Clinical Study Protocol

Full Study Title:	A pilot study of thrombolysis during machine perfusion of circulatory death donor livers to prevent biliary strictures
Short title:	Thrombolysis during machine perfusion of DCD donor livers
Protocol Version:	5.1
Protocol date:	7th February 2023
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IRAS project no	297403
Sponsor's number	A095973
REC number	21/EE/0237

Chief investigator:	Professor Andrew Butler
Co-investigators:	Professor Chris Watson
	Mr Rohit Gaurav
	Professor Vasilis Kosmoliaptsis
	Mr James Richards
	Mr David Nasralla
	Mr Satheesh Iype
	Professor Joerg-Matthias Pollok
	Mr Colin Wilson
	Dr Rebecca Brais
	Dr Sara Upponi
Investigator's address:	Department of Surgery, Box 210, Addenbrookes Hospital, Cambridge CB2 2QQ
Study Locations:	1. Cambridge University Hospitals, Cambridge 2.The Royal Free Hospital, London 3. Freeman Hospital, Newcastle
Study Sponsor:	Cambridge University Hospitals NHS Foundation Trust and the University of Cambridge

1 Table of Contents

1 Study Synopsis

Title of clinical trial	A pilot study of thrombolysis during machine perfusion of circulatory death donor livers to prevent biliary strictures
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Sponsor name	Cambridge University Hospitals NHS Foundation Trust and the University of Cambridge
IRAS project number	297403
Sponsor number	A095973
Trial registry Reference number	ISRCTN
REC number	21//EE/0237
Medical condition or disease under investigation	Cholangiopathy in livers removed from donors following circulatory or brain death
Purpose of clinical trial	To assess whether a thrombolytic regimen during *ex situ* normothermic liver perfusion can prevent biliary strictures
Primary objective	To evaluate the safety and feasibility of adding fresh frozen plasma and TPA to the perfusate
Secondary objective (s)	Prevention of biliary strictures (cholangiopathy) Improved liver graft function
Study Design	Pilot study ascending dose three-arm randomised unblinded study
Study Endpoints	Bleeding intra-operatively Feasibility of treating livers with TPA. Return to theatre within 24 hours for bleeding Incidence of anastomotic and non-anastomotic strictures
Sample Size	60 liver recipients
Summary of eligibility criteria	Adults undergoing a deceased donor liver transplant from a donor after circulatory death
Investigational regimen	Tissue plasminogen activator (alteplase) with fresh frozen plasma added to the perfusate *ex situ.*
Route(s) of administration	*Ex situ* (*ex vivo*) into the perfusate of a liver undergoing *ex situ* perfusion
Maximum duration of treatment of a subject	The recipient is not treated, just the liver. Treatment of the liver lasts 60 minutes

2 General information

2.1 Sponsor details

Cambridge University Hospitals NHS Foundation Trust and the University of Cambridge

2.2 Medical Contact

Prof Watson, email cjew2@cam.ac.uk; Mobile 07515 336053
Deputy Mr Andrew Butler

2.3 Site investigators

2.3.1 Cambridge University Hospitals

Prof Chris Watson (PI)

Mr Andrew Butler (CI)

Mr Rohit Gaurav

Mr Vasilis Kosmoliaptsis

Ms Linda Selves

Dr Rebecca Brais

Dr Sara Upponi

2.3.2 Royal Free Hospital

Mr David Nasralla

Mr Satheesh Iype (PI)

Mr James Richards

Professor Joerg-Matthias Pollok

2.3.3 Freeman Hospital, Newcastle

Mr Colin Wilson (PI)

2.4 Laboratories / Technical departments

MRC Laboratory of Molecular Biology in Cambridge for RNA seq

2.5 Trial Manager

None

2.6 Committees attached to trial

None

2.7 Protocol amendments

2.7.1 Amendment 1, to address REC comments

Protocol 23/11/21. Version 2.0

a) Addition of Dr Rebecca Brais as co-investigator

b) Addition of a secondary endpoint: return to theatre for bleeding within 24 hours

c) Alteration of a secondary endpoint from :
"Total and post reperfusion intra-operative blood transfusion and blood loss"
to:
"Total and post reperfusion intra-operative blood transfusion (including cell saved blood". Note that blood loss is already collected as an endpoint

d) Addition of allergy to latex or gentamicin as exclusion criteria

e) Alter the structure of the study in accord with the independent review to one of three ascending dose groups, 10, 20, and 50mg with each group containing a control liver with FFP and no TPA.

2.7.2 Amendment 2, to alter the perfusion protocol

Protocol 15/03/22, version 3.0

a) Rate of infusion

Following receipt of evidence that the fibrin is present in livers before implantation, and not continuously throughout perfusion, the perfusion regimen has been changed to increase its efficacy in the first hour. Appendix 1 shows the evidence behind the change in perfusion regimen.

Perfusion regimen to change as follows: The initial bolus will be 20% TPA and FFP at the start of perfusion, with the remaining 80% given over the first 60 minutes

	TPA				FFP	
Group	**TPA Bolus dose (mg)**	**Infusion dose (mg)**	**Infusion volume (mls)**	**Infusion rate (mls/hour)**	**Bolus volume (mls)**	**FFP infusion rate (mls/h)**
A	2	8	8	8	50	200
B	4	16	16	16	50	200
C	10	40	40	40	50	200

b) Inclusion of a separate cohort of DBD donor livers

A second group of 30 livers from donors after brain death will be included in the study

Other changes:
Version 3.1, date 21-04-22
Minor typographical errors corrected

Version 3.2, date 07-05-22
Alteration of protocol wording in respect of the changes in amendment one which were not completely reflected by the protocol change in versions 3.0 or 3.1.

2.7.3 Amendment 3, to alter perfusion protocol and add an additional site.

Protocol Version 4, date 18-07-22

a) Addition of Newcastle Liver Transplant Unit at the Freeman hospital as a third site

b) Alteration of infusion protocol

The current protocol involves 3 treatment groups, the first two of 5 alteplase treated liver transplants with a control, and the third group of 15 TPA treated liver transplants .
This will change, and the third group will be divided into three group of TPA treated livers. One group will have 50mg alteplase infused as planned. Following this, D-dimer levels will be measured on perfusions in al groups. The lowest dose to achieve high D-dimer levels will then be chosen for the next two groups. These groups will include one where the infusions go into the reservoir and not directly into the arterial cannula, and one where the perfusion duration is stretched to 2 hours and not one. The table below gives the changes.

	TPA				FFP		Infusion Site
Group	**TPA Bolus dose (mg)**	**Infusion dose (mg)**	**Infusion volume (mls)**	**Infusion rate (mgs/hour)**	**Bolus volume (mls)**	**FFP infusion rate (mls/h)**	
A	2	8	8	8	50	200	Artery cannula
B	4	16	16	16	50	200	Artery cannula
C	10	40	40	40	50	200	Artery cannula
D	TBD				50	200	Reservoir
E	TBD				50	100	TBD

TBD: to be determined by D-dimer level comparisons.

