This thesis has been submitted in fulfilment of the requirements for a postgraduate degree (e. g. PhD, MPhil, DClinPsychol) at the University of Edinburgh. Please note the following terms and conditions of use:

- This work is protected by copyright and other intellectual property rights, which are retained by the thesis author, unless otherwise stated.
- A copy can be downloaded for personal non-commercial research or study, without prior permission or charge.
- This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author.
- The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author.
- When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given.
Translational and Mechanistic Studies of Protection of Human Kidney Cells from Injury

Fiona Anne Ingrid Duthie

MA(Cantab.) MB BChir MRCP(UK)(Nephrology)

A thesis submitted for the degree of Doctor of Philosophy

The University of Edinburgh

2022
Declaration

I declare that the work presented in this thesis is my own and has not previously been published or presented towards another higher degree except where clearly acknowledged as such in the text.
Abstract

Acute kidney injury (AKI) is a common syndrome, affecting up to 14% of patients in hospital and with no specific treatment. It is associated with significant morbidity and mortality and affects patients under the care of all specialities.

Patients who have cardiac surgery are particularly vulnerable to AKI in that they often have multiple risk factors for AKI such as older age, hypertension, and type II diabetes. Planned cardiac bypass surgery gives an opportunity to study ischaemia reperfusion injury (IRI) to the kidney, and allows the testing of potential protective agents and therefore translation from preclinical studies.

The drug Heme Arginate (HA) has been in clinical use for more than 20 years for the treatment of acute porphyria and has a good safety profile. Interestingly, HA treatment induces upregulation of the anti-inflammatory enzyme heme oxygenase-1 (HO-1) and preclinical studies have indicated that HA protects aged mice from renal IRI with macrophage HO-1 expression being critical. Despite encouraging data from Phase I studies in healthy volunteers, HA has not been used in an elderly and comorbid patient population of patients with cardiac disease. There is a known association between iron and inflammation. Therefore, the Heme Arginate in patients planned for Cardiac Surgery (HACS) clinical trial was designed to investigate if HA effectively induces HO-1 expression and if it is safe in this patient group. The HACS trial demonstrated that HO-1 gene expression in peripheral blood mononuclear cells was significantly increased, but that HO-1 protein was not upregulated. There was however a significant increase in HO-1 protein in serum. A recent study of HO-1 expression in the human heart following HA dosing has provided comparable results for peripheral blood mononuclear cells in a similar aged and comorbid patient group. Results will inform future Phase II and Phase III clinical trials.

Continuing the theme of repurposing drugs licensed for human use, dimethyl fumarate (DMF) is a fumaric acid ester which is licensed for the treatment of
multiple sclerosis (MS) and psoriasis. Its exact mechanisms of action are unknown but likely involves activation of the strongly anti-inflammatory nuclear factor erythroid 2–related factor 2 (Nrf2) pathway (of which HO-1 is downstream) and inhibition of the nuclear localisation of the transcription factor NF-κB. Preclinical studies have suggested that macrophages are key to both the development of AKI and subsequent kidney repair. A treatment which could alter macrophage phenotype from pro-inflammatory (classically activated/M1) to anti-inflammatory reparative (alternatively activated/M2) would be of interest in AKI. Experimental work in vitro and in vivo was carried out to examine the effect of DMF on the Nrf2 pathway and to explore the effect of DMF on both murine and human macrophages. Interestingly, DMF reduced phagocytosis of apoptotic cells by murine bone marrow derived macrophages (BMDMs) at high dose, but pilot data suggested that DMF treatment ameliorated the classically activated M1 phenotype of BMDMs as measured by nitrite production. In addition, DMF administration to mice upregulated Nrf2 dependent genes including HO-1 in kidney tissue. Preliminary studies of the effect of DMF upon murine renal IRI did not show protection from AKI although the severity if the injury may have precluded this and further work is required.

It is hoped that HA may offer therapeutic potential in the prevention and treatment of IRI and further clinical trials are ongoing. DMF offers potential as an attractive oral anti-inflammatory therapy and merits further study.
Lay summary

Acute kidney injury (AKI) is a rapid decline in kidney function. AKI is a common syndrome in patients admitted to hospital, and is associated with poor outcomes. Unfortunately there are currently no specific treatments available for patients with this condition.

Patients undergoing heart operations are particularly vulnerable to AKI because they often have multiple risk factors for developing AKI (for example older age, high blood pressure and diabetes). The nature of the operation involves an interruption to the blood supply to the kidneys while the heart is repaired. This predictable injury to the kidneys, in the setting of higher risk of AKI in these patients, makes the heart surgery setting a good place to study medicines which may help to provide some protection from AKI.

The drug Heme arginate (HA) has been in use for over 20 years in the treatment of Acute Porphyria, a condition caused by the lack of an enzyme involved in the breakdown of blood cells. For that reason it is known to have a good safety profile. In experiments in mice, treatment with HA protects the kidney from injury. HA has been shown to increase the levels of an anti-inflammatory enzyme, heme oxygenase-1 (HO-1), in the kidney and this is likely to be at least one of the mechanisms by which HA has its protective effect. Studies in healthy human volunteers suggest that HA can raise HO-1 levels in man too.

The Heme Arginate in Patients Planned for Cardiac Surgery (HACS) trial was designed to ensure that HA is safe in this cohort and that it increases the levels of HO-1 in the blood. The trial showed that the levels of HO-1 protein in the circulating white blood cells were not significantly increased, but that the mRNA (a genetic messenger, which instructs cells to make more protein) levels were higher. The HO-1 levels were also higher in the circulating blood
(serum). This finding is in keeping with another trial done in humans which looked at the levels of HO-1 in the heart at the time of surgery. We hope that this increase in HO-1 will help to inform future clinical trials of HA in vulnerable patients.

Dimethyl fumarate (DMF) is a drug which is licensed for the treatment of multiple sclerosis (MS) and psoriasis. Its exact mechanism of action is unknown but is thought to involve activation of an anti-inflammatory cascade of signals including Nuclear factor-erythroid factor 2-related factor 2 (Nrf2).

Experiments were carried out to examine the effect of DMF on the Nrf2 pathway and to explore the effect of DMF on cells from both mice and human blood. DMF had anti-inflammatory actions on mouse immune cells and initial results indicated that DMF could reduce the activation of harmful inflammatory cells. DMF did not protect mouse kidneys from injury in a study of DMF given prior to injury, but the high severity of the injury may have prevented any possible effect. Further work is needed to look into this in more detail.

It is hoped that HA has potential in preventing and treating AKI, and further clinical trials are ongoing. DMF offers potential as an attractive anti-inflammatory therapy in tablet form and requires further investigation.
Acknowledgements

I am absolutely indebted to my supervisors, Professors Jeremy Hughes and David Kluth, for their support, advice and inspiration both during this period of study and as colleagues in the renal unit in Edinburgh. Their example of being astute and caring clinicians, as well as talented clinician scientists, and inspirational teachers and leaders has been amazing throughout.

I am grateful to the patients who agreed to give up their time and have faith in us to carry out the clinical trial, and to the Cardiac Surgery team who welcomed me into their clinics and supported me to complete the trial recruitment.

The Phagocyte Group at the Centre for Inflammation Research were a very keen and supportive group of researchers – I was lucky to be able to call upon their wide range of skills for developing my knowledge throughout the training fellowship. Their advice, camaraderie and enthusiasm made this work achievable. Carolynn Cairns was an excellent teacher and great support through developing new skills in the laboratory. Jose Meseguer Ripolles and Emily Ostocke were students in the laboratory who took part in some of the experimental work. Gary Borthwick provided excellent technical assistance with the IRI model.

This work was made possible by the award of a Clinical Training Fellowship awarded jointly from the Medical Research Council (MRC) and Kidney Research UK (KRUK), for which I am very grateful.

This work was thoroughly enjoyable, stimulating, and challenging in equal measure. Much has happened in my life during the period of carrying out the initial research and in writing it up. I am so grateful to have Tom, Henry and Cara and I am grateful to them for their patience through all this. This is for them, for our family.
Table of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Aristolochic acid</td>
</tr>
<tr>
<td>AC</td>
<td>Apoptotic cell</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin II Receptor Blocker</td>
</tr>
<tr>
<td>AVR</td>
<td>Aortic valve replacement</td>
</tr>
<tr>
<td>BMDM</td>
<td>Bone marrow derived macrophage</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary Artery Bypass Graft</td>
</tr>
<tr>
<td>CDDO</td>
<td>2-cyano-3,12-dioxolane-1,9-dien-28-oic acid</td>
</tr>
<tr>
<td>CI</td>
<td>Chief Investigator</td>
</tr>
<tr>
<td>CIR</td>
<td>Centre for Inflammation Research</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CO</td>
<td>Carbon Monoxide</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic Obstructive Pulmonary Disease</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CPB</td>
<td>Cardiopulmonary bypass</td>
</tr>
<tr>
<td>CRF</td>
<td>Clinical Research Facility</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTIMP</td>
<td>Clinical trial of an Investigational Medicinal Product</td>
</tr>
<tr>
<td>DMC</td>
<td>Data Monitoring Committee</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethyl fumarate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EPR</td>
<td>Electronic patient record</td>
</tr>
<tr>
<td>FA</td>
<td>Folic acid</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
</tr>
<tr>
<td>HA</td>
<td>Heme arginate</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase 1</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IHD</td>
<td>Ischaemic heart disease</td>
</tr>
<tr>
<td>IL18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischaemia reperfusion injury</td>
</tr>
<tr>
<td>ISF</td>
<td>Investigator site file</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>KDIGO</td>
<td>Kidney Disease: Improving Global Outcomes</td>
</tr>
<tr>
<td>KIM1</td>
<td>Kidney injury molecule 1</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver function tests</td>
</tr>
<tr>
<td>LVEF</td>
<td>Left ventricular ejection fraction</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>MDM</td>
<td>Monocyte derived macrophage</td>
</tr>
<tr>
<td>MI</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>MMF</td>
<td>Monomethyl fumarate</td>
</tr>
<tr>
<td>NCEPOD</td>
<td>National Confidential Enquiry of Patient Outcome and Death</td>
</tr>
<tr>
<td>NF-KB</td>
<td>Nuclear factor kappa B</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase associated lipocalin</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid 2-related factor 2</td>
</tr>
<tr>
<td>NT-ProBNP</td>
<td>B-type Natriuretic Peptide</td>
</tr>
<tr>
<td>NYHA</td>
<td>New York Heart Association</td>
</tr>
<tr>
<td>OSA</td>
<td>Obstructive sleep apnoea</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>QMRI</td>
<td>Queen’s Medical Research Institute</td>
</tr>
<tr>
<td>RIE</td>
<td>Royal Infirmary of Edinburgh</td>
</tr>
<tr>
<td>RIFLE</td>
<td>Risk, Injury, Failure, Loss of Kidney Function, and End Stage Kidney Disease</td>
</tr>
<tr>
<td>RIPA</td>
<td>Radio-immunoprecipitation assay</td>
</tr>
<tr>
<td>ROC</td>
<td>Receiver Operating Characteristic</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious Adverse Event</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic Inflammatory Response Syndrome</td>
</tr>
<tr>
<td>TMG</td>
<td>Trial Management Group</td>
</tr>
<tr>
<td>TSC</td>
<td>Trial Steering Committee</td>
</tr>
<tr>
<td>U&amp;E</td>
<td>Urea and electrolytes</td>
</tr>
<tr>
<td>WCC</td>
<td>White cell count</td>
</tr>
</tbody>
</table>
Table of contents

1 INTRODUCTION .................................................................................................................. 17
  1.1 ACUTE KIDNEY INJURY (AKI) – THE CLINICAL PROBLEM ........................................ 17
     1.1.1 AKI incidence and demography ........................................................................ 17
     1.1.2 AKI leads to an impaired inflammatory response ............................................. 19
     1.1.3 The study of AKI in Cardiac Surgery Patients .................................................. 21
     1.1.4 AKI: a complex syndrome, how best to approach developing new treatments?  21
     1.1.5 Factors involved in pathophysiology of AKI ..................................................... 24
     1.1.6 Factors involved in Cardiac Surgery Associated AKI ...................................... 26
  1.2 THE POTENTIAL OF HEME ARGINATE AS A PROPHYLACTIC THERAPY IN AKI —
     TRANSLATION OF HO-1 PRECLINICAL STUDIES ................................................ 27
     1.2.1 The HO-1 pathway is an attractive target in the treatment of IRI ...................... 27
     1.2.2 Heme arginate is a drug licensed for human use .............................................. 29
     1.2.3 Heme arginate upregulates HO-1 .................................................................. 30
     1.2.4 Heme arginate is protective in IRI in vivo ....................................................... 31
     1.2.5 Heme arginate has been shown to be protective in human IRI ....................... 32
     1.2.6 The need for robust safety data ...................................................................... 33
     1.2.7 Complement ................................................................................................. 34
     1.2.8 Genotyping .................................................................................................. 34
  1.3 FURTHER POTENTIAL THERAPEUTIC TARGETS OF INTEREST: NRF2 PATHWAY/HO-1.... 34
     1.3.2 Current AKI management and Clinical trials .................................................. 50
  1.4 REPURPOSING LICENSED DRUGS........................................................................ 53

2 MATERIALS AND METHODS .......................................................................................... 55
  2.1 A RANDOMISED CLINICAL TRIAL OF HEME ARGINATE IN PATIENTS PLANNED FOR
     CARDIAC SURGERY ....................................................................................................... 55
     2.1.1 End points ........................................................................................................ 55
     2.1.2 Study Drug ....................................................................................................... 57
     2.1.3 Study funding ................................................................................................... 57
     2.1.4 Study Design ................................................................................................... 57
     Inclusion/exclusion criteria ......................................................................................... 63
     2.1.5 Consent .......................................................................................................... 64
     2.1.6 Participant case report form .......................................................................... 64
     2.1.7 Study samples ............................................................................................... 65
2.1.8 Physiological parameters ................................................................. 65
2.1.9 Electrocardiogram ........................................................................ 65
2.1.10 Collection and processing of samples ........................................... 65

2.2 Trial Management and Oversight ..................................................... 66
2.2.1 Trial Management Group ............................................................... 66
2.2.2 Trial Steering Committee ............................................................... 67
2.2.3 Data Monitoring Committee .......................................................... 67
2.2.4 Adverse Event Reporting ............................................................... 67
2.2.5 Laboratory ..................................................................................... 68
2.2.6 Peripheral blood mononuclear cells .............................................. 68
2.2.7 Analysis of samples – blinding to treatment group ....................... 70
2.2.8 Peripheral blood mononuclear cell HO-1 - Analysis ....................... 70
2.2.9 Serum ............................................................................................ 71
2.2.10 Urine collection ........................................................................... 72
2.2.11 Assessment of urinary biomarkers ............................................... 72
2.2.12 Genotyping .................................................................................. 72

2.3 Preparation of Murine Bone Marrow Derived Macrophages (BMDMs) ...................................................................................... 74

2.4 Human MDMs .................................................................................. 74

2.5 Dimethyl Fumarate In Vivo – Murine Experimental Work .................... 75
2.5.1 Dimethyl fumarate in vivo – dose effect on gene expression by qPCR and immunohistochemistry for HO-1 ........................................... 75
2.5.2 Dimethyl fumarate in vivo – dose effect on immunohistochemistry for HO-1 .......................................................... 75
2.5.3 Murine model of renal ischaemia reperfusion injury ........................ 75

2.6 qPCR: Preparation of Samples for Assessment .................................... 77
2.6.1 qPCR – conversion of RNA to cDNA ............................................. 77
2.6.2 qPCR ............................................................................................... 77

2.7 Assessment of Macrophage Phenotype: Phagocytosis Assay (Experiments done in conjunction with Emily Ostoke) .............................. 79
2.7.1 Preparation of apoptotic murine thymocytes .................................. 79
2.7.2 Preparation of BMDMs for phagocytosis assay .............................. 80
2.7.3 Quantification of phagocytosis of apoptotic cells and fluorescent beads by flow cytometry ............................................................ 81
2.7.4 Assessment of macrophage phenotype: nitric oxide measurement .... 82

2.8 Assessment of Cell toxicity by DMF treatment using the LDH Cytotoxicity assay ............................................................................. 82

2.9 Time course of NRF2 pathway by DMF in BMDMs ............................ 82
2.10 Dose response of DMF in BMDMs .................................................... 83
2.11 Immunohistochemistry of murine kidney – Assessment of HO-1 ........ 83
### 3 HACS CLINICAL TRIAL RESULTS – PRIMARY AND SECONDARY OUTCOME MEASURES

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>INTRODUCTION</td>
<td>85</td>
</tr>
<tr>
<td>3.2</td>
<td>PARTICIPANT DEMOGRAPHICS</td>
<td>85</td>
</tr>
<tr>
<td>3.3</td>
<td>HO-1 PROTEIN IN PBMCs</td>
<td>87</td>
</tr>
<tr>
<td>3.4</td>
<td>HO-1 mRNA IN PBMCs</td>
<td>88</td>
</tr>
<tr>
<td>3.5</td>
<td>HO-1 PROTEIN LEVEL IN SERUM</td>
<td>88</td>
</tr>
<tr>
<td>3.6</td>
<td>GENOTYPING</td>
<td>89</td>
</tr>
<tr>
<td>3.7</td>
<td>URINARY BIOMARKERS</td>
<td>90</td>
</tr>
<tr>
<td>3.8</td>
<td>URINARY HO-1</td>
<td>92</td>
</tr>
<tr>
<td>3.9</td>
<td>COMPLEMENT</td>
<td>94</td>
</tr>
<tr>
<td>3.10</td>
<td>SUMMARY OF RESULTS</td>
<td>95</td>
</tr>
</tbody>
</table>

### 4 CHAPTER 4 – SAFETY OF HEME ARGINATE IN HACS TRIAL

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2.1</td>
<td>Surveillance of adverse events</td>
<td>97</td>
</tr>
<tr>
<td>4.3</td>
<td>PROTOCOL DEVIATIONS AND VIOLATIONS</td>
<td>99</td>
</tr>
<tr>
<td>4.4</td>
<td>PHYSIOLOGICAL PARAMETERS ARE NOT SIGNIFICANTLY AFFECTED BY HEME ARGINATE IN ELDERLY PATIENTS PLANNED FOR CARDIAC SURGERY</td>
<td>101</td>
</tr>
<tr>
<td>4.5</td>
<td>MEASURES OF INFLAMMATION</td>
<td>104</td>
</tr>
<tr>
<td>4.6</td>
<td>SUMMARY OF KEY FINDINGS</td>
<td>105</td>
</tr>
</tbody>
</table>

### 5 CHAPTER 5 – DIMETHYL FUMARATE (DMF)

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5.1</td>
<td>DMF BMDM Phagocytosis – both bead and apoptotic cells by flow cytometry</td>
<td>115</td>
</tr>
<tr>
<td>5.5.2</td>
<td>Griess assay</td>
<td>116</td>
</tr>
<tr>
<td>5.6</td>
<td>DIMETHYL FUMARATE IN VIVO – DOSE TITRATION</td>
<td>118</td>
</tr>
<tr>
<td>5.6.1</td>
<td>Dimethyl fumarate in vivo – dose effect on gene expression by qPCR</td>
<td>119</td>
</tr>
<tr>
<td>5.6.2</td>
<td>Dimethyl fumarate in vivo – dose effect on immunohistochemistry for HO-1</td>
<td>120</td>
</tr>
</tbody>
</table>
5.7 DIMETHYL FUMARATE IN VIVO – ISCHAEMIA REPERFUSION INJURY .......................... 124

5.7.1 Experimental injury was too severe to allow for rescue from injury by pre
treatment with DMF – all sections showed severe injury ................................. 124

5.7.2 There was no significant difference in creatinine values for the two groups . 126

5.8 HUMAN MONOCYTE DERIVED MACROPHAGES ................................................. 127

5.9 DISCUSSION AND LIMITATIONS ......................................................................... 129

6 DISCUSSION AND FUTURE WORK ........................................................................ 131

6.1 RESULTS OF THE HACS CLINICAL TRIAL – WHERE THIS MAY LEAD .................. 131

6.2 SHOULD WE BE CONCERNED ABOUT DRUG SAFETY IN THIS PATIENT COHORT? .... 136

6.3 DIMETHYL FUMARATE – ANOTHER POTENTIAL CANDIDATE FOR REPURPOSING DRUGS?

7 REFERENCES ............................................................................................................. 140

8 APPENDIX – CLINICAL TRIAL DOCUMENTS ......................................................... 160

8.1 HACS CLINICAL TRIAL PROTOCOL ................................................................... 160
List of Figures

Figure 1: Causes of Acute Kidney Injury.................................................................19
Figure 2: Risk factors for Acute Kidney Injury in Cardiac Surgery Patients.........................21
Figure 3: Factors contributing to development of Cardiac Surgery Associated Acute Kidney Injury (CS-AKI) ..........................................................................................23
Figure 4: The heme oxygenase-1 pathway ........................................................................29
Figure 5: Fenton and Haber-Weiss Reactions ....................................................................34
Figure 6: Dimethyl fumarate chemical structure ...............................................................35
Figure 7: Nrf2 activation (adapted from a figure by Emily Ostocke). Oxidative stress causes Nrf2 to
dissociate from Keap1 in the cytoplasm and to migrate to the nucleus where it stimulates
transcription of antioxidant genes including heme oxygenase 1 (HMOX1), glutathione reductase
(GSR) and NAD(P)H quinone dehydrogenase 1 (NQO1) ......................................................35
Figure 8: Study Visits for the HACS trial ..........................................................................58
Figure 9: Consort diagram of HACS Clinical trial .............................................................60
Figure 10: Phagocytosis assay by flow cytometry ...............................................................81
Figure 11: HO-1 gene expression in PBMCs. (a) 1mg/kg dose of HA, (b) 3mg/kg dose of HA. Data shown
are median and interquartile range, Wilcoxon matched-pairs signed rank test (**p<0.01)........87
Figure 12: There was a significant increase in HO-1 protein expression in the serum in both doses (a)
1mg/kg dose of HA, (b) 3mg/kg dose of HA. Data shown are median and interquartile range,
Wilcoxon matched-pairs signed rank test. (**p<0.01) .........................................................89
Figure 13: Effect of genotype of HACS trial participants (a) Effect of HA on HO-1 protein in PBMCs
according to HO-1 genotype (b) Effect of HA on HO-1 gene expression compared to the ribosomal
housekeeping gene 18S. LL: homozygous for long GTn repeats (≥27); SS: homozygous for short GTn
repeats (<27); SL heterozygous ................................................................. Error! Bookmark not defined.
Figure 14: Urinary biomarkers (a) NGAL (b)KIM-1 (c)IL-18 adjusted to urinary creatinine concentration
prior to dose administration and at subsequent study visits on day 1 and day 7. There was no
significant difference in any of the urinary biomarkers for either dose (repeated measures one way
ANOVA)................................................................................................................ Error! Bookmark not defined.
Figure 15: Urinary HO-1 concentration adjusted to creatinine prior to HA dose administration and at
subsequent study visits on day 1 and day 7. ................................................. Error! Bookmark not defined.
Figure 16: Serum C1q of the first 6 HACS trial participants. There was no significant reduction in C1q
following infusion of HA (data shown are mean, standard deviation. Paired t test, p=0.3340) ....94
Figure 17: Physiological parameters following study drug administration. (A) Mean arterial pressure (b) pulse rate (c) tympanic temperature (d) oxygen saturations (e) respiratory rate ........................................... 102

Figure 18: There was no effect of HA dose on markers of inflammation and surrogate of HO-1 activity; (A) CRP (b) WCC (c) ferritin (d) Bilirubin. Repeated measures ANOVA, data shown are mean and standard deviation except for ferritin which is median and interquartile range as these data are not normally distributed .................................................................................................................. 105

Figure 19: BMDMs treated with increasing concentrations of DMF vs DMSO control; no outward appearance of cell injury ............................................................................................................. 110

Figure 20 - LDH assay of BMDMs treated with vehicle or DMF up to 50µM. After 24 hours the supernatant was harvested and an LDH assay performed. The LDH level from BMDMs deliberately lysed was deemed to be 100%. Only the 50µM concentration showed a significant increase in cell lysis. Data shown are mean and SEM from 3 individual experiments with 3 replicates in each. P=0.0277 one way ANOVA for vehicle vs 50µM DMF, all others not statistically significant. ..... 111

Figure 21: Increased transcription of key genes in the Nrf2 pathway was evident after treatment with DMF including (A) HMOX1 (Heme oxygenase-1) and (b) GSR (Glutathione reductase). ....................... 112

Figure 22: Time course of dimethyl fumarate treatment of bone marrow derived macrophages in vitro using known downstream genes from Nrf2. Pilot experiment with BMDMs from 3 mice in one experiment .......................................................................................................................... 114

Figure 23: Phagocytosis assay of apoptotic cells. ........................................................................ 117

Figure 24: Phagocytosis assay of fluorescent beads. There was no significant difference in phagocytosis of fluorescent beads by flow cytometry .......................................................................... 118

Figure 25: DMF may ameloriate the M1 phenotype. This was a pilot experiment (n=1) exploring the effect of DMF on the induction of the pro-inflammatory M1 macrophage phenotype by LPS/IFN-gamma. The measurement of nitric oxide concentration in the cell culture supernatant by Griess assay was used to assess the M1 phenotype. ................................................................. 118

Figure 26: Upregulation of Nrf2 pathway genes by DMF in vivo. N=3 per group. (A) HMOX1 (b) NQO1 (c) GSR. Only HMOX1 at a dose of 30 and 100mg/kg reached statistical significance when compared to vehicle (DMSO) by ordinary one-way ANOVA with multiple comparisons (p=0.0114) .............. 120

Figure 26: Immunohistochemistry staining for HO-1 showing the increase in staining by treatment with DMF and HA. A-D; Vehicle control (DMSO), E-H; DMF 10mg/kg, I-L; HA 3mg/kg, A,E,J; heart, B,F,J; liver, C, G, K; spleen, D,H,L kidney. Magnification x50. ................................................................. 122

Figure 28: Injury after ischaemia reperfusion was too severe to allow for potential benefit of DMF pre-treatment. A and B; Vehicle control (DMSO). C and D; DMF 10mg/kg pre-treatment (magnification x100) ........................................................................................................... 125

Figure 28: Creatinine values of mice treated with DMF prior to IRI and vehicle only (DMSO). There was no significant difference in creatinine between the DMSO and DMF treated groups .................................................. 126
Figure 29: Assessment of key genes downstream in NRF2 pathway in human MDMs following treatment with DMF in vitro. N=3 for HMOX1 (A), N=2 for NQO1(b) and GSR (c). There was significant upregulation in HMOX1 for both the 10 and 50μM doses compared to vehicle.
1 Introduction

1.1 Acute Kidney Injury (AKI) – the clinical problem

AKI incidence and demography

AKI is defined as a rapid decline in kidney function. In the UK, the importance of recognition of AKI in patients in hospital was brought to light by the National Confidential Enquiry of Patient Outcome and Death (NCEPOD) report of 2009 (1). This highlighted the need for better awareness of AKI in the clinical setting, as well as encouraging further research into potential novel therapies. Up until that point, there had been much heterogeneity in the definition of AKI used, with criteria such as the RIFLE criteria (Risk, Injury, Failure, Loss, End Stage Kidney Disease) (2) and Acute Kidney Injury Network (AKIN) (3), as well as other arbitrary definitions such as 50% rise in creatinine and the need for renal replacement therapy (RRT) given as end points in clinical trials and cohort studies. Consensus was reached with the publication of the Kidney Disease: Improving Global Outcomes (KDIGO) guideline in 2012 (4), which suggested that AKI should be defined as a creatinine rise of 26.5 µmol/l within 48 hours; or an increase in creatinine to at least 1.5 times baseline within 7 days, or a urine volume of less than 0.5ml/kg/h for at least 6 hours. Stages of AKI were defined (Table 1).

Table 1: AKI Stage by KDIGO criteria

<table>
<thead>
<tr>
<th>AKI Stage</th>
<th>Serum creatinine</th>
<th>Urine output</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5-1.9 times baseline OR ≥ 26µmol/l (≥0.3 mg/dl) increase</td>
<td>&lt;0.5ml/kg/h for 6-12 hours</td>
</tr>
<tr>
<td>2</td>
<td>2-2.9 times baseline</td>
<td>&lt;0.5ml/kg/h for ≥12 hours</td>
</tr>
<tr>
<td>3</td>
<td>3 times baseline OR Increase in serum creatinine to ≥353.6µmol/l (≥4.0mg/dl)</td>
<td>&lt;0.3ml/kg/h for ≥24 hours OR</td>
</tr>
</tbody>
</table>
The consensus of AKI definition including levels of severity has led to further clarity on reporting of incidence and has provided a uniform approach for research studies looking at both modifying risk of developing AKI and its subsequent management (5).

In the UK, AKI is relatively common, with approximately 150 AKI events per 10,000 population per year (1.5% of the population) (6). AKI is common in patients admitted to hospital, with the proportion of inpatients affected in the UK found to be up to 14% (7). AKI is associated with increased morbidity and mortality (8,9), which increases with its severity. Following on from this, it is also expensive for the NHS, accounting for approximately 1% of the annual NHS budget (7). In addition, the risk of future CKD and ESRD is increased with increasing severity(10) and repeated episodes (11) of AKI, significantly adding further morbidity given the long term increased cardiovascular risk associated with CKD (12).

There is currently no proven treatment for AKI, the mainstay of management is supportive care. There is a need for new treatments and there is particular appeal in repurposing licensed drugs which may be of benefit, given their proposed modes of action and established safety profiles (13). Studying new clinical uses for established drugs negates the need for expensive and time-consuming Phase I Studies and could streamline the implementation of bench to bedside translation without compromising patient safety.

The main issue with researching and developing potential new treatments for AKI is that it is a syndrome rather than a specific disease entity, with multiple different causes many of which can be present at once. For simplicity, these
causes are often divided up into pre-renal, renal and post-renal causes (Figure 1).

Figure 1: Causes of Acute Kidney Injury

Whatever the underlying cause, there are several proposed mechanisms by which AKI causes harm. Impairment of acid-base and electrolyte physiology, volume overload and the effects of uraemia are known to lead to complications directly (Reviewed in (14)). Sepsis is the leading cause of death in patients with AKI (15), which is of little surprise given that sepsis is a major risk factor in the development of AKI (16). However, AKI has other effects which continue to be investigated – there is a growing body of evidence that AKI is an independent risk factor for the development of sepsis and the morbidity and mortality that follows (17).

1.1.2 AKI leads to an impaired inflammatory response

AKI is thought to cause an impaired inflammatory response. Preclinical models of AKI show decreased clearance of cytokines, impaired neutrophil recruitment and impaired bacterial clearance (17). A clinical study of patients undergoing cardiac surgery found that AKI was an independent risk factor for
sepsis (18), and can have multiple effects in other organs via circulating factors and immune cells.

Sometimes it can be difficult to determine the sequence of events as AKI itself may lead to other organ dysfunction(19). The effect of AKI on the heart is an area of particular interest, and has (in the face of some controversy) been termed Cardiorenal Syndrome Type 3 (20) (Table 2). It is now understood that as well as fluid overload and a reduced serum pH causing impaired myocardial contractility and electrolyte abnormalities leading to dysrhythmias, there is an associated proinflammatory response which may contribute to cardiac inflammation and subsequent hypertrophy and atherosclerosis (reviewed in (19,20)). This ‘organ cross-talk” is hypothesised to involve circulating inflammatory mediators such as TNF-α and IL-6 – they have been found to be increased in patients with AKI and associated with worsening cardiac failure and mortality in human studies of AKI (21). This is of relevance in patients undergoing cardiac surgery who are at risk of AKI.

Table 2: Cardiorenal Syndrome (CRS) (adapted from (20) Classification of CRS based on the Consensus Conference of the Acute Dialysis Quality Initiative. ACS, Acute coronary syndrome; AHF, acute heart failure; AKI, acute kidney injury; CKD, chronic kidney disease; CRS, cardiorenal syndrome; HF, heart failure; and LVH, left ventricular hypertrophy.

<table>
<thead>
<tr>
<th>Phenotype of CRS</th>
<th>Nomenclature</th>
<th>Description</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>Acute CRS</td>
<td>HF resulting in AKI</td>
<td>ACS leading to cardiogenic shock and AKI, AHF resulting in AKI</td>
</tr>
<tr>
<td>Type 2</td>
<td>Chronic CRS</td>
<td>Chronic HF resulting in CKD</td>
<td>Chronic HF leading to renal hypoperfusion</td>
</tr>
<tr>
<td>Type 3</td>
<td>Acute renocardiac syndrome</td>
<td>AKI resulting in AHF</td>
<td>HF due to AKI (volume overload, uraemic metabolic disturbance, inflammatory surge)</td>
</tr>
<tr>
<td>Type 4</td>
<td>Chronic renocardiac syndrome</td>
<td>CKD resulting in chronic HF</td>
<td>LVH and HF from CKD-associated cardiomyopathy</td>
</tr>
<tr>
<td>Type 5</td>
<td>Secondary CRS</td>
<td>Systemic illness resulting in HF and kidney failure</td>
<td>Amyloidosis, sepsis, cirrhosis</td>
</tr>
</tbody>
</table>
1.1.3 The study of AKI in Cardiac Surgery Patients

Cardiac surgical patients are often recruited into clinical trials of AKI because by nature of their cardiac condition they have many of the main risk factors for its development (including Diabetes Mellitus, CKD, older age, peripheral vascular disease, medications such as ACE inhibitors). The nature of the operation and factors such as cardiogenic shock, haemolysis and blood transfusions can all lead to additive risk (Figure 2). These risk factors together with a planned operation with a known ischaemic hit to the kidneys, offers an environment where patients can be stratified by risk and new treatments may be tested.

