Increase in post-reperfusion sensitivity to tissue plasminogen activator-mediated fibrinolysis during liver transplantation is associated with abnormal metabolic changes and increased blood product utilisation

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Background. Increased systemic fibrinolytic activity can occur in liver transplant recipients after the donor graft is reperfused. However, it remains unclear whether this is related solely to tissue plasminogen activator (t-PA) levels or whether unique metabolic changes can alter t-PA activity and enhance fibrinolytic activity. We hypothesise that an increase in sensitivity to t-PA-mediated fibrinolysis (StF) following liver reperfusion is associated with specific metabolic abnormalities.

Materials and methods. Liver transplant recipients had serial blood samples analysed with a modified thrombelastography assay using exogenous t-PA to measure sensitivity/resistance to fibrinolysis with the lysis 30 min after maximum clot strength (tLY30). Paired plasma samples were analysed with mass spectroscopy-based metabolomics. The tLY30 was correlated to metabolites using Spearman's rho. StF was defined as a tLY30 change of >8.5% from the anhepatic phase to 30 min after reperfusion based on the distribution of tLY30 in a healthy control population.

Results. StF occurred in 53% of patients. Cohorts had similar MELD scores (18 *vs* 16, p=0.876) and tLY30 at baseline (p=0.867) and anhepatic phase of surgery (p=0.463). Thirty min after reperfusion, the tLY30 was 73% in patient with StF *vs* 33% in those without StF 33% (p=0.006). StF was associated with increased red blood cell transfusions (p=0.035), during the first 2 hours of reperfusion. Nine metabolites demonstrated a correlation with tLY30 (p<0.05).

Discussion. StF is a transient event that resolves within 2 hours of graft reperfusion and is associated with increased blood product use. This phenomenon correlates with derangements in citric acid cycle, purine and amino acid metabolism. Future research is needed to determine whether these metabolites are biomarkers or mechanistically linked to increased sensitivity to t-PA-mediated fibrinolytic activity following graft reperfusion.

Keywords: tissue plasminogen activator, liver transplant, metabolomic, reperfusion.

Introduction

Reperfusion of the graft during liver transplantation coincides with a release of metabolically deranged blood from the stagnant portal circulation. There is great variability in how patients respond to this reperfusion event, as anywhere from 12-77% liver transplant recipients will develop a post-reperfusion syndrome[1]. This syndrome is characterised by an acute onset of hypotension[2] and is associated with increased fibrinolytic activity[3]. The severity of the post-reperfusion syndrome has been correlated to the frequency of anti-fibrinolytic use during surgery and blood product use after the liver has been transplanted[3]. However, analysis of post-reperfusion fibrinolysis independent of the post-reperfusion syndrome has received less attention, despite historic literature demonstrating a unique cohort of patients with increased systemic hyperfibrinolysis following reperfusion[4]. Recent biochemical characterisation of plasma from trauma patients has demonstrated a correlation of metabolic abnormalities with increased susceptibility to tissue plasminogen activator (t-PA)-mediated fibrinolysis[5]. These same biological markers may also correlate with changes in sensitivity to t-PA-mediated fibrinolysis early after graft reperfusion.

That metabolites alter fibrinolytic activity is not a new concept, as this was first described in the 1950s[6]. Okatama *et. al.* probed lysine derivatives in an effort to alter fibrinolysis, which produced clinically utilised antifibrinolytics, including aminocaproic acid and tranexamic acid[6]. Since 1978 both ischaemia and reperfusion injury have been linked to metabolic

derangements[7]. Citric acid cycle intermediates, such as succinate, accumulate during ischaemia and although they are rapidly cleared in reperfusion, this accumulation results in the generation of reactive oxygen species and reperfusion damage[7]. Moreover, several of these citric acid cycle metabolites have been correlated with increased sensitivity to t-PA-mediated fibrinolysis in trauma patients[5].

To characterise changes in sensitivity to t-PA-mediated fibrinolysis during surgery we used a modified viscoelastic assay that employs exogenous t-PA to challenge a patient's whole blood fibrinolytic system[5]. The objective of this technique was to quantify changes in sensitivity to t-PA-mediated fibrinolytic activity from the anhepatic phase to 30 min following reperfusion. To evaluate the role of unmeasured metabolites in these changes in sensitivity to t-PA-mediated fibrinolysis, the metabolic profile of liver transplant recipients was characterised during the same time frame. We hypothesise that increased sensitivity to t-PA-mediated fibrinolysis (StF) is common following liver reperfusion and is associated with increased blood product use and with a signature of metabolic abnormalities in the plasma.