Group D: dose to be determined, infused over 1 hour into the portal reservoir
Group E: Dose to be determined, infused over 2 hours either into the portal reservoir or hepatic artery cannula, depending on results of Group D.
Groups A, B, C and D will all get 50mls FFP bolus and 200mls infused over 1 hour, and Group E will get FFP infused over 2 hours as a source of plasminogen

2.7.4 Amendment 4. Change of Chief Investigator

Change of Chief investigator from Prof Watson to Mr Butler

2.7.5 Amendment 5 (declined)

Protocol version 5.1, dated 6/1/23
Added provision to consent retrospectively if the liver is treated following consent and the recipient subsequently changed to a recipient who has not yet been approached about the study. This amendment did not receive a favourable opinion as submitted and the protocol was revised and a separate consent form produced.

2.7.6 Amendment 5 (modified and resubmitted) dated 08/02/2023

Protocol version 5.1, dated 07/02/23

a) Amendment to provide more detailed guidance on what to do if liver is treated, then reallocated.
b) Consent forms for patients being offered a liver that has already been treated.
c) An amendment to protocol in view of fact that 10mg dose given over 60 minutes appears to be efficacious, so 50 mg group (group C) will not be undertaken. 10mg dose will be used for group D.

2.8 Abbreviations

°C	degrees Celsius
ALT	Alanine transaminase
AST	Aspartate transaminase
CTIMP	Clinical trial of an investigational medicinal product
DBD	Donation after brain death
DCD	Donation after circulatory death
EU	European Union
FFP	Fresh frozen plasma
GCP	Good clinical practice
H&E	Haematoxylin and eosin, a histology stain
ICH	International Conference on Harmonisation
ICJME	International Committee of Medical Journal Editors
INR	International normalised ratio
MEAF	Model for early allograft function
MHRA	Medicines and healthcare products regulatory agency
mls	millilitres
MRCP	magnetic resonance cholangiopancreatography
MSB	Martius Scarlet Blue, a histology stain
NaHCO3	Sodium bicarbonate
NAS	Non anastomotic biliary strictures
NESLiP	Normothermic ex situ liver perfusion
NHSBT	National Health Service Blood and Transplant
NMP	Normothermic machine perfusion
pH	$-Log_{10}$ $[H^+]$
REC	Research ethics committee
RIFLE	Risk, Injury, Failure, Loss, and End-stage kidney disease
RNA	Ribonucleic acid
SmPC	Summary of Product Characteristics
SUSAR	Suspected unexpected serious adverse reactions
TPA	Tissue plasminogen activator
UK	United Kingdom

2.9 Trial summary

Livers from donors donating after circulatory arrest (DCD donors) are associated with a high incidence of bile duct strictures after transplantation. This remains the case for liver subject to *ex situ* normothermic perfusion (NESLiP) prior to transplantation, with a rate of around 20% for anastomotic and non-anastomotic strictures. Livers from brain dead donors have a lower incidence of strictures after NESLiP at around is 8%, but they are equally disabling. Recent work in Cambridge has shown that DCD livers during NESLiP develop infarcts in the stromal walls of bile ducts in association with fibrin plugs in the peri-biliary blood supply, suggesting that these fibrin plugs may be the cause of the stromal infarcts, and by extrapolation the bile duct strictures. Subsequent work has shown that by using a similar thrombolytic protocol with tissue plasminogen activator (TPA, alteplase) to that used for clearing clots from the coronary circulation in patients having heart attacks results in no visible fibrin plugs and no bile duct necrosis.

In this study livers will undergo NESLiP and receive an infusion of TPA using a standard thrombolytic regimen for the first 60 minutes of perfusion, with a minimum perfusion time of 2 hours. Alteplase has a half-life of 5 minutes *in vivo* and a 1 hour perfusion will mean there will be at least 60 minutes between the end of the TPA infusion and removing the liver from the machine. Not all DCD donor livers will be viable on testing during NESLiP, but experience suggests that two thirds will meet the viability criteria and be transplanted. It is unlikely that there will be any active TPA in the circuit at the point of removal from the machine. Recipients will then undergo standard follow up with no further research intervention.

This study is a pilot study of 30 DCD and 30 DBD transplants to assess the safety of thrombolysis during *ex situ* perfusion, and to give an idea of is effects on the subsequent outcome of the implanted liver with particular reference to early function and bile duct strictures. The results will be compared with recipients randomised to receive FFP and no TPA. If the study suggests that the intervention is safe and efficacious the protocol will be used in a larger scale multicentre study.

3 Study Flow Chart

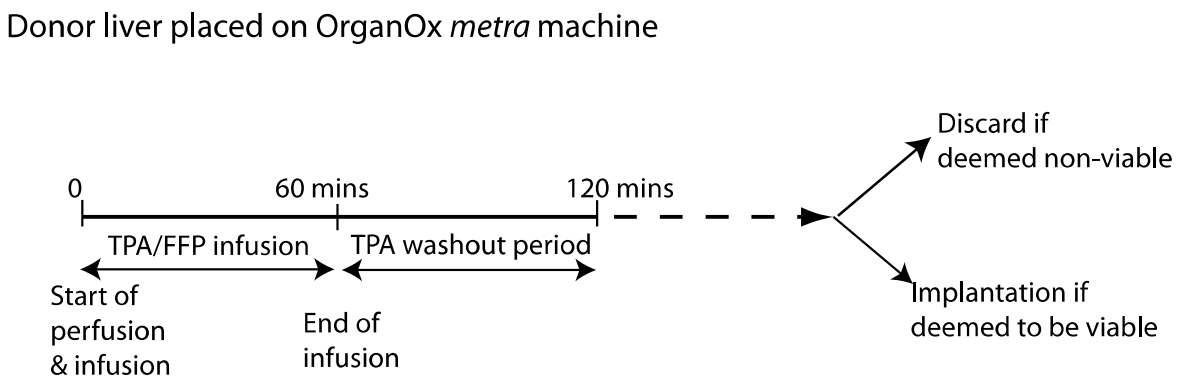

Figure 1: Flow chart for NESLiP assessment of donor livers

4 Background

4.1 Introduction

There are two sorts of deceased organ donors, those certified dead by brain stem criteria (DBD donors) and those certified dead by circulatory criteria (DCD donors). Brain dead donors are confirmed dead and brought to the operating theatre for organ removal while still on a ventilator. The heart is still beating and there is still a blood supply to the organs up to the point they are cooled *in situ* and removed. Circulatory death donors, on the other hand, usually have some sort of brain catastrophe but do not fulfil criteria for brain stem death, but nevertheless future treatment is considered futile and not in their interests. The patients have their treatment withdrawn, ventilation stopped, and after a period of minutes to hours the circulation stops. Death is verified after 5 minutes of circulatory arrest, after which the donor is brought around to the operating theatre, the organs are flushed *in situ* and then removed. Hence there is a period of many minutes where the organs are warm but without a blood supply, a state termed warm ischaemia.

