; 24) | -9 (-29; 11) |
| p-value | | 0.6888 | 0.8590 | 0.7247 | 0.3635 |
| **POD 2** | | | | | |
| Observed mean (SE) | 104 (7) | 95 (7) | 85 (7) | 104 (7) | 80 (7) |
| Difference vs placebo (95% CI) | | -9 (-29; 11) | -19 (-38; 1) | 0 (-20; 21) | -24 (-44; -4) |
| p-value | | 0.3615 | 0.0583 | 0.9769 | **0.0196 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 102 (7) | 94 (7) | 84 (7) | 99 (7) | 78 (7) |
| Difference vs placebo (95% CI) | | -8 (-28; 12) | -19 (-38; 1) | -4 (-24; 17) | -25 (-45; -4) |
| p-value | | 0.4193 | 0.0628 | 0.7339 | **0.0170 *** |
| **eGFR[*] (mL/min/1.73 m$^2$)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 73 (2) | 76 (2) | 78 (2) | 73 (2) | 80 (2) |
| Difference vs placebo (95% CI) | | 4 (-1; 8) | 5 (0; 10) | 0 (-5; 6) | 8 (3; 13) |
| p-value | | 0.1539 | **0.0426 *** | 0.8814 | **0.0035 ** ** |
| **Screening** | | | | | |
| Observed mean (SE) | 77 (4) | 77 (4) | 78 (4) | 79 (4) | 78 (4) |
| Difference vs placebo (95% CI) | | 0 (-10; 11) | 1 (-9; 11) | 2 (-9; 12) | 1 (-10; 11) |
| p-value | | 0.9529 | 0.8863 | 0.7704 | 0.9039 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 76 (4) | 74 (4) | 74 (4) | 78 (4) | 80 (4) |
| Difference vs placebo (95% CI) | | -3 (-13; 8) | -3 (-13; 7) | 2 (-9; 12) | 4 (-7; 14) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| p-value | | 0.6113 | 0.5847 | 0.7529 | 0.5154 |
| **POD 1** | | | | | |
| Observed mean (SE) | 75 (4) | 79 (4) | 78 (4) | 72 (4) | 80 (4) |
| Difference vs placebo (95% CI) | | 4 (-7; 15) | 3 (-8; 13) | -3 (-14; 8) | 5 (-6; 16) |
| p-value | | 0.5045 | 0.6165 | 0.5982 | 0.3466 |
| **POD 2** | | | | | |
| Observed mean (SE) | 66 (4) | 74 (4) | 79 (4) | 67 (4) | 80 (4) |
| Difference vs placebo (95% CI) | | 8 (-3; 19) | 14 (3; 24) | 1 (-10; 12) | 15 (4; 26) |
| p-value | | 0.1309 | **0.0126 *** | 0.8392 | **0.0090 **** |
| **POD 3** | | | | | |
| Observed mean (SE) | 69 (4) | 77 (4) | 80 (4) | 69 (4) | 83 (4) |
| Difference vs placebo (95% CI) | | 8 (-3; 19) | 11 (0; 21) | 0 (-11; 11) | 14 (3; 25) |
| p-value | | 0.1475 | **0.0464 *** | 0.9411 | **0.0107 *** |
| **eGFR[#] (mL/min/1.73 m$^2$)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 78 (2) | 83 (2) | 83 (2) | 79 (2) | 87 (2) |
| Difference vs placebo (95% CI) | | 5 (-1; 11) | 5 (-0; 11) | 1 (-5; 7) | 9 (3; 15) |
| p-value | | 0.0968 | 0.0682 | 0.7673 | **0.0025 **** |
| **Screening** | | | | | |
| Observed mean (SE) | 82 (4) | 83 (5) | 83 (4) | 85 (5) | 84 (5) |
| Difference vs placebo (95% CI) | | 0 (-12; 13) | 1 (-11; 13) | 2 (-10; 15) | 1 (-11; 14) |
| p-value | | 0.9725 | 0.9134 | 0.7360 | 0.8669 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 82 (4) | 81 (5) | 79 (4) | 84 (5) | 86 (5) |
| Difference vs placebo (95% CI) | | -2 (-14; 11) | -3 (-15; 9) | 2 (-11; 14) | 4 (-8; 17) |
| p-value | | 0.7988 | 0.5971 | 0.7932 | 0.5019 |
| **POD 1** | | | | | |
| Observed mean (SE) | 81 (4) | 86 (5) | 83 (4) | 79 (5) | 88 (5) |
| Difference vs placebo (95% CI) | | 5 (-7; 18) | 3 (-10; 15) | -2 (-14; 11) | 8 (-5; 20) |
| p-value | | 0.4085 | 0.6742 | 0.8016 | 0.2363 |
| **POD 2** | | | | | |
| Observed mean (SE) | 71 (4) | 81 (5) | 85 (4) | 72 (5) | 88 (5) |
| Difference vs placebo (95% CI) | | 11 (-2; 23) | 14 (2; 26) | 1 (-12; 14) | 18 (5; 30) |
| p-value | | 0.0963 | **0.0253 *** | 0.8632 | **0.0061 **** |
| **POD 3** | | | | | |
| Observed mean (SE) | 74 (4) | 84 (5) | 86 (4) | 76 (5) | 90 (5) |
| Difference vs placebo (95% CI) | | 10 (-3; 22) | 12 (-0; 24) | 1 (-12; 14) | 16 (4; 29) |
| p-value | | 0.1275 | 0.0513 | 0.8601 | **0.0125 *** |
| **Serum Cystatin (mg/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 1.3 (0.0) | 1.1 (0.0) | 1.2 (0.0) | 1.2 (0.0) | 1.1 (0.0) |
| Difference vs placebo (95% CI) | | -0.1 (-0.3; -0.0) | -0.1 (-0.2; 0.0) | -0.1 (-0.2; 0.0) | -0.2 (-0.3; -0.0) |
| p-value | | **0.0238 *** | 0.1533 | 0.0941 | **0.0100 *** |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 1.2 (0.1) | 1.2 (0.1) | 1.2 (0.1) | 1.2 (0.1) | 1.2 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.2; 0.2) | -0.0 (-0.2; 0.2) | -0.1 (-0.3; 0.2) | -0.0 (-0.2; 0.2) |
| p-value | | 0.7798 | 0.8085 | 0.6229 | 0.8023 |
| **POD 1** | | | | | |
| Observed mean (SE) | 1.2 (0.1) | 1.0 (0.1) | 1.1 (0.1) | 1.1 (0.1) | 1.0 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4; 0.1) | -0.1 (-0.3; 0.1) | -0.1 (-0.3; 0.1) | -0.2 (-0.4; 0.0) |
| p-value | | 0.2052 | 0.4878 | 0.3895 | 0.1005 |
| **POD 3** | | | | | |
| Observed mean (SE) | 1.5 (0.1) | 1.2 (0.1) | 1.3 (0.1) | 1.3 (0.1) | 1.2 (0.1) |
| Difference vs placebo (95% CI) | | -0.3 (-0.5; -0.1) | -0.2 (-0.4; 0.0) | -0.2 (-0.4; 0.0) | -0.3 (-0.5; -0.1) |
| p-value | | **0.0125 *** | 0.1063 | 0.0806 | **0.0054 **** |
| **eGFR$^{+}$ (mL/min/1.73 m$^2$)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 70 (2) | 79 (3) | 75 (3) | 76 (2) | 79 (2) |
| Difference vs placebo (95% CI) | | 9 (2; 17) | 5 (-3; 12) | 6 (-2; 13) | 9 (2; 16) |
| p-value | | **0.0127 *** | 0.1990 | 0.1290 | **0.0095 **** |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 73 (4) | 75 (4) | 75 (4) | 77 (4) | 75 (4) |
| Difference vs placebo (95% CI) | | 2 (-10; 14) | 2 (-10; 13) | 4 (-8; 16) | 2 (-9; 14) |
| p-value | | 0.7537 | 0.7888 | 0.5090 | 0.7007 |
| **POD 1** | | | | | |
| Observed mean (SE) | 64 (4) | 75 (4) | 68 (4) | 70 (4) | 75 (4) |
| Difference vs placebo (95% CI) | | 11 (-1; 23) | 4 (-8; 16) | 6 (-5; 18) | 11 (-1; 23) |
| p-value | | 0.0702 | 0.4890 | 0.2824 | 0.0625 |
| **POD 3** | | | | | |
| Observed mean (SE) | 73 (4) | 88 (5) | 81 (5) | 79 (4) | 87 (4) |
| Difference vs placebo (95% CI) | | 15 (3; 27) | 8 (-4; 20) | 6 (-5; 18) | 15 (3; 26) |
| p-value | | **0.0178 *** | 0.1965 | 0.2766 | **0.0123 *** |
| **Urinary NGAL (mg/mL)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 146.7 (55.5) | 43.4 (67.8) | 46.7 (67.2) | 112.4 (62.2) | 28.0 (63.2) |
| Difference vs placebo (95% CI) | | -103.4 (-281.9; 75.2) | -100.0 (-278.4; 78.3) | -34.3 (-204.3; 135.7) | -118.8 (-290.1; 52.6) |
| p-value | | 0.2467 | 0.2614 | 0.6834 | 0.1675 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 25.5 (92.4) | 19.8 (113.3) | 50.6 (99.5) | 22.9 (104.8) | 12.4 (105.4) |
| Difference vs placebo (95% CI) | | -5.7 (-298.6; 287.1) | 25.0 (-247.5; 297.6) | -2.6 (-282.6; 277.3) | -13.2 (-293.8; 267.5) |
| p-value | | 0.9690 | 0.8546 | 0.9851 | 0.9254 |
| **POD 1** | | | | | |
| Observed mean (SE) | 352.7 (98.0) | 68.6 (113.3) | 55.5 (107.1) | 69.2 (104.8) | 34.5 (105.4) |
| Difference vs placebo (95% CI) | | -284.0 (-584;2, 16.2) | -297.2 (-588.2; -6.2) | -283.4 (-571.0; 4.1) | -318.2 (-606.5; -29.8) |
| p-value | | 0.0632 | **0.0455 *** | 0.0533 | **0.0312 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 62.0 (98.0) | 41.7 (124.1) | 34.1 (127.5) | 245.1 (113.2) | 37.1 (113.7) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | -20.4 (-337.3; 296.5) | -28.0 (-350.4, 294.4) | 183.1 (-116.9; 483.1) | -24.9 (-325.7; 275.8) |
| p-value | | 0.8979 | 0.8626 | 0.2265 | 0.8686 |
| **Urinary NGAL:creatinine ratio (mg/mmol)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 1.48 (0.56) | 0.45 (0.68) | 0.47 (0.69) | 0.93 (0.62) | 0.37 (0.64) |
| Difference vs placebo (95% CI) | | -1.04 (-2.83; 0.76) | -1.02 (-2.85; 0.81) | -0.55 (-2.26; 1.16) | -1.12 (-2.83; 0.60) |
| p-value | | 0.2477 | 0.2660 | 0.5152 | 0.1931 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 0.24 (0.93) | 0.23 (1.14) | 0.51 (1.00) | 0.28 (1.05) | 0.17 (1.06) |
| Difference vs placebo (95% CI) | | -0.02 (-2.96; 2.93) | 0.27 (-2.49; 3.02) | 0.04 (-2.77; 2.85) | -0.08 (-2.89; 2.74) |
| p-value | | 0.9918 | 0.8452 | 0.9770 | 0.9558 |
| **POD 1** | | | | | |
| Observed mean (SE) | 3.73 (0.99) | 0.81 (1.14) | 0.55 (1.08) | 0.73 (1.05) | 0.43 (1.06) |
| Difference vs placebo (95% CI) | | -2.92 (-5.94; 0.09) | -3.18 (-6.12; -0.24) | -3.00 (-5.89; -0.11) | -3.30 (-6.20; -0.41) |
| p-value | | 0.0573 | **0.0348** * | **0.0421** * | **0.0261** * |
| **POD 3** | | | | | |
| Observed mean (SE) | 0.48 (0.99) | 0.31 (1.25) | 0.34 (1.29) | 1.78 (1.14) | 0.50 (1.14) |
| Difference vs placebo (95% CI) | | -0.17 (-3.35; 3.01) | -0.14 (-3.41; 3.13) | 1.30 (-1.71; 4.32) | 0.02 (-2.99; 3.04) |
| p-value | | 0.9157 | 0.9322 | 0.3900 | 0.9871 |
| **Change in creatinine (FAS) (µmol/L), active vs placebo (Mixed effects analysis, REML).** | | | | | |
| **Screening** | | | | | |
| Observed mean | 99.75 | 91.88 | 85.50 | 74.00 | 85.88 |
| Mean difference vs placebo (SE) | | -7.875 (12.63) | -14.25 (11.81) | -25.75 (11.81) | -13.88 (11.51) |
| p-value | | 0.5450 | 0.2562 | 0.0570 | 0.2590 |
| **Pre-operatively** | | | | | |
| Observed mean | 97.63 | 94.38 | 91.38 | 75.29 | 83.00 |
| Mean difference vs placebo (SE) | | -3.25 (9.47) | -6.25 (9.19) | -22.34 (9.22) | -14.63 (8.46) |
| p-value | | 0.7367 | 0.5074 | **0.0310** * | 0.1060 |
| **POD 1** | | | | | |
| Observed mean | 98.38 | 88.38 | 85.25 | 76.29 | 77.75 |
| Mean difference vs placebo (SE) | | -10.00 (11.67) | -13.13 (11.53) | -22.09 (14.99) | -20.63 (10.99) |
| p-value | | 0.4074 | 0.2763 | 0.1663 | 0.0863 |
| **POD 2** | | | | | |
| Observed mean | 107.9 | 99.50 | 83.50 | 74.71 | 77.38 |
| Mean difference vs placebo (SE) | | -8.74 (19.09) | -24.38 (11.69) | -25.73 (14.46) | -28.50 (11.51) |
| p-value | | 0.6689 | 0.0605 | 0.0988 | **0.0310** * |
| **POD 3** | | | | | |
| Observed mean | 99.75 | 94.65 | 85.85 | 76.49 | 80.68 |
| Mean difference vs placebo (SE) | | -0.63 (20.22) | -16.13 (11.66) | -25.04 (12.54) | -22.38 (9.71) |
| p-value | | 0.9759 | 0.1895 | 0.0672 | **0.0469** * |

Abbreviations: AU – arbitrary units; SD – standard deviation; IQR – interquartile range; SE – standard error; CI – confidence interval, REML – restricted maximum likelihood; LMM – linear mixed model

* eGFR by local laboratory; # eGFR – 2021 CKD-EPI; + 2021 CKD-EPI cystatin

## 4.3 Discussion

Bioinformatic analyses presented in this chapter confirm that transcript expression of *HMOX1* is highest within the reticuloendothelial system compared to all other tissues, and specifically enriched within monocytes and tissue resident macrophages. Furthermore, the pattern of expression of *HMOX1* across haematological cells is highly evolutionarily conserved between mouse and human. Analyses have also identified co-expression of *HMOX1* with various genes associated with scavenging and innate immune functions, most notably *CD36* and *CD68*. CD36 is a membrane bound glycoprotein which, when present on phagocytes, acts as a pattern recognition receptor enabling innate immune cells to internalise apoptotic cells, cellular debris and foreign pathogens[321]. CD68 is classically regarded as a marker of circulating monocytes and macrophages; CD68 is a lysosomal membrane associated glycoprotein expressed by macrophages, and its presence in circulating monocytes has been linked with pattern recognition and phagocytosis[322]. Taken together, these findings suggest that HO-1 is expressed in immune cells that sense and respond to haemolysis.

This work also enabled the identification of key immune cells in which to investigate the PD effects of 5-ALA-SFC on HO-1 expression and function. Subsequent *in vitro* work confirmed that 5-ALA-SFC induces dose-dependent increases in HO-1 expression in THP-1 cells and MDMs. Furthermore, by exposing iPSC-CMs to 5-ALA-SFC, it was demonstrated that human cardiomyocytes possess inducible *HMOX1*. Given these data, it is biologically plausible that pre-treatment with 5-ALA-SFC may induce upregulation of HO-1 expression in the circulating monocytes and tissue resident macrophages in addition to cardiomyocytes to confer protection from IRI. These findings are consistent with the results of two studies of intravenous haem arginate (HA) administration which also increased expression of HO-1; HA pre-treatment 24 hours prior to elective AVR resulted in a dose-dependent increase in HO-1 mRNA and protein expression in biopsies from the RA appendage and RV; in the HOT trial recipients of kidney donors were treated with HA pre-operatively and POD2 had a increased expression of HO-1 mRNA in the PBMCs, renal tissue and renal macrophages.

Subsequently, a key focus of the TALEN trial was to obtain data to support selection of the OBD of 5-ALA-SFC for subsequent evaluation in larger, multi-centre clinical trials through careful interrogation of the drug's effect on HO-1 expression and function in circulating immune cells. Work presented in this chapter has shown that HO enzyme activity was reproducibly assayed, using a novel LC-MS/MS quantification of biliverdin method, in human PBMCs for the first time. This method showed that HO activity was increased in response to 5-ALA-SFC in a dose-dependent manner. This, together with higher baseline serum bilirubin levels measured in patients allocated to the highest 5-ALA-SFC dose (see section 3.2.8) in addition to data supporting a dose-response effect for renoprotection (described below), provides evidence for target engagement and validation of the LC-MS/MS HO activity assay findings.

There is growing recognition that, when developing drugs targeting specific enzymes, more nuanced assessment of drug effects on enzymatic function is required to accurately define drug response, particularly during early phase development[323]. The LC-MS/MS assay was developed to address that need for 5-ALA-SFC specifically. Concordant with the enzymatic activity assay findings, a significant 5-ALA-SFC dose-related increase in the proportion of circulating immune cells expressing HO-1, was demonstrated. When considering drug effects at transcript level, no clear treatment related increase in the relative expression of *HMOX1* was found pre-operatively which may reflect the relatively limited sensitivity of this approach to reflect incremental increases in HO-1 expression as a measure of drug PD effect in the context of a clinical trial. However, *HMOX1* was significantly increased on the third post-operative day in patients allocated to receive the highest dose, compared to placebo. This finding is of uncertain significance. Taken together with the observations on renal function/injury biomarkers and inflammatory indices (discussed in the next chapter) the totality of the data are consistent with activation of HO-1 by oral administration of 5-ALA-SFC, with protein expression and enzymatic functional data demonstrating that the greatest degree of activation occurs at the highest dose tested of 300 mg/472 mg.

The potential impact of genetic modulation of HO activity was then investigated, in part to explore the observed higher baseline HO activity was observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group, by investigating whetherthe shorter *HMOX1* promoter (GT)n repeat polymorphism

status of patients in this group may have conferred higher baseline enzyme activity. Genotyping of the promotor allele showed a biphasic spread in (GT)n repeat length frequencies, consistent with that previously described in a cardiac surgical population[249]. Previous literature on the relationship between *HMOX1* promoter genotype (SS vs LL) and HO enzyme activity in lymphoblastic cell lines[235] was consistent with the findings of this study in patient-derived PBMCs.

Technical aspects of the assay must be considered. Cells were cryopreserved at -80°C and there was a delay of some months between preservation of the earliest samples (which included placebo controls) and performing the assay. Cell viability was not assessed prior to performing the assay and data were normalised to the number of viable cells/mL originally cryopreserved. Results should therefore be carefully considered given the known decrement in cell viability over time when stored at -80°C [324]. This study also found an increase in pre-operative serum bilirubin levels, with an apparent 5-ALA-SFC dose-response relationship. As the final product of haem breakdown by HOs, serum bilirubin may represent an accessible marker of enzyme activity, and this inexpensive biochemical readout could be a more feasible and attractive biomarker of drug effect.

Although 5-ALA-SFC treatment was associated with increased relative expression of *NFE2L2* (coding for NRF2), relative expression of canonical NRF2-dependent genes was not equally upregulated. Subtle reductions in post-operative relative expression of *ME1* and *TXN* in 5-ALA-SFC treated patients, may represent drug-related differences in cellular responses to oxidative stress. Reduced levels of *ME1* expression, for example, have been associated with more favourable shifts in carbohydrate oxidation, improving redox state[325].

In addition to 5-ALA-SFC's putative cardioprotective role, this work also sought to investigate the potential ability of this drug to mitigate the negative effects of haemolysis associated with CPB. A temporal relationship between CPB and haemolysis is well described[14,15] and was been observed in this study also. The data presented in this chapter do not clearly support a treatment related reduction in the

extent of haemolytic injury. Haptoglobin levels fluctuate in response to physiological stress (e.g., surgery and CPB) and these data are therefore difficult to interpret[326].

Pre-operative serum iron levels were increased in 5-ALA-SFC treated patients, and there was evidence of dose-related differences between treatment groups, supporting the treatment compliance data. Higher Tsat readings were also seen in patients who had received 5-ALA-SFC, and these data correlated weakly with NTBI levels, a relationship which has been described previously[327]. NTBI was only detected in patients who had received active 5-ALA-SFC, and this peaked in the early hours after CPB. This kinetic profile has been described in cardiac surgery and is related to an acute rise in circulating iron which overcomes transferrin binding capacity[328]. A post-operative decrease in transferrin has been observed and, as with other plasma proteins, this reduction is possibly explained by surgical blood loss and the dilutional effects of peri-operative fluid therapy. Nonetheless, lower systemic iron binding capacity post-operatively would favour the development of an unbound iron pool. Despite the apparent 5-ALA-SFC associated increase in NTBI, data from this study suggested a signal for reduction in iron mediated lipid peroxidation (using MDA as a measurement) by 5-ALA-SFC with a potential dose-dependent response.

Post-operative hypoferraemia was a consistent finding across treatment groups, and this has been well described in cardiac surgical patients[329]. The aetiology of this so-called functional iron deficiency is multifactorial and includes acute blood loss, possible underlying anaemia, and peri-operative fluctuations in the key iron regulatory proteins hepcidin and ferritin[124]. These protein levels are affected by the inflammatory response to cardiac surgery on CPB, and several circulating cytokines (e.g., IL-6) have been linked with increased transcription of these proteins[120,330,331]. This study found a significant 5-ALA-SFC dose related reduction in IL-6 post-operatively (discussed in a subsequent section of this thesis) (Fig. 62 E). Associated changes in hepcidin and ferritin levels were less clear. Although the data suggested a decreased hepcidin concentration in patients treated with the highest 5-ALA-SFC dose, this finding was not statistically significant. The potential association between higher doses of 5-ALA-SFC and lower post-operative hepcidin levels, is particularly notable as high hepcidin levels after cardiac surgery have been linked with AKI[124].

When considering key genes involved in cellular iron handling, data from this study support the hypothesis that 5-ALA-SFC may influence circulating immune cells to enable ferrostasis (i.e., favouring iron uptake, storage, and retention), but this assumption would need further investigation. Relative expression of *CD163* was upregulated post-operatively in all patients. This gene codes for CD163, a membrane bound scavenger protein canonically involved in the uptake of Hb-haptoglobin complexes[332] . This observation points to an innate immune response to haemolysis[154]. The suggestion that 5-ALA-SFC treatment may reduce post-operative expression of *TRFC* is difficult to interpret but could point to an immunomodulatory effect of HO-1 induction. Regulation of *TFRC* transcription is complex and has been shown to be differentially influenced by inflammatory and anti-inflammatory signals, mediated by HIF-1 and NF-κB pathways [333]. The relative expression of *DMT1* was found to be higher during the post-operative period in patients allocated to the active study arm, particularly in patients allocated to the 300 mg/472 mg 5-ALA-SFC treatment group. This gene codes for DMT1 which, together with ZIP14 (encoded by the gene *SLC39A14*), represents an important cellular NTBI uptake mechanism[334]. Monocytes have been shown to express DMT1[151] and 5-ALA-SFC may induce a learned immune response to enable more profound upregulation in response to haemolysis, with increased NTBI uptake. Alongside this, pre-operative relative expression of *FTH* was increased in 5-ALA-SFC treated patients, suggesting an increased capacity to bind NTBI intracellularly[105].

Finally, the work presented in this chapter highlights a renoprotective role of 5-ALA-SFC in the setting of cardiac surgery on CPB with circulating and urine based biomarkers of renal injury consistently favorable for 5-ALA-SFC versus placebo with evidence of a dose-response relationship strongest for the highest dose evaluated. The exact mechanisms by which 5-ALA-SFC may offer this benefit is unknown but should be considered through its ability to increase intracellular haem, as demonstrated in the 'HO-1 in the Heart' and HOT trials, although in neither trial was there a significant impact on biomarkers of tissue injury (troponin I for the former and urinary KIM-1 and urinary NGAL for the latter) suggesting the approach to HO-1 activation (agent, dose, dosing schedule) all matter in providing tissue protection.

Not only can 5-ALA-SFC induce HO-1, as demonstrated in this study, but it is also capable of inducing innate immune memory[114], potentially equipping immune cells to better respond to the haemolytic injury associated with CPB. Although 5-ALA-SFC has not been linked with a reduction in the extent of the haemolytic insult, work presented here informs an immune mediated mechanism in addressing the negative sequalae of haemolysis, a central factor in the aetiology of AKI after cardiac surgery[91,93,132,335]. As the incidence of clinically relevant AKI was low during this study, this hypothesis would need further investigation in future trials potentially enriched with higher risk surgical patients to confirm whether the observed biomarker changes translate into meaningful clinical benefit.

# 5 Chapter 5. The impact of 5-ALA-SFC on the immune-inflammatory response to cardiac surgery requiring cardiopulmonary bypass

## 5.1 Introduction

HO-1, a potent anti-inflammatory and antioxidant protein, is recognised as an important regulator of immune cells, with a specific iron- and redox-regulatory role in cells of the MPS[204]. Work presented in this thesis has demonstrated that 5-ALA-SFC is associated with a degree of renoprotection in cardiac surgical patients. One mechanism by which 5-ALA-SFC may achieve this beneficial effect is through increasing immune cell expression of HO-1, driving a phenotypic commitment towards anti-inflammatory cells capable of maintaining iron homeostasis and restoring redox balance. Therefore, HO-1 induction prior to cardiac surgery may improve outcomes not only by targeting cardiomyocytes to mitigate cardiac IRI, but also through ameliorating the negative effects of CPB-associated haemolysis.

The immunomodulatory potential of 5-ALA-SFC in adult patients undergoing cardiac surgery on CPB is undefined. To address this, the results of the work presented in this chapter had the following specific objectives:

1. Investigate the ability of 5-ALA-SFC to reduce the inflammatory response to cardiac surgery.
2. Examine the impact of 5-ALA-SFC on discrete monocyte subsets by:
    a. Determining the expression of HO-1 as a PD marker of drug effect.
    b. Assessing the phenotype of monocyte subsets, specifically the ability of 5-ALA-SFC to affect their capacity for erythrophagocytosis, antigen processing as well as activation state.
    c. Investigating whether cells expressing both CD68 and HO-1 represent a discrete population in which the effects of 5-ALA-SFC can be further examined.
3. Investigate the potential of 5-ALA-SFC to dampens activation of the sterile inflammasome in response to haemolysis using transcriptomic, flow cytometry and targeted circulating protein evaluation approaches including expression of the canonical downstream cytokines.
4. Assess the impact of 5-ALA-SFC on differential inflammatory protein expression, using a non-biased approach and identification of key biological processes affected by the drug.

## 5.2 Results

### 5.2.1 The impact of 5-ALA-SFC on markers of post-operative inflammation

Routinely used biomarkers of inflammation, C-reactive protein (CRP) and WCC, were assessed in all TALEN trial participants during the peri-operative period. A clear temporal rise in both CRP and WCC was observed in the post-operative period reflecting the impact of surgery, particularly marked for CRP (**Fig. 67 A-B**). Using a LMM, with time as a categorical variable, the most striking treatment-related difference was seen in the CRP dataset (**Fig 67. A**). Patients who had been allocated to the active treatment arm displayed a less profound increase in post-operative CRP levels when compared to placebo with a dose-response relationship. Across the model, a significant treatment-related fixed effect was identified in the 5-ALA-SFC 300 mg/472 mg treatment allocation group with a highly statistically significant (p-value = 0.008) reduction in the observed mean difference of CRP (95% CI) vs. placebo of -42.2 (-72.7; -11.8) mg/L. When considering individual timepoints, CRP continued to rise in all patients and post-operative data visually diverged between 5-ALA-SFC treated patients and placebo as early as POD1. This was most noticeable in the highest dose allocation at POD3 with a substantial reduction in CRP compared to placebo (mean difference [95 % CI] vs. placebo = -83.3 [-128.9; -37.7] mg/L, p-value = 0.0005).

The WCC increased across all treatment groups in response to surgery (Fig. 67 B). A more noticeable increase, suggesting more profound inflammation, is observed in the 225 mg/354 mg treatment allocation group on the first post-operative day (mean difference [95 % CI] vs. placebo = 3.6 [0.7, 6.5] $x10^9$/L cells, p-value = 0.0151) when assessing data from the FAS population. However, when data were re-analysed excluding patients who had experienced significant intra-operative surgical complications (i.e. PAS population), this difference was not statistically significant (mean difference [95 % CI] vs. placebo = 2.2 [-0.6, 5.0] $x10^9$/L cells, p-value = 0.1242). In both the FAS and PAS, data on the third post-operative day showed a tendency to a smaller increase in neutrophil count in 5-ALA-SFC treated patients, but this finding was not statistically significant (**Fig. 67 C**). No significant differences between groups were observed in lymphocyte and monocyte counts (**Fig. 67 D-E**) although a temporal relationship between changes in cell counts and surgery was observed.

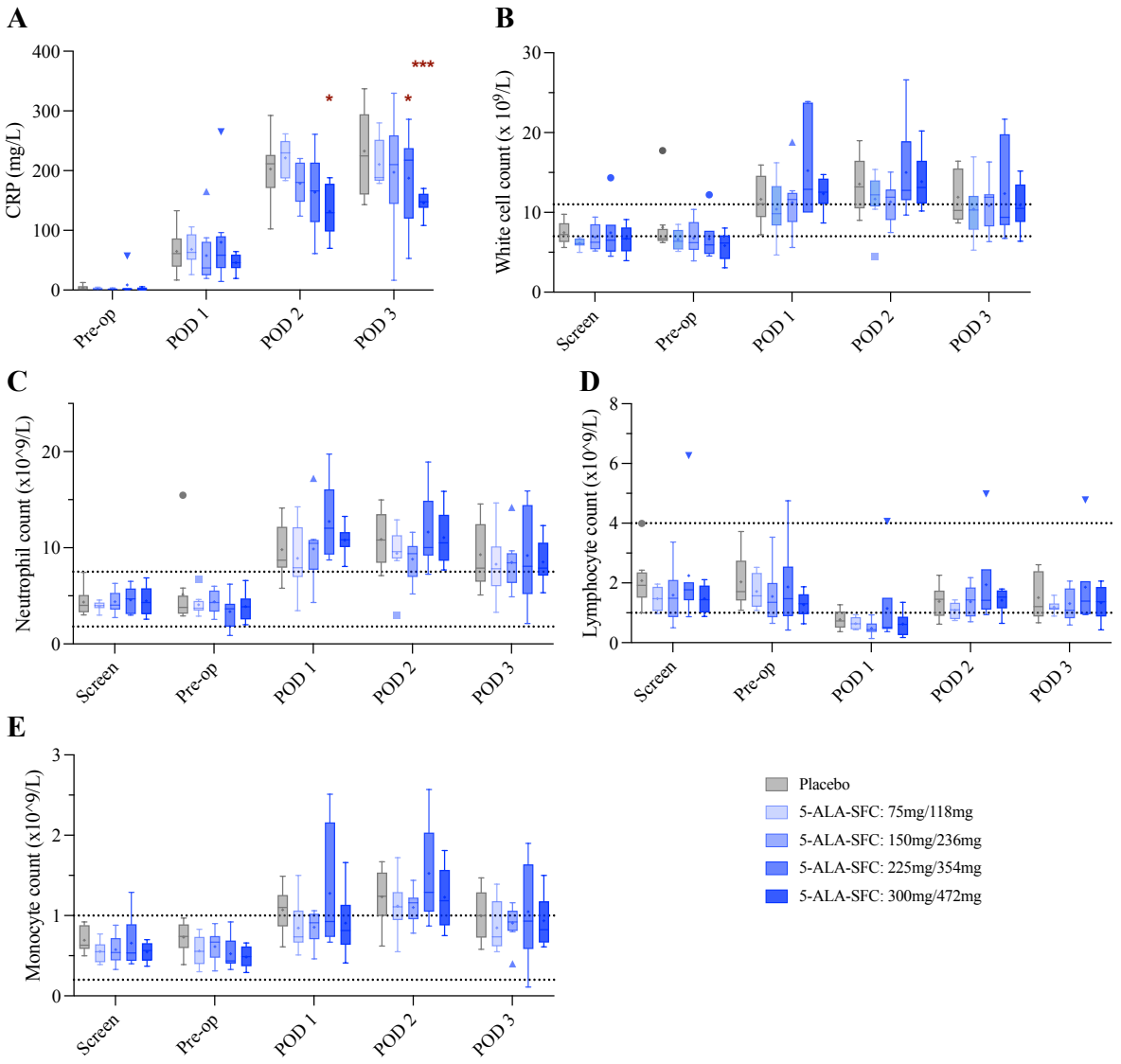



**Figure 67: Peri-operative laboratory markers of inflammation.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values.

Differences between groups assessed using a LMM with time as a categorical variable. * = $p < 0.05$, *** $p = < 0.001$.