Materials and methods

Patients

After obtaining informed consent, liver transplant recipients undergoing cadaveric liver transplant were enrolled in a study approved by the Colorado Multi-Institutional Review Board to prospectively collect blood samples during the peri-operative period. All patients were transplanted at the University of Colorado Hospital, which averages about 100 liver transplant a year. As liver transplantation is not a scheduled procedure, not all liver transplant recipients were enrolled prior to surgery, and during the 8-month study period (July 2015-February 2016) approximately one-third of the patients gave consent and were enrolled. Patients were over 18 years old and recruited in a continuous fashion. Recipients of organs from living, related donors were excluded. Recipient demographics, including age, sex, and co-morbidities, were recorded. The model for end-stage liver disease (MELD) score was calculated on the day of surgery and does not represent the MELD score at listing in the United Network of Organ Sharing (UNOS). Donor characteristics that were recorded included age, sex, comorbidities, and routine laboratory values. Additional variables were the body surface area index of the donor and recipient[8]. The transplants were performed by five different surgeons. Venous bypass was not employed and limited portal flush was performed just prior to graft reperfusion in all cases. During this time-frame of this study 19 patients were successfully enrolled.

Outcomes

Recipient outcomes included StF, defined as an increase in sensitivity to fibrinolysis (with the lysis measured 30 min after maximum clot strength [tLY30]) by 8.5% 30 min after graft reperfusion. This definition is based on a separate study of t-PA thromboelastography (t-PA TEG) in a healthy volunteer population of 160 individuals in which the median tLy30 was 8.5% in response to a 75 ng/mL concentration of t-PA. This concentration of t-PA was previously identified to be the lowest concentration of exogenous t-PA that statistically increased TEG-detected fibrinolysis quantified by tLY30 in a healthy population[5]. Additional outcomes included the total number of blood products used during the first 2 hours of reperfusion, including red blood cells, fresh frozen plasma, cryoprecipitate, platelets, activated factor VII, and antifibrinolytic agents. Total blood product use in the first 24 hours after incision was also compared between groups.

Blood samples for viscoelastic testing and metabolomic analysis

The anaesthesiology team obtained blood samples through a radial arterial line placed after induction of anaesthesia. Blood was stored in 3.5 mL tubes containing 3.2% citrate and transferred directly for analysis by a trained professional research assistant. All viscoelastic assays were completed within 2 hours of the blood being drawn and plasma from the same citrated collection tubes was processed within 30 min of blood drawing. Blood samples were obtained, before the surgical incision (baseline), during the native hepatectomy, during the anhepatic phase (5 min after removal of the native liver from the recipient), 30 and 120 min after reperfusion (determined as the time from unclamping the portal vein), and on post-operative day 1.

Viscoelastic assays

All viscoelastic assays were performed by a single trained research assistant with experience running more than 1,000 assays. Citrated blood collected from the operating room or surgical ward was analysed using the TEG 5000 Thrombelastograph Hemostasis Analyzer (Haemonetics, Niles, IL, USA). The following indices were obtained from the tracings of the TEG: reaction time (R-time) (min), angle (°), maximum amplitude (MA [mm]), and lysis 30 min after the MA (LY30 [%]). A modified assay to quantify sensitivity to fibrinolysis was run in a parallel with a citrated native TEG assay. The same TEG analysis was used for this assay, but blood prior to re-calcification underwent modification by adding exogenous t-PA. This t-PA challenge of whole blood has previously been validated to quantify fibrinolysis sensitivity and

resistance *in vitro* for assessing the effects of different proteins[9,10] and metabolites[9]. Evaluation of clinical samples using this assay has previously been conducted in trauma patients[5]. In short, 500 µL of whole blood were pipetted into a customised vial containing lyophilised t-PA (Molecular Innovation, Novi, MI, USA) to a final concentration of 75 ng/mL t-PA, and mixed by gentle inversion. A 340 µL aliquot of this mixture was then transferred from the vial to a 37 °C TEG cup, preloaded with 20 µL of 0.2 mol/L $CaCl_2$.