In the UK in 2019/20 there were 1580 deceased organ donors, two thirds of which were DBD and a third DCD[1]. Livers were used from 76% of DBD donors and only 27% of DCD donors, largely because of uncertainty regarding the effects of the period of warm ischaemia. DCD donor livers have a higher incidence of never working (primary non function, 4%), more initial poor function (around 30%)[2], and a high incidence of bile duct strictures (scarring of the bile ducts in over 20%)[2], compared to DBD (6-8%). DCD liver recipients also experience more acute kidney injury than recipients of DBD livers[3].

The aetiology of bile duct strictures in donor livers has not been clear but has been thought to be related to clots in the small vessels supplying the bile ducts (the peri-biliary plexus), since the appearance is similar to that seen when the arterial supply to the liver is lost. The peri-biliary thrombi have been assumed to form during the period of hypoperfusion and circulatory arrest, but this is unlikely since they occur in settings where the donor is heparinised before withdrawal of treatment (such as occurs in Belgium).

Recent research with kidneys undergoing normothermic perfusion has shown the upregulation of fibrinogen genes with ischaemia, and the development of fibrin plugs in small vessels of the kidney, which respond to thrombolytic treatment[4]. At the same time, we have demonstrated that DCD livers undergoing NESLiP develop infarcts of the bile duct wall in association with plugs of fibrin in the peribiliary plexus (figure 2), and that treatment with TPA while using fresh frozen plasma as a source of plasminogen, is associated with an absence of such fibrin plugs and no stromal necrosis.

TPA is used clinically to treat thrombotic occlusions of the coronary and cerebral arteries in patients experiencing heart attacks and strokes, respectively. In these settings it known to clear thrombi and restore a circulation. TPA has also been instilled into the hepatic artery of livers undergoing transplantation while the liver is being implanted. Although reported to be effective at reducing cholangiopathy this strategy has also been associated with excess bleeding [5]. The ability to treat livers *ex situ* before implantation affords a safer opportunity for treatment.

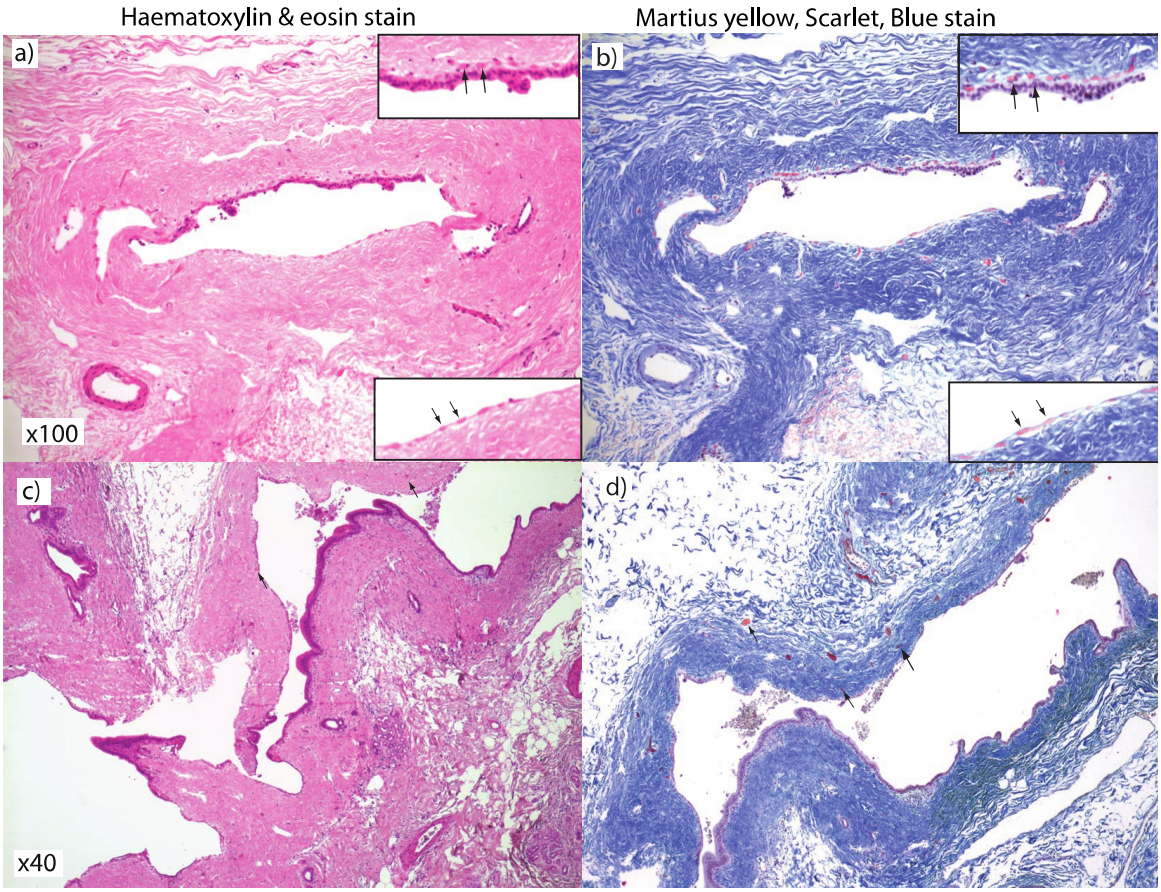

Figure 2: H&E and MSB stained sections of bile ducts from two separate livers showing patchy necrosis of the duct and intravascular clumps of fibrin and erythrocytes. There is widespread stromal necrosis and epithelial loss visible in all 4 panels, best appreciated on the H&E sections. In section (a) eosinophilic inclusions are present in the subendothelial vessels (arrowed). In section (b) these same subendothelial areas are now seen to be intravascular aggregates of fibrin and erythrocytes (staining red and yellow with Martius Scarlet Blue, MSB). The same phenomenon is seen in sections (c) and (d) from a different liver.

4.2 Rationale for Study

Before embarking on a full scale study of thrombolysis during *ex situ* perfusions of DCD livers, we wish to undertake a pilot study to check safety and efficacy.

4.3 Investigational treatment

Alteplase (Actilyse, Boehringer Ingelheim, Germany) is a tissue plasminogen activator used routinely for the treatment of thrombotic occlusive events. *In vivo* it relies on the presence of plasminogen in clots and in the circulation which it breaks down to form plasmin, which is the active lytic component. Alteplase has a very short half-life *in vivo* (5 minutes according to the Summary of Product Characteristics, SmPC [6]).
In the *ex situ* circuit there is no plasminogen circulating, although the liver will make some as time progresses; instead a plasminogen source is required and is readily available in the form of fresh frozen plasma (FFP) which has previously been shown to be effective as a perfusate component in NESLiP [7] as well as being a good source of plasminogen [8].