**Table 33: Descriptive and inferential statistics of clinically relevant markers of inflammation.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **C-reactive protein (CRP) (mg/L)** | | | | | |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.3 (4.4) | 2.1 (1.8) | 1.6 (1.1) | 8.5 (19.6) | 1.9 (2.1) |
| Median (IQR) | 1.4 (0.7; 4.5) | 1.1 (0.7; 3.9) | 1.5 (0.7; 2.4) | 1.6 (0.8; 2.8) | 0.8 (0.5; 3.4) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Min Max | 0.5 12.6 | 0.5 4.9 | 0.4 3.4 | 0.6 57.0 | 0.3 5.5 |
| **POD 1** | | | | | |
| Mean (SD) | 68.5 (37.0) | 68.2 (27.1) | 42.6 (24.0) | 100.7 (112.1) | 45.8 (14.5) |
| Median (IQR) | 66.6 (43.4; 90.6) | 63.2 (51.2; 92.6) | 36.4 (22.4; 61.3) | 61.7 (32.7; 168.7) | 46.0 (38.5; 56.5) |
| Min Max | 16.4 133.1 | 26.0 105.8 | 19.5 87.5 | 14.3 265.0 | 19.6 64.6 |
| **POD 2** | | | | | |
| Mean (SD) | 188.2 (60.1) | 221.5 (31.6) | 184.0 (33.8) | 146.1 (57.8) | 129.1 (44.9) |
| Median (IQR) | 183.7 (149.5; 224.5) | 229.7 (187.1; 246.7) | 185.8 (164.6; 217.7) | 150.5 (104.8; 187.3) | 122.0 (96.2; 186.3) |
| Min Max | 102.5 292.6 | 183.3 261.7 | 123.8 220.3 | 61.0 222.4 | 69.8 188.1 |
| **POD 3** | | | | | |
| Mean (SD) | 228.2 (66.1) | 210.7 (39.7) | 205.7 (99.2) | 163.1 (73.5) | 145.8 (19.0) |
| Median (IQR) | 233.3 (144.2; 287.3) | 188.6 (184.4; 240.4) | 228.0 (160.9; 262.6) | 174.8 (114.0; 222.8) | 148.0 (138.0; 158.0) |
| Min Max | 143.0 298.2 | 178.6 280.1 | 16.5 329.7 | 53.0 239.4 | 108.3 170.3 |
| **White cell count ($x10^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 7.5 (1.4) | 6.1 (0.6) | 6.9 (1.6) | 14.6 (20.4) | 6.7 (1.7) |
| Median (IQR) | 7.2 (6.6; 8.1) | 6.1 (5.9; 6.7) | 6.3 (5.5; 7.8) | 7.1 (5.4; 11.4) | 6.7 (5.6; 8.1) |
| Min Max | 5.6 9.8 | 5.0 6.9 | 5.2 9.4 | 4.5 64.5 | 4.0 9.1 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 8.1 (3.7) | 6.6 (1.3) | 6.8 (2.1) | 12.2 (15.7) | 5.8 (1.7) |
| Median (IQR) | 6.7 (6.5; 7.5) | 6.4 (5.5; 7.8) | 6.2 (5.9; 8.7) | 6.2 (4.9; 10.0) | 6.2 (4.7; 6.9) |
| Min Max | 6.2 17.7 | 5.1 8.5 | 3.9 10.4 | 4.5 50.6 | 3.1 8.1 |
| **POD 1** | | | | | |
| Mean (SD) | 11.7 (3.0) | 10.4 (3.6) | 11.2 (3.6) | 20.8 (16.9) | 12.3 (2.0) |
| Median (IQR) | 11.1 (9.8; 13.9) | 9.9 (8.5; 12.9) | 11.6 (9.4; 12.2) | 13.5 (11.0; 23.8) | 12.5 (11.3; 13.8) |
| Min Max | 7.2 15.9 | 4.7 16.2 | 5.6 18.8 | 10.0 60.2 | 8.7 14.8 |
| **POD 2** | | | | | |
| Mean (SD) | 13.5 (3.4) | 11.7 (3.3) | 11.3 (2.4) | 20.2 (15.6) | 13.9 (3.4) |
| Median (IQR) | 13.2 (11.1; 16.4) | 12.2 (11.1; 13.5) | 11.9 (9.2; 12.5) | 13.2 (11.7; 22.8) | 13.1 (11.5; 15.6) |
| Min Max | 9.0 19.0 | 4.4 15.4 | 7.5 15.1 | 9.7 56.4 | 10.2 20.2 |
| **POD 3** | | | | | |
| Mean (SD) | 11.9 (3.3) | 10.4 (3.5) | 10.8 (3.1) | 18.1 (17.2) | 10.9 (2.9) |
| Median (IQR) | 10.3 (9.1; 15.1) | 10.3 (8.3; 12.0) | 11.9 (9.5; 12.1) | 10.5 (8.6; 20.7) | 10.5 (9.1; 13.2) |
| Min Max | 8.7 16.4 | 5.3 17.0 | 6.3 16.3 | 6.7 58.5 | 6.4 15.2 |
| **Neutrophil count ($x10^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 4.3 (1.4) | 3.9 (0.5) | 4.4 (1.1) | 4.6 (1.4) | 4.5 (1.5) |
| Median (IQR) | 4.0 (3.4; 4.8) | 4.0 (3.7; 4.3) | 4.0 (3.8; 5.1) | 4.7 (3.1; 5.7) | 4.3 (3.3; 5.6) |
| Min Max | 3.0 7.4 | 3.0 4.6 | 2.7 6.3 | 3.0 6.5 | 2.6 6.9 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 5.1 (4.0) | 4.1 (1.2) | 4.4 (1.2) | 3.4 (1.7) | 3.9 (1.5) |
| Median (IQR) | 3.8 (3.2; 4.2) | 3.7 (3.5; 4.3) | 4.3 (3.9; 5.5) | 3.7 (2.0; 4.2) | 3.9 (2.8; 4.5) |
| Min Max | 2.9 15.5 | 2.9 6.7 | 2.6 6.0 | 0.9 6.2 | 2.0 6.6 |
| **POD 1** | | | | | |
| Mean (SD) | 9.8 (2.7) | 8.9 (3.5) | 9.9 (3.5) | 12.7 (3.9) | 10.8 (1.5) |
| Median (IQR) | 8.7 (7.9; 11.8) | 7.9 (7.1; 11.7) | 10.5 (8.5; 10.8) | 12.0 (9.8; 14.9) | 10.8 (10.1; 11.5) |
| Min Max | 5.8 14.1 | 3.5 14.3 | 4.3 17.2 | 8.7 19.7 | 8.1 13.3 |
| **POD 2** | | | | | |
| Mean (SD) | 10.9 (2.8) | 9.4 (2.9) | 8.8 (2.0) | 11.6 (3.9) | 11.1 (2.8) |
| Median (IQR) | 10.8 (8.7; 13.3) | 9.6 (9.1; 11.0) | 9.4 (7.0; 9.9) | 10.0 (9.4; 14.1) | 10.5 (9.1; 12.9) |
| Min Max | 7.1 15.0 | 3.0 12.9 | 5.2 11.6 | 7.3 18.9 | 7.7 15.9 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 3** | | | | | |
| Mean (SD) | 9.3 (3.4) | 8.3 (3.4) | 8.5 (2.7) | 9.2 (5.0) | 8.5 (2.3) |
| Median (IQR) | 7.9 (7.3; 12.0) | 7.8 (6.4; 10.0) | 8.4 (7.4; 9.2) | 8.1 (5.6; 14.0) | 7.9 (7.2; 10.2) |
| Min Max | 5.1 14.5 | 3.3 14.7 | 4.9 14.2 | 2.1 15.9 | 5.3 12.3 |
| **Lymphocyte count (x10$^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 2.1 (0.8) | 1.5 (0.4) | 1.6 (0.9) | 9.1 (19.6) | 1.5 (0.5) |
| Median (IQR) | 1.9 (1.6; 2.3) | 1.5 (1.1; 1.8) | 1.5 (1.2; 1.9) | 1.8 (1.5; 4.1) | 1.4 (1.1; 1.9) |
| Min Max | 1.0 4.0 | 1.0 2.0 | 0.5 3.4 | 0.9 57.4 | 0.9 2.1 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.0 (0.9) | 1.7 (0.6) | 1.5 (0.9) | 7.7 (16.6) | 1.3 (0.4) |
| Median (IQR) | 1.7 (1.5; 2.5) | 1.6 (1.2; 2.3) | 1.4 (0.9; 1.8) | 1.6 (1.1; 3.7) | 1.3 (1.0; 1.6) |
| Min Max | 1.1 3.7 | 1.1 2.5 | 0.7 3.5 | 0.4 48.6 | 0.6 1.9 |
| **POD 1** | | | | | |
| Mean (SD) | 0.8 (0.3) | 0.6 (0.2) | 0.5 (0.2) | 6.8 (16.0) | 0.6 (0.4) |
| Median (IQR) | 0.7 (0.6; 0.9) | 0.6 (0.4; 0.8) | 0.4 (0.4; 0.5) | 0.6 (0.5; 2.8) | 0.6 (0.3; 0.8) |
| Min Max | 0.4 1.3 | 0.4 0.9 | 0.1 0.9 | 0.4 46.3 | 0.2 1.4 |
| **POD 2** | | | | | |
| Mean (SD) | 1.4 (0.5) | 1.1 (0.3) | 1.4 (0.5) | 6.9 (14.2) | 1.4 (0.4) |
| Median (IQR) | 1.5 (1.0; 1.7) | 1.1 (0.8; 1.3) | 1.4 (0.9; 1.6) | 1.5 (1.2; 3.7) | 1.5 (1.2; 1.8) |
| Min Max | 0.6 2.3 | 0.7 1.4 | 0.7 2.2 | 0.9 41.8 | 0.7 1.8 |
| **POD 3** | | | | | |
| Mean (SD) | 1.5 (0.8) | 1.2 (0.2) | 1.3 (0.6) | 7.1 (14.8) | 1.3 (0.6) |
| Median (IQR) | 1.2 (1.0; 2.3) | 1.2 (1.1; 1.3) | 1.1 (1.0; 1.8) | 1.6 (1.1; 3.4) | 1.4 (1.0; 1.8) |
| Min Max | 0.7 2.6 | 0.9 1.6 | 0.6 2.1 | 0.9 43.7 | 0.4 2.1 |
| **Monocyte count (x10$^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.7 (0.2) | 0.6 (0.1) | 0.6 (0.2) | 0.7 (0.3) | 0.5 (0.1) |
| Median (IQR) | 0.6 (0.6; 0.9) | 0.6 (0.4; 0.6) | 0.5 (0.5; 0.7) | 0.5 (0.5; 0.8) | 0.6 (0.4; 0.6) |
| Min Max | 0.5 0.9 | 0.4 0.8 | 0.3 0.9 | 0.4 1.3 | 0.4 0.7 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.7 (0.2) | 0.6 (0.2) | 0.6 (0.2) | 0.5 (0.2) | 0.5 (0.1) |
| Median (IQR) | 0.7 (0.7; 0.8) | 0.6 (0.4; 0.7) | 0.7 (0.5; 0.7) | 0.4 (0.4; 0.6) | 0.5 (0.4; 0.6) |
| Min Max | 0.4 1.0 | 0.3 0.8 | 0.3 0.9 | 0.3 0.9 | 0.3 0.7 |
| **POD 1** | | | | | |
| Mean (SD) | 1.1 (0.3) | 0.8 (0.3) | 0.9 (0.2) | 1.3 (0.8) | 0.9 (0.4) |
| Median (IQR) | 1.1 (1.1; 1.2) | 0.7 (0.7; 1.0) | 0.9 (0.8; 1.0) | 0.9 (0.8; 1.8) | 0.8 (0.7; 1.1) |
| Min Max | 0.6 1.5 | 0.5 1.5 | 0.5 1.1 | 0.7 2.5 | 0.4 1.7 |
| **POD 2** | | | | | |
| Mean (SD) | 1.2 (0.3) | 1.1 (0.3) | 1.1 (0.2) | 1.5 (0.6) | 1.2 (0.4) |
| Median (IQR) | 1.2 (1.0; 1.4) | 1.1 (0.9; 1.3) | 1.2 (1.0; 1.2) | 1.3 (1.1; 2.0) | 1.2 (0.9; 1.6) |
| Min Max | 0.6 1.7 | 0.6 1.7 | 0.8 1.4 | 0.9 2.6 | 0.8 1.8 |
| **POD 3** | | | | | |
| Mean (SD) | 1.0 (0.3) | 0.8 (0.3) | 0.9 (0.2) | 1.0 (0.6) | 0.9 (0.3) |
| Median (IQR) | 1.0 (0.8; 1.1) | 0.7 (0.6; 1.1) | 0.9 (0.8; 1.0) | 0.9 (0.6; 1.6) | 0.8 (0.7; 1.2) |
| Min Max | 0.6 1.5 | 0.6 1.4 | 0.4 1.2 | 0.1 1.9 | 0.6 1.5 |
| **Eosinophil count (x10$^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.20 (0.09) | 0.20 (0.12) | 0.15 (0.13) | 0.21 (0.15) | 0.18 (0.08) |
| Median (IQR) | 0.19 (0.16; 0.23) | 0.18 (0.12; 0.29) | 0.12 (0.08; 0.22) | 0.14 (0.10; 0.34) | 0.20 (0.12; 0.23) |
| Min Max | 0.08 0.39 | 0.05 0.41 | 0.02 0.43 | 0.06 0.49 | 0.04 0.29 |
| **Pre-operatively** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 0.26 (0.21) | 0.20 (0.14) | 0.17 (0.13) | 0.15 (0.12) | 0.17 (0.10) |
| Median (IQR) | 0.19 (0.15; 0.28) | 0.19 (0.09; 0.30) | 0.12 (0.06; 0.33) | 0.14 (0.05; 0.24) | 0.17 (0.07; 0.26) |
| Min Max | 0.09 0.79 | 0.01 0.43 | 0.03 0.34 | 0.05 0.38 | 0.06 0.29 |
| **POD 1** | | | | | |
| Mean (SD) | 0.00 (0.00) | 0.01 (0.01) | 0.01 (0.02) | 0.00 (0.00) | 0.00 (0.00) |
| Median (IQR) | 0.00 (0.00; 0.00) | 0.01 (0.00; 0.01) | 0.00 (0.00; 0.00) | 0.00 (0.00; 0.01) | 0.00 (0.00; 0.00) |
| Min Max | 0.00 0.00 | 0.00 0.04 | 0.00 0.06 | 0.00 0.01 | 0.00 0.00 |
| **POD 2** | | | | | |
| Mean (SD) | 0.04 (0.06) | 0.03 (0.02) | 0.04 (0.05) | 0.03 (0.04) | 0.14 (0.33) |
| Median (IQR) | 0.01 (0.00; 0.06) | 0.03 (0.02; 0.05) | 0.03 (0.01; 0.04) | 0.02 (0.01; 0.05) | 0.03 (0.01; 0.06) |
| Min Max | 0.00 0.18 | 0.00 0.07 | 0.00 0.17 | 0.00 0.12 | 0.00 0.95 |
| **POD 3** | | | | | |
| Mean (SD) | 0.11 (0.20) | 0.08 (0.06) | 0.09 (0.09) | 0.08 (0.07) | 0.10 (0.08) |
| Median (IQR) | 0.01 (0.00; 0.09) | 0.10 (0.02; 0.13) | 0.07 (0.01; 0.12) | 0.07 (0.03; 0.11) | 0.08 (0.03; 0.16) |
| Min Max | 0.00 0.59 | 0.01 0.14 | 0.00 0.26 | 0.00 0.21 | 0.01 0.22 |
| **Basophil count (x$10^9$/L)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.02) | 0.05 (0.03) | 0.04 (0.02) |
| Median (IQR) | 0.04 (0.04; 0.06) | 0.04 (0.04; 0.06) | 0.06 (0.04; 0.06) | 0.06 (0.02; 0.07) | 0.05 (0.02; 0.05) |
| Min Max | 0.03 0.08 | 0.03 0.09 | 0.01 0.09 | 0.01 0.09 | 0.01 0.06 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.05 (0.02) | 0.05 (0.02) | 0.10 (0.15) | 0.04 (0.02) | 0.04 (0.02) |
| Median (IQR) | 0.05 (0.03; 0.06) | 0.05 (0.05; 0.06) | 0.03 (0.03; 0.07) | 0.04 (0.03; 0.06) | 0.04 (0.02; 0.05) |
| Min Max | 0.03 0.07 | 0.01 0.08 | 0.02 0.50 | 0.03 0.08 | 0.01 0.06 |
| **POD 1** | | | | | |
| Mean (SD) | 0.01 (0.01) | 0.03 (0.01) | 0.02 (0.01) | 0.03 (0.03) | 0.02 (0.01) |
| Median (IQR) | 0.02 (0.01; 0.02) | 0.03 (0.02; 0.03) | 0.01 (0.01; 0.02) | 0.02 (0.01; 0.04) | 0.02 (0.01; 0.02) |
| Min Max | 0.00 0.02 | 0.02 0.05 | 0.01 0.04 | 0.01 0.09 | 0.01 0.03 |
| **POD 2** | | | | | |
| Mean (SD) | 0.02 (0.01) | 0.03 (0.02) | 0.03 (0.01) | 0.03 (0.02) | 0.02 (0.01) |
| Median (IQR) | 0.02 (0.01; 0.03) | 0.03 (0.03; 0.05) | 0.03 (0.02; 0.04) | 0.03 (0.02; 0.04) | 0.03 (0.02; 0.03) |
| Min Max | 0.00 0.03 | 0.01 0.06 | 0.02 0.05 | 0.01 0.08 | 0.00 0.03 |
| **POD 3** | | | | | |
| Mean (SD) | 0.02 (0.01) | 0.02 (0.01) | 0.03 (0.02) | 0.05 (0.04) | 0.03 (0.02) |
| Median (IQR) | 0.02 (0.01; 0.03) | 0.03 (0.01; 0.04) | 0.03 (0.02; 0.04) | 0.04 (0.03; 0.06) | 0.03 (0.03; 0.05) |
| Min Max | 0.01 0.04 | 0.01 0.04 | 0.01 0.06 | 0.02 0.14 | 0.01 0.06 |
| **Change in circulating markers of inflammation, active vs placebo (LMM, time as a categorical variable) - FAS** | | | | | |
| **C-reactive protein (mg/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 121.9 (10.7) | 124.7 (10.4) | 107.3 (10.8) | 108.7 (12.2) | 79.6 (10.5) |
| Difference vs placebo (95% CI) | | 2.8 (-27.5, 33.1) | -14.6 (-45.5, 16.3) | -13.2 (-46.3, 19.9) | -42.2 (-72.7, -11.8) |
| p-value | | 0.8517 | 0.3441 | 0.4229 | **0.0080** ** |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 3.0 (15.7) | 1.2 (15.7) | 0.4 (15.7) | 11.3 (15.9) | 0.9 (15.7) |
| Difference vs placebo (95% CI) | | -1.9 (-46.0, 42.3) | -2.6 (-46.8, 41.5) | 8.3 (-36.2, 52.8) | -2.2 (-46.3, 42.0) |
| p-value | | 0.9335 | 0.9061 | 0.7129 | 0.9229 |
| **POD 1** | | | | | |
| Observed mean (SE) | 68.4 (16.7) | 67.3 (15.7) | 41.4 (16.8) | 107.7 (22.2) | 44.7 (15.7) |
| Difference vs placebo (95% CI) | | -1.1 (-46.7, 44.5) | -27.0 (-74.0, 20.1) | 39.3 (-15.9, 94.5) | -23.6 (-69.3, 22.0) |
| p-value | | 0.9624 | 0.2577 | 0.1602 | 0.3059 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 2** | | | | | |
| Observed mean (SE) | 188.1 (16.7) | 220.6 (15.7) | 182.9 (16.8) | 149.3 (18.3) | 128.1 (16.7) |
| Difference vs placebo (95% CI) | | 32.5 (-13.1, 78.1) | -5.2 (-52.2, 41.8) | -38.8 (-88.1, 10.6) | -59.9 (-106.9, -13.0) |
| p-value | | 0.1603 | 0.8267 | 0.1223 | **0.0129 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 228.1 (16.7) | 209.7 (15.7) | 204.5 (16.8) | 166.4 (18.3) | 144.8 (15.7) |
| Difference vs placebo (95% CI) | | -18.3 (-63.9, 27.3) | -23.6 (-70.6, 23.5) | -61.7 (-111.0, -12.3) | -83.3 (-128.9, -37.7) |
| p-value | | 0.4270 | 0.3219 | **0.0149 *** | **0.0005 ***** |
| **White cell count ($x10^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 12.0 (0.7) | 11.5 (0.8) | 11.1 (0.8) | 12.9 (0.8) | 12.0 (0.8) |
| Difference vs placebo (95% CI) | | -0.5 (-2.7, 1.7) | -0.8 (-3.0, 1.3) | 0.9 (-1.4, 3.2) | 0.0 (-2.2, 2.2) |
| p-value | | 0.6598 | 0.4490 | 0.4126 | 0.9805 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 8.8 (1.0) | 8.3 (1.0) | 7.9 (1.0) | 7.2 (1.1) | 7.1 (1.0) |
| Difference vs placebo (95% CI) | | -0.5 (-3.3, 2.4) | -0.9 (-3.7, 1.9) | -1.5 (-4.4, 1.4) | -1.7 (-4.5, 1.1) |
| p-value | | 0.7430 | 0.5207 | 0.2930 | 0.2406 |
| **POD 1** | | | | | |
| Observed mean (SE) | 12.3 (1.0) | 12.1 (1.0) | 12.3 (1.0) | 15.9 (1.1) | 13.6 (1.0) |
| Difference vs placebo (95% CI) | | -0.2 (-3.0, 2.6) | 0.0 (-2.7, 2.8) | 3.6 (0.7, 6.5) | 1.3 (-1.6, 4.1) |
| p-value | | 0.8888 | 0.9803 | **0.0151 *** | 0.3743 |
| **POD 2** | | | | | |
| Observed mean (SE) | 14.2 (1.0) | 13.4 (1.0) | 12.4 (1.0) | 15.2 (1.1) | 15.1 (1.0) |
| Difference vs placebo (95% CI) | | -0.8 (-3.7, 2.0) | -1.8 (-4.5, 1.0) | 1.0 (-1.9, 3.9) | 0.9 (-1.9, 3.8) |
| p-value | | 0.5608 | 0.2073 | 0.4828 | 0.5254 |
| **POD 3** | | | | | |
| Observed mean (SE) | 12.6 (1.0) | 12.1 (1.0) | 11.9 (1.0) | 13.2 (1.1) | 12.2 (1.0) |
| Difference vs placebo (95% CI) | | -0.4 (-3.3, 2.4) | -0.6 (-3.4, 2.1) | 0.6 (-2.2, 3.5) | -0.4 (-3.2, 2.4) |
| p-value | | 0.7654 | 0.6599 | 0.6576 | 0.7842 |
| **Neutrophil count ($x10^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 8.8 (0.7) | 8.0 (0.8) | 7.8 (0.7) | 9.0 (0.8) | 8.4 (0.8) |
| Difference vs placebo (95% CI) | | -0.8 (-2.9, 1.3) | -0.9 (-3.0, 1.1) | 0.3 (-1.8, 2.4) | -0.3 (-2.4, 1.8) |
| p-value | | 0.4648 | 0.3663 | 0.7937 | 0.7587 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 5.1 (0.9) | 4.4 (1.0) | 4.3 (0.9) | 3.2 (1.0) | 3.8 (1.0) |
| Difference vs placebo (95% CI) | | -0.7 (-3.4, 2.0) | -0.8 (-3.4, 1.8) | -2.0 (-4.7, 0.7) | -1.4 (-4.1, 1.3) |
| p-value | | 0.6005 | 0.5540 | 0.1523 | 0.3193 |
| **POD 1** | | | | | |
| Observed mean (SE) | 9.8 (0.9) | 9.2 (1.0) | 9.8 (0.9) | 12.6 (1.0) | 10.7 (1.0) |
| Difference vs placebo (95% CI) | | -0.6 (-3.3, 2.1) | 0.0 (-2.6, 2.6) | 2.8 (0.0, 5.5) | 0.9 (-1.8, 3.6) |
| p-value | | 0.6720 | 0.9873 | **0.0460 *** | 0.5214 |
| **POD 2** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Observed mean (SE) | 10.9 (0.9) | 9.7 (1.0) | 8.8 (0.9) | 11.5 (1.0) | 10.9 (1.0) |
| Difference vs placebo (95% CI) | | -1.1 (-3.8, 1.6) | -2.1 (-4.7, 0.5) | 0.6 (-2.1, 3.3) | 0.1 (-2.6, 2.8) |
| p-value | | 0.4085 | 0.1153 | 0.6719 | 0.9588 |
| **POD 3** | | | | | |
| Observed mean (SE) | 9.3 (0.9) | 8.6 (1.0) | 8.4 (0.9) | 9.0 (1.0) | 8.4 (1.0) |
| Difference vs placebo (95% CI) | | -0.7 (-3.4, 2.1) | -0.8 (-3.4, 1.8) | -0.3 (-3.0, 2.4) | -0.9 (-3.6, 1.8) |
| p-value | | 0.6320 | 0.5313 | 0.8445 | 0.5237 |
| **Monocyte count (x10$^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 0.9 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 1.1 (0.1) | 0.9 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.3, 0.2) | -0.0 (-0.3, 0.2) | 0.1 (-0.1, 0.4) | 0.0 (-0.3, 0.3) |
| p-value | | 0.7094 | 0.7398 | 0.3537 | 0.9848 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 0.7 (0.1) | 0.6 (0.1) | 0.6 (0.1) | 0.5 (0.1) | 0.5 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4, 0.3) | -0.0 (-0.3, 0.3) | -0.2 (-0.5, 0.1) | -0.1 (-0.4, 0.2) |
| p-value | | 0.7421 | 0.9096 | 0.2638 | 0.4370 |
| **POD 1** | | | | | |
| Observed mean (SE) | 1.0 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 1.2 (0.1) | 1.0 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4, 0.2) | -0.1 (-0.4, 0.2) | 0.2 (-0.1, 0.5) | -0.0 (-0.4, 0.3) |
| p-value | | 0.4972 | 0.4433 | 0.1350 | 0.7867 |
| **POD 2** | | | | | |
| Observed mean (SE) | 1.2 (0.1) | 1.2 (0.1) | 1.1 (0.1) | 1.5 (0.1) | 1.3 (0.1) |
| Difference vs placebo (95% CI) | | 0.0 (-0.3, 0.3) | -0.0 (-0.3, 0.3) | 0.3 (0.0, 0.6) | 0.1 (-0.2, 0.4) |
| p-value | | 0.9747 | 0.8360 | **0.0422 *** | 0.4623 |
| **POD 3** | | | | | |
| Observed mean (SE) | 0.9 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 1.0 (0.1) | 1.0 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.4, 0.3) | 0.0 (-0.3, 0.3) | 0.1 (-0.2, 0.4) | 0.1 (-0.3, 0.4) |
| p-value | | 0.8308 | 0.9759 | 0.6073 | 0.7086 |
| **Change in circulating markers of inflammation, active vs placebo (LMM, time as a categorical variable) - PAS** | | | | | |
| **C-reactive protein (mg/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 121.9 (10.8) | 124.7 (10.4) | 107.3 (10.9) | 109.3 (12.6) | 79.6 (10.5) |
| Difference vs placebo (95% CI) | | 2.8 (-27.7, 33.3) | -14.6 (-45.8, 16.5) | -12.5 (-46.4, 21.3) | -42.3 (-72.9, -11.6) |
| p-value | | 0.8534 | 0.3468 | 0.4569 | **0.0084 ** ** |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 3.0 (15.8) | 1.2 (15.8) | 0.4 (15.8) | 12.8 (17.2) | 0.9 (15.8) |
| Difference vs placebo (95% CI) | | -1.9 (-46.2, 42.5) | -2.6 (-47.0, 41.7) | 9.7 (-36.6, 56.1) | -2.2 (-46.5, 42.2) |
| p-value | | 0.9333 | 0.9058 | 0.6777 | 0.9228 |
| **POD 1** | | | | | |
| Observed mean (SE) | 68.4 (16.8) | 67.3 (15.8) | 41.4 (16.8) | 108.1 (22.3) | 44.7 (15.8) |
| Difference vs placebo (95% CI) | | -1.1 (-46.9, 44.7) | -27.0 (-74.2, 20.3) | 39.8 (-15.8, 95.3) | -23.7 (-69.5, 22.2) |
| p-value | | 0.9621 | 0.2595 | 0.1583 | 0.3077 |
| **POD 2** | | | | | |
| Observed mean (SE) | 188.0 (16.8) | 220.5 (15.8) | 182.8 (16.8) | 149.7 (18.5) | 128.1 (16.8) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (95% CI) | | 32.5 (-13.3, 78.3) | -5.2 (-52.5, 42.0) | -38.3 (-88.0, 11.4) | -59.9 (-107.1, -12.8) |
| p-value | | 0.1623 | 0.8269 | 0.1288 | **0.0133 *** |
| **POD 3** | | | | | |
| Observed mean (SE) | 228.0 (16.8) | 209.7 (15.8) | 204.4 (16.8) | 166.8 (18.5) | 144.8 (15.8) |
| Difference vs placebo (95% CI) | | -18.3 (-64.1, 27.5) | -23.6 (-70.9, 23.6) | -61.3 (-111.0, -11.6) | -83.3 (-129.1, -37.5) |
| p-value | | 0.4287 | 0.3237 | **0.0162 *** | **0.0005 ***** |
| **White cell count (x10$^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 11.8 (0.7) | 11.6 (0.7) | 10.7 (0.7) | 12.2 (0.8) | 12.1 (0.7) |
| Difference vs placebo (95% CI) | | -0.2 (-2.2, 1.8) | -1.1 (-3.1, 0.9) | 0.4 (-1.7, 2.6) | 0.3 (-1.7, 2.3) |
| p-value | | 0.8447 | 0.2793 | 0.6849 | 0.7558 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 8.9 (0.9) | 8.4 (0.9) | 7.8 (0.9) | 7.3 (1.0) | 7.2 (0.9) |
| Difference vs placebo (95% CI) | | -0.5 (-3.1, 2.1) | -1.1 (-3.8, 1.5) | -1.6 (-4.3, 1.2) | -1.7 (-4.3, 0.9) |
| p-value | | 0.7168 | 0.3965 | 0.2680 | 0.1999 |
| **POD 1** | | | | | |
| Observed mean (SE) | 12.5 (0.9) | 12.2 (0.9) | 11.5 (0.9) | 14.7 (1.0) | 13.7 (0.9) |
| Difference vs placebo (95% CI) | | -0.3 (-3.0, 2.3) | -1.0 (-3.6, 1.6) | 2.2 (-0.6, 5.0) | 1.1 (-1.5, 3.8) |
| p-value | | 0.8070 | 0.4446 | 0.1242 | 0.3882 |
| **POD 2** | | | | | |
| Observed mean (SE) | 13.5 (0.9) | 13.5 (0.9) | 12.1 (0.9) | 14.6 (1.0) | 15.2 (0.9) |
| Difference vs placebo (95% CI) | | -0.0 (-2.7, 2.6) | -1.4 (-4.0, 1.2) | 1.1 (-1.7, 3.9) | 1.7 (-0.9, 4.3) |
| p-value | | 0.9703 | 0.2857 | 0.4331 | 0.2041 |
| **POD 3** | | | | | |
| Observed mean (SE) | 12.1 (0.9) | 12.2 (0.9) | 11.4 (0.9) | 12.2 (1.0) | 12.3 (0.9) |
| Difference vs placebo (95% CI) | | 0.1 (-2.6, 2.7) | -0.8 (-3.4, 1.9) | 0.0 (-2.8, 2.8) | 0.1 (-2.5, 2.7) |
| p-value | | 0.9539 | 0.5616 | 0.9871 | 0.9370 |
| **Neutrophil count (x10$^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 8.5 (0.7) | 7.9 (0.7) | 7.4 (0.7) | 8.5 (0.8) | 8.4 (0.7) |
| Difference vs placebo (95% CI) | | -0.6 (-2.6, 1.4) | -1.1 (-3.2, 0.9) | -0.1 (-2.1, 2.0) | -0.1 (-2.1, 1.9) |
| p-value | | 0.5543 | 0.2669 | 0.9612 | 0.9283 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 5.3 (0.9) | 4.3 (0.9) | 4.3 (0.9) | 3.1 (1.0) | 3.8 (0.9) |
| Difference vs placebo (95% CI) | | -0.9 (-3.5, 1.7) | -1.0 (-3.6, 1.6) | -2.1 (-4.8, 0.6) | -1.5 (-4.1, 1.1) |
| p-value | | 0.4777 | 0.4528 | 0.1181 | 0.2472 |
| **POD 1** | | | | | |
| Observed mean (SE) | 9.9 (0.9) | 9.2 (0.9) | 9.0 (0.9) | 11.7 (1.0) | 10.7 (0.9) |
| Difference vs placebo (95% CI) | | -0.8 (-3.4, 1.9) | -0.9 (-3.5, 1.7) | 1.7 (-0.9, 4.4) | 0.8 (-1.8, 3.4) |
| p-value | | 0.5659 | 0.4842 | 0.2006 | 0.5628 |
| **POD 2** | | | | | |
| Observed mean (SE) | 10.2 (0.9) | 9.7 (0.9) | 8.5 (0.9) | 11.0 (1.0) | 10.9 (0.9) |
| Difference vs placebo (95% CI) | | -0.6 (-3.2, 2.0) | -1.7 (-4.3, 0.9) | 0.7 (-2.0, 3.4) | 0.7 (-1.9, 3.3) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| p-value | | 0.6697 | 0.1915 | 0.5884 | 0.5927 |
| **POD 3** | | | | | |
| Observed mean (SE) | 8.7 (0.9) | 8.5 (0.9) | 7.8 (0.9) | 8.1 (1.0) | 8.4 (0.9) |
| Difference vs placebo (95% CI) | | -0.1 (-2.8, 2.5) | -0.9 (-3.5, 1.7) | -0.5 (-3.2, 2.1) | -0.3 (-2.9, 2.3) |
| p-value | | 0.9121 | 0.5040 | 0.6869 | 0.8190 |
| **Monocyte count (x10$^9$/L)** | | | | | |
| **Overall** | | | | | |
| Observed mean (SE) | 0.9 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 1.0 (0.1) | 0.9 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.3, 0.2) | -0.1 (-0.3, 0.2) | 0.0 (-0.2, 0.3) | 0.0 (-0.2, 0.3) |
| p-value | | 0.8114 | 0.5491 | 0.7697 | 0.8510 |
| **Pre-operatively** | | | | | |
| Observed mean (SE) | 0.6 (0.1) | 0.6 (0.1) | 0.6 (0.1) | 0.5 (0.1) | 0.5 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.3, 0.3) | -0.0 (-0.3, 0.3) | -0.2 (-0.5, 0.1) | -0.1 (-0.4, 0.2) |
| p-value | | 0.8656 | 0.8446 | 0.2870 | 0.5212 |
| **POD 1** | | | | | |
| Observed mean (SE) | 1.0 (0.1) | 0.9 (0.1) | 0.8 (0.1) | 1.0 (0.1) | 1.0 (0.1) |
| Difference vs placebo (95% CI) | | -0.1 (-0.4, 0.2) | -0.2 (-0.5, 0.1) | 0.0 (-0.3, 0.3) | -0.0 (-0.3, 0.3) |
| p-value | | 0.4746 | 0.2418 | 0.7982 | 0.7810 |
| **POD 2** | | | | | |
| Observed mean (SE) | 1.1 (0.1) | 1.2 (0.1) | 1.1 (0.1) | 1.4 (0.1) | 1.3 (0.1) |
| Difference vs placebo (95% CI) | | 0.1 (-0.2, 0.4) | -0.0 (-0.3, 0.3) | 0.3 (-0.0, 0.6) | 0.2 (-0.1, 0.5) |
| p-value | | 0.7060 | 0.8380 | 0.0531 | 0.2607 |
| **POD 3** | | | | | |
| Observed mean (SE) | 0.9 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 0.9 (0.1) | 1.0 (0.1) |
| Difference vs placebo (95% CI) | | -0.0 (-0.3, 0.3) | -0.0 (-0.3, 0.3) | -0.0 (-0.3, 0.3) | 0.1 (-0.2, 0.4) |
| p-value | | 0.8074 | 0.7606 | 0.8209 | 0.7013 |

Abbreviations: SD – standard deviation; IQR – interquartile range; SE – standard error; CI – confidence interval, REML – restricted maximum likelihood; LMM – linear mixed model, FAS – full analysis set; PAS – primary analysis set.

### 5.2.2 Monocytes as a potential immunomodulatory target for HO-1 induction by 5-ALA-SFC

#### 5.2.2.1 The impact of 5-ALA-SFC on conventional monocyte subsets

Cryopreserved PBMCs from all TALEN trial patients were thawed and analysed using multi-dimensional flow cytometry. Using a conventional gating strategy, monocyte populations were defined as classical (CL) ($CD14^+$ $CD16^-$), intermediate (ITM) ($CD14^+$ $CD16^+$) or non-classical (NC) ($CD14^-$ $CD16^+$) (Fig. 68). The most abundant monocytes at baseline were classical (CL), followed by non-classical (NC) then intermediate (ITM) (**Fig. 69 A-D**). A LMM using REML, correcting for multiple comparisons by controlling the FDR using the two-stage step-up method of Benjamini, Krieger and

Yekutieli, was used to determine whether any differences observed between treatment allocation groups, were statistically significant.

The most striking treatment-related effect was observed in the CL monocyte population. Here, a significant increase in CL monocytes (as a proportion of $Lin^{-}$ $CD45^{+}$ $CD11b^{+}$) was observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group at the pre-operative timepoint, i.e. at the end of dosing but immediately before surgery, plausibly reflecting a drug mediated effect (mean difference vs. placebo [SE] = 12.15 [4.78] %, p-value = 0.0309) (**Fig. 69 A**). During the post-operative period, an increase in the proportion of CL monocytes was observed in all patients and across the model, time was identified as the most significant fixed effect (p-value < 0.0001), consistent with a temporal relationship between this rise and surgery. The data also suggested a sustained increase in CL monocytes in patients allocated to the active arm of the study vs. placebo. For example, on the third post operative day a trend to a higher proportion of CL monocytes was observed in patients allocated to the highest dose of 5-ALA-SFC, vs. placebo (mean difference [SE] vs. placebo = 11.8 [5.87] %, p-value = 0.0738). When data from all patients allocated to the active arm of the trial were pooled and compared to placebo, 5-ALA-SFC's effect on CL monocyte representation was more apparent (**Fig. 69 B**).

Next, given that a possible treatment-related phenotypic shift favouring more CL monocytes (as a proportion of $Lin^{-}$ $CD45^{+}$ $CD11b^{+}$) was identified, the impact of the drug on HO-1 biology in these cells was further evaluated (**Fig. 68 B**). A significant drug related increase in the proportion of $HO\text{-}1^{+}$ CL monocytes (as a proportion of $Lin^{-}$ $CD45^{+}$ $CD11b^{+}$) was observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group at the pre-operative timepoint (mean difference [SE] vs. placebo = 8.37 % [3.26], p-value = 0.0260) (**Fig. 69 E**). Again, within the LMM using REML, time was the most significant fixed effect factor influencing post-operative HO-1 expression in CL monocytes (p-value < 0.0001). Post-operatively, a non-significant trend for a phenotypic shift towards higher HO-1 expression in CL monocytes in patients allocated to receive the active treatment was observed compared to placebo. A comparison of pooled active vs placebo showed an increase in CL monocytes expressing HO-1 at the end of dosing pre-operatively which was sustained in the post-operative period (**Fig. 69 F**).

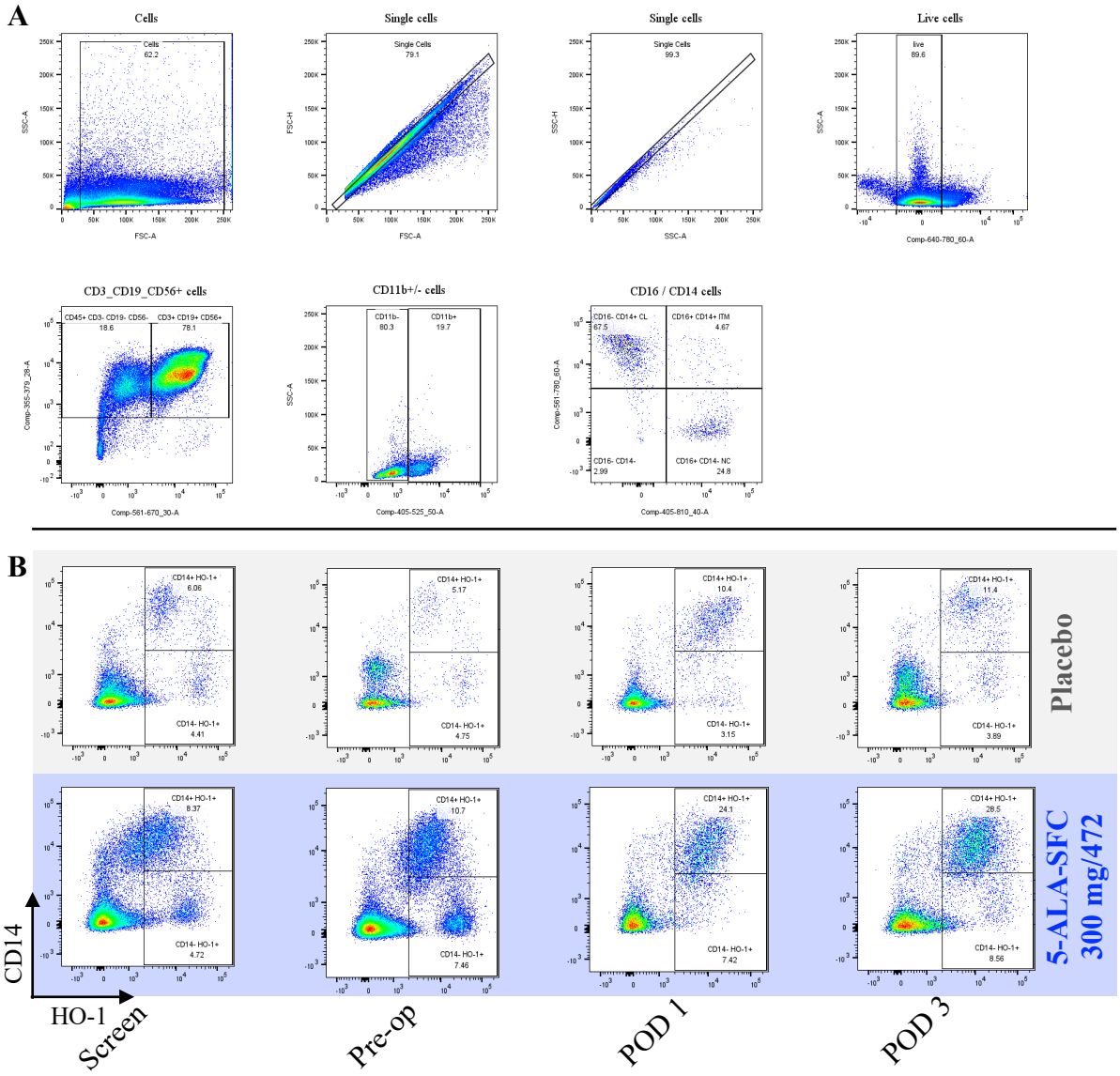



**Figure 68: Gating strategy for conventional classification of monocyte populations.**

Panel A: Representative traces from flow cytometry analysis illustrating the conventional monocyte gating strategy – Live > $CD45^+$ > $CD3^-$ $CD19^-$ $CD56^-$ > $CD11b^+$ > CD14 and CD16.

Panel B: Representative flow cytometry traces demonstrating that the proportion of classical monocytes (CL) as well as $HO\text{-}1^+$ CL monocytes were increased in response to 5-ALA-SFC.

Abbreviations: POD – post-operative day.

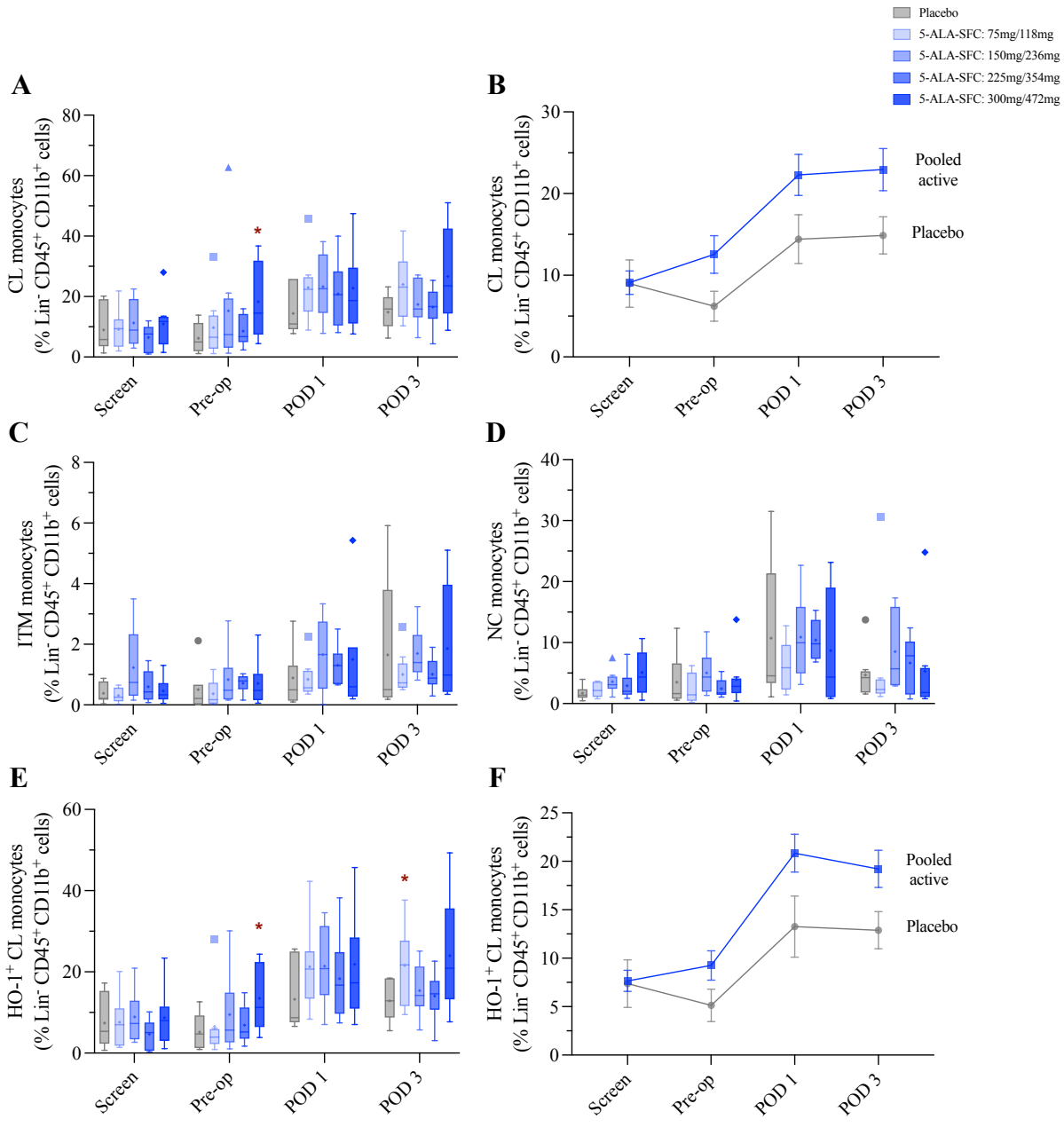



**Figure 69: The impact of 5-ALA-SFC on peri-operative monocyte subsets.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, C-E) and mean (± SE) (panel B, F). Significance from the LMM using REML in red: * = $p < 0.05$.

CL monocytes - $CD14^+$ $CD\ 16^-$ (panel A-B); ITM monocytes – $CD14^+$ $CD16^+$ (panel C); NC monocytes – $CD14^-$ $CD16^+$ (panel D); $HO\text{-}1^+$ CL monocytes (panel E-F). $Lin^-$ cells – excludes natural killer cells, B-cells and T-cells ($CD3^+$ $CD19^+$ $CD56^+$)

Abbreviations: CL – classical; ITM – intermediate; NC – non-classical.

## Table 34: Impact of 5-ALA-SFC on monocyte subsets in the peri-operative period

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **CL monocytes ($CD14^+$ $CD16^-$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 8.97 (7.68) | 9.21 (6.57) | 11.27 (7.50) | 6.48 (4.47) | 10.94 (8.28) |
| Median (IQR) | 5.82 (3.58; 19.15) | 9.37 (3.36; 12.50) | 8.89 (4.35; 19.24) | 7.62 (1.22; 10.02) | 11.74 (4.16; 13.28) |
| Min Max | 1.30 20.13 | 2.03 21.86 | 2.94 22.50 | 0.99 11.97 | 1.49 27.98 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.20 (4.83) | 9.74 (10.49) | 15.25 (20.26) | 8.55 (5.21) | 18.35 (12.49) |
| Median (IQR) | 4.96 (1.92; 11.30) | 6.53 (2.73; 13.76) | 7.42 (3.05; 19.39) | 6.77 (4.89; 14.29) | 14.51 (7.40; 31.90) |
| Min Max | 1.10 13.88 | 1.11 33.25 | 1.25 62.73 | 2.36 15.93 | 4.45 36.72 |
| **POD 1** | | | | | |
| Mean (SD) | 14.42 (7.90) | 22.98 (11.05) | 23.25 (10.49) | 20.83 (11.41) | 22.78 (13.75) |
| Median (IQR) | 10.94 (9.18; 25.84) | 22.41 (15.00; 26.48) | 22.61 (14.51; 33.95) | 20.64 (10.42; 28.34) | 18.64 (11.07; 29.59) |
| Min Max | 7.27 25.87 | 8.89 45.90 | 7.84 38.20 | 8.07 40.00 | 7.63 47.47 |
| **POD 3** | | | | | |
| Mean (SD) | 14.88 (6.02) | 24.03 (10.74) | 17.37 (7.28) | 16.50 (7.00) | 26.69 (15.30) |
| Median (IQR) | 15.87 (10.16; 19.83) | 23.14 (13.29; 31.69) | 15.90 (13.13; 26.31) | 16.73 (12.66; 21.66) | 23.52 (14.36; 42.50) |
| Min Max | 6.27 23.23 | 10.32 41.70 | 6.40 27.11 | 4.34 25.37 | 8.84 51.02 |
| **ITM monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.39 (0.32) | 0.31 (0.23) | 1.23 (1.22) | 0.60 (0.54) | 0.47 (0.41) |
| Median (IQR) | 0.22 (0.17; 0.77) | 0.26 (0.12; 0.57) | 0.74 (0.30; 2.34) | 0.43 (0.18; 1.12) | 0.34 (0.18; 0.72) |
| Min Max | 0.04 0.88 | 0.02 0.65 | 0.16 3.50 | 0.08 1.46 | 0.05 1.30 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.50 (0.75) | 0.37 (0.42) | 0.83 (0.89) | 0.72 (0.31) | 0.71 (0.74) |
| Median (IQR) | 0.22 (0.02; 0.67) | 0.17 (0.04; 0.74) | 0.49 (0.18; 1.23) | 0.79 (0.51; 0.94) | 0.47 (0.17; 1.03) |
| Min Max | 0.00 2.12 | 0.01 1.17 | 0.16 2.77 | 0.16 1.02 | 0.06 2.31 |
| **POD 1** | | | | | |
| Mean (SD) | 0.89 (0.96) | 0.85 (0.63) | 1.66 (1.25) | 1.31 (0.67) | 1.50 (1.87) |
| Median (IQR) | 0.50 (0.14; 1.30) | 0.57 (0.43; 1.13) | 1.66 (0.53; 2.75) | 1.30 (0.69; 1.70) | 0.60 (0.27; 1.90) |
| Min Max | 0.10 2.76 | 0.36 2.24 | 0.00 3.33 | 0.66 2.50 | 0.20 5.43 |
| **POD 3** | | | | | |
| Mean (SD) | 1.65 (2.28) | 1.01 (0.72) | 1.70 (0.85) | 1.01 (0.54) | 1.86 (1.93) |
| Median (IQR) | 0.51 (0.25; 3.80) | 0.73 (0.57; 1.37) | 1.40 (1.08; 2.32) | 0.88 (0.67; 1.45) | 0.98 (0.43; 3.96) |
| Min Max | 0.19 5.92 | 0.50 2.59 | 0.82 3.24 | 0.29 1.90 | 0.35 5.10 |
| **NC monocytes ($CD14^-$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 1.69 (1.11) | 2.27 (1.20) | 3.57 (1.92) | 2.93 (2.62) | 5.09 (3.56) |
| Median (IQR) | 1.37 (0.97; 2.35) | 2.17 (1.20; 3.61) | 3.11 (2.43; 4.46) | 2.02 (1.42; 4.26) | 4.37 (1.77; 8.37) |
| Min Max | 0.47 3.98 | 0.80 3.70 | 1.08 7.53 | 0.87 8.08 | 0.57 10.67 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.51 (4.32) | 2.38 (2.43) | 5.02 (3.53) | 2.46 (1.57) | 3.91 (4.16) |
| Median (IQR) | 1.65 (0.80; 6.57) | 1.46 (0.41; 5.10) | 4.37 (1.95; 7.53) | 1.74 (1.46; 3.83) | 2.84 (1.70; 4.13) |
| Min Max | 0.55 12.34 | 0.18 6.22 | 1.35 11.77 | 1.06 5.25 | 0.44 13.76 |
| **POD 1** | | | | | |
| Mean (SD) | 10.73 (11.27) | 6.14 (4.14) | 10.87 (6.73) | 10.40 (3.58) | 8.68 (8.89) |
| Median (IQR) | 4.56 (3.38; 21.37) | 5.89 (2.30; 9.65) | 9.97 (4.93; 15.88) | 9.79 (7.32; 13.74) | 4.36 (1.06; 19.02) |
| Min Max | 1.08 31.52 | 1.43 12.74 | 3.13 22.68 | 6.81 15.30 | 0.84 23.14 |
| **POD 3** | | | | | |
| Mean (SD) | 4.70 (3.94) | 5.89 (10.03) | 8.53 (6.14) | 6.67 (4.53) | 5.32 (8.11) |
| Median (IQR) | 4.26 (1.80; 5.29) | 2.30 (1.62; 3.97) | 5.72 (3.00; 15.88) | 7.84 (1.48; 10.17) | 1.82 (1.06; 5.85) |
| Min Max | 1.56 13.75 | 1.17 30.58 | 2.91 17.32 | 0.78 12.43 | 0.80 24.79 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **HO-1$^+$ CL monocytes (CD14$^+$ CD16$^-$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 7.39 (6.49) | 7.59 (6.26) | 8.92 (6.20) | 4.65 (3.74) | 8.72 (7.09) |
| Median (IQR) | 5.39 (2.34; 15.32) | 7.00 (1.81; 11.07) | 7.29 (3.37; 12.93) | 5.05 (0.57; 7.56) | 8.01 (3.01; 11.46) |
| Min Max | 0.67 17.27 | 1.38 20.07 | 2.68 20.89 | 0.34 10.11 | 1.11 23.39 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 5.14 (4.40) | 6.58 (8.84) | 9.47 (9.68) | 6.87 (4.79) | 13.51 (7.94) |
| Median (IQR) | 4.70 (1.22; 9.27) | 3.92 (2.20; 5.90) | 5.67 (2.61; 14.89) | 5.21 (3.53; 11.28) | 11.26 (6.41; 22.44) |
| Min Max | 0.88 12.65 | 0.89 27.99 | 1.05 30.12 | 1.71 14.88 | 3.885 24.36 |
| **POD 1** | | | | | |
| Mean (SD) | 13.26 (8.34) | 21.27 (10.26) | 21.42 (9.46) | 18.32 (10.83) | 21.86 (13.24) |
| Median (IQR) | 8.72 (7.58; 25.03) | 20.71 (13.40; 25.08) | 20.79 (14.25; 31.33) | 16.72 (9.67; 24.86) | 17.28 (10.93; 28.49) |
| Min Max | 6.60 25.63 | 8.37 42.31 | 7.03 34.58 | 7.44 38.23 | 7.05 45.71 |
| **POD 3** | | | | | |
| Mean (SD) | 12.89 (5.09) | 21.64 (9.64) | 15.37 (6.43) | 14.07 (6.38) | 24.02 (13.94) |
| Median (IQR) | 12.88 (8.78; 18.44) | 21.73 (11.56; 27.73) | 14.20 (11.49; 21.39) | 14.63 (10.70; 17.81) | 20.90 (13.24; 35.66) |
| Min Max | 5.50 18.48 | 9.46 37.66 | 5.74 25.12 | 3.08 22.65 | 7.67 49.32 |
| **HO-1$^+$ ITM monocytes (CD14$^+$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.35 (0.30) | 0.24 (0.21) | 1.09 (1.15) | 0.92 (0.57) | 0.38 (0.37) |
| Median (IQR) | 0.20 (0.17; 0.73) | 0.18 (0.07; 0.47) | 0.66 (0.20; 2.07) | 0.82 (0.48; 1.42) | 0.32 (0.10; 0.51) |
| Min Max | 0.02 0.77 | 0.02 0.56 | 0.14 3.28 | 0.21 1.83 | 0.02 1.17 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.40 (0.77) | 0.24 (0.26) | 0.52 (0.87) | 0.94 (0.68) | 0.63 (0.72) |
| Median (IQR) | 0.08 (0.00; 0.41) | 0.17 (0.03; 0.40) | 0.19 (0.09; 0.44) | 0.69 (0.49; 1.52) | 0.40 (0.15; 0.90) |
| Min Max | 0.00 2.12 | 0.01 0.77 | 0.06 2.62 | 0.23 2.15 | 0.06 2.24 |
| **POD 1** | | | | | |
| Mean (SD) | 0.71 (0.74) | 0.80 (0.63) | 0.98 (0.89) | 0.63 (0.28) | 1.45 (1.87) |
| Median (IQR) | 0.27 (0.07; 1.30) | 0.57 (0.37; 1.07) | 0.79 (0.13; 1.80) | 0.64 (0.44; 0.85) | 0.52 (0.25; 1.90) |
| Min Max | 0.03 1.85 | 0.27 2.18 | 0.00 2.42 | 0.16 0.98 | 0.15 5.41 |
| **POD 3** | | | | | |
| Mean (SD) | 1.61 (2.23) | 0.98 (0.74) | 1.06 (0.73) | 0.53 (0.51) | 1.82 (1.94) |
| Median (IQR) | 0.48 (0.25; 3.79) | 0.71 (0.49; 1.37) | 0.92 (0.59; 1.74) | 0.37 (0.14; 0.97) | 0.95 (0.34; 3.92) |
| Min Max | 0.18 5.73 | 0.48 2.59 | 0.06 2.26 | 0.06 1.37 | 0.29 5.07 |
| **HO-1$^+$ NC monocytes (CD14$^-$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.11 (0.05) | 0.14 (0.17) | 0.39 (0.29) | 0.14 (0.14) | 0.18 (0.16) |
| Median (IQR) | 0.11 (0.07; 0.14) | 0.09 (0.02; 0.22) | 0.32 (0.14; 0.55) | 0.10 (0.01; 0.27) | 0.14 (0.06; 0.25) |
| Min Max | 0.01 0.18 | 0.02 0.52 | 0.12 0.99 | 0.01 0.37 | 0.01 0.53 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.13(0.15) | 0.09 (0.14) | 0.24 (0.33) | 0.16 (0.10) | 0.11 (0.11) |
| Median (IQR) | 0.08 (0.01; 0.24) | 0.06 (0.01; 0.09) | 0.12 (0.04; 0.34) | 0.18 (0.09; 0.23) | 0.07 (0.03; 0.23) |
| Min Max | 0.00 0.41 | 0.00 0.44 | 0.00 0.99 | 0.00 0.30 | 0.00 0.28 |
| **POD 1** | | | | | |
| Mean (SD) | 0.11 (0.14) | 0.17 (0.10) | 0.09 (0.09) | 0.04 (0.07) | 0.18 (0.21) |
| Median (IQR) | 0.06 (0.00; 0.18) | 0.13 (0.09; 0.27) | 0.07 (0.01; 0.18) | 0.02 (0.00; 0.07) | 0.09 (0.04; 0.21) |
| Min Max | 0.00 0.39 | 0.04 0.31 | 0.00 0.22 | 0.00 0.19 | 0.02 0.62 |
| **POD 3** | | | | | |
| Mean (SD) | 0.09 (0.08) | 0.18 (0.13) | 0.13 (0.15) | 0.14 (0.16) | 0.24 (0.29) |
| Median (IQR) | 0.06 (0.03; 0.19) | 0.16 (0.08; 0.20) | 0.11 (0.00; 0.24) | 0.09 (0.01; 0.29) | 0.10 (0.04; 0.56) |
| Min Max | 0.00 0.21 | 0.08 0.49 | 0.00 0.40 | 0.00 0.40 | 0.02 0.71 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **CL monocytes ($CD14^+$ $CD16^-$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 6.20 | 9.74 | 15.25 | 8.55 | 18.35 |
| Difference vs placebo (SE) | | 3.54 (4.14) | 9.05 (7.39) | 2.35 (2.80) | 12.15 (4.78) |
| p-value | | 0.4115 | 0.2559 | 0.4213 | **0.0309 *** |
| POD 1 | | | | | |
| Observed mean | 14.42 | 22.98 | 23.25 | 20.83 | 22.29 |
| Difference vs placebo (SE) | | 8.56 (4.92) | 8.83 (4.76) | 6.41 (5.53) | 7.86 (5.99) |
| p-value | | 0.1060 | 0.0869 | 0.2771 | 0.1945 |
| POD 3 | | | | | |
| Observed mean | 14.88 | 24.03 | 17.37 | 16.50 | 26.69 |
| Difference vs placebo (SE) | | 9.15 (4.43) | 2.49 (3.57) | 1.62 (3.65) | 11.81 (5.87) |
| p-value | | 0.0626 | 0.5002 | 0.6665 | 0.0738 |
| **ITM monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.50 | 0.37 | 0.83 | 0.72 | 0.71 |
| Difference vs placebo (SE) | | -0.13 (0.32) | 0.33 (0.42) | 0.22 (0.31) | 0.21 (0.39) |
| p-value | | 0.6847 | 0.4506 | 0.5092 | 0.6049 |
| **POD 1** | | | | | |
| Observed mean | 0.89 | 0.85 | 1.66 | 1.31 | 1.50 |
| Difference vs placebo (SE) | | -0.04 (0.43) | 0.77 (0.57) | 0.42 (0.45) | 0.61(0.79) |
| p-value | | 0.9131 | 0.2012 | 0.3750 | 0.4656 |
| **POD 3** | | | | | |
| Observed mean | 1.65 | 1.01 | 1.70 | 1.01 | 1.86 |
| Difference vs placebo (SE) | | -0.64 (0.89) | 0.05 (0.92) | -0.64 (0.88) | 0.21 (1.10) |
| p-value | | 0.4984 | 0.9591 | 0.4960 | 0.8496 |
| **NC monocytes ($CD14^-$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 3.51 | 2.38 | 5.02 | 2.46 | 3.91 |
| Difference vs placebo (SE) | | -1.13 (1.75) | 1.51 (1.97) | -1.05 (1.66) | 0.40 (2.12) |
| p-value | | 0.5329 | 0.4556 | 0.5429 | 0.8498 |
| **POD 1** | | | | | |
| Observed mean | 10.73 | 6.14 | 10.87 | 10.40 | 8.68 |
| Difference vs placebo (SE) | | -4.59 (4.25) | 0.14 (4.64) | -0.33 (4.25) | -2.05 (5.21) |
| p-value | | 0.3082 | 0.9755 | 0.9402 | 0.7013 |
| **POD 3** | | | | | |
| Observed mean | 4.70 | 5.89 | 8.53 | 6.67 | 5.32 |
| Difference vs placebo (SE) | | 1.19 (3.81) | 3.83 (2.71) | 1.97 (2.32) | 0.62 (3.19) |
| p-value | | 0.7625 | 0.1876 | 0.4161 | 0.8514 |
| **$HO\text{-}1^+$ CL monocytes ($CD14^+$ $CD16^-$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 5.14 | 6.58 | 9.47 | 6.87 | 13.51 |
| Difference vs placebo (SE) | | 1.44 (3.54) | 4.33 (3.80) | 1.73 (2.57) | 8.37 (3.26) |
| p-value | | 0.6923 | 0.2807 | 0.5140 | **0.0260 *** |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Observed mean | 13.26 | 21.27 | 21.42 | 18.32 | 21.86 |
| Difference vs placebo (SE) | | 8.01 (4.81) | 8.16 (4.59) | 5.06 (5.43) | 8.60 (5.91) |
| p-value | | 0.1195 | 0.0990 | 0.3745 | 0.1761 |
| **POD 3** | | | | | |
| Observed mean | 12.89 | 21.64 | 15.37 | 14.07 | 24.02 |
| Difference vs placebo (SE) | | 8.75 (3.91) | 2.48 (3.10) | 1.18 (3.24) | 11.13 (5.29) |
| p-value | | **0.0473 *** | 0.4400 | 0.7233 | 0.0646 |
| **HO-1$^+$ ITM monocytes (CD14$^+$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.40 | 0.24 | 0.52 | 0.94 | 0.63 |
| Difference vs placebo (SE) | | -0.16 (0.31) | 0.12 (0.42) | 0.54 (0.40) | 0.23 (0.39) |
| p-value | | 0.6157 | 0.7919 | 0.2086 | 0.5728 |
| **POD 1** | | | | | |
| Observed mean | 0.71 | 0.80 | 0.98 | 0.63 | 1.45 |
| Difference vs placebo (SE) | | 0.09 (0.36) | 0.28 (0.42) | -0.08 (0.30) | 0.74 (0.76) |
| p-value | | 0.8009 | 0.5258 | 0.7947 | 0.3564 |
| **POD 3** | | | | | |
| Observed mean | 1.61 | 0.98 | 1.06 | 0.53 | 1.82 |
| Difference vs placebo (SE) | | -0.63 (0.88) | -0.55 (0.89) | -1.08 (0.89) | 0.21 (1.09) |
| p-value | | 0.4983 | 0.5563 | 0.2558 | 0.8506 |
| **HO-1$^+$ NC monocytes (CD14$^-$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.13 | 0.09 | 0.24 | 0.16 | 0.11 |
| Difference vs placebo (SE) | | -0.04 (0.08) | 0.11 (0.13) | 0.03 (0.07) | -0.02 (0.07) |
| p-value | | 0.6754 | 0.3882 | 0.6082 | 0.7669 |
| **POD 1** | | | | | |
| Observed mean | 0.11 | 0.17 | 0.09 | 0.04 | 0.18 |
| Difference vs placebo (SE) | | 0.06 (0.06) | -0.02 (0.06) | -0.07 (0.06) | 0.07 (0.09) |
| p-value | | 0.3960 | 0.7540 | 0.3044 | 0.5005 |
| **POD 3** | | | | | |
| Observed mean | 0.09 | 0.18 | 0.13 | 0.14 | 0.24 |
| Difference vs placebo (SE) | | 0.09 (0.06) | 0.04 (0.06) | 0.05 (0.07) | 0.15 (0.11) |
| p-value | | 0.1072 | 0.5581 | 0.4541 | 0.195 |

Abbreviations: CL – classical; ITM – intermediate; NC – non-classical; Lin$^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error.

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 5.2.2.2 Classical monocytes as scavengers of damaged erythroid cells in the post-operative period

To characterise the CL monocyte phenotype further, the proportion of cells expressing surface markers associated with phagocytosis (i.e., CD163, CD69, CD64, CD33) was assessed (**Fig. 70**). A similar statistical approach as described above was used. Time was again found to be the most significant fixed effect within the model for all assayed markers with a p-value < 0.0001, suggesting a temporal relationship between surgery and the phenotypic change observed.