Metabolomics

Metabolomic analyses were performed as previously reported[11] on plasma samples of the 15 patients paired to TEG during the anhepatic phase of surgery and 30 min after reperfusion. Briefly, 20 µL of plasma were extracted in 980 µL of ice-cold extraction buffer. After discarding the protein pellets, water and methanol soluble fractions were run through a reversed phase column (250 µL/min - phase A: water, 0.1% formic acid; phase B: acetonitrile, 0.1% formic acid - Phenomenex, Torrance, CA, USA) through an ultra-high performance chromatography system (UHPLC - Vanquish, Thermo Fisher Scientific, San Jose, CA, USA). The UHPLC was coupled on line with a high-resolution quadrupole Orbitrap instrument operated in either polarity modes (QExactive, Thermo Fisher Scientific, Bremen, Germany) at 70,000 resolution (at 200 m/z). Metabolite assignment and peak integration for relative quantitation were performed through the software Maven (Apache Software Foundation, Forest Hill, MD, USA), against the KEGG pathway database and an in-house validated standard library (>800 compounds; SIGMA Aldrich [Saint Louis, MO, USA]; IROATech [Bolton, MA, USA]). Integrated peak areas were exported into Excel (Microsoft, Redmond, CA, USA).

Tissue plasminogen activator and plasminogen activator inhibitor-1 activity and complex levels

Additional plasma samples at the various time points were available for 14 of the 15 patients (n=7 per cohort) to measure t-PA and its inhibitor, plasminogen activator inhibitor-1 (PAI-1). The activity of t-PA and PAI-1 in addition to t-PA/PAI complex levels were quantified by enzyme-linked immunosorbent assay (Molecular Innovations, Novi, MI, USA).

Statistical analysis

Statistical analyses of all clinical variables and outcomes were performed using SPSS version 22 (IBM, Armonk, NY, USA). Normally distributed data were described as mean ± the standard deviation, and abnormally distributed data were described as the median value with the 25th to 75th percentile values (IQR). Correlations between changes of TEG LY30 and metabolites were assessed with Spearman's rho. Categorical data were compared between StF and non-StF organ recipients and donors with a Pearson's chi-square test. Non-normally distributed and ordinal data were compared between groups with a Kruskal-Wallis test. Temporal changes within cohorts (StF *vs* non-StF) between the anhepatic and reperfusion measurements were compared with a Wilcoxon's test. For metabolic analysis, receiver operating characteristic (ROC) curves, partial least square-discriminant analysis and heat maps were elaborated with Metaboanalysis 3.0 software[12] and processed for statistical analysis (t-test, ANOVA) and hierarchical clustering analysis through Graph Pad Prism (Graph Pad Software Inc, La Jolla, CA, USA) and GENE E (Broad Institute, Cambridge, MA, USA), respectively.

Results

Patient and donor characteristics

Fifteen patients were included in the analysis. Four additional patients enrolled during the time frame of the study were excluded (3 because of incomplete blood withdrawal and 1 because of death before reperfusion). The median MELD score of the population of patients was 16 (IQR 11-31), 53% of the patients were female, and the median age was 63 years (IQR 54-67). Hepatocellular carcinoma was diagnosed in 60% of patients prior to surgery. The median age of donors was 25 years old (IQR 20-44), 40% were female, and 73% had trauma as the cause of brain death. There were no living donors and none of the organs to be transplanted were donated after cardiac death. The median number of transfusions in the first 24 hours after incision in the cohort were as follows; red blood cells 5 (IQR 1-9), plasma 4 (IQR 1-12), cryoprecipitate 0 (IQR 0-0), platelets 1 (IQR 0-2).

Increased sensitivity to tissue plasminogen activator-mediated fibrinolysis after reperfusion

StF occurred in 53% of recipients. The non-StF and StF cohorts had similar MELD scores (18 *vs* 15, respectively, p=0.876). The pre-operative coagulation laboratory values were similar in the two groups (Table I), and while not statistically significant, the StF group was predominantly female (75 *vs* 25% in the non-StF group, p=0.136). Donor demographics demonstrated a similar relationship in which female donor livers were more common in the StF group (63 *vs* 13% in the non-StF group, p=0.119). Additional donor characteristics are listed in Table II. Elevated donor sodium and decreased total bilirubin levels were associated with StF (151 *vs* 144 mmol/L, p=0.006 and 0.5 *vs* 1.6 mg/dL, p=0.029). When evaluating combined donor and recipient variables,

Table I - Recipients' characteristics.