4.4 Data from non-clinical studies

The use of plasmin to clear microthrombi in DCD organs was first reported in Japan where rat lungs perfused *ex situ* were treated to clear the pulmonary circulation[9]. This followed work showing how urokinase could be used to achieve a similar effect in a dog DCD lung transplant model [10].

4.5 Data from clinical studies

Clinical studies of TPA relate to its use *in vivo* for cerebral and coronary thromboses, and for the treatment of pulmonary emboli. There are no reports of its use in an *ex situ* clinical setting.

4.6 Risks and benefits

The main risk of thrombolytic therapy is bleeding, but since this treatment will take place *ex situ*, and there will be a 60 minute washout period, there is likely to be little TPA left circulating. The plasma half-life of TPA *in vivo* is 4 to 5 minutes [6]. When the liver is removed from the perfusion machine it is flushed with 2 to 3 litres of preservation solution before being implanted, washing away any last traces of perfusate.

4.7 Dose / Regimen

There are a variety of infusion regimens in the SmPC dependent on body weight and on the disease being managed[6].

The first regimen used in the research studies involved a 10mg bolus at the start of perfusion and an infusion of 40mg over 80 minutes (0.5mg/h) together with a 50ml bolus of FFP and an infusion of 200mls over 80 mins (2.5mls/h). Although this cleared the common duct of one liver had some fibrin plugs within it.

A subsequent regimen involved a 5mg bolus of TPA with 25mls of FFP, and an infusion of 45mg TPA and 225mls FFP over 3 hours.

Alternative regimens have been evaluated. The results suggest a 1 hour infusion is optimal, based on d-dimer release, with 50mls FFP loading at the outset followed by an infusion over an hour.

Following independent protocol review, this study will involve 3 initial treatment groups with total doses of 10, 20 or 50mg alteplase, with a 20% loading dose and 80% infusion given over 1 hour. There will be 6 transplanted livers in each of these groups, with at least one in each group receiving FFP and no alteplase. Following these groups D-dimer levels on perfusates over the first 2 hours will be run to determine the efficacy in fibrinolysis. Following that the smallest dose associated with optimal fibrinolysis will be chosen for the next two groups. The fourth group will comprise an infusion into the portal reservoir, rather than into the hepatic artery cannula to assess efficacy of this approach, and the fith group will be over 2 hours instead of one, to assess whether this gives additional benefit in terms of D-dimer release.

Group A: 2mg alteplase bolus and 8mg infused over 1 hour
Group B: 4mg alteplase bolus and 16mg infused over 1 hour
~~Group C: 10mg alteplase bolus and 40mg infused over 1 hour~~ – cancelled in light of efficacy of 10mg dose

Group D: 10mg alteplase, infused over 1 hour into the portal reservoir
Group E: 10mg alteplase, infused over 2 hours either into the portal reservoir or hepatic artery cannula, depending on results of Group D.

Groups A, B, and D will all get 50mls FFP bolus and 200mls infused over 1 hour, and Group E will get FFP infused over 2 hours as a source of plasminogen.

4.8 Population

Patients with liver disease awaiting transplantation who have agreed to receive a liver from a DCD or DBD donor.

4.9 Trial objective and purpose

4.9.1 Primary objective

To evaluate the safety and feasibility of thrombolytic therapy delivered *ex situ, viz:*

- Is it possible to safely treat livers on the perfusion machine with a clot busting regimen?
- Is it associated with excess bleeding in the recipients?

4.9.2 Secondary objectives

- To assess the effects of thrombolytic therapy on early liver function
- To evaluate the efficacy of delivering a thrombolytic treatment *ex situ* to clear fibrin/red cell plugs and prevent symptomatic biliary strictures and obtain data on efficacy to power a lager study

5 Trial Design

5.1 Statement of design

This is a three arm open label pilot study

5.2 Number of Centres

Three centres

5.3 Number of Subjects

Around 60 transplant patients will be recruited. However, since approximately a third of DCD livers undergoing NESLiP will not pass viability criteria, approximately 45 DCD livers will need to be perfused. Similarly, around 15% of DBD livers undergoing perfusion do not pass viability criteria so around 35 DBD livers will need to be perfused.

5.4 Sample size determination

Previous experience with back-to-base perfusion of DCD livers has been associated with a 20% incidence of anastomotic strictures and a 20% incidence of non-anastomotic bile duct strictures (unreported Cambridge data). In a cohort of 20 patients receiving a DCD liver treated by NESLiP it is likely that one will die, five will undergo retransplantation and four will develop non-anastomotic strictures. Similarly, the incidence of non-anastomotic strictures in DBD livers undergoing NESLiP in Cambridge is 8%, with a further 15% suffering anastomotic strictures.

A sample size of 30 DBD and 30 DCD should allow us to evaluate the safety of this therapy. It may also help determine whether the thrombolytic regimen increases the proportion of perfused livers that get transplanted and abolishes non-anastomotic strictures, and therefore whether it is worth proceeding to a formal randomised study and how to power that study

5.5 Study duration

The study will continue until all the subjects are recruited. In a normal year, Cambridge perfuse 25 DCD livers and 25 DBD livers and the Royal Free a similar number. Recruitment should thus be complete within a year and possibly sooner, allowing for patients declining consent and livers not being viable.

5.6 Study endpoints

5.6.1 Primary endpoint

- Post reperfusion intra-operative blood loss
- Feasibility of adding TPA and FFP to livers undergoing normothermic perfusion before transplantation

5.6.2 Secondary endpoint

- Return to theatre in first 24 hours for bleeding
- Total and post reperfusion intra-operative blood transfusion
- Liver utilisation: proportion of liver perfusions resulting in a transplant
- Incidence of symptomatic anastomotic and non-anastomotic strictures at 6 months post-transplant
- Incidence of any anastomotic or non-anastomotic stricture excluding those related to hepatic artery thrombosis
- Incidence of "clinically relevant" non-anastomotic strictures, using the van Rijn definition[11]
- Incidence of post reperfusion syndrome: 30% fall in mean BP lasting at least a minute in the first 5 minutes post reperfusion or the need for adrenaline or doubling of noradrenaline to support the circulation.
- Early allograft function (Olthoff criteria [12] and MEAF score [13])
- Incidence of hepatic artery thrombosis in the first 6 months
- Incidence of acute kidney injury (RIFLE criteria)
- D-dimer release into perfusate compared to controls

5.7 Trial treatments

Infusion of TPA and fresh frozen plasma into the perfusate during DCD liver perfusion.

5.8 Criteria for Discontinuation

5.8.1 Individual liver

5.8.1.1 Transplant criteria

Criteria favouring transplantation of liver include:

Perfusate:

- ALT<6000u/L

- Lactate fall to <2 in 2 hours
- Glucose falling hour on hour after 2 hours
- Producing bile
- Need for <40mls 8.4% $NaHCO_3$ in the first 4 hours to maintain a perfusate pH>7.2 as measured on near patient blood gas machine (not the Organox *metra* reading)

Bile

- pH>7.6
- Bile glucose <2.ommol/L or >10mmol/L less than perfusate.