Figure 2: Risk factors for Acute Kidney Injury in Cardiac Surgery Patients

<table>
<thead>
<tr>
<th>Patient factors</th>
<th>Perioperative factors</th>
<th>Postoperative risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Older age</td>
<td>Duration of cardiopulmonary bypass</td>
<td>Sepsis</td>
</tr>
<tr>
<td>Female gender</td>
<td>Cross clamp time</td>
<td>Drugs</td>
</tr>
<tr>
<td>Comorbidity</td>
<td>Perioperative hypotension</td>
<td>Cardiogenic shock</td>
</tr>
<tr>
<td>• Diabetes mellitus</td>
<td>Blood transfusion</td>
<td>Hypotension</td>
</tr>
<tr>
<td>• Hypertension</td>
<td>Type of procedure</td>
<td>Hypovolaemia</td>
</tr>
<tr>
<td>• Chronic Kidney Disease</td>
<td>Emergency surgery</td>
<td>Atheroembolism</td>
</tr>
<tr>
<td>• COPD</td>
<td>Haemolysis</td>
<td>Intense</td>
</tr>
<tr>
<td>• Cardiac failure</td>
<td>Haemodilution</td>
<td>vasoconstriction</td>
</tr>
<tr>
<td>• Ischaemic heart disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Peripheral vascular disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous cardiac surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Need for IABP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAAS inhibition</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.1.4 AKI: a complex syndrome, how best to approach developing new treatments?
AKI is a clinical syndrome, with many different causes each requiring different treatments. As mentioned these are often divided into pre-renal, renal and post-renal causes (Figure 1), but in reality, AKI is often multifactorial. There is a huge amount of heterogeneity in patients who experience AKI, which includes their susceptibility to injury as well as the different causes of such injury which might include sepsis, toxins, hypovolaemia or other more specific conditions such as myeloma or glomerulonephritis.

Much of the understanding of the pathophysiology of AKI in general comes from animal models, which to an extent limits the translation of treatments developed in the laboratory given the multifactorial complexity of most cases of human AKI. Animal models tend to involve very predictable, simple insults such as toxic injury eg cisplatin (24) or folic acid (25) in rodent models of AKI. Experiments are often carried out in young male mice of a particular genetic strain with normal renal function, whereas the reality of human AKI is far more complex with older individuals of both sexes with multiple risk factors usually being affected, even if there is an additional predictable insult such as ischaemia reperfusion injury during cardiac or vascular surgery. This may explain why there has been some success in ameliorating experimental AKI but these treatments have shown limited promise in randomised controlled trials (26).

Focusing in on AKI associated with cardiac surgery still requires consideration of heterogeneity within this patient group and in aetiology of AKI; this can be mitigated to a degree by the ability to select patients for study prior to their elective surgery. This allows risk stratification for AKI and for its study in a situation where patients have a planned ischaemic insult to their kidneys.
The major contributing factors to the development of AKI in patients undergoing cardiac surgery includes renal ischaemia-reperfusion injury, haemolysis, inflammation and toxins (Figure 3).

There may in some cases be sepsis or hypovolaemia due to haemorrhage or indeed hypoperfusion due to pump failure (cardiogenic shock)(22)

Figure 3 Factors contributing to development of Cardiac Surgery Associated Acute Kidney Injury (CS-AKI)

### 1.1.4.1 Understanding pathogenesis of AKI: Insights from preclinical models

Ischaemia reperfusion injury is caused by the restriction of an organ’s blood supply followed by its restoration. There is cellular injury caused by the restriction of oxygen (hypoxia) and nutrients, which worsens during the reperfusion phase due to the presence of free radicals and oxidative stress. Hypoxia causes harm by impairing ATP synthesis by mitochondrial oxidative
phosphorylation and therefore restricts ATP-dependent processes. This leads to a shift to anaerobic metabolism which causes a decrease in intracellular pH.

1.1.4.1.1 Murine models of acute kidney injury

Given its good blood supply, the kidney is susceptible to insults from toxins. Several different animal models of toxic injury are in widespread use for the study of AKI such as folic acid (FA) (25) cisplatin (27) and aristolochic acid (AA) (28). FA crystallises in tubular cells at high doses. This results in direct epithelial cell injury with intratubular obstruction by crystals, inducing tubular cell apoptosis as well as proliferation, recruitment of inflammatory cells and subsequently fibrosis (29). Cisplatin is directly toxic to renal epithelial cells and its nephrotoxicity is associated with inflammation and oxidative stress (27). Although the mechanisms of AA induced renal injury are not fully understood, apoptosis, mitochondrial damage and subsequent oxidative stress are also believed to play key roles (28).

1.1.5 Factors involved in pathophysiology of AKI

1.1.5.1 Microcirculation dysfunction

The kidneys receive 20% of cardiac output and their blood supply is autoregulated. This is achieved by tubuloglomerular feedback, where an increase in sodium concentration in the tubules leads to changes in the vascular tone of the afferent arteriole. This is carried out by several mechanisms including activation of the sympathetic nervous system, release of vasoconstrictors such as endothelin, angiotensin II and adenosine, and vasodilators such as nitric oxide (NO) and prostaglandin E2 (30). Renal oxygenation therefore depends on renal blood flow, glomerular filtration rate and sodium delivery to the tubules. In sustained periods of hypoperfusion,
experimental models have shown that these protective mechanisms can be lost (31) leading to injury. The relationship between renal perfusion and the development of AKI is therefore a complex one; the renal microcirculation is as important as perfusion pressure. In AKI associated with cardiac surgery, cardiopulmonary bypass is associated with low-flow non pulsatile perfusion with haemodilution. There may be haemorrhage during the operation or afterwards which may contribute to renal hypoperfusion. In addition, in the post operative period, low cardiac output in the recovering heart can reduce renal perfusion compounding the period of ischaemia (32). In sepsis, even in the absence of renal hypoperfusion there is shunting of blood which reduces glomerular filtration rate and endothelial dysfunction ensues (16). Afferent arteriolar vasoconstriction due to tubulo-glomerular feedback is thought to be secondary to tubular dysfunction rather than its cause (33)

1.1.5.2 Endothelial dysfunction

Endothelial cells maintain the natural barrier between the intravascular and extravascular spaces (34). They produce vasodilators such as NO and prostacyclin to regulate the tone of arterioles and venules, helping to manage glomerular filtration rate. When intact they inhibit platelet aggregation maintaining laminar blood flow. The glycocalyx is a network of glycoproteins that lines the extracellular surface of the endothelial cells and maintains the endothelial barrier’s integrity. After ischaemic injury, the lack of oxygen means there is less ATP synthesis by mitochondrial oxidative phosphorylation and therefore restriction of ATP-dependent processes (35). The endothelium undergoes structural changes with endothelial cell apoptosis and cell death resulting in loss of cell-cell contact and disruption of the glycocalyx leading to increased vascular permeability. The injured cells upregulate expression of adhesion molecules leading to increased adhesion of leukocytes and leukocyte transmigration to the interstitium. The injured tubular and endothelial
cells continue to release pro-inflammatory cytokines which propagate the inflammatory process.

1.1.5.3 Microvascular thrombi
In steady state, endothelial cells inhibit blood coagulation via protein C and thrombomodulin. During inflammation, these natural anticoagulants are degraded or their production is interrupted. In addition, the damaged endothelial cells apoptose and cytokines are released leading to the formation of micro-thrombi which causes capillary occlusion.

1.1.5.4 Inflammation and oxidative stress
Endothelial or tubular cell injury and cell death leads to production of reactive oxygen species (ROS) (36) and subsequent upregulation of pro-inflammatory transcription factors. Cytokines and chemokines cause activation of resident inflammatory cells and the recruitment and subsequent invasion of monocytes, neutrophils and dendritic cells. These cells are thought to propagate the injury process. Other cells are likely protective such as regulatory T cells. The role of macrophages in inflammation is not fully understood; there is a spectrum of activation from pro-inflammatory (traditionally called M1) macrophages to anti-inflammatory/pro-repair (traditionally called M2) macrophages (37). M1 macrophages are thought to contribute to inflammation in the early phase but M2 macrophages have anti-inflammatory functions and help to facilitate renal recovery and repair (38). Treatments to alter macrophage phenotype from M1 to predominantly M2 may improve longer term kidney outcomes by promoting repair rather than ongoing inflammation (39).

1.1.6 Factors involved in Cardiac Surgery Associated AKI

1.1.6.1 Haemolysis
The development of intravascular haemolysis is a consequence of CPB and is characterised by a rise in free haemoglobin. This is a likely cause of direct toxicity to the renal tubules, as well as increased intravascular NO
consumption leading to increased vasoconstriction and reduced renal perfusion(40). Increased free circulating labile catalytic iron from both haemoglobin and injured cells due to oxidative stress can damage tubular epithelial cells, leading to impaired cell proliferation, increased lipid peroxidation and protein oxidation(40). Complement activation via the alternative pathway is another mechanism by which CPB can have an additive effect on AKI(41).

1.1.6.2 Drugs and mechanical insults

Patients having cardiac surgery are often given medications which may affect their renal function such as antibiotics (including aminoglycosides) and non steroidal inflammatory agents (NSAIDs) both before and after their procedures. Angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs) are commonly prescribed drugs for cardiac failure and can reduce GFR by causing efferent arteriolar vasodilatation (42). Cholesterol emboli can occur during and after cardiac surgery due to disruption of atherosclerotic plaques. This can cause AKI due to direct occlusion of branches of the renal artery, arterioles and glomerular capillaries, as well as subsequent complement activation (43).

1.2 The potential of Heme Arginate as a prophylactic therapy in AKI – translation of HO-1 preclinical studies

1.2.1 The HO-1 pathway is an attractive target in the treatment of IRI

Free heme molecules are released by injured cells in ischaemia reperfusion injury. This sets off a cascade of release of free radicals, which leads to oxidative stress and further cell injury and recruitment of inflammatory cells (reviewed in (35)). The heme oxygenase enzymes play a key role in the response to cell injury by the breakdown of free heme to free iron (Fe2+) and carbon monoxide (CO) and bilirubin. CO and bilirubin have anti-apoptotic and
immunomodulatory properties (44) which adds to the appeal of HO-1 as a target for IRI treatments.

Heme oxygenase-2 (HO-2) is a constitutive enzyme, whereas HO-1 is readily inducible.
Figure 4). HO-1 is a heat shock protein (HSP) that is expressed in many cell types including endothelial, epithelial, and mononuclear cells and is induced by a variety of stimuli including oxidative stress (45). This induction is caused by transcriptional modulation of the HO-1 gene.

There are case reports of human HO-1 deficiency, where the patients had widespread inflammation including renal tubulo-interstitial nephritis and died at a young age (46–48). Impaired HO-1 production leads to progressive monocyte dysfunction, unregulated macrophage activation and endothelial cell dysfunction, with subsequent systemic inflammatory response syndrome (SIRS) (48).

A drug which can induce the enzyme HO-1 could therefore have effects on both the immune system and the microcirculation, which makes this an appealing area of study in the context of ischaemia reperfusion injury.
1.2.2 Heme arginate is a drug licensed for human use

Heme arginate has been in clinical use for over 30 years as a treatment for acute intermittent porphyria (49). The porphyrias are a group of rare inherited diseases which in the main are caused by enzyme deficiencies in the haem biosynthetic pathway (50). Porphyria tends to cause acute episodes, or ‘attacks’, which present in a variety of different ways including acute abdominal pain, skin lesions, hypertension and behavioural disturbance. The administration of heme inhibits the formation of harmful metabolites of porphyrin synthesis and corrects the haem deficiency by inhibiting the induction of β-aminolaevulinic acid synthase(51). Heme arginate is a human derived blood product; a stable haem compound of human hemin (which is ferriporphyrin with iron in the ferric state, Fe^{3+}(52)). This concentrated hemin solution is stabilised by the formation of a complex with arginine, allowing it to be more stable at physiological pH. Its chemical formula is C_{40}H_{46}FeN_{6}O_{6}^{2+} and has a molecular weight of 792.7 ((53):}
Figure 5). 

Figure 5: Heme arginate Chemical Structure. Heme consists of a tetrapyrrole ring with an iron (Fe) atom bound in the centre, coordinated to the pyrrole rings. The addition of arginine increases stability in solution.

The dose for this condition is 3mg/kg (up to 250mg) daily for 5 days. Heme arginate is a human derived blood product. Although relatively expensive at £400 per 250mg vial (at the time of study in 2016), it has a good safety profile.

1.2.3 Heme arginate upregulates HO-1

Heme arginate (HA) has been shown to upregulate HO-1 \textit{in vitro} (54,55). Other heme preparations have been too expensive to prepare, or were
lacking in suitable stability at physiological pH to be used in clinical practice. The development of HA allowed the design and execution of pre clinical studies of HA in models of IRI.

1.2.4 Heme arginate is protective in IRI in vivo

Pre-clinical studies in a murine model of IRI carried out by the Hughes group have established that 12-month-old aged mice, like aged humans, develop more severe AKI than young mice following renal IRI (56) despite the absence of CKD. The aged mice exhibit minimal HO-1 upregulation in the renal medulla following renal IRI in comparison to young mice that strongly express HO-1 in both interstitial macrophages (Mϕ) and renal tubules. Importantly, when considering HO-1 as a potential therapeutic target, treatment of aged mice with a single dose of HA (30mg/kg) 24 hours prior to IRI conferred strong protection from AKI, with a significant reduction in both the serum creatinine and acute tubular necrosis (ATN) scoring on sections from the outer medulla 24 hours post IRI.

Transgenic CD11b-DTR mice were used to examine the role of monocyte/Mϕ in the protection afforded by HA, as diphtheria toxin (DT) administration to these mice selectively ablates monocytes and renal Mϕ (57–59). Although monocyte/Mϕ ablation in CD11b-DTR mice did not confer protection from renal IRI (60), DT treatment completely abolished HA-mediated protection (58) suggesting that monocyte/Mϕ HO-1 expression is key to HA-mediated renoprotection in vivo. This provided the pre clinical data leading to the
consideration of HA as a potential prophylactic treatment for patients at risk of renal IRI.

1.2.5 Heme arginate has been shown to be protective in human IRI

The ability of HA to induce HO-1 in humans has been investigated in preliminary Phase I clinical trials. A dose escalation Phase I study in healthy young subjects examined the effect of HA on HO-1 mRNA levels and protein expression in peripheral blood monocytes at doses of 0.3mg/kg, 1mg/kg and 3mg/kg (61). This showed a dose dependent response, with the HA dose of 1mg/kg increasing HO-1 protein expression by approximately 2-fold, while 3mg/kg led to a 5-fold increase. HO-1 gene expression (mRNA) was increased by over 1.5-fold with 1mg/kg, and approximately 2-fold with 3mg/kg. In another Phase I study in healthy male subjects, a single dose of 1mg/kg HA improved reperfusion patterns measured by blood oxygen dependent functional MRI following calf muscle ischaemia (62). In both studies the drug was well tolerated and there were no significant adverse events to raise safety concerns.

More recently, in a trial carried out after HACS, HO-1 has been shown to be upregulated by HA in the human heart and in the PBMCs of patients undergoing cardiac surgery (63). This trial aimed to inform future studies of HA as a prophylactic treatment in the prevention of IRI in the myocardium during cardiac surgery, and importantly showed that HA was safe in this comorbid cohort.
In nephrology, the HOT study demonstrated that a dose of HA caused HO-1 to be upregulated in PBMCs of patients undergoing renal transplant and in their transplant kidney biopsy tissue at day 5 post operatively(64). Renal transplant is another example of renal IRI – a predictable interruption in renal blood supply and subsequent reperfusion at the time of transplant, and these findings provide further evidence that HA could be a potential prophylactic treatment in renal IRI. A Phase III trial, HOT2, which aimed to determine if HA could reduce delayed graft function (a consequence of IRI) in patients undergoing renal transplant, was underway but has been terminated due to the Coronavirus pandemic and the resulting difficulties in trial recruitment (Clinicaltrials.gov NCT03646344).

1.2.6 The need for robust safety data
Catalytic iron is not bound to protein or transferrin and is capable of causing cellular damage via the free radicals generated by the Fenton and Haber-Weiss reactions (Figure 6). Release of intracellular iron stores at the time of tissue injury may therefore cause further inflammation. Free iron generated by heme oxygenase 1 is generally sequestered by iron binding proteins such as ferritin (65). The dose of heme arginate in a clinical trial could in theory increase the amount of free iron therefore giving potential for unwarranted side effects. This reinforces the need for a clinical trial of HA in this cohort of patients who are clinically vulnerable.
Some studies have shown that Heme interacts with C1q and inhibits classical pathway complement deposition (66). C1q activity increases with age (67), so there is a hypothesis that one way in which HA could have its protective effect in IRI is by depleting C1q levels or activity.

1.2.7 Complement

A GTₙ dinucleotide repeat polymorphism has been identified in the proximal promoter region of the HO-1 gene(68). The polymorphism is highly polymorphic and modulates gene transcription. Longer GTₙ repeats are associated with lower transcriptional activity of the HO-1 promoter and with susceptibility to different diseases such as coronary artery disease and emphysema(69). The GTₙ repeats show a bimodal distribution with peaks at 22-23 and 27-30 repeats. Alleles are usually classed as either Long (L) or Short (S) with GTₙ repeats divided as being ≥25 or ≥27, <25 or 27 respectively.

The significance of the number of GT repeats for individuals at risk of AKI is yet to be determined. The HACS trial is not powered to detect a difference, but data on genotype were obtained for further information.

1.3 Further potential therapeutic targets of interest: Nrf2 pathway/HO-1

Exploring inflammation as a potential target for the prevention and treatment of AKI, Dimethyl fumarate (C₆H₈O₄, Figure 7) is a drug which is licensed for
the treatment of multiple sclerosis and psoriasis (70). Its mechanism of action is not fully understood but it is known to activate the Nrf2 pathway, which is upregulated in response to oxidative stress (Figure 8). Upon oral administration, dimethyl fumarate (DMF) is converted to its active metabolite monomethyl fumarate (MMF) and MMF binds to Nrf2.

Figure 7: Dimethyl fumarate chemical structure

Figure 8: Nrf2 activation (adapted from a figure by Emily Ostocke). Oxidative stress causes Nrf2 to dissociate from Keap1 in the cytoplasm and to migrate to the nucleus where it stimulates transcription of antioxidant genes including heme oxygenase 1 (HMOX1), glutathione reductase (GSR) and NAD(P)H quinone dehydrogenase 1 (NQO1)
DMF has been shown to be protective in a murine model of cardiac ischaemia reperfusion injury at a dose of 15mg/kg by oral gavage twice daily for 5 days. The effect was lost in Nrf-2 knockout mice, indicating that the role of this pathway was key(71). Similar findings of cardioprotection were shown in a model of cardiac ischaemia in the rat, where it was shown to reduce NF-κB(72). Similarly, in a rat model of hepatic IRI, DMF given twice daily by gavage ameliorated ischaemic injury and was associated with lower levels of key inflammatory mediators such as NF-κB as well as higher levels of antioxidant enzymes such as catalase and Cyclooxygenase-2 (COX-2) although there was no significant difference in HO-1 or NQO-1 protein expression in the liver by western blot (73).

In vitro, DMF has been shown to protect neuronal cells from oxidative glutamate toxicity in a model of endogenous oxidative stress (74). This effect was concentration dependent and was associated with increased Nrf-2 protein concentrations and nuclear localisation. There was no difference in NF-κB translocation in these studies. In a murine model of intracranial haemorrhage, sulforaphane, an activator of the Nrf2 pathway, induced the scavenger receptor CD36 expression on microglia and improved haematoma clearance in rats and mice, but not in Nrf-2 knockout mice(75). The potential effect of DMF on the phenotype of macrophages is of great interest.

In the kidney, the upregulation of Nrf2 has been shown to mitigate cisplatin induced nephrotoxicity in a murine model of AKI by upregulating antioxidant enzymes(76). The absence of Nrf2 (in Nrf2 knockout mice) led to more pronounced renal injury following cisplatin exposure. Further elegant studies in a murine model of IRI have shown that Nrf2 activation increased gene expression of antioxidant enzymes and ameliorated the injury in the outer medulla and the progression of cortical tubular damage (77). Short term treatment with the Nrf2 inducer 2-cyano-3,12-dioxolane-1,9-
dien-28-oic acid (CDDO) at the time of injury reduced the severity of tubular damage.

There is also evidence that DMF has anti-fibrotic effects in the kidney, having been shown to reduce fibrosis in the murine unilateral ureteric obstruction model (78). This was not shown to be mediated by the antioxidant Nrf2 target genes such as HO-1, NQO1 and glutathione S transferase however, indicating that there are likely to be other additional effects. It was found that DMF increased nuclear levels of Nrf2, and inhibited TGF-β/Smad3 signalling.

Given that DMF is an oral tablet rather than an intravenous infusion, it is appealing as a potential prophylactic treatment for the prevention of AKI in susceptible individuals undergoing cardiac surgery. These studies in vitro and in vivo aimed to understand better the potential role of DMF in these patients prior to Phase II clinical trials. This was through studying the effect of DMF on the Nrf2 pathway in vitro, and by carrying out in vivo experiments to understand its effects in a murine model of renal ischaemia reperfusion injury.

DMF has been shown to ameliorate obstructive sleep apnoea (OSA), another condition with inflammatory pathogenesis, in a randomised controlled trial (79). The trial was informed by retrospective data from MS patients showing an association between DMF treatment and improved OSA symptoms(80). There are currently no published clinical trials of DMF in humans testing the hypothesis that it might ameliorate ischaemia reperfusion injury. The first step may be to look retrospectively at larger patient cohorts prescribed DMF such as those with MS or psoriasis, identifying whether or not there is a signal of reduced incidence or duration of AKI.

1.3.1.1 Clinical: Insights from clinical studies

Patients undergoing cardiac surgery often have multiple risk factors for AKI including advanced age, diabetes mellitus, CKD, and prescribed medications such as angiotensin converting enzyme (ACE) inhibitors (32). There are
different surgical risk scores in use for informing risk of morbidity and mortality perioperatively. These aid clinical decision making and inform discussions with patients. Prior to undertaking the HACS study, a retrospective analysis of the data from over 4000 NHS Lothian cardiac surgery patients demonstrated that as with other similar studies (81,82), any post-operative AKI was associated with increased mortality and increased length of stay in hospital (83). The overall incidence of AKI was 12.8% over the 5 years studied, with 1.4% of all patients requiring renal replacement therapy.

On multivariate analysis, the additive EuroSCORE-1 (84) was the best predictor of incidence of AKI in patients undergoing cardiac surgery (83) with each point equating to an 8% increase in relative risk of AKI. The score includes patient demographics such as age and sex, as well as comorbidity such as chronic obstructive pulmonary disease (COPD) and vascular disease. The addition of cardiac related factors such as left ventricular dysfunction and recent myocardial infarct are also incorporated, along with operation related factors such as urgency of procedure. The utility of EuroSCORE-1 alone as a predictor of AKI post operatively was unlikely to be of use at this stage however, as on receiver operating characteristic (ROC) analysis of the EuroSCORE-1 as a predictor for post-operative AKI, the area under the curve is 0.634 (95% CI 0.609-0.658, p<0.001) while the Lemeshow test value was 0.08. The hunt for a means to risk stratify patients prior to selection into clinical trials for AKI is ongoing.

Cardiac surgery offers the opportunity to study the effect of an ischaemic insult on the kidneys in a planned manner. There is complexity involved, in that there are other factors at play in the cumulation of risk of AKI (pre operative known patient and surgical risk factors, as well as unexpected complications such as infection and technical difficulties). Despite this, there is an appeal to studying the effect of pharmacological (and non-pharmacological) interventions in this group to determine the effect on AKI rates. Many have led on from promising pre-clinical studies. Clinical trials registered on www.clinicaltrials.gov with the
aim of studying the prevention or treatment of AKI in cardiac surgery are summarised in Table 3 below.
Table 3: Clinical trials of Investigational Medical Products in the prevention/treatment of AKI in Cardiac Surgery patients.

Abbreviations: NYHA; New York heart Association, LVEF; Left Ventricular Ejection Fraction, COPD; Chronic Obstructive Pulmonary Disease, IV; intravenous, ACE; Angiotensin Converting Enzyme, ARB; Angiotensin II Receptor Blocker, CABG; Coronary Artery Bypass Graft, CKD; Chronic Kidney Disease, NT-ProBNP; B-type natriuretic peptide, CRP; C-reactive protein, CK; creatine kinase, LDH; lactate dehydrogenase, CPB; Cardiopulmonary bypass, NO; nitric oxide, NGAL; Neutrophil gelatinase-associated lipocalin

<table>
<thead>
<tr>
<th>Agent</th>
<th>Trial details</th>
<th>Participants</th>
<th>Phase</th>
<th>Dosing regime</th>
<th>N</th>
<th>Outcome measures</th>
<th>Results</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>THR-184 (Bone morphogenic protein (BMP-7) receptor agonist)</td>
<td>NCT01830920 (May 2013-Sep 2015) 42 centres in USA and Canada</td>
<td>Elective patients for cardiac surgery requiring cardiopulmonary bypass age 18-85 At least one risk factor for AKI: • eGFR&lt;60 • age ≥75 • higher risk surgery • NYHA 3 or 4 • LVEF ≤35% • Diabetes with insulin or proteinuria • Hb&lt; 10mg/dl</td>
<td>2b</td>
<td>1 IV dose pre op (3 varied dosages) and 3 doses post op</td>
<td>452</td>
<td>Primary: • AKI by KDIGO criteria in 7 days post op Secondary: • Severity of AKI (7 days) • Duration of AKI • Composite of death, dialysis, sustained impaired renal function by day 30 Maximum change in creatinine from baseline</td>
<td>No significant difference</td>
<td>Himmelfarb 2018 (85)</td>
</tr>
<tr>
<td>ABT mela melanocyte-stimulating hormone analog</td>
<td>NCT01777165 (Feb 2013-Mar 2014) 41 centres in USA and Denmark</td>
<td>Elective patients for cardiac surgery age ≥18 requiring cardiopulmonary bypass, with eGFR ≥16 and ≤59</td>
<td>2b</td>
<td>6 IV doses of either placebo or 3 different doses of the study drug, given pre and perioperatively, up to 48 hours post op</td>
<td>231</td>
<td>Primary: • AKI by AKIN criteria in 7 days post op Secondary • Composite end point: death, RRT, ≥25% reduction in GFR (to day 90)</td>
<td>No significant difference</td>
<td>McCullogh 2016 (86)</td>
</tr>
<tr>
<td>Study</td>
<td>Identifier</td>
<td>Study Design</td>
<td>Study Population</td>
<td>Intervention</td>
<td>Outcomes</td>
<td>Notes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------</td>
<td>---------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Levosimendan        | NCT02531724      | Single centre study in Sweden         | Patients aged >18 with AKI by KDIGO criteria within 48 hours of elective cardiac surgery requiring cardiopulmonary bypass | Infusion of levosimendan or placebo (bolus 12µg/kg followed by 0.1µg/kg/min infusion over 5 hours) | Primary: Renal blood flow  
Secondary: Glomerular filtration Rate  
Serum creatinine over 4 days post op  
Compared to the placebo group, levosimendan increased RBF ($p=0.011$) and decreased RVR ($p=0.043$). No difference in GFR | Tholen 2021 (87)                                                                                                                                 |
| QPI-1002 (Teprasiran) | NCT02610283     | Multiple centres study in USA, Canada, and Germany | Patients aged ≥45 undergoing elective cardiac surgery. Must have at least one pre-specified risk factor for AKI:  
- Age >70 years  
- eGFR<60ml/min/1.73m²  
- diabetes  
- proteinurina  
- history of heart failure | Single dose 10mg/kg teprasiran vs placebo | Primary: AKI by AKIN criteria (up to day 5)  
Secondary: Composite end point: (death, RRT, ≥25% reduction in eGFR at 90 days)  
AKI incidence was 37% for teprasiran vs 50% for placebo; 12.8% risk reduction, $P=0.02$; odds ratio, 0.58 (95% CI 0.37–0.92). 2.5% of teprasiran vs 6.7% of placebo had grade 3 AKI; 7% teprasiran vs 13% placebo had AKI lasting for 5 days | Thielmann 2021 (88)                                                                                                                                 |
| Atorvastatin        | NCT00791648      | Single centre in USA                  | Patients with eGFR<60ml/min/1.73m² (both statin naive and patients already on statins were studied) | Atorvastatin vs placebo (both statin naïve and patients already on statins were studied) | Primary: AKI by AKIN criteria  
Secondary: Maximum creatinine increase from baseline to 48 hours post surgery  
Incidence and duration of delirium  
Myocardial injury  
No significant difference in AKI, study stopped early due to futility | Billings 2016 (89)                                                                                                                                 |
<table>
<thead>
<tr>
<th>Study Title</th>
<th>NCT Number</th>
<th>Study Details</th>
<th>Primary:</th>
<th>Secondary:</th>
<th>Result:</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Patients aged 18-80 undergoing elective cardiac surgery involving cardiopulmonary bypass</td>
<td>Secondary: Oliguria (up to 12 hours post op)</td>
<td>Incidence of AKI (defined by elevated NGAL at 3 hours post cardiopulmonary bypass)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Three 5-minute intervals of leg ischaemia induced by tourniquet prior to initiation of cardiopulmonary bypass</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remote Ischaemic Preconditioning</td>
<td>NCT03205410 (Jan 2014-Dec 2014)</td>
<td>Single centre in Poland</td>
<td>Incidence of AKI by AKIN criteria</td>
<td>Secondary: Length of hospital stay, Length of ICU stay, Time of mechanical ventilation, Occurrence of atrial fibrillation, Days of RRT Death (up to 2 years)</td>
<td>Fewer patients in RIPC group developed AKI (29% vs 93%, p=0.003) The median NGAL at 3 hours post surgery was lower in the RIPC group (124 vs 176.7, p=0.0003)</td>
<td>Stokfisz 2020 (91)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patients aged ≥18 undergoing cardiac surgery off-pump cardiopulmonary bypass</td>
<td>Primary: Incidence of AKI by AKIN criteria, NGAL level 3 hours post surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Three 5-minute intervals of ischaemia induced by inflating upper arm blood pressure cuff to 200mmHg</td>
<td>Secondary: Length of hospital stay, Length of ICU stay, Time of mechanical ventilation, Occurrence of atrial fibrillation, Days of RRT Death (up to 2 years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium Bicarbonate</td>
<td>NCT00921518 (Jan 2009-Jan 2012)</td>
<td>Single centre study in USA</td>
<td>Incidence of AKI by AKIN criteria (up to day 5 post op)</td>
<td>No significant difference</td>
<td></td>
<td>Kristeller 2013 (92)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patients aged ≥18 undergoing cardiac surgery by cardiopulmonary bypass with eGFR ≤60 pre operatively</td>
<td>Primary: AKI by AKIN criteria (up to day 5 post op)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Sodium bicarbonate infusion pre- and peri-operatively (sodium chloride infusion as placebo)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>92</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minocycline</td>
<td>NCT00556491 (Dec 2007-Dec 2013)</td>
<td>2 centres in USA</td>
<td>Development of AKI by AKIN criteria within 5 days post op</td>
<td>No significant difference</td>
<td></td>
<td>Golestaneh 2014 (93)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Patients aged &gt;18 planned for cardiac surgery with cardiopulmonary bypass</td>
<td>Primary: Development of AKI by AKIN criteria within 5 days post op</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 Minocycline or placebo orally for up to 7 days pre operatively</td>
<td>Secondary: Post operative length of stay, Time on ventilation, Infections, Stroke, Re-operation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>38</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Trial ID</td>
<td>Country</td>
<td>Age</td>
<td>Inclusion Criteria</td>
<td>Intervention</td>
<td>Sample Size</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>---------</td>
<td>-----</td>
<td>-------------------</td>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>NCT01909739</td>
<td>South Korea</td>
<td>≥20</td>
<td>Undergoing elective valvular heart surgery</td>
<td>Atorvastatin or placebo orally from the day prior to the operation and for 4 days afterwards</td>
<td>198</td>
</tr>
<tr>
<td>Erythropoetin</td>
<td>NCT01758861</td>
<td>South Korea</td>
<td>≥20</td>
<td>Undergoing elective complex valvular heart surgery with ≥2 risk factors for AKI: Female gender, COPD, preoperative creatinine &gt;106 µmol/l, NYHA IV, LVEF&lt;35%, Age &gt; 65 years, peripheral vascular disease</td>
<td>Single dose IV epo vs placebo at induction of anaesthesia</td>
<td>98</td>
</tr>
<tr>
<td>RenalGuard®</td>
<td>NCT02974946</td>
<td>UK</td>
<td>≥1</td>
<td>Undergoing cardiac surgery with ≥1 risk factor for AKI: Diabetes, eGFR 20-60ml/min/1.73m², bypass time predicted longer than 120 min, Haemoglobin level ≤12.5g/dl, Logistic Euroscore of ≥5</td>
<td>Forced diuresis with low dose (0.25–0.5 mg/kg) furosemide along with IV fluid at a rate that is matched to the urine output versus standard care</td>
<td>220</td>
</tr>
<tr>
<td>AKI Care Bundle</td>
<td>NCT03244514</td>
<td>N/A</td>
<td>18 to 90</td>
<td>Undergoing cardiac</td>
<td>Implementation of bundle vs usual care</td>
<td>276</td>
</tr>
<tr>
<td>Preoperative calorie restriction</td>
<td>NCT01534364 (Jan 2012-Feb 2015)</td>
<td>Patients aged ≥18 with cardiac surgery using cardiopulmonary bypass with at least one risk factor for AKI: CKD Type II diabetes Peripheral vascular disease NYHA III-IV Combined CABG and heart valve surgery</td>
<td>N/A</td>
<td>Implementation of calorie restriction (60% of calculated daily requirement) for 7 days pre op vs usual care</td>
<td>76</td>
<td>Primary Increase in serum creatinine 24 hours after cross clamping compared to baseline Secondary Urine NGAL 8h after induction of ischaemia cf baseline Parameters comparing baseline with 24h after induction of ischaemia: CRP Leukocyte count CK Troponin T</td>
</tr>
</tbody>
</table>