The most significant apparent drug-related effect was observed in $CD68^+$ CL monocytes (as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$) at the pre-operative timepoint (**Fig. 70 C-D**). Here, patients that had received 5-ALA-SFC 300 mg/472 mg had a higher observed mean abundance of $CD68^+$ CL monocytes when compared to placebo (mean [SE] = 19.93 % [4.67]) and this was statistically significant (p-value = 0.0297). In all groups that had been allocated to receive the active treatment, a non-significant but congruent trend for an increase in the expression of $CD68^+$ CL monocytes was observed; this phenotypic commitment continued into the post-operative period and in patients allocated to the highest 5-ALA-SFC dose a mean (SE) of 11.56 (5.81) % higher abundance than the placebo group was observed on the third post-operative day (p-value = 0.0767).

When considering CD163, a marker of specific interest given its defined role in erythrophagocytosis, the data showed a trend to a treatment-related increase in pre-operative expression of $CD163^+$ CL monocytes (as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$) in patients allocated to the highest dose of 5-ALA-SFC (mean difference [SE] vs. placebo = 5.35 [2.65] %) (p-value = 0.0710) (**Fig. 70 A-B**). This A striking increase in the proportion of $CD163^+$ CL monocytes is observed on the first and third post-operative days across all treatment allocation groups. On the third post-operative day there was a sustained increase in this population in the 5-ALA-SFC 300 mg/472 mg treatment allocation group which approached nominal statistical significance (mean difference [SE] vs placebo = 6.16 [3.17] %, p-value = 0.0761).

Treatment with 5-ALA-SFC was associated with an increased proportion of $CD64^+$ and $CD33^+$ CL monocytes both pre-operatively and in a sustained fashion in the post-operative period (**Fig. 70 E-H**).

This signal was most apparent when considering the proportion of $CD64^+$ CL monocytes on the third post-operative day. Patients who had received the lowest 5-ALA-SFC dose had a higher proportion of $CD64^+$ CL monocytes at this time point (mean difference [SE] vs. placebo = 9.78 [4.31] %, p-value = 0.0446). A similar trend was observed in all groups allocated to the active treatment arm, and in the 300 mg/472 mg dose group this was observed to be 9.06 % higher than the placebo group but with wide variance to the data. Findings in the other treatment groups were not statistically significant and no clear 5-ALA-SFC dose-related phenotypic changes were apparent.

Overall, the data demonstrate a significant increase in the proportion of CL monocytes displaying a phagocytic phenotype in response to cardiac surgery. Furthermore, a possible drug-associated increase in the pre-operative expression of CD68 and CD163, with a sustained increase over placebo in the post-operative period, was observed. Pooled data from patients allocated to receive 5-ALA-SFC consistently showed a drug associated shift towards a phagocytic phenotype (**Fig. 70 B,D,F,H**). No post-*hoc* analysis was performed on pooled data.

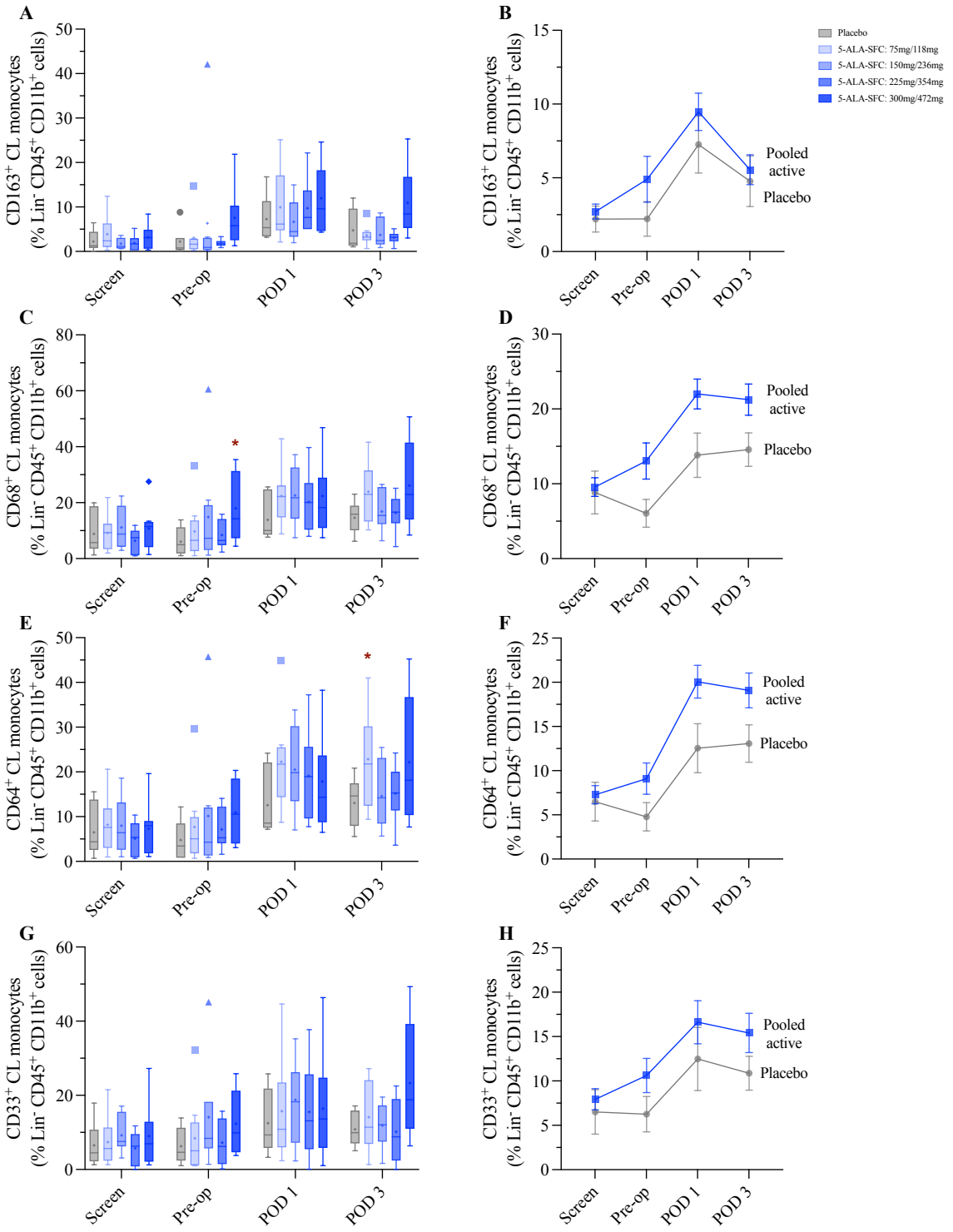



**Figure 70: The impact of 5-ALA-SFC on the ability of classical monocytes to acts as erythrophagocytosis cells.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, C-E) and mean (± SE) (panel B, F). Significance from the LMM using REML in red: * = $p < 0.05$.

CL monocytes - $CD14^+$ $CD16^-$; $Lin^-$ cells – excludes natural killer cells, B-cells and T-cells ($CD3^+$ $CD19^+$ $CD56^+$).

Abbreviations: CL – classical.

**Table 35: Impact of 5-ALA-SFC on expression of surface markers associated with phagocytosis on classical monocytes**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **$CD163^+$ CL monocytes ($CD14^+$ $CD16^-$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 2.21 (2.33) | 3.86 (4.09) | 1.74 (1.28) | 1.89 (1.82) | 3.17 (2.73) |
| Median (IQR) | 1.29 (0.76; 4.41) | 2.39 (1.01; 6.31) | 1.21 (0.67; 3.07) | 1.73 (0.25; 2.98) | 3.13 (0.63; 4.88) |
| Min Max | 0.03 6.45 | 0.28 12.44 | 0.60 3.64 | 0.23 5.17 | 0.32 8.40 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.22 (3.09) | 3.11 (4.80) | 6.32 (14.51) | 1.85 (0.83) | 7.57 (6.74) |
| Median (IQR) | 0.80 (0.32; 3.01) | 1.63 (0.49; 2.87) | 0.92 (0.30; 3.07) | 1.74 (1.31; 2.33) | 5.77 (2.53; 10.31) |
| Min Max | 0.79 8.84 | 0.21 14.69 | 0.16 42.12 | 0.85 3.32 | 1.30 21.86 |
| **POD 1** | | | | | |
| Mean (SD) | 7.26 (5.11) | 9.92 (8.13) | 6.68 (4.67) | 9.73 (6.51) | 11.95 (7.74) |
| Median (IQR) | 5.35 (3.42; 11.33) | 6.17 (4.73; 17.14) | 4.46 (3.27; 11.03) | 7.62 (5.03; 13.72) | 9.59 (4.58; 18.27) |
| Min Max | 3.18 16.78 | 2.15 25.13 | 1.95 14.98 | 5.0 0 22.15 | 4.35 24.62 |
| **POD 3** | | | | | |
| Mean (SD) | 4.77 (4.53) | 3.62 (2.26) | 3.70 (3.18) | 3.09 (1.43) | 10.93 (7.53) |
| Median (IQR) | 1.86 (1.29; 9.62) | 3.25 (2.48; 4.40) | 2.45 (1.56; 7.85) | 3.20 (2.28; 3.97) | 8.43 (5.26; 16.77) |
| Min Max | 1.03 12.01 | 0.67 8.44 | 0.88 8.67 | 0.66 5.08 | 3.02 25.31 |
| **$CD68^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 8.85 (7.56) | 9.19 (6.56) | 11.12 (7.42) | 6.41 (4.44) | 10.76 (8.16) |
| Median (IQR) | 5.71 (3.58; 18.76) | 9.32 (3.34; 12.49) | 8.78 (4.26; 18.92) | 7.48 (1.22; 9.98) | 11.47 (4.09; 13.13) |
| Min Max | 1.30 19.96 | 2.03 21.82 | 2.94 22.39 | 0.96 11.91 | 1.46 27.52 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.05 (4.93) | 9.70 (10.46) | 14.90 (19.55) | 8.42 (5.20) | 17.98 (12.11) |
| Median (IQR) | 4.96 (1.84; 11.22) | 6.51 (2.70; 13.75) | 7.29 (3.04; 19.17) | 6.49 (4.87; 14.17) | 14.29 (7.31; 31.31) |
| Min Max | 1.04 13.88 | 1.07 33.11 | 1.25 60.61 | 2.36 15.91 | 4.41 35.37 |
| **POD 1** | | | | | |
| Mean (SD) | 13.82 (7.84) | 22.37 (10.18) | 22.60 (10.12) | 20.30 (11.24) | 22.37 (13.54) |
| Median (IQR) | 10.03 (8.58; 24.73) | 22.16 (14.75; 26.15) | 21.78 (14.29; 32.64) | 20.09 (10.34; 27.03) | 18.26 (10.97; 46.81) |
| Min Max | 7.68 25.66 | 8.81 42.82 | 7.50 37.14 | 7.94 39.75 | 7.49 46.81 |
| **POD 3** | | | | | |
| Mean (SD) | 14.57 (5.92) | 23.93 (10.70) | 16.81 (7.14) | 16.37 (6.93) | 26.13 (15.16) |
| Median (IQR) | 15.87 (10.15; 18.94) | 23.00 (13.26; 31.51) | 15.42 (12.31; 25.54) | 16.66 (12.60; 21.34) | 22.95 (13.96; 41.49) |
| Min Max | 6.22 23.05 | 10.29 41.63 | 6.40 26.51 | 4.29 25.17 | 8.42 50.68 |
| **$CD64^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 6.50 (5.79) | 8.20 (6.42) | 7.97 (6.23) | 5.11 (3.80) | 7.29 (6.06) |
| Median (IQR) | 4.396 (2.56; 13.82) | 7.59 (2.99; 11.81) | 6.41 (2.58; 13.20) | 5.34 (0.93; 8.53) | 7.97 (1.80; 9.04) |
| Min Max | 0.71 15.55 | 0.97 20.62 | 1.03 18.61 | 0.69 10.34 | 1.07 19.61 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 4.78 (4.23) | 7.70 (9.43) | 10.15 (15.04) | 7.13 (4.72) | 10.93 (6.90) |
| Median (IQR) | 3.43 (0.82; 8.50) | 5.05 (1.79; 9.91) | 4.32 (1.29; 12.02) | 5.29 (4.03; 12.26) | 10.54 (4.02; 18.54) |
| Min Max | 0.82 12.19 | 0.71 29.57 | 0.86 45.76 | 1.61 14.09 | 3.08 20.34 |
| **POD 1** | | | | | |
| Mean (SD) | 12.55 (7.33) | 22.26 (10.84) | 20.52 (9.28) | 19.14 (10.47) | 17.83 (11.06) |
| Median (IQR) | 8.59 (7.51; 22.14) | 21.75 (14.31; 25.53) | 19.85 (13.44; 30.27) | 18.95 (9.57; 25.62) | 14.32 (8.76; 23.69) |
| Min Max | 7.17 24.20 | 8.73 44.87 | 7.03 33.83 | 7.78 37.22 | 6.51 38.24 |
| **POD 3** | | | | | |
| Mean (SD) | 13.07 (5.58) | 22.85 (10.62) | 14.60 (7.45) | 15.19 (6.81) | 22.15 (14.04) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Median (IQR) | 14.62 (7.94; 17.45) | 21.77 (12.42; 30.21) | 14.19 (8.44; 23.15) | 15.32 (11.36; 20.06) | 18.14 (10.36; 36.71) |
| Min Max | 5.55 20.85 | 9.43 40.99 | 5.66 25.48 | 3.64 24.26 | 7.67 45.24 |
| **$CD33^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 6.51 (6.11) | 7.40 (6.74) | 9.20 (5.15) | 5.72 (4.48) | 9.00 (8.72) |
| Median (IQR) | 4.50 (2.19; 10.75) | 5.68 (2.37; 11.34) | 7.53 (6.21; 15.59) | 6.27 (0.85; 9.58) | 6.92 (2.13; 12.88) |
| Min Max | 1.30 17.90 | 1.27 21.53 | 3.14 17.09 | 0.02 11.74 | 1.23 27.26 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.25 (4.89) | 8.41 (10.61) | 14.10 (14.76) | 7.23 (6.14) | 12.31 (8.46) |
| Median (IQR) | 4.65 (2.41; 11.28) | 5.07 (1.14; 12.73) | 8.41 (5.66; 18.27) | 6.24 (1.44; 13.77) | 9.87 (4.63; 21.27) |
| Min Max | 1.04 13.88 | 1.01 32.15 | 1.42 45.15 | 0.16 15.75 | 3.78 25.82 |
| **POD 1** | | | | | |
| Mean (SD) | 12.48 (8.73) | 15.72 (13.91) | 18.75 (11.29) | 15.51 (13.46) | 16.45 (15.22) |
| Median (IQR) | 9.31 (5.83; 21.84) | 10.86 (5.96; 23.50) | 18.26 (7.20; 26.25) | 13.15 (5.47; 25.70) | 13.64 (5.83; 24.75) |
| Min Max | 3.30 25.80 | 2.36 44.62 | 2.31 35.25 | 0.07 37.72 | 1.02 46.37 |
| **POD 3** | | | | | |
| Mean (SD) | 10.87 (4.68) | 14.09 (9.28) | 11.88 (6.35) | 10.21 (9.28) | 23.28 (15.72) |
| Median (IQR) | 9.92 (7.03; 15.90) | 11.42 (6.88; 24.09) | 12.05 (7.51; 17.32) | 8.82 (2.39; 19.05) | 18.80 (10.99; 39.26) |
| Min Max | 5.07 17.13 | 1.33 27.22 | 1.64 19.54 | 0.00 22.52 | 6.37 49.32 |
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **$CD163^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 2.22 | 3.11 | 6.32 | 1.85 | 7.57 |
| Difference vs placebo (SE) | | 0.89 (2.06) | 4.10 (5.26) | -0.37 (1.22) | 5.35 (2.65) |
| p-value | | 0.6723 | 0.4583 | 0.7740 | 0.0710 |
| **POD 1** | | | | | |
| Observed mean | 7.26 | 9.92 | 6.68 | 9.73 | 11.95 |
| Difference vs placebo (SE) | | 2.66 (3.46) | -0.58 (2.54) | 2.47 (3.28) | 4.69 (3.50) |
| p-value | | 0.4574 | 0.8219 | 0.4714 | 0.2092 |
| **POD 3** | | | | | |
| Observed mean | 4.77 | 3.62 | 3.70 | 3.09 | 10.93 |
| Difference vs placebo (SE) | | -1.14 (1.89) | -10.7 (2.09) | -1.68 (1.81) | 6.16 (3.17) |
| p-value | | 0.5596 | 0.6177 | 0.3831 | 0.0761 |
| **$CD68^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 6.05 | 9.70 | 14.90 | 8.42 | 17.98 |
| Difference vs placebo (SE) | | 3.65 (4.14) | 8.85 (7.16) | 2.37 (2.82) | 11.93 (4.67) |
| p-value | | 0.3992 | 0.2516 | 0.4202 | **0.0297 *** |
| **POD 1** | | | | | |
| Observed mean | 13.82 | 22.37 | 22.60 | 20.36 | 22.37 |
| Difference vs placebo (SE) | | 8.55 (4.66) | 8.78 (4.65) | 6.48 (5.46) | 8.55 (5.91) |
| p-value | | 0.0902 | 0.0818 | 0.2671 | 0.1801 |
| **POD 3** | | | | | |
| Observed mean | 14.57 | 23.93 | 16.81 | 16.37 | 26.13 |
| Difference vs placebo (SE) | | 9.36 (4.40) | 2.24 (3.51) | 1.80 (3.61) | 11.56 (5.81) |
| p-value | | 0.0562 | 0.5358 | 0.6283 | 0.0767 |
| **$CD64^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Pre-operatively** | | | | | |
| Observed mean | 4.78 | 7.70 | 10.15 | 7.13 | 10.93 |
| Difference vs placebo (SE) | | 2.92 (3.70) | 5.37 (5.55) | 2.35 (2.51) | 6.15 (2.92) |
| p-value | | 0.4485 | 0.3611 | 0.3702 | 0.0573 |
| **POD 1** | | | | | |
| Observed mean | 12.55 | 22.26 | 20.52 | 19.14 | 17.83 |
| Difference vs placebo (SE) | | 9.71 (4.73) | 7.97 (4.30) | 6.59 (5.09) | 5.28 (5.02) |
| p-value | | 0.0620 | 0.0867 | 0.2289 | 0.3162 |
| **POD 3** | | | | | |
| Observed mean | 13.07 | 22.85 | 14.60 | 15.19 | 22.15 |
| Difference vs placebo (SE) | | 9.78 (4.31) | 1.53 (3.52) | 2.12 (3.49) | 9.06 (5.39) |
| p-value | | **0.0446 *** | 0.6730 | 0.5582 | 0.1255 |
| **$CD33^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 6.25 | 8.41 | 14.10 | 7.23 | 12.31 |
| Difference vs placebo (SE) | | 2.16 (4.25) | 7.85 (5.93) | 0.98 (3.20) | 6.06 (3.60) |
| p-value | | 0.6218 | 0.2240 | 0.7675 | 0.1187 |
| **POD 1** | | | | | |
| Observed mean | 12.48 | 15.72 | 18.75 | 15.51 | 16.45 |
| Difference vs placebo (SE) | | 3.24 (6.07) | 6.27 (5.56) | 3.03 (6.55) | 3.97 (6.77) |
| p-value | | 0.6040 | 0.2835 | 0.6559 | 0.5709 |
| **POD 3** | | | | | |
| Observed mean | 10.87 | 14.09 | 11.88 | 10.21 | 23.28 |
| Difference vs placebo (SE) | | 3.22 (3.80) | 1.01 (3.22) | -0.66 (4.24) | 12.41 (5.88) |
| p-value | | 0.4147 | 0.7598 | 0.8798 | 0.065 |

Abbreviations: CL – classical; ITM – intermediate; NC – non-classical; $Lin^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error.

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 5.2.2.3 The impact of 5-ALA-SFC on CL monocyte activation state and antigen processing

Next, the impact of the drug on CL monocyte activation and antigen processing ability was assessed. In all patients allocated to the active 5-ALA-SFC treatment arm, the proportion of HLA-DR$^+$ CL monocytes was increased at the pre-operative time point vs. placebo (**Fig. 71 A-B**);this change from baseline was most marked in the 5-ALA-SFC 300 mg/472 mg treatment allocation group (mean [SE] vs. placebo = 0.63 [2.56] %, p-value = 0.0607). By the third post operative day, although data from patients allocated to the highest 5-ALA-SFC dose still had a higher proportion of HLA-DR$^+$ cells; this was less marked than at the pre-operative timepoint and trending downwards. No clear dose-related changes in HLA-DR expression were observed. No apparent 5-ALA-SFC dose-related differences in the proportion of CD86$^+$ CL monocytes was observed (**Fig 71 C-D**).

The proportion of CCR2$^+$ CL monocytes increased in response to surgery in all patients and within the statistical model time was identified as the most significant fixed effect (p-value < 0.0001) (**Fig. 71 E-F**). A similar temporal relationship to surgery was observed in CD11c$^+$ CL monocytes, but this was not statistically significant (p = 0.0582) (**Fig. 71 G-H**). A treatment-associated increase in the proportion of CCR2$^+$ CL monocytes was demonstrated at the pre-operative timepoint, potentially reflecting a drug-related response. In the highest dose allocation group the mean (SE) difference vs placebo was 1.79 (0.59) % which was statistically significant (p-value = 0.0157). The phenotypic commitment towards CCR2$^+$ cells was sustained on the first post-operative day, but by the third day after surgery this was less clear. There was a non-significant increase in CD11c$^+$ CL monocytes in 5-ALA-SFC treated groups compared to placebo pre-operatively and in the post-operative period. Again, patients allocated to the 300 mg/472 mg treatment allocation group had the most marked increase (mean difference [SE] vs. placebo = 6.07 % [2.97], p-value = 0.0716).

Pooled data suggest a treatment-related phenotypic shift, favouring CL monocyte expression of HLA-DR, CCR2 and CD11c, and less markedly CD86.

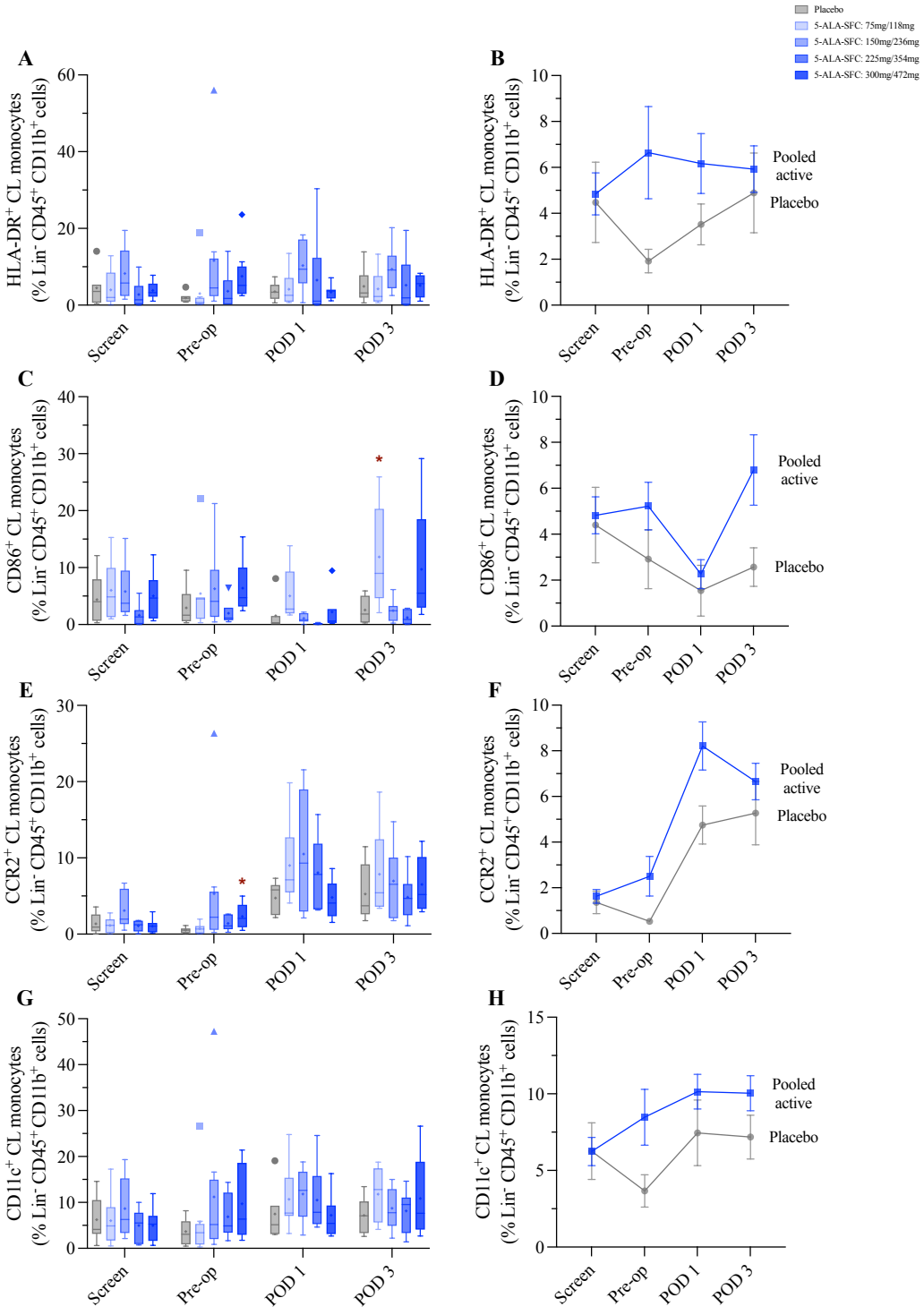



**Figure 71: Monocyte activation state and antigen processing.,**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, C,E,G) and mean (± SE) (panel B,D,F,H). Significance from the LMM using REML in red: * = $p < 0.05$.

CL monocytes - $CD14^+$ $CD\ 16^-$; $Lin^-$ cells – excludes natural killer cells, B-cells and T-cells ($CD3^+$ $CD19^+$ $CD56^+$).

Abbreviations: CL – classical.

**Table 36: Impact of 5-ALA-SFC on markers of activation and antigen processing in classical monocytes**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **HLA-DR$^+$ CL monocytes (CD14$^+$ CD16$^-$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 4.48 (4.62) | 4.00 (4.87) | 8.26 (6.78) | 2.75 (3.78) | 3.86 (2.20) |
| Median (IQR) | 3.56 (0.66; 5.38) | 2.00 (0.90; 8.50) | 5.77 (2.36; 14.24) | 1.40 (0.25; 5.05) | 3.29 (2.32; 5.61) |
| Min Max | 0.28 14.05 | 0.07 12.87 | 1.54 19.50 | 0.03 9.94 | 1.03 7.79 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.92 (1.36) | 3.04 (6.41) | 11.58 (18.41) | 3.65 (5.32) | 7.55 (7.09) |
| Median (IQR) | 1.79 (0.93; 2.34) | 0.70 (0.23; 1.96) | 4.51 (2.32; 12.16) | 1.75 (0.19; 6.52) | 5.17 (2.94; 10.08) |
| Min Max | 0.77 4.70 | 0.12 18.80 | 1.05 56.06 | 0.17 14.07 | 2.46 23.62 |
| **POD 1** | | | | | |
| Mean (SD) | 3.52 (2.36) | 4.15 (4.52) | 10.35 (6.26) | 6.52 (11.93) | 3.39 (2.02) |
| Median (IQR) | 3.39 (1.66; 5.30) | 2.58 (0.89; 7.16) | 9.40 (5.68; 17.14) | 1.05 (0.17; 12.39) | 3.70 (1.76; 4.01) |
| Min Max | 0.61 7.38 | 0.62 13.49 | 0.64 18.29 | 0.05 30.38 | 1.16 7.15 |
| **POD 3** | | | | | |
| Mean (SD) | 4.89 (4.59) | 4.24 (4.53) | 9.40 (6.15) | 5.19 (7.55) | 5.13 (2.81) |
| Median (IQR) | 3.16 (1.96; 7.81) | 2.26 (0.96; 7.66) | 9.17 (4.41; 12.92) | 1.89 (0.14; 10.61) | 5.60 (2.00; 7.79) |
| Min Max | 0.60 13.89 | 0.60 13.26 | 2.54 20.22 | 0.03 19.50 | 1.05 8.28 |
| **CD86$^+$ CL monocytes (CD14$^+$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 4.40 (4.34) | 6.01 (5.14) | 5.82 (4.77) | 1.65 (2.01) | 5.02 (4.08) |
| Median (IQR) | 4.01 (0.66; 7.93) | 4.88 (1.32; 9.96) | 3.73 (2.17; 9.49) | 1.34 (0.07; 2.56) | 4.66 (1.05; 7.82) |
| Min Max | 0.33 12.10 | 0.99 15.31 | 1.62 15.14 | 0.03. 5.06 | 0.65 12.25 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.91 (3.40) | 5.43 (7.00) | 6.28 (6.92) | 1.97 (2.23) | 6.40 (4.61) |
| Median (IQR) | 1.63 (0.58; 5.34) | 4.47 (1.01; 4.79) | 4.07 (1.34; 9.65) | 1.12 (0.74; 2.98) | 4.72 (3.15; 10.01) |
| Min Max | 0.32 9.55 | 0.32 22.11 | 0.47 21.24 | 0.50 6.41 | 2.43 15.40 |
| **POD 1** | | | | | |
| Mean (SD) | 1.54 (2.92) | 5.04 (4.69) | 1.11 (0.82) | 0.15 (0.12) | 2.22 (3.31) |
| Median (IQR) | 0.32 (0.07; 1.51) | 2.71 (1.95; 9.32) | 0.82 (0.53; 2.01) | 0.14 (0.06; 0.23) | 0.63 (0.33; 2.74) |
| Min Max | 0.07 8.08 | 1.67 13.85 | 0.00 2.17 | 0.00 0.35 | 0.23 9.45 |
| **POD 3** | | | | | |
| Mean (SD) | 2.57 (2.22) | 11.88 (8.85) | 2.43 (1.96) | 1.24 (1.30) | 9.69 (10.24) |
| Median (IQR) | 1.86 (0.34; 5.05) | 8.99 (4.62; 20.33) | 2.43 (0.61; 3.27) | 0.88 (0.07; 2.77) | 5.49 (2.97; 18.51) |
| Min Max | 0.29 5.92 | 2.09 25.92 | 0.27 6.15 | 0.00 2.87 | 1.77 29.15 |
| **CCR2$^+$ CL monocytes (CD14$^+$ CD16$^+$) as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 1.36 (1.31) | 1.15 (0.99) | 3.08 (2.43) | 1.08 (0.71) | 1.06 (0.91) |
| Median (IQR) | 0.93 (0.36; 2.58) | 1.15 (0.13; 1.94) | 1.98 (1.30; 5.96) | 1.23 (0.33; 1.76) | 1.05 (0.23; 1.47) |
| Min Max | 0.03 3.57 | 0.02 2.79 | 0.53 6.69 | 0.04 1.80 | 0.16 2.93 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.53 (0.37) | 0.71 (0.66) | 5.30 (8.75) | 1.40 (1.00) | 2.32 (1.61) |
| Median (IQR) | 0.53 (0.14; 0.74) | 0.66 (0.08; 1.07) | 2.22 (0.55; 5.58) | 1.08 (0.65; 2.60) | 2.08 (0.89; 3.84) |
| Min Max | 0.11 1.14 | 0.01 1.96 | 0.21 26.36 | 0.23 2.64 | 0.50 5.00 |
| **POD 1** | | | | | |
| Mean (SD) | 4.75 (2.20) | 9.00 (5.60) | 10.51 (7.74) | 8.07 (4.72) | 4.81 (2.52) |
| Median (IQR) | 5.79 (2.49; 6.43) | 7.13 (5.47; 12.72) | 9.32 (2.96; 18.98) | 7.83 (3.28; 11.88) | 4.08 (2.36; 6.63) |
| Min Max | 2.17 7.32 | 4.09 19.86 | 2.11 21.56 | 3.17 15.70 | 1.53 8.60 |
| **POD 3** | | | | | |
| Mean (SD) | 5.27 (3.68) | 7.86 (5.60) | 6.98 (4.64) | 4.83 (3.05) | 6.52 (3.61) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Median (IQR) | 3.72 (2.60; 9.15) | 5.43 (3.58; 12.46) | 6.54 (2.06; 10.04) | 4.68 (2.47; 6.60) | 5.21 (3.31; 10.13) |
| Min Max | 1.77 11.47 | 3.40 18.64 | 1.76 14.74 | 1.11 10.17 | 2.93 12.19 |
| **$CD11c^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 6.26 (4.89) | 6.05 (5.57) | 8.67 (6.41) | 4.96 (3.58) | 4.96 (3.69) |
| Median (IQR) | 4.12 (3.14; 10.54) | 4.90 (1.71; 9.01) | 6.27 (3.34; 15.28) | 5.53 (0.90; 7.77) | 5.25 (1.64; 7.08) |
| Min Max | 0.63 14.58 | 0.54 17.27 | 2.15 19.31 | 0.75 10.00 | 0.65 11.92 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.66 (2.79) | 5.68 (8.70) | 11.22 (15.45) | 6.86 (4.92) | 9.73 (7.86) |
| Median (IQR) | 3.05 (0.88; 5.91) | 3.43 (0.82; 5.37) | 5.20 (2.00; 47.27) | 4.87 (3.41; 12.15) | 6.38 (2.93; 18.59) |
| Min Max | 0.47 8.18 | 0.32 26.70 | 0.86 47.27 | 1.66 14.36 | 1.75 21.41 |
| **POD 1** | | | | | |
| Mean (SD) | 7.46 (5.67) | 10.70 (6.92) | 11.84 (5.49) | 10.54 (7.46) | 7.23 (4.74) |
| Median (IQR) | 5.15 (3.12; 9.31) | 7.68 (7.07; 15.39) | 12.62 (6.88; 16.71) | 7.86 (5.26; 15.78) | 5.44 (3.10; 9.33) |
| Min Max | 3.05 19.06 | 3.25 24.80 | 2.88 18.81 | 4.63 24.58 | 2.68 16.26 |
| **POD 3** | | | | | |
| Mean (SD) | 7.18 (3.79) | 11.78 (5.80) | 8.72 (4.64) | 8.16 (4.72) | 10.86 (8.66) |
| Median (IQR) | 7.10 (3.32; 10.24) | 12.82 (5.92; 17.43) | 7.76 (4.90; 12.92) | 9.54 (3.34; 11.05) | 7.65 (4.10; 18.86) |
| Min Max | 2.58 13.44 | 4.14 18.78 | 2.21 15.01 | 1.41 14.63 | 2.70 26.60 |
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **HLA-$DR^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 1.92 | 3.04 | 11.58 | 3.65 | 7.55 |
| Difference vs placebo (SE) | | 1.12 (2.32) | 9.66 (6.53) | 1.73 (2.23) | 5.63 (2.56) |
| p-value | | 0.6416 | 0.1817 | 0.4685 | 0.0607 |
| **POD 1** | | | | | |
| Observed mean | 3.52 | 4.15 | 10.35 | 6.52 | 3.39 |
| Difference vs placebo (SE) | | 0.63 (1.83) | 6.83 (2.38) | 3.00 (4.95) | -0.13 (1.17) |
| p-value | | 0.7377 | 0.0182 | 0.5686 | 0.9165 |
| **POD 3** | | | | | |
| Observed mean | 4.89 | 4.24 | 9.40 | 5.19 | 5.13 |
| Difference vs placebo (SE) | | -0.65 (2.36) | 4.51 (2.90) | 0.30 (3.54) | 0.24 (2.00) |
| p-value | | 0.7878 | 0.1480 | 0.9340 | 0.9059 |
| **$CD86^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 2.91 | 5.43 | 6.28 | 1.97 | 6.40 |
| Difference vs placebo (SE) | | 2.52 (2.79) | 3.37 (2.76) | -0.96 (1.57) | 3.49 (2.07) |
| p-value | | 0.3874 | 0.2506 | 0.5605 | 0.1171 |
| **POD 1** | | | | | |
| Observed mean | 1.54 | 5.04 | 1.11 | 0.15 | 2.22 |
| Difference vs placebo (SE) | | 3.50 (1.99) | -0.43 (1.14) | -1.39 (1.11) | 0.68 (1.67) |
| p-value | | 0.1055 | 0.7159 | 0.2543 | 0.6927 |
| **POD 3** | | | | | |
| Observed mean | 2.57 | 11.88 | 2.43 | 1.24 | 9.69 |
| Difference vs placebo (SE) | | 9.31 (3.24) | -0.14 (1.12) | -1.33 (0.99) | 7.12 (3.72) |
| p-value | | **0.0206 *** | 0.9039 | 0.2121 | 0.0929 |
| **$CCR2^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Pre-operatively** | | | | | |
| Observed mean | 0.53 | 0.71 | 5.30 | 1.40 | 2.32 |
| Difference vs placebo (SE) | | 0.18 (0.27) | 4.76 (3.10) | 0.87 (0.43) | 1.79 (0.59) |
| p-value | | 0.5105 | 0.1666 | 0.0891 | **0.0157 *** |
| **POD 1** | | | | | |
| Observed mean | 4.75 | 9.00 | 10.51 | 8.07 | 4.81 |
| Difference vs placebo (SE) | | 4.25 (2.07) | 5.76 (2.86) | 3.32 (2.10) | 0.06 (1.27) |
| p-value | | 0.0684 | 0.0776 | 0.1582 | 0.9643 |
| **POD 3** | | | | | |
| Observed mean | 5.27 | 7.86 | 6.98 | 4.83 | 6.52 |
| Difference vs placebo (SE) | | 2.59 (2.42) | 1.72 (2.24) | -0.44 (1.87) | 1.25 (1.89) |
| p-value | | 0.3053 | 0.4590 | 0.8181 | 0.5186 |
| **$CD11c^+$ CL monocytes ($CD14^+$ $CD16^+$) as a proportion of $Lin^-$ $CD45^+$ $CD11b^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 3.66 | 5.68 | 11.22 | 6.86 | 9.73 |
| Difference vs placebo (SE) | | 2.02 (3.25) | 7.56 (5.56) | 3.20 (2.27) | 6.07 (2.97) |
| p-value | | 0.5511 | 0.2139 | 0.1978 | 0.0716 |
| **POD 1** | | | | | |
| Observed mean | 7.46 | 10.70 | 11.84 | 10.54 | 7.23 |
| Difference vs placebo (SE) | | 3.24 (3.25) | 4.38 (2.89) | 3.06 (3.72) | -0.23 (2.79) |
| p-value | | 0.3360 | 0.1540 | 0.4282 | 0.9382 |
| **POD 3** | | | | | |
| Observed mean | 7.18 | 11.78 | 8.72 | 8.16 | 10.86 |
| Difference vs placebo (SE) | | 4.60 (2.50) | 1.54 (2.27) | 0.98 (2.40) | 3.68 (3.38) |
| p-value | | 0.0903 | 0.5089 | 0.6910 | 0.3023 |

Abbreviations: CL – classical; $Lin^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 5.2.2.4 A novel gating strategy to identify bulk Lin$^-$ immune cells expressing HO-1

Considering that bioinformatics analyses of human RNAseq data highlighted that monocytes enrich for both HO-1 and CD68, a gating strategy focussing on CD68 and HO-1 double positive cells was investigated as a means of identifying immune cell subsets in which to more precisely study the putative effects of 5-ALA-SFC on HO-1 biology. Co-expression of CD68 and HO-1 was apparent in Lin$^-$ CD45$^+$ cells. Using representative contour plots of flow cytometry data from a post-operative sample, CD68$^+$ HO-1$^+$ cells were further analysed based on the presence of CD14 and CD16 (**Fig. 72 A**). This demonstrated that, although most cells expressing both CD68 and HO-1 were CL monocytes, there was a reasonably sized CD14$^-$ CD16$^-$ population (19.1% in this sample), pointing to a substantial non-monocyte population which expresses HO-1. CD68$^-$ HO-1$^-$ cells were predominantly CD14$^-$ CD16$^-$ (64 %), and a smaller proportion of these cells were found to be NC monocytes (34.7%). When this CD68-based gating strategy was applied to investigate the impact of 5-ALA-SFC on these cells, a large increase in the HO-1$^+$ CD68$^+$ population is appreciated (**representative flow cytometry traces in Fig. 72 B**). Comparing the 5-ALA-SFC 300 mg/472 mg sample to placebo, this population increased from 7.21 % of Lin$^-$ CD45$^+$ cells at the screening timepoint to 17.8% (vs. 12.8 % in placebo) and rose to 33.8 % (vs.19.3% in placebo), by the third post-operative day.

Considering data from all TALEN trial participants, an increase in the proportion of Lin$^-$ CD45$^+$ cells expressing CD68, and HO-1 is appreciated at the pre-operative time point in patients allocated to the active intervention arm. Across the model, this was most apparent in patients allocated to the highest dose of 5-ALA-SFC (mean difference [SE] vs. placebo = 12.09 [4.47] %, p-value = 0.0197) (**Fig. 73 A**), suggesting a treatment related effect at this dose. Interestingly, this was accompanied by a reciprocal and statistically significant treatment-related decrease in the proportion of CD68$^-$ HO-1$^-$ cells in patients receiving the highest 5-ALA-SFC dose (mean difference [SE] vs. placebo = -18.51 [6.46] %, p-value = 0.0163) (Fig. 73 C). This suggests a transient population of cells, capable of assuming a CD68$^+$ HO-1$^+$ in response to an appropriate stimulus (e.g., surgery). This phenotypic commitment of Lin$^-$ CD45$^+$ cells persisted into the post-operative period, and pooled data suggested a possible 5-ALA-SFC-associated enhancement of this cell population (**Fig. 73 B**).

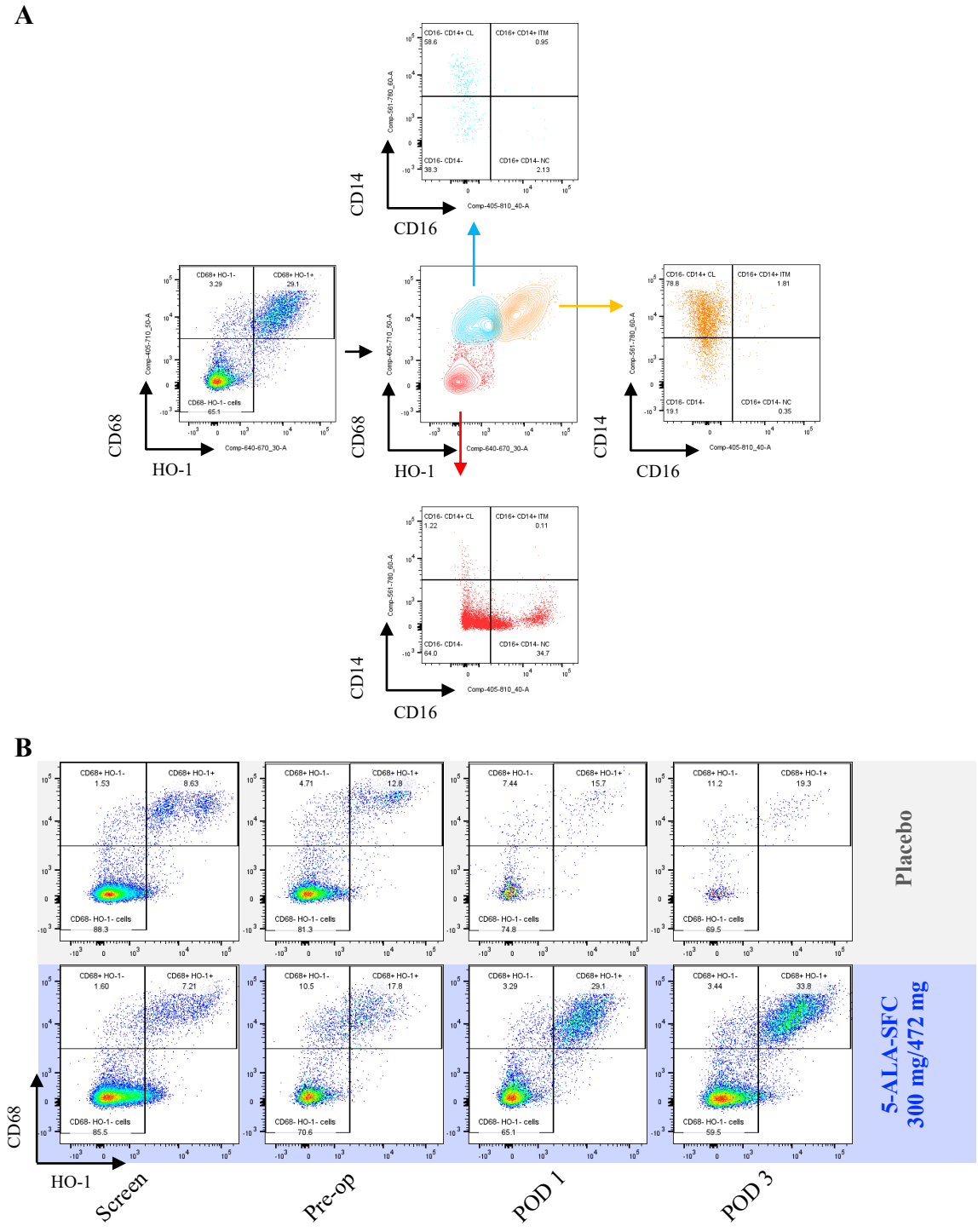



**Figure 72: Characterising novel immune cell subsets expressing HO-1.**

Panel A – representative flow cytometry trace and contour analysis showing that most $CD68^{+}$ $HO\text{-}1^{+}$/$Lin^{-}$ $CD45^{+}$ (in orange) were CL monocytes (78.8%) and non-monocyte (19.9%). Most $CD68^{-}$ $HO\text{-}1^{-}$/ $Lin^{-}$ $CD45^{+}$ (in red) were non-monocyte (64%) and NC monocyte (34.7%).

Panel B – representative flow cytometry traces showing an increase in $CD68^{+}$ $HO\text{-}1^{+}$/$Lin^{-}$ $CD45^{+}$ and a possible treatment-related difference (5-ALA-SFC 300 mg/472 mg vs. placebo).

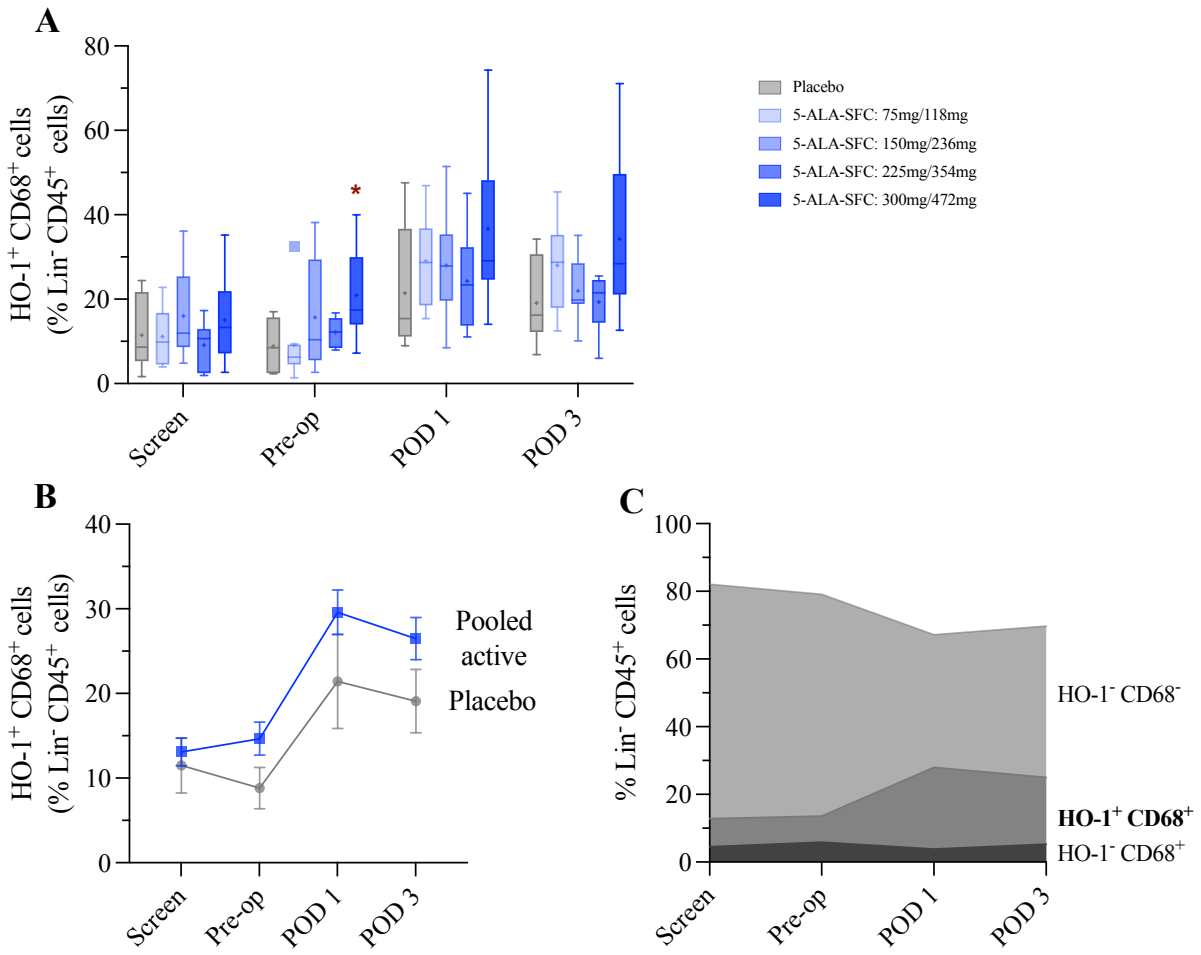



**Figure 73: The impact of 5-ALA-SFC on HO-1 expression in CD68+ cells – a novel marker of drug effect.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A), mean (± SE) (panel B) and mean (panel C). Significance from the LMM using REML in red: * = $p < 0.05$.

$Lin^-$ cells – excludes natural killer cells, B-cells and T-cells ($CD3^+$ $CD19^+$ $CD56^+$).