Criteria favouring non-use of liver include

Perfusate

- ALT>10000iu/L
- Lactate>2.5 at 2 hours
- Glucose failing to fall after the second hour
- Not producing bile
- Requirement for >60mls 8.4% $NaHCO_3$ in first 4 hours of perfusion

Bile

- *pH<7.5*
- Bile glucose >2.0mmol/L or <10mmol/L less than perfusate

The above criteria reveal an area of uncertainty with livers between the definitely usable and definitely unusable. There are currently no internationally accepted viability criteria, but those proposed by ourselves are in accord with most other criteria, and are based on the largest published experience to date [14,15]. That said, a recent case, and the reason for amendment 5, has suggested that assessment at 2 or 4 hours may be too soon and a longer period of perfusion may be advisable in some livers to distinguish between a liver that will not recover, and one that is stunned and recovers after 4 hours.

5.8.1.2 Livers outside criteria with late recovery of function

Where the criteria are outside those favouring transplantation, but the liver appears to recover function as perfusion continues, a decision to use the liver will be taken in consultation with a member of the transplant team who is not involved in the research (typically both the on call hepatologist and surgeon), and the appropriateness of the proposed recipient will also be reviewed. It may be necessary to change recipient if there remains doubt over the liver

5.8.1.3 Change of recipient after liver treatment has begun

Any decision to change recipient will need to go through the national liver offering protocol, that is, it will need to be offered back to all other centres as a “Fast Track” offer and only if they decline its use can it be reallocated locally (unless it was originally accepted as a Fast Track with no named recipient when there is no need to offer the liver back).

If the liver is declined by all centres above Cambridge on the Fast Track offering, then the second recipient will be the person deemed most appropriate on clinical grounds by the medical team (hepatologist and surgeon) on call. If either of the individuals are study members, then someone outside the study should be involved in their stead. The second

recipient should be informed that the liver has been treated as part of the study, as well as being informed of the suboptimal nature of the perfusion parameters and the risk that using the liver for transplantation entails, that is, the risk that it may not function satisfactorily or at all.

5.8.2 Individual subject

Once the liver has been transplanted there is no further trial intervention so there are no criteria for withdrawing patient from study

5.8.3 Trial

The trial will end if:

- 30 DBD and 30 DCD livers have been transplanted
- Evidence of residual thrombolytic activity in the livers post transplant. However, it is common in DCD liver transplantation for the patient to become coagulopathic post implant with evidence of fibrinolysis
- ≥5 cases of anastomotic or non-anastomotic strictures within six months of transplant

5.9 Ancillary / sub studies

5.9.1 Liver biopsies

Biopsies (20mm core biopsies) will be taken pre-perfusion, at the end of perfusion, and one hour after reperfusion in the recipient. They will be divided into two, with half in formalin for histological examination, and half in RNA later for transcriptomic studies. The histological examination will be done using haematoxylin and Eosin (H&E) stained sections and Martius Scarlet Blue (MSB) stained sections.

5.9.2 Bile duct

The end of the bile duct is usually trimmed to remove the crushed area where the cannula draining bile on the machine was tied. This end of bile duct will also be stained with H&E and MSB.

5.9.3 Perfusate samples

Perfusate samples will be taken before perfusion begins, at 30, 60, 90, 120, 180 and 240 minutes. 10mls will be taken and spun down and the cell free perfusate frozen for later analysis including assays of fibrin breakdown and markers of reperfusion injury.

6 Eligibility

6.1 Inclusion Criteria

A recipient of a liver from a deceased donor

6.2 Exclusion Criteria

- Inability to give informed consent
- Age <18 years
- Allergy to Latex or gentamicin by donor or recipient
- Hypersensitivity to TPA by donor or recipient

6.3 Criteria for withdrawal from trial treatment

Subjects cannot withdraw from study treatment as it is completed by the time the liver is transplanted. They can withdraw from the study and their data will be removed from the study files.

7 Randomisation and enrolment

This is a five arm pilot study. In all arms one transplanted liver in six will be randomised to receive FFP and no TPA.
Randomisation sequence will be determined by random number generation using an excel spreadsheet, and concealed in an opaque envelope at the lead site.

8 Treatment regimens

8.1 Liver perfusate

The OrganOx perfusate will be the same as routinely used in the centre, and will comprise 3 units of packed red cells, a plasma substitute (human albumin in Cambridge, Gelofusine at the Royal Free) together with the standard additives and infusions.

8.2 Trial material / source of drugs / presentation of drugs

Alteplase will be reconstituted with sterile water: 10mls for 10mg dose; 20mls for 20mg dose, and 50mls for the 50mg dose, to make a 1mg/ml solution. It should be drawn up into a 50ml syringe to which Normal saline will be added (10mg and 20mg doses only) to achieve a final volume of 50mls. The syringe containing alteplase will be placed on a syringe driver and connected to a three-way tap attached to the arterial cannula within the liver container for the first 3 groups, and on the portal reservoir for the 4th group. The site of infusion for the 5th group will be determined from a D-dimer analysis after completion of the first 4 groups.

One unit of fresh frozen plasma (approx. 250mls) will be run through a giving set or divided into five 50mls syringes; 50mls will be added to the soft shell reservoir, the remaining 200mls will be delivered over 60 mins using an infusion pump or syringe driver to the second port of the three-way tap.

Once the liver is placed on the circuit **and as perfusion is begun,** 20% of the total dose of alteplase for the study group (see below) is infused through the arterial cannula (groups A, B, and C) or portal reservoir (group D). The remainder of the dose delivered to the arterial cannula / portal reservoir infused over one hour.

Group	Alteplase bolus dose (mg)	Alteplase infusion dose (mg)	Infusion site
A	2	8	Hepatic artery cannula
B	4	16	Hepatic artery cannula
~~**C**~~	~~10~~	~~40~~	~~Hepatic artery cannula~~
D	2	8	Portal reservoir
E	10	0	To be decided

Addendum: *The initial analysis of the 10mg dose data suggest this is more than adequate, so there will be no 50mg treatment arm (Group C).*

It is important not to mix the FFP and alteplase before they are added by infusion since the plasminogen in FFP will be rapidly converted to plasmin, and this plasmin will itself be broken down rapidly afterwards.

8.3 Known drug reactions

Immune mediated hypersensitivity to alteplase is associated with sensitivity to gentamicin (traces may be present) and latex (rubber in the bung of the glass vial). Angio-oedema is also reported. It is unclear whether these will have any manifestation in an isolated perfused liver or the recipient of the same.

8.4 Legal status of the drug

Alteplase is licensed for thrombolytic treatment in acute myocardial infarction, thrombolytic treatment in acute massive pulmonary embolism with haemodynamic instability, and fibrinolytic treatment of acute ischaemic stroke.

8.5 Drug storage and supply

Once reconstituted, alteplase can be stored for 24 hours in a fridge at 2°C to 8°C, or 8 hours at 25°C. Once thawed, fresh frozen plasma can be stored for 24 hours at 4°C.