<p>| surgery with cardiopulmonary bypass Urinary [TIMP-2][IGFBP7]≥0.3 4 hours after cardiopulmonary bypass | Bundle: • Discontinuation of nephrotoxic drugs • Optimisation of volume and haemodynamics • Close monitoring of creatinine and fluid balance • Avoiding hyperglycaemia • Consideration of alternatives to contrast • Discontinuation of ACE inhibitors/ARBs perioperatively • Avoidance of HES, gelatine and chloride-rich solutions | AKI incidence up to 72 hours post op Severity of AKI Days without vasopressors/ventilation Renal recovery Mortality ICU and hospital length of stay RRT | compared to controls: (55.1 vs. 71.7%); ARR 16.6% (95 CI 5.5–27.9%); p = 0.004 |  |</p>
<table>
<thead>
<tr>
<th>Intervention</th>
<th>Study ID / Details</th>
<th>Participants</th>
<th>Outcomes</th>
<th>Results/Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Further surgery after CABG or valve surgery</td>
<td></td>
<td></td>
<td>LDH, NT-ProBNP, Lactate, RRT, In-hospital mortality, Time until fit for discharge, Length of stay, LVEF, AKI by KDIGO criteria within 10 days post op</td>
<td>No significant difference in any of the other secondary outcome measures.</td>
</tr>
<tr>
<td>Dexmedetomidine</td>
<td>NCT01886079 (May 2013-May 2015) Single centre in South Korea</td>
<td>Patients aged 20-90 having cardiac surgery with cardiopulmonary bypass</td>
<td>200 Primary Incidence of AKI (AKIN criteria) Secondary Composite of morbidity (stroke, haemostatic re-exploration, AKI, ventilation &gt;48h, deep sternal wound infection) Length of ICU stay In-hospital mortality</td>
<td>AKI was lower in dexmedetomidine group than control (14 vs 33%, p=0.002). The incidence of composite of morbidity was lower in the dexmedetomidine group (21 vs 38%, p=0.008) and ICU stay was shorter</td>
</tr>
<tr>
<td>Chloride limited perioperative fluid regime</td>
<td>NCT02020538 (Feb 2014-Feb 2016) Single centre in Australia</td>
<td>Patients aged ≥18 undergoing cardiac surgery</td>
<td>113 Primary Maximum change in serum creatinine from baseline by 5 days post op AKI stage 2 or more by creatinine based KDIGO criteria by 5 days Secondary Mortality RRT ICU length of stay Hospital length of stay Time to extubation Red cell transfusion FFP transfusion Platelet transfusion</td>
<td>There was no significant difference in primary or secondary outcomes</td>
</tr>
</tbody>
</table>

Dex = Dexmedetomidine; LDH = Lactate Dehydrogenase; NT-ProBNP = N-terminal Pro-B-type Natriuretic Peptide; RRT = Renal Replacement Therapy; LVEF = Left Ventricular Ejection Fraction; AKI = Acute Kidney Injury; AKIN = Acute Kidney Injury Network; KDIGO = Kidney Disease Improving Global Outcomes; ICU = Intensive Care Unit.
<table>
<thead>
<tr>
<th>Drug</th>
<th>NCT number</th>
<th>Study details</th>
<th>Participants</th>
<th>Dose</th>
<th>Primary</th>
<th>Secondary</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium bicarbonate</td>
<td>NCT01920126</td>
<td>(Aug 13-Aug 16) Single centre in South Korea</td>
<td>Patients aged 20-90 scheduled for open heart surgery for infective endocarditis</td>
<td>Sodium bicarbonate 0.5mmol/kg for 1 hour (started at induction of anaesthesia), then 0.15mmol/kg/h for 23 hours. Placebo group: normal saline</td>
<td>Difference in peak serum creatinine levels between groups during first 48 hours post op</td>
<td>AKI in first 48 hours post op by AKIN criteria</td>
<td>Electrolyte abnormalities</td>
<td>ICU length of stay</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>NCT00188630</td>
<td>(Jul 2003-Jul 2007) Single centre in Canada</td>
<td>Patients aged &gt;18 having CABG and/or valve replacement/repair surgery under cardiopulmonary bypass, with preoperative creatinine clearance ≤60ml/min</td>
<td>IV NAC 100mg/kg bolus at the start of the operation followed by a 10 mg/kg/hr infusion until 4 hours after completion of surgery Placebo: 5% dextrose solution in the equivalent volume</td>
<td>Maximum percent decline in calculated creatinine clearance in first 72 hours post surgery</td>
<td>RRT, Mortality, Atrial fibrillation, Vasoactive medications, Adverse Effects</td>
<td>There was no significant difference in creatinine clearance. All-cause mortality was lower in the N-acetylcysteine group (0% vs 8%, p=0.007)</td>
<td>Wijeysundera 2007 (102)</td>
</tr>
<tr>
<td>Fenoldopam</td>
<td>NCT00621790</td>
<td>(Feb 2008-June 2013) 19 centres in Italy</td>
<td>Patients who have developed AKI after cardiac surgery (≥50% increase in serum creatinine or oliguria for ≥6 hours)</td>
<td>Fenoldopam 0.1ug/kg min for up to 4 days vs placebo (0.9% sodium chloride)</td>
<td>Rate of RRT administration in ICU</td>
<td>Mortality, Time on mechanical ventilation, Length of ICU stay, Length of hospital stay, Peak serum creatinine, Incidence of AKI (RIFLE criteria)</td>
<td>Stopped early for futility, no difference in primary or secondary end points. Increased hypotension in fenoldopam group</td>
<td>Bove 2014 (103)</td>
</tr>
<tr>
<td>Drug</td>
<td>Trial ID</td>
<td>Study Duration</td>
<td>Setting</td>
<td>Inclusion Criteria</td>
<td>Randomised Group</td>
<td>Outcome Measures</td>
<td>Results</td>
<td>Comments</td>
</tr>
<tr>
<td>------</td>
<td>----------</td>
<td>----------------</td>
<td>---------</td>
<td>--------------------</td>
<td>------------------</td>
<td>------------------</td>
<td>---------</td>
<td>----------</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NCT03527381 (Sep 2015-Dec 2016)</td>
<td>Single centre in Russia</td>
<td>Age ≥18 having elective cardiac surgery using CPB (including CABG, valve surgery, surgical reconstruction left ventricle)</td>
<td>2 Nitric oxide supplementation via CPB circuit or usual care</td>
<td>96 Primary • AKI incidence (KDIGO criteria) within 48h of surgery Secondary • Urine output during CPB • Urine NGAL at 4h after surgery • Free Hb • Concentrations of NO metabolites (NO2- and NO3-) and total NO metabolites in plasma Inflammatory and anti-inflammatory mediators</td>
<td>NO administration was associated with significantly less AKI: 20.8% vs 41.6% (p=0.023). Urine output was better in NO group. NGAL was lower in NO group. There was no difference in Free Hb or inflammatory mediators</td>
<td>Kamenshchikov 2020 (104)</td>
<td></td>
</tr>
<tr>
<td>Erythropoetin</td>
<td>NCT00654992 (Feb 2008-Feb 2009)</td>
<td>Single centre in South Korea</td>
<td>Age ≥18 having elective CABG</td>
<td>3 300iu/kg EPO or placebo (0.9% sodium chloride) intravenously before surgery</td>
<td>71 Primary • AKI incidence in first 5 days post op (defined as 50% rise in creatinine from baseline) Secondary Change in eGFR in the first 5 days post op</td>
<td>The incidence of AKI was significantly lower (8%) in the EPO group than in the placebo group (29%, p=0.035).</td>
<td>Song 2009 (105)</td>
<td></td>
</tr>
<tr>
<td>Dexmedetomidine</td>
<td>NCT02607163 (Sep 2015-Mar 2019)</td>
<td>Single centre in Korea</td>
<td>Patients aged 20-100 undergoing ascending, arch and/or proximal descending aorta surgery with cardiopulmonary bypass</td>
<td>2 0.4µg/kg/h dexmedetomidine vs placebo (0.9% sodium chloride) intravenously started after anaesthetic induction and continued for 24 hours post op</td>
<td>108 Primary AKI incidence in first 7 days post op defined by KDIGO criteria up to 7 days post op Secondary Major morbidity (AKI, stroke, ventilation &gt;24h, deep wound infection, mortality) Postoperative delirium Drug related adverse events (hypotension, bradycardia, vasopressor use, inotropes, temporary pacing or arrhythmia)</td>
<td>The incidence of AKI was significantly lower in the dexmedetomidine group than in the placebo group (31% vs 13%, p=0.026), and the length of hospital stay was shorter (12 days vs 15 days, p=0.039).</td>
<td>Soh 2020 (106)</td>
<td></td>
</tr>
<tr>
<td>Remote Ischaemic preconditioning</td>
<td>NCT02981680 (Nov 2013-Feb 2017)</td>
<td>Single centre in Iran</td>
<td>Patients aged 18 to 85 undergoing emergency or elective on pump coronary artery bypass grafting</td>
<td>2 RIPC group: 3 cycles of 5 min ischaemia and 5 min reperfusion in the upper arm after induction of</td>
<td>180 Primary Incidence of AKI within 72 hours after surgery Secondary Hospital stay</td>
<td>No significant difference in AKI rates, nor in secondary outcome measures</td>
<td>Bagheri 2018 (107)</td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>NCT ID</td>
<td>Location</td>
<td>Participants (age, comorbidities)</td>
<td>Timepoints/Interventions</td>
<td>Sample Size</td>
<td>Primary Endpoints</td>
<td>Secondary Endpoints</td>
<td>Results/Outcomes</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>-------------------------</td>
<td>---------------------------</td>
<td>-----------------------------------</td>
<td>--------------------------</td>
<td>-------------</td>
<td>-------------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>N-acetylcysteine and Fenoldopam</td>
<td>NCT00122018 (May 2002–Mar 2008)</td>
<td>Single centre in USA</td>
<td>Patients aged ≥18 with CKD (creatinine clearance ≤40ml/min)</td>
<td>2</td>
<td>80</td>
<td>Length of hospital stay&lt;br&gt;Length of critical care stay&lt;br&gt;Creatinine clearance days 3 and 14 post op and nadir&lt;br&gt;Days to post-op creatinine clearance nadir&lt;br&gt;Intra-operative and post-op vasopressor use&lt;br&gt;Hospital costs&lt;br&gt;Mortality</td>
<td>The change in creatinine clearance from preoperative to postoperative day 3 was statistically less for group 1 and group 2 compared with controls</td>
<td>Barr 2008 (108)</td>
</tr>
<tr>
<td>EA-230 (Human Chorionic Gonadotrophin Hormone-Derivative)</td>
<td>NCT03145220 (Jun 2016-Oct 2018)</td>
<td>Single centre in the Netherlands</td>
<td>Patients aged ≥18 undergoing CABG+/valve replacement surgery</td>
<td>2</td>
<td>180</td>
<td>Safety and tolerability&lt;br&gt;IL-6 in plasma (0h, start of CPB, 2h, 4h and 6h after stop of CPB, and day 1 post op)&lt;br&gt;Secondary&lt;br&gt;Measured GFR (Iohexol)&lt;br&gt;Urinary biomarkers of AKI&lt;br&gt;Other cytokines/chemokines&lt;br&gt;Leukocyte count</td>
<td>There was no difference in adverse events.&lt;br&gt;There was no difference in IL-6 or other inflammatory markers&lt;br&gt;There was a statistically significant rise in GFR (and reduction in creatinine in the EA-230 group</td>
<td>Van Groenendaal 2021 (109)</td>
</tr>
<tr>
<td>Fenoldopam</td>
<td>NCT00747331 (Sep 2008-Apr 2009)</td>
<td>Single centre in Italy</td>
<td>Patients aged ≥18 undergoing complex cardiac surgery (with expected CPB time &gt;90 mins)</td>
<td>4</td>
<td>80</td>
<td>Peak blood lactate during CPB&lt;br&gt;Urine output during CPB&lt;br&gt;Peak blood lactate in first 48h post op</td>
<td>The incidence of AKI was significantly lower in the fenoldopam group (0%) than in</td>
<td>Ranucci 2010 (110)</td>
</tr>
<tr>
<td>of the operation vs 0.9% sodium chloride</td>
<td>Peak serum creatinine level in first 48h post op AKI (RIFLE criteria) sustained by more than 24h within the first 72h post op</td>
<td>the placebo group (10%) p=0.045</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.3.2 Current AKI management and Clinical trials

The current mainstay of treatment of AKI is supportive care. The heterogeneity of the syndrome of AKI has made translation from preclinical studies to the clinical arena very challenging, with multiple potential therapeutic agents lacking in efficacy in Phase II clinical trials. Cardiac surgery, as a predictive ischaemic insult to the kidney, offers the opportunity to minimise some of that heterogeneity by selecting a cohort of patients with known risk factors and a known interruption in renal perfusion to study.

The main target for developing treatments for AKI has for many years been improving renal perfusion. Low dose dopamine (up to 5µg/kg/min) had been shown in animal models and in trials of healthy volunteers to improve renal perfusion by selective renal vasodilatation by inducing dopaminergic and β-adrenergic effects, however it had no effect on the occurrence of AKI in a large randomised clinical trial of patients with systemic inflammatory response syndrome (SIRS) (111) in a subsequent meta-analysis which included 61 clinical trials of 3359 patients either with or at risk of AKI (112). This meta-analysis gathered data from clinical trials of patients undergoing cardiac surgery, but also vascular surgery, general surgery and trials of patients receiving intravenous contrast and other patient groups such as those with SIRS. There was no benefit in renal function, requirement for renal replacement therapy or mortality. There was an increased urine output of 24% (CI 14-35%) on the first day of dopamine treatment, which was not sustained. There is no evidence that low dose dopamine has a place in preventing or treating AKI.

Other vasodilators such as fenoldopam(103,113) and levosimendan (87,114) have also failed to show benefit in these patients.

Fenoldopam mesylate is a selective dopamine receptor D₁ agonist, which induces selective vasodilatation of the renal, peripheral, mesenteric and coronary arteries. In theory, because it has no affinity for D2 receptors, it...
should induce greater vasodilatation in the renal medulla than the cortex. In small studies, it had shown some benefit in reduction of AKI incidence in cardiac surgery patients (110), with a reduction of AKI rates by RIFLE criteria in this single centre study from 10 to 0% (p=0.045). Unfortunately, a large multicentre randomised trial had to be stopped early due to futility of the treatment and the signal of increased hypotension in the fenoldopam group (103). The drug was administered to patients who had AKI defined as an increase in serum creatinine by ≥50% from baseline, and/or a ≤0.5ml/kg/h urine output for 6 hours. This may have meant that the degree of renal injury was such that the fenoldopam was administered too late in the disease course and that by giving this earlier, there may have been some benefit.

Levosimendan is a drug primarily used for heart failure which has multiple effects including improved cardiac contractility due to calcium sensitisation, increased vasodilatation and cardiac protection by opening potassium ATP-channels. In the kidney, it has been shown to increase renal blood flow through renal vasodilatation with preference for the afferent arterioles, leading to higher filtration rates. It has shown promise in improving renal blood flow in cardiac patients with AKI in a small randomised trial (87), however when a large RCT was carried out to assess its effect on mortality in patients having cardiac surgery requiring vasopressor or inotrope support, the study had to be stopped due to futility (114). As with many of these studies, it may be that patient selection was the issue here and that a well powered clinical trial with risk stratification carried out to determine those most at risk, a benefit may be demonstrated.

Loop diuretics such as furosemide block the K/Na/2Cl cotransporter in the ascending limb of the loop of Henle, therefore their use may offer renal protection by decreasing the energy and oxygen demand in the tubules. This has been the subject of several clinical trials but has yet to be shown to confer significant benefit (115,116). More recently, a trial of furosemide as part of a
forced diuresis with accompanied intravenous fluid replacement (RenalGuard®) has been more promising, with a reduction in AKI rates by day 3 post operatively by RIFLE criteria from 20.9 to 10%(96). This was a small study (110 patients randomised to RenalGuard®, 110 to standard care) and further Phase III trials are necessary.

More recently it has been recognised that inflammation plays an important role in AKI and that therefore therapies targeting the inflammatory response may be of benefit. Dexmedetomidine is a selective -2 adrenoceptor agonist, and may reduce AKI because of its anti-inflammatory and antioxidative effects, as well as its action on the sympathetic nervous system(106). In a small randomised trial of aortic surgery, drug administration pre and post operatively by continuous infusion reduced the rate of AKI compared to placebo. There is still work to be done to determine the optimum dose and timings, and patient selection, but a meta-analysis of 10 published RCTs also showed an overall benefit in AKI rates compared to placebo with an odds ratio of 0.65; confidence interval of 0.45-0.92, (p=0.02).

Aside from pharmacological therapies, remote ischaemic preconditioning (RIPC) is a technique by which an ischaemia reperfusion injury is carried out in a planned way at a remote site in advance of a predicted, more significant ischaemic insult.

This technique has been used in clinical trials of cardiac surgery patients; often using a non-invasive blood pressure cuff to induce short repeated cycles of upper arm ischaemia prior to planned surgery, with the aim of myocardial and renal protection. Unfortunately, despite promising results in a trial of 240 patients at high risk of AKI undergoing cardiac surgery(117) in which RIPC treated patients had significantly less AKI within the first 3 post operative days than patients undergoing a sham procedure (AKI incidence 37.5% vs. 52.5%,
p=0.02; RR, 0.71 [95% CI 0.54–0.95]), this has not been replicated in other larger randomised controlled trials (118,119). Part of the reason for this may be the use of propofol anaesthesia in the large RCTs, as this is thought to attenuate the benefit of RIPC as it has been shown to reduce markers of renal IRI in animal models (120,121) and to reduce the risk of AKI when compared to sevofluorane when used for anaesthesia during CABG operations (121). This is however an important finding given the practicality of trial design; propofol is a commonly used anaesthetic agent and volatile anaesthesia has its own disadvantages. It may be that more time was needed between intervention and surgical procedure if the effect were mediated by inflammatory pathways. Further RCTs are ongoing (NCT02997748).

1.4 Repurposing licensed drugs
In developing new treatments, rather than depending on entirely new compounds there is great benefit of using licensed drugs for new indications. Drug repurposing (also known as drug repositioning) bypasses the need for lengthy drug development, avoiding the cost and risk associated with Phase I clinical trials in healthy volunteers as these have been carried out previously. As disease mechanisms become better understood, it is easier to hypothesise which treatments may be suitable and therefore test potential therapies.

The most recent notable example of this is the use of dexamethasone and IL-6 blockers in the treatment for Covid-19. These anti-inflammatory drugs had been licensed for use for many years for a wide variety of indications in different patient groups, and therefore the Phase II studies such as the RECOVERY trial could be commenced much more quickly (122).

Another well known example is thalidomide, which was developed as a sedative and as treatment for hyperemesis gravidarum, but unfortunately was later found to be teratogenic (123). It was found to have anti-angiogenic properties, which inhibited foetal development in the womb and led to the
devastating side effects such as miscarriage and abnormal limb development. However, the same effects of inhibition of angiogenesis in a different context led to successful repurposing of the drug to another use. Plasma cell myeloma is a condition of increased bone marrow microvascular density, and treatment with thalidomide has shown improvement in remission from myeloma and patient survival (124).

The work outlined in this thesis had the following aims:

1. Develop HA as a potential prophylactic treatment for acute kidney injury associated with cardiac surgery by testing the efficacy and safety of 2 doses of HA (1mg/kg and 3mg/kg) in an elderly comorbid population of patients awaiting cardiac surgery. The primary outcome measure was the difference in HO-1 protein expression from baseline in the circulating peripheral blood mononuclear cells.

2. Explore the potential for DMF to upregulate nrf2-dependent genes including HO-1 in primary murine and human macrophages and beneficially modulate macrophage phenotype and behaviour.

3. Explore the potential for DMF to upregulate nrf2-dependent genes including HO-1 in the murine kidney and limit experimental renal IRI.
2 Materials and Methods

2.1 A randomised clinical trial of Heme Arginate in Patients planned for Cardiac Surgery

The design, organisation and delivery of this clinical trial was the main focus of this clinical training fellowship. At the time of writing the fellowship application, the hope and expectation was that a clinical trial of HA in patients at the time of cardiac surgery, with incidence of AKI as primary end point, would be feasible based on the preliminary results of the HOT trial (64). This demonstrated that when compared to placebo, HA significantly upregulated HO-1 protein in PBMCs of patients undergoing renal transplant, and in renal macrophages at day 5 post operatively. However, in the early stages of designing this proposed trial, it was felt that there were insufficient data to be confident that in an older, more comorbid population, HA would have the same effect, nor that it would be safe to give in the context of major cardiac surgery.

With these reasons in mind, the project changed to designing a trial to demonstrate efficacy and safety in this elderly comorbid population at risk of AKI. The timing of the study drug administration was set deliberately to be remote from the timing of the operation, by a minimum of 2 weeks. This was to avoid the study drug, or perhaps more realistically any adverse events which could have been attributed to the drug, being present at the timing of the operation itself. A further Phase II study of HA would be needed to test the hypothesis that HA could confer renal protection at the time of cardiac surgery.

2.1.1 End points

2.1.1.1 Primary end point

The primary outcome measure was the difference in HO-1 protein expression from baseline in the circulating peripheral blood mononuclear cells (PBMCs) of participants following a single dose of 1mg/kg or 3mg/kg HA (up to a maximum 250mg) at 24 hours post dose
2.1.1.2 Secondary end points

2.1.1.2.1 HO-1 mRNA induction in PBMCs
The difference in HO-1 mRNA expression from baseline in the circulating PBMCs of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.1.1.2.2 HO-1 protein level in serum
The difference in HO-1 enzyme levels from baseline in the serum of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.1.1.2.3 HO-1 protein level in urine
The difference in HO-1 enzyme levels from baseline in the urine of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.1.1.2.4 Safety
The safety of HA in this elderly and comorbid population will be established by recording of adverse events, as well as accepted measurements of physiological systems such as liver function, renal function, full blood count, C-reactive protein and ferritin.

2.1.1.2.5 Urinary Biomarkers
Urine will be collected to examine the urinary sediment (by urine dipstick) and to quantify microalbuminuria and urinary biomarkers associated with AKI (NGAL, KIM-1 and IL18) to establish if HA dosing has any adverse effect per se upon the kidney. If there is no adverse impact then this will inform future trials of HA in AKI where urinary biomarkers will be used as surrogate markers of AKI.

2.1.1.2.6 HO-1 Genotyping
The pre-dose blood sample will also be used to genotype recruited participants for the short or long (or heterozygous) repeat HO-1 promoter polymorphism to investigate the potential effect of this polymorphism on HO-1 induction by HA.

2.1.1.2.7 Complement
The difference in the complement cascade components C1q level from baseline in the participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose
2.1.2 Study Drug

The study drug was Heme arginate (trade name Normosang) 25mg/ml, concentrate for solution for infusion. This was manufactured up to the primary packaging stage at EVER Pharma Jena GmbH, Otto Schott Strasse 15, 07745 Jena, Germany and was secondary packaged and QP released by Orphan Europe. Once the HA had been received from the supplier, it was labelled in Pharmacy at the RIE to identify it as the trial drug, and it was stored in the pharmacy department in a refrigerator (2-8°C) protected from light.

2.1.3 Study funding

The HACS study was funded by the MRC/KRUK Clinical Training Fellowship. The HA was provided by Orphan Europe free of charge. The drug company had no input in the design of the clinical trial, nor did they have rights to the intellectual property.

2.1.4 Study Design

2.1.4.1 Study Design in brief

This was a single centre randomised trial of HA in elderly patients who were planned to have elective cardiac surgery in the coming weeks to months. The study cohort was patients who were planned for elective cardiac surgery and were aged 60 and above at the time of consent. Participation in the trial involved 3 study visits in addition to their usual clinical care (Figure 9). The time points are given as 0 hours (just prior to study drug administration), 6 hours post study drug, 24 hours post study drug and and 7 days post study drug for the purpose of simplicity. However it was stated in the study protocol that the 6 hour time point for blood sampling was 6 hours ± 30 minutes, with follow up at 24 hours ± 180 minutes and 7 days ± 24 hours. This allowed the participants some flexibility given these were additional visits to the clinical research facility.
The randomisation process was by a permuted block web-based programme in conjunction with the Edinburgh Clinical Trials Unit (ECTU), subsequently embedded in the secure REDCap database. The appropriate dose of study drug, in accordance with the randomisation result, was prepared by a member of the trial team.

Enrolled participants were randomised to receive one of:

- 3mg/kg heme arginate (up to a maximum dose of 250mg)
- 1mg/kg heme arginate

The study drug was given as a single dose by intravenous infusion, and the participant was observed by a member of the trial team for 6 hours following this to measure physiological parameters and monitor for adverse effects given the high risk patient
study cohort. Blood samples were taken immediately prior to administration of the drug, and at 6 hours following the dose. At this point the patient was discharged home. A further blood sample was taken at a return visit the following day, (at 24 hours post dose), and another at a final study visit on day 7.

Once the blood had been taken, the routine blood tests (U&E, LFT, FBC, ferritin, CRP) were processed at the hospital laboratory at the RIE. The study blood samples for HO-1 were processed at the CIR and stored there for later batch analysis. The pre-dose blood sample was also used for HO-1 genotype assessment for each participant.

Urine samples were collected prior to the dose of HA being given and again at 24 hours and 7 days post dose. After the urine was tested for blood and protein on dipstick, an aliquot was sent for microalbuminuria testing at the RIE biochemistry laboratory. The remaining urine was then frozen and later analysed for urinary biomarkers of acute kidney injury (NGAL, KIM-1 and IL18) by ELISA.

Patients were considered members of the trial from the time of consent to the end of the final study visit or the resolution of any ongoing adverse event. There were no drop outs and all study visits were completed as planned (Figure 10).
Figure 10: Consort diagram of HACS Clinical trial

CONSORT 2010 Flow Diagram

Enrollment
Assessed for eligibility (n=105)
Excluded (n=84)
- Not meeting inclusion criteria (n=0)
- Declined to participate (n=76)
- Other reasons (n=8)
Consented and Randomized (n=21)
Low dose (1mg/kg) Heme arginate infusion (n=11)
Allocation
High dose (3mg/kg) Heme arginate infusion (n=10)
Follow-Up
No participants lost to follow up
All received planned intervention
Analysis
Analysed (n=11)
Analysed (n=10)
2.1.4.2 Power calculation

The HOT clinical trial results demonstrated a 9 fold increase in monocyte HO-1 protein expression at 24 hours measured by enzyme linked immunosorbent assay (ELISA): placebo group $2.1 \pm 10.3$, 3mg/kg HA group $18.8 \pm 22.4$ (p=0.0006, mean±SD)

Based on these data, calculations carried out together with the Statistician Catriona Graham from the Clinical Trials Unit (ECTU) at the Western General Hospital, indicated that a study group of 10 patients would enable the detection of a difference of 10.3 (from 2.1 to 12.4) in mean monocyte HO-1 protein expression if the baseline HO-1 expression level were comparable to the placebo group of the HOT study. However, it was noted that the HOT participants had end stage renal disease and were receiving renal replacement therapy, with patients receiving haemodialysis for up to 4 hours at variable times (0-3 days) prior to participation in the trial. Such dialysis treatment would have exposed the circulating monocytes to artificial membranes and may have modulated the monocyte HO-1 levels separately to the study drug itself.

As patients recruited into the HACS trial would not be receiving dialysis, it was felt that the baseline level of monocyte HO-1 expression would be less variable than in HOT so that the study of 10 patients would be able to detect a lower level of HO-1 upregulation following the administration of HA at 3mg/kg. For example, using a two-sided paired t-test with 5% level of significance and 80% power we would be able to detect a baseline to 24-hour difference in HO-1 protein of 6.0 (from 2.1 to 8.1, a 4-fold increase) if the standard deviation of the differences was 6 (see column 4 of Table 4 below). Columns 3 to 6 of the table demonstrate the calculations repeated using standard deviation values from 8 down to 2. As the standard deviation decreases so do the size of the mean difference required to demonstrate a statistically significant baseline to 24-hour change (highlighted in bold). Therefore, based on these calculations,
we were confident that a sample size of 10 would be sufficient to show a statistically significant change in HO-1 protein expression following the administration of HA (3mg/kg).

Table 4: Power calculation showing paired t-test of mean difference equal to zero

<table>
<thead>
<tr>
<th></th>
<th>Data from HOT study HA (3mg/kg) treatment arm</th>
<th>Data from HOT study placebo arm</th>
<th>Calculations based upon various levels of the reduced variance predicted to be evident in proposed patients for recruitment to this study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test significance level, ( \alpha )</td>
<td>0.050</td>
<td>0.050</td>
<td>0.050</td>
</tr>
<tr>
<td>1 or 2 sided test?</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean difference, ( \mu_d = \mu_1 - \mu_2 )</td>
<td>22.310</td>
<td>10.259</td>
<td>7.968</td>
</tr>
<tr>
<td>Standard deviation of differences, ( \sigma_d )</td>
<td>22.400</td>
<td>10.300</td>
<td>8.000</td>
</tr>
<tr>
<td>Effect size, ( \delta =</td>
<td>\mu_d</td>
<td>/ \sigma_d )</td>
<td>0.996</td>
</tr>
<tr>
<td>Power (( % ))</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>( n )</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>


Inclusion/exclusion criteria

Male and female participants were included, and there was no upper age limit.

Inclusion criteria:

Age $\geq 60$ (no upper age limit)

Planned for elective cardiac surgical procedure comprising either:

- Aortic valve replacement (AVR) or
- Coronary artery bypass grafting (CABG)

Exclusion criteria

Participants were not eligible for inclusion in the study if they met any of the following criteria:

1. Inability to give informed consent
2. Enrolment in other clinical trials
3. Undergoing renal replacement therapy
4. Planned for elective cardiac surgical procedure other than CABG or AVR (including combined CABG and AVR)
5. Less than 2 weeks until planned cardiac surgery at time of study drug administration
6. Symptoms or signs suggestive of active infection
7. Contraindications (or relative contraindications) to HA including:

- Hypersensitivity to HA
- Liver disease
- Alcoholism
- Epilepsy
- Brain injury
8. Women who were pregnant, breastfeeding or of child-bearing potential (women who have experienced menarche, are pre-menopausal and have not been sterilised) would not have been enrolled into the trial as participants were at least 60 years old.

2.1.5 Consent

Potential trial participants were identified by their clinical team that was either a specialist nurse or consultant cardiothoracic surgeon. If they expressed interest in the study they were invited to the Clinical Research Facility (CRF) at the RIE. The chief investigator clarified that they met the inclusion criteria, and if consent was given then the participant was randomised to receive heme arginate at a dose of either 1mg/kg or 3mg/kg (up to a maximum dose of 250mg).

2.1.6 Participant case report form

Potential participants were consented to participate in the study if they met the inclusion criteria but not the exclusion criteria. Following randomisation they were asked a series of standardised questions to complete the participant case report form. This included information on ethnicity, past medical history, medications, and smoking. The same case report form was used to record details of the infusion, physiological parameters of safety including blood pressure and pulse, and to note any possible adverse events. Participants were asked specifically about possible adverse events at all study visits. Data were then copied to the anonymised secure online REDCap database. Data were checked by the sponsor to ensure that any possible transcribing errors were detected.
2.1.7 Study samples

40ml blood was taken for extraction of peripheral blood mononuclear cells (PBMCs) and serum prior to administration of the study drug and at 24 hours post dose. Samples were held either in the fridge in the Clinical Research Facility or on ice in transit until they were able to be analysed after each participant had been discharged home from their study visit.

2.1.8 Physiological parameters

Routine observations (pulse, blood pressure, oxygen saturations, respiratory rate and tympanic temperature) were taken at initial examination, and then just prior to the study drug being administered. The observations were repeated at 15 minute intervals until 2 hours post dose, then hourly until 6 hours post dose. These observations were carried out to help detect any haemodynamic or other adverse effect of the study drug on the participant.