## Table 37: HO-1$^+$ CD68$^+$ immune cells as candidate cells for PD marker of drug effect.

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **HO-1$^+$ CD68$^+$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 11.49 (8.59) | 11.12 (6.89) | 16.04 (10.79) | 9.12 (5.85) | 15.10 (10.50) |
| Median (IQR) | 8.63 (5.34; 21.72) | 9.84 (4.49; 16.80) | 11.96 (8.66; 25.44) | 10.65 (2.48; 12.98) | 13.33 (7.12; 21.89) |
| Min Max | 1.66 24.41 | 3.94 22.76 | 4.83 36.11 | 1.92 17.28 | 2.64 21.89 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 8.82 (6.43) | 9.24 (9.74) | 15.69 (13.23) | 12.16 (3.54) | 20.91 (10.62) |
| Median (IQR) | 8.51 (24.9; 15.65) | 6.28 (4.49; 9.27) | 10.40 (5.51; 29.42) | 12.25 (8.40; 15.56) | 17.44 (13.99; 29.98) |
| Min Max | 2.33 17.07 | 1.38 32.44 | 2.70 38.18 | 7.98 16.78 | 7.25 40.00 |
| **POD 1** | | | | | |
| Mean (SD) | 21.43 (14.70) | 29.03 (10.63) | 28.00 (12.77) | 24.27 (12.03) | 36.68 (20.13) |
| Median (IQR) | 15.43 (11.15; 36.71) | 28.66 (18.56; 36.83) | 27.89 (19.64; 35.36) | 23.41 (13.69; 32.33) | 29.08 (24.63; 48.21) |
| Min Max | 8.99 47.59 | 15.38 46.92 | 8.48 51.46 | 11.07 45.06 | 14.03 48.74.29 |
| **POD 3** | | | | | |
| Mean (SD) | 19.10 (9.94) | 28.01 (10.78) | 21.96 (7.92) | 19.34 (7.13) | 34.27 (19.40) |
| Median (IQR) | 16.20 (12.25; 30.67) | 28.73 (17.92; 35.23) | 19.82 (18.85; 28.54) | 21.52 (14.41; 24.57) | 28.40 (21.12; 49.66) |
| Min Max | 6.89 34.25 | 12.50 45.39 | 10.15 35.15 | 6.01 25.49 | 12.61 71.09 |
| **HO-1$^-$ CD68$^+$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 3.67 (2.69) | 4.69 (2.49) | 4.60 (3.26) | 4.48 (1.89) | 4.94 (2.92) |
| Median (IQR) | 2.69 (1.54; 5.46) | 3.87 (3.51; 4.19) | 3.78 (1.86; 6.45) | 5.01 (2.91; 5.95) | 4.92 (1.90; 7.61) |
| Min Max | 1.05 5.46 | 3.44 10.79 | 1.26 11.13 | 1.17 6.35 | 1.57 9.38 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.20 (2.04) | 7.23 (6.20) | 4.13 (3.27) | 4.79 (2.13) | 9.17 (8.53) |
| Median (IQR) | 4.42 (1.27; 4.73) | 4.21 (2.13; 13.09) | 2.78 (1.05; 8.21) | 4.21 (3.23; 6.90) | 5.65 (3.13; 14.26) |
| Min Max | 0.48 5.49 | 1.11 17.43 | 0.86 8.86 | 2.22 7.99 | 2.37 27.12 |
| **POD 1** | | | | | |
| Mean (SD) | 2.83 (2.24) | 4.79 (1.40) | 3.73 (2.09) | 5.53 (5.15) | 2.52 (0.85) |
| Median (IQR) | 2.35 (1.11; 3.56) | 5.03 (3.39; 5.96) | 3.27 (1.87; 5.60) | 3.02 (2.27; 9.98) | 2.47 (1.74; 3.29) |
| Min Max | 0.96 7.44 | 2.50 6.33 | 1.40 6.84 | 1.41 14.81 | 1.31 3.67 |
| **POD 3** | | | | | |
| Mean (SD) | 5.16 (3.46) | 5.67 (1.88) | 4.75 (2.56) | 5.77 (2.37) | 4.96 (3.11) |
| Median (IQR) | 4.35 (2.40; 7.44) | 5.53 (4.47; 6.27) | 3.82 (2.69; 6.54) | 5.48 (3.67; 7.48) | 3.93 (3.51; 6.48) |
| Min Max | 1.49 11.21 | 3.23 9.52 | 2.49 9.62 | 3.28 9.86 | 1.29 11.57 |
| **HO-1$^-$ CD68$^-$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 83.51 (11.33) | 82.94 (7.23) | 78.25 (13.25) | 85.64 (7.50) | 78.00 (12.02) |
| Median (IQR) | 88.46 (70.49; 92.24) | 81.29 (78.14; 90.14) | 82.92 (63.95; 88.86) | 83.94 (80.33; 93.30) | 78.68 (73.14; 85.47) |
| Min Max | 65.59 94.86 | 72.36 92.03 | 56.67 91.23 | 75.44 96.27 | 54.40 95.02 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 86.83 (7.84) | 82.03 (13.50) | 74.48 (24.42) | 81.95 (3.76) | 68.32 (16.24) |
| Median (IQR) | 88.95 (77.69; 94.75) | 86.04 (77.24; 90.99) | 85.13 (60.53; 91.64) | 82.62 (78.59; 85.06) | 7348 (51.11; 80.51) |
| Min Max | 77.64 94.75 | 43.08 95.56 | 22.15 93.42 | 75.98 86.39 | 43.53 88.26 |
| **POD 1** | | | | | |
| Mean (SD) | 72.95 (15.84) | 63.44 (12.22) | 66.63 (14.35) | 68.52 (13.55) | 58.04 (20.92) |
| Median (IQR) | 77.86 (59.44; 85.52) | 63.09 (55.01; 75.22) | 66.79 (59.28; 75.93) | 66.27 (57.94; 82.88) | 65.22 (46.94; 72.84) |
| Min Max | 43.98 88.38 | 43.08 80.16 | 39.79 88.56 | 50.38 85.87 | 19.30 81.36 |
| **POD 3** | | | | | |
| Mean (SD) | 74.70 (11.17) | 65.11 (12.35) | 72.19 (8.91) | 74.02 (8.96) | 59.04 (20.45) |
| Median (IQR) | 77.17 (61.45; 83.27) | 64.82 (55.10; 76.06) | 75.10 (51.15; 76.92) | 72.45 (67.78; 79.40) | 65.65 (39.19; 73.49) |
| Min Max | 60.73 59.95 | 45.96 83.23 | 58.13 82.35 | 64.09 90.40 | 23.78 82.33 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **HO-1$^+$ CD68$^+$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 8.82 | 9.24 | 15.69 | 12.16 | 20.91 |
| Difference vs placebo (SE) | | 0.42 (4.21) | 6.87 (5.27) | 3.34 (2.83) | 12.09 (4.47) |
| p-value | | 0.9222 | 0.2206 | 0.2662 | **0.0197 *** |
| **POD 1** | | | | | |
| Observed mean | 21.43 | 29.03 | 28.00 | 24.27 | 36.68 |
| Difference vs placebo (SE) | | 7.60 (6.71) | 6.57 (7.16) | 2.84 (7.41) | 15.25 (9.42) |
| p-value | | 0.2817 | 0.3768 | 0.7091 | 0.1338 |
| **POD 3** | | | | | |
| Observed mean | 19.10 | 28.01 | 21.96 | 19.34 | 34.27 |
| Difference vs placebo (SE) | | 8.91 (5.32) | 2.86 (4.80) | 0.24 (4.75) | 15.17 (7.82) |
| p-value | | 0.1196 | 0.5629 | 0.9610 | 0.0792 |
| **HO-1$^-$ CD68$^+$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 3.20 | 7.23 | 4.13 | 4.79 | 9.17 |
| Difference vs placebo (SE) | | 4.03 (2.32) | 0.93 (1.46) | 1.59 (1.16) | 5.97 (3.11) |
| p-value | | 0.1176 | 0.5403 | 0.1996 | 0.0920 |
| **POD 1** | | | | | |
| Observed mean | 2.83 | 4.79 | 3.73 | 5.53 | 2.52 |
| Difference vs placebo (SE) | | 1.96 (0.98) | 0.90 (1.13) | 2.70 (2.27) | -0.31 (0.91) |
| p-value | | 0.0733 | 0.4391 | 0.2738 | 0.7441 |
| **POD 3** | | | | | |
| Observed mean | 5.16 | 5.67 | 4.75 | 5.77 | 4.96 |
| Difference vs placebo (SE) | | 0.51 (1.47) | -0.41 (1.63) | 0.61 (1.63) | -0.20 (1.71) |
| p-value | | 0.7330 | 0.8085 | 0.7158 | 0.9120 |
| **HO-1$^-$ CD68$^-$ cells as a proportion of Lin$^-$ CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 86.83 | 82.03 | 74.48 | 81.95 | 68.32 |
| Difference vs placebo (SE) | | -4.80 (5.62) | -12.35 (9.13) | -4.88 (3.34) | -18.51 (6.46) |
| p-value | | 0.4103 | 0.2108 | 0.1782 | **0.0163 *** |
| **POD 1** | | | | | |
| Observed mean | 72.95 | 63.44 | 66.63 | 68.52 | 58.04 |
| Difference vs placebo (SE) | | -9.51 (7.38) | -6.32 (7.85) | -4.43 (8.15) | -14.91 (9.92) |
| p-value | | 0.2238 | 0.4366 | 0.5984 | 0.1606 |
| **POD 3** | | | | | |
| Observed mean | 74.70 | 65.11 | 72.19 | 74.02 | 59.04 |
| Difference vs placebo (SE) | | -9.59 (6.07) | -2.51 (5.40) | -0.68 (5.59) | -15.66 (8.37) |
| p-value | | 0.1382 | 0.6505 | 0.9051 | 0.0881 |

Lin$^-$: excludes natural killer cells and lymphocytes (CD3$^-$ CD19$^-$ CD56$^-$)

Abbreviations: Lin$^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 5.2.2.5 $CD68^+$ $HO\text{-}1^+$ $Lin^-$ $CD45^+$ cells as erythrophagocytes

Next, investigating the impact of 5-ALA-SFC on $CD68^+$ immune cells specifically capable of erythrophagocytosis was explored. In patients allocated to receive 5-ALA-SFC at a dose of 300 mg/472 mg, a significant increase in the proportion of $CD163^+$ cells (% $CD68^+$ $HO\text{-}1^+$ $Lin^-$ $CD45^+$ cells) was observed at the pre-operative time point (mean difference [SE] vs. placebo = 4.68 [2.15] %, p-value = 0.0498), suggesting a plausible drug mediated effect at this dose (**Fig. 74 A-B**). In this treatment allocation group, the effect on CD163 expression was sustained post-operatively (on post-operative day 3: mean difference [SE] vs. placebo = 6.16 [3.81] %), but this was not statistically significant (p-value = 0.1309). Within the statistical model however, pre-operative 5-ALA-SFC treatment was identified as exerting a significant fixed effect (p-value = 0.0199), implying a drug-associated change in peri-operative CD163 expression. A drug-related increase in CD163 expression was not as clear with lower doses of 5-ALA-SFC.

An increase in $CD64^+$ cells (% $CD68^+$ $HO\text{-}1^+$ $Lin^-$ $CD45^+$ cells) was observed at the pre-operative timepoint (mean difference [SE] vs. placebo = 5.53 [2.76] %), but this was not statistically significant (p-value = 0.0681). Pooled data suggested a treatment-related increase in expression of CD64. At the third post-operative day this was significant only in the 5-ALA-SFC 75 mg/118 mg treatment allocation group (mean difference [SE] vs. placebo = 10.53 [4.69] %, p-value = 0.0461) (**Fig. 74 C-D**). No clear 5-ALA-SFC dose-related increases in CD64 expression were observed when examined on an individual dose level basis. An increase in cells expressing CD33 was observed in all patients allocated to the active treatment arm pre-operatively and on the first post-operative day, but this was not statistically significant.

Across the statistical model, and for all markers, time was shown to be the most important fixed effect factor (p-value < 0.0001), highlighting an important temporal relationship with surgery and their expression.

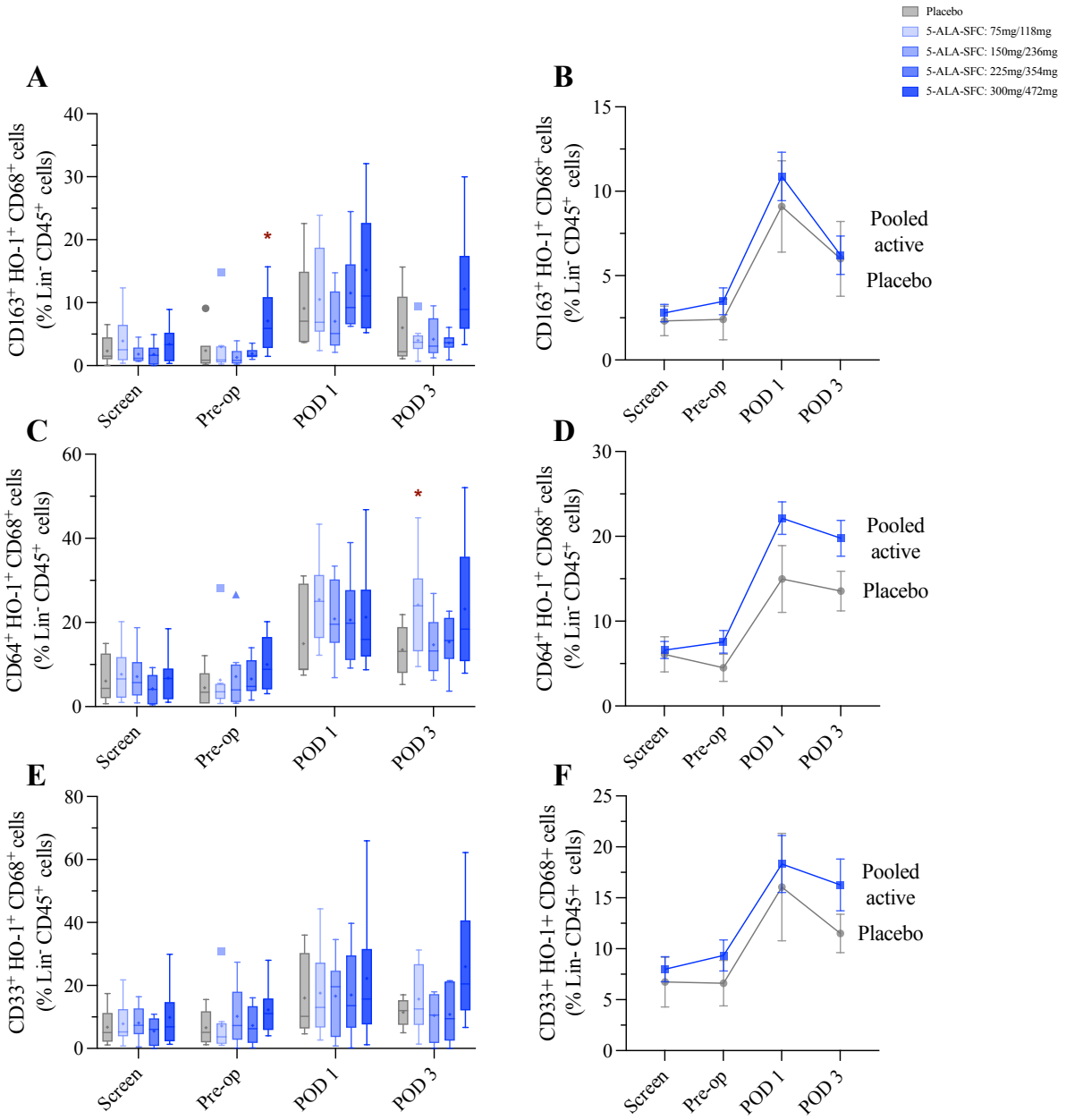



**Figure 74: HO-1+ CD68 + cells and phenotypic commitment towards erythrophagocytosis.**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A, C, E), mean (± SE) (panel B, D, F). Significance from the LMM using REML in red: * = $p < 0.05$.

$Lin^-$ cells – excludes natural killer cells, B-cells and T-cells ($CD3^+$ $CD19^+$ $CD56^+$).

## Table 38: CD68+ HO-1 + phagocytosis

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **$CD163^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 2.32 (2.31) | 3.88 (4.11) | 1.83 (1.41) | 1.77 (1.78) | 3.40 (2.90) |
| Median (IQR) | 1.50 (0.99; 4.49) | 2.48 (0.80; 6.46) | 1.16 (0.73; 2.90) | 1.65 (0.16; 2.86) | 3.34 (0.69; 5.25) |
| Min Max | 0.04 6.51 | 0.36 12.33 | 0.66 4.53 | 0.00 4.94 | 0.35 8.93 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.41 (3.19) | 2.96 (4.90) | 1.29 (1.38) | 1.90 (0.90) | 7.09 (5.03) |
| Median (IQR) | 0.85 (0.36; 3.17) | 0.87 (0.47; 3.05) | 0.84 (0.31; 2.36) | 1.61 (1.34; 2.51) | 5.90 (2.80; 10.90) |
| Min Max | 0.20 9.09 | 0.24 14.79 | 0.23 3.96 | 1.01 3.57 | 1.46 15.71 |
| **POD 1** | | | | | |
| Mean (SD) | 9.13 (7.16) | 10.47 (7.89) | 7.03 (4.70) | 11.53 (6.97) | 15.20 (9.81) |
| Median (IQR) | 7.07 (3.73; 14.93) | 6.88 (5.32; 18.72) | 5.11 (3.16; 11.80) | 9.23 (6.57; 16.11) | 11.06 (5.90; 22.68) |
| Min Max | 3.66 22.59 | 2.37 23.87 | 2.11 14.75 | 6.26 24.48 | 5.24 32.09 |
| **POD 3** | | | | | |
| Mean (SD) | 6.00 (5.85) | 3.97 (2.54) | 4.20 (3.15) | 3.62 (1.65) | 12.16 (8.46) |
| Median (IQR) | 2.23 (1.44; 10.96) | 3.66 (2.58; 4.81) | 3.11 (1.94; 7.54) | 3.65 (2.82; 4.49) | 8.92 (5.83; 17.42) |
| Min Max | 1.09 15.67 | 0.67 9.39 | 1.25 9.51 | 0.91 6.08 | 3.35 30.00 |
| **$CD64^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 6.10 (5.51) | 7.65 (6.43) | 7.11 (5.85) | 4.23 (3.49) | 6.81 (5.74) |
| Median (IQR) | 4.34 (2.01; 12.63) | 6.53 (2.04; 11.77) | 5.70 (2.61; 10.57) | 4.08 (0.53; 7.52) | 6.69 (1.76; 9.09) |
| Min Max | 0.71 15.02 | 0.92 20.15 | 0.87 18.78 | 0.43 9.27 | 1.03 18.50 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 4.51 (4.21) | 6.26 (9.02) | 7.12 (8.66) | 6.60 (4.55) | 10.03 (6.36) |
| Median (IQR) | 3.43 (0.76; 7.93) | 3.49 (1.78; 5.39) | 3.97 (1.06; 10.05) | 4.80 (3.71; 11.03) | 8.87 (4.09; 16.54) |
| Min Max | 0.71 7.93 | 0.71 28.18 | 0.87 26.64 | 1.56 14.00 | 3.08 20.21 |
| **POD 1** | | | | | |
| Mean (SD) | 14.98 (10.42) | 25.37 (9.95) | 20.87 (8.94) | 20.58 (10.60) | 21.28 (13.22) |
| Median (IQR) | 8.83 (8.79; 29.25) | 24.96 (16.25; 31.25) | 19.61 (15.14; 30.26) | 4.80 (3.71; 11.03) | 15.98 (11.94; 27.82) |
| Min Max | 7.48 31.05 | 12.18 43.33 | 6.86 33.42 | 9.17 38.99 | 8.75 46.81 |
| **POD 3** | | | | | |
| Mean (SD) | 13.55 (6.78) | 24.08 (11.49) | 14.70 (7.14) | 15.38 (6.71) | 23.18 (15.27) |
| Median (IQR) | 13.15 (7.98; 18.94) | 23.89 (13.07; 30.46) | 13.24 (8.39; 20.15) | 15.74 (11.29. 21.09) | 18.42 (10.79; 35.66) |
| Min Max | 5.26 21.89 | 9.46 44.82 | 6.25 26.92 | 3.69 22.69 | 7.95 52.04 |
| **$CD33^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 6.74 (5.98) | 7.84 (6.85) | 8.12 (5.14) | 5.54 (4.31) | 9.84 (9.63) |
| Median (IQR) | 5.06 (2.17; 11.26) | 5.26 (3.81; 12.52) | 7.34 (4.51; 12.75) | 6.09 (0.76; 9.57) | 6.86 (2.27; 14.71) |
| Min Max | 1.11 17.47 | 0.80 21.78 | 0.53 16.46 | 0.03 10.87 | 1.34 29.94 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 6.62 (5.47) | 7.17 (9.92) | 10.19 (9.32) | 7.26 (6.05) | 12.26 (7.65) |
| Median (IQR) | 5.11 (1.95; 11.79) | 3.70 (1.37; 8.04) | 7.25 (2.75; 18.08) | 6.27 (1.71; 13.43) | 11.09 (5.84; 15.86) |
| Min Max | 1.21 15.58 | 0.96 30.81 | 0.92 27.39 | 0.20 16.06 | 4.04 28.03 |
| **POD 1** | | | | | |
| Mean (SD) | 16.05 (12.91) | 17.56 (14.04) | 16.63 (11.94) | 16.97 (14.19) | 22.26 (21.52) |
| Median (IQR) | 10.20 (6.34; 30.28) | 13.04 (6.58; 27.28) | 19.59 (3.63; 24.73) | 13.57 (6.51; 29.56) | 15.65 (7.63; 31.62) |
| Min Max | 4.61 36.01 | 2.66 44.36 | 0.61 34.58 | 0.17 39.75 | 1.17 65.93 |
| **POD 3** | | | | | |
| Mean (SD) | 11.51 (4.63) | 15.67 (10.58) | 10.49 (7.33) | 10.83 (9.61) | 26.00 (18.87) |
| Median (IQR) | 11.99 (7.51; 15.31) | 12.57 (7.39; 26.80) | 10.63 (1.72; 17.31) | 9.48 (2.47; 21.28) | 20.51 (12.08; 40.70) |
| Min Max | 4.98 17.01 | 1.40 31.28 | 0.05 17.96 | 0.02 21.53 | 6.65 62.25 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **$CD163^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 2.41 | 2.96 | 1.29 | 1.90 | 7.09 |
| Difference vs placebo (SE) | | 0.55 (2.11) | -1.12 (1.32) | -0.51 (1.26) | 4.68 (2.15) |
| p-value | | 0.7960 | 0.4197 | 0.7008 | **0.0498 *** |
| **POD 1** | | | | | |
| Observed mean | 9.10 | 10.47 | 7.03 | 11.53 | 15.20 |
| Difference vs placebo (SE) | | 1.37 (3.89) | -2.07 (3.18) | 2.43 (3.93) | 6.10 (4.59) |
| p-value | | 0.7299 | 0.5276 | 0.5492 | 0.2112 |
| **POD 3** | | | | | |
| Observed mean | 6.00 | 3.96 | 4.20 | 3.62 | 12.16 |
| Difference vs placebo (SE) | | -2.04 (2.39) | -1.80 (2.51) | -2.38 (2.31) | 6.16 (3.81) |
| p-value | | 0.4207 | 0.4919 | 0.3375 | 0.1309 |
| **$CD64^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 4.51 | 6.26 | 7.12 | 6.60 | 10.03 |
| Difference vs placebo (SE) | | 1.75 (3.56) | 2.61 (3.45) | 2.09 (2.45) | 5.52 (2.76) |
| p-value | | 0.6338 | 0.4661 | 0.4114 | 0.0681 |
| **POD 1** | | | | | |
| Observed mean | 14.98 | 25.37 | 20.87 | 20.58 | 21.28 |
| Difference vs placebo (SE) | | 10.38 (5.28) | 5.88 (5.05) | 5.60 (5.85) | 6.29 (6.36) |
| p-value | | 0.0715 | 0.2661 | 0.3594 | 0.3428 |
| **POD 3** | | | | | |
| Observed mean | 13.55 | 24.08 | 14.70 | 15.38 | 23.18 |
| Difference vs placebo (SE) | | 10.53 (4.69) | 1.15 (3.57) | 1.83 (3.60) | 9.63 (5.88) |
| p-value | | **0.0461 *** | 0.7520 | 0.6226 | 0.1340 |
| **$CD33^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 6.62 | 7.17 | 10.19 | 7.26 | 12.26 |
| Difference vs placebo (SE) | | 0.55 (4.16) | 3.57 (3.98) | 0.64 (3.33) | 5.64 (3.51) |
| p-value | | 0.8983 | 0.3885 | 0.8520 | 0.1337 |
| **POD 1** | | | | | |
| Observed mean | 16.05 | 17.56 | 16.63 | 16.97 | 22.26 |
| Difference vs placebo (SE) | | 1.51 (7.24) | 0.58 (6.75) | 0.92 (7.83) | 6.21 (9.69) |
| p-value | | 0.8387 | 0.9333 | 0.9087 | 0.5362 |
| **POD 3** | | | | | |
| Observed mean | 11.51 | 15.67 | 10.49 | 10.83 | 26.00 |
| Difference vs placebo (SE) | | 4.16 (4.19) | -1.02 (3.35) | -0.68 (4.36) | 14.49 (6.94) |
| p-value | | 0.3444 | 0.7662 | 0.8805 | 0.0698 |

Abbreviations: $Lin^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

#### 5.2.2.6 Activation state and antigen processing profile of $Lin^{-}$ $CD45^{+}$ $CD68^{+}$ $HO\text{-}1^{+}$ cells

In all patients allocated to the active treatment arm of the study, there was an increase in the proportion of $CD68^{+}$ $HO\text{-}1^{+}$ $Lin^{-}$ $CD45^{+}$ cells expressing HLA-DR, CCR2 and CD11c at the pre-operative timepoint. By fitting a LMM using REML, this was most marked in the 5-ALA-SFC 300 mg/472 mg treatment allocation group, where there was a statistically significant increase in the proportion of cells expressing HLA-DR (mean difference [SE] vs. placebo = 5.69 [2.30] %, p-value = 0.0354) and CCR2 (mean difference [SE] vs. placebo = 1.35 [0.38] %, p-value = 0.0073). The proportion of cells expressing CD11c at the pre-operative time point tended to be increased in the 225 mg/354 mg treatment allocation group (mean difference [SE] vs placebo = 4.64 [21.14] %, p-value = 0.0533). This phenotypic shift extended into the post-operative period (**Fig. 75**).

Considering HLA-DR expression specifically, on the first post-operative day all patients that had been allocated to receive active 5-ALA-SFC had an increase in the proportion of cells expressing this marker. This finding was significant in the 150 mg/236 mg dosage group (mean difference [SE] vs. placebo = 7.41 [2.72] %, p-value = 0.024). By the third post-operative day the increase in HLA-DR expression was not as consistent between groups, approaching the response of the placebo group. Across the statistical model, treatment with 5-ALA-SFC was identified as the most significant fixed effect factor, with a p-value of 0.0533. This suggests a significant treatment-related increase in HLA-DR expression in $Lin^{-}$ $CD45^{+}$ $CD68^{+}$ $HO\text{-}1^{+}$ cells (**Fig. 75 A-B**). CD86 expression was not consistently affected by the administration of 5-ALA-SFC. CD86 expression was increased in the 75 mg/118 mg and 300 mg/472 mg treatment allocation groups, pre- and post-operatively. This was significant on the third post-operative day in the lower dose treatment allocation group (mean difference (SE) vs. placebo = 9.56 (3.41) %, p-value = 0.0219). No clear 5-ALA-SFC dose-related differences in expression were seen.

Across the model, time was identified as the most significant fixed effect factor for CD86 (p-value = 0.0051), CCR2 (p-value < 0.0001) and CD11c (p-value = 0.0050) expression, suggesting that the surgery had the most profound effect on their expression.

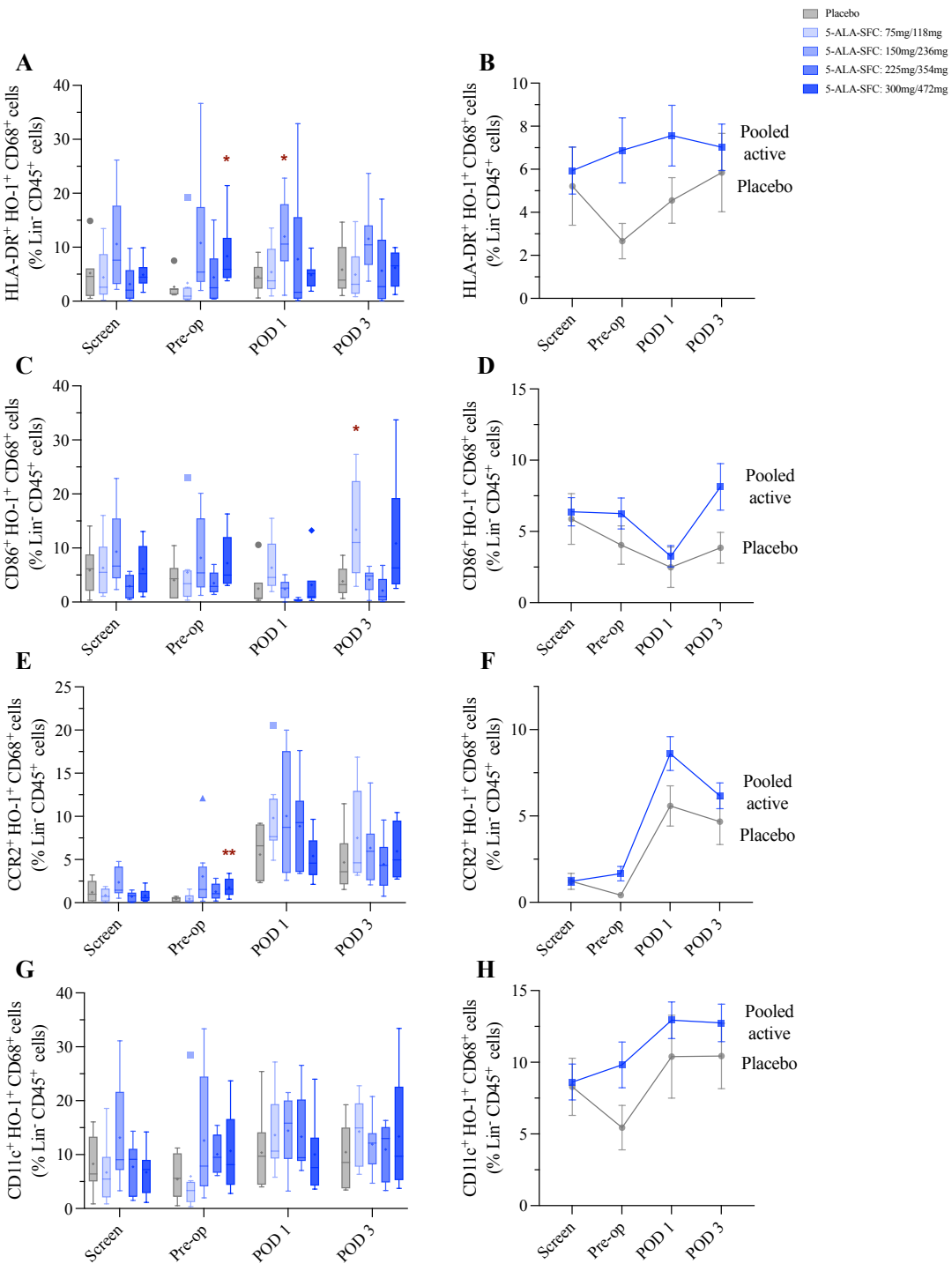



***Figure 75: Activation state of HO-1+ CD68+ cells.***

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values (panel A,C,E,G), mean (± SE) (panel B,D,F,H).Significance from the LMM using REML in red: * = $p < 0.05$, ** = $p < 0.005$.

$Lin^-$ cells – excludes natural killer cells, T-cells and B-cells ($CD3^+$ $CD19^+$ $CD56^+$).

## Table 39: Activation state and antigen processing of HO-1+ CD68+ cells.

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **HLA-DR$^+$ HO-1$^+$ CD68$^+$ cells as a proportion of Lin$^-$CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 5.21 (4.79) | 4.42 (4.88) | 10.59 (8.55) | 3.16 (3.70) | 4.91 (2.51) |
| Median (IQR) | 4.59 (0.95; 6.08) | 2.61 (1.20; 8.75) | 7.30 (3.17; 17.73) | 2.03 (0.45; 5.76) | 4.46 (3.27; 6.31) |
| Min Max | 0.51 14.89 | 0.17 13.46 | 2.19 26.17 | 0.12 9.80 | 1.65 9.92 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 2.66 (2.18) | 3.36 (6.49) | 10.78 (11.95) | 4.41 (5.62) | 8.35 (6.07) |
| Median (IQR) | 2.20 (1.26; 2.40) | 0.94 (0.29; 2.51) | 5.40 (3.53; 17.46) | 2.47 (0.47; 7.95) | 5.91 (4.29; 11.74) |
| Min Max | 1.17 7.50 | 0.21 19.23 | 1.97 36.67 | 0.38 15.06 | 3.79 21.41 |
| **POD 1** | | | | | |
| Mean (SD) | 4.55 (2.81) | 5.39 (4.57) | 11.96 (7.07) | 7.78 (12.84) | 4.83 (2.69) |
| Median (IQR) | 4.29 (2.32; 6.37) | 3.78 (2.20; 9.74) | 10.59 (7.35; 18.01) | 1.63 (0.46; 15.60) | 5.08 (2.73; 5.90) |
| Min Max | 0.61 9.06 | 0.98 13.56 | 1.10 22.81 | 0.10 32.91 | 1.83 9.83 |
| **POD 3** | | | | | |
| Mean (SD) | 5.85 (4.82) | 4.93 (4.86) | 11.58 (6.38) | 5.63 (7.26) | 6.17 (3.26) |
| Median (IQR) | 3.91 (2.34; 10.04) | 3.12 (1.37; 8.31) | 10.46 (6.69; 14.07) | 2.70 (0.38; 11.40) | 6.55 (2.68; 9.02) |
| Min Max | 1.04 14.65 | 0.84 14.75 | 3.71 23.68 | 0.09 18.93 | 1.24 9.94 |
| **CD86$^+$ HO-1$^+$ CD68$^+$ cells as a proportion of Lin$^-$CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 5.87 (4.70) | 6.31 (5.22) | 9.34 (7.16) | 2.92 (2.16) | 6.10 (4.55) |
| Median (IQR) | 6.15 (2.08; 8.81) | 5.50 (1.63; 10.29) | 6.45 (4.37; 15.49) | 2.91 (0.65; 5.06) | 5.27 (1.79; 10.38) |
| Min Max | 0.36 14.09 | 1.05 16.04 | 2.33 22.89 | 0.48 5.67 | 1.00 13.06 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 4.05 (3.56) | 5.85 (7.44) | 8.16 (7.10) | 3.51 (2.15) | 7.18 (5.04) |
| Median (IQR) | 4.32 (0.67; 6.30) | 3.39 (0.98; 5.87) | 5.42 (2.68; 15.50) | 2.88 (1.82; 5.47) | 5.00 (3.38; 12.04) |
| Min Max | 0.67 10.46 | 0.34 23.06 | 1.25 20.15 | 1.39 6.95 | 3.05 16.32 |
| **POD 1** | | | | | |
| Mean (SD) | 2.49 (3.75) | 6.35 (4.96) | 2.38 (1.75) | 0.41 (0.26) | 3.16 (4.62) |
| Median (IQR) | 0.74 (0.55; 3.61) | 4.55 (2.97; 10.80) | 2.60 (0.74; 3.75) | 0.39 (0.18; 0.56) | 1.00 (0.68; 3.97) |
| Min Max | 0.34 10.59 | 1.93 15.51 | 0.00 5.05 | 0.16 0.87 | 0.28 13.25 |
| **POD 3** | | | | | |
| Mean (SD) | 3.85 (2.87) | 13.39 (9.14) | 4.15 (2.15) | 2.11 (2.60) | 10.87 (11.33) |
| Median (IQR) | 3.22 (1.64; 6.18) | 11.04 (5.35; 22.41) | 4.83 (2.21; 5.48) | 1.02 (0.26; 4.29) | 6.32 (3.26; 19.26) |
| Min Max | 0.67 8.66 | 2.89 27.35 | 0.30 6.62 | 0.07 6.78 | 2.51 33.71 |
| **CCR2$^+$ HO-1$^+$ CD68$^+$ cells as a proportion of Lin$^-$CD45$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 1.22 (1.22) | 0.87 (0.76) | 2.36 (1.72) | 0.71 (0.57) | 0.82 (0.75) |
| Median (IQR) | 0.99 (0.16; 2.53) | 0.73 (0.14; 1.64) | 1.46 (1.09; 4.19) | 0.81 (0.05; 1.18) | 0.57 (0.20; 1.37) |
| Min Max | 0.06 3.22 | 0.02 1.87 | 0.53 4.78 | 0.02 1.46 | 0.15 2.28 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.42 (0.27) | 0.46 (0.55) | 3.04 (3.96) | 1.29 (0.98) | 1.77 (1.04) |
| Median (IQR) | 0.53 (0.09; 0.64) | 0.23 (0.05; 0.85) | 1.54 (0.51; 4.21) | 1.05 (0.49; 2.21) | 1.57 (0.89; 2.79) |
| Min Max | 0.07 0.73 | 0.02 1.58 | 0.17 12.12 | 0.21 2.82 | 0.42 3.40 |
| **POD 1** | | | | | |
| Mean (SD) | 5.59 (3.08) | 9.81 (4.94) | 10.05 (3.96) | 8.85 (5.21) | 5.41 (2.57) |
| Median (IQR) | 6.58 (2.49; 9.07) | 7.66 (7.19; 12.05) | 8.72 (3.44; 17.57) | 9.28 (3.56; 11.82) | 4.59 (3.20; 7.22) |
| Min Max | 2.32 9.20 | 4.93 20.58 | 2.60 20.00 | 3.41 17.62 | 2.14 9.67 |
| **POD 3** | | | | | |
| Mean (SD) | 4.68 (3.52) | 7.51 (5.41) | 6.33 (3.97) | 4.46 (3.02) | 5.97 (3.26) |
| Median (IQR) | 3.59 (2.12; 6.89) | 4.63 (3.43; 12.96) | 5.95 (2.59; 8.01) | 4.32 (1.97; 6.46) | 4.97 (2.93; 9.51) |
| Min Max | 1.53 11.47 | 3.20 16.87 | 2.06 13.87 | 0.76 9.59 | 2.74 10.44 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **$CD11c^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 8.28 (5.27) | 6.69 (5.81) | 13.14 (9.62) | 7.76 (4.89) | 6.68 (4.23) |
| Median (IQR) | 6.43 (5.02; 13.33) | 5.48 (2.04; 9.59) | 9.08 (7.12; 21.65) | 9.13 (2.19; 11.10) | 7.20 (2.84; 8.98) |
| Min Max | 0.87 16.08 | 0.88 18.58 | 3.29 31.11 | 1.49 14.33 | 1.11 14.17 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 5.44 (4.10) | 5.99 (9.24) | 12.62 (11.81) | 10.08 (3.62) | 10.66 (7.26) |
| Median (IQR) | 5.59 (2.20; 10.25) | 3.33 (1.17; 4.98) | 7.88 (4.11; 24.52) | 9.51 (6.61; 13.72) | 8.10 (4.37; 16.58) |
| Min Max | 0.53 11.23 | 0.36 28.47 | 1.97 33.33 | 6.11 15.42 | 2.75 23.96 |
| **POD 1** | | | | | |
| Mean (SD) | 10.39 (7.65) | 13.64 (7.11) | 14.45 (6.58) | 13.34 (7.54) | 10.01 (7.09) |
| Median (IQR) | 9.70 (4.43; 14.13) | 10.65 (9.26; 19.40) | 15.84 (9.18; 20.08) | 9.42 (8.81; 20.25) | 7.59 (4.21; 13.13) |
| Min Max | 4.01 25.42 | 5.79 27.18 | 3.22 21.51 | 7.07 26.58 | 3.56 23.96 |
| **POD 3** | | | | | |
| Mean (SD) | 10.44 (6.03) | 14.28 (6.01) | 11.90 (5.03) | 10.95 (5.39) | 13.31 (10.68) |
| Median (IQR) | 8.55 (3.74; 15.03) | 14.97 (7.75; 19.52) | 12.16 (8.17; 14.00) | 12.99 (4.82; 15.12) | 9.68 (5.23; 22.57) |
| Min Max | 3.45 19.27 | 6.36 22.79 | 4.71 20.79 | 3.33 16.33 | 3.72 33.37 |
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| **$HLA\text{-}DR^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 2.66 | 3.36 | 10.78 | 4.41 | 8.35 |
| Difference vs placebo (SE) | | 0.70 (2.44) | 8.12 (4.31) | 1.75 (2.44) | 5.69 (2.30) |
| p-value | | 0.7823 | 0.0984 | 0.4989 | **0.0354 *** |
| **POD 1** | | | | | |
| Observed mean | 4.55 | 5.39 | 11.96 | 7.78 | 4.83 |
| Difference vs placebo (SE) | | 0.84 (1.93) | 7.41 (2.72) | 3.23 (5.35) | 0.28 (1.47) |
| p-value | | 0.6736 | **0.0224 *** | 0.5705 | 0.8522 |
| **POD 3** | | | | | |
| Observed mean | 5.85 | 4.96 | 11.58 | 5.63 | 6.17 |
| Difference vs placebo (SE) | | -0.89 (2.50) | 5.73 (3.02) | -0.22 (3.48) | 0.32 (2.16) |
| p-value | | 0.7215 | 0.0840 | 0.9519 | 0.8836 |
| **$CD86^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 4.05 | 5.48 | 8.16 | 3.51 | 7.18 |
| Difference vs placebo (SE) | | 1.43 (2.95) | 4.11 (2.85) | -0.54 (2.85) | 3.13 (2.23) |
| p-value | | 0.6368 | 0.1772 | 0.7436 | 0.1845 |
| **POD 1** | | | | | |
| Observed mean | 2.49 | 6.35 | 2.38 | 0.41 | 3.16 |
| Difference vs placebo (SE) | | 3.86 (2.25) | -0.11 (1.55) | -2.08 (1.42) | 0.67 (2.25) |
| p-value | | 0.1105 | 0.9454 | 0.1932 | 0.7398 |
| **POD 3** | | | | | |
| Observed mean | 3.85 | 13.39 | 4.15 | 2.11 | 10.87 |
| Difference vs placebo (SE) | | 9.56 (3.41) | 0.30 (1.35) | -1.74 (1.52) | 7.02 (4.15) |
| p-value | | **0.0219 *** | 0.8336 | 0.2753 | 0.1293 |
| **$CCR2^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 0.42 | 0.46 | 3.04 | 1.29 | 1.77 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Difference vs placebo (SE) | | 0.04 (0.22) | 2.62 (1.40) | 0.87 (0.41) | 1.35 (0.38) |
| p-value | | 0.8433 | 0.1037 | 0.0841 | **0.0073** ** |
| **POD 1** | | | | | |
| Observed mean | 5.59 | 9.81 | 10.50 | 8.85 | 5.41 |
| Difference vs placebo (SE) | | 4.22 (2.10) | 4.91 (2.69) | 3.26 (2.43) | -0.18 (1.52) |
| p-value | | 0.0675 | 0.1281 | 0.2162 | 0.9073 |
| **POD 3** | | | | | |
| Observed mean | 4.68 | 7.51 | 6.33 | 4.46 | 5.97 |
| Difference vs placebo (SE) | | 2.83 (2.33) | 1.65 (2.00) | -0.22 (1.81) | 1.29 (1.76) |
| p-value | | 0.2467 | 0.4254 | 0.9082 | 0.4759 |
| **$CD11c^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Pre-operatively** | | | | | |
| Observed mean | 5.44 | 5.99 | 12.62 | 10.08 | 10.66 |
| Difference vs placebo (SE) | | 0.55 (3.62) | 7.18 (4.45) | 4.64 (2.14) | 5.22 (3.00) |
| p-value | | 0.8841 | 0.1422 | 0.0533 | 0.1090 |
| **POD 1** | | | | | |
| Observed mean | 10.39 | 13.64 | 14.45 | 13.34 | 10.01 |
| Difference vs placebo (SE) | | 3.25 (3.83) | 4.06 (3.71) | 2.95 (4.22) | -0.38 (3.94) |
| p-value | | 0.4111 | 0.2947 | 0.4993 | 0.9261 |
| **POD 3** | | | | | |
| Observed mean | 10.44 | 14.28 | 11.90 | 10.95 | 13.31 |
| Difference vs placebo (SE) | | 3.84 (3.12) | 1.46 (2.97) | 0.51 (3.17) | 2.87 (4.41) |
| p-value | | 0.2402 | 0.6317 | 0.8751 | 0.5271 |

$Lin^-$: excludes natural killer cells and lymphocytes ($CD3^-$ $CD19^-$ $CD56^-$)

Abbreviations: $Lin^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error.

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 5.2.3 The impact of 5-ALA-SFC on peri-operative inflammasome activity

The ability of 5-ALA-SFC to modulate activation of the sterile inflammasome in the context of cardiac surgery was assessed at transcript and protein levels, as well as by assessing expression levels of canonical downstream pro-inflammatory cytokines. Across a LMM using REML, 5-ALA-SFC did not appear to impact relative expression of *NLRP3* and *CASP1* at the pre-operative timepoint and post-operatively there was some suggestion that patients allocated to the higher 5-ALA-SFC doses had higher relative gene expression related to the inflammasome, compared to placebo (**Fig. 76 A-B**). For example, on the first post operative day patients who had been allocated to the 5-ALA-SFC 300 mg/472 mg treatment allocation group had higher expression of *NLRP3*, relative to *ACTB* (mean difference (SE) vs.

placebo = 0.45 (0.16) AU, p-value = 0.0170). Although not statistically significant, it was also observed that all patients allocated to the active treatment arm had increased relative gene expression of *CASP1* by the third post-operative day, compared to placebo. In the 5-ALA-SFC 300 mg/472 mg treatment allocation group there was a mean difference (SE) vs. placebo of 0.13 (0.24) AU with a p-value of 0.6176.

The proportion of immune cells expressing NLRP3 increased in response to surgery, with marked increases on the first post-operative day noted in CL monocytes as well as in $CD68^{+}$ $HO\text{-}1^{+}$ $Lin^{-}$ $CD45^{+}$ cells (F**ig. 76 C-D**). Here, there was some suggestion that the proportion of cells expressing NLRP3 was increased in patients who had received 5-ALA-SFC at the pre-operative timepoint, implying a drug associated phenotypic change. For example, in the 5-ALA-SFC 300 mg/472 mg treatment allocation group there was a mean difference (SE) vs. placebo of 3.18 (2.24) %. This finding was non-significant (p-value = 0.1799) but a similar increase in NLRP3 expression was seen post-operatively, both in CL monocytes and in $CD68^{+}$ $HO\text{-}1^{+}$ $Lin^{-}$ $CD45^{+}$ cells, in 5-ALA-SFC treated patients when compared to placebo.

Next, using protein expression data from the Olink® Explore inflammation panel, IL-1ß and IL-18 levels were assessed (**Fig. 76 E-F**). Within a LMM using REML, no statistically significant differences related to 5-ALA-SFC treatment were found but the data did suggest a dampened sterile-inflammasome interleukin response to surgery in patients receiving the active treatment, particularly in the highest dose treatment allocation group. This was already noticeable at the pre-operative time point. Here, lower levels of IL-1ß (mean difference (SE) vs. placebo = -0.58 (0.45) NPX, p-value = 0.2253) and IL-18 (mean difference (SE) vs. placebo = -0.22 (0.24) NPX, p-value = 0.3898) were observed in patients allocated to the 5-ALA-SFC 300 mg/472 mg treatment allocation group. No clear 5-ALA-SFC dose -related changes to expression of these ILs were observed.

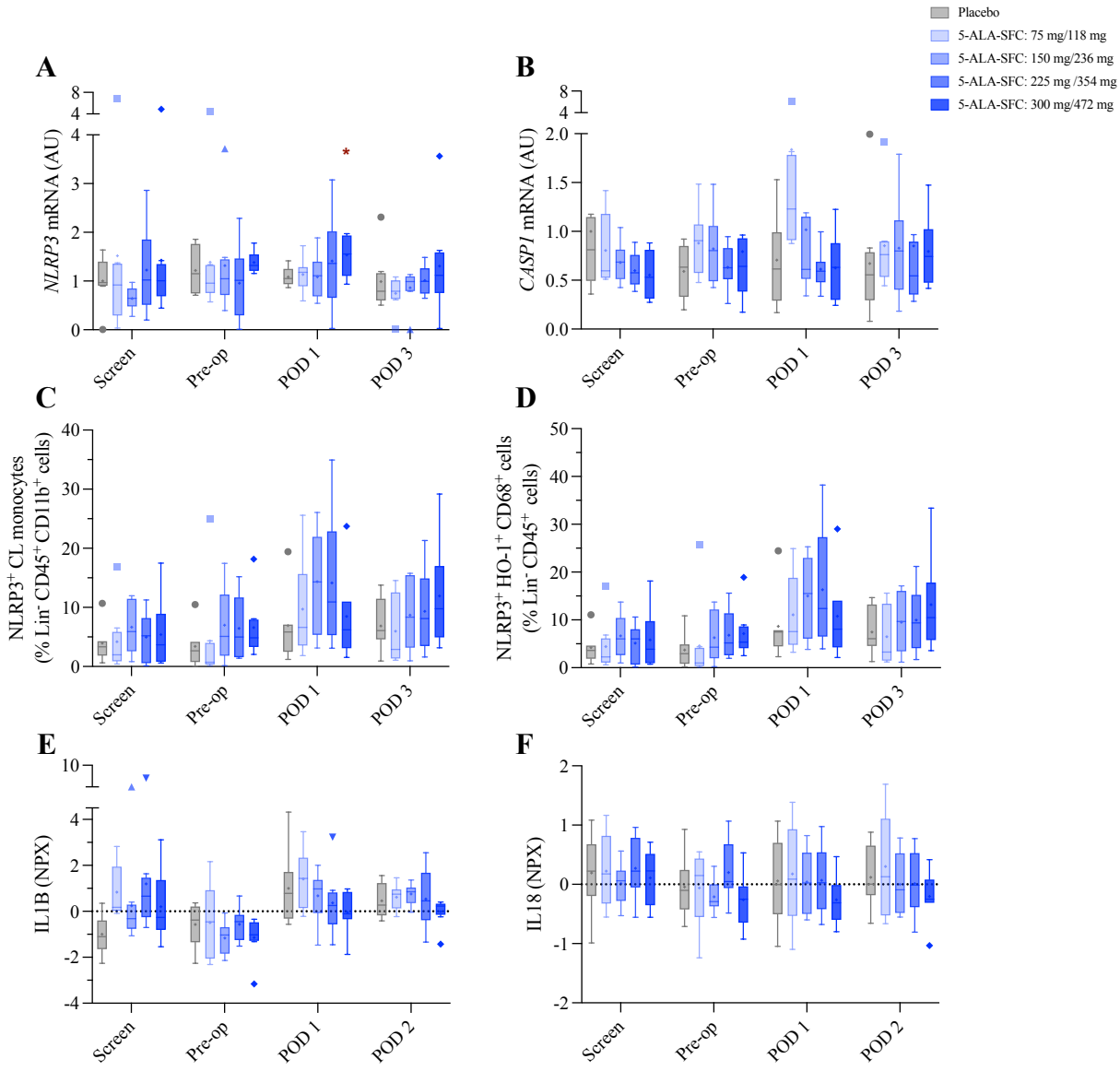



**Figure 76: Inflammasome activation. Data**

Data presented as median, IQR, Tukey's upper and lower adjacent values with outside values. Significance from LMM using REML * = $p < 0.05$.