Recipient	Non-StF (n=7)	StF (n=8)	p value
Age (years)	64 (55-68)	63 (56-67)	0.955
Female	29%	75%	0.132
MELD score	19 (8-31)	15 (12-34)	0.799
INR	1.8 (1.1-3.0)	1.7 (1.3-2.6)	0.867
Platelet count (1,000)	99 (68-117)	78 (50-106)	0.281
Haematocrit (%)	36 (30-45)	31 (29-40)	0.867
R time (min)	9.3 (7.4-12.4)	11.0 (7.4-14.7)	0.999
Angle (degrees)	52.7 (49.9)	47.9 (31.2-55.5)	0.536
MA (mm)	54.4 (34-63)	46.5 (36.1-58.9)	0.694
LY30 (%)	0.1 (0-1.6)	0.5 (0-1.9)	0.999
tLY30 (%)	11.8 (2.0-23.0)	7.1 (2.7-19.0)	0.867

StF: sensitivity to tissue-plasminogen activator (t-PA) mediated fibrinolysis; MELD: model for end-stage liver disease; INR: international normalised ratio of prothrombin time; R: reaction time for TEG; MA: maximum amplitude of thromboelastography (TEG); LY30: the percent lysis 30 min after clot achieves MA; tLY30: LY30 of t-PA TEG.

Table II - Donor characteristics.

Donor	Non-StF	StF	p value
Age (years)	24 (19-41)	34 (22-52)	0.463
Female	14%	63%	0.119
Body mass index	24 (22-31)	25 (23-27)	0.779
Sodium (mmol/L)	144 (139-147)	151 (148-154)	0.006
Total bilirubin (mg/dL)	1.6 (0.8-2.6)	0.5 (0.3-1.5)	0.029
AST (U/L)	134 (45-188)	44 (33-130)	0.281
ALT (U/L)	74 (34-150)	64 (20-111)	0.613
Albumin (g/dL)	2.4 (2.1-3.1)	2.5 (2.1-2.7)	0.867
INR	1.3 (1.2-1.4)	1.3 (1.1-1.4)	0.779
Creatinine (mg/dL)	0.9 (0.8-1.2)	0.9 (0.8-1.3)	0.999
Platelet count (1,000×/µL)	81 (54-84)	85 (72-132)	0.281
Haematocrit (%)	29 (24-30)	28 (26-31)	0.867
Diabetes	0%	25%	0.267
HTN	0%	25%	0.267
Trauma death	86%	50%	0.182
Cigarette use	14%	0%	0.467
Heavy alcohol use	14%	25%	0.554
IV drug use	14%	13%	0.733
Hepatitis C	0	25%	0.533
Cytomegalovirus	57%	63%	0.622
Epstein-Barr virus	100%	100%	0.999

StF: sensitivity to tissue-plasminogen activator mediated fibrinolysis; AST: aspartate aminotransferase; ALT: alanine aminotransferase; INR: international normalised ratio of prothrombin time; HTN: history of documented hypertension; IV: intravenous.

Table III - Matched donor recipients variables and outcomes.

	Non StF	StF	p value
Female to female	0%	50%	0.077
Male to male	57%	13%	0.119
Mixed sex	43%	47%	0.999
Donor-recipient BSA ratio	0.89 (0.86-0.95)	1.02 (0.98-1.08)	0.021
Cold ischaemia time (min)	493 (369-543)	517 (367-603)	0.567
Warm ischaemia time (min)	40 (32-44)	40 (34-46)	0.772
RBC units in reperfusion	0 (0-1)	4 (1-4)	0.035
RBC units in 24 h	1 (1-9)	6 (1-17)	0.560
Plasma units in reperfusion	1 (0-3)	4 (1-5)	0.138
Plasma units in 24 h	4 (1-9)	5 (1-15)	0.816
Cryoprecipitate units in reperfusion	0 (0-0)	0 (0-0)	0.350
Cryoprecipitate units in 24 h	0 (0-0)	0 (0-1)	0.171
Platelet units in reperfusion	0 (0-0)	1 (0-2)	0.759
Platelet units in 24 h	1 (0-2)	1 (0-3)	0.094

StF: sensitivity to t-PA mediated fibrinolysis; BSA: body surface area; RBC: red blood cell. Reperfusion represents blood product transfusions in the first 120 min after donor graft reperfusion, 24 h total blood product within 24 hours of surgery.