2.1.9 Electrocardiogram

An electrocardiogram (ECG) was recorded at initial assessment and checked by the study team. The main reason for this was to have a baseline ECG to allow comparison with any that would be required if the participant developed chest pain during the study.

2.1.10 Collection and processing of samples

Venepuncture was performed at time points 0, 6 hours, 24 hours and 7 days post dose (Table 5). Study samples were taken at 0 and 24 hours only. Samples for routine safety data were taken at 0, 6, 24 hours and 7 days and were analysed at the laboratory at the Royal Infirmary of Edinburgh as per their usual laboratory protocols, and published in the patient’s electronic patient record. A urine sample was taken prior to receiving the study drug and at 24 hours. A urine dipstick was performed and a sample sent for determination of the urine albumin:creatinine ratio at the RIE laboratory as above.
Table 5: Study visits and data collection

<table>
<thead>
<tr>
<th></th>
<th>Visit 1 (Day 0) T=0hrs</th>
<th>Visit 1 (Day 0) T=6hrs</th>
<th>Visit 2 (Day 1)</th>
<th>Visit 3 (Day 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Discussion with trial member</strong> (including AE reporting)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><strong>Routine physical examination</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Electrocardiogram (ECG)</strong></td>
<td></td>
<td></td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td><strong>Blood tests</strong></td>
<td>Assessment of HO-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Routine bloods</strong></td>
<td>(FBC, U&amp;E, LFT, CRP, ferritin)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><strong>Genotyping</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Urine sample</strong></td>
<td></td>
<td></td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td><strong>Routine observations</strong></td>
<td>(pulse, blood pressure, temperature, respiratory rate, oxygen saturations)</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
</tbody>
</table>

2.2 Trial Management and Oversight

2.2.1 Trial Management Group

The Trial Management Group (TMG), consisting of the grant holders/ co-investigators, myself the chief investigator (CI) and the trial statistician, coordinated the trial.

Given the small nature of the study, many of the roles were undertaken by myself (as CI) with delegation on the rare occasion when I was not available. I was responsible for checking the CRFs for completeness, accuracy and consistency.

A Delegation Log was prepared detailing the responsibilities of each member of staff working on the trial. This specified trial team members permitted to prepare the drug and those permitted to administer the study drug to participants.
2.2.2 Trial Steering Committee

The trial steering committee (TSC) was established to oversee the conduct and progress of the trial, and consisted of the TMG and a representative from ACCORD.

2.2.3 Data Monitoring Committee

There was no independent Data Monitoring Committee (DMC) in this trial due to its small nature and relatively short duration. The TSC was therefore tasked with overseeing safety of participants.

2.2.4 Adverse Event Reporting

All Adverse Events (AEs) Serious Adverse Events (SAEs) were recorded from the time the participant signed the consent form to take part in the study until the end of the 3rd study visit (ie day 7 post dose). Participants were asked about the occurrence of AEs/SAEs at every visit during the study. Open-ended and non-leading verbal questioning of the participant were used to enquire about AE/SAE occurrence. Participants were asked if they have been admitted to hospital, had any accidents, used any new medicines or changed their medication regimens. If there was any doubt as to whether a clinical observation was an AE, the event was be recorded.
2.2.5 Laboratory

Blood and urine samples were analysed at the RIE biochemistry and haematology laboratories as per the usual laboratory protocols with the results published in the patient’s electronic patient record.

2.2.6 Peripheral blood mononuclear cells

40ml of blood was collected into a 50ml falcon tube containing 4ml 3.8% sodium citrate (made up as 3.8g sodium citrate tribasic dihydrate, Sigma 25116 in 100ml sterile bottled water Baxter UKF7114). The tube was inverted gently to mix. This was then placed in the CRF refrigerator at 4°C while the trial participant received the study dose and was observed for 6 hours afterwards.

Upon returning to the lab after the participant had been discharged home, the falcon tube of blood was centrifuged at 350g for 20 minutes at 4°C (acceleration 5, deceleration 0) to separate erythrocytes and leucocytes from plasma. The plasma was then aspirated with a pipette into glass vials and incubated in a water bath at 37°C for 1 hour with 200µl of 1M calcium chloride added for every 10ml of plasma. Serum could then be separated from the resultant platelet precipitate, aliquotted and frozen at -20°C until required.

The leucocytes were separated from erythrocytes by dextran sedimentation. 6ml of 6% Dextran (Pharmacosmos 551005008007 in 0.9% NaCl (Baxter UKF7124)) was added to the cell layer and then made up to 50ml total volume with 0.9% sterile NaCl. The tube was inverted gently 2-3 times to resuspend the cells. The top of the falcon tube was opened and any large bubbles burst with the tip of a sterile needle. The lid was then placed back on the tube, and cells were allowed to sediment for 1 hour in the fridge.

Following sedimentation, the cells in the upper layer (predominantly leucocytes) were removed with a pipette and transferred to a fresh 50ml
Falcon tube. This was then placed in the centrifuge at 350g for 6 minutes to form a cell pellet. The erythrocytes were discarded.

In order to separate the PBMCs from granulocytes and any remaining erythrocytes, a Percoll gradient was used. This was created by diluting Percoll (GE healthcare 17-0891-02) with 10x Phosphate buffered Saline (PBS) (without Ca$^{2+}$/Mg$^{2+}$, PAA) to different concentrations. The gradient itself was formed by placing 3ml of 81% Percoll in the bottom of a 15ml polystyrene tube (Falcon) followed by carefully layering 3ml of 68% Percoll on top, using a 5ml stripette. The top layer is formed by discarding the supernatant from the cell pellet and resuspending the cells in 3ml of 55% Percoll, mixing well. This was then carefully added to the gradient to form the top layer. The gradient was then placed in the centrifuge at 720g for 20 minutes. Following this, mononuclear cells could be harvested from the 55/68 interface, and granulocytes from the 68/82 interface.

The cells were washed twice in phosphate buffered saline (PBS) to remove the Percoll by resuspending in 50ml PBS and then spinning at 300g for 5 minutes. The supernatant was then discarded. The cells were then resuspended in 10ml PBS and counted using a haemocytometer. The cells could then be divided for later analysis. The number of cells varied among trial participants but at least 2 sets of 4x10$^6$ cells were resuspended in 350µl lysis buffer for later RNA analysis (NucleoSpin® RNA kit, Macherey-Nagel) and immediately frozen on dry ice then transferred to a freezer at -80°C.

At least 2 sets of 1x10$^7$ cells were lysed in Radio-immunoprecipitation assay (RIPA) buffer (Sigma) with protease inhibitor (complete mini tabs, Roche) and phosphatase inhibitor (PhosStop, Roche) and immediately frozen on dry ice prior to being transferred to a freezer at -80°C.
2.2.7 Analysis of samples – blinding to treatment group

Participant samples (including aliquots of serum, urine and peripheral blood mononuclear cells) were labelled with the participant study number only. Once all the samples had been collected at the end of the study, the samples were blinded by Dr Jamie Smith, a researcher independent of the trial who had Good Clinical Practice (GCP) training. This meant that the investigator analysing the samples was always blind to the treatment group. The data were collected and analysed after all the samples had finally been processed. The raw data were sent in encoded form to the trial monitors, at which point the data were unblinded and the results could be discovered and shared.

2.2.8 Peripheral blood mononuclear cell HO-1 - Analysis

2.2.8.1 Protein quantification for standardisation

PBMC protein lysates were defrosted on ice and concentration of protein was quantified using the Bio-Rad Protein Assay Kit, based on the Bradford method. This method uses a dye binding assay in which the colour change of dye correlates to protein concentration. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue dye shifts from 465nm to 595nm when bound to protein.

In brief, a standard curve of bovine serum albumin (BSA) was prepared in triplicate with deionised water with a range of 200-1400µg/ml, 100µl in each well. Trial participant samples were pipetted in triplicate into adjacent wells of the same 96 well plate. 200µl of dye reagent was added to each well and incubated at room temperature for at least 5 minutes. Absorbance was measured using a Spectrophotometer at 595nm and protein concentration determined for each participant sample using the standard curve.

2.2.8.2 ELISA for HO-1 quantification

HO-1 protein in PBMCs was then quantified by Enzyme-linked immunosorbent assay kit (ELISA) using the kit according to the manufacturer’s instructions
All samples were standardised to 350µg/ml of total protein, and analysed in duplicate.

In brief, a mouse monoclonal antibody specific for HO-1 was already pre-coated on the wells of the HO-1 immunoassay plate. After the addition of the study samples, HO-1 was captured by the immobilised antibody and detected with an HO-1 specific, rabbit polyclonal antibody. This polyclonal antibody was bound by horseradish peroxidase conjugated anti-rabbit IgG secondary antibody. The assay was then developed with tetramethylbenzidine substrate and the blue colour was generated in proportion to the concentration of captured HO-1. The colour development was halted by the addition of an acidic stop solution which converted the colour to yellow. The intensity of the colour was quantified by a microplate reader at 450nm, and compared to absorbance readings from the standard curve which was generated from the protein standard provided in the kit.

2.2.8.3 Quantification of HO-1 gene expression

HO-1 gene expression was quantified according to the protocol given below. 18S was used as a housekeeping gene (18s (human) Taqman gene expression assay Hs99999901_s1, Thermofisher Scientific) and HMOX1 (Hmox1 (human) Taqman gene expression assay Hs01110250_m1, Thermofisher Scientific) was used to measure HO-1 gene expression. Quantification was carried out using the standard curve method as described below.

2.2.9 Serum

Serum, prepared as above, was allowed to defrost from -80 to room temperature slowly over ice. Protein was then quantified using the Bio-Rad Protein Assay Kit, based on the Bradford method for later standardisation for ELISA.
2.2.10 Urine collection
Urine was collected in a 50ml polystyrene tube (Falcon) and then refrigerated at 4°C in the CRF, prior to being transported on ice back to the laboratory. The urine was placed in the centrifuge at 220g for 5 minutes, the supernatant was frozen on dry ice in 1ml aliquots, stored at -20°C until required for analysis and the cell pellet discarded.

2.2.11 Assessment of urinary biomarkers
Urine was defrosted on ice prior to analysis. Samples had protein quantification by the Bio-Rad Protein Assay Kit, based on the Bradford method and then ELISAs were carried out according to the manufacturers’ instructions.
NGAL: Human NGAL Monomer ELISA kit (Alpha laboratories)
KIM1: Human TIM-1/KIM-1/HAVCR Duoset ELISA kit (R&D systems)
IL18: Human IL-18/IL-1F4 ELISA kit (R&D systems)
Urinary creatinine concentration was also determined by ELISA, using the Parameter creatinine assay (R&D systems)

2.2.12 Genotyping

2.2.12.1 Extraction of genomic DNA
1ml of whole blood was defrosted from storage at -80°C on ice and vortexed to mix. DNA was extracted using the DNeasy blood and tissue kit (Qiagen, 69504) according to the manufacturer’s instructions. DNA quality and final concentration was determined spectrophotometrically (Nanodrop).

2.2.12.2 Polymerase chain reaction
The 5’ flanking region of the HO-1 gene, containing a (GT)\text{n} repeat, was then amplified using RedTaq SuperPak DNA polymerase (Sigma, D6063). Primers were used at a concentration of 0.2µM, and DNA samples were first diluted 1:10 in DNAse and RNAse free water prior to adding the volume of sample
required for 10ng DNA for each participant. The following primers were used: FAM labelled 5'-AGAGCCTGCAGCTTCTCAGA-3’ (forward) and 5'-ACAAAGTCTGGCCATAGGAC-3’ (reverse).

PCR was performed on a Thermocycler at 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute. The PCR products were then stored at 4°C overnight prior to running the samples on a 1% agarose gel to ensure that a PCR product was present as expected.

2.2.12.3 Sequencing analysis
1µl DNA PCR product were then added in duplicate to a 96 well plate provided by Edinburgh Genomics (GenePool). 9µl of master mix was then added (made up of 10µl GeneScan 500 LIZ size standard and 1ml HiDi formamide, both Applied Biosystems). Samples were then given to Edinburgh Genomics for capillary electrophoresis to determine the size of the amplified PCR product, using the laser based DNA capillary sequencer ABI 3730. Data were then analysed at the QMRI using Peak Scanner Software to examine each individual electropherogram and assess the size of the PCR products. As the HO-1 promoter sequence is 67 base pairs long, the number of GT repeats was determined by subtracting 67 from the PCR product to give the relevant length, then divided by 2 to give the number of GT repeats. If not a whole number then this was rounded to the nearest whole number. As samples were analysed in duplicate, an average was used to give the GT repeat for each trial participant. Allelic repeats were then classified according to the standard of <27 GT\textsubscript{n} being allele class short (S), and \geq 27 being long (L)(61,68). Participants were identified as either being homozygous or heterozygous.
2.3 Preparation of Murine bone marrow derived macrophages (BMDMs)

All procedures were carried out according to Home Office Guidelines (Animals (Scientific Procedures) Act 1986).

Male C57Bl/6 mice were bred on site and culled at age 6-8 weeks. Skinned mouse legs were provided in PBS (Gibco) and the femurs were dissected out using a sterile technique. The femur was washed in 70% ethanol to reduce the risk of bacterial contamination. The femur was then cut at both ends, and marrow extracted by flushing the bone through with warm macrophage medium (DMEM/F-12, GlutMAX (Gibco) supplemented with 10% heat inactivated fetal bovine serum (FBS), (Gibco), 1% antibiotic antimycotic solution (ABAM), (Sigma) and 20% L929 medium. L929 is a source of macrophage colony-stimulating factor. A 10ml syringe was used with a 23 guage needle to flush each femur’s bone marrow into a 50ml Teflon pot. This was made up to 40ml macrophage medium and the cells were gently pipetted to suspend them fully. The cells were incubated at 37\(^\circ\)C, 5% CO\(_2\) for 7 days to allow them to differentiate into macrophages. 20ml of medium was replaced every 48 hours.

At the end of the 7 day period, the cells were checked for contamination before placing them into 50ml sterile tubes (Falcon) and spinning the cells down in the centrifuge at 300g for 5 minutes at 4\(^\circ\)C. The cell pellets were then resuspended in 15ml macrophage medium and counted by haemocytometer prior to use.

2.4 Human MDMs

Human blood was taken from healthy volunteers as per protocol at the Centre for Inflammation Research, with written participant consent.

PBMCs were extracted as above, counted using a haemocytometer and were then diluted with Iscove’s Modified Dulbecco’s Medium (IMDM (Gibco)) supplemented with 10% autologous serum and 1% penicillin-streptomycin solution (Gibco). They were immediately plated into 24 well plates. Cells were
plated at $2 \times 10^6$ cells/ml in order to achieve a monocyte concentration of approximately $8 \times 10^5$ cells/ml. Cells were allowed to mature by selective adherence, and so were left at $37^\circ C$, 5% CO$_2$ for 6 days in order to facilitate this.

2.5 Dimethyl fumarate in vivo – murine experimental work

Experiments were performed on young male mice (aged 8-12 weeks) from in house colonies of (C57BL6) mice. All animals were kept in accordance with Home Office guidelines.

2.5.1 Dimethyl fumarate in vivo – dose effect on gene expression by qPCR and immunohistochemistry for HO-1

In parallel with in vitro experiments a series of in vivo studies were undertaken.

2.5.2 Dimethyl fumarate in vivo – dose effect on immunohistochemistry for HO-1

The first study involved a dose titration of DMF followed by an assessment of the renal expression of 3 well described nrf2 dependent genes 24 hours later. DMF was administered to mice by gavage once daily for 3 days and then animals were culled 24 hours after their final dose. There were 3 animals in each group and groups included a PBS control and a DMSO vehicle control. DMF was administered at doses of 10, 30 and 100mg/kg in each experimental group.

2.5.3 Murine model of renal ischaemia reperfusion injury

This experiment was carried out to test the hypothesis that pre-treatment with DMF could confer protection in an animal model of acute kidney injury. The experimental groups were devised as follows (Table 6). Animals were dosed with the study drug by daily gavage for 3 days by a trained animal technician. Mice were administered either DMSO (the vehicle for DMF) or with 30mg/kg DMF (dissolved in DMSO). There were 8 animals per experimental group.
Unilateral IRI surgery was performed 24 hours after the third dose of study drug. There were also sham surgery control groups: DMF 30mg/kg (dissolved in DMSO) and DMSO alone (vehicle control for DMF), both with 3 animals per group.

For the IRI model, isoflurane anaesthesia was used with buprenorphine analgesia. Mice underwent laparotomy and unilateral nephrectomy followed by clamping of the renal artery to the remaining kidney for 22 minutes. Body temperature was maintained using a homeostatically controlled blanket. After reperfusion, the peritoneum was closed with a 5/0 suture and then clips to the skin. 1ml sterile 0.9% sodium chloride was given subcutaneously prior to and after surgery, and the animals were kept in an incubator overnight.

Animals were monitored on the afternoon of surgery and the following morning. The animals were culled by schedule 1 method 24 hours post surgery. Blood was taken for analysis of serum creatinine. Kidneys were removed for histological assessment of kidney injury (acute tubular necrosis) and gene expression of the NRF2 pathway. The heart, liver and spleen were collected for subsequent analyses.

Table 6: Murine model of ischaemia reperfusion injury; Experimental groups

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental condition</td>
<td>Ischaemia reperfusion injury</td>
<td>Ischaemia reperfusion injury</td>
<td>Sham</td>
<td>Sham</td>
</tr>
<tr>
<td>Study drug administered</td>
<td>DMF</td>
<td>Vehicle control</td>
<td>DMF</td>
<td>Vehicle control</td>
</tr>
<tr>
<td>Number of animals per group</td>
<td>8</td>
<td>8</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>
2.5.3.1 Assessment of Renal function

Blood samples were taken by cardiac puncture at the time of the cull. Plasma samples were prepared from whole blood by centrifugation, and creatinine concentration subsequently determined by the Jaffe method (Alpha laboratories Ltd, UK) on a Cobras Fara Centrifugal Analyser (Roche Diagnostics, UK) according to the manufacturer's instructions by Dr Forbes Howie, MRC Centre for Reproductive Health.

2.6 qPCR: Preparation of samples for assessment

2.6.1 qPCR – conversion of RNA to cDNA

Cells were washed and then lysed with 350µl RA1 Lysis buffer (NucleoSpin RNA kit, Machery-Nagel). Samples were snap frozen on dry ice and then stored at -80°C until required for analysis. RNA was extracted using the NucleoSpin RNA kit according to the manufacturer's instructions. After extraction, concentration of the RNA was measured, and quality assured, using a NanoDrop 1000 Spectrophotometer. The extracted RNA was then stored at -80°C until required for further processing or immediately used for reverse transcription. Samples were diluted to a standard concentration of 500µg RNA in 10µl sterile water. The High Capacity cDNA Reverse Transcription Kit with RNAse inhibitor (Applied Biosystems) was then used according to the manufacturer’s instructions, and samples were transferred to the thermal cycler (Applied Biosystems) for reverse transcription. This involved a programme of 25°C for 10 minutes, 37 ºC for 120 minutes and 85°C for 5 minutes. The cDNA could then be frozen at -20°C for later use.

2.6.2 qPCR

cDNA was either taken directly from the thermal cycler or defrosted on ice. A standard curve was prepared by taking 2µl from each sample and then diluting this mix down to 1:8 using RNAse free water. This was then further diluted in subsequent wells in triplicate from a concentration of 1:8 to 1:512. cDNA
samples were diluted 1:20 and added to a 384 well plate in triplicate using a multichannel pipette, along with the standard curve. The mastermix was added to each well so that each contained 5µl probe master (PerfeCTa® FastMix® II, Quanta Biosciences), 0.25µl gene expression assay and 2.75µl RNAse free water.

The plate was sealed and spun at 4000RPM for 2 minutes to settle the samples into the base of the wells. The plate was stored in the dark until PCR commenced, with cycles of 95°C for 2 minutes, 95°C for 10 seconds and 60°C for 60 seconds on an ABI Prism 7900 PCR machine (Applied Biosystems). The threshold at which each gene became detectable was recorded and the relative concentration compared to the housekeeping gene was quantified.

Key readouts of the Nrf2 inflammatory pathway and their respective housekeeping genes were chosen for murine experiments (Table 7) and for those using human MDMs (Table 8).

Table 7: Genes of interest for qPCR in murine experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1 (Hypoxanthine</td>
<td>Housekeeping gene</td>
<td>Hprt1 (mouse) Taqman® gene expression assay</td>
</tr>
<tr>
<td>Phosphoribosyltransferase 1</td>
<td></td>
<td>Mm00446968_m1, Thermofisher Scientific</td>
</tr>
<tr>
<td>GAPDH (Glyceraldehyde-3-</td>
<td>Housekeeping gene</td>
<td>Gapdh (mouse) Taqman gene expression assay</td>
</tr>
<tr>
<td>phosphate dehydrogenase)</td>
<td></td>
<td>Mm99999915_g1, Thermofisher Scientific</td>
</tr>
<tr>
<td>HMOX1 (Heme oxygenase-1)</td>
<td>Anti-inflammatory</td>
<td>Hmox1 (mouse) Taqman gene expression assay</td>
</tr>
<tr>
<td>GSR (Glutathione reductase)</td>
<td>Anti-inflammatory (reduces glutathione)</td>
<td>Gsr (mouse) Taqman gene expression assay</td>
</tr>
<tr>
<td>NQO1 (NAD(P)H Quinone</td>
<td>Anti-inflammatory (quinone detoxification,</td>
<td>Nqo1 (mouse) Taqman® gene expression assay</td>
</tr>
<tr>
<td>Dehydrogenase)</td>
<td>among other actions)</td>
<td></td>
</tr>
</tbody>
</table>

79
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT1 (Hypoxanthine Phosphoribosyltransferase 1)</td>
<td>Housekeeping gene</td>
<td>Hprt1 (human) Taqman® gene expression assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hs02800695_m1, Thermofisher Scientific</td>
</tr>
<tr>
<td>GAPDH (Glyceraldehyde-3-phosphate dehydrogenase)</td>
<td>Housekeeping gene</td>
<td>Gapdh (human) Taqman® gene expression assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hs02758991_g1, Thermofisher Scientific</td>
</tr>
<tr>
<td>18S</td>
<td>Housekeeping (ribosomal RNA)</td>
<td>18s (human) Taqman® gene expression assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hs99999901_s1, Thermofisher Scientific</td>
</tr>
<tr>
<td>HMOX1 (Heme oxygenase-1)</td>
<td>Anti-inflammatory</td>
<td>Hmox1 (human) Taqman® gene expression assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hs01110250_m1, Thermofisher Scientific</td>
</tr>
</tbody>
</table>

Table 8: Genes of interest for qPCR in human experiments

2.7 Assessment of macrophage phenotype: phagocytosis Assay
(experiments done in conjunction with Emily Ostocke)

2.7.1 Preparation of apoptotic murine thymocytes
The thymus was removed from three C57Bl/6 mice aged 4-6 weeks after culling by cervical dislocation. Thymocytes were extracted by compressing each thymus between two sterilised glass slides. Thymocytes were added to 30ml RPMI 1640 medium (Gibco) supplemented with 1% penicillin/streptomycin (penicillin 10 units/ml, streptomycin 10mg/ml) in a 75cm³ culture flask. Thymocytes were then incubated for 60 minutes at 37°C,
5% CO\(_2\) with 10µl of CellTracker Green CMFDA Dye (ThermoFisher Scientific) to stain the cells. Following this, 1ml FCS and 10µl of 10µM dexamethasone (Organon Laboratories Limited) were added to induce cell apoptosis. After making up the total volume to 100ml with RPMI 1640 medium, the flask was returned to the incubator at 37\(^\circ\)C, 5% CO\(_2\) for 24 hours. Cells were then spun at 300g for 5 minutes to create a pellet and the supernatant discarded. Cells were then resuspended in 20ml PBS -/- to wash and then spun again. The cells were then resuspended in 20ml medium and counted using a haemocytometer. The cells were then diluted to a concentration of 2.5x10\(^6\) cells/ml in macrophage media, ready to be used in the macrophage phagocytosis assay.

2.7.2 Preparation of BMDMs for phagocytosis assay

BMDMs were washed and counted and resuspended in medium at a concentration of 2.5x10\(^5\) cells per well on a 24 well plate. Cells were plated for 24 hours prior to the experiment to allow for the cells to settle. The supernatant was then discarded and wells were washed with 1ml PBS/-/. This was replaced with 1ml of medium containing 2.5x10\(^6\)/ml thymocytes or fluorescent beads (Fluoresbrite YG microspheres 3µm, Polysciences). Plates were incubated at 37\(^\circ\)C, 5% CO\(_2\) for 30 minutes to allow for phagocytosis to occur. At that point, plates were put on ice to halt any further phagocytic activity and washed three times with PBS/-/- at 4\(^\circ\)C, leaving the final wash in situ. Cells were lifted using a cell scraper and aspirated into FACS tubes, which were spun for 5 minutes at 300g, at 4\(^\circ\)C. The supernatant was then discarded and the cell pellet was resuspended in 50µl antibody solution (1% mouse F4/80 APC conjugate, Invitrogen), 10% mouse serum (azide free, AbD Sertec), 89% PBS). In the isotype control, the antibody was RAT IgG2b APC conjugate (Invitrogen) and an unstained control had no antibody stain. Samples were incubated in the dark for 30 minutes and then analysed by flow cytometry on the FACSCalibur (bd biosciences).
2.7.3 Quantification of phagocytosis of apoptotic cells and fluorescent beads by flow cytometry

10,000 events were collected per sample. Using Flowjo software, the macrophage population was identified using forward and side scatter and then gated by F4/80 staining (Figure 11). The number of events which both F4/80 and CM green/bead fluorescence positive (indicating cells undertaking phagocytosis of such particles) were divided by the total number of macrophages (F4/80 positive events). This gave the percentage of macrophages that were likely to have internalised the particle of interest.

Statistical analysis was performed using one-way ANOVA with Tukey’s test for multiple comparisons.

Figure 11: Phagocytosis assay by flow cytometry
2.7.4 Assessment of macrophage phenotype: nitric oxide measurement
Murine BMDMs were prepared as detailed above. On day 7, BMDMs were plated on a 24 well plate at 250,000 cells per well with conditioned media. Cells were treated with lipopolysaccharide (LPS) (100ng/ml) and interferon-gamma (IFN-γ) (10ng/ml) in Gibco’s DMEM/F12 Glutmax + 1% ABAM for 24 hours after pre-treatment with 20 or 50µM DMF (control vehicle DMSO). After 24 hours the cell culture supernatant was removed and NO generation assessed by the Griess assay (Promega) as per the manufacturer’s instructions. Untreated M0 macrophages served as control for any spontaneous NO release by cultured BMDMs.

2.8 Assessment of Cell toxicity by DMF treatment using the LDH cytotoxicity assay
BMDMs were plated at day 7 into 12 well plates at a concentration of 4x10^5 cells per well. After 24 hours, cells were treated with increasing concentrations of DMF in parallel. Supernatants were removed at different time points and LDH concentration in the supernatant was assessed using the Pierce LDH cytotoxicity Assay Kit (Thermo Scientific) as per the manufacturer’s instructions. Cytotoxicity was expressed as a percentage of maximal LDH release.

2.9 Time course of Nrf2 pathway by DMF in BMDMs
BMDMs were plated at day 7 into 12 well plates at a concentration of 4x10^5 cells per well. After 24 hours, cells were treated with 50µM of DMF and the cells were lifted at 3, 6, 12, 24 and 48h. DMSO alone and medium alone controls were collected at the 48h time point only. qPCR of the anti-inflammatory genes HMOX1, GSR and NQO1 was carried out with GAPDH as a housekeeping gene.
2.10 Dose response of DMF in BMDMs

BMDMs were plated at day 7 into 12 well plates at a concentration of \(4 \times 10^5\) cells per well. After 24 hours, cells were treated with increasing concentrations of DMF (5, 10, 20, and 50 \(\mu\)M DMF) with DMSO alone and medium alone as negative controls. After 6 hours of incubation with DMF, the cells were lifted for qPCR of the anti-inflammatory genes HMOX1, GSR and NQO1. GAPDH was used as a housekeeping gene.

2.11 Immunohistochemistry of murine kidney – assessment of HO-1

Whole kidneys were fixed in methyl-Carnoy solution (60% methanol, 30% chloroform and 10% acetic acid) prior to being embedded in paraffin by the QMRI histopathology department. Blocks were cut into 4\(\mu\)m thickness tissue sections. These were stained with haematoxylin and eosin if required for assessment of injury following IRI.

For assessment of HO-1 expression in renal tissue, tissue sections were deparaffinised with xylene, rehydrated with decreasing concentrations of ethanol (100%, 90%, 80%, 70%) and then immersed in PBS. Any potential endogenous peroxidase activity was blocked by immersion for 15 minutes in 2% H$_2$O$_2$. After washing with PBS, slides were then loaded into Sequenza racks (Thermo Scientific 72110017). Staining was then begun, by first using the Avidin-Biotin Complex (ABC) staining method to avoid non-specific interaction of the tissue (Avidin/Biotin Blocking Kit – Vector Labs Cat SP-2001). Avidin and biotin were blocked for 10 minutes each with PBS wash in between. A protein block was then added (DAKO Cat X0909) for a further 10 minutes.

After this the primary antibody was added (Rabbit anti-mouse HO-1 polyclonal IgG, (Stressgen UK) in 125\(\mu\)l antibody diluent (DAKO REAL Ab diluent S2022). This was initially at a dilution of 1/250 but on subsequent attempts at
increasing concentrations of 1/100 and 1/25. This was left overnight at 4°C and then washed twice with PBS. The secondary antibody was then added: biotinylated anti-rabbit antibody 1/100 dilution in 125µl antibody diluent. This was incubated for 1 hour at room temperature and then washed twice with PBS. Vectastain RTU Elite (Vector labs- PK-7100) was then added for 30 minutes, and washed 3 times with PBS. 125µl diaminobenzidine (DAB) solution was then added (liquid DAB + substrate chromogen system – DAKO K3468). The slides were then washed twice in PBS and transferred to a rack for immersion in haematoxylin for 20 seconds for counterstaining. After rinsing in PBS, the slides were then dehydrated through baths of increasing concentrations of ethanol solution for 1 minute each prior to being immersed in xylene. They then had coverslips applied to prevent degradation. Brown staining by light microscopy detected HO-1 protein. Appropriate isotype antibodies were used as negative controls.

2.12 Assessment of murine renal function

Plasma samples were analysed for creatinine concentration using the Jaffe method (Alpha laboratories ltd, UK) on a Cobras Centrifugal Analyser (Roche diagnostics, UK) as per the manufacturer’s instructions by Dr Forbes Howie, department of Clinical Chemistry, QMRI.
3 HACS Clinical Trial Results – Primary and Secondary Outcome Measures

3.1 Introduction
Participants were recruited from the Cardiac surgery clinic at RIE and invited to the CRF for trial participation. As the trial was designed to study patients remote from the time of surgery, there had to be a minimum of 2 weeks prior to surgery at the time of study drug administration. All randomised participants received the study drug. There were no withdrawals and no participants were lost to follow up.

3.2 Participant demographics
Participants were randomised to receive either 1mg/kg or 3mg/kg HA up to the maximum licensed dose of 250mg. The participant demographics (
Table 9) were typical of this patient population in Scotland with a high proportion of individuals with hypertension, Type II diabetes and a history of smoking. It is important to note that this was an elderly group of participants with a median age of 73 years and 74 days at the time of infusion (range 61-83) years. This is in marked contrast to other Phase I and Phase II clinical trials of HA.
Table 9: Demographic data of HACS participants. AVR, aortic valve replacement; CABG, coronary artery bypass graft; IHD, ischaemic heart disease; MI, myocardial infarction; CKD, chronic kidney disease; BMI, body mass index

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>1mg/kg HA</th>
<th>3mg/kg HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Age (median, range)</td>
<td>69 (61-83)</td>
<td>73.5 (62-79)</td>
</tr>
<tr>
<td>Female</td>
<td>0 (0%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>AVR</td>
<td>1 (9.1%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>CABG</td>
<td>10 (90.9%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Caucasian</td>
<td>11 (100%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>IHD</td>
<td>10 (90.9%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>History of MI</td>
<td>1 (9.1%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>History of stroke</td>
<td>0 (0%)</td>
<td>2 (20%)</td>
</tr>
<tr>
<td>Type II Diabetes</td>
<td>5 (54.5%)</td>
<td>3 (30%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>6 (54.5%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>CKD</td>
<td>3 (27.3%)</td>
<td>1 (10%)</td>
</tr>
<tr>
<td>Additive Euroscore</td>
<td>2.80 (1.63)</td>
<td>3.00 (1.05)</td>
</tr>
<tr>
<td>(mean, sd)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (mean, sd)</td>
<td>29.04 (3.67)</td>
<td>29.29 (4.13)</td>
</tr>
<tr>
<td>Statin</td>
<td>10 (90.9%)</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>Smoking</td>
<td>7 (63.6%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Alcohol use</td>
<td></td>
<td></td>
</tr>
<tr>
<td>moderate/heavy</td>
<td>5 (45.5%)</td>
<td>5 (50%)</td>
</tr>
</tbody>
</table>
3.3 HO-1 protein in PBMCs

There was no significant increase in heme oxygenase 1 protein expression in PBMCs for either dose of HA compared to baseline expression levels (Figure 12). For the 1mg/kg dose, the HO-1 protein in PBMCs changed from a median of 0.80 (0.42-4.48) to 0.85 (0.24-3.32) ng/ml, p=0.56 and for the 3mg/kg dose from a median of 0.91 (0.38-2.31) to 1.76 (0.88-2.14) ng/ml, (p=0.1602).