9 Study procedure and assessments

9.1 Informed consent

An information sheet will be sent to all recipients on the liver transplant waiting list who expressed a willingness to accept a deceased donor liver. Informed consent will be taken in hospital/clinic while on the waiting list although there may be occasions when the recipient is called in for a transplant having received the information letter but not been formally consented. They will be approached on the ward and given at least an hour to make a decision regarding participation.

9.2 Unconsented recipients

On rare occasions treatment of the liver according to the randomisation will begin with the consent of one patient, but a decision may be made to change recipient. Examples of such circumstances are as follows:

- The liver function during perfusion is such that the first recipient is not deemed appropriate, e.g., a suboptimal liver and a recipient likely to get a better liver soon on the National Liver Offering Scheme.
- The intended recipient is not able to receive the liver, for example, if he suffers an anaesthetic reaction before explant of the transplant and his operation must be abandoned
- A recipient of greater urgency requires the liver instead of the intended recipient. For example, when two livers are accepted, and the intended liver for the urgent case turns out to be unusable, such that the liver on the machine is required for the urgent case.

In such circumstances the actual recipient should be informed, before the transplant (unless they are unconscious or encephalopathic) that the liver has been part of a trial. They should then be asked if they wish to participate or not and shown the patient

information sheet prepared for this eventuality. If they decide not to participate, no further trial intervention (e.g. taking biopsies for RNA analysis) will be undertaken.

9.3 Recipient who cannot consent

It is the intention to only enrol consenting subjects into this trial. However, in the circumstance above, where the liver has commenced perfusion and the trial treatment has begun, and is then switched from an intended recipient who has consented to an urgent patient who is unable to consent (e.g. he is unconscious or encephalopathic), and in the absence of the next of kin, a clinician independent from the study team will be required to give their approval. This could be a consultant hepatologist, consultant surgeon, or the medical director. They should record their agreement in the patient's notes. In this setting the transplant will proceed as "normal care" and no research samples will be taken, no research biopsies taken, and no research data recorded. In this circumstance, the patient may be approached once he has recovered and retrospective consent sought for data collection.

9.4 Baseline data

All patients will have a full medical history taken and a clinical examination as part of the admission process. The following routine tests are to be recorded:

a) Weight
b) Sex
c) Age
d) Liver disease and indication for transplant
e) Full blood count (including platelets and differential white cell count)
f) INR
g) Biochemical series (including creatinine, sodium, bilirubin, alkaline phosphatase, AST/ALT) to allow calculation of UKELD, MELD and MELDNa scores

9.5 Study assessments

9.5.1 Perfusate and biopsy samples

	Liver biopsy*	Perfusate sample (10ml)	Recipient blood sample
Liver benchwork	x		
Pre-perfusion		x	
30 min		x	
60 mins		x	
90 mins		x	
120 mins		x	
180 mins		x	
240 mins		x	
Immediately before removal from machine	x	x	x
60 – 120 mins after reperfusion in recipient (at close)	x		x

* Liver biopsies will involve dividing the routine clinical biopsies into two, sending one half for histopathology and the other will be stored in RNALater for RNA analysis.

9.5.2 Peri reperfusion blood pressure

Mean blood pressure in the 5 minutes before and in the 5 minutes after reperfusion.
Was adrenaline given?
Were other inotropes given or increased in dosage?

9.5.3 Recordings in the first week

- ALT days 1 to 10
- INR days 1 to 10
- Bilirubin days 1 to 10
- Platelets days 1 to 10
- Peak creatinine in first 7 days

9.5.4 Assessment at 6 months

- Graft survival
- Patient survival
- Result of MRCP if one has been done

10 Statistics

10.1 Study statistician

Statistical analysis will be undertaken by a member of the research team, either Professor Watson or designated deputy.

10.2 Number of Subjects to be enrolled

60 subjects in total, 30 DBD and 30 DCD. This will represent around 45 DCD and 35 DBD livers needing to be perfused. It is anticipated this will be 35-45 per site.

10.3 Sample size considerations

This is a pilot study of the safety and efficacy of *ex situ* thrombolytic therapy to assess whether a full clinical trial of the treatment is warranted. The TPA perfused livers and the number of transplants will be compared to the control perfusions .

10.4 Response criteria

10.4.1 Bile duct strictures

In a review of 82 DCD livers perfused in Cambridge between 1/2/2018 and 31/12/2020, 29 (35%) livers were not deemed suitable for transplantation. Of the 53 that were transplanted, 11 (21%) developed either anastomotic, hilar or peripheral strictures, and many developed both (table 1). MRCPs were done in 20/53 = 38% of cases, indicated by clinical concern, and read separately by two radiologists, who agreed on the presence of strictures in all cases but differed in their assessment of severity.

Liver number	Anastomotic stricture	Hilar stricture	First order duct stricture	Second order duct stricture	Peripheral duct stricture
134750	no	yes	yes	yes	yes
135596	no	yes	yes	yes	yes
137384	yes	yes	yes	yes	yes
138054	yes	yes	yes	no	no

138608	yes	Yes	yes	yes	yes
138925	Yes	Yes	Yes	Yes	Yes
141319	no	yes	yes	yes	yes
142228	yes	yes	yes	yes	no
142459	yes	no	no	no	no
143102	yes	yes	yes	yes	Yes
Totals	7/53 = 13%	10/53 = 19%	10/53 = 19%	9/11 = 17%	7/53 = 13%

Given these data it is estimated that 20 transplanted livers would allow sufficient assessment of efficacy and safety to decide whether to proceed to a formal randomised trial; assessment of the number of livers perfused which resulted in a transplant may give an indication of comparative utilisation rates.

An incidence of 10% or less of strictures without complications would be a good indication to proceed with a trial. The current standard of care is likely to change in the next 6 months with the recent publication of the results of a study of hypothermic oxygenated perfusion which reported clinically significant non-anastomotic biliary stricture (NAS) rate in DCD livers of 6%, although they also reported radiological NAS rate of 65%, and a 29% anastomotic stricture rate[11].

10.4.2 Post reperfusion syndrome

Defined as a fall in mean arterial pressure of 30% for one minute in the first 5 minutes post reperfusion of the liver compared to the 5 minutes before reperfusion, or the requirement for adrenaline in that period.

10.4.3 Early allograft dysfunction

Early allograft dysfunction will be measured by two methods. In the Olthoff method, it is defined by either a rise in transaminases (ALT or AST) to over 2000u/L between 1 and 7 days post-transplant, or an INR>1.6, or a bilirubin >10mg/dL (171 µmol/L) on day 7[12]. Treatment and comparator groups will also be compared by the model for early allograft function (MEAF) score, a continuous score out of 10 reflecting variables measured in the first 3 days [13].