**Table 40: Inflammasome activation.**

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| ***NLRP3* mRNA (AU)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 1.0 (0.49) | 1.52 (2.20) | 0.64 (0.23) | 1.23 (0.88) | 1.43 (0.43) |
| Median (IQR) | 0.67 (0.91; 1.40) | 0.92 (0.29; 1.36) | 0.65 (0.47; 0.85) | 1.02 (0.51; 1.85) | 1.01 (0.69; 1.35) |
| Min Max | 0.01 1.64 | 0.03 6.82 | 0.27 0.97 | 0.20 2.86 | 0.44 4.89 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 1.22 (0.48) | 1.39 (1.27) | 1.32 (1.03) | 0.86 (0.75) | 1.38 (0.22) |
| Median (IQR) | 0.15 (0.75; 1.76) | 0.96 (0.75; 1.33) | 1.05 (0.71; 1.45) | 1.02 (0.30; 1.46) | 1.32 (1.21; 1.55) |
| Min Max | 0.71 1.86 | 0.57 4.44 | 0.39 3.72 | 0.01 2.29 | 1.12 1.78 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Mean (SD) | 1.09 (0.19) | 1.14 (0.34) | 1.08 (0.45) | 1.41 (0.95) | 1.54 (0.40) |
| Median (IQR) | 1.05 (0.94; 1.25) | 1.18 (0.89; 1.29) | 1.11 (0.68; 1.40) | 1.36 (0.65; 1.46) | 1.56 (1.11; 1.94) |
| Min Max | 0.86 0.42 | 0.59 1.29 | 0.54 1.89 | 0.03 3.08 | 0.94 1.97 |
| **POD 3** | | | | | |
| Mean (SD) | 0.99 (0.58) | 0.75 (0.34) | 0.86 (0.37) | 1.01 (0.30) | 1.31 (1.05) |
| Median (IQR) | 0.79 (0.60; 1.16) | 0.79 (0.63; 1.01) | 0.99 (0.79; 1.10) | 0.99 (0.74; 1.26) | 1.12 (0.76; 1.59) |
| Min Max | 0.51 2.31 | 0.02 1.08 | 0.01 1.13 | 0.65 1.49 | 0.03 3.56 |
| ***CASP1* mRNA (AU)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 1.00 (0.76) | 0.81 (0.36) | 0.68 (0.20) | 0.60 (0.17) | 0.55 (0.24) |
| Median (IQR) | 0.81 (0.49; 1.15) | 0.60 (0.52; 1.18) | 0.69 (0.51; 0.81) | 0.57 (0.46; 0.76) | 0.53 (0.31; 0.81) |
| Min Max | 0.36 2.75 | 0.51 1.42 | 0.43 1.04 | 0.38 0.89 | 0.27 0.88 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 0.59 (0.26) | 0.88 (0.33) | 0.82 (0.36) | 0.63 (0.22) | 0.79 (0.61) |
| Median (IQR) | 0.63 (0.33; 0.85) | 0.91 (0.57; 1.07) | 0.80 (0.49; 1.06) | 0.63 (0.51; 0.83) | 0.64 (0.38; 0.93) |
| Min Max | 0.20 0.92 | 0.48 1.49 | 0.43 1.48 | 0.26 0.95 | 0.17 2.17 |
| **POD 1** | | | | | |
| Mean (SD) | 0.71 (0.45) | 1.84 (1.73) | 1.02 (0.97) | 0.61 (0.19) | 0.63 (0.34) |
| Median (IQR) | 0.61 (0.29; 0.99) | 1.23 (0.91; 1.79) | 0.61 (0.51; 1.15) | 0.59 (0.48; 0.69) | 0.63 (0.30; 0.88) |
| Min Max | 0.17 1.53 | 0.88 6.03 | 0.34 3.31 | 0.34 1.00 | 0.24 1.23 |
| **POD 3** | | | | | |
| Mean (SD) | 0.67 (0.58) | 0.85 (0.47) | 0.83 (0.51) | 0.85 (0.92) | 0.80 (0.35) |
| Median (IQR) | 0.55 (0.30; 0.79) | 0.76 (0.53; 0.90) | 0.80 (0.40; 1.11) | 0.54 (0.35; 0.89) | 0.74 (0.47; 1.02) |
| Min Max | 0.08 1.99 | 0.44 1.92 | 0.18 1.79 | 0.28 3.06 | 0.42 1.47 |
| **$NLRP3^+$ CL monocytes as a proportion of $Lin^- CD45^+ CD11b^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 3.93 (3.25) | 4.12 (5.51) | 6.66 (4.34) | 4.95 (4.16) | 5.41 (5.81) |
| Median (IQR) | 3.33 (1.86; 4.29) | 1.95 (0.83; 5.85) | 5.92 (2.55; 11.47) | 5.20 (0.56; 8.20) | 3.67 (0.81; 8.92) |
| Min Max | 0.63 10.70 | 0.39 16.84 | 0.83 11.99 | 0.12 11.26 | 0.61 17.49 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.39 (3.49) | 4.30 (8.45) | 6.98 (6.03) | 6.46 (5.43) | 6.57 (5.12) |
| Median (IQR) | 2.67 (0.77; 4.19) | 0.68 (0.27; 4.00) | 5.11 (1.80; 12.18) | 4.99 (1.56; 11.71) | 4.86 (3.29; 7.94) |
| Min Max | 0.09 10.50 | 0.08 24.88 | 0.15 17.46 | 1.41 15.19 | 2.07 18.16 |
| **POD 1** | | | | | |
| Mean (SD) | 6.92 (5.94) | 9.65 (8.14) | 14.33 (8.63) | 14.13 (11.52) | 8.50 (7.65) |
| Median (IQR) | 5.86 (2.49; 7.08) | 6.58 (3.52; 15.64) | 14.32 (5.36; 21.98) | 10.93 (5.29; 22.86) | 6.22 (3.09; 11.01) |
| Min Max | 1.21 19.44 | 1.82 25.54 | 3.12 26.07 | 3.09 34.94 | 1.56 23.74 |
| **POD 3** | | | | | |
| Mean (SD) | 6.84 (4.37) | 5.93 (5.69) | 8.69 (5.58) | 9.34 (7.16) | 11.92 (8.50) |
| Median (IQR) | 6.07 (4.59; 11.46) | 2.86 (1.32; 12.52) | 8.37 (3.15; 15.46) | 8.12 (3.48; 14.93) | 9.76 (4.94; 16.97) |
| Min Max | 0.92 13.76 | 1.02 14.52 | 0.96 15.77 | 1.62 21.32 | 3.16 29.18 |
| **$NLRP3^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^- CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 4.23 (3.33) | 4.42 (5.49) | 6.65 (4.41) | 5.15 (3.98) | 5.81 (6.04) |
| Median (IQR) | 3.61 (1.90; 6.34) | 2.27 (1.09; 6.15) | 6.02 (2.65; 10.45) | 6.05 (0.66; 8.06) | 3.90 (0.95; 9.77) |
| Min Max | 0.79 11.08 | 0.60 17.02 | 1.01 13.72 | 0.15 10.59 | 0.73 18.14 |
| **Pre-operatively** | | | | | |
| Mean (SD) | 3.70 (3.62) | 4.58 (8.67) | 6.31 (5.20) | 6.80 (5.18) | 7.14 (5.18) |
| Median (IQR) | 2.92 (0.82; 4.88) | 1.00 (0.31; 4.18) | 4.29 (1.95; 12.23) | 5.19 (2.60; 11.41) | 5.34 (4.07; 8.66) |
| Min Max | 0.19 10.87 | 0.09 25.69 | 0.22 13.73 | 1.96 15.62 | 2.51 18.91 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **POD 1** | | | | | |
| Mean (SD) | 8.64 (7.25) | 11.06 (8.02) | 15.03 (8.44) | 16.33 (12.65) | 10.78 (9.19) |
| Median (IQR) | 7.46 (4.48; 7.87) | 7.54 (4.77; 18.80) | 15.53 (6.07; 23.02) | 12.37 (6.49; 27.35) | 8.07 (4.24; 14.02) |
| Min Max | 2.32 24.44 | 3.22 24.90 | 3.82 25.29 | 3.96 38.23 | 2.12 29.01 |
| **POD 3** | | | | | |
| Mean (SD) | 7.49 (4.77) | 6.52 (6.09) | 9.50 (5.89) | 9.99 (7.12) | 13.18 (9.57) |
| Median (IQR) | 6.09 (4.55; 13.23) | 3.29 (1.47; 13.41) | 9.66 (3.40; 16.08) | 9.40 (4.10; 15.28) | 10.48 (5.79; 17.82) |
| Min Max | 1.30 14.65 | 1.22 15.61 | 1.18 17.13 | 1.72 21.20 | 3.58 33.40 |
| **IL1B (NPX)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | -1.00 (0.83) | 0.84 (1.13) | 0.24 (1.80) | 1.20 (2.37) | 0.21 (1.53) |
| Median (IQR) | -1.10 (-1.65; -0.39) | 0.17 (0.02; 1.96) | -0.31 (-0.76; 0.30) | 0.66 (-0.26; 1.47) | -0.27 (-0.81; 1.36) |
| Min Max | -2.26 0.35 | -0.09 2.82 | -1.07 4.55 | -0.71 6.75 | -1.54 3.11 |
| **Pre-operatively** | | | | | |
| Mean (SD) | -0.57 (0.92) | -0.49 (1.61) | -1.16 (0.70) | -0.56 (0.72) | -1.15 (0.89) |
| Median (IQR) | -0.38 (-1.34; 0.21) | -0.46 (-0.206; 0.93) | -1.03 (-1.85; -0.69) | -0.45 (-1.25; -0.15) | -1.02 (-1.29; -0.48) |
| Min Max | -2.26 0.36 | -2.32 2.17 | -2.14 -0.07 | -1.51 0.67 | -3.16 -0.34 |
| **POD 1** | | | | | |
| Mean (SD) | 1.00 (1.60) | 1.40 (1.27) | 0.68 (1.10) | 0.37 (1.38) | -0.04 (0.92) |
| Median (IQR) | 0.79 (-0.32; 1.71) | 1.43 (0.14; 2.34) | 0.98 (-0.08; 1.37) | 0.25 (-0.57; 0.83) | -0.11 (-0.35; 0.85) |
| Min Max | -0.56 4.32 | -0.22 3.46 | -1.47 2.01 | -1.45 3.23 | -1.87 0.98 |
| **POD 2** | | | | | |
| Mean (SD) | 0.45 (0.73) | 0.62 (0.54) | 0.76 (0.46) | 0.54 (1.29) | -0.01 (0.51) |
| Median (IQR) | 0.27 (-0.17; 1.23) | 0.75 (0.12; 0.96) | 0.86 (0.35; 1.04) | 0.46 (-0.39; 1.68) | 0.23 (-0.15; 0.34) |
| Min Max | -0.42 1.56 | -0.23 1.46 | -0.04 1.36 | -1.34 2.55 | -1.43 0.40 |
| **IL18 (NPX)** | | | | | |
| **Screening** | | | | | |
| Mean (SD) | 0.19 (0.64) | 0.22 (0.61) | 0.02 (0.35) | 0.27 (0.50) | 0.11 (0.47) |
| Median (IQR) | 0.22 (-0.20; 0.68) | 0.18 (-0.32; 0.82) | 0.06 (-0.28; 0.23) | 0.22 (-0.05; 0.78) | 0.23 (-0.35; 0.51) |
| Min Max | -1.00 1.08 | -0.55 1.17 | -0.53 0.56 | -0.56 0.96 | -0.56 0.71 |
| **Pre-operatively** | | | | | |
| Mean (SD) | -0.04 (0.52) | -0.06 (0.62) | -0.21 (0.27) | 0.20 (0.51) | -0.26 (0.45) |
| Median (IQR) | -0.10 (-0.42; 0.24) | 0.15 (-0.55; 0.44) | -0.19 (-0.37; 0.00) | 0.05 (-0.06; 0.68) | -0.25 (-0.65; -0.03) |
| Min Max | -0.71 0.93 | -1.24 0.55 | -0.56 0.30 | -0.48 1.07 | -0.92 0.53 |
| **POD 1** | | | | | |
| Mean (SD) | 0.06 (0.70) | 0.18 (0.85) | 0.04 (0.56) | 0.07 (0.56) | -0.26 (0.40) |
| Median (IQR) | -0.00 (-0.5; 0.70) | 0.09 (-0.43; 0.93) | 0.02 (-0.50; 0.53) | 0.03 (-0.41; 0.54) | -0.31 (-0.60; -0.01) |
| Min Max | -1.05 1.07 | -1.10 1.39 | -0.60 0.82 | -0.48 0.98 | -0.80 0.47 |
| **POD 3** | | | | | |
| Mean (SD) | 0.12 (0.51) | 0.30 (0.88) | 0.00 (0.52) | 0.01 (0.53) | -0.20 (0.42) |
| Median (IQR) | 0.00 (-0.18; 0.65) | 0.13 (-0.53; 1.11) | -0.09 (-0.48; 0.52) | -0.01 (-0.39; 0.53) | -0.25 (-0.31; 0.08) |
| Min Max | -0.66 0.88 | -0.66 1.69 | -0.55 0.78 | -0.80 0.77 | -1.03 0.42 |
| **Change in circulating monocyte populations, active vs placebo (Mixed effects analysis, REML)** | | | | | |
| ***NLRP3* mRNA (AU)** | | | | | |
| **Screening** | | | | | |
| Observed mean | 1.00 | 1.52 | 0.64 | 1.23 | 1.43 |
| Difference vs placebo (SE) | | 0.52 (0.80) | -0.36 (0.19) | 0.23 (0.36) | 0.43 (0.54) |
| p-value | | 0.5324 | 0.0930 | 0.5404 | 0.4456 |
| **Pre-operatively** | | | | | |
| Observed mean | 1.22 | 1.39 | 1.32 | 0.96 | 1.38 |
| Difference vs placebo (SE) | | 0.17 (0.48) | 0.10 (0.40) | -0.26 (0.31) | 0.16 (0.19) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| p-value | | 0.7282 | 0.8083 | 0.4284 | 0.3903 |
| **POD 1** | | | | | |
| Observed mean | 1.09 | 1.14 | 1.08 | 1.41 | 1.54 |
| Difference vs placebo (SE) | | 0.05 (0.14) | -0.01 (0.17) | 0.32 (0.34) | 0.45 (0.16) |
| p-value | | 0.7199 | 0.9923 | 0.3694 | **0.0170 *** |
| **POD 3** | | | | | |
| Observed mean | 0.99 | 0.75 | 0.86 | 1.01 | 1.31 |
| Difference vs placebo (SE) | | -0.24 (0.24) | -0.13 (0.24) | 0.02 (0.23) | 0.32 (0.42) |
| p-value | | 0.3301 | 0.6143 | 0.9248 | 0.4681 |
| ***CASP1* mRNA (AU)** | | | | | |
| **Screening** | | | | | |
| Observed mean | 1.00 | 0.81 | 0.68 | 0.60 | 0.55 |
| Difference vs placebo (SE) | | -0.19 (0.30) | -0.32 (0.28) | -0.40 (0.28) | -0.45 (0.28) |
| p-value | | 0.5282 | 0.2860 | 0.1847 | 0.1498 |
| **Pre-operatively** | | | | | |
| Observed mean | 0.59 | 0.88 | 0.52 | 0.63 | 0.79 |
| Difference vs placebo (SE) | | 0.29 (0.15) | -0.07 (0.16) | 0.04 (0.12) | 0.20 (0.23) |
| p-value | | 0.0732 | 0.1680 | 0.7327 | 0.4113 |
| **POD 1** | | | | | |
| Observed mean | 0.71 | 1.84 | 1.02 | 0.61 | 0.63 |
| Difference vs placebo (SE) | | 1.13 (0.63) | 0.31 (0.38) | -0.10 (0.17) | -0.08 (0.20) |
| p-value | | 0.1114 | 0.4293 | 0.6039 | 0.6954 |
| **POD 3** | | | | | |
| Observed mean | 0.67 | 0.85 | 0.83 | 0.85 | 0.80 |
| Difference vs placebo (SE) | | 0.18 (0.26) | 0.16 (0.27) | 0.18 (0.38) | 0.13 (0.24) |
| p-value | | 0.5058 | 0.5781 | 0.6488 | 0.6176 |
| **NLRP3$^+$ CL monocytes as a proportion of Lin$^-$ CD45$^+$ CD11b$^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Observed mean | 3.93 | 4.16 | 6.66 | 4.95 | 5.41 |
| Difference vs placebo (SE) | | 0.23 (2.30) | 2.73 (1.97) | 1.02 (2.09) | 1.48 (2.39) |
| p-value | | 0.9225 | 0.1897 | 0.6398 | 0.5503 |
| **Pre-operatively** | | | | | |
| Observed mean | 3.39 | 4.30 | 6.98 | 6.46 | 6.57 |
| Difference vs placebo (SE) | | 0.91 (3.27) | 3.59 (2.51) | 3.07 (2.58) | 3.18 (2.24) |
| p-value | | 0.7877 | 0.1788 | 0.2673 | 0.1799 |
| **POD 1** | | | | | |
| Observed mean | 6.92 | 9.65 | 14.33 | 14.13 | 8.50 |
| Difference vs placebo (SE) | | 2.73 (3.65) | 7.41 (3.79) | 7.21 (5.21) | 1.58 (3.61) |
| p-value | | 0.4674 | 0.0734 | 0.2079 | 0.6748 |
| **POD 3** | | | | | |
| Observed mean | 6.84 | 5.93 | 8.69 | 9.34 | 11.92 |
| Difference vs placebo (SE) | | -0.91 (2.60) | 1.85 (2.68) | 2.50 (3.36) | 5.08 (3.43) |
| p-value | | 0.7318 | 0.5039 | 0.4777 | 0.1675 |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **$NLRP3^+$ $HO\text{-}1^+$ $CD68^+$ cells as a proportion of $Lin^-$ $CD45^+$ cells (%)** | | | | | |
| **Screening** | | | | | |
| Observed mean | 4.23 | 4.42 | 6.65 | 5.15 | 5.81 |
| Difference vs placebo (SE) | | 0.19 (2.31) | 2.42 (2.00) | 0.92 (2.05) | 1.58 (2.48) |
| p-value | | 0.9365 | 0.2491 | 0.6649 | 0.5374 |
| **Pre-operatively** | | | | | |
| Observed mean | 3.70 | 4.58 | 6.31 | 6.80 | 7.14 |
| Difference vs placebo (SE) | | 0.88 (3.36) | 2.61 (2.29) | 3.10 (2.52) | 3.44 (2.29) |
| p-value | | 0.7981 | 0.2769 | 0.2509 | 0.1573 |
| **POD 1** | | | | | |
| Observed mean | 8.64 | 11.06 | 15.03 | 16.33 | 10.78 |
| Difference vs placebo (SE) | | 2.42 (3.94) | 6.39 (2.86) | 7.69 (5.85) | 2.14 (4.43) |
| p-value | | 0.5513 | 0.1391 | 0.2264 | 0.6392 |
| **POD 3** | | | | | |
| Observed mean | 7.49 | 6.52 | 9.50 | 9.99 | 13.18 |
| Difference vs placebo (SE) | | -0.97 (2.81) | 2.01 (2.86) | 2.50 (3.42) | 5.69 (3.83) |
| p-value | | 0.7348 | 0.4977 | 0.4850 | 0.1668 |
| **IL1B (NPX)** | | | | | |
| **Screening** | | | | | |
| Observed mean | -1.00 | 0.84 | 0.24 | 1.20 | 0.21 |
| Difference vs placebo (SE) | | 1.84 (0.50) | 1.24 (0.70) | 2.20 (0.89) | 1.21 (0.61) |
| p-value | | 0.002 | 0.1096 | 0.0363 | 0.0763 |
| **Pre-operatively** | | | | | |
| Observed mean | -0.57 | -0.49 | -1.16 | -0.56 | -1.15 |
| Difference vs placebo (SE) | | 0.08 (0.66) | -0.59 (0.41) | 0.01 (0.41) | -0.58 (0.45) |
| p-value | | 0.9029 | 0.1761 | 0.9772 | 0.2253 |
| **POD 1** | | | | | |
| Observed mean | 1.00 | 1.40 | 0.68 | 0.37 | -0.04 |
| Difference vs placebo (SE) | | 0.40 (0.72) | -0.32 (0.69) | -0.63 (0.75) | -1.04 (0.65) |
| p-value | | 0.5871 | 0.6463 | 0.4129 | 0.1391 |
| **POD 2** | | | | | |
| Observed mean | 0.45 | 0.61 | 0.76 | 0.54 | -0.01 |
| Difference vs placebo (SE) | | 0.16 (0.32) | 0.31 (0.31) | 0.09 (0.53) | -0.46 (0.34) |
| p-value | | 0.6203 | 0.3373 | 0.8735 | 0.1912 |
| **IL18 (NPX)** | | | | | |
| **Screening** | | | | | |
| Observed mean | 0.19 | 0.22 | 0.02 | 0.27 | 0.11 |
| Difference vs placebo (SE) | | 0.03 (0.31) | -0.17 (0.26) | 0.08 (0.29) | -0.08 (0.28) |
| p-value | | 0.9248 | 0.5143 | 0.7897 | 0.7671 |
| **Pre-operatively** | | | | | |
| Observed mean | -0.04 | -0.06 | -0.21 | 0.20 | -0.26 |
| Difference vs placebo (SE) | | -0.02 (0.29) | -0.17 (0.21) | 0.26 (0.26) | -0.22 (0.24) |
| p-value | | 0.9517 | 0.4418 | 0.3582 | 0.3898 |
| **POD 1** | | | | | |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg/354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Observed mean | 0.06 | 0.18 | 0.04 | 0.07 | -0.26 |
| Difference vs placebo (SE) | | 0.12 (0.39) | -0.02 (0.32) | 0.01 (0.32) | -0.32 (0.29) |
| p-value | | 0.7661 | 0.9539 | 0.9728 | 0.2900 |
| **POD 2** | | | | | |
| Observed mean | 0.12 | 0.30 | 0.00 | 0.01 | -0.20 |
| Difference vs placebo (SE) | | 0.18 (0.36) | -0.12 (0.26) | -0.11 (0.26) | -0.32 (0.23) |
| p-value | | 0.6160 | 0.6581 | 0.6944 | 0.1949 |

$Lin^-$: excludes natural killer cells and lymphocytes ($CD3^-$ $CD19^-$ $CD56^-$)

Abbreviations: CL – classical; ITM – intermediate; NC – non-classical; $Lin^-$ – lineage negative; SD – standard deviation; IQR – interquartile range; SE – standard error; REML – restricted maximum likelihood.

LMM with REML - Linear Mixed Model with Restricted Maximum Likelihood, correcting for multiple comparisons by controlling the false discovery rate (FDR) using the two-stage step-up method of Benjamini, Krieger and Yekutieli.

### 5.2.4 The impact of 5-ALA-SFC on the inflammatory proteome and associated biological processes – the Olink Explore® 384 Inflammation 1 panel

#### 5.2.4.1 Quality control and principal component analysis

All samples submitted for analysis underwent quality control (QC) checks as part of the Olink pipeline and if warnings were raised these samples were excluded from analysis before unblinding to treatment allocation (**Fig. 77**). A principal component analysis (PCA), based on data from all measured proteins, from all patients and at all timepoints, was also performed and outliers were identified. Samples were regarded as outliers if the data were > 3 SD from either the IQR or median. Samples that had not passed QC or that had been identified as outliers were excluded from subsequent downstream analysis. A total of 2.2% submitted samples were excluded.

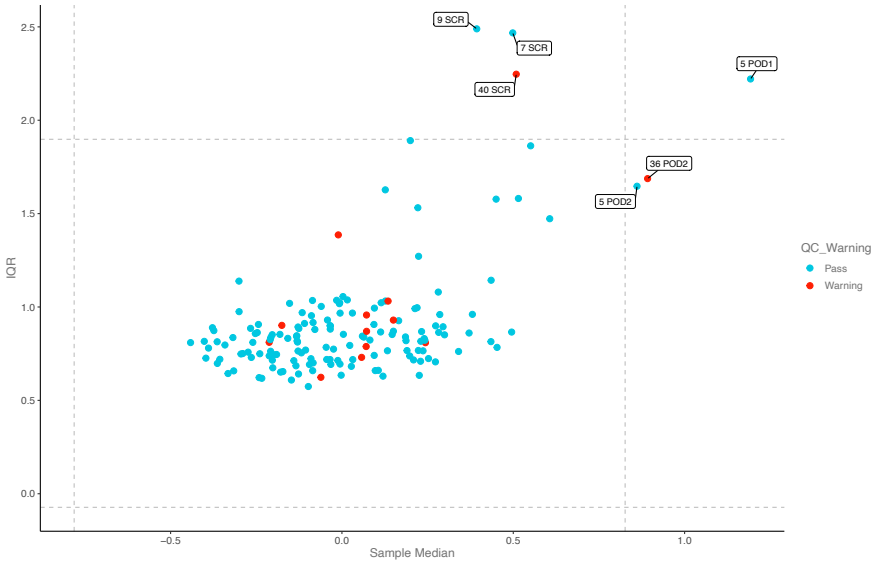



**Figure 77: O-link quality control plot (IQR vs. median).**

Datapoints coloured by QC warning, dashed lines = ± 3 SD from IQR and median respectively. Samples in red and/or labelled excluded from subsequent downstream analysis.

#### 5.2.4.2 The impact of 5-ALA-SFC on inflammatory plasma protein expression

The most significant factor affecting the levels of inflammatory proteins was timepoint, an expected response to the surgical insult. In total, 288 proteins differed significantly between timepoints. This PCA highlighted this temporal relationship with surgery with expression clustering by pre-operative or post-operative timepoint (**Fig. 78**).

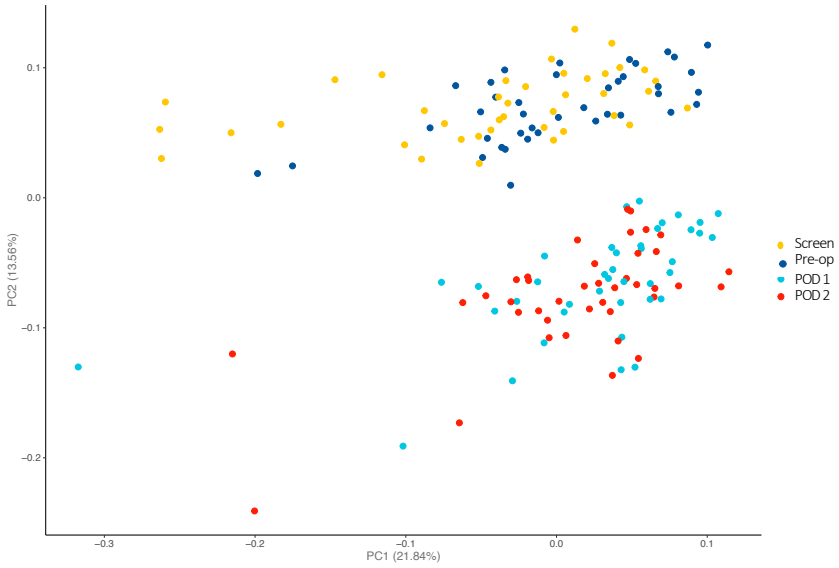



**Figure 78: Principal component analysis grouped by timepoint showing clustering of post-operative samples.**

These proteins are summarised by time point (compared to screening) in **table 41**. Up to 12 significantly up- or down-regulated proteins are highlighted in red and blue, respectively. Several proteins differed significantly at the pre-operative timepoint which could represent a direct drug-mediated effect. At this timepoint, the most profoundly down regulated proteins included platelet derived growth factor (PDGFB), IL-1ß and TIMP metallopeptidase inhibitor 3 (TIMP3). The most profoundly upregulated protein at this time point was aldehyde dehydrogenase 3 family member A1 (ALDH3A1), with an observed mean difference (95 % CI) at the pre-operative time point vs. screening of 1.52 NPX (0.98; 20.05). This was a highly statistically significant finding with a p-value of < 0.0001. In the post-operative period, there were similarities between significant differences in protein expression on post-operative day 1 and 2. Notably, at both timepoints IL-6 displayed the most profoundly increased predicted mean compared to the screening timepoint, followed by EPO. At both timepoints IL-10 was also increased. When considering significant differences in the most down-regulated proteins in the post-operative period, some similarities are seen with the pre-operative timepoint (e.g., PDGFB and epidermal growth factor [EGF]), suggesting that 5-ALA-SFC either induces changes in expression that persist into the post-operative period, or that 5-ALA-SFC therapy induces changes similar to surgery.

**Table 41: Significant differences in protein expression by timepoint.**

| Screening to pre-operatively | | | | | Screening to post-operative day 1 | | | | | Screening to post-operative day 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Protein** | **Estimate** | **95 % CI** | | **p-value[+]** | **Protein** | **Estimate** | **95 % CI** | | **p-value[+]** | **Protein** | **Estimate** | **95 % CI** | | **p-value[+]** |
| PDGFB | -1.04 | -1.64 | -0.44 | 0.0001 | CTRC | -2.94 | -3.28 | -2.61 | < 0.0001 | CTRC | -2.57 | -2.91 | -2.23 | < 0.0001 |
| IL1B | -1.01 | -1.70 | -0.33 | 0.0010 | CCL13 | -2.47 | -2.82 | -2.12 | < 0.0001 | IL4 | -2.56 | -3.94 | -1.19 | < 0.0001 |
| TIMP3 | -0.97 | -1.54 | -0.40 | 0.0001 | PDGFB | -2.45 | -3.04 | -1.85 | < 0.0001 | TNFSF11 | -2.44 | -2.79 | -2.10 | < 0.0001 |
| CD40LG | -0.96 | -1.42 | -0.50 | < 0.0001 | TNFSF11 | -2.15 | -2.49 | -1.81 | < 0.0001 | CCL13 | -1.87 | -2.23 | -1.52 | < 0.0001 |
| ANGPT1 | -0.82 | -1.36 | -0.29 | 0.0006 | IFNG | -2.15 | -3.14 | -1.16 | < 0.0001 | PDGFB | -1.75 | -2.35 | -1.14 | < 0.0001 |
| EGF | -0.77 | -1.41 | -0.12 | 0.0123 | IL4 | -2.07 | -3.46 | -0.68 | 0.0010 | TNFSF10 | -1.66 | -1.89 | -1.43 | < 0.0001 |
| LAT | -0.76 | -1.29 | -0.23 | 0.0016 | EGF | -1.98 | -2.62 | -1.33 | < 0.0001 | SH2D1A | -1.65 | -2.00 | -1.30 | < 0.0001 |
| FGF19 | -0.71 | -1.16 | -0.25 | 0.0005 | ANGPT1 | -1.93 | -2.47 | -1.40 | < 0.0001 | CLEC4C | -1.57 | -1.76 | -1.39 | < 0.0001 |
| CCL26 | -0.69 | -1.12 | -0.26 | 0.0004 | CD40LG | -1.79 | -2.25 | -1.33 | < 0.0001 | IFNG | -1.56 | -2.54 | -0.58 | 0.0004 |
| MMP1 | -0.68 | -1.11 | -0.25 | 0.0005 | CLEC4C | -1.65 | -1.84 | -1.47 | < 0.0001 | EGF | -1.54 | -2.18 | -0.89 | < 0.0001 |
| OSM | -0.66 | -1.14 | -0.17 | 0.0033 | CCL11 | -1.64 | -1.86 | -1.42 | < 0.0001 | LTA | -1.52 | -1.70 | -1.34 | < 0.0001 |
| CCL17 | -0.63 | -1.04 | -0.22 | 0.0005 | CCL26 | -1.62 | -2.04 | -1.19 | < 0.0001 | EPCAM | -1.51 | -1.72 | -1.30 | < 0.0001 |
| FABP9 | 0.19 | 0.01 | 0.37 | 0.0331 | CXCL8 | 1.51 | 1.14 | 1.89 | < 0.0001 | IL4R | 1.42 | 1.15 | 1.69 | < 0.0001 |
| ANGPTL4 | 0.28 | 0.10 | 0.45 | 0.0003 | PTX3 | 1.53 | 1.30 | 1.76 | < 0.0001 | IL15 | 1.47 | 1.31 | 1.62 | < 0.0001 |
| IL33 | 0.42 | 0.15 | 0.69 | 0.0007 | IL1RN | 1.58 | 1.15 | 2.00 | < 0.0001 | HGF | 1.57 | 1.06 | 2.08 | < 0.0001 |
| CXCL17 | 0.50 | 0.19 | 0.81 | 0.0004 | IL17C | 1.61 | 0.93 | 2.29 | < 0.0001 | CCL20 | 1.62 | 0.99 | 2.26 | < 0.0001 |
| FABP1 | 0.66 | 0.03 | 1.29 | 0.0371 | OSM | 1.65 | 1.17 | 2.13 | < 0.0001 | OSM | 1.63 | 1.15 | 2.10 | < 0.0001 |
| ALDH3A1 | 1.52 | 0.98 | 2.05 | < 0.0001 | CCL7 | 1.67 | 1.30 | 2.03 | < 0.0001 | CCL23 | 1.68 | 1.45 | 1.90 | < 0.0001 |
| | | | | | IL4R | 1.73 | 1.46 | 2.00 | < 0.0001 | IL5RA | 1.71 | 1.52 | 1.91 | < 0.0001 |
| | | | | | CCL23 | 1.87 | 1.64 | 2.09 | < 0.0001 | IL10 | 1.92 | 1.29 | 2.54 | < 0.0001 |
| | | | | | IL10 | 3.20 | 2.57 | 3.83 | < 0.0001 | CSF3 | 2.16 | 1.70 | 2.62 | < 0.0001 |
| | | | | | CSF3 | 3.66 | 3.20 | 4.12 | < 0.0001 | CCL7 | 2.26 | 1.89 | 2.62 | < 0.0001 |
| | | | | | EPO | 4.73 | 3.97 | 5.50 | < 0.0001 | EPO | 4.88 | 4.11 | 5.64 | < 0.0001 |
| | | | | | IL6 | 5.77 | 5.30 | 6.25 | < 0.0001 | IL6 | 5.24 | 4.76 | 5.72 | < 0.0001 |

[+] Adjusted p-values. Upregulated proteins shaded in red, down regulated proteins shaded in blue.

Next, the effect on treatment allocation (i.e., active vs. placebo) across all time points was assessed. A total of 22 proteins differed significantly between groups and these are summarised in **table 42**. The most striking finding was reduced expression of IL-6 in patients who received the active treatment. Patients in this group had an IL-6 predicted mean (95 % CI) of -0.92 NPX (-2.07; 0.23) vs. placebo with a p-value of 0.0118. EGF was significantly upregulated in patients who had received 5-ALA-SFC, compared to placebo across all time points with a mean difference (95 % CI) of 0.94 NPX (0.21; 1.68) and a p-value of 0.0132. Post-hoc analysis, comparing differences in protein expression combing timepoint and treatment allocation, was also undertaken and significant between groups are summarised in **table 43**. Notably, there were no significant differences in protein expression in the placebo group when considering the pre-operative timepoint, and in patients allocated to 5-ALA-SFC, down-regulation of IL-1ß expression is observed.

**Table 42: Significant differences in protein expression related to 5-ALA-SFC.**

| Protein | Estimate | 95 % CI | | p-value [+] |
|---|---|---|---|---|
| IL6 | -0.92 | -2.07 | 0.23 | 0.0118 |
| CXCL17 | -0.73 | -1.14 | -0.32 | 0.0008 |
| KRT19 | -0.70 | -1.20 | -0.21 | 0.0063 |
| IL10 | -0.70 | -1.38 | -0.02 | 0.0428 |
| IL33 | -0.67 | -1.20 | -0.14 | 0.0139 |
| TFF2 | -0.62 | -1.24 | 0.01 | 0.0053 |
| IL17C | -0.55 | -1.27 | 0.16 | 0.0127 |
| CDSN | -0.46 | -0.94 | 0.02 | 0.0062 |
| CCL3 | -0.45 | -0.92 | 0.01 | 0.0057 |
| CCL7 | -0.41 | -0.96 | 0.13 | 0.0139 |
| CD276 | -0.39 | -0.73 | -0.05 | 0.0251 |
| CXCL8 | -0.28 | -0.71 | 0.14 | 0.0186 |
| JCHAIN | -0.27 | -0.53 | -0.01 | 0.0438 |
| WNT9A | -0.27 | -0.52 | -0.02 | 0.0339 |
| CTSC | -0.23 | -0.73 | 0.27 | 0.0365 |
| PREB | -0.16 | -0.33 | 0.02 | 0.0080 |
| RAB6A | 0.26 | -0.10 | 0.62 | 0.0152 |
| ARNT | 0.39 | -0.27 | 1.04 | 0.0243 |
| BANK1 | 0.68 | 0.05 | 1.32 | 0.0347 |
| SKAP2 | 0.72 | 0.06 | 1.38 | 0.0333 |
| ARHGEF12 | 0.87 | 0.33 | 1.41 | 0.0022 |
| EGF | 0.94 | 0.21 | 1.68 | 0.0132 |

[+] Adjusted p-values. Upregulated proteins shaded in red, down regulated proteins shaded in blue.

**Table 43: Significant differences in inflammatory protein expression by treatment allocation and timepoint.**

| Protein | Estimate | 95 % CI | | p-value [+] | Protein | Estimate | 95 % CI | | p-value [+] |
|---|---|---|---|---|---|---|---|---|---|
| **Active: Screening to pre-operatively** | | | | | **Placebo: Screening to pre-operatively** | | | | |
| IL1B | -1.39 | -2.25 | -0.53 | 0.0001 | | | | | |
| NBN | -0.92 | -1.66 | -0.17 | 0.0059 | | | | | |
| ROBO1 | -0.22 | -0.37 | -0.06 | 0.0006 | | | | | |
| NME3 | -0.20 | -0.39 | -0.01 | 0.0372 | | | | | |
| IL10RB | -0.18 | -0.33 | -0.04 | 0.0040 | | | | | |
| NELL2 | -0.14 | -0.27 | -0.01 | 0.0332 | | | | | |
| **Active: Screening to post-operative day 1** | | | | | **Placebo: Screening to post-operative day 1** | | | | |
| TNFSF10 | -1.38 | -1.66 | -1.10 | < 0.0001 | TNFSF10 | -1.91 | -2.46 | -1.36 | < 0.0001 |
| NBN | -0.89 | -1.66 | -0.12 | 0.0125 | EPHA1 | -1.10 | -1.55 | -0.65 | < 0.0001 |
| EPHA1 | -0.57 | -0.80 | -0.34 | < 0.0001 | NME3 | -0.77 | -1.15 | -0.39 | < 0.0001 |
| ROBO1 | -0.51 | -0.66 | -0.35 | < 0.0001 | ROBO1 | -0.69 | -1.00 | -0.39 | < 0.0001 |
| NME3 | -0.44 | -0.63 | -0.24 | < 0.0001 | IL10RB | -0.63 | -0.92 | -0.34 | < 0.0001 |
| NELL2 | -0.29 | -0.43 | -0.16 | < 0.0001 | NELL2 | -0.61 | -0.87 | -0.35 | < 0.0001 |
| IL10RB | -0.27 | -0.42 | -0.12 | < 0.0001 | | | | | |
| PGF | 0.20 | 0.02 | 0.38 | 0.0182 | SIGLEC10 | 0.53 | 0.13 | 0.92 | 0.0021 |
| TNFRSF11A | 0.28 | 0.03 | 0.53 | 0.0147 | IL1B | 2.00 | 0.31 | 3.69 | 0.0093 |
| SCG3 | 0.28 | 0.12 | 0.45 | < 0.0001 | CCL20 | 2.01 | 0.42 | 3.59 | 0.0038 |
| SIGLEC10 | 0.60 | 0.39 | 0.81 | < 0.0001 | | | | | |
| PROK1 | 0.63 | 0.32 | 0.95 | < 0.0001 | | | | | |
| CCL20 | 1.24 | 0.43 | 2.05 | 0.0002 | | | | | |
| **Active: Screening to post-operative day 2** | | | | | **Placebo: Screening to post-operative day 2** | | | | |
| TNFSF10 | -1.54 | -1.82 | -1.26 | < 0.0001 | TNFSF10 | -2.11 | -2.66 | -1.56 | < 0.0001 |
| NBN | -1.07 | -1.83 | -0.31 | 0.0008 | ENPP7 | -1.23 | -1.95 | -0.50 | < 0.0001 |
| ROBO1 | -0.52 | -0.68 | -0.37 | < 0.0001 | EPHA1 | -0.74 | -1.19 | -0.29 | < 0.0001 |
| NME3 | -0.34 | -0.54 | -0.15 | < 0.0001 | ROBO1 | -0.64 | -0.95 | -0.33 | < 0.0001 |
| EPHA1 | -0.25 | -0.48 | -0.02 | 0.0202 | NME3 | -0.55 | -0.93 | -0.17 | 0.0004 |
| NELL2 | -0.18 | -0.32 | -0.05 | 0.0012 | NELL2 | -0.37 | -0.63 | -0.10 | 0.0010 |
| PGF | 0.29 | 0.11 | 0.47 | 0.0001 | TNFRSF11A | 0.72 | 0.23 | 1.21 | 0.0003 |
| TNFSF13 | 0.41 | 0.18 | 0.64 | < 0.0001 | TNFSF13 | 0.88 | 0.43 | 1.33 | < 0.0001 |
| SCG3 | 0.41 | 0.25 | 0.58 | < 0.0001 | SIGLEC10 | 1.29 | 0.89 | 1.69 | < 0.0001 |
| TNFRSF11A | 0.45 | 0.20 | 0.70 | < 0.0001 | PROK1 | 1.49 | 0.89 | 2.10 | < 0.0001 |
| PROK1 | 0.77 | 0.46 | 1.08 | < 0.0001 | CCL20 | 2.83 | 1.25 | 4.42 | < 0.0001 |
| SIGLEC10 | 0.85 | 0.65 | 1.06 | < 0.0001 | | | | | |
| CCL20 | 1.30 | 0.48 | 2.11 | 0.0001 | | | | | |

[+] Adjusted p-values. Upregulated proteins shaded in red, down regulated proteins shaded in blue.

To determine whether any 5-ALA-SFC dose-dependent differences in protein expression were present a further analysis, comparing treatment allocation groups (i.e., dose), was performed. In total 55 proteins significantly differed and when only comparisons between active treatment and placebo were considered

this decreased. The top 12 proteins that were either significantly upregulated or downregulated are summarised in **table 44**. There were no significant differences in inflammatory protein expression found in the 5-ALA-SFC 150 mg/236 mg treatment allocation group, vs. placebo. The only protein that was similarly affected across all the remaining 5-ALA-SFC treatment allocation groups was Rho Guanine Nucleotide Exchnage Factor 12 (ARHGEF12). 5-ALA-SFC, administered at the lower two doses, was associated with similar downregulation of in Dual Adaptor of Phosphotyrosine And 3-Phosphoinositides 1 (DAPP1), Mevalonate Kinase (MVK) and Protein Tyrosine Phosphatase Non-Receptor Type 6 (PTPN6).

**Table 44: 5-ALA-SFC dose related differences in inflammatory protein expression.**

| Protein | Estimate | 95 % CI | | p-value [+] |
|---|---|---|---|---|
| **5-ALA-SFC 75 mg/118 mg vs. placebo** | | | | |
| DAPP1* | -2.38 | -3.58 | -1.18 | < 0.0001 |
| FKBP1B | -2.00 | -3.13 | -0.86 | 0.0001 |
| MANF | -1.86 | -3.10 | -0.63 | 0.0010 |
| CXCL3 | -1.60 | -2.50 | -0.70 | 0.0001 |
| MVK* | -1.56 | -2.46 | -0.67 | 0.0001 |
| SKAP2 | -1.56 | -2.49 | -0.63 | 0.0002 |
| EGF | -1.54 | -2.68 | -0.41 | 0.0031 |
| IRAK4 | -1.54 | -2.41 | -0.68 | < 0.0001 |
| ARHGEF12* | -1.52 | -2.30 | -0.73 | < 0.0001 |
| PTPN6* | -1.48 | -2.35 | -0.61 | 0.0002 |
| CLIP2 | -1.46 | -2.51 | -0.41 | 0.0024 |
| MGLL | -1.44 | -2.28 | -0.61 | 0.0001 |
| NTF3 | 0.75 | 0.16 | 1.34 | 0.0066 |
| **5-ALA-SFC 225 mg/354 mg vs. placebo** | | | | |
| DAPP1* | -1.24 | -2.45 | -0.02 | 0.0443 |
| MVK* | -1.15 | -2.03 | -0.26 | 0.0061 |
| PTPN6* | -1.07 | -1.93 | -0.20 | 0.0089 |
| ARHGEF12* | -0.93 | -1.72 | -0.13 | 0.0149 |
| SPINK4 | 1.26 | 0.18 | 2.35 | 0.0150 |
| **5-ALA-SFC 300 mg/472 mg vs. placebo** | | | | |
| ARNT | -1.23 | -2.21 | -0.25 | 0.0078 |
| BANK1 | -0.87 | -1.74 | -0.01 | 0.0480 |
| ARHGEF12* | -0.81 | -1.59 | -0.03 | 0.0387 |
| CTSC | 0.72 | 0.08 | 1.36 | 0.0214 |
| CD276 | 0.77 | 0.25 | 1.30 | 0.0014 |
| IL17C | 1.16 | 0.05 | 2.27 | 0.0362 |

[+] Adjusted p-values. * Proteins with similar differences vs. placebo across groups. Upregulated proteins shaded in red, down regulated proteins shaded in blue.

Finally, a heatmap was generated from all significant proteins across all terms (i.e., timepoint, treatment allocation and dose) to evaluate for to co-expressions in different subjects, samples, cohort, allocation, and timepoints groups (**Fig. 79**).

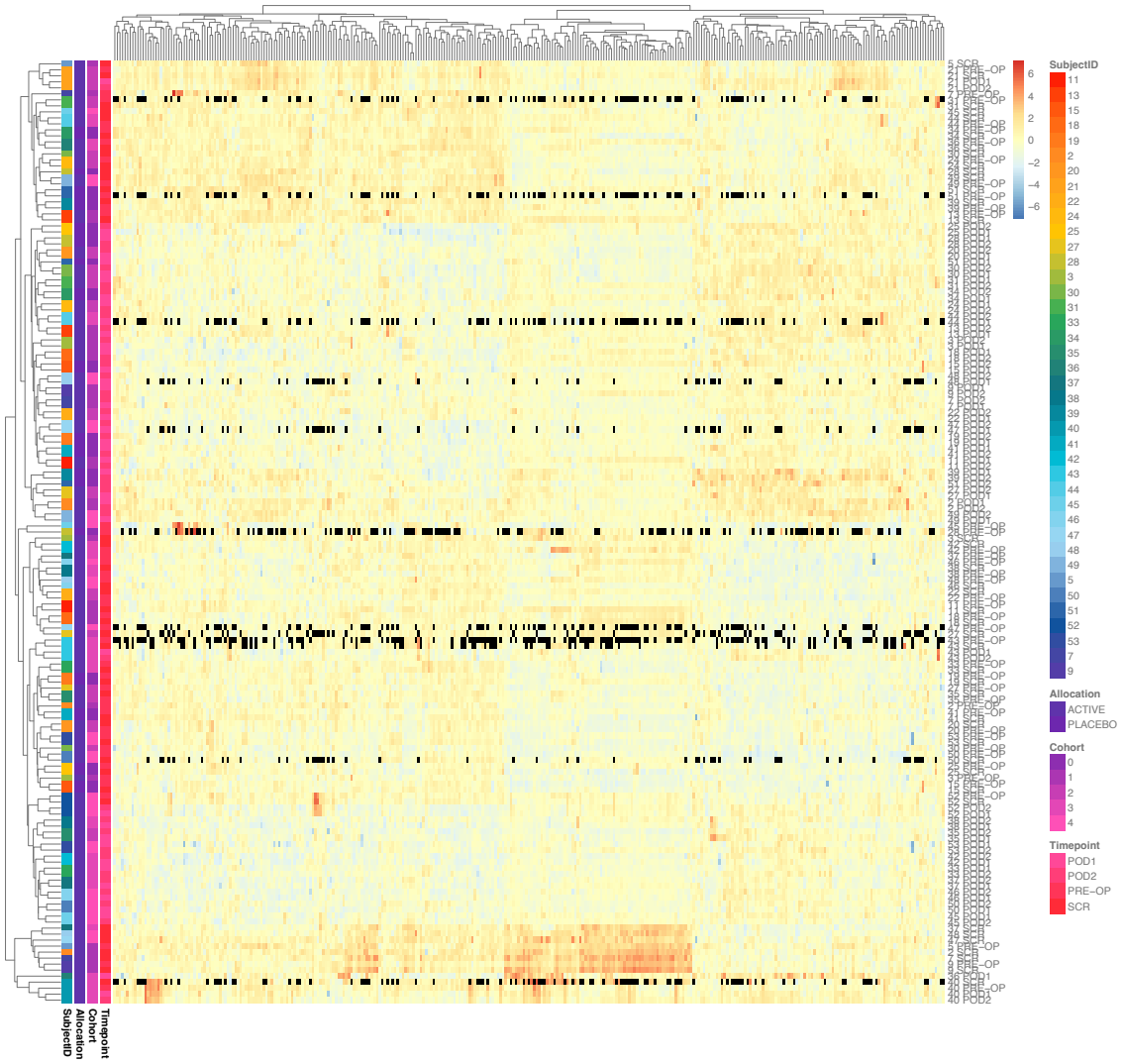



**Figure 79: Heatmap with structured hierarchical clustering of inflammatory proteins with significant differences across all metrics.**

#### 5.2.4.3 The impact of 5-ALA-SFC on inflammatory biological processes

Using findings of significant differences in inflammatory protein expression, gene ontology analyses were undertaken using the PANTHER classification system. The results of these analyses are summarised in **Table 45**, with no overlap in the three different sets of significant pathway enrichment. Broadly, 5-ALA-SFC enriches pathways involved in both the innate and adaptive immune system including macrophage activation. Treatm,ent with 5-ALA-SFC was found to be associated with changes in pathways involved in DNA replication, VEGF, MAP kinase signalling, angiogenesis and fever across different time points. Notably (particularly given the phenotype of humans with HO-1 loss of function), different doses of 5-ALA-SFC were observed to affect pathways involved in the endothelium. It should be noted that after correction for multiple testing, there were no biological processes that were significantly enriched, likely reflecting the small sample size available for analysis combined with intrinsic biological and extrinsic factor variability.