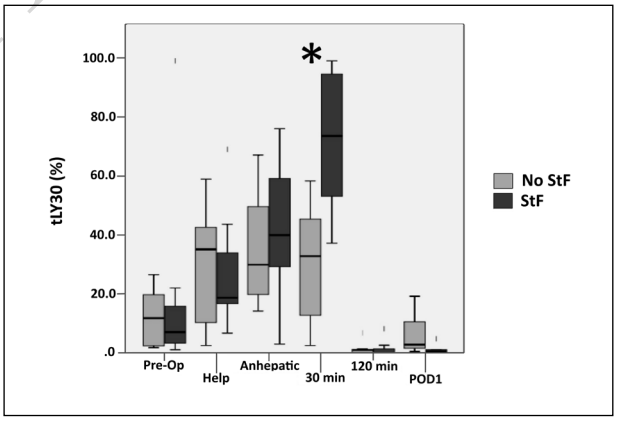

Figure 1 - Changes in fibrinolysis during the first 24 hours of surgery.

The x axis represents time points during the operation. The y axis represents the percent lysis quantified by t-PA thromboelastography. The only time point at which StF patients had significantly higher tLY30 was 30 min following reperfusion of the donor graft.

t-PA: tissue-plasminogen activator; tLY30: sensitivity to exogenous t-PA-mediated fibrinolysis with the lysis 30 min after maximum clot strength; StF: post-reperfusion fibrinolysis; Pre-op: blood collected before surgical incision (baseline); Help: blood drawn during native hepatectomy; Anhepatic: blood drawn during the anhepatic period; 30 min: blood drawn 30 min after graft reperfusion; 120 min: blood drawn 120 min after graft reperfusion; POD1: blood drawn on post-operative day 1.

all female recipients of organs from female donors developed StF and accounted for 50% of all cases of StF (Table III). A larger donor body surface area to recipient ratio was associated with StF (1.02 *vs* 0.89, p=0.021). Patients with StF required more red blood cell units during reperfusion (0 *vs* 4, p=0.035) (Table III). However, overall there were no significant differences in total blood product use at 24 hours.

Changes in fibrinolysis during the peri-operative period

The t-PA TEG LY30 was similar in the groups for the duration of the surgery with the exception of the measurement at 30 min following reperfusion (p=0.006, Figure 1). By 2 hours of reperfusion both cohorts had similar levels of fibrinolysis as measured by t-PA TEG (p=0.397) and continued to do so through post-operative day 1 (p=0.101). Table IV presents the viscoelastic LY30 data of both t-PA TEG and native TEG in association with t-PA and PAI-1 activity levels with t-PA/PAI-1 complex levels. In a non-t-PA TEG, the change in LY30 between the anhepatic period and after 30 min of reperfusion was significantly different between StF and non-StF patients (StF +11.5 [3.2 to 72.70] *vs* −4.5 [−24.1 to −1.6] in non-StF patients) confirming large differences in fibrinolytic activity between cohorts. Comparing the temporal changes of LY30 within groups from the anhepatic phase to reperfusion, the StF cohort had significant increases in tLY30 (p=0.012) and native LY30 after reperfusion, whereas in the non-StF group only native LY30 was increased significantly (p=0.046) while tLY30 was not (p=0.345). The StF cohort t-PA activity levels remained stable from the anhepatic period to reperfusion whereas the non-StF cohort had a reduction in t-PA activity (StF −0.53 IU/mL [−3.81 to 3.52] *vs* non-StF −3.28 [−10.86 to −2.88], p=0.038). Changes in PAI-1 activity (0.073) and t-PA/PAI-1 complex levels (0.999) did not differ significantly between groups during the anhepatic to 30-minute reperfusion periods.

Metabolomic correlation to tLY30 during reperfusion

Over 9,000 features were monitored, resulting in 140 named metabolites. Hierarchical clustering analysis and partial least square-discriminant analysis were used to identify metabolic signatures and clustering of samples on the basis of metabolic phenotypes, respectively (Online Supplementary Content, Figure S1). During the transition from the anhepatic period to 30 min reperfusion, nine metabolites had a strong correlation with change in tLY30 during this same time frame (Online Supplementary Content, Table SI). Four of these metabolites (allantoate, glutamate, N-N dimethylglycine, and 6-thioxanthine5-monophosphate) correlated more strongly with changes in tLY30 than with t-PA activity. ROC curves were generated from metabolites obtained at reperfusion to identify their performance for predicting StF using the area under the curve. Citric acid cycle intermediates (succinate), purine oxidation (allantoate), and bioactive lipids (sphingosine 1-phosphate) performed well for identifying StF (Figure 2).