10.4.4 Acute kidney injury

Acute kidney injury will be defined by the RIFLE criteria as a creatinine in the first 7 days post-transplant which has risen more than two-fold higher than the pre-transplant value, or the need for haemofiltration or dialysis. The peak to baseline values will also be compared.
The ratio of peak (d1-7) over baseline creatinine will also be recorded and compared with historical comparators; the absolute percentage where the ratio is ≥2.0 will also be compared.

10.4.5 Survival (patient and graft)

These will be measured from the date of transplant and will be reported for all deaths and graft failures both due to rejection and due to all causes.

10.5 Statistical methods to be employed

Descriptive data will be presented as median, interquartile range or range (continuous variables) or number, percentage (categorical variables) and compared using the Kruskal-Wallis test (continuous variables) and Fisher's exact test (categorical variables). Survival will be compared with log rank analysis.

10.6 Analysis plan

Transplant recipients in the pilot study will be compared with twice the number of recipients of DCD livers that were machine perfused in the recipient centres in the previous 2 years. They will be matched for agonal phase duration, cold ischaemia time and UK donor liver index [16].

10.7 Trial supervision

As the trial is a small pilot study of only 60 subjects in two centres both very experienced in perfusing donor livers *ex situ,* external supervision of the study processes is not considered necessary.

Because the treatment intervention is novel an internet meeting (e.g. Zoom or Teams) will be held between sites to review each of the first 4 cases, then the next 6, and then the last 10.

11 Assessment of Safety

Contact with the Medicines and Healthcare products Regulatory Agency (MHRA) has been made. They stated that "requirement for MHRA notification would depend on the manufacturer's intended use of the device". This trial will use the machine for the manufacturer's intended use, and indeed it is trying to enhance its efficacy. They are content that this is not a clinical trial of a medicinal product (CTIMP).

11.1 Definitions

The following definitions of adverse events and reactions apply to clinical trials of investigational medicinal products, **which this is not**; it has been modified accordingly. Nevertheless, we will use the terms adverse events/reaction for incidents explicable by the use of the TPA/FFP in this study.

11.1.1 Adverse event

Any untoward medical occurrence in a patient or clinical trial subject which does not necessarily have a causal relationship with this treatment. An adverse event would be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with study treatment, whether or not considered related to the investigational medicinal product

11.1.2 Adverse reaction of an investigational medicinal product (AR)

All untoward and unintended responses to the treatment. All adverse events judged by either the reporting investigator or the sponsor as having a reasonable causal relationship to the treatment qualify as adverse reactions. The expression reasonable causal relationship means to convey in general that there is evidence or argument to suggest a causal relationship

11.1.3 Unexpected adverse reaction

An adverse reaction, the nature, or severity of which is not consistent with the applicable product information (e.g. investigator's brochure for an unapproved investigational product or summary of product characteristics (SmPC) for an authorised product). When the outcome of the adverse reaction is not consistent with the applicable product information this adverse reaction should be considered as unexpected.

The term "severe" is often used to describe the intensity (severity) of a specific event. This is not the same as "serious," which is based on patient/event outcome or action criteria.

11.1.4 Serious adverse event or serious adverse reaction

Any untoward medical occurrence or effect that:

- results in death,
- is life-threatening
- requires hospitalisation or prolongation of existing inpatients´ hospitalisation,
- results in persistent or significant disability or incapacity,
- is a congenital anomaly or birth defect.

Life-threatening in the definition of a serious adverse event or serious adverse reaction refers to an event in which the subject was at risk of death at the time of event; it does not refer to an event which hypothetically might have caused death if it were more severe.

11.2 Expected adverse drug reactions

No drug is being given to the recipient so no adverse reactions would be expected.

11.3 Expected Serious Adverse Events

Liver transplantation is a life-saving procedure but is associated with serious life-threatening complications. It is expected that many of these will be encountered during the study and which we do not propose to report as AEs or SUSARs to the REC/Sponsors.

- Peri-operative death (within 48h) ~ 1 to 2%
- Death from graft failure or sepsis or other complication in the first year: 5%
- Primary non function: 4% for DCD livers; 1% for DBD livers
- Early allograft dysfunction (Olthoff criteria): 40% DCD, 25% DBD.
- Hepatic artery thrombosis: 4-15%, depending whether anomalous arterial supply required reconstruction or not
- Post reperfusion syndrome: 30%
- Biliary anastomotic breakdown 5%
- Post-operative haemorrhage requiring reoperation: 10%
- Acute rejection: 20%
- Acute kidney injury (Rifle definition): 50% DCD, 35% DBD.
- Chest complications: sepsis; effusion; paralysed right hemidiaphragm: common
- Ascites: universal if pre-existing ascites, common if not pre-existing.

- Complication associated with operative techniques in particular, or surgery in general (e.g. adhesions, adhesive obstruction, wound infection, wound hernia, wound dehiscence)
- Line sepsis
- Biliary anastomotic stricture: 20% DCD, 15% DBD
- Non-anastomotic strictures: 20% DCD, 8% DBD
- Retransplantation in first year: 17% DCD, 11% DBD.

11.4 Recording and evaluation of adverse events

Individual adverse events will be evaluated by the investigator and, where indicated, will be reported to the sponsor for evaluation. This includes the evaluation of its seriousness and the causality between the therapy and the adverse event.

The sponsor has to keep detailed records of all AEs reported by the investigator(s) and to perform an evaluation with respect to seriousness, causality and expectedness.

11.4.1 Assessment of seriousness

Mild: The subject is aware of the event or symptom, but the event or symptom is easily tolerated

Moderate: The subject experiences sufficient discomfort to interfere with or reduce his or her usual level of activity

Severe: Significant impairment of functioning; the subject is unable to carry out usual activities and / or the subject's life is at risk from the event.

11.4.2 Assessment of causality

Probable: A causal relationship is clinically / biologically highly plausible and there is a plausible time sequence between onset of the AE and administration of the investigational medicinal product and there is a reasonable response on withdrawal.

Possible: A causal relationship is clinically / biologically plausible and there is a plausible time sequence between onset of the AE and administration of the investigational medicinal product.

Unlikely: A causal relation is improbable and another documented cause of the AE is most plausible.

Unrelated: A causal relationship can be definitely excluded and another documented cause of the AE is most plausible.

11.5 Reporting adverse events

The sponsor is responsible for the prompt notification to all concerned investigator(s), the Research Ethics Committee and competent authority (e.g. MHRA) of each concerned Member State of findings that could adversely affect the health of subjects, impact on the conduct of the trial or alter the competent authority's authorisation to continue the trial in accordance with Directive 2001/20/EC.

11.6 Reporting of Suspected Unexpected Serious Adverse Reactions (SUSARs)

All suspected adverse reactions related to the treatment which occur in the concerned trial, and that are both unexpected and serious (SUSARs) are subject to expedited

reporting.

11.6.1 Who should report and whom to report to?

The sponsor should report all the relevant safety information previously described to the concerned competent authorities and to the Ethics Committee concerned. The sponsor shall inform all investigators concerned of relevant information about SUSARs that could adversely affect the safety of subjects.