Figure 12: There is no significant difference in HO-1 protein in PBMCs from baseline when measured 24 hours after HA dose. (a) 1mg/kg dose, (b) 3mg/kg dose. Data shown are median and interquartile range, Wilcoxon matched-pairs signed rank test (not significant)
3.4 HO-1 mRNA in PBMCs
There was a significant increase in HO-1 mRNA expression in PBMCs at 24 hours post HA infusion when compared with the housekeeping gene 18S (Figure 13). For the 1mg/kg dose, the HO-1 mRNA expression in PBMCs increased by a median of 1.413 fold (1.13-1.80), p=0.014 and for the 3mg dose by a median of 2.271 fold (1.78-2.85) p=0.004.

Figure 13: HO-1 gene expression in PBMCs. (a) 1mg/kg dose of HA, (b) 3mg/kg dose of HA. Data shown are median and interquartile range, Wilcoxon matched-pairs signed rank test (*p<0.05, **p<0.01).

3.5 HO-1 protein level in serum
There was a significant increase in HO-1 protein in serum (Figure 14) from a median of 0.41 (0.37-0.61) to 0.61ng/ml (0.45-0.95) for the 1mg/kg dose, and from 0.26 (0.18-0.42) to 0.63ng/ml (0.45-0.75) for the 3mg/kg dose. P=0.002 and p=0.006 respectively.
Figure 14: There was a significant increase in HO-1 protein expression in the serum in both doses (a) 1mg/kg dose of HA, (b) 3mg/kg dose of HA. Data shown are median and interquartile range, Wilcoxon matched-pairs signed rank test. (**p<0.01)

3.6 Genotyping
The GT length polymorphism (GT<sub>n</sub>) dinucleotide repeat in the promoter region of the HO-1 gene was analysed for each participant. 11 participants were homozygous for LL, 2 were homozygous for SS and 8 were heterozygous (SL). Participants were grouped by genotype rather than dose due to the small numbers but there was no significant difference in HO-1 gene expression or HO-1 protein expression in PBMCs between the groups (Figure 15).
Figure 15: Effect of genotype of HACS trial participants (a) Effect of HA on HO-1 protein in PBMCs according to HO-1 genotype (b) Effect of HA on HO-1 gene expression compared to the ribosomal housekeeping gene 18S. LL: homozygous for long GTn repeats

Genotype

Genotype (RNA)

3.7 Urinary biomarkers

There was no significant difference in the measured urinary biomarkers NGAL, KIM-1 and IL-18 in participant urine over the course of the study (Figure 16). This result will allow these biomarkers to be used for patient risk stratification.
in recruitment to future Phase IIb and Phase III clinical trials of HA in this cohort of patients, knowing that their result has not been affected by HA. Statistical analysis was by repeated measures one way ANOVA.

Figure 16: Urinary biomarkers (a) NGAL (b)KIM-1 (c)IL-18 adjusted to urinary creatinine concentration prior to dose administration and at subsequent study visits on day 1 and day 7. There was no significant difference in any of the urinary biomarkers for either dose.
3.8 Urinary HO-1

There was no significant difference in urinary HO-1 levels on days 1 or 7 for either the 1mg/kg or 3mg/kg doses (Figure 17). HO-1 has been shown to be a potential biomarker for oxidative stress in the kidney(125) and therefore it is of
interest to note that HA administration at these doses does not affect urinary HO-1 levels. Statistical analysis was by repeated measures one way ANOVA.

Figure 17: Urinary HO-1 concentration adjusted to creatinine prior to HA dose administration and at subsequent study visits on day 1 and day 7.
3.9 Complement

There was no significant difference in C1q levels in the serum of 6 participants tested before and 24 hours after their dose of heme arginate. Fewer than the 21 samples were analysed because of cost constraints but the samples remain in storage for future work.

**Figure 18** Serum C1q of the first 6 HACS trial participants. There was no significant reduction in C1q following infusion of HA (data shown are mean, standard deviation. Paired t test, p=0.3340)
3.10 Summary of results

- HA did not significantly upregulate HO-1 protein in PBMCs of HACS study participants
- HA did significantly upregulate HO-1 gene expression in PBMCs
- HA significantly upregulated HO-1 protein in participant serum
- There was no effect of genotype of the GT length polymorphism (GT<sub>n</sub>) dinucleotide repeat in the promoter region of the HO-1 gene on HO-1 levels in this cohort – the caveat being that numbers are small
- HA did not affect levels of urinary biomarkers of AKI or HO-1 levels in the urine
- HA did not affect complement C1q levels in 6 patients receiving HA
4 Chapter 4 – Safety of Heme arginate in HACS trial

4.1 Introduction

There were no safety concerns reported in the Phase I studies in healthy individuals (61,62) and it is reassuring that the Phase II HOT study (64)did not report any serious adverse events given its cohort of patients with renal disease undergoing kidney transplantation. However, the nature of cardiac disease requiring cardiac bypass operations (coronary artery bypass grafting and valve replacement) means that these patients have more comorbidity such as Type II Diabetes, hypertension, and peripheral vascular disease. There are more perioperative risks than in procedures such as kidney transplantation.

Although HA has been used for many years in the treatment of acute porphyria, there has been little study of its effects in patients who are elderly or who have significant comorbidity. There is a theoretical risk of inflammation caused by administering iron. The maximum dose of HA (250mg) contains 22mg of iron and although this would be given as a single dose rather than repeated over a period of days, we felt it was important to quantify any potential risk. There are possible risks of worsening infection (90,91), and oxidative stress. The risks of iron administration and its possible association with vascular calcification (128) is not thought to be of relevance in this setting as this have been associated with longer term rather than short term dosing.

It was felt that giving this drug at the time of surgery to a cohort of relatively elderly and comorbid patients with cardiac disease may be considered too risky for a Phase IIb study, therefore further data were required in order to inform perioperative risk assessment. The aim of HACS would be to inform a large Phase IIb study of HA as a prophylactic treatment of patients at higher risk of AKI undergoing cardiac surgery.
4.2 Adverse events

4.2.1 Surveillance of adverse events

Safety was determined by the recording of adverse reactions, adverse events and by measurement of physiological parameters and standard laboratory measures of inflammation. Study team members were encouraged to report any possible adverse events and participants were directly questioned on occurrence of possible adverse events at every study visit. Phlebitis was not recorded as an adverse event as this is a known side effect of HA infusions (129). Only one of the 21 participants experienced this.

There were no serious adverse events, but there were 7 adverse events reported in 5 patients (Table 10). These included upper respiratory tract symptoms, light-headedness on standing (in the absence of demonstrable postural hypotension), polyuria and intermittent abdominal pain.

Table 10: Adverse Events of the HACS study. SAE, serious adverse event; CTIMP, clinical trial of an investigational medicinal product

<table>
<thead>
<tr>
<th>Study number</th>
<th>Dose of HA (mg/kg)</th>
<th>Description of event</th>
<th>SAE</th>
<th>Causality due to CTIMP</th>
<th>Severity</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>3</td>
<td>Upper respiratory tract symptoms</td>
<td>No</td>
<td>Unrelated</td>
<td>Mild</td>
<td>7 days</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Left sided abdominal pain</td>
<td>No</td>
<td>Unrelated</td>
<td>Mild</td>
<td>1 day</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Light-headedness on standing</td>
<td>No</td>
<td>Possibly related</td>
<td>Mild</td>
<td>1 day</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>Urinary tract infection</td>
<td>No</td>
<td>Possibly related</td>
<td>Moderate</td>
<td>6 days</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>Coryzal symptoms</td>
<td>No</td>
<td>Unrelated</td>
<td>Mild</td>
<td>3 days</td>
</tr>
<tr>
<td>12</td>
<td>3</td>
<td>Lower respiratory tract infection</td>
<td>No</td>
<td>Possibly related</td>
<td>Mild</td>
<td>5 days</td>
</tr>
<tr>
<td>20</td>
<td>1</td>
<td>Polyuria</td>
<td>No</td>
<td>Possibly related</td>
<td>Mild</td>
<td>12 hours</td>
</tr>
</tbody>
</table>
Of the 5 patients who experienced adverse events, only 2 of them received the higher dose of HA. Participant 3 experienced upper respiratory tract symptoms (sore throat, runny nose) which he stated started, in retrospect, prior to the infusion. He also experienced some left sided abdominal pain 5 days after the infusion, which lasted only a few hours. He had experienced this several times previously and was not concerned. Physical examination of his respiratory and abdominal systems was normal. CRP was normal throughout (4, 4, 5 on visits 1, 2 and 3 respectively) as was WCC (4.5, 4.7, and 4.4 on visits 1, 2 and 3 respectively).

Participant 6 reported some symptoms of light-headedness on standing which began the same day as the infusion (although after the 6 hour observation period) and lasted several hours. There was no collapse or loss of consciousness. At the 24 hour review appointment, the symptoms had resolved, physical examination was normal and there was no postural drop in blood pressure. At the third study visit (day 7) participant 6 reported a second adverse event, of a urinary tract infection. This was diagnosed by his general practitioner (GP) based on symptoms of dysuria and urinary frequency and for which he was given antibiotics. His symptoms had completely resolved by this review. His CRP was only mildly elevated at 38 on visit 3 (up from 2 at both previous visits) and his WCC was elevated at 13.6 having been 8.1 at visit 1 and 8.9 at visit 2.

Participant 9 reported some coryzal symptoms of sore throat, mild headache, and cough which were improving by the time of assessment at visit 3 (day 7 post dose). His inflammatory markers (WCC, CRP) were not elevated.

Participant 12 reported that he had developed a cough productive of green sputum and had received a course of antibiotics from his GP between study visits 2 and 3. His symptoms were much improved by visit 3 and his
inflammatory markers were only mildly elevated (CRP 45 up from 2 at visit 1, 3 at visit 2). His WCC did increase slightly although not outwith the normal range (5.3, 9.2, and 6.8 on visits 1, 2 and 3 respectively).

Participant 20 had symptoms of polyuria which lasted only a few hours after the infusion. It was unclear if there was a causative link for this with HA, there was no change in his urine dipstick to suggest infection and no change in serum sodium.

4.3 Protocol deviations and violations

There were 9 protocol deviations in 8 participants (Table 11). None were felt to have had any impact on either the safety of the trial participants or the study outcomes.

Table 11: Protocol Deviations

<table>
<thead>
<tr>
<th>Study number</th>
<th>Description of deviation</th>
<th>Could this occurrence have an impact on patient safety?</th>
<th>Could this occurrence have an impact on study outcomes?</th>
<th>Site corrective or preventative actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>serum albumin not processed by laboratory</td>
<td>No</td>
<td>No</td>
<td>More rigorous checking of samples in progress</td>
</tr>
<tr>
<td>6</td>
<td>Serum ferritin not processed by laboratory</td>
<td>No</td>
<td>No</td>
<td>More rigorous checking of samples in progress</td>
</tr>
<tr>
<td>6</td>
<td>Participant visit 3 outwith stated time window (24 hours late)</td>
<td>No</td>
<td>No</td>
<td>Arranging visits to fit around patient schedule</td>
</tr>
<tr>
<td></td>
<td>Description</td>
<td>Correct?</td>
<td>Action?</td>
<td>Additional Information</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------------------------------------------------</td>
<td>----------</td>
<td>---------</td>
<td>-------------------------------------------------------------</td>
</tr>
<tr>
<td>7</td>
<td>180 minute time point observations taken 3 minutes late</td>
<td>No</td>
<td>No</td>
<td>More rigorous attention to timekeeping</td>
</tr>
<tr>
<td>8</td>
<td>Participant visit 3 outwith stated time window (24 hours early)</td>
<td>No</td>
<td>No</td>
<td>Arranging visits to fit around patient schedule</td>
</tr>
<tr>
<td>9</td>
<td>Urine ACR not analysed by the laboratory</td>
<td>No</td>
<td>No</td>
<td>More rigorous checking of samples in progress</td>
</tr>
<tr>
<td>10</td>
<td>Full blood count visit 2 – insufficient sample</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Participant 2 hours late for visit 2</td>
<td>No</td>
<td>No</td>
<td>Arranging visits to fit around patient schedule</td>
</tr>
<tr>
<td>12</td>
<td>Full blood count visit 1, 6 hour time point sample clotted</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
</tbody>
</table>

There were two protocol violations. Participant 9 was found, at visit 3, to have already been enrolled in another clinical trial (High-Sensitivity Troponin in the Evaluation of Patients with Acute Coronary Syndrome: HighSTEACS, clinicaltrials.gov NCT01852123). This was not a CTIMP and neither the integrity of the HACS trial nor HighSTEACS, nor the safety of the patient involved were compromised. However, enrolment in other clinical trials was an exclusion criterion and therefore upon discussion with the trial monitors this was deemed to be a protocol violation. The error had occurred because the patient themselves did not realise that HighSTEACS was a clinical trial, and had forgotten that at the time of his myocardial infarction that he had consented to his participation. The paper medical notes were not available at the time of drug administration, and the trial team did not see the alert of trial participation on his electronic patient record (EPR). Following this violation, paper notes were obtained and reviewed more thoroughly prior to dose administration and alerts were double checked on the EPR.
The second protocol violation was in participant 12. This was an incorrect recording of the patient weight on the drug prescription sheet. The weight was recorded in the paper case report form as 110kg, but on the drug prescription sheet as 100kg. In this case, the participant was randomised to receive the larger dose of 3mg/kg and so there was no drug administration error (they received the maximum dose of 250mg). However, if they had been randomised to receive 1mg/kg they would have been underdosed by 10mg. This violation was detected on review of the trial ISF by the monitor at the end of the study. There were no other such errors detected.

4.4 Physiological parameters are not significantly affected by heme arginate in elderly patients planned for cardiac surgery

Pharmacodynamic safety surveillance included the measure of blood pressure, pulse rate, oxygen saturations, respiratory rate and tympanic temperature in order to monitor any physiological effects of the infusion of HA. Direct medical supervision was carried out for the 6 hours following the study drug infusion. For individual participants, there were no clinically concerning changes in any of these parameters during the study to prompt concerns about safety at the time of infusion or in the hours of observation that followed. Physiological parameters for each group were analysed following the study, statistical analysis was by repeated measures one way ANOVA. There was no significant difference in mean arterial pressure following the dose, \( p=0.0537 \) for 1mg/kg and \( p=0.1834 \) for 3mg/kg group. Similarly, there was no significant difference in pulse rate (\( p=0.2486 \) for 1mg/kg and \( p=0.187 \) for 3mg/kg) or temperature (\( p=0.0662 \) for 1mg/kg and \( p=0.0635 \) for 3mg/kg) for either dose. There was no significant difference in oxygen saturations for the 1mg/kg dose (\( p=0.0702 \)), but there was a difference in saturations from baseline measurement at certain time point for the 3mg/kg dose. This was significant at 30 minutes, 45 minutes, 2 hour and 5 hour time points (\( p=0.0103, 0.0223, 0.0341 \) and 0.0246 respectively). The greatest difference was from the
baseline mean of 96.9% (SD 1.449) to 94.3% (SD 2.111) at 30 minutes post dose.

There was a statistically significant difference in respiratory rate from baseline for the 1mg/kg group at timepoint 45 minutes (p=0.0435) and in the 3mg/kg group at 1 hour 15 minutes (p=0.0358) and at 4 hours (p=0.0239).

The difference in respiratory rate may be explained by the effect of relative relaxation following a period of anxiety at the time of study drug administration and this, together with the effect of the study participants sitting up at 45 degrees in a bed, rather than upright in a chair or standing, could have affected their vital capacity enough to cause the small decrease in oxygen saturations. Importantly, this was not sustained and there were no respiratory symptoms at the time.

Figure 19: Physiological parameters following study drug administration. (a) mean arterial pressure (b) pulse rate (c) tympanic temperature (d) oxygen saturations (e) respiratory rate

Mean arterial pressure during follow up

- 1mg/kg
- 3mg/kg

Time post dose
oxygen saturations

![Graph showing oxygen saturations (%) over time post dose for 1mg/kg and 3mg/kg doses.]

respiratory rate

![Graph showing respiratory rate (breaths per minute) over time post dose for 1mg/kg and 3mg/kg doses.]

Time post dose
4.5 Measures of inflammation

As part of the safety assessment in this relatively vulnerable group of patients, routine measures of inflammatory markers and other routine blood tests were collected at time points 0h (immediately pre dose of study drug), 6 hours post dose, 24 hours post dose and 7 days post dose (Figure 9). There was no significant difference in C-reactive protein (CRP) in either group, p=0.3296 for the 1mg/kg and p=0.2550 for the 3mg/kg group, repeated measures one way ANOVA (Figure 20).

There was no significant difference in white cell count, another marker of inflammation. Ferritin was measured as a surrogate of iron load and of inflammation. Again, there was no significant difference in ferritin at each time point for either dose (p=0.2511 for 1mg/kg, 0.5012 for 3mg/kg)

Bilirubin was measured given it is a product in the breakdown of heme by the enzyme HO-1, as a crude measure of enzyme activity. There was no significant difference in bilirubin at any time point from baseline in either group (p=0.4841 for 1mg/kg, p=0.3594 for 3mg/kg)

Figure 20: There was no effect of HA dose on markers of inflammation and surrogate of HO-1 activity; (a) CRP (b) WCC (c) ferritin (d) Bilirubin . Repeated measures ANOVA, data
shown are mean and standard deviation except for ferritin which is median and interquartile range as these data are not normally distributed.

**CRP**

**White cell count**
4.6 Summary of key findings

- There were no serious adverse events in this trial of patients known to be at high risk of significant comorbidity
- HA was well tolerated in this patient group
- The adverse events were varied in nature and there was no signal of harm
- There was no physiological upset caused by the study drug
- The theoretical risk of increased susceptibility to worsening infection remains to be clarified; larger randomised controlled trials would potentially help
- There were 2 protocol violations, neither of which compromised either patient safety or the study results but they highlighted the need for vigilance in clinical trial recruitment and management
5 Chapter 5 – Dimethyl fumarate (DMF)

5.1 Introduction

The transcription factor Nrf2 is activated by cell injury including the effects of IRI and leads to the induction of many genes that combat oxidative stress (134) (Figure 8). Dimethyl fumarate, a drug licensed for use in multiple sclerosis and psoriasis, is known to be an Nrf2 agonist (131) and preclinical data in models of myocardial infarction showed that DMF treatment could protect against ischaemic injury in the heart (71,72). DMF can modify macrophage behaviour and phenotype; limiting their pro-inflammatory actions while also improving their anti-inflammatory capacity such as increasing phagocytosis(75). As DMF is in wide clinical use for other indications with a good safety profile, it is an attractive candidate in the search for a new agent to either treat AKI or prevent it in higher risk patients.

The work described in this chapter tested the following hypotheses:

- HO-1 will upregulate expression of Nrf2 dependent genes, including HO-1, in cultured murine bone marrow derived macrophages (BMDMs)
- DMF will increase the phagocytic capacity of cultured BMDMs for apoptotic cells
- DMF administration to mice will upregulate the expression of Nrf2 dependent genes, including HO-1, in the murine kidney
- DMF administration to mice will confer protection from renal ischaemia reperfusion injury
- DMF will upregulate Nrf2 genes including HO-1 in human monocyte derived macrophages (MDMs)

The aim of this work is to act as the next step in the development of DMF as a potential prophylactic treatment for high risk individuals to reduce their risk of AKI when undergoing cardiac surgery.
5.2 BMDMs 24 hour treatment with DMF

BMDMs in culture were treated with increasing concentrations of DMF and examined after cytospin and “Diff-Quick” staining at 24 hours (Figure 21). For all concentrations of DMF the cells appeared to be healthy at 24 hours, with good confluence and adherence to the culture dish with no change in morphology. A lactate dehydrogenase (LDH) assay was performed to quantify any potential cytotoxicity and was only significant for the 50µM concentration with an increase from mean 2.87(SEM 1.72) to 9.71(1.89)% cell lysis after 24 hours (Figure 22).

Figure 21: BMDMs treated with increasing concentrations of DMF vs DMSO control; no outward appearance of cell injury

![Image of cytospin images showing BMDMs treated with different concentrations of DMF compared to control](image-url)
Figure 22 - LDH assay of BMDMs treated with vehicle or DMF up to 50µM. After 24 hours the supernatant was harvested and an LDH assay performed. The LDH level from BMDMs deliberately lysed was deemed to be 100%. Only the 50µM concentration showed a significant increase in cell lysis. Data shown are mean and SEM from 3 individual experiments with 3 replicates in each. P=0.0277 one way ANOVA for vehicle vs 50µM DMF, all others not statistically significant.

**LDH assay**

5.3 DMF dose response in BMDMs *in vitro*

Murine bone marrow derived macrophages (BMDMs) were incubated for 6 hours with differing concentrations of DMF and the transcription of key genes
in the Nrf2 pathway was assessed by qPCR (Figure 23). HO-1 mRNA expression was increased at all concentration of DMF used with a dose dependent effect being clearly seen. The nrf2-dependent gene glutathione reductase (GSR) was also increased at all concentration of DMF used but there was no dose dependency evident. This was a pilot experiment with 3 replicates and could not be repeated due to time constraints, hence there is no statistical analysis.

Figure 23: Increased transcription of key genes in the Nrf2 pathway was evident after treatment with DMF including (a) HMOX1 (Heme oxygenase-1) and (b) GSR (Glutathione reductase).
5.4 DMF time course: Pilot experimental data

The optimum time for treatment of BMDM cells was determined in an experiment to understand gene expression of genes in the Nrf2 pathway following different duration of exposure to DMF \textit{in vitro} (Figure 24).
Figure 24: Time course of dimethyl fumarate treatment of bone marrow derived macrophages \textit{in vitro} using known downstream genes from Nrf2. Pilot experiment with BMDMs from 3 mice in one experiment.
In this pilot experiment, the time taken for upregulation of the key readouts of the Nrf2 pathway appeared to be maximal at 6 hours, and returned to baseline by 48 hours. For that reason, 6 hours of treatment with DMF was used for future work.

5.5 Exploration of the effect of DMF on macrophage phenotype

Previous studies have shown that DMF may influence macrophage phenotype by making the cells switch from a more pro-inflammatory to anti-inflammatory phenotype (75). This could occur through the expression of nrf2-dependent anti-inflammatory genes or through scavenger receptors such as CD36 that are involved in macrophage phagocytosis of apoptotic cells (132,133). As the phagocytosis of apoptotic cells by macrophages can also promote the acquisition of the anti-inflammatory, reparative M2 phenotype, an experiment was designed to determine the phagocyte activity of BMDMs after pre-treatment with DMF.
Phagocytosis of apoptotic cells is a key macrophage function and promotes the switch to the more anti-inflammatory (M2) phenotype of macrophages via release of IL-10 and TGFß. Clearance of injured cells before cell necrosis occurs prevents inflammatory mediators being released, which propagate inflammation and maladaptive repair. A treatment which enhances the clearance of apoptotic cells would be predicted to be beneficial.

DMF was added to cultured BMDMs for 24 hours prior to performing an assay of phagocytosis using apoptotic murine thymocytes which had been stained with fluorescent green dye. Thus, following macrophage detachment any macrophages that had ingested green apoptotic cells could be detected by flow cytometric analysis. The flow cytometric phagocytosis assay was used to test the hypothesis that DMF would increase macrophage capacity to ingest apoptotic cells. This was based on previous data in murine models of neuroinflammation (75) and malaria (133).

In order to demonstrate that the phagocytic capacity of macrophages could be increased some BMDMs were treated with dexamethasone as previous work indicated that corticosteroids significantly augmented apoptotic cells ingestion by macrophages(134). In addition, a control phagocytosis assay was used employing the ingestion of fluorescent beads with flow cytometric quantification. If DMF did exert an enhancing effect then this control would enable an assessment of whether the increase was a general increase in phagocytic capacity for any particle or whether it was specific for apoptotic cells as their clearance is key to the resolution of inflammation in vivo.

As can be seen in Figure 25 there was significantly less apoptotic cell phagocytosis by macrophages quantified after treatment with 50µM DMF.
There was no significant difference in apoptotic cell phagocytosis at the lower concentrations of DMF. In addition, dexamethasone that was included as positive control but did not increase apoptotic cell phagocytosis. This is a high level of AC phagocytosis for this assay and this may make it hard to see an increase as the assay could be saturated. Future work would require an assay with a lower level of AC phagocytosis (by having a shorter duration, reduced AC number) aiming for a 35-40% phago signal so that an increase would then be more readily identified.

Figure 25: Phagocytosis assay of apoptotic cells.
Figure 26: Phagocytosis assay of Fluorescent beads. There was no significant difference in phagocytosis of fluorescent beads by flow cytometry.

![BMDM Bead Phagocytosis](image)

5.5.2 Griess assay

Figure 27: DMF may ameliorate the M1 phenotype. This was a pilot experiment (n=1) exploring the effect of DMF on the induction of the pro-inflammatory M1 macrophage phenotype by LPS/IFN-gamma. The measurement of nitric oxide concentration in the cell culture supernatant by Griess assay was used to assess the M1 phenotype.
Although macrophage phagocytosis is key to the resolution of inflammation a pilot study explored whether DMF could modulate the generation of nitric oxide (NO) by BMDMs induced to adopt a classical pro-inflammatory M1 phenotype. BMDMs were treated with LPS/IFN-gamma in the presence or absence of 20 or 50µM DMF (control vehicle DMSO). After 24 hours the cell culture supernatant was removed and NO generation assessed by the Griess assay. Untreated M0 macrophages served as control for any spontaneous NO release by cultured BMDMs.

Figure 27 depicts the pilot data generated. Treatment with both 20µM and 50µM DMF reduced NO generation to levels comparable to M0 macrophages with no discernible effect of the vehicle DMSO. Although striking, this is preliminary pilot data only and further detailed experiments are required to first determine if this effect if reproducible. If the anti-inflammatory effect of DMF upon the induction of M1 macrophages and subsequent NO release is reproducible then full time course and dose dependency studies need to be performed.
5.6 Dimethyl fumarate in vivo – dose titration

DMF was given to mice by gavage once daily for 3 days and then animals were culled 24 hours after their final dose. There were 3 animals in each group, with a PBS control, DMSO as vehicle control, DMF at 10, 30 and 100mg/kg doses in each experimental group.

5.6.1 Dimethyl fumarate in vivo – dose effect on gene expression by qPCR

In parallel with in vitro experiments a series of in vivo studies were undertaken. The first study involved a dose titration of DMF followed by an assessment of the renal expression of 3 well described nrf2 dependent genes 24 hours later. DMF was administered to mice by gavage once daily for 3 days and then animals were culled 24 hours after their final dose. There were 3 animals in each group and groups included a PBS control and a DMSO vehicle control. DMF was administered at doses of 10, 30 and 100mg/kg in each experimental group. Expression of key genes in the Nrf2 pathway was determined via qPCR (Figure 28)

Figure 28: Upregulation of Nrf2 pathway genes by DMF in vivo. N=3 per group. (a) HMOX1 (b) NQO1 (c) GSR. Only HMOX1 at a dose of 30 and 100mg/kg reached statistical
significance when compared to vehicle (DMSO) by ordinary one-way ANOVA with multiple comparisons (p=0.0114)

5.6.2 Dimethyl fumarate in vivo – dose effect on immunohistochemistry for HO-1

A preliminary experiment was carried out to ensure that DMF upregulated HO-1 in the kidney. Comparison was made with splenic tissue which is known to be rich in HO-1 given its role in breaking down red blood cells. Heme arginate
treatment was used as a positive control given previous data from carrying out IRI experiments with HA (56).

Figure 29: Immunohistochemistry staining for HO-1 showing the increase in staining by treatment with DMF and HA. A-D; Vehicle control (DMSO), E-H; DMF 10mg/kg, I-L; HA 3mg/kg. A,E,I; heart, B,F,J; liver, C, G, K; spleen, D,H,L kidney. Magnification x50.
5.7 Dimethyl fumarate \textit{in vivo} – ischaemia reperfusion injury

Preliminary experiments were performed on young male C57BL6 mice (aged 8-12 weeks) to explore whether pre-treatment with DMF could confer protection in a translationally relevant animal model of acute kidney injury. Experimental mice were dosed with 30mg/kg DMF (dissolved in DMSO) by daily gavage for 3 days by a trained animal technician. Control mice were administered DMSO (the vehicle for DMF). There were 8 animals per group. Unilateral IRI surgery was performed 24 hours after the third dose of DMF. There were also sham surgery control groups: DMF 30mg/kg (dissolved in DMSO) and DMSO alone (vehicle control for DMF), both with 3 animals per group.

Renal tissue was examined and unfortunately the injury was too severe in all cases to make any assessment of DMF effect (Figure 30).

5.7.1 Experimental injury was too severe to allow for rescue from injury by pre-treatment with DMF – all sections showed severe injury
The experimental model called for an ischaemic time of 22 minutes which was felt to be a modest injury in our laboratory practice. However, upon inspection of the renal tissue it became clear that the degree of injury was too severe to allow for rescue of the injury. This could not be repeated because of time constraints but in the future the experiment would be repeated with another attempt at clarifying the optimum time of ischaemia to cause modest injury, followed by a repeat of the experiment looking at the effect of DMF.

Specimens were prepared for immunohistochemistry to determine if HO-1 had been upregulated by the addition of DMF. Samples were prepared for qPCR of genes of interest but unfortunately there was not enough time to allow for analysis of these samples. This would be a priority in future work.
There was no significant difference in creatinine values for the two groups

Blood was analysed for creatinine values. There was no significant difference in creatinine in the DMSO group vs the DMF group (Figure 31) which may reflect the fact that the injury was too severe to ameliorate.

Figure 31: Creatinine values of mice treated with DMF prior to IRI and vehicle only (DMSO). There was no significant difference in creatinine between the DMSO and DMF treated groups.
5.8 Human monocyte derived macrophages

Human MDMs were treated with DMF to determine what dose in vitro would upregulate the Nrf2 pathway (Figure 32). Cells were plated for 24 hours and then treated with DMF or control for 6 hours. The upregulation of HMOX1 was statistically significant at both concentrations vs DMSO control (p=0.0004, ordinary one way ANOVA). NQO1 and GSR appeared to be upregulated but could not be analysed as only 2 experiments were carried out.
Figure 32: assessment of key genes downstream in Nrf2 pathway in human MDMs following treatment with DMF in vitro. N=3 for HMOX1 (a), N=2 for NQO1(b) and GSR (c). There was significant upregulation in HMOX1 for both the 10 and 50µM doses compared to vehicle.
5.9 Discussion and limitations

Much of the work described in the chapter is of a preliminary nature as a result of limited time for experiments. However, there are some signals that are encouraging and form the basis for further work.

- The work suggests that doses of DMF below 50µM are non-toxic to BMDMs with no discernible effect upon cell morphology or LDH levels in a cytotoxicity assay. Thus the interpretation of data generated with the 50µM DMF dose should be in the knowledge that there may be adverse effects upon macrophage health and function. The work does however suggest that DMF is able to upregulate the expression of nrf2-dependent genes, including HO-1, in cultured BMDMs at doses that are non-toxic.

- Study of the effect of DMF upon the phagocytic capacity of cultured BMDMs for apoptotic cells was complicated by several factors. First, the level of phagocytosis was very high and this can make the detection of an augmentation of phagocytosis of apoptotic cells a challenge. Indeed, dexamethasone was added to BMDMs as a positive control to indicate that the BMDMs were able to increase their phagocytic capacity but this proved ineffective. Interestingly the treatment of BMDMs with 50µM appeared to exert a marked effect upon apoptotic cell ingestion and this may be related to a toxic effect as phagocytosis is a complex process that involves dramatic changes in the cytoskeleton and energy expenditure by the cell.

- The phagocytosis of beads was at a much lower level than apoptotic cells and no augmentation was evident suggesting that phagocytosis
for inert particles was not modulated by DMF. Indeed, similar to the ingestion of apoptotic cells, the ingestion of beads appeared to be reduced by DMF at the 50µM concentration and may reflect a cytotoxic effect of DMF at this concentration.

- Macrophage phenotype (a spectrum from pro-inflammatory, ‘M1’, to pro-repair, ‘M2’) is important in tissue injury and repair and NO generation is a marker of the pro-inflammatory M1 phenotype. It is thus of interest that very preliminary study (n=1) of the effect of DMF upon NO generation by LPS stimulated M1 macrophages showed an inhibitory effect at both the 20µM and 50µM concentrations. As indicated previously all experiments that used the 50µM concentration should be interpreted with caution but the 20µM concentration of DMF appeared to be non-toxic. Significant further work is required in this area.

- Preliminary studies were undertaken using human MDMs to explore the effect of DMF upon nrf2-dependent genes. Interestingly, both 10µM and 50µM DMF significantly upregulated HO-1 gene expression. Upregulation of both NQO1 and GSR was seen but further studies are required as only 2 biological replicates were performed due to time constraints.