**Table 45: Biological processes affected by 5-ALA-SFC based on differences in inflammatory protein expression.**

| Gene Ontology Biological Process (top level) | Unadjusted p-value | | |
|---|---|---|---|
| | Allocation | Allocation and timepoint | Allocation, timepoint and dose |
| Negative regulation of B cell activation | 0.008 | | |
| Positive regulation of MHC class II biosynthetic process | 0.011 | | |
| B cell activation | 0.013 | 0.530 | |
| Type 2 immune response | 0.016 | | |
| Negative regulation of type II interferon production | 0.020 | | |
| Negative regulation of inflammatory response | 0.025 | | |
| Positive regulation of ubiquitin-dependent protein catabolic process | 0.026 | | |
| Positive regulation of macrophage activation | 0.032 | | |
| Regulation of receptor signalling pathway via JAK-STAT | 0.038 | 0.374 | 0.222 |
| Negative regulation of production of molecular mediator of immune response | 0.038 | | 0.222 |
| Negative regulation of interleukin-6 production | 0.045 | | 0.241 |
| Morphogenesis of a branching epithelium | 0.045 | | |
| Antigen receptor-mediated signalling pathway | 0.045 | | |
| Regulation of binding | 0.045 | | |
| Regulation of DNA replication | | 0.011 | |
| Positive regulation of vascular endothelial growth factor receptor signalling pathway | | 0.017 | |
| Positive regulation of lipid catabolic process | | 0.017 | |
| Positive regulation of cell division | | 0.025 | |
| Positive regulation of fever generation | | 0.026 | 0.117 |
| Temperature homeostasis | | 0.026 | |
| Positive regulation of MAP kinase activity | 0.350 | 0.030 | 0.297 |
| Positive regulation of lipid biosynthetic process | | 0.035 | 0.139 |
| Angiogenesis | 0.199 | 0.038 | 0.144 |
| Negative regulation of protein kinase activity | | 0.046 | |
| Positive regulation of prostaglandin secretion | | 0.046 | |
| Positive regulation of angiogenesis | 0.408 | 0.048 | 0.350 |
| Endothelial cell apoptotic process | 0.114 | | 0.005 |
| Natural killer cell mediated cytotoxicity | | | < 0.001 |
| Negative regulation of blood vessel endothelial cell migration | | | 0.008 |
| Negative regulation of endothelial cell proliferation | | | 0.011 |
| Cellular response to amyloid-beta | | | 0.011 |
| Regulation of endothelial cell apoptotic process | | | 0.031 |

Significant interactions with top-level biological processes shaded in green, non-significant interactions with processes shaded in grey.

## 5.3 Discussion

The ability of any intervention in cardiac surgery to mitigate the negative impact of the immune-inflammatory response to surgery and CPB, is of significant interest and potential clinical benefit. The totality of data presented in this chapter provide evidence for an impact of 5-ALA-SFC on the extent of the inflammatory response to surgery, most robustly in the form of an attenuated post-operative serum CRP rise. Patients allocated to the active treatment arm had less marked post-operative increases in CRP levels when compared to placebo, and a dose-response relationship was evident, consistent with an anti-inflammatory effect of 5-ALA-SFC. CRP, a hallmark acute-phase response protein, is rapidly produced in the liver under the influence of various pro-inflammatory cytokines (e.g., IL-6, IL-1ß, TNF)[336]. As an inexpensive and highly sensitive, but non-specific, marker of inflammation it is widely used in clinical practice, often to gauge treatment response to certain interventions (e.g., the effectiveness of antibiotics in treating infections).

An increase in CRP is common after cardiac surgery and this rise is due to a combination of factors such as direct surgical trauma, endotoxemia, immune activation in response to contact with the extracorporeal CPB circuit and in some cases, post-operative infections[337]. The extent of CRP rise has been linked with overall surgical trauma and immune activation, and one study describes a non-significant difference when comparing CABG cases to other surgeries (e.g., valve procedures), with the former group registering higher CRP levels on admission to ICU[338]. Although the placebo group in the present study represents a balanced group in terms of surgical intervention, the context specific effect of the surgical intervention and CPB-duration on CRP should not be overlooked. Higher peri-operative CRP levels have been linked with poorer outcomes following cardiac surgery and, in some cases, has predicted specific complications such as post-operative arrhythmias and infections[339,340]. Therapies capable of effectively targeting the overall inflammatory insult therefore remain attractive.

Importantly, no significant treatment-related changes in the overall white cell- or differential-count were found in this study, i.e. indicative that 5-ALA-SFC is not an immunosuppressant. Intriguing observations in terms of phenotypic shifts in particular of CL monocytes were identified. As evidence of target

engagement and the ability of 5-ALA-SFC to induce HO-1 expression in key effector immune cells, an increase in the proportion of CL monocytes expressing HO-1 at the highest dose of 5-ALA-SFC was observed. Further analysis suggested that these HO-$1^+$ CL monocytes possess a phagocytic phenotype, reflected by the presence of CD68 [322]. The presence of CD163 on these cells further suggests a specific role in Hb scavenging; indeed, an increase in circulating cells expressing CD163 in response to cardiac surgery has been described[154]. The potential link between 5-ALA-SFC and an increased post-operative capacity for erythrophagocytosis, becomes clearer when considering cells co-expressing CD68 and HO-1. This study has shown that within these cells, statistically significant pre-operative changes occur in response to 5-ALA-SFC treatment, particularly for CD163 expression and for the highest dose evaluated. Whilst a clear 5-ALA-SFC dose-responsive induction of a phenotypic shift favouring erythrophagocytosis has not been shown, there is a compelling directionality to the data (particularly when comparing pooled active vs placebo) that suggests a specific effect of the drug on classical monocytes and, especially, $CD68^+$ $Lin^-$ immune cells.

Increased HLA-DR expression in CL monocytes, and especially in HO-$1^+$ $CD68^+$ cells was observed in 5-ALA-SFC treated patients pre-operatively and, to a lesser degree, during the post operative period. HLA-DR, a major histocompatibility complex (MHC) class II molecule with an important role in antigen presentation and processing, has been linked with immune competency[341], and lower levels post-operatively have been associated with poorer outcomes in paediatric cardiac surgical patients[342]. A reduction in HLA-DR after cardiac surgery is expected[343], so the suggestion that this does not occur in 5-ALA-SFC treated patients may be important. Furthermore, 5-ALA-SFC at a dose of 300 mg/472 mg may cause an increase in CCR2 expression pre-operatively in CL monocytes as well as HO-$1^+$ $CD68^+$ cells. This receptor mediates monocyte chemotaxis into injured tissue (e.g., cardiac IRI), where cells may differentiate into tissue resident macrophages directing specific immune responses[344,345]. Overall, the data support the hypothesis that pre-operative 5-ALA-SFC may increase circulating HO-$1^+$ $CD68^+$ $CD163^+$ immune competent (HLA-$DR^+$) cells in patients undergoing cardiac surgery on CPB, and that this phenotypic commitment persists into the post-operative period. The findings presented in this

chapter also highlight that $CD68^+$ $Lin^-$ $CD45^+$ cells may the most sensitive immune cell subpopulation in which to investigate the cellular effects of 5-ALA-SFC and subsequent HO-1 induction.

Using an unbiased approach to analyse inflammatory proteomic data, a significant treatment-related effect on a range of pro-inflammatory cytokines was found (Table 42). Patients allocated to 5-ALA-SFC had a reduced expression of IL-6 – a key pleiotropic, pro-inflammatory cytokine - across all timepoints and given the significantly increased expression of IL-6 post-operatively identified during the timepoint analysis Ttable 41), collectively indicating that pre-operative 5-ALA-SFC use was associated with lower levels of IL-6 post-operatively. IL-6, induced by TNF-α through NFκB and MAPK signalling, is released in response to various stimuli and increased levels after cardiac surgery have been linked with poorer patient outcomes, including prolonged mechanical ventilation[346]. The ability of IL-6 to direct a profound sterile inflammatory response in the setting of cardiac surgery, has led to significant interest in developing specific therapies aimed at reducing its expression[347]. This study has shown that 5-ALA-SFC is associated with negative regulation of interleukin-6 production, possibly as a result of positive MAPK regulation which is highlighted as a key biological process affected by the interaction between 5-ALA-SFC and timepoint (Table 45). Importantly, the main limitation from pathway analysis was that after correction for multiple testing, there was no significant enrichment of any biological process, and as such any conclusions derived must be regarded as hypothesis generating. With this caveat in mind, 5-ALA-SFC treatment was associated with B-cell activation, decreased IL-6 production and negative regulation of the type II interferon response and inflammatory response with involvement of the JAK-STAT pathway. These findings are consistent with the role of IL-6 as a pleiotropic cytokine that negatively regulates the immune response and works, in part, through the JAK-STAT pathway. Several pro-survival biological processes were highlighted during this analysis and further bioinformatic work may inform the specific role of 5-ALA-SFC on these.

Taken together, the findings presented in this chapter highlight an important immunomodulatory role for 5-ALA-SFC, with compelling congruence across multiple endpoints assessed through a variety of methodologies. These findings support the potential of 5-ALA-SFC to ameliorate the systemic

inflammatory response to cardiac surgery on CPB and induce phenotypic changes in dedicated immune cell subcompartments to enhance their ability to manage a haem load which may translate to reduced organ dysfunction post-surgery. Much of the work in this chapter should be viewed as hypothesis generating and would need further exploration and validation in orthogonal larger patient cohorts as well as with respect to clinically relevant outcome measures.

# 6 Chapter 6. Discussion and future work

## 6.1 Principal findings

### 6.1.1 Safety and tolerability profile of 5-ALA-SFC in adult patients undergoing cardiac surgery on CPB

TALEN successfully evaluated treatment with 5-ALA-SFC in an adult cardiac surgical population for the first time, based on a strong rationale for HO-1 as a biological target to improve outcomes in cardiac surgery. Successful clinical development of a therapeutic strategy targeting HO-1 modulation in a cardiac surgical population will need to confirm the investigational agent is activating the HO-1 system as intended, in addition to defining the risk-benefit profile of the investigational agent. To the latter end, a primary focus of the work presented in this thesis was to obtain preliminary data to understand the tolerability and safety profile of 5-ALA-SFC across a range of dose levels when administered pre-operatively to cardiac surgical patients.

In this trial, 5-ALA-SFC, up to doses of 300 mg/472 mg for a total of seven days before surgery, was well tolerated in the studied patient population. The study treatment was taken exactly as instructed in more than > 80% patients, and 100% patients took at least the protocol-specified doses required to be evaluable for the primary analysis of pharmacodynamic effect. In the few isolated cases where doses were missed, most patients reported that they had failed to take a dose for reasons apparently unrelated to 5-ALA-SFC (e.g., 'forgot to take a dose'). Reflecting the highly invasive nature of cardiac surgery on CPB, 64% patients experienced at least one AE, but only 12% of all reported AEs were attributed to the study treatment by study investigators. All TRAEs were judged to be mild, did not require any medical intervention and were self-limiting. They reflected primarily well described TRAEs for 5-ALA-SFC and were related to gastrointestinal disturbance (e.g., nausea). There was no dose-related increase in the frequency of TRAEs and only one TRAE resulted in a dose interruption and subsequent withdrawal, with the event (headache) occurring in a participant with a history of migraine. Across the trial there were no SUSARs or DLTs. All occurrences of SAEs reflected events well-described in the context of cardiac surgery and had been pre-specified in the study protocol as anticipated events in the context of cardiac surgery – the frequency and magnitude of these were not judged to be more marked than expected

for a cardiac surgical population and none were attributed to 5-ALA-SFC. As this was an early phase translational study with a mechanistic focus, a limited sample size was required. Subsequent, expansion phase research investigating 5-ALA-SFC will be needed to further understand its safety profile and identify potential AEs of lower frequency.

The enrolled study population represented a group of patients with a median age of 69 years, most of whom had one or more medical comorbidities for which they were taking concomitant medication. The finding that 5-ALA-SFC was well tolerated in this population adds significantly to our knowledge of the safe clinical use of this compound. The pharmacokinetic profile of this drug may be part of the reason it seems to have been well tolerated[275]. The drug is rapidly absorbed after oral absorption, redistributes and is taken up into cells where it is incorporated into the haem biosynthesis pathway. Therefore, clearance does not rely on organ-dependent metabolism/excretion, and only minimal amounts of unaltered drug will be excreted renally. The drug has a very short half-life and appears to be devoid of any significant drug-drug interactions. These are all properties which are attractive for potential future use in patients who are at risk of underlying organ dysfunction or have limited physiological reserve, take multiple concomitant medications and will receive a general anaesthetic to facilitate major invasive cardiac surgery, sometimes complicated by significant organ dysfunction.

In terms of trial logistics, a few important observations have been made. First, the impact of the SARS-CoV-2019 (COVID) pandemic on clinical research in the UK cannot be understated. Whilst the initial trial design focused on a more homogeneous patient population of participants with underlying ischaemic heart disease listed for CABG on CPB, a pragmatic decision was made to include patients scheduled for valve procedures in addition to isolated CABG. This ultimately facilitated recruitment and enabled more timely completion of the trial. Inevitably this made the overall enrolled population more heterogeneous, with implications on interpretation of specific findings in the study. The specific surgery performed is intrinsically linked with certain co-morbidities (e.g., higher proportion of diabetes mellitus in a CABG-only population)[348], differences in the duration of CPB[349] and discrete differences in the extent of PMI as quantified using cardiac biomarkers[3]. The placebo group does constitute a balanced control. On the

other hand, the broader inclusion criteria represent a strength of the study, in that 5-ALA-SFC was trialled in a diverse cardiac surgical population, increasing external validity with the resultant safety, tolerability (and efficacy) data more generalisable to broader clinical practice. Furthermore, demonstration of the ability of 5-ALA-SFC to increase HO-1 expression/function in circulating immune cell subsets was confirmed regardless of surgical indication.

Detailed clinical data collection and biological sampling was used for this trial. Using an electronic medical records system (EMR) enabled accurate data collection of particularly anaesthetic, surgical and intensive care data. Electronic requests for research blood sampling allowed accurate sampling in the post-operative period, when samples were required to be taken frequently and at specific times often out of hours. This also minimised the risk of missing data and for hs-TropI, > 95% protocol defined datapoints, spanning 72 hours from aortic cross clamp removal, were fully captured in this trial. In a cardiac surgical population, CMR is rarely practical in the initial post-operative period, and accordingly blood-based biomarker-based quantification of PMI remains important[77]; the feasibility of obtaining an intense biomarker sampling frequency for quantification of PMI was demonstrated. Lastly, a pragmatic approach to expedited safety reporting was taken in this study. Given the complexity of the post-operative state in cardiac surgical patients and very high occurrence of AEs, a detailed list of anticipated AEs in this context was pre-defined in the approved study protocol. This still allowed for AE attribution to be assigned by investigators and expedited reporting if required but in the setting that the AE was judged to be simply related to cardiac surgery a serious AE (not in keeping with that specified in the Reference Safety Information of the 5-ALA-SFC investigator brochure) would not automatically trigger expedited reporting to regulatory authorities. The SRC reviewed all SAE cases and confirmed attribution. This allowed for streamlined safety reporting whilst ensuring accurate capture and representation of potential safety signals.

### 6.1.2 5-ALA-SFC activates HO-1 in key circulating immune cell subsets

5-ALA-SFC, is incorporated into the haem biosynthetic pathway with a resultant rise in intracellular haem. This potently activates *HMOX1* transcription through an NRF2 dependent mechanism, increasing

HO-1 expression and activity[253]. The drug therefore relies on harnessing the physiological handling of ALA, an essential amino acid, and iron. What is less clearly understood is the effect of increasing intracellular haem on signalling more broadly (i.e., haem related NRF2 pathway activation outside of the canonical activation of *HMOX1* transcription). The work presented in this thesis has attempted to address this gap in our understanding of the use of 5-ALA-SFC.

This study did not find clear, statistically significant treatment-related effects on four other NRF2-related genes. *NQO1* relative expression was non-significantly increased in response to the highest 5-ALA-SFC dose pre-operatively, implying a potential drug-related effect on other NRF2-dependent pathways. The relative expression of *ME1* and *TXN* was subtly reduced, particularly on post-operative day 1, in patients who had been treated with 5-ALA-SFC. The biological significance of this is unclear. However, the association between reductions in the expression of these genes and favourable shifts in carbohydrate oxidation improving redox state[325], together with an emerging understanding of the role of HO-1 in immunometabolism and redox regulation[204], points to a possible immunometabolic intersection of haem-associated increased intracellular HO-1, and regulation of other NRF2 dependent pathways which merits further investigation.

Unbiased analyses of publicly available transcriptomic datasets identified monocytes as enriching for *HMOX1*. Accordingly, PBMCs (i.e., monocytes and dendritic cells) were selected as 'bulk' immune cells in which to evaluate for 5-ALA-SFC target engagement through interrogation of HO-1 expression and HO activity. Previous experience with 5-ALA-SFC in humans (largely healthy volunteer based) has relied on transcriptomic and proteomic data to confirm PD drug effect. To date there is no evidence that 5-ALA-SFC increases enzymatic function in humans, a key PD metric when developing drugs targeting enzymatic catalytic activity[323].The development and successful application of a novel, first-in-human LC-MS/MS based HO activity assay through biliverdin quantification addresses this shortcoming. The data presented in this thesis confirm drug-related activation of HO function consistent with 5-ALA-SFC inducible HO-1 activation, with evidence to suggest a dose-response relationship. A challenge in interpreting the HO enzyme activity data (beyond lack of specificity for HO-1 alone) was the observation

of higher baseline enzyme function observed in the 5-ALA-SFC 300 mg/472 mg treatment allocation group. Evaluation of patients randomly allocated to this treatment group revealed a predominance of a shorter *HMOX1* (GT)n repeat polymorphism, which is associated with higher baseline HO-1 activity. Furthermore, these patients also had higher baseline serum bilirubin levels, measured by an independent hospital clinical laboratory. Importantly, significant increases in HO activity were identified with this dose level from a bulk PBMC population (which may have limited sensitivity compared to immune cell subsets) using an analysis (ANCOVA) which accounts for baseline activity values and when adjusted for *HMOX1* polymorphism status. Additional technical factors to consider with the assay include the potential impact of prolonged storage of PBMCs at -80°C (rather than in liquid nitrogen) and, although the placebo group acts as a reasonable control for this, the assay would require further validation for use in larger studies.

Confirmation of increased HO-1 in response to 5-ALA-SFC has typically been demonstrated using bulk immune cell analysis techniques (e.g., PBMC protein extraction and ELISA)[257]. As an improvement to this, high dimensional flow cytometry was employed to identify increases initially in HO-1 expression in bulk PBMCs ($Lin^-$ $CD45^+$) and, secondarily, to identify specific immune cell populations most optimal for study of the effect of 5-ALA-SFC on HO-1 expression, function and downstream cellular signalling. In patients treated with 5-ALA-SFC, a significant dose-related increase in % $HO\text{-}1^+$ $Lin^-$ $CD45^+$ cells was observed pre-operatively. Furthermore, as initial flow cytometry analysis had revealed a drug treatment-associated expansion of the CL monocyte population, CL monocytes were further evaluated. A PD drug response was again seen with a substantial increase in the proportion of $HO\text{-}1^+$ CL monocytes in response to 5-ALA-SFC, with data suggesting a dose-related increase vs. placebo. Given the striking co-expression of *HMOX1* and *CD68* in immune cells across multiple $RNA_{seq}$ datasets, using CD68 as a starting point to identify cells which may express HO-1, a significant $HO\text{-}1^+$ $CD14^-$ $CD16^-$ (i.e., non-monocyte) population was observed, and in these cells a similar 5-ALA-SFC dose-dependent increase in HO-1 expression was appreciated. This phenotypic switch, favouring increased HO-1 expression in 5-ALA-SFC treated patients vs. placebo, persisted in the post-operative period consistently across bulk PBMCs, CL monocytes and $CD68^+$ $Lin^-$ $CD45^+$ cells.

At odds with compelling evidence of both increased protein expression and activity, relative expression of *HMOX1* transcript using qPCR did not display similar pre-operative increases. Cellular responses to drugs are temporally dynamic when considering effects at transcript, protein and post-assembly levels and this finding may reflect a temporal disparity between the effects on gene vs. protein expression[350], technical factors (lability of mRNA) and sensitivity to drug effect of mRNA from a bulk immune cell population. The suggestion that patients exposed to 5-ALA-SFC have increased expression of *HMOX1* post-operatively is of uncertain significance but may be advantageous in promoting antioxidant responses and mitigating inflammatory stress beyond the immediate post-operative day.

Overall, the findings from orthogonal analyses using the HO activity assay and flow cytometry are statistically significant and concordant, pointing to dose-dependent ability of 5-ALA-SFC to increase HO-1 activity. The most significant increase in HO-1 expression/HO function was seen at the highest dose, supporting the use of this drug at a dose of 300 mg/472 mg twice daily pre-operatively for maximal PD effect.

### 6.1.3 HO-1 induction through pre-operative 5-ALA-SFC as a strategy to reduce AKI in adult patients undergoing cardiac surgery on CPB

AKI in cardiac surgery is common and has an attributable mortality rate of as high as 30%[131]. Developing strategies specifically targeting improved renal outcomes in cardiac surgery therefore represents an important unmet clinic need. The incidence of clinically relevant AKI (according to the KDIGO classification) in this study was 14%. Although TALEN was not powered to make any meaningful deductions on a reduction in AKI, a striking and clinically meaningful signal for renal protection has been observed. When considering multiple biochemical assays assessing renal function (e.g., eGFR calculated CKD-EPI equations, including serum cystatin C) and injury (e.g., urinary NGAL), together with consistent dose-response effects, the data strongly suggest the potential for 5-ALA-SFC to exert renoprotective effects in the context of cardiac surgery on CPB, although the degree to which the magnitude of mitigation in change in renal biomarkers would translate to clinical hard outcomes (e.g.

requirement for temporary or permanent renal replacement therapy or significant durable loss of renal excretory capacity) is unknown and would require evaluation in a much larger, adequately powered trial. The most profound signal for renal protection with respect to biomarkers was observed in patients allocated to the highest dose of 5-ALA-SFC.

Given the concordance of data suggesting benefit from HO-1 activation, this thesis also aimed to investigate potential mechanisms by which 5-ALA-SFC may offer clinically relevant protection against organ injury such as acute kidney injury after cardiac surgery on CPB. The exposition of literature around AKI in cardiac surgery has shown that the aetiology is multi-factorial[135,140]. The haemolytic insult, and subsequent increases in systemic cf-Hb and NTBI associated with CPB appears to be central to its pathogenesis[14,120,138]. The presence of *HMOX1* in cells and tissues of the reticuloendothelial system (discussed in chapter 4), the central role of HO-1 in mounting an inducible cellular response to increases in haem[155], the established regulatory function of MPS cells in iron homeostasis and redox control[204], all suggest that 5-ALA-SFC may directly influence this established homeostatic system to mitigate the negative sequelae of haemolysis.

Although there is evidence linking increased HO-1 function with reduced haemolysis after cardiac surgery[249], the findings of the work presented in this thesis, with the caveat of a relatively limited patient sample size, do not clearly support this. The degree of haemolysis, assessed using an absorbance assay detecting cf-Hb, was comparable across all treatment allocation groups and no clear 5-ALA-SFC dose-related changes were observed. Surgery on CPB was temporally associated with significant haemolysis and it is likely that the mechanical injury caused by the extracorporeal circuit is so significant, that any drug would have a limited ability to overcome this. Furthermore, NTBI peaked after surgery and was detected most frequently in patients who had been treated with the higher doses of 5-ALA-SFC. Despite this finding, patients allocated to the highest 5-ALA-SFC dose had lower mean and median MDA measurements vs. placebo during the post-operative period, suggesting less profound lipid peroxidation, a hallmark of ferroptosis. Taken together, these findings suggest that drug-induced HO-1 activation does not significantly reduce the extent of the haemolytic injury and (in the context of concomitant exogenous

iron in the form of SFC) potentially increases systemic NTBI transiently. Due to the low frequency of patients with quantifiable NTBI in this study, the latter finding would need further investigation. Reassuringly, the signal suggesting reduced ferroptosis in patients treated with 5-ALA-SFC possibly points towards improved erythrophagocytosis and immune ferrostasis as mechanisms to avoid cellular injury.

Multiple lines of evidence presented in this thesis support the hypothesis that 5-ALA-SFC associated increases in HO-1 expression in immune cells, prime them to enable more efficient innate immune responses to haemolysis. First, an induction of HO-1 was demonstrated in both CL monocytes and $CD68^+$ $Lin^-$ $CD45^+$ mononuclear cells. A striking increase was observed in the % $HO\text{-}1^+$ $C68^+$ $CD163^+$ cells in patients treated with 5-ALA-SFC at the highest dose vs. placebo, both pre-operatively and in the post-operative period, suggesting a fixed phenotypic commitment favouring immune cells possessing a specific dedicated role in erythrophagocytosis. Although there was a temporal response between an increase in this population and surgery in all patients, this was more profound in patients treated with 5-ALA-SFC vs. placebo, suggesting that the drug may enhance an existing innate immune response to haemolysis. There is an emerging understanding that circulating mononuclear cells may have a significant role to play in the innate response to haemolysis, regulating iron homeostasis and redox balance[151]. The findings presented in this thesis add *in vivo* data to support this.

Furthermore, transcriptomic data point to an increased capacity for NTBI uptake by immune cells following 5-ALA-SFC treatment, suggested by significantly increased relative expression of *SLC11A2* (coding for DMT1) on the first post-operative day in patients allocated to the highest 5-ALA-SFC dose. Although not statistically significant, there was an apparent dose-related increase in *FTH* expression (coding for ferritin heavy chain 1, or H-type ferritin) evident before surgery. H-type ferritin is highly capable of oxidising $Fe^{2+}$, thereby neutralising redox-active iron and allowing for safe sequestration of inert $Fe^{3+}$ in immune cells[351]. Relative expression of *SLC40A1* (coding for the iron exporter ferroportin) was apparently reduced in 5-ALA-SFC treated patients and this was most noticeable on the third day. Although this finding was not statistically significant, it could be explained by lower levels of

intracellular labile iron, recognised as a regulator of FPN transcription[352]. These findings are not conclusive, but hypothesis generating in the context of this trial and would require further investigation. Nevertheless, the signal is consistent with an enhanced capacity for uptake, neutralisation, and storage of NTBI by circulating immune cells attendant upon 5-ALA-SFC exposure.

Overall, the ability of 5-ALA-SFC to improve renal protection in cardiac surgery is plausibly linked to an enhanced innate immune response to haemolysis to mitigate haem-induced nephrotoxicity.

### 6.1.4 5-ALA-SFC is an immunomodulatory drug

Although increased HO-1 activity has been linked with potent antioxidant effects, immunomodulation and favourable immunometabolic shifts[204], the impact of pharmacological induction of this enzyme on immune function has not been studied in a clinical setting of acute inflammation associated with surgery. In the TALEN trial, using data from 5-ALA-SFC treated patients undergoing cardiac surgery on CPB, which represents a profound sterile inflammatory stimulus, the effect of pharmacological induction of HO-1 on a profound subsequent inflammatory response has been studied for the first time. The results of the work presented in this thesis address an important gap in our understanding of the ability of acute pharmacological HO-1 induction to result in a clinically favourable immunomodulatory profile in patients.

The most striking evidence to suggest that 5-ALA-SFC induced upregulation of HO-1 positively regulates inflammation after cardiac surgery was the dose-dependent reduction in post-operative CRP levels. This was highly statistically significant, particularly by the third post-operative day, apparent even with the lowest dose of 5-ALA-SFC assessed across a broad range of biomarkers and represented large absolute and relative reductions compared to the degree of CRP rise observed with placebo. As a highly sensitive marker for inflammation, these data suggest a lower degree of systemic inflammation in patients allocated to the study drug despite a broadly comparable inciting stimulus across treatment groups. A parallel decrease in IL-6 was also demonstrated, and through non-biased proteomic analysis IL-6 was identified as the most significantly down regulated inflammatory protein in patients allocated

to the active study treatment vs. placebo. Taken together, these two findings are highly complementary and concordant with a discrete anti-inflammatory effect of 5-ALA-SFC. Considering the mechanisms by which HO-1 activation may reduce IL-6 and CRP expression, unbiased analyses of inflammatory proteomic data have identified several biological processes that may be implicated, representing important hypotheses for the further study of the drug in this context.

Using multi-dimensional flow cytometry, functional assessment of monocytes identified consistent signals for drug-associated phenotypic changes. Although not all findings were statistically significant, concordant observations were made across multiple markers related to inflammatory activation state, scavenging, chemotaxis, and antigen presentation capacity of monocytes. The most significant finding was that 5-ALA-SFC caused monocyte polarisation towards the CL monocyte phenotype. This occurred prior to cardiac surgery in patients who had received the drug, with evidence for a dose-response effect. Although it is accepted that monocytes represent highly diverse innate immune cells with vast heterogeneity in terms of function[146], CL monocytes are regarded as important innate immune cells with monitoring (e.g., sensing PAMPs and DAMPs), phagocytic and antigen presenting functions[353]. The effects of the drug on HLA-DR and CCR2 expression, suggested the potential for improved immune competency as well as an increased ability for tissue migration and scavenging, although the changes did not meet statistical significance and should be considered exploratory and hypothesis generating. Taken together, the directionality of the data, along with assessments of pooled active drug vs. placebo patients, suggest the ability of 5-ALA-SFC to induce a fixed phenotypic commitment of CL monocytes which may mediate a more effective innate immune response to surgery on CPB. It is hoped that further research with an appropriately powered trial using a similar approach to immunophenotyping monocytes may help validate these findings.

The precise molecular mechanism by which 5-ALA-SFC affects immune regulation is unclear. The central role of HO-1 activation has been discussed and this is certainly a reasonable contributor/driver. Furthermore, it is possible that small, non-lethal increases in intracellular haem induce long-lasting epigenetic changes to enact trained innate immunity[114].

### 6.1.5 Concluding remarks

Considering the relevant tolerability, safety, and efficacy data, the findings from the TALEN study identify pre-operative 5-ALA-SFC at a dose of 300 mg/472 mg twice daily for three days for further investigation in larger trials to improve outcomes after cardiac surgery, potentially with the consideration of an additional lower dose level. This recommendation is made based on evidence for this dose being associated most consistently with evidence of increased HO activity, as well as increased immune cell expression of HO-1 at this dose. Further confidence in the dose selection is provided by multiple orthogonal biological signals that could drive clinical benefit, including the most convincing evidence of renal protection and immunomodulation at this higher dose. Primary efficacy data from future trials could include assessment of enzymatic HO function and HO-1 expression in $CD68^{+}$ $Lin^{-}$ $CD45^{+}$ mononuclear cells, in addition to any bulk analyses (e.g., PBMC HO-1 by ELISA). The findings from this work lead to the recommendation that future trials of 5-ALA-SFC are powered to A) detect a meaningful impact on AKI after cardiac surgery and B) investigate 5-ALA-SFC as a strategy for cardioprotection.

## 6.2 Significance of work

The work presented in this thesis reflects the first trial of 5-ALA-SFC as a pharmacological means of targeting HO-1 activation, as a strategy for improving outcomes in adult patients undergoing cardiac surgery on CPB. The primary objective of this work has been met, and a recommendation for an OBD for future clinical trials can be made. Furthermore, important design considerations in this study may be implemented in subsequent research and these include pragmatic approaches to AE reporting, as well as electronic prescription of requests for biological sampling to limit the incidence of missing blood-based biomarker data. A novel LC-MS/MS based HO functional assay for PBMCs has been developed and successfully used to assess drug PD effect. This method was proven to be reproducible and could be used in further evaluation of this compound in the clinical setting. This research has also added to our understanding of the use of 5-ALA-SFC in adult patients, and specific contributions have been highlighted above. Most significantly, in addition to a biologically plausible role in cardioprotection, this

work has shown that HO-1 induction has the potential to improve renal outcomes after cardiac surgery and any future investigation into this compound should investigate this. Finally, early progress has been made on understanding the immunomodulatory role of HO-1 induction through 5-ALA-SFC and this should represent an important focus of further research.

### 6.3 Strengths and limitations of this work

As a single centre study, TALEN was able to ensure excellent compliance with trial requirements and interventions. Thorough biological sampling was possible, ensuring minimal missing data across the trial. Conduct of CPB was standardised, reducing the confounding impact of wide variations in CPB practice. Unfortunately, as a single centre study this does limit external validity. The study population was primarily male and Caucasian, and further research would need to confirm preliminary signals for safety and drug efficacy in female and non-Caucasian patients. The variation in surgical indications adds complexity in reaching conclusions on certain important endpoints (e.g., PMI). This does, however, enable certain safety and efficacy extrapolations to be made to a broader cardiac surgical population.

This study has employed multiple readouts of HO-1 biology, including a novel enzyme activity assay, confirming 5-ALA-SFC target engagement which should enable more confident selection of an OBD. Furthermore, the intensive collection of relevant clinical endpoints and especially biological profiling of inflammation, organ injury and function represents a comprehensive dataset and multiple signals for potential drug benefit, some in which a dose-response relationship was apparent. This could add further certainty to the selection of the dose of 5-ALA-SFC for future multi-centre trials.

Finally, due to the small study population in which multiple dosages of the study drug have been investigated, this study was underpowered for all clinical endpoints. Furthermore, as a primarily translational early phase study, multiple comparisons were made without corrections in the majority of cases and thus most of the p-values must be considered nominal. Consequently, most of the mechanistic/exploratory findings in this trial should be viewed cautiously and require external validation in larger, well-powered multi-centre trials.

### 6.4 Future work

It is hoped that further late phase clinical research into 5-ALA-SFC will expand on certain important contributions made during the conduct of this research:

1. Further clinical research into this molecule in cardiac surgical patients will hopefully confirm the preliminary safety and tolerability findings made in this study, especially in female and non-Caucasian patients. A similar, pragmatic approach to AE reporting is advised to ensure that all AE data is collected, regardless of whether it is anticipated in the context of cardiac surgery or not.

2. Specific clinically relevant signals for improved outcomes after surgery have been proposed by the work presented in this thesis, and it is hoped that further clinical research into this drug's utility in adult cardiac surgical patients should focus on these specific themes:
    a. A signal for renal protection – this would require trials specifically powered to detect clinically relevant reductions in AKI endpoints such as AKI by the KDIGO classification or MAKE. This should be accompanied by a simar approach to in-depth sampling, including biomarkers of renal function and injury, to ensure early detection of AKI.
    b. A rationale for cardioprotection – pooled data by surgical indication in this study point to a possible signal for cardiac protection and note was made of lower incidence of arrhythmia and inopressor requirement in the 5-ALA-SFC groups versus placebo but these observations are made post-hoc. Nonetheless, the biological rationale for a cardioprotective role for HO-1 induction is sound and should be explored focussing on detecting and quantifying PMI using serial troponin measures, consideration of CMR quantification of infarct size and improved cardiac tissue sampling to confirm organ specific 5-ALA-SFC target engagement.
    c. A signal for immunomodulation – although most of the findings made on this theme are viewed as hypothesis generating, the biological plausibility as well as striking directionality to multiple endpoints related to the immune-inflammatory response to cardiac surgery on CPB, in particular an apparent discrete effect on innate immune cells with polarisation towards a specific phenotype for erythrophagocytosis, deserves exploration. The possibility of developing a compound that

can mitigate the negative inflammatory impact of acute haemolysis by harnessing an innate, homeostatic mechanisms, is attractive and of potential clinical importance. This could be explored through multiple approaches including single cell RNAseq focussing on HO-$1^{+}$ $CD68^{+}$ $Lin^{-}$ $CD45^{+}$ immune cells. Further bioinformatics analyses of available proteomic data, including pathway enrichment analyses and potentially further proteomic profiling would generate additional mechanistic insights. Finally, the ability of increases in intracellular haem to induce trained innate immunity should be investigated using ChIP-Seq or ATAC-seq to examine the epigenetic impact of this drug.

3. The mechanism of action of 5-ALA-SFC and its haem mediated effect on NRF2 associated pathways should be explored further. This could be addressed through determining intracellular levels of haem in immune cells through absorption spectroscopy/LC-MS/MS, more extensive transcriptomic analyses (e.g., single cell RNAseq) focussing on pathway analyses related to NRF2 activation.

## 7 References


1. Robinson, L. A., Schwarz, G. D., Goddard, D. B., Fleming, W. H. & Galbraith, T. A. Myocardial protection for acquired heart disease surgery: results of a national survey. *Ann Thorac Surg* **59**, 361–372 (1995).
2. Croal, B. L. *et al.* Relationship Between Postoperative Cardiac Troponin I Levels and Outcome of Cardiac Surgery. *Circulation* **114**, 1468–1475 (2006).
3. Devereaux, P. J. *et al.* High-Sensitivity Troponin I after Cardiac Surgery and 30-Day Mortality. *New England Journal of Medicine* **386**, 827–836 (2022).
4. Algarni, K. D., Maganti, M. & Yau, T. M. Predictors of low cardiac output syndrome after isolated coronary artery bypass surgery: Trends over 20 years. *Annals of Thoracic Surgery* **92**, 1678–1684 (2011).
5. Salis, S. *et al.* Cardiopulmonary Bypass Duration Is an Independent Predictor of Morbidity and Mortality After Cardiac Surgery. *J Cardiothorac Vasc Anesth* **22**, 814–822 (2008).
6. Pahwa, S. *et al.* Impact of postoperative complications after cardiac surgery on long-term survival. *J Card Surg* **36**, 2045–2052 (2021).
7. Roques, F. *et al.* Risk factors and outcome in European cardiac surgery: analysis of the EuroSCORE multinational database of 19030 patients. *Eur J Cardiothorac Surg* **15**, 813–816 (1999).
8. Mehaffey, J. H. *et al.* Cost of individual complications following coronary artery bypass grafting. *Journal of Thoracic and Cardiovascular Surgery* **155**, 875-882.e1 (2018).
9. Hausenloy, D. J. *et al.* Remote Ischemic Preconditioning and Outcomes of Cardiac Surgery. *N Engl J Med* **373**, 1408–1417 (2015).
10. Cleland, W. P. The evolution of cardiac surgery in the United Kingdom. *Thorax* **38**, 887–896 (1983).
11. Machin, D. & Allsager, C. Principles of cardiopulmonary bypass. *Continuing Education in Anaesthesia, Critical Care and Pain* **6**, 176–181 (2006).
12. Coulson, T. & Falter, F. Principles of Cardiopulmonary Bypass. in *Core Topics in Cardiac Anaesthesia* vol. Third edition 30–44 (2020).
13. Warren, O. J. *et al.* The Inflammatory Response to Cardiopulmonary Bypass: Part 1-Mechanisms of Pathogenesis. *J Cardiothorac Vasc Anesth* **23**, 223–231 (2009).
14. Vermeulen Windsant, I. C. *et al.* Hemolysis during cardiac surgery is associated with increased intravascular nitric oxide consumption and perioperative kidney and intestinal tissue damage. *Front Physiol* **14**, 1–9 (2014).
15. Vercaemst, L. Hemolysis in Cardiac Surgery Patients Undergoing Cardiopulmonary Bypass: A Review in Search of a Treatment Algorithm. *J Extra Corpor Technol* **40**, 257–267 (2008).
16. Kunst, G. *et al.* 2019 EACTS/EACTA/EBCP guidelines on cardiopulmonary bypass in adult cardiac surgery. *Br J Anaesth* **123**, 713–757 (2019).
17. Garg, A. X. *et al.* Coronary Artery Bypass Grafting Surgery Off- or On-pump Revascularisation Study (CORONARY): Kidney substudy analytic protocol of an international randomised controlled trial. *BMJ Open* **2**, (2012).
18. Mentzer, R. M. J. Myocardial protection in heart surgery. *J Cardiovasc Pharmacol Ther* **16**, 290–297 (2011).
19. Xia, Z., Li, H. & Irwin, M. G. Myocardial ischaemia reperfusion injury: the challenge of translating ischaemic and anaesthetic protection from animal models to humans. *Br J Anaesth* **117 Suppl**, ii44–ii62 (2016).
20. Di Lisa, F. & Bernardi, P. Mitochondria and ischemia-reperfusion injury of the heart: Fixing a hole. *Cardiovasc Res* **70**, 191–199 (2006).
21. Domanski, M. J. *et al.* Association of Myocardial Enzyme Elevation and Survival Following Coronary Artery Bypass Graft Surgery. *JAMA* **305**, 585–591 (2011).
22. Lurati Buse, G. A. *et al.* The prognostic value of troponin release after adult cardiac surgery - a meta-analysis. *European Journal of Cardio-thoracic Surgery* **37**, 399–406 (2010).
23. Paparella, D. *et al.* Myocardial damage influences short- and mid-term survival after valve surgery: A prospective multicenter study. *Journal of Thoracic and Cardiovascular Surgery* **148**, 2373-2379.e1 (2014).
24. Thygesen, K. *et al.* Fourth Universal Definition of Myocardial Infarction (2018). *J Am Coll Cardiol* **72**, 2231–2264 (2018).
25. Simonetti, O. P. *et al.* An improved MR imaging technique for the visualization of myocardial infarction. *Radiology* **218**, 215–223 (2001).

26. Pegg, T. J. *et al.* Utility of cardiac biomarkers for the diagnosis of type V myocardial infarction after coronary artery bypass grafting: insights from serial cardiac MRI. *Heart* **97**, 810–816 (2011).
27. Bøtker, H. E. *et al.* Practical guidelines for rigor and reproducibility in preclinical and clinical studies on cardioprotection. *Basic Res Cardiol* **113**, (2018).
28. Croal, B. L. *et al.* Relationship between postoperative cardiac troponin I levels and outcome of cardiac surgery. *Circulation* **114**, 1468–1475 (2006).
29. Quinn, D. W. *et al.* Improved myocardial protection during coronary artery surgery with glucose-insulin-potassium: a randomized controlled trial. *J Thorac Cardiovasc Surg* **131**, 34–42 (2006).
30. Maganti, M. D., Rao, V., Borger, M. A., Ivanov, J. & David, T. E. Predictors of low cardiac output syndrome after isolated aortic valve surgery. *Circulation* **112**, i448-452 (2005).
31. Chiari, P. *et al.* Multimodal cardioprotective strategy in cardiac surgery (the ProCCard trial): Study protocol for a multicenter randomized controlled trial. *Trials* **20**, (2019).
32. Turer, A. T. & Hill, J. A. Pathogenesis of Myocardial Ischemia-Reperfusion Injury and Rationale for Therapy. *Am J Cardiol* **106**, 360–368 (2010).
33. Murry, C. E., Jennings, R. B. & Reimer, K. A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* **74**, 1124–1136 (1986).
34. Heusch, G. Myocardial ischaemia–reperfusion injury and cardioprotection in perspective. *Nat Rev Cardiol* **17**, 773–789 (2020).
35. Jenkins, D. P. *et al.* Ischaemic preconditioning reduces troponin T release in patients undergoing coronary artery bypass surgery. *Heart* **77**, 314–318 (1997).
36. Zhao, Z.-Q. *et al.* Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. *Am J Physiol Heart Circ Physiol* **285**, 579–588 (2003).
37. Hausenloy, D. J. *et al.* Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. *Lancet* **370**, 575–579 (2007).
38. Heusch, G. Molecular basis of cardioprotection signal transduction in ischemic pre-, post-, and remote conditioning. *Circ Res* **116**, 674–699 (2015).
39. Heusch, G., Boengler, K. & Schulz, R. Cardioprotection: Nitric oxide, protein kinases, and mitochondria. *Circulation* **118**, 1915–1919 (2008).
40. Boengler, K., Lochnit, G. & Schulz, R. Mitochondria "THE" target of myocardial conditioning. *American Journal of Physiology-Heart and Circulatory Physiology* **315**, H1215–H1231 (2018).
41. Hendrickson, S. C., St Louis, J. D., Lowe, J. E. & Abdel-aleem, S. Free fatty acid metabolism during myocardial ischemia and reperfusion. *Mol Cell Biochem* **166**, 85–94 (1997).
42. Liedtke, A. J. Alterations of carbohydrate and lipid metabolism in the acutely ischemic heart. *Prog Cardiovasc Dis* **23**, 321–336 (1981).
43. Drury, N. E. *et al.* The effect of perhexiline on myocardial protection during coronary artery surgery: a two-centre, randomized, double-blind, placebo-controlled trial. *European Journal of Cardio-Thoracic Surgery* **47**, 464–472 (2015).
44. Hagerman, A., Schorer, R., Putzu, A., Keli-Barcelos, G. & Licker, M. Cardioprotective Effects of Glucose-Insulin-Potassium Infusion in Patients Undergoing Cardiac Surgery: A Systematic Review and Meta-Analysis. *Semin Thorac Cardiovasc Surg* (2022) doi:10.1053/j.semtcvs.2022.11.002.
45. Howell, N. J. *et al.* Glucose-insulin-potassium reduces the incidence of low cardiac output episodes after aortic valve replacement for aortic stenosis in patients with left ventricular hypertrophy: results from the Hypertrophy, Insulin, Glucose, and Electrolytes (HINGE) trial. *Circulation* **123**, 170–177 (2011).
46. Czibik, G., Steeples, V., Yavari, A. & Ashrafian, H. Citric Acid Cycle Intermediates in Cardioprotection. *Circ Cardiovasc Genet* **7**, 711–719 (2014).
47. Sapieha, P. *et al.* The succinate receptor GPR91 in neurons has a major role in retinal angiogenesis. *Nat Med* **14**, 1067–1076 (2008).
48. Hochachka, P. W., Owen, T. G., Allen, J. F. & Whittow, G. C. Multiple end products of anaerobiosis in diving vertebrates. *Comp Biochem Physiol B* **50**, 17–22 (1975).
49. Taegtmeyer, H. Metabolic responses to cardiac hypoxia. Increased production of succinate by rabbit papillary muscles. *Circ Res* **43**, 808–815 (1978).

50. Kula-Alwar, D., Prag, H. A. & Krieg, T. Targeting Succinate Metabolism in Ischemia/Reperfusion Injury. *Circulation* **140**, 1968–1970 (2019).
51. Kleinbongard, P., Heusch, G. & Kleinbongard, P. Extracellular signalling molecules in the ischaemic/reperfused heart-druggable and translatable for cardioprotection? *Br J Pharmacol* **172**, 2010–2025 (2010).
52. Ross, A. M., Gibbons, R. J., Stone, G. W., Kloner, R. A. & Alexander, R. W. A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). *J Am Coll Cardiol* **45**, 1775–1780 (2005).
53. Savarese, G. *et al.* A meta-analysis reporting effects of angiotensin-converting enzyme inhibitors and angiotensin receptor blockers in patients without heart failure. *J Am Coll Cardiol* **61**, 131–142 (2013).
54. Her, A. Y. *et al.* The impact of angiotensin-converting-enzyme inhibitors versus angiotensin receptor blockers on 3-year clinical outcomes in patients with acute myocardial infarction without hypertension. *PLoS One* **15**, (2020).
55. Zhao, X. *et al.* Early ACEI/ARB use and in-hospital outcomes of acute myocardial infarction patients with systolic blood pressure <100 mmHg and undergoing percutaneous coronary intervention: Findings from the CCC-ACS project. *Front Cardiovasc Med* **9**, (2022).
56. Roth, S. *et al.* Pharmacological conditioning of the heart: An update on experimental developments and clinical implications. *Int J Mol Sci* **22**, 1–19 (2021).
57. Irwin, M. G. & Wong, G. T. C. Remifentanil and Opioid-Induced Cardioprotection. *J Cardiothorac Vasc Anesth* **29**, S23–S26 (2015).
58. Kato, R., Ross, S. & Foëx, P. Fentanyl protects the heart against ischaemic injury via opioid receptors, adenosine A1 receptors and K(ATP) channel linked mechanisms in rats. *Br J Anaesth* **84**, 204–214 (2000).
59. Melo, Z., Ishida, C., Goldaraz, M. de la P., Rojo, R. & Echavarria, R. Novel roles of non-coding RNAs in opioid signaling and cardioprotection. *Noncoding RNA* **4**, (2018).
60. Zaugg, M., Lucchinetti, E., Behmanesh, S. & Clanachan, A. Anesthetic cardioprotection in clinical practice from proof-of-concept to clinical applications. *Curr Pharm Des* **20**, 5706–5726 (2014).
61. Zhu, D. *et al.* Nitrate-functionalized patch confers cardioprotection and improves heart repair after myocardial infarction via local nitric oxide delivery. *Nat Commun* **12**, (2021).
62. Farah, C., Michel, L. Y. M. & Balligand, J. L. Nitric oxide signalling in cardiovascular health and disease. *Nat Rev Cardiol* **15**, 292–316 (2018).
63. Sanada, S. *et al.* Cardioprotective Effect Afforded by Transient Exposure to Phosphodiesterase III Inhibitors The Role of Protein Kinase A and p38 Mitogen-Activated Protein Kinase. *Circulation* **104**, 705–710 (2001).
64. Kukreja, R. C. *et al.* Cardioprotection with phosphodiesterase-5 inhibition - A novel preconditioning strategy. *J Mol Cell Cardiol* **36**, 165–173 (2004).
65. Torregroza, C. *et al.* Perioperative Cardioprotection: General Mechanisms and Pharmacological Approaches. *Anesth Analg* **131**, 1765–1780 (2020).
66. Yadav, M., Kumari, P., Yadav, V. & Kumar, S. Pharmacological preconditioning with phosphodiestrase inhibitor: an answer to stem cell survival against ischemic injury through JAK/STAT signaling. *Heart Fail Rev* **25**, 355–366 (2020).
67. Lemoine, S., Tritapepe, L., Hanouz, J. L. & Puddu, P. E. The mechanisms of cardio-protective effects of desflurane and sevoflurane at the time of reperfusion: Anaesthetic post-conditioning potentially translatable to humans? *Br J Anaesth* **116**, 456–475 (2016).
68. Lotz, C. & Kehl, F. Volatile anesthetic-induced cardiac protection: Molecular mechanisms, clinical aspects, and interactions with nonvolatile agents. *J Cardiothorac Vasc Anesth* **29**, 749–760 (2015).
69. Wu, J. *et al.* Sevoflurane postconditioning protects the myocardium against ischemia/reperfusion injury via activation of the JAK2-STAT3 pathway. *PeerJ* **2017**, (2017).
70. Wang, B. *et al.* Propofol induces cardioprotection against ischemia-reperfusion injury via suppression of transient receptor potential vanilloid 4 channel. *Front Pharmacol* **10**, (2019).
71. Li, Y. mei *et al.* Propofol-mediated cardioprotection dependent of microRNA-451/HMGB1 against myocardial ischemia-reperfusion injury. *J Cell Physiol* **234**, 23289–23301 (2019).
72. Yu, X. *et al.* Propofol attenuates myocardial ischemia reperfusion injury partly through inhibition of resident cardiac mast cell activation. *Int Immunopharmacol* **54**, 267–274 (2018).