Discussion

Over half (53%) of liver transplant recipients evaluated in this study had an increase in sensitivity to t-PA-mediated fibrinolysis after reperfusion. StF is associated with an expected increase in systemic fibrinolytic activity measured by a non-t-PA TEG, but not an increase in endogenous t-PA activity. This increased sensitivity to t-PA-mediated fibrinolysis is also associated with increased blood product use during the first 2 hours of graft reperfusion. Recipient baseline coagulation status had no association with StF. Several unique metabolic changes are associated with StF and can be attributed to alterations in the citric acid cycle, and both purine and amino acid metabolism.

Post-reperfusion syndrome had previously been associated with increased fibrinolysis and increased blood product use[3,13]. However, these studies were

Table IV - Fibrinolysis measurements from the anhepatic to reperfusion period.

Assay	Anhepatic period		Reperfusion	
	Non-StF	*StF*	*Non-StF*	*StF*
nLY30 %	8.4 (1.9-33.0)	10.1 (1.0-41.5)	2.4 (0.3-5.2)	36.8 (10.2-90.3)
tLY30 %	29.9 (16.1-58.2)	40.0 (28.7-63.8)	32.8 (4-49.7)	73.6 (49.0-96.8)
t-PA activity IU/mL	10.87 (3.88 - 12.06)	7.00 (1.21 - 8.56)	0.85 (0.24 - 4.09)	6.63 (3.18 - 8.00)
PAI-1 activity IU/mL	0.23 (0.00-0.58)	0.84 (0.20-1.82)	0.55 (0.00 - 2.81)	0.34 (0.27-0.74)
t-PA/PAI-1 complex ng/mL	3.25 (2.2-6.3)	3.39 (0.84-4.61)	3.88 (2.60-5.73)	2.53 (2.14-3.02)

StF: sensitivity to t-PA mediated fibrinolysis; t-PA: tissues plasminogen activator; nTEG: native thromboelastography (TEG); tTEG: t-PA TEG; PAI-1: plasminogen activator inhibitor 1; IU: international activity units.