11.6.2 When to report?

11.6.2.1 Fatal or life-threatening SUSARs

The Research Ethics Committee should be notified as soon as possible but no later than 7 calendar days after the sponsor has first knowledge of the minimum criteria for expedited reporting.
In each case relevant follow-up information should be sought and a report completed as soon as possible. It should be communicated to the Ethics Committee within an additional eight calendar days.

11.6.2.2 Non-fatal and non life-threatening SUSARs

All other SUSARs and safety issues must be reported to the Ethics Committee in the concerned Member States as soon as possible but no later than 15 calendar days after the sponsor has first knowledge of the minimum criteria for expedited reporting. Further relevant follow-up information should be given as soon as possible.

11.6.3 How to report?

11.6.3.1 Minimum criteria for initial expedited reporting of SUSARs

Information on the final description and evaluation of an adverse reaction report may not be available within the required time frames for reporting. For regulatory purposes, initial expedited reports should be submitted within the time limits as soon as an adverse event is assessed as serious and unexpected, and for which there is a reasonable suspected causal relationship.

11.6.3.2 Follow-up reports of SUSARs

In case of incomplete information at the time of initial reporting, all the appropriate information for an adequate analysis of causality should be actively sought from the reporter or other available sources. The sponsor should report further relevant information after receipt as follow-up reports.
In certain cases, it may be appropriate to conduct follow-up of the long-term outcome of a particular reaction.

12 Data handling and record keeping

Data will be collated on a password controlled study database held on an encrypted partition of computer hard disc, or trust or university server.

13 Direct access to source data / documents

The investigators will permit trial related monitoring, audits, REC review, regulatory inspections.

14 Publications policy

Authorship will follow the guidelines of the International Committee of Medical Journal Editors (ICJME)[1] based on satisfying the following 4 criteria

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work;
- Drafting the work or revising it critically for important intellectual content;
- Final approval of the version to be published;
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

15 Finance

Since the intervention is to be in patients already having a DCD liver which is to undergo *ex situ* perfusion, the only study related expense is the provision of alteplase and fresh frozen plasma. This cost will be borne out of local funds at each participating hospital.

Subsequent analyses on biopsies and perfusate will be funded from future grants.

16 Ethical considerations

The investigators do not envisage any unusual ethical issues.

16.1 Consent

All patients will freely give their informed consent to participate in the study. A patient may decide to withdraw from the study at any time without prejudice to their future care.

16.2 Ethical committee review

The study protocol is to be seen and approved by the appropriate ethical review committee(s) . Copies of the letters of approval are to be filed in the study file.

16.3 Declaration of Helsinki and ICH Good Clinical Practise

The study is to be carried out in conformation with the spirit and the letter of the declaration of Helsinki, and in accord with the ICH Good Clinical Practice Guidelines

17 Regulatory approval

The Medicines and Healthcare products Regulatory Agency have been consulted regarding the regulatory status of this study. They are content that this is not a clinical trial of an investigational medicinal product (CTIMP).

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19 Appendix 1. Rationale for change of infusion regimen.

A cohort of research livers infused with different protocols of TPA, varying from 20 to 50mg, over 80 to 180 minutes were reviewed. Perfusate samples were assayed for d-dimers, a marker of fibrin degradation. Figure A1 below shows that beyond an hour the d-dimer concentration does not increase, suggesting that in spite of infusions up to 3 hours most of the fibrin was degraded in the first hour.

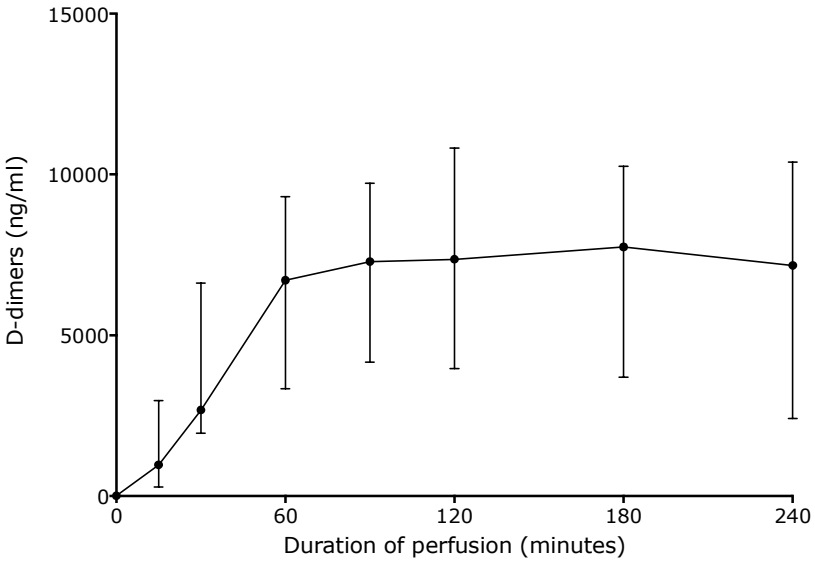

Figure A1. D-dimer concentrations in perfusate at different time points following the onset of perfusion.

This study was extended and a cohort of 20 livers, DBD and DCD, were flushed with 2L Hartmann's solution before being placed on the machine. D-dimers were assayed in the effluent. Figure A2 below shows that several livers had significant concentrations of d-dimers in the effluent, suggesting the clot was in the liver before the liver was placed on the machine. Clot was present in both DBD and DCD livers, with two DCD livers being particularly affected. Liver A failed to work, and was replaced on day 2; liver B has venous outflow complications, both reflecting the clot burden.

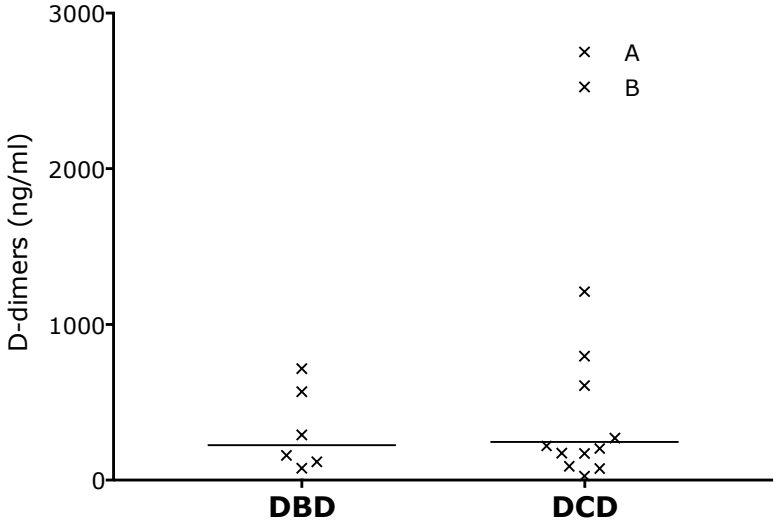

Figure A2. D-dimer concentrations in the effluent preservation solution washed out of the liver before being placed on the machine