73. Flick, M. *et al.* Helium postconditioning regulates expression of caveolin-1 and -3 and induces RISK pathway activation after ischaemia/reperfusion in cardiac tissue of rats. *Eur J Pharmacol* **791**, 718–725 (2016).
74. Smit, K. F. *et al.* Helium alters the cytoskeleton and decreases permeability in endothelial cells cultured in vitro through a pathway involving Caveolin-1. *Sci Rep* **8**, (2018).
75. Schilling, J. M., Head, B. P. & Patel, H. H. Caveolins as regulators of stress adaptation. *Mol Pharmacol* **93**, 277–285 (2018).
76. Society for Cardiothoracic Surgery of Great Britain and Ireland. Recently published trials. *https://scts.org/professionals/research/recently_published_trials.aspx* (2023).
77. Heusch, G. Critical Issues for the Translation of Cardioprotection. *Circ Res* **120**, 1477–1486 (2017).
78. Bøtker, H. E. The Future of Cardioprotection—Pointing Toward Patients at Elevated Risk as the Target Populations. *J Cardiovasc Pharmacol Ther* **25**, 487–493 (2020).
79. Xie, J. *et al.* Effect of Remote Ischemic Preconditioning on Outcomes in Adult Cardiac Surgery: A Systematic Review and Meta-analysis of Randomized Controlled Studies. *Anesth Analg* **127**, 30–38 (2018).
80. Pierce, B., Bole, I., Patel, V. & Brown, D. L. Clinical outcomes of remote ischemic preconditioning prior to cardiac surgery: A meta-analysis of randomized controlled trials. *J Am Heart Assoc* **6**, (2017).
81. Usman, M. S. *et al.* The need for increased pragmatism in cardiovascular clinical trials. *Nat Rev Cardiol* **19**, 737–750 (2022).
82. Heusch, G. & Gersh, B. J. ERICCA and RIPHeart: two nails in the coffin for cardioprotection by remote ischemic conditioning? Probably not! *Eur Heart J* **37**, 200–202 (2016).
83. Thielmann, M. *et al.* Cardioprotective and prognostic effects of remote ischaemic preconditioning in patients undergoing coronary artery bypass surgery: a single-centre randomised, double-blind, controlled trial. *Lancet* **382**, 597–604 (2013).
84. Candilio, L. *et al.* Effect of remote ischaemic preconditioning on clinical outcomes in patients undergoing cardiac bypass surgery: a randomised controlled clinical trial. *Heart* **101**, 185–192 (2015).
85. Zheng, Z. *et al.* Perioperative Rosuvastatin in Cardiac Surgery. *N Engl J Med* **374**, 1744–1753 (2016).
86. Meybohm, P. *et al.* A Multicenter Trial of Remote Ischemic Preconditioning for Heart Surgery. *N Engl J Med* **373**, 1397–1407 (2015).
87. Vail, E. A. *et al.* Use of Vasoactive Medications after Cardiac Surgery in the United States. *Ann Am Thorac Soc* **18**, 103–111 (2021).
88. Ko, W.-J. *et al.* Extracorporeal membrane oxygenation support for adult postcardiotomy cardiogenic shock. *Ann Thorac Surg* **73**, 538–545 (2002).
89. Koponen, T. *et al.* Vasoactive-inotropic score and the prediction of morbidity and mortality after cardiac surgery. *Br J Anaesth* **122**, 428–436 (2019).
90. Rudolph, J. L. *et al.* Measurement of post-operative cognitive dysfunction after cardiac surgery: A systematic review. *Acta Anaesthesiol Scand* **54**, 663–677 (2010).
91. Mariscalco, G., Lorusso, R., Dominici, C., Renzulli, A. & Sala, A. Acute kidney injury: a relevant complication after cardiac surgery. *Ann Thorac Surg* **92**, 1539–1547 (2011).
92. Herdman, M. *et al.* Development and preliminary testing of the new five-level version of EQ-5D (EQ-5D-5L). *Quality of Life Research* **20**, 1727–1736 (2011).
93. Leaf, D. E. *et al.* Increased plasma catalytic iron in patients may mediate acute kidney injury and death following cardiac surgery. *Kidney Int* **87**, 1046–1054 (2015).
94. Schaer, D. J., Buehler, P. W., Alayash, A. I., Belcher, J. D. & Vercellotti, G. M. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. *Blood* **121**, 1276–1284 (2013).
95. Vallelian, F., Buehler, P. W. & Schaer, D. J. Hemolysis, free hemoglobin toxicity, and scavenger protein therapeutics. *Blood* **140**, 1837–1844 (2022).
96. Rother, R. P., Bell, L., Hillmen, P. & Gladwin, M. T. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. *JAMA* **293**, 1653–1662 (2005).
97. Meegan, J. E., Bastarache, J. A. & Ware, L. B. Toxic effects of cell-free hemoglobin on the microvascular endothelium: Implications for pulmonary and nonpulmonary organ dysfunction. *Am J Physiol Lung Cell Mol Physiol* **321**, L429–L439 (2021).

98. Gladwin, M. T., Crawford, J. H. & Patel, R. P. The biochemistry of nitric oxide, nitrite, and hemoglobin: Role in blood flow regulation. *Free Radic Biol Med* **36**, 707–717 (2004).
99. Studt, J. D. *et al.* Fatal congenital thrombotic thrombocytopenic purpura with apparent ADAMTS13 inhibitor: In vitro inhibition of ADAMTS13 activity by hemoglobin. *Blood* **105**, 542–544 (2005).
100. Nakai, K. *et al.* Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. *J Lab Clin Med* **132**, 313–319 (1998).
101. Schaer, C. A. *et al.* Haptoglobin Preserves Vascular Nitric Oxide Signaling during Hemolysis. *Am J Respir Crit Care Med* **193**, 1111–1122 (2016).
102. Nyakundi, B. B. *et al.* Formation and Detection of Highly Oxidized Hemoglobin Forms in Biological Fluids during Hemolytic Conditions. *Oxid Med Cell Longev* **2020**, (2020).
103. Bunn, H. & Jandl, J. Exchange of Heme among Hemoglobins and between Hemoglobin and Albumin. *J Biol Chem* **243**, (1968).
104. Sawicki, K. T., Chang, H.-C. & Ardehali, H. Role of heme in cardiovascular physiology and disease. *J Am Heart Assoc* **4**, 1–14 (2015).
105. Balla, J. *et al.* Haem, haem oxygenase and ferritin in vascular endothelial cell injury. *Mol Nutr Food Res* **49**, 1030–1043 (2005).
106. Nath, K. A. *et al.* Intracellular targets in heme protein-induced renal injury. *Kidney Int* **53**, 100–111 (1998).
107. Sawicki, K. T. *et al.* Increased Heme Levels in the Heart Lead to Exacerbated Ischemic Injury. *J Am Heart Assoc* **4**, e002272 (2015).
108. Alvarado, G. *et al.* Heme-induced contractile dysfunction in Human cardiomyocytes caused by oxidant damage to thick filament proteins. *Free Radic Biol Med* **89**, 248–262 (2015).
109. Khechaduri, A., Bayeva, M., Chang, H. C. & Ardehali, H. Heme levels are increased in human failing hearts. *J Am Coll Cardiol* **61**, 1884–1893 (2013).
110. Immenschuh, S., Vijayan, V., Janciauskiene, S. & Gueler, F. Heme as a Target for Therapeutic Interventions. *Front Pharmacol* **8**, 146 (2017).
111. Figueiredo, R. T. *et al.* Characterization of heme as activator of toll-like receptor 4. *Journal of Biological Chemistry* **282**, 20221–20229 (2007).
112. Dutra, F. F. *et al.* Hemolysis-induced lethality involves inflammasome activation by heme. *PNAS* **111**, E4110–E4118 (2014).
113. Wegiel, B., Hauser, C. J. & Otterbein, L. E. Heme as a danger molecule in pathogen recognition. *Free Radic Biol Med* **89**, 651–661 (2015).
114. Jentho, E. *et al.* Trained innate immunity, long-lasting epigenetic modulation, and skewed myelopoiesis by heme. *PNAS* **118**, 1–10 (2021).
115. Chen, G. *et al.* Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood* **123**, 3818–3827 (2014).
116. Brinkmann, V. *et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532–1535 (2004).
117. Qi, Y. *et al.* Perioperative Elevation in Cell-Free DNA Levels in Patients Undergoing Cardiac Surgery: Possible Contribution of Neutrophil Extracellular Traps to Perioperative Renal Dysfunction. *Anesthesiol Res Pract* **2016**, 2794364 (2016).
118. Merkle, J., Daka, A., Deppe, A. C., Wahlers, T. & Paunel-Gorgulu, A. High levels of cell-free DNA accurately predict late acute kidney injury in patients after cardiac surgery. *PLoS One* **14**, e0218548 (2019).
119. Pimstone, N. R. *et al.* Inducible heme oxygenase in the kidney: a model for the homeostatic control of hemoglobin catabolism. *J Clin Invest* **50**, 2042–2050 (1971).
120. van Swelm, R. P. L., Wetzels, J. F. M. & Swinkels, D. W. The multifaceted role of iron in renal health and disease. *Nat Rev Nephrol* **16**, 77–98 (2020).
121. Nemeth, E. & Ganz, T. Annual Review of Medicine Hepcidin and Iron in Health and Disease. *Annu. Rev. Med. 2023* **74**, 261–277 (2022).
122. Qiao, B. *et al.* Hepcidin-induced endocytosis of ferroportin is dependent on ferroportin ubiquitination. *Cell Metab* **15**, 918–924 (2012).
123. Anderson, S. A. *et al.* The IRP1-HIF-2α axis coordinates iron and oxygen sensing with erythropoiesis and iron absorption. *Cell Metab* **17**, 282–290 (2013).

124. Karkouti, K., Yip, P., Chan, C., Chawla, L. & Rao, V. Pre-operative anaemia, intra-operative hepcidin concentration and acute kidney injury after cardiac surgery: a retrospective observational study. *Anaesthesia* **73**, 1097–1102 (2018).
125. Koppenol, W. H. & Hider, R. H. Iron and redox cycling. Do's and don'ts. *Free Radic Biol Med* **133**, 3–10 (2019).
126. Leaf, D. E. *et al.* Iron, hepcidin, and death in human AKI. *Journal of the American Society of Nephrology* **30**, 493–504 (2019).
127. Brissot, P., Ropert, M., Le Lan, C. & Loréal, O. Non-transferrin bound iron: A key role in iron overload and iron toxicity. *Biochim Biophys Acta Gen Subj* **1820**, 403–410 (2012).
128. Jin, X. *et al.* Ferroptosis: Emerging mechanisms, biological function, and therapeutic potential in cancer and inflammation. *Cell Death Discov* **10**, 45 (2024).
129. Roghi, A. *et al.* Role of Non-Transferrin-Bound Iron in the pathogenesis of cardiotoxicity in patients with ST-elevation myocardial infarction assessed by Cardiac Magnetic Resonance Imaging. *Int J Cardiol* **199**, 326–332 (2015).
130. Mumby, S., Koh, T. W., Pepper, J. R. & Gutteridge, J. M. C. Risk of iron overload is decreased in beating heart coronary artery surgery compared to conventional bypass. *Biochim Biophys Acta* **1537**, 204–210 (2001).
131. Hu, J. *et al.* Global Incidence and Outcomes of Adult Patients With Acute Kidney Injury After Cardiac Surgery: A Systematic Review and Meta-Analysis. *J Cardiothorac Vasc Anesth* **30**, 82–89 (2016).
132. Cheruku, S. R., Raphael, J., Neyra, J. A. & Fox, A. A. Acute Kidney Injury after Cardiac Surgery: Prediction, Prevention, and Management. *Anesthesiology* **139**, 880–898 (2023).
133. Billings, F. T., Ball, S. K., Roberts, L. J. & Pretorius, M. Postoperative acute kidney injury is associated with hemoglobinemia and an enhanced oxidative stress response. *Free Radic Biol Med* **50**, 1480–1487 (2011).
134. Kellum, J. A. & Lameire, N. Diagnosis, evaluation, and management of acute kidney injury: a KDIGO summary (Part 1). *Crit Care* **17**, 204 (2013).
135. O'Neal, J. B., Shaw, A. D. & Billings, F. T. Acute kidney injury following cardiac surgery: current understanding and future directions. *Crit Care* **20**, 187 (2016).
136. Choi, N. *et al.* Early intraoperative iron-binding proteins are associated with acute kidney injury after cardiac surgery. *J Thorac Cardiovasc Surg* **157**, 287-297.e2 (2019).
137. Ni, L., Yuan, C. & Wu, X. Targeting ferroptosis in acute kidney injury. *Cell Death Dis* **13**, 182 (2022).
138. Leaf, D. E., Rajapurkar, M., Lele, S. S., Mukhopadhyay, B. & Waikar, S. S. Plasma catalytic iron, AKI, and death among critically ill patients. *Clinical Journal of the American Society of Nephrology* **9**, 1849–1856 (2014).
139. Englberger, L. *et al.* Validation of Clinical Scores Predicting Severe Acute Kidney Injury After Cardiac Surgery. *American Journal of Kidney Diseases* **56**, 623–631 (2010).
140. Meersch, M. *et al.* Prevention of cardiac surgery-associated AKI by implementing the KDIGO guidelines in high risk patients identified by biomarkers: the PrevAKI randomized controlled trial. *Intensive Care Med* **43**, 1551–1561 (2017).
141. Guilliams, M. *et al.* Dendritic cells, monocytes and macrophages: A unified nomenclature based on ontogeny. *Nat Rev Immunol* **14**, 571–578 (2014).
142. Kratofil, R. M., Kubes, P. & Deniset, J. F. Monocyte conversion during inflammation and injury. *Arterioscler Thromb Vasc Biol* **37**, 35–42 (2017).
143. Lambert, C., Preijers, F. W. M. B., Yanikkaya Demirel, G. & Sack, U. Monocytes and macrophages in flow: an ESCCA initiative on advanced analyses of monocyte lineage using flow cytometry. *Cytometry B Clin Cytom* **92**, 180–188 (2017).
144. Guilliams, M., Mildner, A. & Yona, S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* **49**, 595–613 (2018).
145. Rigamonti, A. *et al.* Distinct responses of newly identified monocyte subsets to advanced gastrointestinal cancer and COVID-19. *Front Immunol* **13**, (2022).
146. Ong, S. M. *et al.* A Novel, Five-Marker Alternative to CD16-CD14 Gating to Identify the Three Human Monocyte Subsets. *Front Immunol* **10**, 1761 (2019).

147. Pfefferlé, M. *et al.* Hemolysis transforms liver macrophages into antiinflammatory erythrophagocytes. *Journal of Clinical Investigation* **130**, 5576–5590 (2020).
148. Klei, T. R. L., Meinderts, S. M., van den Berg, T. K. & van Bruggen, R. From the cradle to the grave: The role of macrophages in erythropoiesis and erythrophagocytosis. *Front Immunol* **8**, (2017).
149. Winn, N. C., Volk, K. M. & Hasty, A. H. Regulation of tissue iron homeostasis: The macrophage "ferrostat". *JCI Insight* **5**, e132964 (2020).
150. Corna, G. *et al.* The Repair of Skeletal Muscle Requires Iron Recycling through Macrophage Ferroportin. *The Journal of Immunology* **197**, 1914–1925 (2016).
151. Haschka, D. *et al.* Classical and intermediate monocytes scavenge non-transferrin-bound iron and damaged erythrocytes. *JCI Insight* **4**, e98867 (2019).
152. Theurl, I. *et al.* On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nat Med* **22**, 945–951 (2016).
153. Philippidis, P. *et al.* Anti-inflammatory haemoglobin scavenging monocytes are induced following coronary artery bypass surgery. *European Journal of Cardio-thoracic Surgery* **37**, 1360–1366 (2010).
154. Quimby, K. R., Greenidge, A., Harris, A. & Landis, R. C. Phenotypic commitment of monocytes towards a protective hemoglobin scavenging phenotype (CD14posCD163highHLA-DR low) following cardiopulmonary bypass. *Cytometry B Clin Cytom* **78**, 357–360 (2010).
155. Grochot-Przeczek, A., Dulak, J. & Jozkowicz, A. Haem oxygenase-1: non-canonical roles in physiology and pathology. *Clin Sci (Lond)* **122**, 93–103 (2012).
156. Chiabrando, D., Vinchi, F., Fiorito, V., Mercurio, S. & Tolosano, E. Heme in pathophysiology: a matter of scavenging, metabolism and trafficking across cell membranes. *Front Pharmacol* **5**, 61 (2014).
157. Alam, J. & Cook, J. L. How many transcription factors does it take to turn on the heme oxygenase-1 gene? *Am J Respir Cell Mol Biol* **36**, 166–174 (2007).
158. Hull, T. D. *et al.* Heme oxygenase-1 regulates mitochondrial quality control in the heart. *JCI Insight* **1**, e85817 (2016).
159. Piantadosi, C. A., Carraway, M. S., Babiker, A. & Suliman, H. B. Heme oxygenase-1 regulates cardiac mitochondrial biogenesis via Nrf2-mediated transcriptional control of nuclear respiratory factor-1. *Circ Res* **103**, 1232–1240 (2008).
160. Fredenburgh, L. E., Merz, A. A. & Cheng, S. Haemeoxygenase signalling pathway: Implications for cardiovascular disease. *Eur Heart J* **36**, 1512–1518 (2015).
161. Yachie, A. *et al.* Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* **103**, 129–135 (1999).
162. Radhakrishnan, N. *et al.* Human heme oxygenase-1 deficiency presenting with hemolysis, nephritis, and asplenia. *J Pediatr Hematol Oncol* **33**, 74–78 (2011).
163. Ohta, K. *et al.* Tubular injury as a cardinal pathologic feature in human heme oxygenase-1 deficiency. *Am J Kidney Dis* **35**, 863–870 (2000).
164. Chau, A. S. *et al.* Heme oxygenase-1 deficiency presenting with interstitial lung disease and hemophagocytic flares. *Pediatr Rheumatol Online J* **18**, 80 (2020).
165. Motterlini, R. & Otterbein, L. E. The therapeutic potential of carbon monoxide. *Nat Rev Drug Discov* **9**, 728–743 (2010).
166. Ryter, S. W., Otterbein, L. E., Morse, D. & Choi, A. M. K. Heme oxygenase/carbon monoxide signaling path-ways: Regulation and functional significance. *Mol Cell Biochem* **234–235**, 249–263 (2002).
167. Ryter, S. W. & Otterbein, L. E. Carbon monoxide in biology and medicine. *BioEssays* **26**, 270–280 (2004).
168. Foresti, R., Bani-Hani, M. G. & Motterlini, R. Use of carbon monoxide as a therapeutic agent: Promises and challenges. *Intensive Care Med* **34**, 649–658 (2008).
169. Wu, L. & Wang, R. Carbon monoxide: Endogenous production, physiological functions, and pharmacological applications. *Pharmacol Rev* **57**, 585–630 (2005).
170. Nowaczyk, A., Kowalska, M., Nowaczyk, J. & Grześk, G. Carbon monoxide and nitric oxide as examples of the youngest class of transmitters. *Int J Mol Sci* **22**, 6029 (2021).
171. Morita, T., Perrellat, M. A., Leeti, M.-E., Kourembanas, S. & Avery, M. E. Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *PNAS* **92**, 1475–1479 (1995).
172. Sarady, J. K. *et al.* Carbon monoxide protection against endotoxic shock involves reciprocal effects on iNOS in the lung and liver. *FASEB* **18**, 854–856 (2004).

173. Scragg, J. L., Dallas, M. L., Wilkinson, J. A., Varadi, G. & Peers, C. Carbon monoxide inhibits L-type Ca2+ channels via redox modulation of key cysteine residues by mitochondrial reactive oxygen species. *Journal of Biological Chemistry* **283**, 24412–24419 (2008).
174. Russo, I., Barale, C., Melchionda, E., Penna, C. & Pagliaro, P. Platelets and Cardioprotection: The Role of Nitric Oxide and Carbon Oxide. *Int J Mol Sci* **24**, 6107 (2023).
175. Suliman, H. B., Carraway, M. S., Tatro, L. G. & Piantadosi, C. A. A new activating role for CO in cardiac mitochondrial biogenesis. *J Cell Sci* **120**, 299–308 (2007).
176. Zhang, X. *et al.* Carbon monoxide inhibition of apoptosis during ischemia-reperfusion lung injury is dependent on the p38 mitogen-activated protein kinase pathway and involves caspase 3. *Journal of Biological Chemistry* **278**, 1248–1258 (2003).
177. Su, W. C., Liu, X., Macias, A. A., Baron, R. M. & Perrella, M. A. Heme oxygenase-1-derived carbon monoxide enhances the host defense response to microbial sepsis in mice. *Journal of Clinical Investigation* **118**, 239–247 (2008).
178. Chen, B. *et al.* Carbon monoxide rescues heme oxygenase-1-deficient mice from arterial thrombosis in allogeneic aortic transplantation. *American Journal of Pathology* **175**, 422–429 (2009).
179. Wegiel, B. *et al.* Heme oxygenase-1 derived carbon monoxide permits maturation of myeloid cells. *Cell Death Dis* **5**, e1139 (2014).
180. Tenhunen, R., Ross, M., Marver, H. & Schmid, R. Reduced Nicotinamide-Adenine Dinucleotide Phosphate Dependent Biliverdin Reductase: Partial Purification and Characterization. *Biochemistry* **9**, 298–303 (1970).
181. Sedlak, T. W. & Snyder, S. H. Bilirubin benefits: cellular protection by a biliverdin reductase antioxidant cycle. *Pediatrics* **113**, 1776–1782 (2004).
182. Neuzil, J. & Stocker, R. Free and Albumin-bound Bilirubin Are Efficient Co-antioxidants for a-Tocopherol, Inhibiting Plasma and Low Density Lipoprotein Lipid Peroxidation. *J Biol Chem* **269**, 16712–16719 (1994).
183. Jansen, T. *et al.* Conversion of biliverdin to bilirubin by biliverdin reductase contributes to endothelial cell protection by heme oxygenase-1-evidence for direct and indirect antioxidant actions of bilirubin. *J Mol Cell Cardiol* **49**, 186–195 (2010).
184. Mazzone, G. L. *et al.* Bilirubin inhibits the TNFα-related induction of three endothelial adhesion molecules. *Biochem Biophys Res Commun* **386**, 338–344 (2009).
185. Clark, J. E. *et al.* Heme oxygenase-1-derived bilirubin ameliorates postischemic myocardial dysfunction. *Am J Physiol Heart Circ Physiol* **278**, 643–651 (2000).
186. Novotny, L., Novotny, N. & Vítek, L. Inverse Relationship Between Serum Bilirubin and Atherosclerosis in Men: A Meta-Analysis of Published Studies. *Exp Biol Med* **228**, 568–571 (2003).
187. Djoussé, L., Rothman, K. J., Cupples, L. A., Levy, D. & Ellison, R. C. Effect of serum albumin and bilirubin on the risk of myocardial infarction (the Framingham Offspring Study). *American Journal of Cardiology* **91**, 485–488 (2003).
188. Hopkins, P. N. *et al.* Higher Serum Bilirubin Is Associated With Decreased Risk for Early Familial Coronary Artery Disease. *Arterioscler Thromb Vasc Biol* **16**, 250–255 (1996).
189. Balla, G. *et al.* Ferritin: A Cytoprotective Antioxidant Strategem of Endothelium. *J Biol Chem* **267**, 18148–18153 (1992).
190. Choi, B. M. *et al.* Overexpression of heme oxygenase (HO)-1 renders jurkat t cells resistant to fas-mediated apoptosis: Involvement of iron released by HO-1. *Free Radic Biol Med* **36**, 858–871 (2004).
191. Hori, R. *et al.* Gene transfection of H25A mutant heme oxygenase-1 protects cells against hydroperoxide-induced cytotoxicity. *Journal of Biological Chemistry* **277**, 10712–10718 (2002).
192. Dennery, P. A. Signaling function of heme oxygenase proteins. *Antioxid Redox Signal* **20**, 1743–1753 (2014).
193. Lin, Q. *et al.* Heme oxygenase-1 protein localizes to the nucleus and activates transcription factors important in oxidative stress. *Journal of Biological Chemistry* **282**, 20621–20633 (2007).
194. Bomsztyk, K., Seuningen, I. Van, Suzuki, H., Denisenko, O. & Ostrowski, J. Diverse molecular interactions of the hnRNP K protein. *FEBS Lett* **403**, 113–115 (1997).

195. Bindu, S. *et al.* Translocation of heme oxygenase-1 to mitochondria is a novel cytoprotective mechanism against non-steroidal anti-inflammatory drug-induced mitochondrial oxidative stress, apoptosis, and gastric mucosal injury. *Journal of Biological Chemistry* **286**, 39387–39402 (2011).
196. Yang, G. *et al.* Heme oxygenase-1 regulates postnatal lung repair after hyperoxia: Role of β-catenin/hnRNPK signaling. *Redox Biol* **1**, 234–243 (2013).
197. Salinas, M. *et al.* Protein kinase Akt/PKB phosphorylates heme oxygenase-1 in vitro and in vivo. *FEBS Lett* **578**, 90–94 (2004).
198. Jagadeesh, A. S. V. *et al.* Non-canonical vs. Canonical Functions of Heme Oxygenase-1 in Cancer. *J Cancer Prev* **27**, 7–15 (2022).
199. Lee, G. R., Shaefi, S. & Otterbein, L. E. HO-1 and CD39: It Takes Two to Protect the Realm. *Front Immunol* **10**, 1765 (2019).
200. Corriden, R. & Insel, P. A. Basal Release of ATP- An Autocrine- Paracrine Mechanism for Cell Regulation. *Science Signalling* **3**, 1–12 (2010).
201. Sáez, P. J. *et al.* ATP promotes the fast migration of dendritic cells through the activity of pannexin 1 channels and P2X 7 receptors. *Science Signalling* **10**, 1–11 (2017).
202. Kurashima, Y. *et al.* Extracellular ATP mediates mast cell-dependent intestinal inflammation through P2X7 purinoceptors. *Nat Commun* **3**, (2012).
203. Correa-Costa, M. *et al.* Carbon monoxide protects the kidney through the central circadian clock and CD39. *Proc Natl Acad Sci U S A* **115**, E2302–E2310 (2018).
204. Campbell, N. K., Fitzgerald, H. K. & Dunne, A. Regulation of inflammation by the antioxidant haem oxygenase 1. *Nat Rev Immunol* **21**, 411–425 (2021).
205. Wegiel, B. *et al.* Heme oxygenase-1 derived carbon monoxide permits maturation of myeloid cells. *Cell Death Dis* **5**, e1139–e1139 (2014).
206. Naito, Y., Takagi, T. & Higashimura, Y. Heme oxygenase-1 and anti-inflammatory M2 macrophages. *Arch Biochem Biophys* **564**, 83–88 (2014).
207. Boyle, J. J. *et al.* Heme induces heme oxygenase 1 via Nrf2: Role in the homeostatic macrophage response to intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* **31**, 2685–2691 (2011).
208. Chauveau, C. *et al.* Heme oxygenase-1 expression inhibits dendritic cell maturation and proinflammatory function but conserves IL-10 expression. *Blood* **106**, 1694–1702 (2005).
209. Vitali, S. H. *et al.* Heme oxygenase-1 dampens the macrophage sterile inflammasome response and regulates its components in the hypoxic lung. *Am J Physiol Lung Cell Mol Physiol* **318**, 125–134 (2020).
210. Swanson, K. V., Deng, M. & Ting, J. P. Y. The NLRP3 inflammasome: molecular activation and regulation to therapeutics. *Nat Rev Immunol* **19**, 477–489 (2019).
211. McKee, C. M. & Coll, R. C. NLRP3 inflammasome priming: A riddle wrapped in a mystery inside an enigma. *J Leukoc Biol* **108**, 937–952 (2020).
212. Netea, M. G. *et al.* Differential requirement for the activation of the inflammasome for processing and release of IL-1 in monocytes and macrophages. *Blood* **113**, 2324–2335 (2009).
213. Gritsenko, A. *et al.* Priming Is Dispensable for NLRP3 Inflammasome Activation in Human Monocytes In Vitro. *Front Immunol* **11**, (2020).
214. Shi, J. *et al.* Inflammatory caspases are innate immune receptors for intracellular LPS. *Nature* **514**, 187–192 (2014).
215. Dutra, F. F. *et al.* Hemolysis-induced lethality involves inflammasome activation by heme. *PNAS* **111**, E4110–E4118 (2014).
216. May, O. *et al.* The receptor for advanced glycation end products is a sensor for cell-free heme. *FEBS Journal* **288**, 3448–3464 (2021).
217. Bolívar, B. E. *et al.* Noncanonical Roles of Caspase-4 and Caspase-5 in Heme-Driven IL-1β Release and Cell Death. *The Journal of Immunology* **206**, 1878–1889 (2021).
218. Salgar, S., Bolívar, B. E., Flanagan, J. M., Anum, S. J. & Bouchier-Hayes, L. The NLRP3 inflammasome fires up heme-induced inflammation in hemolytic conditions. *Translational Research* **252**, 34–44 (2023).
219. Fortes, G. B. *et al.* Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. *Blood* **119**, 2368–2375 (2012).
220. Lin, Y. *et al.* Bilirubin alleviates alum–induced peritonitis through inactivation of NLRP3 inflammasome. *Biomedicine and Pharmacotherapy* **116**, 108973 (2019).

221. Jung, S. S. *et al.* Carbon monoxide negatively regulates NLRP3 inflam-masome activation in macrophages. *Am J Physiol Lung Cell Mol Physiol* **308**, 1058–1067 (2015).
222. Luo, Y. P. *et al.* Hemin inhibits NLRP3 inflammasome activation in sepsis-induced acute lung injury, involving heme oxygenase-1. *Int Immunopharmacol* **20**, 24–32 (2014).
223. Li, H., Zhou, X. & Zhang, J. Induction of heme oxygenase-1 attenuates lipopolysaccharide-induced inflammasome activation in human gingival epithelial cells. *Int J Mol Med* **34**, 1039–1044 (2014).
224. Coll, R., O'Neill, L. & Schroder, K. Questions and controversies in innate immune research: what is the physiological role of NLRP3? *Cell Death Discov* **2**, 16019 (2016).
225. Liu, X. *et al.* Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. *FASEB J* **20**, 207–216 (2006).
226. Liu, X. *et al.* Preemptive heme oxygenase-1 gene delivery reveals reduced mortality and preservation of left ventricular function 1 yr after acute myocardial infarction. *Am J Physiol Heart Circ Physiol* **293**, H48-59 (2007).
227. Vulapalli, S. R., Chen, Z., Chua, B. H. L., Wang, T. & Liang, C.-S. Cardioselective overexpression of HO-1 prevents I/R-induced cardiac dysfunction and apoptosis. *Am J Physiol Heart Circ Physiol* **283**, H688-94 (2002).
228. Akamatsu, Y. *et al.* Heme oxygenase-1-derived carbon monoxide protects hearts from transplant associated ischemia reperfusion injury. *FASEB J* **18**, 771–772 (2004).
229. Tsuchihashi, S., Zhai, Y., Bo, Q., Busuttil, R. W. & Kupiec-Weglinski, J. W. Heme oxygenase-1 mediated cytoprotection against liver ischemia and reperfusion injury: inhibition of type-1 interferon signaling. *Transplantation* **83**, 1628–1634 (2007).
230. Shen, X.-D. *et al.* Toll-like receptor and heme oxygenase-1 signaling in hepatic ischemia/reperfusion injury. *Am J Transplant* **5**, 1793–1800 (2005).
231. Chok, M. K. *et al.* Renoprotective potency of heme oxygenase-1 induction in rat renal ischemia-reperfusion. *Inflamm Allergy Drug Targets* **8**, 252–259 (2009).
232. Chao, X. D. *et al.* Up-regulation of heme oxygenase-1 attenuates brain damage after cerebral ischemia via simultaneous inhibition of superoxide production and preservation of NO bioavailability. *Exp Neurol* **239**, 163–169 (2013).
233. Hinkel, R. *et al.* Heme Oxygenase-1 Gene Therapy Provides Cardioprotection Via Control of Post-Ischemic Inflammation: An Experimental Study in a Pre-Clinical Pig Model. *J Am Coll Cardiol* **66**, 154–165 (2015).
234. Exner, M., Minar, E., Wagner, O. & Schillinger, M. The role of heme oxygenase-1 promoter polymorphisms in human disease. *Free Radic Biol Med* **37**, 1097–1104 (2004).
235. Hirai, H. *et al.* Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. *Blood* **102**, 1619–1621 (2003).
236. Chen, Y.-H. *et al.* Microsatellite polymorphism in promoter of heme oxygenase-1 gene is associated with susceptibility to coronary artery disease in type 2 diabetic patients. *Hum Genet* **111**, 1–8 (2002).
237. Chen, Y.-H., Hung, S.-C. & Tarng, D.-C. Length polymorphism in heme oxygenase-1 and cardiovascular events and mortality in hemodialysis patients. *Clin J Am Soc Nephrol* **8**, 1756–1763 (2013).
238. Dick, P. *et al.* Haem oxygenase-1 genotype and cardiovascular adverse events in patients with peripheral artery disease. *Eur J Clin Invest* **35**, 731–737 (2005).
239. Freystaetter, K. *et al.* The recipient's heme oxygenase-1 promoter region polymorphism is associated with cardiac allograft vasculopathy. *Transpl Int* **30**, 510–518 (2017).
240. Pechlaner, R. *et al.* Heme oxygenase-1 gene promoter microsatellite polymorphism is associated with progressive atherosclerosis and incident cardiovascular disease. *Arterioscler Thromb Vasc Biol* **35**, 229–236 (2015).
241. Hsu, L.-A. *et al.* Microsatellite polymorphism in the heme oxygenase-1 gene promoter and the risk of atrial fibrillation in Taiwanese. *PLoS One* **9**, e108773–e108773 (2014).
242. Køllgaard, T. *et al.* GTn repeat polymorphism in heme oxygenase-1 (HO-1) correlates with clinical outcome after myeloablative or nonmyeloablative allogeneic hematopoietic cell transplantation. *PLoS One* **11**, 1–8 (2016).
243. Katana, E. *et al.* Association between the heme oxygenase-1 promoter polymorphism and renal transplantation outcome in Greece. *Transplant Proc* **42**, 2479–2485 (2010).

244. Buis, C. I. *et al.* Heme oxygenase-1 genotype of the donor is associated with graft survival after liver transplantation. *Am J Transplant* **8**, 377–385 (2008).
245. Saraf, S. L. *et al.* HMOX1 and acute kidney injury in sickle cell anemia. *Blood* **132**, 1621–1625 (2018).
246. Bean, C. J. *et al.* Heme oxygenase-1 gene promoter polymorphism is associated with reduced incidence of acute chest syndrome among children with sickle cell disease. *Blood* **120**, 3822–3828 (2012).
247. Lagan, A. L., Quinlan, G. J. & Evans, T. W. Haem oxygenase-1 polymorphism and ARDS, friend and foe?. *Intensive Care Med* **35**, 1325–1327 (2009).
248. Vilander, L. M. *et al.* Heme oxygenase-1 repeat polymorphism in septic acute kidney injury. *PLoS One* **14**, e0217291 (2019).
249. Leaf, D. E. *et al.* Length Polymorphisms in Heme Oxygenase-1 and AKI after Cardiac Surgery. *J Am Soc Nephrol* **27**, 3291–3297 (2016).
250. Donadee, C. *et al.* Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. *Circulation* **124**, 465–476 (2011).
251. Thomas, R. A. B. *et al.* Hemin Preconditioning Upregulates Heme Oxygenase-1 in Deceased Donor Renal Transplant Recipients: A Randomized, Controlled, Phase IIB Trial. *Transplantation* **100**, 176–183 (2016).
252. Andreas, M. *et al.* Intravenous Heme Arginate Induces HO-1 (Heme Oxygenase-1) in the Human Heart. *Arterioscler Thromb Vasc Biol* **38**, 2755–2762 (2018).
253. Ito, H. *et al.* Oral administration of 5-aminolevulinic acid induces heme oxygenase-1 expression in peripheral blood mononuclear cells of healthy human subjects in combination with ferrous iron. *Eur J Pharmacol* **833**, 25–33 (2018).
254. Saito, K. *et al.* Dynamics of absorption, metabolism, and excretion of 5-aminolevulinic acid in human intestinal Caco-2 cells. *Biochem Biophys Rep* **11**, 105–111 (2017).
255. Yien, Y. Y. & Perfetto, M. Regulation of Heme Synthesis by Mitochondrial Homeostasis Proteins. *Front Cell Dev Biol* **10**, (2022).
256. Igarashi, K. & Sun, J. The Heme-Bach1 Pathway in the Regulation of Oxidative Stress Response and Erythroid Differentiation. *Antioxidants and Redox Signalling* **8**, 107–118 (2006).
257. Nishio, Y. *et al.* 5-Aminolevulinic acid combined with ferrous iron enhances the expression of heme oxygenase-1. *Int Immunopharmacol* **19**, 300–307 (2014).
258. Chen, Q. M. & Maltagliati, A. J. Nrf2 at the heart of oxidative stress and cardiac protection. *Physiol Genomics* **50**, 77–97 (2018).
259. Tonelli, C., Chio, I. I. C. & Tuveson, D. A. Transcriptional Regulation by Nrf2. *Antioxid Redox Signal* **29**, 1727–1745 (2018).
260. Itoh, K. *et al.* Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes and Development* **13**, 76–86 (1999).
261. Itoh, K. *et al.* Keap1 regulates both cytoplasmic-nuclear shuttling and degradation of Nrf2 in response to electrophiles. *Genes to Cells* **8**, 379–391 (2003).
262. Ahmed, S. M. U., Luo, L., Namani, A., Wang, X. J. & Tang, X. Nrf2 signaling pathway: Pivotal roles in inflammation. *Biochim Biophys Acta Mol Basis Dis* **1863**, 585–597 (2017).
263. Nioi, P., Nguyen, T., Sherratt, P. J. & Pickett, C. B. The Carboxy-Terminal Neh3 Domain of Nrf2 Is Required for Transcriptional Activation. *Mol Cell Biol* **25**, 10895–10906 (2005).
264. Rada, P. *et al.* SCF/β-TrCP Promotes Glycogen Synthase Kinase 3-Dependent Degradation of the Nrf2 Transcription Factor in a Keap1-Independent Manner. *Mol Cell Biol* **31**, 1121–1133 (2011).
265. Shah, N. M., Rushworth, S. A., Murray, M. Y., Bowles, K. M. & Macewan, D. J. Understanding the role of NRF2-regulated miRNAs in human malignancies. *Oncotarget* **4**, 1130–1142 (2013).
266. Fukunaga, N. *et al.* Protective role of Nrf2 against ischemia reperfusion injury and cardiac allograft vasculopathy. *American Journal of Transplantation* **20**, 1262–1271 (2020).
267. Rubio-Navarro, A. *et al.* Nrf2 Plays a Protective Role Against Intravascular Hemolysis-Mediated Acute Kidney Injury. *Front Pharmacol* **10**, 740 (2019).
268. Liu, C. *et al.* 5-aminolaevulinic acid (ALA), enhances heme oxygenase (HO)-1 expression and attenuates tubulointerstitial fibrosis and renal apoptosis in chronic cyclosporine nephropathy. *Biochem Biophys Res Commun* **508**, 583–589 (2019).
269. Liu, C. *et al.* 5-ALA/SFC enhances HO-1 expression through the MAPK/Nrf2 antioxidant pathway and attenuates murine tubular epithelial cell apoptosis. *FEBS Open Bio* **9**, 1928–1938 (2019).

270. Shimura, M. *et al.* Effects of 5-aminolevulinic acid and sodium ferrous citrate on fibroblasts from individuals with mitochondrial diseases. *Sci Rep* **9**, 10549 (2019).
271. Hou, J. *et al.* 5-Aminolevulinic acid with ferrous iron induces permanent cardiac allograft acceptance in mice via induction of regulatory cells. *J Heart Lung Transplant* **34**, 254–263 (2015).
272. Zhao, M. *et al.* 5-Aminolevulinic acid combined with sodium ferrous citrate ameliorates H2O2-induced cardiomyocyte hypertrophy via activation of the MAPK/Nrf2/ HO-1 pathway. *Am J Physiol Cell Physiol* **308**, 665–672 (2015).
273. MEDAC, Gliolan - Summary of Product Characteristics. 2014, European Medicines Agency: 4–6 https://www.ema.europa.eu/en/documents/product-information/gliolan-epar-product-information_en.pdf.
274. Cozzens, J. W. *et al.* A Phase 1 Dose-Escalation Study of Oral 5-Aminolevulinic Acid in Adult Patients Undergoing Resection of a Newly Diagnosed or Recurrent High-Grade Glioma. *Neurosurgery* **81**, 46–55 (2017).
275. Brooks, A. et al. *SBI Pharmaceuticls Study SPP1C301., 5-ALA-SFC: A Phase I, Double-Blind, Placebo-Controlled, Single and Multiple Oral Dose, Safety and Tolerability and Pharmacokinetic Study in Healthy Caucasian and Japanese Subjects. SBI Pharmaceuticals Co., Ltd.: Tokyo, .* (2013).
276. Saitama, I. *SBI Pharmaceuticls Study SPP-003., Phase II Clinical Study of SPP-003 for Chemotherapy-Induced Anemia. SBI Pharmaceuticals Co., Ltd.: Tokyo, Japan (2016).*
277. Ito, H. *et al.* Oral administration of 5-aminolevulinic acid induces heme oxygenase-1 expression in peripheral blood mononuclear cells of healthy human subjects in combination with ferrous iron. *Eur J Pharmacol* **833**, 25–33 (2018).
278. Billings, F. T. 4th & Shaw, A. D. Clinical trial endpoints in acute kidney injury. *Nephron Clin Pract* **127**, 89–93 (2014).
279. Ely, E. W. *et al.* Delirium in mechanically ventilated patients: validity and reliability of the confusion assessment method for the intensive care unit (CAM-ICU). *JAMA* **286**, 2703–2710 (2001).
280. Lorch, U., O'Kane, M. & Taubel, J. Three steps to writing adaptive study protocols in the early phase clinical development of new medicines. *BMC Med Res Methodol* **14**, 84 (2014).
281. Smedley, D. *et al.* BioMart - Biological queries made easy. *BMC Genomics* **10**, 22 (2009).
282. GTEx Consortium. The GTEx Consortium atlas of genetic regulatory effects across human tissues The GTEx Consortium*. *Science (1979)* **369**, 1318–1330 (2020).
283. Lattin, J. E. *et al.* Expression analysis of G Protein-Coupled Receptors in mouse macrophages. *Immunome Res* **4**, 5 (2008).
284. Shay, T. *et al.* Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proc Natl Acad Sci U S A* **110**, 2946–2951 (2013).
285. Novershtern, N. *et al.* Densely interconnected transcriptional circuits control cell states in human hematopoiesis. *Cell* **144**, 296–309 (2011).
286. Heng, T., Painter, M. & The Immunological Genome Project Consortium. The Immunological Genome Project: networks of gene expression in immune cells. *Nat Immunol* **9**, 1091–1094 (2008).
287. Thul, P. J. & Lindskog, C. The human protein atlas: A spatial map of the human proteome. *Protein Science* **27**, 233–244 (2018).
288. Schmiedel, B. J. *et al.* Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell* **175**, 1701-1715.e16 (2018).
289. Monaco, G. *et al.* RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Rep* **26**, 1627-1640.e7 (2019).
290. Uhlen, M. *et al.* A genome-wide transcriptomic analysis of protein-coding genes in human blood cells. *Science (1979)* **366**, (2019).
291. Bolstad, B. M., Irizarry, R. A., Astrand, M. & Speed, T. P. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185–193 (2003).
292. Saldanha, A. J. Java Treeview—extensible visualization of microarray data. *Bioinformatics* **20**, 3246–3248 (2004).
293. Chanput, W., Mes, J. J. & Wichers, H. J. THP-1 cell line: an in vitro cell model for immune modulation approach. *Int Immunopharmacol* **23**, 37–45 (2014).
294. Birney, E. *et al.* An overview of Ensembl. *Genome Res* **14**, 925–928 (2004).

295. Gill, A. J., Garza, R., Ambegaokar, S. S., Gelman, B. B. & Kolson, D. L. Heme oxygenase-1 promoter region (GT)n polymorphism associates with increased neuroimmune activation and risk for encephalitis in HIV infection. *J Neuroinflammation* **15**, 70 (2018).
296. Schutzman, D. L., Gatien, E., Ajayi, S. & Wong, R. J. Heme oxygenase-1 genetic variants and the conundrum of hyperbilirubinemia in African-American newborns. *J Perinatol* **38**, 345–350 (2018).
297. Chang, P.-F., Lin, Y.-C., Liu, K., Yeh, S.-J. & Ni, Y.-H. Heme oxygenase-1 gene promoter polymorphism and the risk of pediatric nonalcoholic fatty liver disease. *Int J Obes (Lond)* **39**, 1236–1240 (2015).
298. Askenazi, D. J. *et al.* Genetic polymorphisms of heme-oxygenase 1 (HO-1) may impact on acute kidney injury, bronchopulmonary dysplasia, and mortality in premature infants. *Pediatr Res* **77**, 793–798 (2015).
299. Doberer, D. *et al.* Haem arginate infusion stimulates haem oxygenase-1 expression in healthy subjects. *Br J Pharmacol* **161**, 1751–1762 (2010).
300. Saukkonen, K. *et al.* Heme oxygenase 1 polymorphisms and plasma concentrations in critically ill patients. *Shock* **34**, 558–564 (2010).
301. Seu, L. *et al.* Variations in the heme oxygenase-1 microsatellite polymorphism are associated with plasma CD14 and viral load in HIV-infected African-Americans. *Genes Immun* **13**, 258–267 (2012).
302. Roy, J. G., McElhaney, J. E. & Verschoor, C. P. Reliable reference genes for the quantification of mRNA in human T-cells and PBMCs stimulated with live influenza virus. *BMC Immunol* **21**, 4 (2020).
303. Iwamori, S. *et al.* A novel and sensitive assay for heme oxygenase activity. *Am J Physiol Renal Physiol* **309**, 667–671 (2015).
304. Gils, C., Hansen, D. L., Nybo, M. & Frederiksen, H. Elevated Hemolysis Index is associated with higher risk of cardiovascular diseases. *Clin Chem Lab Med* **61**, 1497–1505 (2023).
305. Zhang, D., Okada, S., Kawabata, T. & Yasuda, T. An improved simple colorimetric method for quantitation of non-transferrin-bound iron in serum. *Biochem Mol Biol Int.* **35**, 635–641 (1995).
306. Wik, L. *et al.* Proximity Extension Assay in Combination with Next-Generation Sequencing for High-throughput Proteome-wide Analysis. *Molecular and Cellular Proteomics* **20**, (2021).
307. Mi, H., Muruganujan, A., Casagrande, J. T. & Thomas, P. D. Large-scale gene function analysis with the PANTHER classification system. *Nat Protoc* **8**, 1551–1566 (2013).
308. Schulz, K. F., Altman, D. G. & Moher, D. CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials. *Trials* **11**, (2010).
309. O'Brien, S. M. *et al.* The Society of Thoracic Surgeons 2008 Cardiac Surgery Risk Models: Part 2-Isolated Valve Surgery. *Annals of Thoracic Surgery* **88**, (2009).
310. Yatabe, T. *et al.* 5-Aminolevulinic acid-induced severe hypotension during transurethral resection of a bladder tumor: a case report. *JA Clin Rep* **5**, (2019).
311. Kaufmann, J. & Kung, E. Factors Affecting Cardiovascular Physiology in Cardiothoracic Surgery: Implications for Lumped-Parameter Modeling. *Frontiers in Surgery* vol. 6 Preprint at https://doi.org/10.3389/fsurg.2019.00062 (2019).
312. Nagele, P. *et al.* Postoperative QT Interval Prolongation in Patients Undergoing Noncardiac Surgery under General Anesthesia. *Anesthesiology* **117**, 321–328 (2012).
313. Hofmann, B. *et al.* Positive impact of retrograde autologous priming in adult patients undergoing cardiac surgery: A randomized clinical trial. *J Cardiothorac Surg* **13**, (2018).
314. Lako, S. *et al.* Hematological Changes in Patients Undergoing Coronary Artery Bypass Surgery: a Prospective Study. *Med Arch* **69**, 181–186 (2015).
315. Berbel-Franco, D. *et al.* The influence of postoperative albumin levels on the outcome of cardiac surgery. *J Cardiothorac Surg* **15**, (2020).
316. Polderman, K. H. & Girbes, A. R. Severe electrolyte disorders following cardiac surgery: a prospective controlled observational study. **8**, (2004).
317. Liskaser, F. J. & Bellomo, R. Role of Pump Prime in the Etiology and Pathogenesis of Cardiopulmonary Bypass-associated Acidosis. *Anesthesiology* **93**, 1170–1173 (2000).
318. Najmaii, S., Redford, D. & Larson, D. F. Hyperglycemia as an Effect of Cardiopulmonary Bypass: Intra-operative Glucose Management. *J Extra Corpor Technol* **38**, 168–173 (2006).
319. Minton, J. & Sidebotham, D. A. Hyperlactatemia and Cardiac Surgery. *J Extra Corpor Technol* **49**, 7–15 (2017).