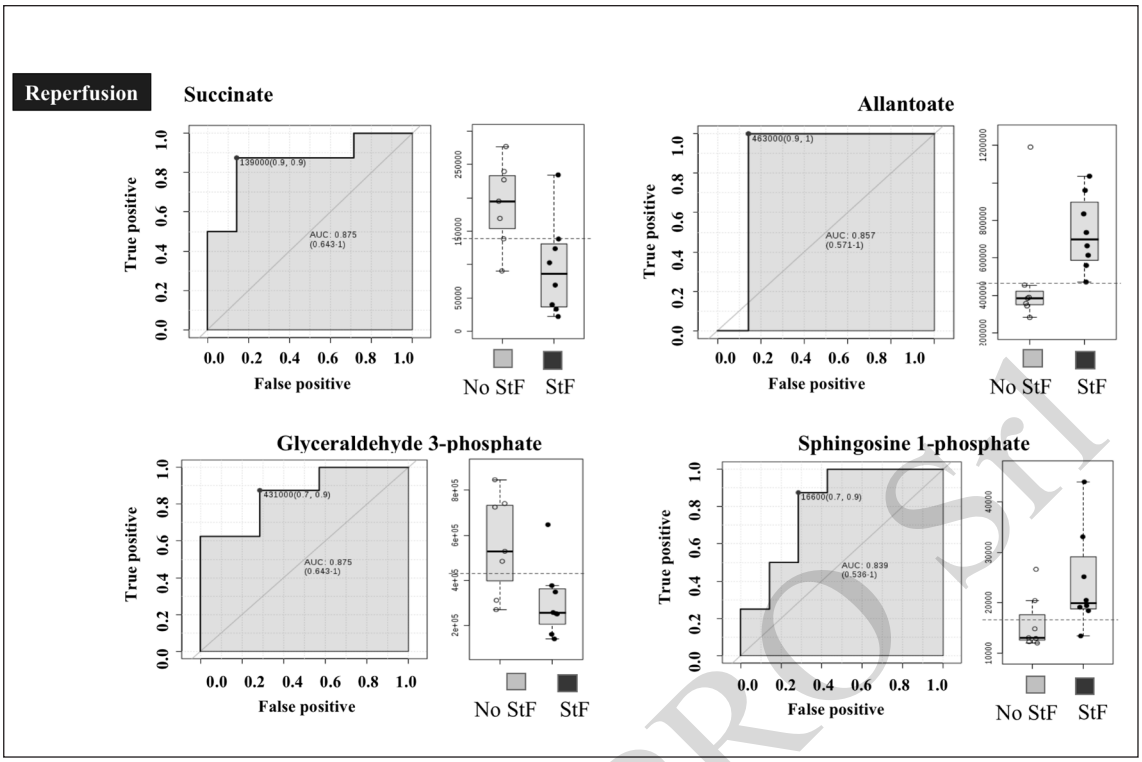

Figure 2 - Performance of metabolites in predicting sensitivity to t-PA mediated fibrinolysis. Receiver operating characteristics curves identifying small molecule biomarker predictors of StF based on their circulating levels in plasma of liver transplant patients at the reperfusion stage following liver transplantation. StF: post-reperfusion fibrinolysis.

not conducted to identify changes in fibrinolytic activity from the anhepatic period to early reperfusion; rather, they defined fibrinolysis associated with post-reperfusion syndrome for the possible use of an antifibrinolytic[13] or as a simple descriptor at 30 min following graft implantation without a unit of measure[3]. The results of our study are concordant with these prior observations that increased systemic fibrinolysis after reperfusion is associated with increased blood product use. Historically increased t-PA levels following reperfusion were implicated in driving an increase in fibrinolysis[4]. However, in our study, t-PA activity levels did not increase significantly in the StF group after reperfusion. In fact, the t-PA activity levels were nearly the same, with no change in its inhibitor levels, supporting the hypothesis that StF after reperfusion is not driven by increasing t-PA levels.

Several donor variables were associated with StF. Elevated donor serum sodium was a risk factor for StF and has been previously associated with adverse liver transplant outcomes[14,15]. Strategies to normalise sodium levels in donors prior to transplantation have been associated with clinical improvements[16]. The presumed mechanism for poor function of the allografts in hypernatraemic patients is oedema of the transplanted livers following transplantation into patients with low or normal sodium levels[17]. This is consistent with the observation that StF is a transient event, and by 2 hours of equilibration, systemic hyperfibrinolysis and StF have resolved (Figure 1). StF was also associated with female to female liver donation. Female donors have been associated with worse outcomes in liver transplantation[18,19], and animal data suggest that "female livers" are more prone to hypoxia and reperfusion injury compared to livers from males[20]. Both hypernatraemia and female donor have a biological mechanism to explain a transient delay in liver function.

These risk factors may also be related to the size of the liver transplanted. A large donor to recipient body surface area ratio (>1.4) has previously been associated with reperfusion syndrome[8]. A larger body surface area index was also associated with StF, despite no patient having an index >1.4. The proposed mechanism of worse outcomes with a large liver is attributed to inadequate blood flow to the allograft, which can be associated with necrosis in extreme cases[8]. The time required to perfuse a larger liver adequately may be representative of the transient coagulopathy observed in our patients. Although these findings are preliminary, they provide a novel insight that a slightly smaller donor liver may in

fact perform better early after reperfusion because there is less graft parenchyma to resuscitate.