Preliminary in vivo studies demonstrated that DMF administration (30mg/kg x3) to mice upregulate the expression of nrf2-dependent genes, including HO-1, in the murine kidney. However, it was not possible to determine whether DMF administration to mice might confer protection from subsequent renal IRI. This was because of several factors including marked severity in the level if functional renal injury and the possibility that the DMF was nephrotoxic at the dose administered. Significant further work is required.
6 Discussion and Future Work

6.1 Results of the HACS clinical trial – where this may lead

The primary outcome of protein expression in PBMCs showed that a dose of either 1mg/kg or 3mg/kg HA did not significantly upregulate HO-1 at the protein level in this patient group. This was somewhat unexpected given published preclinical studies (56,60) and clinical trials in healthy volunteers (61) and renal transplant recipients (64). Regarding the latter study, it is noteworthy that, although the renal transplant recipients had end stage renal failure, they were younger (mean age 52 years) than the patients in this study. Furthermore, it is interesting that this result is in keeping with another recent clinical trial which studied the effect of HA treatment on HO-1 protein expression in the heart. Andreas et al showed that HO-1 protein was significantly upregulated in cardiac myocytes but not in circulating PBMCs (63). The mean age of participants in that study was 73±7 years (Table 12) which is similar to the elderly HACS participants. Indeed, the study by Andreas et al also showed that PBMC HO-1 gene transcription was significantly increased, measured as a significant increase in HO-1 mRNA expression evident.

In both this study and the trial undertaken by Andreas et al, it may be that the 24 hour time period was not sufficient to allow for a significant increase in protein expression in this elderly cohort as the translation of certain genes has been shown to be slower in the elderly. This is a field of ongoing research but a recent study of murine liver and kidney tissue has demonstrated that protein synthesis and ribosome biogenesis machinery can become dysregulated with age, specifically at the translational level (135). In addition, although there is reduction in translation overall with increasing age (Reviewed in (136)), some genes may in fact be preferentially translated, including those which promote cellular maintenance and repair. The role of post transcriptional mechanisms (including selective translation) in the control of gene expression in the stress response of aged cells remain controversial, however there is increasing
evidence of this. In a study of *Saccharomyces cerevisiae* yeast, there was a decreased response capacity to osmotic stress with age. Using single cell sequencing of gene transcription and quantification of protein translation, it was demonstrated that the decline in capacity was mainly due to the reduction in rate of translation rather than the transcription of the relevant genes of interest(137).

In animal models, it has been shown that the Nrf2 signalling pathway is disrupted during aging and leads to a decreased antioxidant response(138). In a study of human bronchial epithelial cells, Nrf2-regulated antioxidant genes were compared between participants aged 20-29 and those aged 60-69(139). This study showed that not only was there a lower basal expression of the Nrf2 regulated genes NAD(P)H and quinone oxioreductase 1 (NQO1) in the older age group, but that the induction of these genes by treatment with the Nrf2 agonist sulforaphane was blunted when compared to the young controls. They also demonstrated that there was higher expression of the Nrf2 suppressor genes Bach1 and c-Myc in the aged participants, indicating this could be another mechanism by which there is less inducibility with age. These findings are pertinent as HO-1 gene transcription has been shown to be induced by the Nrf2 pathway.

Another human study examined HO-1 gene expression and other Nrf2 induced genes in peripheral blood mononuclear cells(140). Exercise is known to induce oxidative and metabolic stress (reviewed in (141)) and this study demonstrated that aerobic exercise induced HO-1 gene expression in the PBMCs of younger individuals (mean age 23) but not older subjects (mean age 63). Interestingly, in the older participants there was impaired nuclear import of Nrf2 (required for the key function of nrf2 as a transcription factor). There was no significant difference in HO-1 protein in PBMCs after exercise by Western blot in either age group.
HO-1 protein has been shown to be upregulated with HA at a dose of 3mg/kg in both young and aged mice, and is associated with the protection of aged mice from renal ischaemia reperfusion injury(56). However, the aged mice used in this study were 12-months old and can be regarded as middle-aged rather than elderly. It is unknown whether HA robustly induces HO-1 expression in very aged mice such as mice that are 2 years old.

One criticism of this study would be that the outcome measures have been related to measurements of the expression level of PBMC HO-1 protein or mRNA but not HO-1 function. This was due to practical limitations of feasibility and expense. In other studies, muscle perfusion has been shown to be increased at these doses of HA in young healthy subjects (62) and carboxyhaemoglobin (a measure of carbon monoxide production) was shown by Andreas et al to be increased 24 hours post dose in elderly patients with cardiac disease(63). In another study of healthy volunteers, HA has been shown to increase plasma HO-1 protein concentration and HO-1 activity (as measured by bilirubin production) at 24 hours post 3mg/kg dose(61).

The significant increase in serum HO-1 protein at 24 hours post dose is interesting and suggests that other cells such as parenchymal or endothelial cells may exhibit increased HO-1 expression. Further studies would need to consider whether serum HO-1 is within microvesicles or exosomes and whether it is biologically active within the serum or possibly within cells that may take up microvesicles from the circulation. The relevance of serum HO-1 protein to function would be helpful as the isolation and measurement of serum HO-1 is easier, quicker and cheaper than the measurement of PBMC HO-1 expression so would potentially inform further clinical studies.

This result highlights the challenge in translating Phase I clinical trials into the clinical arena where physiology, and factors such as age and prescription of medicines may play a role in determining the effect of a studied drug. The search for an effective prophylactic treatment or intervention in AKI is ongoing.
AKI in particular is a good example of a condition where there are multiple factors can determine its onset, severity, and duration and therefore identifying a particular therapeutic regimen is challenging.

It is still possible that HA will offer a preventative treatment option to those who are at high risk of developing AKI after planned cardiac surgery. Ongoing clinical studies of HA in cardiac and renal transplant IRI, together with this clinical trial, will inform future proposed phase II trials in this patient group. As with all trials in AKI, the challenge lies in selecting the most appropriate patients to study as AKI is such a varied condition with multiple aetiologies. We would hope that future trials would involve more sophisticated methods of patient selection and adaptive trial design, including the use of better biomarkers for AKI, and repeated risk stratification to guide ongoing treatment.

Table 12: Results from previous Phase I and Phase II clinical trials of heme arginate in the evaluation of heme oxygenase 1 in peripheral blood mononuclear cells. All data given are for dosage of 3mg/kg HA. HO-1, heme oxygenase 1; AVR, aortic valve replacement

<table>
<thead>
<tr>
<th>Trial</th>
<th>Participants</th>
<th>Age</th>
<th>HO-1 protein at 24 hours</th>
<th>HO-1 mRNA at 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doberer 2010 (45)</td>
<td>Healthy volunteers</td>
<td>Range 22-43 years</td>
<td>5 fold increase</td>
<td>2 fold increase</td>
</tr>
<tr>
<td>Thomas 2016 (48)</td>
<td>Renal transplant recipients</td>
<td>Mean 52.4 years, range 25-79</td>
<td>Median 11ng/ml increase (IQR 1.0-37.0)</td>
<td>2.73 fold increase (1.8-3.2)</td>
</tr>
<tr>
<td>Andreas 2018 (47)</td>
<td>Patients undergoing AVR</td>
<td>Mean age 73 +/- 7 years</td>
<td>No significant difference</td>
<td>2 fold increase</td>
</tr>
<tr>
<td>HACS</td>
<td>Patients planned for AVR or CABG</td>
<td>Median 73 years, range 61 to 83</td>
<td>No significant difference</td>
<td>2.27 fold increase</td>
</tr>
</tbody>
</table>
Genotyping of the HMOX promoter region was carried out because polymorphisms in the number of guanosine thymidine dinucleotide repeats \(((\text{GT})_n)\) have been shown to be inversely associated with HMOX1 mRNA expression and HO-1 activity\(^{(69)}\). In previous studies, long repeats (LL, 27 or more GT repeats) have been associated with increased cardiovascular events and mortality in patients with both end stage kidney disease on dialysis and those with peripheral artery disease \(^{(142)}\). In addition, in renal transplant patients, transplant survival is associated with donor (but not recipient) HO-1 genotype; those with the S allele had reduced risk of graft failure when compared to LL, particularly in those with prolonged cold ischaemic time and acute rejection \(^{(143)}\). More recently, patients with longer GT repeats were found to have a 1.58 fold increased risk of AKI (LL when compared with SS) when having cardiac surgery \(^{(144)}\). It is however worth noting that in a different population of patients with sepsis in critical care, the L allele was in fact associated with lower HO-1 concentration in serum, and those patients were less likely to develop AKI than those with the S allele \(^{(145)}\). The study was not powered to detect the effect of patient genotype on response to a dose of HA and this means that it is not possible to draw conclusions from the available HACS data. Further larger scale studies could gather data on HO-1 genotype in order to inform which patients might respond better to HA treatment.

Overall there is a signal that the response to HA in the older population undergoing cardiac surgery may not be as robust as among younger patients. However, how this translates to function is still to be determined. There are further studies of HA in cardiac IRI planned which would inform this question further. Including renal end points in future studies of HA in cardiac IRI would be of interest. The outcome of the HACS2 trial should hopefully also help inform the benefit of HA in the prevention of renal IRI and would provide more evidence to pursue a larger Phase IIb clinical trial of HA at the time of cardiac surgery in patients who are at increased risk of acute kidney injury.
6.2 Should we be concerned about drug safety in this patient cohort?

There were infection associated adverse events in the HACS trial but the potential role of the drug in causation is difficult to determine. There is a theoretical risk of worsening infection with iron overload as iron has been shown to be required for pathogen growth in vitro, and therefore caution is exercised with administration of iron containing compounds in patients known to have infections.

There have been case reports of iron accumulation in patients who receive HA for acute intermittent porphyria leading to secondary haemochromatosis with liver fibrosis (129), however this was always in the context of repeated porphyria attacks requiring frequent dosing of HA over many years. Serum ferritin was checked at each study visit as this has previously been shown to correlate with the number of doses of HA given (146). In patients receiving heme arginate treatment for porphyria, a regime which requires daily infusions of 3mg/kg daily for 4 days, the median ferritin was 208µg/l (range 21-3165µg/l). In this study there was no significant increase in ferritin at any time point which is very reassuring in this regard.

In the HOT study (64) which involved 2 doses of 3mg/kg HA, only serious adverse events were described in the published data, none of which included any infective issues Admission to intensive care for pulmonary oedema and post operative bleeding events were described specifically, and it was stated that there was no difference in number or type of events between placebo and HA groups.

In the manuscript of the Andreas clinical study of HA 24 hours prior to aortic valve replacement, the adverse events are not described in detail but it is stated that none were related to the study medication(147). In this relatively elderly and comorbid population which was similar to the HACS participants, there was a significant increase in leucocytes, CRP, creatinine kinase and
troponin T after surgery (all $p<0.001$) but no significant difference after infusion of HA. Plasma reactive oxygen species were also measured and were not found to be affected by the study drug administration but were significantly increased by surgery ($p<0.001$). Ferritin or other measures of iron metabolism were not described.

It is difficult to ascertain whether the adverse events in this study were related to the trial drug or not, but it would seem unlikely. The infective symptoms of upper respiratory tract infection would be more likely to be viral than bacterial, and occurred at the time of year when such viruses were prevalent. It is reassuring that the PIVOTAL trial of high dose intravenous iron supplementation in haemodialysis patients did not show a significant increase in adverse events associated with infection, as this trial was in patients who were similarly comorbid as in HACS (high incidence of diabetes and cardiovascular disease, and older than other studies with a mean age 62.7 years (SD 14.9 years)). The risk of worsening infection would need to be assessed further in any subsequent Phase IIb trial of HA in patients undergoing cardiac surgery. Reassuringly, a recent publication of pre-determined secondary end points of PIVOTAL showed that in those patients randomised to high dose iron, there was a reduction in the number of patients with myocardial infarction (148), indicating that increased iron dose does not confer increased cardiovascular risk in patients undergoing haemodialysis, a particularly high risk group.

6.3 Dimethyl fumarate – another potential candidate for repurposing drugs?

DMF certainly has appeal as a drug with potential for ameliorating renal ischaemia reperfusion injury given that it has been in clinical use for many years with a good safety profile. The preliminary studies in this work need to be replicated and expanded; for example there would need to be better dose finding studies as it may be that the doses used in these experiments were too high to have a beneficial effect and in fact DMF in too high a dose may be toxic
to cells in culture and to mice in vivo. Very high concentration of DMF (50µM) did lead to a mean of 10% cell lysis (by Griess assay) but the morphology of the cells appeared to be normal. Indeed, the dose of 50µM may have been too high to promote phagocytosis.

Time constraints meant that several questions were asked but the evidence needs to be backed up with more replicates and more experiments. As a therapy, the oral route of administration makes DMF particularly attractive as it can be given at home prior to admission to hospital and patients prefer it. Bardoxolone was another oral medication which was shown to activate the Nrf2 pathway. It showed promise in the treatment of CKD and Diabetes but a large randomised controlled trial, the BEACON trial (148), was stopped early due to heart failure complications, thought to be related to salt and water retention. A more recent subgroup analysis demonstrates that when patients are selected appropriately there may well be a benefit in slowing CKD progression (149).

Some new treatments, particularly those with anti-inflammatory properties, show promise (88,150). Again, the stumbling blocks tend to be heterogeneity of the patient group and the need for better risk stratification to identify patients most at risk and the time at which any potential treatment may show most benefit.

This highlights the need for more clinical trials in the field of AKI, and trial design has been an increasing area of interest. Given the heterogeneity of the condition, any signals of benefit can be lost even in large randomised trials as the number needed to treat can be much greater. Pragmatic trial design including careful patient selection by quantifying risk, further stratification with new tools such as urinary biomarkers (151,152) may change the trial landscape (5,153). We have an increasingly elderly, comorbid and therefore vulnerable population who are living longer, and more data are coming through about the longer term effects of AKI including the risk of CKD (154) and all the
cardiovascular comorbidity that comes with it. The need for more drug development in AKI is pressing. We would hope that the future treatment for AKI will involve the use of drugs with a better evidence base, tailored to the individual patient and administered at the right time in their disease trajectory.
7 References


57. Cailhier JF, Sawatzky D a, Kipari T, Houlberg K, Walbaum D, Watson S, et al. Resident pleural macrophages are key orchestrators of


90. Zimmerman RF, Ezeanuna PU, Kane JC, Cleeland CD, Kempananjappa TJ, Lucas FL, et al. Ischemic preconditioning at a remote site prevents acute kidney injury in patients following cardiac


112. Friedrich JO, Neill Adhikari D, Herridge MS, Beyene J. Meta-Analysis: Low-Dose Dopamine Increases Urine Output but Does Not Prevent Renal Dysfunction or Death Background: Surveys have documented the continued popular [Internet]. 2005. Available from: www.annals.org


144. Leaf DE, Body SC, Muehlschlegel JD, McMahon GM, Lichtner P, Collard CD, et al. Length polymorphisms in heme oxygenase-1 and


8 Appendix – Clinical trial documents

8.1 HACS Clinical Trial Protocol
Study Protocol

A Randomised Clinical Trial of Heme Arginate in Patients Planned for Cardiac Surgery

HACS

| Co-sponsors              | University of Edinburgh & NHS Lothian ACCORD
|                         | The Queen’s Medical Research Institute
|                         | 47 Little France Crescent
|                         | Edinburgh
|                         | EH16 4TJ
| Funder                  | Medical Research Council and Lothian Health Board Endowment Fund
| Funding Reference Number | SE01047
| Chief Investigator      | Dr Fiona Al Duthie
| EudraCT Number          | 2014-001021-32
| REC Number              | 14/SS/1007
| ClinicalTrials.gov identifier | NCT02142699
| Version Number and Date | 1.6 02/02/2016

<table>
<thead>
<tr>
<th>Chief Investigator</th>
<th>Co-sponsor Representative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Fiona Duthie</td>
<td>Dr Annya Smyth</td>
</tr>
<tr>
<td>MRC/University of Edinburgh Centre for Inflammation Research</td>
<td>Clinical Research Facilitator</td>
</tr>
<tr>
<td>Queen’s medical Research Institute</td>
<td>University of Edinburgh, Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ</td>
</tr>
<tr>
<td>47 Little France Crescent</td>
<td>Tel: 0131 242 3325</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>Email: <a href="mailto:Annya.Smyth@ed.ac.uk">Annya.Smyth@ed.ac.uk</a></td>
</tr>
<tr>
<td>EH16 4TJ</td>
<td></td>
</tr>
<tr>
<td>Tel: 0131 242 6671</td>
<td></td>
</tr>
<tr>
<td>Email: <a href="mailto:fduthie@staffmail.ed.ac.uk">fduthie@staffmail.ed.ac.uk</a></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Co-Investigator</th>
<th>Co-Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Professor Jeremy Hughes</td>
<td>Dr David Kluth</td>
</tr>
<tr>
<td>MRC/University of Edinburgh Centre for Inflammation Research</td>
<td>MRC/University of Edinburgh Centre for Inflammation Research</td>
</tr>
<tr>
<td>Queen’s Medical Research Institute</td>
<td>Queen’s Medical Research Institute</td>
</tr>
<tr>
<td>47 Little France Crescent</td>
<td>47 Little France Crescent</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>Edinburgh</td>
</tr>
<tr>
<td>EH16 4TJ</td>
<td>EH16 4TJ</td>
</tr>
<tr>
<td>Tel: 0131 242 6683</td>
<td>Tel: 0131 242 6684</td>
</tr>
<tr>
<td>Email: <a href="mailto:jeremy.hughes@ed.ac.uk">jeremy.hughes@ed.ac.uk</a></td>
<td>Email: <a href="mailto:david.kluth@ed.ac.uk">david.kluth@ed.ac.uk</a></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Co-Investigator</th>
<th>Co-Investigator</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr Vipin Zamvar</td>
<td>Ms Catriona Graham</td>
</tr>
<tr>
<td>Cardiothoracic Unit</td>
<td>Lead Statistician</td>
</tr>
<tr>
<td>Royal Infirmary of Edinburgh</td>
<td>Wellcome Trust Clinical Research Facility</td>
</tr>
<tr>
<td>51 Little France Crescent</td>
<td>Western General Hospital</td>
</tr>
<tr>
<td>Edinburgh</td>
<td>Crewe Road South</td>
</tr>
<tr>
<td>EH16 4SA</td>
<td>Edinburgh</td>
</tr>
<tr>
<td>Tel: 0131 536 1000</td>
<td>EH4 2XU</td>
</tr>
<tr>
<td>Email: <a href="mailto:vipin.zamvar@luht.scot.nhs.uk">vipin.zamvar@luht.scot.nhs.uk</a></td>
<td>Tel: 0131 537 3350</td>
</tr>
<tr>
<td></td>
<td>Email: <a href="mailto:c.graham@ed.ac.uk">c.graham@ed.ac.uk</a></td>
</tr>
</tbody>
</table>
CONTENTS

1 INTRODUCTION .................................................................................................................. 13
  1.1 BACKGROUND ............................................................................................................... 13
  1.2 RATIONALE FOR STUDY ............................................................................................ 14

2 STUDY OBJECTIVES ......................................................................................................... 15
  2.1 OBJECTIVES ................................................................................................................ 15
      2.1.1 Primary Objective .................................................................................................. 15
      2.1.2 Secondary Objectives ........................................................................................ 15
  2.2 ENDPOINTS .................................................................................................................. 16
      2.2.1 Primary Endpoint ................................................................................................ 16
      2.2.2 Secondary Endpoints ........................................................................................ 16

3 STUDY DESIGN .................................................................................................................. 17

4 STUDY POPULATION ......................................................................................................... 19
  4.1 NUMBER OF PARTICIPANTS ....................................................................................... 19
  4.2 INCLUSION CRITERIA ................................................................................................. 19
  4.3 EXCLUSION CRITERIA ................................................................................................. 19

5 PARTICIPANT SELECTION AND ENROLMENT ................................................................. 20
  5.1 IDENTIFYING PARTICIPANTS ..................................................................................... 20
  5.2 CONSENTING PARTICIPANTS ................................................................................... 20
  5.3 SCREENING FOR ELIGIBILITY ................................................................................... 21
  5.4 INELIGIBLE AND NON-RECRUITED PARTICIPANTS .............................................. 21
  5.5 RANDOMISATION ....................................................................................................... 21
      5.5.1 Randomisation Procedures ................................................................................ 21
      5.5.2 Treatment Allocation ......................................................................................... 21
      5.5.3 Emergency Unblinding Procedures ................................................................... 21
      5.5.4 Withdrawal of Study Participants ..................................................................... 21

6 INVESTIGATIONAL MEDICINAL PRODUCT AND PLACEBO ........................................ 23
  6.1 STUDY DRUG ............................................................................................................... 23
      6.1.1 Study Drug Identification .................................................................................... 23
      6.1.2 Study Drug Manufacturer .................................................................................. 23
      6.1.3 Marketing Authorisation Holder ........................................................................ 23
      6.1.4 Labelling and Packaging .................................................................................... 23
      6.1.5 Storage ................................................................................................................ 23
      6.1.6 Summary of Product Characteristics or Investigators Brochure ...................... 23
  6.2 PLACEBO ..................................................................................................................... 23
  6.3 DOSING REGIME ......................................................................................................... 23
  6.4 DOSE CHANGES .......................................................................................................... 24
  6.5 PARTICIPANT COMPLIANCE ..................................................................................... 24
  6.6 OVERDOSE .................................................................................................................. 24
  6.7 OTHER MEDICATIONS ............................................................................................... 25
      6.7.1 Non-Investigational Medicinal Products .............................................................. 25
      6.7.2 Permitted Medications ......................................................................................... 25
      6.7.3 Prohibited Medications ....................................................................................... 25

7 STUDY ASSESSMENTS ....................................................................................................... 26
  7.1 SAFETY ASSESSMENTS .............................................................................................. 26
7.2 STUDY ASSESSMENTS ................................................................. 26
8 DATA COLLECTION ........................................................................... 27
9 STATISTICS AND DATA ANALYSIS ............................................. 29
  9.1 SAMPLE SIZE CALCULATION ...................................................... 29
  9.2 PROPOSED ANALYSES ............................................................... 28
10 ADVERSE EVENTS ........................................................................... 29
  10.1 DEFINITIONS ........................................................................... 29
  10.2 IDENTIFYING AEs AND SAEs ................................................... 30
  10.3 RECORDING AEs AND SAEs ..................................................... 30
  10.4 ASSESSMENT OF AEs AND SAEs ............................................. 30
     10.4.1 Assessment of Seriousness ................................................. 30
     10.4.2 Assessment of Causality ..................................................... 30
     10.4.3 Assessment of Expectedness .............................................. 31
     10.4.4 Assessment of Severity ..................................................... 31
  10.5 REPORTING OF SAEs/SARs/SUSARs ........................................ 31
  10.6 REGULATORY REPORTING REQUIREMENTS ............................ 32
  10.7 FOLLOW UP PROCEDURES ....................................................... 32
11 PREGNANCY .................................................................................. 32
12 TRIAL MANAGEMENT AND OVERSIGHT ARRANGEMENTS ............... 32
  12.1 TRIAL MANAGEMENT GROUP .................................................. 32
  12.2 TRIAL STEERING COMMITTEE ............................................... 33
  12.3 DATA MONITORING COMMITTEE ............................................ 33
  12.4 INSPECTION OF RECORDS ..................................................... 33
  12.5 RISK ASSESSMENT ................................................................. 33
  12.6 STUDY MONITORING AND AUDIT .......................................... 33
13 GOOD CLINICAL PRACTICE .......................................................... 33
  13.1 ETHICAL CONDUCT ............................................................... 33
  13.2 REGULATORY COMPLIANCE ................................................... 34
  13.3 INVESTIGATOR RESPONSIBILITIES ......................................... 34
     13.3.1 Informed Consent ............................................................ 34
     13.3.2 Study Site Staff ............................................................... 34
     13.3.3 Data Recording .............................................................. 34
     13.3.4 Investigator Documentation .............................................. 35
     13.3.5 GCP Training ................................................................. 35
     13.3.6 Confidentiality ............................................................... 35
     13.3.7 Data Protection .............................................................. 35
14 STUDY CONDUCT RESPONSIBILITIES ........................................... 36
  14.1 PROTOCOL AMENDMENTS ..................................................... 36
  14.2 PROTOCOL VIOLATIONS AND DEVIATIONS ............................ 36
  14.3 SERIOUS BREACH REQUIREMENTS ....................................... 36
  14.4 STUDY RECORD RETENTION .................................................. 36
  14.5 END OF STUDY ....................................................................... 36
  14.6 CONTINUATION OF DRUG FOLLOWING THE END OF STUDY .... 37
  14.7 INSURANCE AND INDEMNITY ................................................ 37
15 REPORTING, PUBLICATIONS AND NOTIFICATION OF RESULTS .... 37
  15.1 AUTHORSHIP POLICY ............................................................. 37
15.2 PUBLICATION ................................................................. 38
15.3 PEER REVIEW ............................................................... 38
16 REFERENCES ........................................................................ 38
PROTOCOL APPROVAL

Randomised Controlled Trial of Heme Arginate in Cardiac Surgery Patients
EudraCT number 2014-001021-32

Signatures

Dr Fiona Duthie  
Chief Investigator/Principal Investigator  
Signature  
Date

Ms Catriona Graham  
Trial Statistician  
Signature  
Date

Dr Fiach O'Mahony  
Sponsor(s) Representative  
Signature  
Date

Prof Jeremy Hughes  
Co-Investigator  
Signature  
Date

Dr David Kluth  
Co-investigator  
Signature  
Date

Mr Vipin Zamvar  
Co-investigator  
Signature  
Date
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCORD</td>
<td>Academic and Clinical Central Office for Research &amp; Development - Joint office for University of Edinburgh and NHS Lothian</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse Event</td>
</tr>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>AKIN</td>
<td>Acute Kidney Injury Network</td>
</tr>
<tr>
<td>AR</td>
<td>Adverse Reaction</td>
</tr>
<tr>
<td>AVR</td>
<td>Aortic valve replacement</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass graft</td>
</tr>
<tr>
<td>CIR</td>
<td>Centre for Inflammation Research</td>
</tr>
<tr>
<td>CRF</td>
<td>Case Report Form</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTA</td>
<td>Clinical trial authorisation</td>
</tr>
<tr>
<td>CV</td>
<td>Curriculum vitae</td>
</tr>
<tr>
<td>DSUR</td>
<td>Development safety update report</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>ECTU</td>
<td>Edinburgh Clinical Trials Unit</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FBC</td>
<td>Full blood count</td>
</tr>
<tr>
<td>GCP</td>
<td>Good Clinical Practice</td>
</tr>
<tr>
<td>HA</td>
<td>Heme arginate</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hemeoxygenase-1</td>
</tr>
<tr>
<td>ICH</td>
<td>International Conference on Harmonisation</td>
</tr>
<tr>
<td>IL18</td>
<td>Interleukin 18</td>
</tr>
<tr>
<td>IMP</td>
<td>Investigational Medicinal Product</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischaemia reperfusion injury</td>
</tr>
<tr>
<td>ISF</td>
<td>Investigator Site File</td>
</tr>
<tr>
<td>KIM-1</td>
<td>Kidney injury molecule-1</td>
</tr>
<tr>
<td>LFT</td>
<td>Liver function test</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose binding lectin</td>
</tr>
<tr>
<td>MHRA</td>
<td>Medicines and healthcare products regulatory agency</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase associated lipocalin</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>QMRI</td>
<td>Queen’s Medical Research Institute</td>
</tr>
<tr>
<td>QP</td>
<td>Qualified person</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>R&amp;D</td>
<td>Research and development</td>
</tr>
<tr>
<td>REC</td>
<td>Research ethics committee</td>
</tr>
<tr>
<td>RIE</td>
<td>Royal Infirmary of Edinburgh</td>
</tr>
<tr>
<td>RIECRF</td>
<td>Royal Infirmary of Edinburgh Clinical Research Facility</td>
</tr>
<tr>
<td>RTqPCR</td>
<td>Quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious Adverse Event</td>
</tr>
<tr>
<td>SAR</td>
<td>Serious Adverse Reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SmPC</td>
<td>Summary of product characteristics</td>
</tr>
<tr>
<td>SOP</td>
<td>Standard Operating Procedure</td>
</tr>
<tr>
<td>SUSAR</td>
<td>Suspected Unexpected Serious Adverse Reaction</td>
</tr>
<tr>
<td>TMF</td>
<td>Trial Master File</td>
</tr>
<tr>
<td>TMG</td>
<td>Trial monitoring group</td>
</tr>
<tr>
<td>TSC</td>
<td>Trial steering committee</td>
</tr>
<tr>
<td>UAR</td>
<td>Unexpected Adverse Reaction</td>
</tr>
<tr>
<td>U&amp;E</td>
<td>Urea and electrolytes</td>
</tr>
</tbody>
</table>
SUMMARY

Lay Summary

A recent analysis of over 4500 cardiac surgical patients at the Royal Infirmary of Edinburgh has confirmed that acute kidney injury (AKI) is not only a relatively common post-operative complication but is associated with prolonged hospital stay, and increased risk of death. There is currently no specific therapy available except supportive care.

In laboratory studies, heme arginate (HA), a drug licensed for human use, has been shown to upregulate the anti-inflammatory enzyme hemeoxygenase-1 (HO-1) and protect aged mice from acute kidney injury.

This study will bring this research into the human arena. It will aim to evaluate the minimum effective dose of HA and verify its safety in this specific group of patients. This will be the next step in investigating if HA could be a potential protective treatment for reducing AKI in patients about to have cardiac surgery.

Patients who are due to have cardiac surgery and are aged 60 or above will be approached for inclusion in the study. If agreeable, they will be randomly assigned to receive HA either at a dose of 1mg/kg or 3mg/kg. There will be 10 patients in each group.

Blood tests will be taken just before the study drug is given, and at 6 hours, 24 hours and 7 days post dose. These samples will be used to examine the effect of HA on HO-1 expression at different doses, and will verify drug safety. Any adverse effects of the drug will be evaluated, although HA has an excellent safety profile and is currently licensed for the treatment for acute porphyria. Urine samples will also be collected to assess kidney inflammation and quantify urinary biomarkers of AKI. This will set the scene for a randomised clinical trial of HA in cardiac surgery patients at high risk of AKI.

Professional Summary

AKI is a common condition which carries significant morbidity and mortality and has no current treatment except supportive care(1).

Cardiac surgery remains the standard of care for the revascularisation of the most severe coronary artery disease despite improvements in percutaneous coronary intervention(2). The nature of such surgery confers an ischaemic renal insult and it follows on that AKI is a relatively common complication. However, the reported incidence of AKI post cardiac surgery varies widely depending on the diagnostic criteria used. A recent retrospective analysis of 4572 cardiac surgical patients at the Royal Infirmary of Edinburgh over a 5 year period showed that 12.4% developed post-operative AKI by Acute Kidney Injury Network (AKIN) criteria post operatively(3). This was associated with a prolonged inpatient stay and increased risk of death.

Pre-clinical studies in murine models of ischaemia reperfusion injury (IRI) have demonstrated that the anti-inflammatory enzyme HO-1 is of interest as a potential
target for therapeutic intervention(4). Heme arginate (HA) is a drug licensed for human use, as a treatment for acute porphyria (an inherited condition of abnormal haem synthesis). HA upregulates HO-1, and ameliorates IRI when given before injury in murine models of IRI(5). In Phase I clinical trials, HA has been shown to upregulate HO-1 in humans and is well tolerated(6, 7).

This is a single-blinded randomised trial of Heme arginate (HA) in patients who are scheduled to undergo elective cardiac surgery. The aim of the study is to investigate the efficacy of HA in HO-1 upregulation and to confirm its safety in this specific patient cohort. This will inform a subsequent randomised controlled clinical trial of the effectiveness of pre-operative HA administration as a preventative treatment for AKI in patients who are at high risk of AKI following cardiac surgery.

Methods

Potential participants will be patients who are planned for elective cardiac surgery, identified at their Cardiothoracic outpatient clinic assessment, aged 60 or above and meet the eligibility criteria.

HA (3mg/kg) upregulates HO-1 protein up to 5-fold at 24 hours post dose in healthy young volunteers(6), and up to 7-fold in participants in the recently completed HOT study (Induction of HO-1; a Therapeutic Approach to Reduce Ischaemia Reperfusion Injury (IRI) Following Deceased Donor Renal Transplantation) carried out at our institution (publication in preparation). Power calculations based on the results of the HOT study have determined that 20 patients will be recruited (10 per group).

Patients who agree to participate will be invited up for a study visit to the Clinical Research Facility at the Royal Infirmary of Edinburgh. If consent is given, participants will be randomised to receive HA at a dose of either 1 or 3mg/kg (maximum dose will be 250mg). Prior to the dose being given, routine observations (blood pressure, pulse rate, respiratory rate, oxygen saturations and temperature) will be taken, and the trial team member will perform a routine clinical examination of the cardiac and respiratory systems. An electrocardiogram (ECG) will be performed for reference.