320. Bhirowo, Y. P. *et al.* Hemolysis and cardiopulmonary bypass: meta-analysis and systematic review of contributing factors. *J Cardiothorac Surg* **18**, (2023).
321. Silverstein, R. L. & Febbraio, M. CD36,a Scavenger Receptor Involved in Immunity, Metabolism, Angiogenesis, and Behavior. *Science Signalling* **2**, re3 (2009).
322. Möller, A. *et al.* The roles of toll-like receptor 4, CD33, CD68, CD69, or CD147/EMMPRIN for monocyte activation by the DAMP S100A8/S100A9. *Front Immunol* **14**, 1110185 (2023).
323. Holdgate, G. A., Meek, T. D. & Grimley, R. L. Mechanistic enzymology in drug discovery: a fresh perspective. *Nat Rev Drug Discov* **17**, 115–132 (2018).
324. Whaley, D. *et al.* Cryopreservation: An Overview of Principles and Cell-Specific Considerations. *Cell Transplant* **30**, (2021).
325. Lahey, R. *et al.* Enhanced Redox State and Efficiency of Glucose Oxidation With miR Based Suppression of Maladaptive NADPH-Dependent Malic Enzyme 1 Expression in Hypertrophied Hearts. *Circ Res* **122**, 836–845 (2018).
326. Warkentin, D., Marchand, A. & Van Lente, F. Serum haptoglobin concentrations in concurrent hemolysis and acute-phase reaction. *Clin Chem.* **33**, 1265–1266 (1987).
327. Infanti, L. *et al.* Indices of iron homeostasis in asymptomatic subjects with HFE mutations and moderate ferritin elevation during iron removal treatment. *Blood Cells Mol Dis* **97**, (2022).
328. Pepper, J., Mumby, S. & Gutteridge, J. Transient Iron-Overload with Bleomycin-Detectable Iron Present During Cardiopulmonary Bypass Surgery. *Free Radic Res* **21**, 53–58 (1994).
329. Xu, H. *et al.* Intravenous Iron Versus Placebo in the Management of Postoperative Functional Iron Deficiency Anemia in Patients Undergoing Cardiac Valvular Surgery: A Prospective, Single-Blinded, Randomized Controlled Trial. *J Cardiothorac Vasc Anesth* **33**, 2941–2948 (2019).
330. Kernan, K. F. & Carcillo, J. A. Hyperferritinemia and inflammation. *Int Immunol* **29**, 401–409 (2017).
331. Lee, P., Peng, H., Gelbart, T., Wang, L. & Beutler, E. Regulation of hepcidin transcription by interleukin-1 and interleukin-6. *PNAS* **102**, 1906–1910 (2005).
332. Etzerodt, A. & Moestrup, S. K. CD163 and inflammation: Biological, diagnostic, and therapeutic aspects. *Antioxid Redox Signal* **18**, 2352–2363 (2013).
333. Tacchini, L., Gammella, E., De Ponti, C., Recalcati, S. & Cairo, G. Role of HIF-1 and NF-κB transcription factors in the modulation of transferrin receptor by inflammatory and anti-inflammatory signals. *Journal of Biological Chemistry* **283**, 20674–20686 (2008).
334. Ingrassia, R., Garavaglia, B. & Memo, M. DMT1 Expression and Iron Levels at the Crossroads Between Aging and Neurodegeneration. *Front Neurosci* **13**, (2019).
335. Billings, F. T. 4th, Yu, C., Byrne, J. G., Petracek, M. R. & Pretorius, M. Heme Oxygenase-1 and Acute Kidney Injury following Cardiac Surgery. *Cardiorenal Med* **4**, 12–21 (2014).
336. Rhodes, B., Fürnrohr, B. G. & Vyse, T. J. C-reactive protein in rheumatology: biology and genetics. *Nat Rev Rheumatol* **7**, 282–289 (2011).
337. Paparella, D. Cardiopulmonary bypass induced inflammation: pathophysiology and treatment. An update. *European Journal of Cardio-Thoracic Surgery* **21**, 232–244 (2002).
338. Piccoli, M. *et al.* 'Lone' increase in C-reactive protein after cardiac surgery: prevalence, clinical characteristics, in-hospital course, and prognostic value. *European Journal of Cardiovascular Prevention & Rehabilitation* **15**, 482–487 (2008).
339. Bruins, P. *et al.* Activation of the Complement System During and After Cardiopulmonary Bypass Surgery. *Circulation* **96**, 3542–3548 (1997).
340. Boeken, U. Increased preoperative C-reactive protein (CRP)-values without signs of an infection and complicated course after cardiopulmonary bypass (CPB) – operations. *European Journal of Cardio-Thoracic Surgery* **13**, 541–545 (1998).
341. Joshi, I., Carney, W. P. & Rock, E. P. Utility of monocyte HLA-DR and rationale for therapeutic GM-CSF in sepsis immunoparalysis. *Front Immunol* **14**, (2023).
342. Allen, M. L. *et al.* Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care*. *Crit Care Med* **30**, 1140–1145 (2002).
343. Oczenski, W. *et al.* HLA-DR as a marker for increased risk for systemic inflammation and septic complications after cardiac surgery. *Intensive Care Med* **29**, 1253–1257 (2003).

344. Zhang, H. *et al.* Role of the CCL2-CCR2 axis in cardiovascular disease: Pathogenesis and clinical implications. *Front Immunol* **13**, (2022).
345. Bajpai, G. *et al.* Tissue Resident CCR2− and CCR2+ Cardiac Macrophages Differentially Orchestrate Monocyte Recruitment and Fate Specification Following Myocardial Injury. *Circ Res* **124**, 263–278 (2019).
346. Bauer, A. *et al.* EuroScore and IL-6 predict the course in ICU after cardiac surgery. *Eur J Med Res* **26**, 29 (2021).
347. Geisler, D. *et al.* Impact of CytoSorb® on interleukin-6 in cardiac surgery. *Front Cardiovasc Med* **10**, (2023).
348. Mullan, C. W. *et al.* United States national trends in comorbidity and outcomes of adult cardiac surgery patients. *J Card Surg* **35**, 2248–2253 (2020).
349. Bartoszko, J. *et al.* Impact of cardiopulmonary bypass duration on efficacy of fibrinogen replacement with cryoprecipitate compared with fibrinogen concentrate: a post hoc analysis of the Fibrinogen Replenishment in Surgery (FIBRES) randomised controlled trial. *Br J Anaesth* **129**, 294–307 (2022).
350. Selevsek, N. *et al.* Network integration and modelling of dynamic drug responses at multi-omics levels. *Commun Biol* **3**, 573 (2020).
351. Mesquita, G. *et al.* H-Ferritin is essential for macrophages' capacity to store or detoxify exogenously added iron. *Sci Rep* **10**, 3061 (2020).
352. Ward, D. M. & Kaplan, J. Ferroportin-mediated iron transport: Expression and regulation. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1823**, 1426–1433 (2012).
353. Yang, J., Zhang, L., Yu, C., Yang, X.-F. & Wang, H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res* **2**, 1 (2014).

# 8 Appendices

## 8.1 Protocol defined adaptive design features

| Element | Adaptive features | Boundaries |
|---|---|---|
| Dosing regimen | Dosing regimens may be determined and adapted in accordance with safety, tolerability, dose compliance and/or PD data (as applicable) collected up to the decision-making time point.<br><br>The term dosing regimen includes both the dose level of 5-ALA-SFC, frequency and duration of administration. These can be adjusted individually or in combination.<br><br>The number of dosing regimens investigated may be adjusted.<br><br>Any dosing regimen may be repeated in full. | 1. The maximum dose that may be administered is 5-ALA-SFC 300mg/470mg twice daily<br>2. The maximum dosing frequency of 5-ALA-SFC or placebo is twice daily<br>3. The maximum duration of continual pre-operative dosing for 5-ALA-SFC is 7 days (BD dosing for 6 consecutive days with an additional 1 or 2 doses on day 7 depending on time of scheduled surgery)<br>4. The dosing regimen may be adapted between dosing cohorts<br>5. The minimum number of dosing levels to be investigated, safety and tolerability permitting, is 2<br>6. The maximum number of dosing levels to be investigated, safety and tolerability permitting, is 4<br>7. A maximum number of dosing regimens investigated is not pre-specified but the total number of evaluable patients in TALEN will not exceed 55 without protocol amendment |
| Sample size | The number of subjects in a dosing cohort may be decreased.<br><br>Additional patients may be enrolled to a dose cohort to ensure the minimum number of evaluable patients is obtained. Subjects may be replaced if non-evaluable, or judged non-compliant with study procedures, or who withdraw due to AE's judged to be mild and unrelated to the IMP, or in the setting of a consented patient's cardiac surgery being cancelled or subject to prolonged or indefinite | 1. The minimum number of subjects in a dosing cohort will be 8 evaluable subjects, including a minimum of 6 patients treated with 5-ALA-SFC to allow dose escalation<br>2. The maximum number of evaluable patients in a dosing cohort will be 10 (other than the optional cohort where it will be up to 15)<br>3. If patient's cardiac surgery is cancelled or subject to prolonged or indefinite deferral, patients may be replaced or, where appropriate and feasible, re-allocated from their current planned cohort to a a different/higher dose cohort and on more than 1 occasion if necessary. If allocated to a higher dose cohort, the dosing of the patient will not commence until the SRC has approved enrolment to that dose level/cohort |

| | | |
|---|---|---|
| | deferral (whether on one or more occasions for the individual patient). | 4. Replacement patients for a dose cohort may be enrolled to an ongoing cohort or be enrolled after initial closure of a dosing cohort |
| Selection criteria | The eligibility criteria pertaining to presence of recent acute myocardial infarction (AMI) may be adapted. | 1. Patients with a recent diagnosis of AMI awaiting CABG may be permitted to be enrolled at the discretion of the investigator upon discussion and agreement by the chief investigator on a case by case basis provided that: all other eligibility criteria are met, that they are clinically stable (i.e. no evidence of haemodynamic instability, no resting angina, no ongoing requirement for parenteral nitrates, no requirement for intra-aortic balloon pump, no refractory ischaemia, or decompensated heart failure); and that there is sufficient time to enable full dosing with study drug (i.e. ≥3 days to time of planned CABG surgery). Kinetics of troponin release will additionally be evaluated separately in any such patients<br>2. Patients undergoing emergency CABG (i.e. unscheduled surgery required in the next available theatre on the same day due to refractory angina or cardiac compromise) will remain ineligible |
| Cohort allocation | Patients may be assigned to a different dosing cohort to that which they are initially or currently assigned or randomised to (e.g. to the next dose level) and on more than one occasion if necessary.<br><br>This includes in the setting of a patient's cardiac surgery being cancelled or subject to prolonged or indefinite deferral or postponement (whether on one or more occasions for the individual patient). | 1. The patient must not have commenced dosing in their current cohort prior to any re-allocation<br>2. The approach to dose escalation will otherwise remain unchanged (see Section 8.2)<br>3. If a patient is re-allocated to a higher dose level, they will not commence dosing at the higher dose level until the respective cohort is opened for dose escalation following SRC review |
| Optional cohort | The optional dose cohort may be utilised at any point during the trial after completion of the first dosing cohort. | 1. The maximum number of evaluable patients in the optional dosing cohort will be 15 |

| | | |
|---|---|---|
| | The optional dose cohort may evaluate more than 1 dose regimen. | 2. No lower limit is set on the minimum number of evaluable patients in this cohort<br>3. The IMP (5-ALA-SFC):placebo ratio is not pre-specified for the optional cohort and may consist of all actives or all placebos<br>4. The dosing regimen boundaries outlined above (including maximal dose and duration) will apply to the optional dose cohort |
| Samples and assessments | Additional blood samples for safety may be obtained.<br><br>Additional or less samples for PD assessment may be taken.<br><br>The timing of any samples may be adapted<br><br>Changes may be made to the methodology or technique used for analysis of PD endpoints | 1. The maximum volume of blood obtained from each participant is estimated to be 279 mL and will not in any case exceed 300mL<br>2. The minimum safety data requirements for progression between dosing levels must be met<br>3. Adaptability is permitted for any PD samples, including for all exploratory and translational objectives, including volumes, specific tubes, sample processing steps and timing of samples |

Experimental Therapeutics
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**A Research study to see if 5-AminoLEvuliNic acid might make cardiac surgery better**

## KEY FACTS SUMMARY

We are inviting you to take part in our research study because you are due to undergo cardiac surgery. Participating in this study is entirely your decision and you can withdraw this at any time.

First, we want to highlight key points that we think you should know before making a decision. If you are interested, we'll then get into more detail in the accompanying Participant Information Sheet.

**Why we are conducting this study**

- This study uses a new treatment, 5-ALA (5aminolevulinic acid) with iron, given for several days before your heart operation. 5-ALA is an amino acid present naturally in food and drink, such as green tea and red wine.
- Giving 5-ALA with iron can boost the levels of a protective enzyme in our cells called haem oxygenase-1 ("HO-1"). In response to stress, such as during an operation, HO-1 switches on protective mechanisms to protect cells and organs from damage.
- This study aims to find whether 5-ALA and iron given before cardiac surgery can increase HO-1 levels and tests different doses to work out which is best.
- If successful, this will allow a future study to test whether 5-ALA and iron can protect the heart and other organs to reduce the risk of serious complications.

**Your choices**

- You can choose to not take part in the study, in which case your operation and care will proceed in the standard way.
- Or you can choose to take part in the study, in which case you will be asked to take the study treatment before your operation.
- Separately, you will be asked whether you consent to provide a small sample of heart tissue, call a heart biopsy. This is obtained during the operation taken by your cardiac surgeon. This is an entirely voluntary separate aspect of the study and you can still take part in the study without undergoing a biopsy.

**Possible major benefits and harms**



- 5-ALA is used as an authorised oral medicine called Gliolan that can help specific brain tumours glow red under a certain light. It is given before brain surgery to help neurosurgeons better visualise a tumour to remove it. There is no upper age limit to Gliolan. We are using 5-ALA for a different purpose in this trial and the doses being tested in this study are lower than used in Gliolan.

| | **Possible important benefits and harms** |
|---|---|
| **Standard treatment: cardiac surgery alone** | Possible benefits – improve heart blood supply. Improve symptoms (chest pain and breathlessness). Lower risk of heart attack.<br>Possible major harms – stroke, heart attack, kidney failure and need for dialysis, irregular heart beat, wound infection, bleeding. |
| **This trial: cardiac surgery + 5-ALA with iron or placebo** | Those listed for standard treatment (cardiac surgery) above and:<br>Additional possible benefits – better protection of the heart, kidneys, brain and other vital organs. Lower risk of needing organ support or dialysis after surgery.<br>Additional possible harms – Side-effects are rare with 5-ALA. Those reported include: sensitivity to light causing a skin rash, nausea, low blood pressure, abnormalities in blood results related to liver function which are transient and do not normally cause symptoms. |

**Briefly, what will it entail?**

- If you consent to take part, you will receive either 5-ALA with iron (4 in 5 chance) or a placebo (1 in 5 chance). This will be decided at random, so you will not be guaranteed to receive 5-ALA. Different doses of 5-ALA with iron will be used during the study and you will not know what dose you are taking. We will ask you to take the study drug by mouth twice a day for several days (in most cases for 3 consecutive days) just before your operation.
- Your usual medication and the cardiac operation will continue as normal. If you provide specific consent, cardiac biopsy may be taken during the time of your operation by your surgeon to allow measuring the effect of 5-ALA on the heart.
- You will undergo additional blood tests before and after the operation to test the effects of the study drug on HO-1 levels and markers of organ function and injury. We will time these tests as far as possible to take place when you are visiting the hospital for pre-operative assessment, on the intensive care unit/ward after surgery and at your outpatient follow-up appointment.

If you would like, we can now explain more about the study in the accompanying Participant Information Sheet.

Radcliffe Department of Medicine | University of Oxford | TALEN | Oxford University Hospitals NHS Foundation Trust

Experimental Therapeutics
Radcliffe Department of Medicine
University of Oxford
John Radcliffe Hospital
01865 231454
01865 225636

*Site ID Code:* *Participant identification number:*

**CONSENT FORM**

**Study Title: T**reatment with 5-**A**mino**LE**vuli**N**ic acid before cardiac surgery **(TALEN)**

Principal Investigator: Dr Arash Yavari

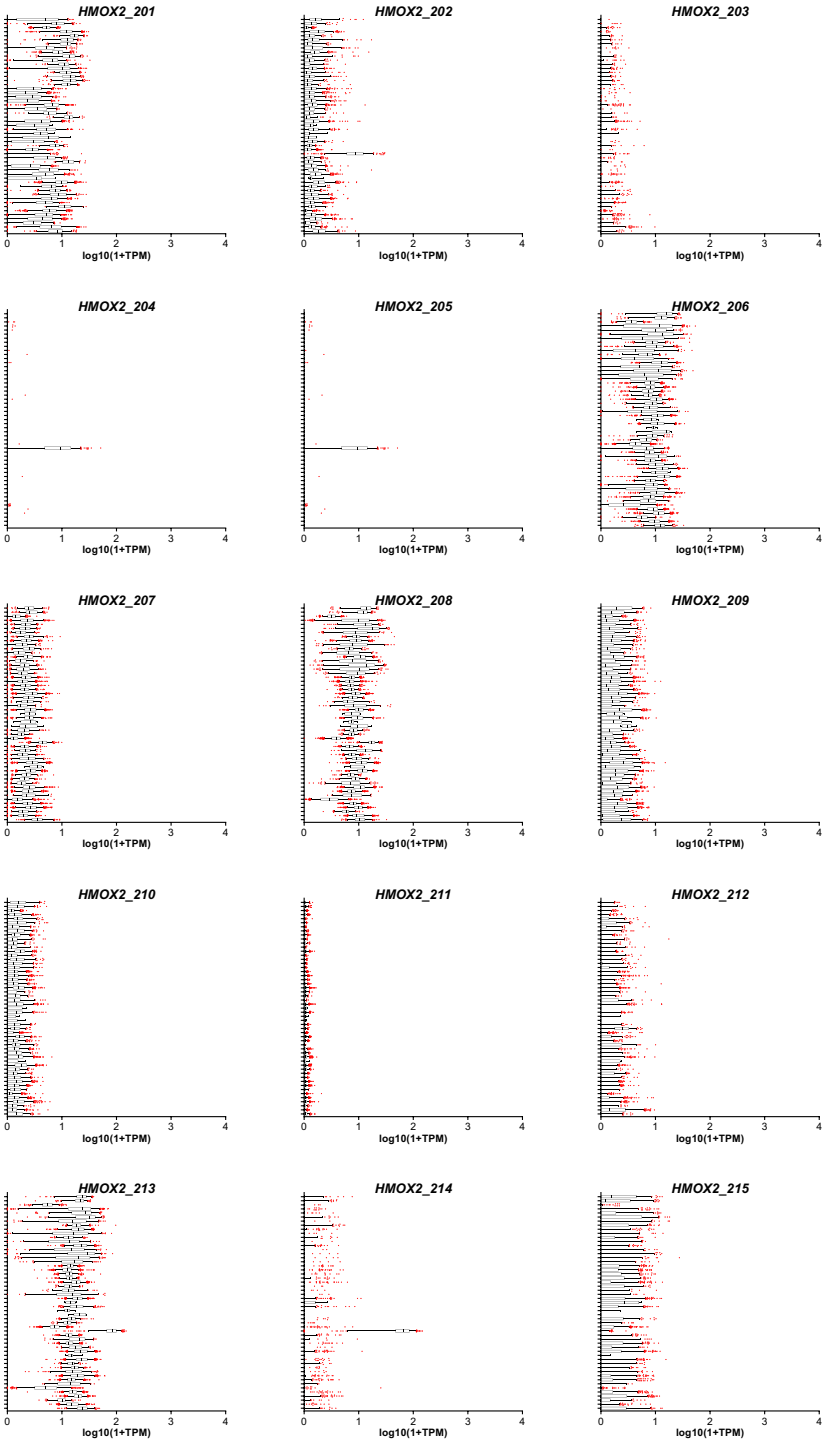
***If you agree, please initial box***

| | | |
|---|---|---|
| 1. | I confirm that I have read the information sheet TALEN_PIL_V2.0_04July2021.docx for this 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 I may be randomly allocated to the placebo arm of the trial and may therefore not receive the active treatment (5-ALA-SFC). | |
| 4. | I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Oxford, from regulatory authorities and from the NHS Trust(s), study monitors and auditors where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. | |
| 5. | I agree for the research team to collect my information for the purpose of the research study. | |
| 6. | I agree that blood and urine samples will be taken and stored for research within this study. | |
| 7. | I agree for the surgeon to take biopsies from my heart during my operation and for it to be stored for research within this study. | Yes / No |
| 8. | I agree to donate blood and urine samples. I consider these samples a gift to the University of Oxford. If a commercial product were developed as a result of this study, I will not gain any direct personal or financial benefit from it. | |
| 9. | I understand that the information held and maintained by NHS Digital / NHS Central Register may be used to help contact me or provide information about my health status. | |
| 10. | I give permission for the research team to share my blood and tissue samples in de-identified format, with other academic, NHS or commercial organisations for the purposes of this research, in the UK, EU or outside the EU, including the US. | |
| 11. | I consent to my GP being informed of my study participation. My GP will also (with my permission) be informed of the results of medical tests performed as part of the research, which are important for my health care. | |
| 12. | I consent for research team members or medical staff appointed by them to visit me at my personal residence for some study activities and/or for study treatment to be directly sent to me if for any reason my personal visit to study site is not possible. | |
| 13. | I understand and agree that my samples will be used in research aimed at understanding the genetic influences on disease and response to surgery and that the results of these investigations are unlikely to have any implications for me personally. | |
| 14. | I agree to take part in this study. | |
| | Additional: | |
| 15. | I agree for my de-identified samples to be used in future research, here or abroad, which has ethics approval. I understand this research may involve commercial organisations. | Yes / No |

| | | |
|---|---|---|
| _____ | _____ | _____ |
| *Name of Participant* | *Date* | *Signature* |
| _____ | _____ | _____ |
| *Name of Person taking Consent* | *Date* | *Signature* |

**1 copy for participant; 1 copy for researcher site file; 1 (original) to be kept in medical notes.*

## 8.3 Bio-informatics analysis – supplementary figures.



### 8.4 Adverse events – Full listing

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Any events** | 12 6 (66.7%) | 9 3 (37.5%) | 17 8 (88.9%) | 12 5 (62.5%) | 6 5 (62.5%) |
| **Vascular disorders** | 3 3 (33.3%) | 2 1 (12.5%) | 7 4 (44.4%) | 1 1 (12.5%) | 0 0 (0.0%) |
| Hypotension | 2 2 (22.2%) | 2 1 (12.5%) | 6 4 (44.4%) | 1 1 (12.5%) | 0 0 (0.0%) |
| Hypertension | 1 1 (11.1%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Cardiac disorders** | 2 2 (22.2%) | 0 0 (0.0%) | 1 1 (11.1%) | 2 1 (12.5%) | 3 3 (37.5%) |
| Atrial fibrillation | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 1 1 (12.5%) |
| Myocardial infarction | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |
| Pericardial effusion | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 2 2 (25.0%) |
| Tachyarrhythmia | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Ventricular fibrillation | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |
| **Gastrointestinal disorders** | 1 1 (11.1%) | 0 0 (0.0%) | 5 4 (44.4%) | 1 1 (12.5%) | 1 1 (12.5%) |
| Dyspepsia | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 1 1 (12.5%) |
| Nausea | 0 0 (0.0%) | 0 0 (0.0%) | 3 2 (22.2%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Abdominal discomfort | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Abdominal pain | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Ileus | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Nervous system disorders** | 1 1 (11.1%) | 2 2 (25.0%) | 1 1 (11.1%) | 1 1 (12.5%) | 2 2 (25.0%) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Transient ischaemic attack | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 1 1 (12.5%) |
| Cerebral artery occlusion | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Headache | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) |
| Hypoaesthesia | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |
| Meralgia paraesthetica | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Taste disorder | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Injury, poisoning and procedural complications** | 1 1 (11.1%) | 2 2 (25.0%) | 1 1 (11.1%) | 2 2 (25.0%) | 0 0 (0.0%) |
| Post procedural hypotension | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 2 2 (25.0%) | 0 0 (0.0%) |
| Post procedural complication | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Post procedural constipation | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Post procedural haemorrhage | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Renal and urinary disorders** | 2 2 (22.2%) | 1 1 (12.5%) | 1 1 (11.1%) | 2 2 (25.0%) | 0 0 (0.0%) |
| Acute kidney injury | 2 2 (22.2%) | 1 1 (12.5%) | 0 0 (0.0%) | 2 2 (25.0%) | 0 0 (0.0%) |
| Urinary retention | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| **Metabolism and nutrition disorders** | 1 1 (11.1%) | 2 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Hyperglycaemia | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Hypocalcaemia | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Hypokalaemia | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Respiratory, thoracic and mediastinal disorders** | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 2 2 (25.0%) | 0 0 (0.0%) |
| Dyspnoea | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |
| Pleural effusion | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |
| **Eye disorders** | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Vision blurred | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **General disorders and administration site conditions** | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| Discharge | 1 1 (11.1%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) |
| **Musculoskeletal and connective tissue disorders** | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |

| | Placebo (n=9) | 75 mg/118 mg (n=8) | 150 mg/236 mg (n=9) | 225 mg /354 mg (n=8) | 300 mg/472 mg (n=8) |
|---|---|---|---|---|---|
| Muscular weakness | 0 0 (0.0%) | 0 0 (0.0%) | 0 0 (0.0%) | 1 1 (12.5%) | 0 0 (0.0%) |

## 8.5 Haemodynamic and respiratory observations

| Measurement (unit)<br>Timepoint | Placebo<br>(N=9) | 75 mg 5-ALA/<br>118 mg SFC<br>(N=8) | 150 mg 5-ALA/<br>236 mg SFC<br>(N=9) | 225 mg 5-ALA/<br>354 mg SFC<br>(N=8) | 300 mg 5-ALA/<br>472 mg SFC<br>(N=8) |
|---|---|---|---|---|---|
| **SBP (mmHg)** | | | | | |
| Screening | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 143 (15) | 140 (30) | 140 (15) | 121 (22) | 139 (24) |
| Median (Q1; Q3) | 141 (133; 155) | 135 (119; 164) | 140 (126; 147) | 113 (109; 134) | 147 (125; 152) |
| min max | 117 161 | 96 186 | 121 164 | 95 164 | 96 168 |
| Before induction of anaesthesia | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 144 (35) | 152 (24) | 147 (19) | 148 (26) | 155 (14) |
| Median (Q1; Q3) | 154 (128; 172) | 155 (132; 172) | 148 (136; 168) | 149 (130; 170) | 155 (143; 168) |
| min max | 80 188 | 116 180 | 114 168 | 108 180 | 140 172 |
| T1- 1H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 102 (18) | 109 (7) | 113 (15) | 104 (11) | 116 (20) |
| Median (Q1; Q3) | 103 (93; 115) | 112 (102; 114) | 110 (104; 124) | 101 (100; 108) | 108 (102; 130) |
| min max | 68 128 | 100 120 | 88 136 | 88 124 | 96 153 |
| T2- 3H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 116 (18) | 108 (8) | 112 (17) | 118 (17) | 112 (10) |
| Median (Q1; Q3) | 118 (111; 128) | 109 (103; 114) | 114 (99; 128) | 117 (110; 130) | 110 (105; 122) |
| min max | 80 142 | 97 120 | 89 133 | 88 142 | 99 126 |
| T5- 24H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 108 (18) | 115 (15) | 117 (11) | 112 (17) | 111 (15) |
| Median (Q1; Q3) | 110 (98; 115) | 111 (105; 130) | 118 (106; 123) | 107 (101; 124) | 111 (101; 123) |
| min max | 77 134 | 94 134 | 101 136 | 91 138 | 88 133 |
| T6- 48H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 118 (24) | 120 (15) | 118 (13) | 109 (12) | 109 (19) |
| Median (Q1; Q3) | 105 (101; 134) | 121 (113; 131) | 120 (106; 130) | 107 (103; 116) | 113 (96; 124) |
| min max | 94 163 | 93 140 | 98 134 | 89 130 | 75 131 |
| T7- 72H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 126 (8) | 120 (13) | 122 (18) | 108 (18) | 113 (12) |
| Median (Q1; Q3) | 127 (126; 131) | 122 (113; 130) | 121 (110; 137) | 107 (93; 118) | 112 (105; 115) |
| min max | 111 135 | 96 138 | 91 143 | 87 140 | 99 140 |
| **DBP (mmHg)** | | | | | |
| Screening | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 77 (9) | 78 (16) | 77 (9) | 71 (14) | 78 (10) |
| Median (Q1; Q3) | 75 (73; 79) | 77 (66; 93) | 79 (74; 82) | 72 (64; 77) | 79 (75; 86) |

| Measurement (unit)<br>Timepoint | Placebo<br>(N=9) | 75 mg 5-ALA/<br>118 mg SFC<br>(N=8) | 150 mg 5-ALA/<br>236 mg SFC<br>(N=9) | 225 mg 5-ALA/<br>354 mg SFC<br>(N=8) | 300 mg 5-ALA/<br>472 mg SFC<br>(N=8) |
|---|---|---|---|---|---|
| min max | 70 98 | 56 98 | 64 93 | 44 94 | 58 89 |
| Before induction of anaesthesia | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 66 (15) | 64 (13) | 66 (10) | 67 (11) | 74 (12) |
| Median (Q1; Q3) | 68 (50; 72) | 62 (57; 68) | 60 (58; 76) | 64 (59; 74) | 71 (64; 84) |
| min max | 46 88 | 48 90 | 56 84 | 52 88 | 60 92 |
| T1- 1H after AXC removal | | | | | |
| n | 9 | 8 | 8 | 8 | 8 |
| Mean (SD) | 57 (9) | 57 (8) | 59 (6) | 59 (9) | 62 (8) |
| Median (Q1; Q3) | 56 (54; 60) | 56 (52; 63) | 60 (54; 62) | 59 (53; 64) | 62 (56; 68) |
| min max | 40 73 | 42 68 | 52 68 | 46 76 | 50 74 |
| T2- 3H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 61 (10) | 59 (8) | 55 (7) | 65 (12) | 60 (6) |
| Median (Q1; Q3) | 62 (56; 67) | 59 (53; 64) | 54 (51; 56) | 69 (55; 75) | 60 (55; 65) |
| min max | 45 76 | 49 72 | 48 67 | 46 80 | 54 71 |
| T5- 24H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 53 (7) | 61 (14) | 58 (12) | 57 (5) | 55 (6) |
| Median (Q1; Q3) | 56 (50; 57) | 60 (53; 63) | 55 (55; 56) | 56 (54; 60) | 55 (50; 60) |
| min max | 38 63 | 47 93 | 48 88 | 53 66 | 49 62 |
| T6- 48H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 57 (10) | 64 (15) | 60 (13) | 60 (13) | 59 (18) |
| Median (Q1; Q3) | 56 (50; 66) | 63 (49; 80) | 57 (51; 66) | 62 (49; 69) | 58 (46; 66) |
| min max | 43 69 | 44 81 | 47 87 | 41 77 | 40 95 |
| T7- 72H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 62 (8) | 71 (14) | 64 (11) | 63 (11) | 62 (5) |
| Median (Q1; Q3) | 64 (59; 68) | 69 (62; 78) | 65 (60; 70) | 66 (58; 68) | 63 (59; 66) |
| min max | 50 71 | 55 98 | 42 80 | 43 81 | 54 69 |
| **Oxygen saturation (%)** | | | | | |
| Screening | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 97 (2) | 98 (1) | 98 (1) | 92 (17) | 98 (0) |
| Median (Q1; Q3) | 97 (96; 98) | 98 (97; 99) | 98 (98; 98) | 97 (95; 99) | 98 (98; 98) |
| min max | 95 100 | 96 99 | 97 100 | 51 100 | 97 98 |
| Before induction of anaesthesia | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 99 (0) | 99 (1) | 99 (1) | 99 (1) | 99 (1) |
| Median (Q1; Q3) | 99 (99; 99) | 99 (98; 99) | 99 (99; 99) | 99 (99; 99) | 100 (98; 100) |
| min max | 99 100 | 97 100 | 97 100 | 98 100 | 97 100 |
| T1- 1H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 98 (3) | 98 (3) | 99 (1) | 99 (1) | 99 (2) |

| Measurement (unit)<br>Timepoint | Placebo<br>(N=9) | 75 mg 5-ALA/<br>118 mg SFC<br>(N=8) | 150 mg 5-ALA/<br>236 mg SFC<br>(N=9) | 225 mg 5-ALA/<br>354 mg SFC<br>(N=8) | 300 mg 5-ALA/<br>472 mg SFC<br>(N=8) |
|---|---|---|---|---|---|
| Median (Q1; Q3) | 98 (98; 100) | 100 (98; 100) | 99 (99; 99) | 99 (99; 100) | 99 (98; 100) |
| min max | 92 100 | 92 100 | 98 100 | 97 100 | 95 100 |
| T2- 3H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 99 (1) | 99 (2) | 99 (1) | 99 (1) | 99 (1) |
| Median (Q1; Q3) | 98 (98; 100) | 100 (98; 100) | 100 (98; 100) | 100 (100; 100) | 100 (98; 100) |
| min max | 96 100 | 96 100 | 98 100 | 96 100 | 97 100 |
| T5- 24H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 96 (3) | 96 (1) | 98 (2) | 96 (3) | 97 (3) |
| Median (Q1; Q3) | 97 (96; 98) | 97 (96; 98) | 97 (97; 100) | 97 (94; 99) | 98 (96; 99) |
| min max | 90 98 | 94 98 | 93 100 | 93 100 | 92 100 |
| T6- 48H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 95 (3) | 96 (2) | 97 (2) | 96 (2) | 96 (2) |
| Median (Q1; Q3) | 96 (95; 97) | 96 (94; 98) | 98 (97; 98) | 95 (94; 97) | 97 (95; 98) |
| min max | 91 99 | 94 98 | 94 100 | 94 100 | 94 99 |
| T7- 72H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 96 (3) | 95 (1) | 97 (1) | 96 (2) | 97 (2) |
| Median (Q1; Q3) | 97 (94; 98) | 94 (94; 96) | 97 (96; 98) | 96 (94; 97) | 98 (95; 99) |
| min max | 93 100 | 93 96 | 95 98 | 94 100 | 94 100 |
| **Heart rate (bpm)** | | | | | |
| Screening | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 62 (13) | 62 (10) | 65 (11) | 65 (10) | 67 (9) |
| Median (Q1; Q3) | 65 (57; 70) | 61 (56; 66) | 66 (64; 69) | 67 (56; 74) | 67 (63; 74) |
| min max | 39 78 | 51 83 | 42 82 | 50 75 | 50 78 |
| Before induction of anaesthesia | | | | | |
| n | 9 | 8 | 9 | 8 | 7 |
| Mean (SD) | 70 (20) | 64 (10) | 73 (14) | 78 (13) | 69 (18) |
| Median (Q1; Q3) | 72 (54; 84) | 64 (57; 70) | 72 (68; 80) | 75 (68; 89) | 64 (57; 75) |
| min max | 40 104 | 50 80 | 48 92 | 60 96 | 55 108 |
| T1- 1H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 76 (9) | 83 (12) | 84 (6) | 88 (9) | 81 (14) |
| Median (Q1; Q3) | 78 (72; 80) | 84 (78; 94) | 84 (80; 86) | 90 (80; 92) | 78 (74; 92) |
| min max | 60 92 | 60 96 | 76 96 | 76 104 | 60 101 |
| T5- 24H after AXC removal | | | | | |
| n | 7 | 8 | 9 | 8 | 8 |
| Mean (SD) | 76 (13) | 79 (12) | 73 (13) | 80 (14) | 77 (14) |
| Median (Q1; Q3) | 76 (66; 87) | 81 (67; 87) | 68 (65; 84) | 82 (70; 89) | 82 (67; 88) |
| min max | 59 96 | 63 99 | 56 90 | 59 101 | 54 93 |
| **p:F Ratio** | | | | | |

| Measurement (unit)<br>Timepoint | Placebo<br>(N=9) | 75 mg 5-ALA/<br>118 mg SFC<br>(N=8) | 150 mg 5-ALA/<br>236 mg SFC<br>(N=9) | 225 mg 5-ALA/<br>354 mg SFC<br>(N=8) | 300 mg 5-ALA/<br>472 mg SFC<br>(N=8) |
|---|---|---|---|---|---|
| T1- 1H after AXC removal | | | | | |
| n | 8 | 8 | 6 | 7 | 8 |
| Mean (SD) | 387 (385) | 272 (159) | 636 (502) | 510 (347) | 513 (187) |
| Median (Q1; Q3) | 286 (181; 392) | 289 (148; 397) | 500 (224; 972) | 487 (272; 662) | 539 (397; 652) |
| min max | 81 1300 | 25 484 | 155 1468 | 61 1167 | 178 748 |
| T2- 3H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 218 (70) | 249 (111) | 279 (139) | 208 (92) | 288 (134) |
| Median (Q1; Q3) | 235 (162; 283) | 224 (155; 369) | 284 (202; 301) | 213 (131; 281) | 259 (169; 406) |
| min max | 120 316 | 117 383 | 63 538 | 81 335 | 144 495 |
| T5- 24H after AXC removal | | | | | |
| n | 9 | 8 | 9 | 8 | 8 |
| Mean (SD) | 264 (78) | 325 (90) | 402 (139) | 280 (66) | 304 (90) |
| Median (Q1; Q3) | 225 (205; 311) | 318 (254; 377) | 373 (301; 486) | 276 (228; 335) | 302 (217; 387) |
| min max | 189 390 | 212 491 | 211 648 | 190 375 | 195 422 |
| T6- 48H after AXC removal | | | | | |
| n | 5 | 4 | 2 | 1 | 6 |
| Mean (SD) | 262 (96) | 258 (89) | 409 (152) | 195 (-NA-) | 346 (77) |
| Median (Q1; Q3) | 271 (205; 288) | 234 (197; 319) | 409 (301; 516) | 195 (195; 195) | 322 (302; 419) |
| min max | 147 401 | 180 383 | 301 516 | 195 195 | 252 457 |
| T7- 72H after AXC removal | | | | | |
| n | 4 | 2 | 1 | 1 | 1 |
| Mean (SD) | 278 (97) | 338 (74) | 387 (-NA-) | 188 (-NA-) | 390 (-NA-) |
| Median (Q1; Q3) | 255 (203; 353) | 338 (285; 390) | 387 (387; 387) | 188 (188; 188) | 390 (390; 390) |
| min max | 197 405 | 285 390 | 387 387 | 188 188 | 390 390 |

## 8.6 Coagulation data

| Time point<br>Readout | Placebo<br>(n=9) | 75 mg/118 mg<br>(n=8) | 150 mg/236<br>mg (n=9) | 225 mg/354<br>mg (n=8) | 300 mg/472<br>mg (n=8) |
|---|---|---|---|---|---|
| **Screening** | | | | | |
| **PT (s)** | | | | | |
| N | 6 | 4 | 4 | 4 | 8 |
| Mean (SD) | 13.0 (5.1) | 10.7 (0.5) | 10.7 (1.4) | 10.3 (0.6) | 12.1 (4.6) |
| Median (IQR) | 10.9 (10.6; 11.4) | 10.7 (10.3; 11.1) | 10.2 (9.9; 11.6) | 10.5 (9.9; 10.8) | 10.6 (10.1; 11.3) |
| Min Max | 10.6 23.4 | 10.2 11.2 | 9.8 12.8 | 9.5 10.9 | 9.8 23.5 |
| **INR (ratio)** | | | | | |
| N | 6 | 4 | 4 | 4 | 8 |
| Mean (SD) | 1.25 (0.56) | 1.03 (0.05) | 1.00 (0.14) | 0.98 (0.05) | 1.19 (0.54) |
| Median (IQR) | 1.00 (1.00; 1.10) | 1.00 (1.00; 1.05) | 0.95 (0.90; 1.10) | 1.00 (0.95; 1.00) | 1.00 (0.95; 1.10) |
| Min Max | 1.00 2.40 | 1.00 1.10 | 0.90 1.20 | 0.90 1.00 | 0.90 2.50 |
| **APTT (sec)** | | | | | |
| N | 6 | 4 | 3 | 4 | 8 |
| Mean (SD) | 27.1 (3.3) | 24.5 (2.6) | 26.4 (0.8) | 25.1 (0.9) | 27.5 (4.7) |
| Median (IQR) | 26.2 (24.7; 29.5) | 25.4 (22.8; 26.3) | 26.7 (25.5; 26.9) | 25.0 (24.5; 25.7) | 26.9 (23.9; 29.2) |
| Min Max | 23.9 32.4 | 20.7 26.5 | 25.5 26.9 | 24.0 26.3 | 23.1 37.3 |
| **Pre-operatively** | | | | | |
| **PT (s)** | | | | | |
| N | 4 | 4 | 2 | 3 | 1 |
| Mean (SD) | 10.7 (0.3) | 11.1 (0.8) | 10.2 (0.3) | 9.9 (0.1) | 10.7 (-NA-) |
| Median (IQR) | 10.7 (10.5; 10.9) | 11.0 (10.4; 11.8) | 10.2 (10.0; 10.4) | 9.9 (9.8; 10.0) | 10.7 (10.7; 10.7) |
| Min Max | 10.3 11.1 | 10.3 12.0 | 10.0 10.4 | 9.8 10.0 | 10.7 10.7 |
| **INR (ratio)** | | | | | |
| N | 4 | 4 | 2 | 3 | 1 |

| Time point<br>Readout | Placebo<br>(n=9) | 75 mg/118 mg<br>(n=8) | 150 mg/236<br>mg (n=9) | 225 mg/354<br>mg (n=8) | 300 mg/472<br>mg (n=8) |
|---|---|---|---|---|---|
| Mean (SD) | 1.03 (0.05) | 1.08 (0.10) | 0.95 (0.07) | 0.90 (0.00) | 1.00 (-NA-) |
| Median (IQR) | 1.00 (1.00; 1.05) | 1.05 (1.00; 1.15) | 0.95 (0.90; 1.00) | 0.90 (0.90; 0.90) | 1.00 (1.00; 1.00) |
| Min Max | 1.00 1.10 | 1.00 1.20 | 0.90 1.00 | 0.90 0.90 | 1.00 1.00 |
| **APTT (sec)** | | | | | |
| N | 4 | 4 | 2 | 3 | 1 |
| Mean (SD) | 24.9 (1.8) | 24.3 (2.0) | 23.8 (3.0) | 23.0 (1.6) | 27.1 (-NA-) |
| Median (IQR) | 25.0 (23.5; 26.4) | 24.6 (23.1; 25.5) | 23.8 (21.6; 25.9) | 22.8 (21.6; 24.7) | 27.1 (27.1; 27.1) |
| Min Max | 22.8 26.8 | 21.6 26.4 | 21.6 25.9 | 21.6 24.7 | 27.1 27.1 |
| **POD 1** | | | | | |
| **PT (s)** | | | | | |
| N | 5 | 7 | 2 | 7 | 8 |
| Mean (SD) | 11.3 (0.4) | 11.3 (0.6) | 11.3 (0.9) | 11.0 (0.8) | 11.4 (1.1) |
| Median (IQR) | 11.4 (11.2; 11.4) | 11.3 (10.8; 11.9) | 11.3 (10.6; 11.9) | 10.7 (10.5; 11.7) | 11.3 (10.7; 11.5) |
| Min Max | 10.7 11.9 | 10.4 12.1 | 10.6 11.9 | 9.9 12.2 | 10.4 14.0 |
| **INR (ratio)** | | | | | |
| N | 5 | 7 | 2 | 7 | 8 |
| Mean (SD) | 1.08 (0.04) | 1.07 (0.08) | 1.05 (0.07) | 1.04 (0.10) | 1.10 (0.13) |
| Median (IQR) | 1.10 (1.10; 1.10) | 1.10 (1.00; 1.10) | 1.05 (1.00; 1.10) | 1.00 (1.00; 1.10) | 1.10 (1.00; 1.10) |
| Min Max | 1.00 1.10 | 1.00 1.20 | 1.00 1.10 | 0.90 1.20 | 1.00 1.40 |
| **APTT (sec)** | | | | | |
| N | 5 | 7 | 2 | 7 | 8 |
| Mean (SD) | 42.5 (37.6) | 24.3 (1.6) | 22.8 (0.4) | 24.7 (3.8) | 25.0 (3.1) |
| Median (IQR) | 26.1 (25.3; 26.7) | 24.7 (23.4; 25.4) | 22.8 (22.5; 23.1) | 23.8 (21.6; 26.0) | 25.3 (22.2; 27.7) |
| Min Max | 24.6 109.7 | 21.6 26.4 | 22.5 23.1 | 21.6 32.5 | 20.7 29.4 |

| Time point<br>Readout | Placebo<br>(n=9) | 75 mg/118 mg<br>(n=8) | 150 mg/236<br>mg (n=9) | 225 mg/354<br>mg (n=8) | 300 mg/472<br>mg (n=8) |
|---|---|---|---|---|---|
| **POD 2** | | | | | |
| **PT (s)** | | | | | |
| N | 4 | 4 | 2 | 4 | 7 |
| Mean (SD) | 12.4 (1.2) | 11.2 (0.7) | 10.9 (0.3) | 11.4 (1.3) | 11.7 (1.8) |
| Median (IQR) | 12.7 (11.7; 13.2) | 11.3 (10.6; 11.8) | 10.9 (10.7; 11.1) | 10.9 (10.5; 12.2) | 11.1 (10.6; 12.1) |
| Min Max | 10.7 13.6 | 10.4 11.9 | 10.7 11.1 | 10.4 13.3 | 10.6 15.6 |
| **INR (ratio)** | | | | | |
| N | 4 | 4 | 2 | 4 | 7 |
| Mean (SD) | 1.18 (0.13) | 1.05 (0.06) | 1.05 (0.07) | 1.10 (0.14) | 1.13 (0.18) |
| Median (IQR) | 1.20 (1.10; 1.25) | 1.05 (1.00; 1.10) | 1.05 (1.00; 1.10) | 1.05 (1.00; 1.20) | 1.10 (1.00; 1.20) |
| Min Max | 1.00 1.30 | 1.00 1.10 | 1.00 1.10 | 1.00 1.30 | 1.00 1.50 |
| **APTT (sec)** | | | | | |
| N | 4 | 4 | 2 | 3 | 7 |
| Mean (SD) | 31.7 (5.4) | 24.3 (0.7) | 31.2 (3.7) | 29.0 (3.8) | 30.5 (8.0) |
| Median (IQR) | 30.2 (27.9; 35.5) | 24.1 (23.9; 24.7) | 31.2 (28.5; 33.8) | 27.1 (26.5; 33.4) | 28.9 (24.6; 32.1) |
| Min Max | 27.1 39.3 | 23.8 25.3 | 28.5 33.8 | 26.5 33.4 | 24.0 47.2 |
| **POD 3** | | | | | |
| **PT (s)** | | | | | |
| N | 3 | 2 | 1 | 2 | 4 |
| Mean (SD) | 11.8 (0.2) | 10.6 (0.8) | 11.4 (-NA-) | 11.4 (1.3) | 10.7 (0.3) |
| Median (IQR) | 11.7 (11.7; 12.1) | 10.6 (10.0; 11.1) | 11.4 (11.4; 11.4) | 11.4 (10.5; 12.3) | 10.7 (10.5; 10.9) |
| Min Max | 11.7 12.1 | 10.0 11.1 | 11.4 11.4 | 10.5 12.3 | 10.3 11.1 |
| **INR (ratio)** | | | | | |
| N | 3 | 2 | 1 | 2 | 4 |
| Mean (SD) | 1.13 (0.06) | 1.00 (0.14) | 1.10 (-NA-) | 1.10 (0.14) | 1.03 (0.05) |

| Time point<br>Readout | Placebo<br>(n=9) | 75 mg/118 mg<br>(n=8) | 150 mg/236<br>mg (n=9) | 225 mg/354<br>mg (n=8) | 300 mg/472<br>mg (n=8) |
|---|---|---|---|---|---|
| Median (IQR) | 1.10 (1.10; 1.20) | 1.00 (0.90; 1.10) | 1.10 (1.10; 1.10) | 1.10 (1.00; 1.20) | 1.00 (1.00; 1.05) |
| Min Max | 1.10 1.20 | 0.90 1.10 | 1.10 1.10 | 1.00 1.20 | 1.00 1.10 |
| **APTT (sec)** | | | | | |
| N | 3 | 2 | 1 | 2 | 4 |
| Mean (SD) | 32.6 (5.2) | 23.7 (1.6) | 35.4 (-NA-) | 31.7 (10.5) | 26.0 (1.8) |
| Median (IQR) | 31.9 (27.7; 38.1) | 23.7 (22.6; 24.8) | 35.4 (35.4; 35.4) | 31.7 (24.3; 39.1) | 25.7 (24.6; 27.4) |
| Min Max | 27.7 38.1 | 22.6 24.8 | 35.4 35.4 | 24.3 39.1 | 24.3 28.2 |

Abbreviations: SD – standard deviation; IQR – interquartile range; Min – minimum; Max – maximum

## 8.7 DNA spectrophotometry data

| Sample ID | Concentration (ng/ul) | 260/280 ratio | 260/230 ratio |
|---|---|---|---|
| 1 | 36.74 | 1.97 | 1.81 |
| 2 | 37.34 | 1.86 | 1.36 |
| 3 | 55.07 | 1.85 | 1.58 |
| 4 | 69.91 | 1.89 | 1.87 |
| 5 | 26.7 | 1.92 | 1.85 |
| 6 | 55.06 | 1.98 | 1.07 |
| 7 | 37.13 | 1.89 | 1.34 |
| 8 | 37.93 | 1.88 | 1.95 |
| 9 | 47.35 | **1.76** | 0.9 |
| 10 | 38.91 | 1.9 | 1.17 |
| 11 | 42.55 | 1.83 | 1.46 |
| 12 | 17.93 | 1.88 | 1.94 |
| 13 | 49.2 | **1.63** | 1.31 |
| 14 | 30.35 | 1.88 | 2.5 |
| 15 | 37.5 | 1.95 | 2.47 |
| 16 | 53.22 | **1.68** | 0.93 |
| 18 | 90.82 | **1.65** | 0.91 |
| 19 | 43.78 | 1.83 | 1.39 |
| 20 | 56.57 | 1.86 | 1.67 |
| 21 | 33.36 | 1.82 | 1.09 |
| 22 | 53.68 | 1.87 | 1.76 |
| 23 | 55.94 | 1.85 | 1.59 |
| 24 | 44.33 | 1.8 | 1.32 |
| 25 | 36.39 | **1.79** | 1.11 |
| 26 | 58.65 | 1.95 | 1.77 |
| 27 | 55.58 | 1.81 | 1.41 |
| 28 | 53.26 | 1.86 | 1.57 |
| 29 | 36.5 | 1.9 | 1.84 |
| 30 | 30.37 | 1.88 | 1.01 |
| 31 | 46.72 | 2 | 1.01 |
| 32 | 40.23 | 1.87 | 1.22 |
| 33 | 24.08 | 2.13 | 1.36 |
| 34 | 29.1 | 1.98 | 1.31 |
| 35 | 27.85 | 1.97 | 1.12 |
| 36 | 27.13 | **1.77** | 0.9 |
| 37 | 44.28 | 1.84 | 1.74 |
| 38 | 40.6 | 1.94 | 1.13 |
| 39 | 41.29 | 1.85 | 1.5 |
| 40 | 80.64 | 1.86 | 1.65 |
| 41 | 42.27 | 1.88 | 1.28 |
| 42 | 44.76 | 1.91 | 1.45 |
| 42 | 33.16 | 1.94 | 1.24 |
| 43 | 33.92 | 1.91 | 1.24 |

| | | | |
|---|---|---|---|
| 44 | 18.06 | 2.1 | 0.62 |
| 44 | 21.08 | 2 | 0.65 |
| 45 | 32.66 | **1.77** | 0.98 |
| 46 | 29.81 | **1.78** | 2.38 |
| 47 | 48.36 | 1.89 | 2.14 |
| 48 | 14.51 | 2.07 | 1.83 |
| 49 | 35.92 | 1.94 | 1.25 |
| 50 | 26.17 | 1.86 | 1.51 |
| 51 | 29.87 | **1.79** | 0.7 |
| 52 | 50.71 | 1.86 | 1.07 |
| 53 | 19.87 | 2.12 | 3.17 |

Samples in **red** below acceptable quality