Blood samples to assess HO-1 expression in circulating monocytes and in serum will be taken prior to the administration of the study drug and at 24 hours post dose. Samples for routine blood tests (full blood count (FBC), urea and electrolytes (U&E), C-reactive protein (CRP), liver function tests (LFT), and ferritin) will be taken alongside those for monocyte isolation, with further sets at 6 hours and 7 days post dose. Serum will be frozen for batch analysis and monocytes will be isolated by in-house protocols. Routine blood tests will be processed at the biochemistry and haematology laboratories at the RIE as per their usual methods. Serum will be analysed for complement cascade components C1q and mannose binding lectin (MBL).

Urine samples will be collected before the study drug is given, and at 24 hours and 7 days post dose. Urinalysis will be performed to assess for haematuria and proteinuria, and microalbuminuria will be quantified. Urine will be frozen for batch analysis of HO-1 enzyme levels and for biomarkers of renal tubular injury (neutrophil gelatinase associated lipocalin (NGAL), interleukin 18 (IL-18) & kidney injury molecule 1 (KIM-1)), quantified by ELISA(8).
Routine clinical observations will be carried out for 6 hours post dose, at which time the patient will be allowed home in the absence of any adverse events such as chest pain. A second study visit at the Clinical Research Facility at the RIE will be scheduled for 24 hours post dose (day 1), with a third study visit on day 7. The second and third study visits will allow reporting of any adverse events, as well as an opportunity to perform routine observations and take further blood samples for HO-1 induction in monocytes and for routine measures of organ function in order to analyse safety.

All participants will be genotyped for the short or long repeat HO-1 promoter polymorphism (either homozygous or heterozygous) though this has not been shown to affect HO-1 induction by HA in previous studies(6).

The RIE Cardiac Surgery unit performs approximately 1000 cases per year, with just less than 80% aged 60 or over from our analysis of 4572 patients(3). Therefore we are confident that recruitment of 20 patients into this trial will be readily achievable.
1 INTRODUCTION

1.1 BACKGROUND
Acute kidney injury (AKI) is a common problem that affects patients worldwide and carries significant morbidity and mortality (1). There are no specific treatments currently available for AKI except supportive care.

Ischaemia-reperfusion injury (IRI) is an important cause of acute kidney injury (AKI) (9) that can occur in a wide range of clinical conditions and has no specific treatment. The pathogenesis of renal IRI is not fully understood but oxidative stress and inflammation play key roles with involvement of both the innate and adaptive immune response (10). Cardiac surgery causes a predictive interruption to renal perfusion and therefore offers an opportunity for the study of IRI in humans, and the investigation of potential therapeutic agents.

AKI is a relatively common complication of cardiac surgery, and is well known to be associated with increased morbidity and mortality (11), as well as increased costs (12,13). AKI is an independent risk factor for death post cardiac surgery (14).

Of particular relevance to the NHS given the ageing population, elderly patients are more susceptible to developing AKI (15) and are also at higher risk of developing subsequent chronic kidney disease (CKD) in the longer term (16).

There are currently no specific treatments for AKI. Following on from pre-clinical studies, the anti-inflammatory enzyme heme oxygenase-1 (HO-1) offers potential as a therapeutic target. Heme is a ubiquitous protein that is released upon cell injury. HO-1 cleaves the porphyrin ring of heme, generating free iron, biliverdin (which is subsequently converted to the anti-oxidant bilirubin) and carbon monoxide (CO), which has multiple anti-inflammatory properties including inhibition of platelet aggregation and cell death (4). Ferritin is also upregulated by HO-1, so that iron-mediated damage is prevented (17). Consequently not only does HO-1 degrade the potentially cytotoxic heme but has further anti-inflammatory properties downstream.

The drug heme arginate (HA) is a licensed treatment for acute porphyria (18,19), an inherited condition of abnormal haem synthesis. A single dose of HA has been shown to induce HO-1 in young healthy subjects (6,7). Pre-clinical studies in a murine model of IRI have established that 12-month-old aged mice, like aged humans, develop more severe AKI than young mice following renal IRI (5) despite the absence of CKD. The aged mice exhibit minimal HO-1 upregulation in the renal medulla following renal IRI in comparison to young mice, which in contrast express HO-1 strongly in both interstitial macrophages (Mϕ) and in renal tubules. Importantly when considering HO-1 as a potential therapeutic target, treatment of aged mice with a single dose of HA 24 hours prior to IRI conferred strong protection from AKI.

In further preclinical studies, transgenic mice have been used to examine the role of monocyte/Mϕ in the protection afforded by HA, as diphtheria toxin (DT) administration to these mice ablates monocytes and renal Mϕ (20–22). Although monocyte/Mϕ ablation alone has not been shown to be protective in this model of AKI (23), DT treatment completely abolished HA-mediated protection (5) suggesting that monocyte/Mϕ HO-1 expression is key to HA-mediated renoprotection in vivo.
Therefore the upregulation of HO-1 in participant monocytes will be assessed. In addition, HO-1 in participant serum will be measured in order to investigate whether this correlates with monocyte HO-1. This would inform the end points of future clinical studies.

An additional potential mechanism of HA protection in IRI is via the complement cascade, an important pathway of the innate immune system. Heme, in the form of hemin, has been shown to inhibit classical pathway complement deposition and to interact with the complement system component C1q(24). C1q activity has been shown to increase with age, and there is evidence of complement deposition in the kidney following injury in vivo(25). It is therefore possible that heme administration in this cohort of patients over the age of 60 could confer protection from IRI via depletion of C1q. The effect of HA on complement cascade C1q and MBL levels will be assessed in comparison to baseline.

The ability of HA to induce HO-1 has been investigated in preliminary Phase I clinical trials. A dose escalation study examined the effect of HA on HO-1 mRNA levels and protein expression in peripheral blood monocytes at doses of 0.3mg/kg, 1mg/kg and 3mg/kg(6). This showed a dose dependent response, with the dose of 1mg/kg increasing HO-1 protein expression by approximately 2-fold, while 3mg/kg led to a 5-fold increase. HO-1 gene expression (mRNA) was increased by over 1.5-fold with 1mg/kg, and approximately 2-fold with 3mg/kg. In a separate Phase I study in healthy male subjects, a single dose of 1mg/kg HA improved reperfusion patterns measured by blood oxygen dependent functional MRI following calf muscle ischaemia(7). In both studies the drug was well tolerated and there were no concerns about safety.

Taking this into the clinical arena, published data from the Cardiothoracic Unit in the Royal Infirmary of Edinburgh indicates that AKI affects 12.4% of patients undergoing cardiac surgery by AKIN criteria(3). In this cohort, there was an inpatient mortality of 2.2% in those without AKI compared with 13.5% in those with AKI of any severity (p<0.05). There was also an association with prolonged inpatient stay, with a median length of stay of 7 days (IQR 4) in those who did not develop AKI, compared to 9 days (IQR 11) in those with AKI of any severity (p<0.05). The clinical significance of the development of AKI on patient outcomes highlights the need for further research into AKI prevention and management. The EuroSCORE, a cardiac surgical risk score (Appendix 3), outperformed any other single risk factor in the prediction of post-operative AKI risk and therefore has potential to be used as a pre-operative risk assessment tool for AKI.

Given the encouraging pre-clinical studies of HA in murine models of IRI, and Phase I studies of HA in human subjects, there is a need to proceed to a Phase II trial in order to further evaluate HA as a potential preventative treatment for AKI. The age of 60 has been chosen because the effect of HA on HO-1 has been demonstrated in young, healthy individuals in Phase I studies(6,7), and there is data from pre-clinical studies of murine models of ischaemia reperfusion injury that HO-1 upregulation by HA is less effective in older subjects(5). Therefore, given that the majority (approximately 80%) of patients planned for cardiac surgery are aged 60 or over, we are keen to study this cohort.

1.2 RATIONALE FOR STUDY
In the UK, the importance of AKI has been emphasised following the publication of the National Confidential Enquiry of Patient Outcomes and Deaths report in 2009(26). This highlighted the need for better awareness of AKI, prompt recognition and regular reassessment of patients in order to tackle its significant associated mortality. In light of the ageing population, clinical trials to investigate potential therapies for AKI are needed given that elderly patients are more susceptible to developing AKI(15) and are also at higher risk of developing chronic kidney disease (CKD) thereafter(16).

This study follows on from promising pre-clinical work in murine models of IRI, and aims to inform a clinical trial of Heme arginate (HA) as a specific preventative treatment for AKI. HA is an established therapy for acute porphyria, which has been used for over 20 years and has an excellent safety profile. HA has been proven to upregulate HO-1 mRNA and protein expression in Phase I clinical trials.

The primary aim of this study is to investigate the effect of HA on the upregulation of HO-1 in this patient population with cardiovascular disease. It will also aim to provide robust safety data for HA in this specific patient cohort. The proposed single-blinded, two arm randomised trial will establish the efficacy and confirm the safety of a single dose of HA (1mg/kg or 3mg/kg, up to a maximum dose of 250mg) in the induction of HO-1 in patients awaiting elective cardiac surgery who are aged 60 and over.

The study will examine the upregulation of HO-1 gene and protein expression in circulating monocytes in order to determine the minimum effective dose of HA required in further clinical trials. Monocytes will be purified from study blood samples and HO-1 expression and bioactivity quantified by techniques including qPCR and ELISA(27).

The results of this study would inform further clinical trials of HA as a potential prophylactic therapy in patients at high risk of acute kidney injury, a serious condition that carries significant morbidity and mortality.

2 STUDY OBJECTIVES

2.1 OBJECTIVES

2.1.1 Primary Objective

In elderly patients who are planned for elective cardiac surgery, what is the efficacy of heme arginate (HA) in the upregulation of hemeoxygenase-1 (HO-1) protein expression from baseline in the participants’ monocytes at 24 hours when given at a dose of 3mg/kg (maximum 250mg) or 1mg/kg?

2.1.2 Secondary Objectives
In elderly patients who are planned for elective cardiac surgery, what is the efficacy of heme arginate (HA) in the upregulation of hemeoxygenase-1 (HO-1) mRNA from baseline in the participants’ monocytes and serum at 24 hours when given at a dose of 3mg/kg (maximum 250mg) or 1mg/kg?

What is the safety profile of HA dosing of 1mg/kg and 3mg/kg (maximum 250mg) in this patient cohort?

Does HA affect accepted measurements of physiological systems such as liver function, renal function, the full blood count, C-reactive protein and ferritin?

Does HA affect the levels of urinary biomarkers for acute kidney injury?

Does the patient’s genotype of the HO-1 promoter (homozygous for short, long or heterozygous) affect the upregulation of HO-1 by HA?

Does HA affect the complement cascade components C1q and MBL in serum?

2.2 ENDPOINTS

2.2.1 Primary Endpoint

The primary outcome measure is the difference in HO-1 protein expression from baseline in the circulating monocytes of participants following a single dose of 1mg/kg or 3mg/kg (up to a maximum of 250mg) HA at 24 hours post dose

2.2.2 Secondary Endpoints

2.2.2.1 HO-1 mRNA induction in monocytes
The difference in HO-1 mRNA expression from baseline in the circulating monocytes of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.2.2.2 HO-1 in serum
The difference in HO-1 enzyme levels from baseline in the serum of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.2.2.3 HO-1 in urine
The difference in HO-1 enzyme levels from baseline in the urine of participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose

2.2.2.4 Safety
The safety of HA in this elderly and comorbid population will be established by recording of adverse events, as well as accepted measurements of physiological systems such as liver function, renal function, full blood count, C-reactive protein and ferritin.

2.2.2.5 Urinary Biomarkers
Urine will be collected to examine urinary sediment (by urine dipstick) and to quantify microalbuminuria and urinary biomarkers of AKI (NGAL, KIM-1 and IL18) to establish if HA dosing has any effect. This will inform future trials of HA in AKI where urinary biomarkers will be used as surrogate markers of AKI.
2.2.2.6 Genotyping
The pre dose blood sample will also be used to genotype for the short or long (or heterozygous) repeat HO-1 promoter polymorphism to investigate the potential effect on HO-1 induction by HA.

2.2.2.7 Complement
The difference in the complement cascade components C1q and mannose binding lectin (MBL) levels from baseline in the participants receiving 1mg/kg or 3mg/kg (up to a maximum of 250mg) at 24 hours post dose
STUDY DESIGN

This is a single centre randomised trial of HA in elderly patients. The study cohort is patients who are planned for elective cardiac surgery and are aged 60 and above. The study is expected to take at most 3 months to complete from the first patient recruited to the end of the last participant’s final visit.

The randomisation will be by a permuted block web-based programme in conjunction with the Edinburgh Clinical Trials Unit (ECTU). The appropriate dose of study drug, in accordance with the randomisation result, will be prepared by a member of the trial team. Randomisation information and the study drug batch number will be recorded.

Enrolled participants will be randomised to receive one of:
- 3mg/kg heme arginate (up to a maximum dose of 250mg)
- 1mg/kg heme arginate

The study drug will be given as a single dose by intravenous infusion, and the participant will be observed by a member of the trial team for six hours following this. Blood samples will be taken immediately prior to administration of the drug, and at 6 hours following the dose. If there are no concerns about adverse effects at this point, the patient will be discharged home. A further blood sample will be taken at a return visit the following day, (at 24 hours post dose), and another at a final study visit on day 7.

Once the blood has been taken, the routine blood tests (U&E, LFT, FBC, ferritin, CRP) will be processed at the laboratory at the RIE. The study blood samples for HO-1 will be processed at the QMRI and stored there for later batch analysis. The pre-dose blood sample will also be used for HO-1 genotype assessment.

Urine samples will be taken prior to the dose being given and again at 24 hours and 7 days post dose. After the urine is tested for blood and protein on dipstick, an aliquot will be sent for microalbuminuria testing at the RIE biochemistry laboratory. The urine will then be stored and later analysed for urinary biomarkers (NGAL, KIM-1 and IL18) of acute kidney injury by ELISA.

Patients will be considered a member of the trial from the time of consent to the end of the final study visit or the resolution of any ongoing adverse event. If the adverse event persists beyond 30 days post study drug administration, the usual care team will be made aware of this and this event will no longer be followed up by the trial team.
Timeline for Study

**Clinic Visit**
- Initial discussion with clinical team member

**Study Visit 1**
- Discussion
- Consent
- Routine observations
  - Blood test
  - Urine sample
- **Study drug given**
  - 6 hrs observation
  - Blood test

**Study Visit 2** (24 hrs after dose)
- Discussion
- Routine observations
  - Blood test
  - Urine sample

**Study Visit 3** (7 days after dose)
- Discussion
- Routine observations
  - Blood test
  - Urine sample
3  STUDY POPULATION

3.1  NUMBER OF PARTICIPANTS
20 participants aged 60 or older will be recruited (10 in each treatment group) from the Outpatient clinic of the Cardiothoracic Surgery Unit at the RIE. The patients will be from all over South East Scotland. Recruitment will be continued until at least 20 patients have been recruited, which is predicted to take less than 3 months.

3.2  INCLUSION CRITERIA
Male and female patients will be included, and there is no upper age limit. Other inclusion criteria are as follows:

Age ≥60
Planned for elective cardiac surgical procedure:
- Aortic valve replacement
- CABG

3.3  EXCLUSION CRITERIA
Patients will not be eligible for inclusion in the study if they meet any of the following criteria:

1. Inability to give informed consent
2. Enrolment in other clinical trials
3. Undergoing renal replacement therapy
4. Planned for elective cardiac surgical procedure other than CABG or AVR (including combined CABG and AVR)
5. Less than 2 weeks until planned cardiac surgery at time of study drug administration
6. Symptoms or signs suggestive of active infection
7. Contraindications (or relative contraindications) to HA including:
   - Hypersensitivity to HA
   - Liver disease
   - Alcoholism
   - Epilepsy
   - Brain injury
8. Women who are pregnant, breastfeeding or of child-bearing potential (women who have experienced menarche, are pre-menopausal and have not been sterilised) will not be enrolled into the trial
3.4 CO-ENROLMENT

Co-enrolment will not be permitted. If an individual has recently participated in another clinical trial, a minimum time of 3 months since the end of their participation will be permitted prior to recruitment into this trial.

4 PARTICIPANT SELECTION AND ENROLMENT

4.1 IDENTIFYING PARTICIPANTS

A member of the patient's usual care team will discuss the trial with those who meet the study criteria during their Cardiotoracic outpatient clinic assessment. A patient information sheet will be provided for them to take home and consider in their own time, and they will be given the option to decline to participate in the trial at this point and if so they will not be contacted again.

4.2 CONSENTING PARTICIPANTS

If verbal consent for contact is given in the clinic, potential participants will be contacted by telephone by a member of the study team in the week following their initial visit to discuss the trial further and answer any questions they may have. Those who agree to participate in the study will be invited to the Royal Infirmary of Edinburgh Clinical Research Facility (RIECRF), to meet a member of the trial team. They will go through the details of the trial again and answer any questions. If participation in the trial is agreed, written consent will be taken by the trial team member.

The patient and investigator will each sign the consent form and two copies will be made. The original will be filed in the site file, one copy will be given to the participant to keep, and one copy will be filed in the participant’s medical notes. A letter will be sent to the patient's GP, with the patient's consent to do so, to inform them of their participation.

4.3 SCREENING FOR ELIGIBILITY

Potential participants will be screened for eligibility by their usual clinical care team. At their outpatient Cardiac Surgery clinic visit, their clinical care team will discuss the trial and further evaluate them for inclusion/exclusion criteria. If verbal consent is given, those potential participants identified will be contacted subsequently by the trial team by telephone. If agreeable, the individual will be invited up to the RIECRF to participate in the trial and written consent will be obtained. The inclusion/exclusion criteria will be reviewed again prior to the study drug being given by a qualified member of the trial team.

4.4 INELIGIBLE AND NON-RECRUITED PARTICIPANTS

All potential participants who meet the inclusion criteria will have anonymous consort data (age, sex and planned procedure only) recorded on a pre-screening log. In the event of ineligibility or consent not being given, the reason for this will be documented and stored securely with the participant CRFs.
4.5 RANDOMISATION

4.5.1 Randomisation Procedures
Randomisation will be by a permuted block web-based regime in conjunction with the Edinburgh Clinical Trials Unit (ECTU), with an equal number of subjects in both treatment arms. The participants will be blinded to the dose of study drug administered. Due to the nature of HA, the member of the trial team who is making up and administering the drug cannot be blinded. The dose of the drug will be checked by a nurse in the RIECRF in order to avoid drug errors.

An independent laboratory member will anonymise the samples for HO-1 assessment prior to processing by the investigator, and no patient identifying details will be stored with study samples or with data collection sheets. Therefore, although the investigator will know the treatment received by the participant at the time of administration, they will be blinded at the time of processing the study samples. The participant will remain blinded throughout.

4.5.2 Treatment Allocation
There are 2 arms to the study:

Arm A: 3mg/kg heme arginate (up to a maximum dose of 250mg)
This group will receive a single infusion of 3mg/kg HA over 30 minutes into a peripheral vein

Arm B: 1mg/kg heme arginate
This group will receive a single infusion of 1mg/kg HA over 30 minutes into a peripheral vein

HA in solution has a distinctive orange colour. As two concentrations of active drug are being used, despite the visible drug colour the participant will not be aware of which dose they are receiving. Only the investigator responsible for making up and administering the trial drug will be aware of its dose.

The study drug will be purchased from Orphan Europe, and held by pharmacy at the RIE. The dose will be calculated according to the patient’s weight. The drug will be prepared immediately prior to its administration by a member of the trial team. The drug volume of administration will be 100ml in all cases. A further flush of 100ml 0.9% saline will be administered into the peripheral cannula following the study drug, and a giving set with an inline filter will be used as per the manufacturer’s recommendations.

4.5.3 Emergency Unblinding Procedures
The Chief Investigator will hold login details for a web-based randomisation programme provided by the ECTU and will perform emergency unblinding if required. All requests for unblinding will go through the Chief Investigator. As both treatment groups involve active drug (Heme arginate), participants would be presumed to have the higher dose and treated as such.
4.5.4 Withdrawal of Study Participants

The participant can withdraw from the trial at any point at their request. Participants who consent and withdraw prior to the day 1 study blood sample for HO-1 protein and gene expression will be replaced (by recruiting new participants under new study numbers) and their data will be collected as minimum consort data. The participant will continue to be monitored (with their permission) for AEs/SAEs if they withdraw following administration of the study drug.

In the event that there are not sufficient monocytes extracted to provide the primary outcome measure of the difference of heme oxygenase-1 induction from baseline, then another participant will be recruited to allow for this. Their data will be collected as minimum consort data. The original participant will continue to be monitored (with their permission) for AEs/SAEs and secondary end points.

5 INVESTIGATIONAL MEDICINAL PRODUCT AND PLACEBO

5.1 STUDY DRUG

5.1.1 Study Drug Identification

The study drug is Heme arginate (trade name Normosang) 25mg/ml, concentrate for solution for infusion

5.1.2 Study Drug Manufacturer

Normosang will be manufactured up to the primary packaging stage at EVER Pharma Jena GmbH, Otto Schott Strasse 15, 07745 Jena, Germany and will be secondary packaged and QP released by Orphan Europe.

5.1.3 Marketing Authorisation Holder

Orphan Europe, Immeuble "Le Wilson", 70 avenue du Général de Gaulle, F-92800 Puteaux, France
MA No: PL 15266/0008

5.1.4 Labelling and Packaging

Once the HA has been received from the supplier, it will be labelled in pharmacy to identify it as the trial drug, and stored in the pharmacy department as detailed below. During pharmacy working hours, a member of the trial team will request vials of HA on an individual basis. The need for access to HA out with these hours is not anticipated as patients will be seen during the working day. The drug will be prepared by a member of the trial team in the RIECRF. The drug labels will comply with the Medicines for Human Use (Clinical Trials) Regulations 2004 (appendix 4).

5.1.5 Storage

Heme arginate will be stored in a refrigerator (2-8°C) protected from light and in a secure area within the Pharmacy department at the RIE.

5.1.6 Summary of Product Characteristics or Investigators Brochure
The Summary of Product Characteristics (SmPC) is given at [http://www.medicines.org.uk/emc/medicine/20795/SPC/normosang/](http://www.medicines.org.uk/emc/medicine/20795/SPC/normosang/)

Licensed indications for HA are the treatment of acute attacks of hepatic porphyria (acute intermittent porphyria, porphyria variegata, and hereditary coproporphyria). HA will be used out with its licensed indications in this study.

### 5.2 PLACEBO

There is no placebo in this trial.

### 5.3 DOSING REGIME

HA will be given as a single infusion in the RIECRF. Participants will be randomised to receive either HA at 1mg/kg or 3mg/kg (to a maximum dose of 250mg), diluted in 0.9% sodium chloride.

The HA dose will be calculated according to the participant’s admission weight in kg.

In order to minimise pain at the site of injection, or subsequent phlebitis, the maximum concentration of HA administered will be 2.5mg/ml. Therefore, the total volume of infusion will be standardised at 100ml (therefore at a concentration of 2.5mg/ml for those receiving the maximum dose of 250mg). This will be followed by a flush of 100ml of normal saline over 15 minutes in all patients.

Therefore the dosing regime in the two trial arms will be:

**Arm A (3mg/kg HA)**

100ml of diluted HA (3mg/kg HA made up to 100ml solution with 0.9% sodium chloride) over 30 minutes, immediately followed by a separate infusion of 100ml 0.9% sodium chloride over 15 minutes

**Arm B (1mg/kg HA)**

100ml of diluted HA (1mg/kg HA made up to 100ml solution with 0.9% sodium chloride) over 30 minutes, immediately followed by a separate infusion of 100ml 0.9% sodium chloride over 15 minutes

### 5.4 DOSE CHANGES

As HA is given as a single dose in this study there will be no dose changes.

### 5.5 PARTICIPANT COMPLIANCE

Once the individual has consented to participation in the trial, the drug will be given as a single dose by a member of the trial team who will remain throughout the duration of the infusion. Therefore participant compliance with taking the study drug is unlikely to be an issue.

### 5.6 OVERDOSE

See Section 4.9 of SmPC, as detailed below. The risk of overdose will be minimised by the fact that the maximum dose in this trial is one vial (250mg) of Normosang, of which all the trial team will be aware. At least 2 members of the trial team will check the calculation and preparation of the dose of the study drug on every occasion prior to administration.
In animal experiments with Normosang the acute toxic effects after high dosage were directed to the liver. Ten times higher total doses than the recommended human posology also decreased blood pressure in rats. High doses may cause disturbances in hemostasis.

Normosang contains 4000 mg of propylene glycol per ampoule of 10 ml. Propylene glycol in high doses may cause central nervous system side-effects, lactic acidosis, kidney and liver toxicity, increase in plasma osmolarity, and haemolytic reactions.

Cases of overdosage with Normosang have been reported. For example, one patient had slight vomiting, pain and tenderness over the forearm (at the site of infusion) and made uneventful recovery. One other patient who received 10 ampoules of Normosang (2500 mg of human hemin) in a single infusion developed fulminant hepatic failure and one patient with a medical history of chronic hepatic failure who received 4 ampoules of Normosang (1000 mg of human hemin) experienced acute liver failure requiring liver transplantation. One patient received 12 ampoules of Normosang (3000 mg of human hemin) over 2 days which resulted in hyperbilirubinemia, anemia and a generalised haemorrhagic diathesis. The effects lasted for several days after administration, but the patient then improved without consequences.

Also, a high dose (1000 mg) of haematin, another form of haem, has been reported to have caused transitory renal failure in one patient.

Blood coagulation parameters, hepatic, renal and pancreatic functions should be carefully monitored until their normalisation.

Cardiovascular monitoring should also be performed (possibility of arrythmias).

Therapeutic measures in the event of an overdose:

- Albumin infusions should be administered to fix the freely-circulating and potentially reactive hemin.
- The administration of activated charcoal will enable the enterohepatic recirculation of the haem to be interrupted.
- Haemodialysis is necessary to eliminate the propylene glycol.

### 5.7 OTHER MEDICATIONS

#### 5.7.1 Non-Investigational Medicinal Products

Normal saline (0.9% Sodium chloride) will be used to dilute the HA to the uniform volume of 100ml, and will be administered as a flush in all participants.

Summary of product characteristics may be found at: [http://www.medicines.org.uk/emc/medicine/23601/spc](http://www.medicines.org.uk/emc/medicine/23601/spc)

#### 5.7.2 Permitted Medications

The participant should take all their usual routine medication. Their regular prescribed medications will not be altered during their participation in the trial.

#### 5.7.3 Prohibited Medications

There are no specific drugs that should not be prescribed in combination with heme arginate. HA may increase cytochrome p450 activity, thereby potentially increasing
the metabolism of drugs such as oestrogens, barbiturates and steroids, leading to lower effective drug levels. Given that a single dose is being administered, this is not considered to be of clinical concern given that the half-life (±SD) is relatively short, given as 10.8±1.6hrs.

6 STUDY ASSESSMENTS

6.1 SAFETY ASSESSMENTS

There are no specific safety assessments required. General safety assessments will include routine blood tests as a measure of any effect on physiological systems, and measurement of parameters such as blood pressure and pulse. A clinical examination of the cardiac, respiratory and abdominal systems will be performed prior to administration of the study drug and repeated if clinically indicated. Given that this patient cohort has known cardiovascular disease, an ECG will be performed prior to the study drug being given to ensure that a recent example is available for comparison in the event of the patient developing symptoms such as chest pain. In the event of the development of such symptoms, an additional ECG will be performed, for comparison with the pre-treatment example.

6.2 STUDY ASSESSMENTS

7.2.1 Blood tests
Venepuncture will be performed prior to the administration of the study drug. Further blood samples will be taken at 6 hours post dose (±30 minutes), and on return at 24 hours (±180 minutes) and 7 days (±24 hours) post dose.

The blood pre dose (60ml) will be taken for HO-1 genotype assessment, monocyte isolation for analysis of HO-1 expression, and for routine blood tests (full blood count (FBC), urea and electrolytes (U&E), liver function tests (LFTs) C-reactive protein (CRP) and ferritin). The repeat sample at 6 hours (±30 minutes) post dose (10ml) will be for routine blood tests (FBC, U&E, LFT, ferritin, CRP).

The further sample at 24 hours (±180 minutes) post dose (50ml) will be taken for monocyte isolation for analysis of HO-1 expression as well as repeat routine tests (FBC, U&E, LFT, ferritin, CRP). Repeat samples at 7 days (±24 hours) post dose (10ml) will be routine tests only (FBC, U&E, LFT, ferritin, CRP).

7.2.2 Urine samples
Urine will be collected at the first study visit prior to the study drug being given, at 24 hours (±180 minutes) and at 7 days (±24 hours) post dose. The urine samples will be tested for the presence of blood and protein by dipstick analysis (Siemens Multistix), and then an aliquot will be sent for routine laboratory quantification of microalbuminuria. The rest of the sample will be placed on ice immediately, followed by centrifugation to remove cell content and then stored at -80°C. The urine will later be analysed for the urinary biomarkers KIM-1, IL-18 and NGAL using commercially available ELISAs.

7.2.3 Clinical Assessment
The patient will have routine observations taken (pulse rate, blood pressure, oxygen saturations, respiratory rate and temperature) and will have a clinical examination of the cardiac, respiratory and abdominal systems by a member of the trial team prior to the drug being given. The routine observations will be repeated at 15-minute (±5 minute) intervals for the first two hours after initiation of the dose, followed by hourly
(±10 minutes) thereafter until 6 hours (±30 minutes) post dose. The observations will be repeated sooner if there are any new symptoms experienced or there is any cause for clinical concern. Routine observations will be repeated at the follow up visits at 24 hours (±180 minutes) and 7 days (±24 hours) post dose.

7.2.4 Leukocyte Analysis
The primary outcome measure is HO-1 expression in monocytes. Peripheral blood taken prior to the study drug and at 24 hours (±180 minutes) will be analysed. Blood will be taken in an EDTA tube using a leukolock filter for RNA studies, and a lithium-heparin sample for leukocyte isolation and protein extraction.

HO-1 expression will be assessed as follows:
- HO-1 protein expression will be assessed by Western blot on isolated monocytes with formal quantification undertaken by densitometry (arbitrary units) and/or by ELISA
- HO-1 mRNA will be assessed via quantitative reverse transcription polymerase chain reaction (RTqPCR) on isolated monocytes

7.2.5 HO-1 Genotype assessment
Blood will be collected for HO-1 promoter polymorphism analysis prior to administration of the study drug. Genomic DNA will be extracted from peripheral blood mononuclear cells (PBMCs) using commercially available kits, as per the vendors’ protocols. This will be amplified by PCR. The repeat length for each participant will be determined and classified as per the system previously described: alleles with short (S) repeats with <27 GT repeats, and long (L) repeats with ≥27 GT repeats. Participants can therefore be either genotype SS, SL or LL. This data will be collected for information, although the study is not powered to determine if genotype is a confounding variable in HO-1 expression.
### 7 DATA COLLECTION

#### 8.1 Study period
The study will commence when the first patient is consented, and will finish when the last patient is discharged following their day 7 follow up visit, or upon resolution of any ongoing adverse event (AE). If the adverse event persists beyond 30 days post study drug administration, the usual care team will be made aware of this and this event will no longer be followed up by the trial team.

#### 8.2 Baseline information
The CI (or a delegated member of the trial team) will obtain patient demographics and past medical history from discussion with the patient themselves, and by accessing their electronic patient record (EPR) and medical notes following consent. This will include weight and height, current medication, smoking and alcohol history, past medical and surgical history. The nature of the planned surgical procedure will also be documented. The EuroSCORE will be calculated and documented for reference (Appendix 3).

Patient data collected during the study will be recorded on a paper case report form (CRF), which will be held securely in a locked filing cabinet in a locked office in the CIR. The study ID will be the only identifier on a secure anonymised electronic auditable database designed in conjunction with the Edinburgh Clinical Trials Unit.
8 STATISTICS AND DATA ANALYSIS

8.1 SAMPLE SIZE CALCULATION

The recent HOT study (Induction of HO-1; a Therapeutic Approach to Reduce Ischaemia Reperfusion Injury (IRI) Following Deceased Donor Renal Transplantation) was carried out in the Royal Infirmary of Edinburgh (publication in preparation). Its primary end point was HO-1 protein expression in monocytes at 24 hours post study drug dose (3mg/kg HA vs placebo, n=20/group). The results from the HOT trial demonstrated a significant 9-fold increase in monocyte HO-1 protein expression levels determined by Enzyme linked immunosorbent assay (ELISA): placebo group 2.1±10.3 (mean±SD) and treatment group (3mg/kg HA) 18.8±22.4 (p=0.0006).

Based on these data, calculations indicate that study of a group of 10 patients will enable detection of a baseline to 24-hour difference of 10.3 (from 2.1 to 12.4, a 6-fold increase) in mean monocyte HO-1 expression if the baseline expression level is comparable to the placebo group of the HOT study. However, it should be noted that the HOT participants were patients with end stage renal disease on renal replacement therapy with patients receiving haemodialysis (3-4 hours) at variable times (range 0-3 days) prior to the blood samples being taken. Such dialysis treatment exposes circulating monocytes to artificial membranes etc and may modulate monocyte HO-1 levels separately to the study drug. As patients recruited in this study will not be receiving dialysis, we believe that the baseline level of monocyte HO-1 expression will be less variable that that found in the HOT study so that study of 10 patients will be able to significantly detect a lower level of HO-1 upregulation following the administration of HA (3mg/kg). For example, using a two-sided paired t-test with 5% level of significance and 80% power we would be able to detect a baseline to 24-hour difference of 6.0 (from 2.1 to 8.1, a 4-fold increase) if the standard deviation of the differences was 6 (see column 4 of Table below). Columns 3 to 6 of the table above demonstrate the calculations repeated using standard deviation values from 8 down to 2. As the standard deviation decreases so do the size of the mean difference required to demonstrate a statistically significant baseline to 24-hour change (highlighted in bold in Table). Therefore we are confident that a sample size of 10 will be sufficient to show a statistically significant change in HO-1 protein expression following the administration of HA (3mg/kg).

<table>
<thead>
<tr>
<th>Test significance level, α</th>
<th>0.050</th>
<th>0.050</th>
<th>0.050</th>
<th>0.050</th>
<th>0.050</th>
<th>0.050</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 or 2 sided test?</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mean difference, ( \mu_d = \mu_1 - \mu_2 )</td>
<td>22.310</td>
<td>10.259</td>
<td>7.968</td>
<td>5.976</td>
<td>3.984</td>
<td>1.992</td>
</tr>
<tr>
<td>Standard deviation of differences, ( \sigma_d )</td>
<td>22.400</td>
<td>10.300</td>
<td>8.000</td>
<td>6.000</td>
<td>4.000</td>
<td>2.000</td>
</tr>
<tr>
<td>Effect size, ( \delta = \frac{\mu_d}{\sigma_d} )</td>
<td>0.996</td>
<td>0.996</td>
<td>0.996</td>
<td>0.996</td>
<td>0.996</td>
<td>0.996</td>
</tr>
<tr>
<td>Power ( % )</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

No data is available for the 1mg/kg dose of HA in patients aged over 60 but this dose has previously been shown to induce a 3-fold increase in the HO1 protein expression.
of peripheral blood mononuclear cells in young healthy male subjects (6). Although we are not directly comparing the 2 doses to be used in this study, we do wish to determine whether the lower dose of HA does effectively and significantly upregulate monocyte HO1 expression compared to baseline.

Approximately 1000 operations are carried out per year at the RIE, of whom approximately 80% are aged 60 or over. Therefore we are confident that this sample size will be readily achievable.

8.2 PROPOSED ANALYSES

In order to compare the change in HO-1 protein and gene expression within each treatment group (i.e. baseline to 24 hours post dose), a paired t-test or a non-parametric equivalent will be used as appropriate. As this is a small study the pattern of change over time of individual physiological measures of safety will be examined (4 samples from each participant of liver function tests, renal function, full blood count, CRP and ferritin). In addition, we will also analyse the data by ANOVA or a non-parametric equivalent as appropriate. Urinary biomarkers (3 samples for each participant) will be compared by ANOVA or a non-parametric equivalent will be used as appropriate.

9 ADVERSE EVENTS

The Investigator is responsible for the detection and documentation of events meeting the criteria and definitions detailed below.

Full details of contraindications and side effects that have been reported following administration of the IMP can be found in the relevant Summary of Product Characteristics (SmPC).

Participants will be instructed to contact their Investigator at any time after consenting to join the trial if any symptoms develop. All adverse events (AE) that occur after joining the trial must be reported in detail in the Case Report Form (CRF) or AE form, with the exception of headache and localised symptoms of discomfort at the injection site or phlebitis which are known side effects of HA. In the case of an AE, the Investigator should initiate the appropriate treatment according to their medical judgment. Specific possible adverse events in this elderly, comorbid group with known cardiovascular disease would be chest pain or breathlessness. Participants with AEs present at the last visit must be followed up until resolution of the event, or until 30 days post dose at which time this event will be reported to the usual care team and no longer followed up by the trial team.

9.1 DEFINITIONS

An adverse event (AE) is any untoward medical occurrence in a clinical trial participant which does not necessarily have a causal relationship with an investigational medicinal product (IMP).

An adverse reaction (AR) is any untoward and unintended response to an IMP which is related to any dose administered to that participant.
A serious adverse event (SAE), serious adverse reaction (SAR). Any AE or AR that at any dose:

- results in death of the clinical trial participant;
- is life threatening*;
- requires in-patient hospitalisation^ or prolongation of existing hospitalisation;
- results in persistent or significant disability or incapacity;
- consists of a congenital anomaly or birth defect;
- results in any other significant medical event not meeting the criteria above.

*Life-threatening in the definition of an SAE or SAR refers to an event where 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.

^Any hospitalisation that was planned prior to randomisation will not meet SAE criteria. Any hospitalisation that is planned post randomisation will meet the SAE criteria.

A suspected unexpected serious adverse reaction (SUSAR) is any AR that is classified as serious and is suspected to be caused by the IMP, that it is not consistent with the information about the IMP in the Summary of Product Characteristics (SmPC) or Investigators Brochure.

9.2 IDENTIFYING AEs AND SAEs

All AEs and SAEs will be recorded from the time a participant signs the consent form to take part in the study until the end of the 3rd study visit (ie day 7 post dose).

Participants will be asked about the occurrence of AEs/SAEs at every visit during the study. Open-ended and non-leading verbal questioning of the participant will be used to enquire about AE/SAE occurrence. Participants will also be asked if they have been admitted to hospital, had any accidents, used any new medicines or changed concomitant medication regimens. If there is any doubt as to whether a clinical observation is an AE, the event will be recorded.

AEs and SAEs may also be identified via information from support departments e.g. laboratories.

9.3 RECORDING AEs AND SAEs

When an AE/SAE occurs, it is the responsibility of the Investigator to review all documentation (e.g. hospital notes, laboratory and diagnostic reports) related to the event. The Investigator will then record all relevant information in the CRF and on the SAE form (if the AE meets the criteria of serious).

Information to be collected includes dose, type of event, onset date, Investigator assessment of severity and causality, date of resolution as well as treatment required, investigations needed and outcome.

9.4 ASSESSMENT OF AEs AND SAEs

Seriousness, causality, severity and expectedness will be assessed by the Principal Investigator. For randomised double blind studies, AEs will be assessed as though the participant is taking active IMP. Cases that are considered serious, possibly, probably or definitely related to IMP and unexpected (i.e. SUSARs) will be unblinded.

The Investigator is responsible for assessing each AE.
The Chief Investigator (CI) may not downgrade an event that has been assessed by an Investigator as an SAE or SUSAR, but can upgrade an AE to an SAE, SAR or SUSAR if appropriate.

9.4.1 Assessment of Seriousness

The Investigator will make an assessment of seriousness as defined in Section 10.1.

9.4.2 Assessment of Causality

The Investigator will make an assessment of whether the AE/SAE is likely to be related to the IMP according to the definitions below.

- **Unrelated**: where an event is not considered to be related to the IMP.
- **Possibly Related**: The nature of the event, the underlying medical condition, concomitant medication or temporal relationship make it possible that the AE has a causal relationship to the study drug. The assessment of causality will be made against the reference safety information found in the Summary of Product Characteristics.

Where non Investigational Medicinal Products (NIMPs) e.g. rescue/escape drugs are given: if the AE is considered to be related to an interaction between the IMP and the NIMP, or where the AE might be linked to either the IMP or the NIMP but cannot be clearly attributed to either one of these, the event will be considered as an AR. Alternative causes such as natural history of the underlying disease, other risk factors and the temporal relationship of the event to the treatment should be considered and investigated. The blind should not be broken for the purpose of making this assessment.

9.4.3 Assessment of Expectedness

If an event is judged to be an AR, the evaluation of expectedness will be made based on knowledge of the reaction and the relevant product information documented in the SmPC.

The event may be classed as either:
- **Expected**: the AR is consistent with the toxicity of the IMP listed in the SmPC.
- **Unexpected**: the AR is not consistent with the toxicity in the SmPC.

9.4.4 Assessment of Severity

The Investigator will make an assessment of severity for each AE/SAE and record this on the CRF or SAE form according to one of the following categories:

- **Mild**: an event that is easily tolerated by the participant, causing minimal discomfort and not interfering with everyday activities.
- **Moderate**: an event that is sufficiently discomforting to interfere with normal everyday activities.
- **Severe**: an event that prevents normal everyday activities.

Note: the term ‘severe’, used to describe the intensity, should not be confused with ‘serious’ which is a regulatory definition based on participant/event outcome or action criteria. For example, a headache may be severe but not serious, while a minor stroke is serious but may not be severe.
9.5 REPORTING OF SAEs/SARs/SUSARs

Once the Investigator becomes aware that an SAE has occurred in a study participant, the information will be reported to the ACCORD Research Governance & QA Office immediately or within 24 hours. If the Investigator does not have all information regarding an SAE, they should not wait for this additional information before notifying ACCORD. The SAE report form can be updated when the additional information is received.

The SAE report will provide an assessment of causality and expectedness at the time of the initial report to ACCORD according to Sections 10.4.2, Assessment of Causality and 10.4.3, Assessment of Expectedness.

The SAE form will be transmitted by fax to ACCORD on +44 (0)131 242 9447 or may be transmitted by hand to the office or submitted via email to Safety.Accord@ed.ac.uk. Only forms in a pdf format will be accepted by ACCORD via email.

Where missing information has not been sent to ACCORD after an initial report, ACCORD will contact the investigator and request the missing information.

All reports faxed to ACCORD and any follow up information will be retained by the Investigator in the Investigator Site File (ISF).

In addition, the Co-Sponsors will provide Orphan with details of all SAEs thought to be related to the Investigational Medicinal Product/SUSARs within fifteen (15) days of first notification of the SAE. Initial and follow-up reports shall be sent directly to Orphan by facsimile on 00 33 1 49 00 18 00 using the ACCORD SAE report form.

9.6 REGULATORY REPORTING REQUIREMENTS

The ACCORD Research Governance & QA Office is responsible for pharmacovigilance reporting on behalf of the co-sponsors (Edinburgh University and NHS Lothian).

The ACCORD Research Governance & QA Office has a legal responsibility to notify the regulatory competent authority and relevant ethics committee (Research Ethics Committee (REC) that approved the trial). Fatal or life threatening SUSARs will be reported no later than 7 calendar days and all other SUSARs will be reported no later than 15 calendar days after ACCORD is first aware of the reaction.

ACCORD will inform Investigators at participating sites of all SUSARs and any other arising safety information.

An Annual Safety Report/Development Safety Update Report will be submitted, by ACCORD, to the regulatory authorities and RECs listing all SARs and SUSARs.

9.7 FOLLOW UP PROCEDURES

After initially recording and reporting an SAE, the Investigator will follow each participant until resolution or death of the participant. Follow up information on an SAE will be reported to the ACCORD office.

After initially recording an AE, the investigator will follow each participant until resolution or until no longer medically indicated. If the AE has not resolved 30 days post drug administration, the follow up of the AE will be handed over to the participant’s usual care provider for ongoing follow up as required.
10 PREGNANCY

Pregnancy is not considered an AE or SAE. Pregnancy in female participants will not be possible given their age at recruitment and their imminently planned cardiac procedure. The investigators will collect pregnancy information for any female partners of male participants who become pregnant while participating in the study. The Investigator will record the information on a Pregnancy Notification Form and submit this to the ACCORD office within 14 days of being made aware of the pregnancy. All pregnant partners of male participants would be followed up until following the outcome of the pregnancy.

11 TRIAL MANAGEMENT AND OVERSIGHT ARRANGEMENTS

11.1 TRIAL MANAGEMENT GROUP

The Trial Management Group (TMG), consisting of the grant holders/co-investigators, the CI and the trial statistician, will coordinate the trial.

Given the small nature of the study, many of the roles are being undertaken by the Chief Investigator with delegation when CI is not available. The Grant Holders will oversee the study and the Chief Investigator will be accountable to them. The CI will be responsible for checking the CRFs for completeness, plausibility and consistency. Any queries will be resolved by the CI or delegated member of the trial team.

A Delegation Log will be prepared detailing the responsibilities of each member of staff working on the trial. This will specify trial team members permitted to prepare the drug and those permitted to administer the study drug to participants.

11.2 TRIAL STEERING COMMITTEE

A Trial Steering Committee (TSC) will be established to oversee the conduct and progress of the trial. The TSC comprises the TMG and a representative from ACCORD. The terms of reference of the Trial Steering Committee, the draft template for reporting and the names and contact details are detailed in Appendix 2.

11.3 DATA MONITORING COMMITTEE

An independent Data Monitoring Committee (DMC) will not be convened for this study. This is a small study that is aiming to inform a larger randomised clinical trial of HA in cardiac surgical patients at high risk of AKI. The TSC will also be tasked with overseeing the safety of participants for the duration of the study.

11.4 INSPECTION OF RECORDS

Investigators and institutions involved in the study will permit trial related monitoring and audits on behalf of the sponsor, REC review, and regulatory inspection(s). In the event of an audit or monitoring, the Investigator agrees to allow the representatives of the sponsor direct access to all study records and source documentation. In the event of regulatory inspection, the Investigator agrees to allow inspectors direct access to all study records and source documentation.
11.5 RISK ASSESSMENT

An independent risk assessment will be performed by an ACCORD Clinical Trials Monitor to determine if monitoring is required and if so, at what level. An independent risk assessment will also be carried out by the ACCORD Quality Assurance Group to determine if an audit should be performed before/during/after the study and if so, at what locations and at what frequency.

11.6 STUDY MONITORING AND AUDIT

An ACCORD Clinical Trials Monitor or an appointed monitor will visit the Investigator site prior to the start of the study and during the course of the study if required, in accordance with the monitoring plan if required. Risk assessment will determine if audit, by the ACCORD QA group, is required. Details will be captured in an audit plan. Audit of Investigator sites, study management activities and study collaborative units, facilities and 3rd parties may be performed.

12 GOOD CLINICAL PRACTICE

12.1 ETHICAL CONDUCT

The study will be conducted in accordance with the principles of the International Conference on Harmonisation Tripartite Guideline for Good Clinical Practice (ICH GCP).

A favorable ethical opinion will be obtained from the appropriate REC and local R&D approval will be obtained prior to commencement of the study.

12.2 REGULATORY COMPLIANCE

The study will not commence until a Clinical Trial Authorisation (CTA) is obtained from the appropriate Regulatory Authority. The protocol and study conduct will comply with the Medicines for Human Use (Clinical Trials) Regulations 2004, as amended.

12.3 INVESTIGATOR RESPONSIBILITIES

The Investigator is responsible for the overall conduct of the study at the site and compliance with the protocol and any protocol amendments. In accordance with the principles of ICH GCP, the following areas listed in this section are also the responsibility of the Investigator. Responsibilities may be delegated to an appropriate member of study site staff. Details of who will be undertaking each of the duties will be documented in the Delegation Log and signed by all those undertaking study-related duties.

12.3.1 Informed Consent

The Investigator is responsible for ensuring informed consent is obtained before any protocol specific procedures are carried out. The decision of a participant to participate in clinical research is voluntary and should be based on a clear understanding of what is involved.

Participants must receive adequate oral and written information – appropriate Participant Information and Informed Consent Forms will be provided. The oral
explanation to the participant will be performed by the Investigator or qualified delegated person, and must cover all the elements specified in the Participant Information Sheet and Consent Form.

The participant must be given every opportunity to clarify any points they do not understand and, if necessary, ask for more information. The participant must be given sufficient time to consider the information provided. It should be emphasised that the participant may withdraw their consent to participate at any time without loss of benefits to which they otherwise would be entitled.

The participant will be informed and agree to their medical records being inspected by regulatory authorities and representatives of the sponsor(s) but understand that their name will not be disclosed outside the hospital.

The Investigator or delegated member of the trial team and the participant will sign and date the Informed Consent Form(s) to confirm that consent has been obtained. The participant will receive a copy of this document and a copy filed in the Investigator Site File (ISF) and participant’s medical notes.

12.3.2 Study Site Staff

The Investigator must be familiar with the IMP, protocol and the study requirements. It is the Investigator’s responsibility to ensure that all staff assisting with the study are adequately informed about the IMP, protocol and their trial related duties.

12.3.3 Data Recording

The Chief Investigator is responsible for the quality of the data recorded in the CRF. The source data plan identifies which source data correspond to CRF data and states which data are recorded directly into the CRF.

12.3.4 Investigator Documentation

Prior to beginning the study, each Investigator will be asked to provide particular essential documents to the ACCORD Research Governance & QA Office, including but not limited to:

• An original signed Investigator’s Declaration (as part of the Clinical Trial Agreement documents);

• Curriculum vitae (CV) signed and dated by the Investigator indicating that it is accurate and current.

The ACCORD Research Governance & QA Office will ensure all other documents required by ICH GCP are retained in a Trial Master File (TMF), where required, and that appropriate documentation is available in local ISFs.

12.3.5 GCP Training

All study staff must hold evidence of appropriate GCP training.

12.3.6 Confidentiality

All laboratory specimens, evaluation forms, reports, and other records must be identified in a manner designed to maintain participant confidentiality. All records must be kept in a secure storage area with limited access. Clinical information will not be released without the written permission of the participant. The Investigator
and study site staff involved with this study may not disclose or use for any purpose other than performance of the study, any data, record, or other unpublished, confidential information disclosed to those individuals for the purpose of the study. Prior written agreement from the sponsor or its designee must be obtained for the disclosure of any said confidential information to other parties.

12.3.7 Data Protection

All Investigators and study site staff involved with this study must comply with the requirements of the Data Protection Act 1998 and the NHS Lothian Privacy and Data Protection Policy with regard to the collection, storage, processing and disclosure of personal information and will uphold the Act’s core principles. Access to collated participant data will be restricted to those clinicians treating the participants, representatives of the sponsor(s) and representatives of regulatory authorities.

Computers used to collate the data will have limited access measures via user names and passwords.

Published results will not contain any personal data that could allow identification of individual participants.

13 STUDY CONDUCT RESPONSIBILITIES

13.1 PROTOCOL AMENDMENTS

Any changes in research activity, except those necessary to remove an apparent, immediate hazard to the participant in the case of an urgent safety measure, must be reviewed and approved by the Chief Investigator.

Amendments to the protocol must be submitted in writing to the appropriate REC, Regulatory Authority and local R&D for approval prior to participants being enrolled into an amended protocol.

13.2 PROTOCOL VIOLATIONS AND DEVIATIONS

Prospective protocol deviations, i.e. protocol waivers, will not be approved by the sponsors and therefore will not be implemented, except where necessary to eliminate an immediate hazard to study participants. If this necessitates a subsequent protocol amendment, this should be submitted to the REC, Regulatory Authority and local R&D for review and approval if appropriate.

Protocol deviations will be recorded in a protocol deviation log and logs will be submitted to the sponsors every 3 months. Each protocol violation will be reported to the sponsor within 24 hours of becoming aware of the violation.

13.3 SERIOUS BREACH REQUIREMENTS

A serious breach is a breach, which is likely to effect to a significant degree:

(a) the safety or physical or mental integrity of the participants of the trial; or
(b) the scientific value of the trial.
If a potential serious breach is identified by the Chief investigator, Principal Investigator or delegates, the co-sponsors (accord.seriousbreach@ed.ac.uk) must be notified within 24 hours. It is the responsibility of the co-sponsors to assess the impact of the breach on the scientific value of the trial, to determine whether the incident constitutes a serious breach and report to regulatory authorities and research ethics committees as necessary.

13.4 STUDY RECORD RETENTION

All study documentation will be kept for a minimum of 5 years from the protocol defined end of study point. When the minimum retention period has elapsed, study documentation will not be destroyed without permission from the sponsor.

13.5 END OF STUDY

The end of study is defined as the end of the last participant's final visit or the resolution of any ongoing adverse event at that time.

The Investigators and/or the trial steering committee and/or the co-sponsor(s) have the right at any time to terminate the study for clinical or administrative reasons.

The end of the study will be reported to the REC, the local R+D Office(s) and Regulatory Authority within 90 days, or 15 days if the study is terminated prematurely. The Investigators will inform participants of the premature study closure and ensure that the appropriate follow up is arranged for all participants involved.

A summary report of the study will be provided to the REC, the local R+D Office(s) and Regulatory Authority within 1 year of the end of the study.

13.6 CONTINUATION OF DRUG FOLLOWING THE END OF STUDY

The drug will be given as a once only dose and will not be continued following the end of the study.

13.7 INSURANCE AND INDEMNITY

The co-sponsors are responsible for ensuring proper provision has been made for insurance or indemnity to cover their liability and the liability of the Chief Investigator and staff.

The following arrangements are in place to fulfil the co-sponsors' responsibilities:

- The Protocol has been designed by the Chief Investigator and researchers employed by the University and collaborators. The University has insurance in place (which includes no-fault compensation) for negligent harm caused by poor protocol design by the Chief Investigator and researchers employed by the University.

- Sites participating in the study will be liable for clinical negligence and other negligent harm to individuals taking part in the study and covered by the duty of care owed to them by the sites concerned. The co-sponsors require individual sites participating in the study to arrange for their own insurance or indemnity in respect of these liabilities.

- Sites which are part of the United Kingdom's Nation Health Service will have the benefit of NHS Indemnity.
• Sites out with the United Kingdom will be responsible for arranging their own indemnity or insurance for their participation in the study, as well as for compliance with local law applicable to their participation in the study.

• The manufacturer supplying IMP has accepted limited liability related to the manufacturing and original packaging of the study drug and to the losses, damages, claims or liabilities incurred by study participants based on known or unknown Adverse Events which arise out of the manufacturing and original packaging of the study drug, but not where there is any modification to the study drug (including without limitation re-packaging and blinding).

14 REPORTING, PUBLICATIONS AND NOTIFICATION OF RESULTS

14.1 AUTHORSHIP POLICY

Ownership of the data arising from this study resides with the study team. On completion of the study, the study data will be analysed and tabulated, and a clinical study report will be prepared in accordance with ICH guidelines.

14.2 PUBLICATION

The clinical study report will be used for publication and presentation at scientific meetings. Investigators have the right to publish orally or in writing the results of the study.

Summaries of results will also be made available to Investigators for dissemination within their clinics (where appropriate and according to their discretion).

14.3 PEER REVIEW

The proposal for the investigation of Heme arginate as a potential preventative treatment for AKI in the context of elective cardiac surgery has been subject to peer review by the Medical Research Council (MRC) upon successful application for a Clinical Research Fellowship. The Lothian Health Board Endowment Fund has also reviewed the proposal and agreed to contribute to its funding.

15 REFERENCES


APPENDIX 1: Summary of Product Characteristics

Normosang
http://www.medicines.org.uk/emc/medicine/20795/SPC/normosang/

Normal saline (0.9% sodium chloride)
http://www.medicines.org.uk/emc/medicine/23601/spc
APPENDIX 2: Trial Steering Committee

The Trial Steering Committee has been established with the following terms of reference:

A) Terms of Reference

1. To monitor and supervise the progress of the trial towards its objectives;

2. To review at regular intervals relevant information from other sources (e.g. other related trials)

3. In the light of 1, 2 to inform the Funder and Ethical committee on the progress of the trial;

4. To decide on publicity and the presentation of all aspects of the trial.

5. To ensure that the trial is conducted in keeping with the guidelines for Good Clinical Practice.

6. Review of SAEs, participant safety and any necessary action

B) Membership

<table>
<thead>
<tr>
<th>Independent Chairman</th>
<th>Professor Neil Turner, Professor of Nephrology, University of Edinburgh</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chief Investigator</td>
<td>Dr Fiona Duthie, Clinical Research Fellow, University of Edinburgh</td>
</tr>
<tr>
<td>Co-Investigator</td>
<td>Professor Jeremy Hughes, Professor of Experimental Nephrology, University of Edinburgh</td>
</tr>
<tr>
<td>Co-Investigator</td>
<td>Dr David Kluth, Senior Lecturer in Nephrology, University of Edinburgh</td>
</tr>
<tr>
<td>Co-Investigator</td>
<td>Mr Vipin Zamvar, Consultant Cardiothoracic Surgeon, Royal Infirmary of Edinburgh</td>
</tr>
<tr>
<td>Sponsor representative</td>
<td>ACCORD University of Edinburgh, Queen’s Medical Research Institute, Edinburgh</td>
</tr>
<tr>
<td>Trial Statistician</td>
<td>Ms Catriona Graham, Lead Statistician, University of Edinburgh</td>
</tr>
</tbody>
</table>
C) Notes

Meetings
A meeting of the TSC will be held prior to the start of the trial to finalise the protocol. Subsequently the TSC will meet after 6 participants have been recruited, and again after 12, to review safety information. Meetings will be called and organised by the chief investigator. Papers for meetings shall be circulated well in advance of the meeting and an accurate minute of the meeting will be prepared by the chief investigator and agreed by all members.

Trial Steering and Management
The role of the TSC is to provide overall supervision of the trial. The TSC will concentrate on progress of the trial, adherence to the protocol, patient safety and the consideration of new information. Day-to-day management of the trial is the responsibility of the chief investigator.

Patient Safety
In all the deliberations of the TSC the rights, safety and well being of the trial participants are the most important considerations and should prevail over the interests of science and society. The TSC will ensure that the protocol demands freely given informed consent from every trial participant.

Progress of the Trials
It is the role of the TSC to monitor the progress of the trial and to maximise the chances of completing the study within the proposed time scale. At the first TSC meeting, targets for recruitment, data collection, and compliance shall be agreed. These targets will not be “set in stone” but are designed to act as a gauge of trial progress. The TSC will agree a set of data, based on the targets set that should be presented at each meeting.

Data Monitoring Committee
An independent Data Monitoring Committee (DMC) will not be convened for this study. This is a small study which is aiming to inform a larger randomised clinical trial of HA in cardiac surgical patients at high risk of AKI. The TSC will also be tasked with overseeing the safety of participants for the duration of the study.

Adherence to Protocol
The full protocol will be presented as an agenda item at the first TSC meeting. If the investigators need to make any changes to the protocol during the course of the trial, approval will be sought from the TSC, the Sponsor, Lothian REC and MHRA if appropriate.

Consideration of New Information
The TSC should consider new information relevant to the trial including the results of other studies. It is the responsibility of the chief investigator and the chairman and other members of the TSC to bring to the attention of the TSC any results from other studies that may have a direct bearing on future conduct of the trial.
On consideration of this information the TSC should recommend appropriate action, such as changes to the trial protocol, additional patient information or stopping of the study. The rights, safety and well-being of the trial participants should be the most important considerations in these deliberations.

It is the responsibility of the investigators to notify the TSC and relevant regulatory authority (if applicable) of any unexpected serious adverse events during the course of the study.
# TRIAL STEERING COMMITTEE CONTACT DETAILS

<table>
<thead>
<tr>
<th>Role</th>
<th>Name</th>
<th>Contact Information</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Independent Chairman</strong></td>
<td>Prof Neil Turner</td>
<td><a href="mailto:Neil.turner@ed.ac.uk">Neil.turner@ed.ac.uk</a> 0131 242 9167</td>
</tr>
<tr>
<td></td>
<td>Professor of Nephrology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Royal Infirmary of Edinburgh, 51 Little France Crescent, EH16 4SA</td>
<td></td>
</tr>
<tr>
<td><strong>Chief Investigator</strong></td>
<td>Dr Fiona Duthie</td>
<td><a href="mailto:fduthie@staffmail.ed.ac.co.uk">fduthie@staffmail.ed.ac.co.uk</a> 0131 242 6671 07764946050</td>
</tr>
<tr>
<td></td>
<td>Clinical Research Fellow</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Investigator</strong></td>
<td>Prof Jeremy Hughes</td>
<td><a href="mailto:Jeremy.hughes@ed.ac.uk">Jeremy.hughes@ed.ac.uk</a></td>
</tr>
<tr>
<td></td>
<td>Professor of Experimental Nephrology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Investigator</strong></td>
<td>Dr David Kluth</td>
<td><a href="mailto:David.kluth@ed.ac.uk">David.kluth@ed.ac.uk</a></td>
</tr>
<tr>
<td></td>
<td>Senior Lecturer in Nephrology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Investigator</strong></td>
<td>Mr Vipin Zamvar</td>
<td><a href="mailto:Vipin.zamvar@luht.scot.nhs.uk">Vipin.zamvar@luht.scot.nhs.uk</a></td>
</tr>
<tr>
<td></td>
<td>Consultant Cardiothoracic Surgeon</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Royal Infirmary of Edinburgh, 51 Little France Crescent, EH16 4SA</td>
<td></td>
</tr>
<tr>
<td><strong>Co-Sponsor representative</strong></td>
<td>A Representative of the Co-Sponsors</td>
<td></td>
</tr>
<tr>
<td></td>
<td>University of Edinburgh, Queen's Medical Research Institute, 47 Little France Crescent, Edinburgh EH16 4TJ</td>
<td></td>
</tr>
<tr>
<td><strong>Trial Statistician</strong></td>
<td>Ms Catriona Graham</td>
<td><a href="mailto:C.Graham@ed.ac.uk">C.Graham@ed.ac.uk</a> 0131 537 3350</td>
</tr>
<tr>
<td></td>
<td>Lead Statistician</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wellcome Trust Clinical Research Facility</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Western General Hospital</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crewe Road South</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Edinburgh</td>
<td></td>
</tr>
<tr>
<td></td>
<td>EH4 2XU</td>
<td></td>
</tr>
</tbody>
</table>
Appendix 3: The Additive EuroSCORE I scoring system

Patients are assessed and scored on pre-operative factors to generate a total Additive EuroSCORE-1(28). Online calculators can be accessed at http://www.euroscore.org/calcold.html. (reproduced with permission of Sam Nashef).

<table>
<thead>
<tr>
<th>Patient-related factors</th>
<th>Criteria</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>(per 5 years or part thereof over 60 years)</td>
<td>1</td>
</tr>
<tr>
<td>Sex</td>
<td>Female</td>
<td>1</td>
</tr>
<tr>
<td>Chronic pulmonary disease</td>
<td>longterm use of bronchodilators or steroids for lung disease</td>
<td>1</td>
</tr>
<tr>
<td>Extracardiac arteriopathy</td>
<td>any one or more of the following: claudication, carotid occlusion or &gt;50% stenosis, previous or planned intervention on the abdominal aorta, limb arteries or carotids</td>
<td>2</td>
</tr>
<tr>
<td>Neurological dysfunction</td>
<td>severely affecting ambulation or day-to-day functioning</td>
<td>2</td>
</tr>
<tr>
<td>Previous cardiac surgery</td>
<td>requiring opening of the pericardium</td>
<td>3</td>
</tr>
<tr>
<td>Serum creatinine</td>
<td>&gt;2.3mg/dL preoperatively</td>
<td>2</td>
</tr>
<tr>
<td>Active endocarditis</td>
<td>patient still under antibiotic treatment for endocarditis at the time of surgery</td>
<td>3</td>
</tr>
<tr>
<td>Critical preoperative state</td>
<td>any one or more of the following: ventricular tachycardia or fibrillation or aborted sudden death, preoperative cardiac massage, preoperative ventilation before arrival in the anaesthetic room, preoperative inotropic support, intraaortic balloon counterpulsation or preoperative acute renal failure (anuria or oliguria&lt;10 ml/hour)</td>
<td>3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cardiac-related factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Unstable angina</td>
<td>rest angina requiring iv nitrates until arrival in the anaesthetic room</td>
<td>2</td>
</tr>
<tr>
<td>LV dysfunction</td>
<td>moderate or LVEF30-50%</td>
<td>1</td>
</tr>
<tr>
<td>Recent myocardial infarct</td>
<td>(&lt;90 days)</td>
<td>3</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>Systolic PA pressure&gt;60 mmHg</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Operation-related factors</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Emergency</td>
<td>carried out on referral before the beginning of the next working day</td>
<td>2</td>
</tr>
<tr>
<td>Other than isolated CABG</td>
<td>major cardiac procedure other than or in addition to CABG</td>
<td>2</td>
</tr>
<tr>
<td>Surgery on thoracic aorta</td>
<td>for disorder of ascending, arch or descending aorta</td>
<td>3</td>
</tr>
<tr>
<td>Post-infarct septal rupture</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>
Appendix 4: Drug labels

For Clinical Trial Use Only RIE HACS Study

**EudraCT No:** 2014-001021-32
**Chief Investigator:** Dr Fiona Duthie

Heme arginate infusion (3mg/kg) OR Heme arginate infusion (1mg/kg) in 100ml of Sodium Chloride 0.9%

Infusion rate: 200 ml / hour
Subject Name: Subject ID Number:
Date of Birth: Hospital Number:
Date & Time (24h clock) prepared:
Date & Time (24h clock) expires:
Prepared By: Royal Infirmary of Edinburgh

Sponsor: NHS Lothian & University of Edinburgh, Edinburgh Clinical Trials Unit
Keep Out of Reach and Sight of Children

Label for infusion of study drug

For Clinical Trial Use Only RIE HACS Study

**EudraCT No:** 2014-001021-32
**Chief Investigator:** Dr Fiona Duthie

100ml of Sodium Chloride 0.9%

Infusion rate: 400 ml / hour
Subject Name: Subject ID Number:
Date of Birth: Hospital Number:
Date & Time (24h clock) prepared:
Date & Time (24h clock) expires:
Prepared By: Royal Infirmary of Edinburgh

Sponsor: NHS Lothian & University of Edinburgh, Edinburgh Clinical Trials Unit
Keep Out of Reach and Sight of Children

Label for infusion of 100ml 0.9% saline flush over 15 minutes