While our data do not implicate t-PA, or a depletion of its inhibitor PAI-1, as the cause of StF, there are numerous proteins regulating fibrinolysis[21] that were not measured in this study and may play a mechanistic role. Ignoring the potential role of metabolites on coagulation would be short-sighted as it is becoming increasing appreciated that these small molecules can have a direct impact on coagulation[22] and the most commonly used antifibrinolytic is an amino acid derivative[6]. To our knowledge, prior studies have not assessed the association of metabolites on fibrinolysis after reperfusion in liver transplant patients. We have identified several unique metabolic changes that correlate with changes in sensitivity to t-PA-mediated fibrinolysis (Online Supplementary Content, Table SI), some of which can independently predict StF (Online Supplementary Content, Figure S1). In a case study of a liver transplant recipient who had a failed graft, several similar metabolic changes were identified as potential biomarkers of graft failure including derangements of purine and amino acid metabolism[23]. However, many of the metabolites evaluated in our study have not been previously associated with clinical bleeding. Previous investigations into metabolites from liver ischaemia have demonstrated that bile acids can directly increase t-PA- and non-t-PA-mediated fibrinolysis[24-26]. However, the metabolites with the highest correlation with t-PA-mediated fibrinolysis (Online supplementary content, Figure S1) have not been described to drive coagulation changes. The majority of these metabolites are related to purine metabolism. Recent work has identified that uric acid can promote t-PA-mediated fibrinolysis by quenching oxidative stress[27]. Therefore, the effect of metabolites on coagulation may be situational on the patient's systemic level of oxidative stress. Using healthy volunteer blood donors to test the impact of these metabolites on coagulation without additional conditional modifications to the blood may reflect why these metabolites have not previously been described to alter fibrinolysis, and warrants future investigation.

Metabolic derangements may also alter fibrinolysis through modifying cellular activity. Succinate can directly activate platelets[28] and reverse pharmacologic inhibition[29]. In our study, succinate depletion was associated with StF, and could result in impaired platelet function, but this remains to be validated *in vitro*. However, platelet inhibition following liver reperfusion has been speculated to exacerbate fibrinolysis[30] and lacks a clear mechanism. Additional cellular mechanisms related to modification of t-PA-mediated activity could explain these differences between the StF and non-StF cohort. Recently, glutamate has been implicated in increased expression of the annexin A2 receptor and increasing fibrinolytic activity in neuronal cells[31]. Annexin A2 is heavily expressed in liver endothelial cells co-localising with t-PA and plasmin while preventing inhibition from their circulating inhibitors[32]. This could help explain why t-PA levels remain stable in StF patients 30 min after reperfusion and yet there is a 2-fold increase in tLY30 and 4-fold increase in native LY30. This potential mechanism is interesting as glutamate had a stronger correlation with t-PA LY30 than with t-PA activity level (Online Supplementary Content, Table SI) supporting that concept that glutamate modified the fibrinolytic effects of exogenous t-PA, but this requires *in vitro* validation.

The correlations between these metabolites and reperfusion coagulopathy may represent biomarkers of transient graft dysfunction, rather than causality for increased fibrinolysis after reperfusion. This would still have clinical relevance as a specific metabolite could be used to risk-stratify patients for StF and guide treatment for antifibrinolytic use after reperfusion. For example, oxidative stress associated with reperfusion has been associated with purine catabolism from adenosine to urate[33]. Consistent with this hypothesis, reperfusion levels of allantoate (a urate oxidation product) were predictive of StF. While mass spectrometry was successful in identifying a cluster of metabolites associated with StF, use of this technique is not clinically feasible because of the time frame required to obtain results. Measuring metabolites can be accomplished in a shorter time frame than quantifying fibrinolysis with current point-of-care (POC) devices. POC measurements of lactate levels, available within min, have been demonstrated to reduce mortality in sepsis though earlier treatment intervention times[34]. In theory, a POC assay for a targeted metabolite during early reperfusion could guide the use of antifibrinolytics.

Future validation for the chosen definition of StF will be needed in a larger cohort. A limitation of measuring StF is the requirement of a modified TEG assay. While the native LY30 was also markedly elevated in the StF group we did not design this study to validate one assay's superiority over another. It remains unclear whether StF and systemic fibrinolysis measured by a non-t-PA TEG can be interchanged. In the StF group both native LY30 and tLY30 were increased during reperfusion. In the non-StF group only native LY30 was increased and tLY30 remained the same. The differences in these assays warrants an ongoing investigation with a larger population of patients to identify whether these assays are measuring different haemostatic properties. However, during reperfusion both of these assays required on average an hour or more to provide LY30 results. This limits their clinical

utility for guiding tranexamic acid treatment during reperfusion, as all of the patients had corrected StF by 2 hours after graft reperfusion. The future use of TEG to identify StF will, therefore, probably be limited to quantifying fibrinolysis during reperfusion for future research endeavours. Another important finding of this study is that the ischaemia-reperfusion metabolic changes in human liver transplantation do not parallel reports of metabolic changes following injury[5] and rodent models of ischaemia-reperfusion[35]. These are important limitations to consider with regards to future translation of our observations.

Conclusions

Currently unmeasured metabolic changes during liver transplantation are associated with increased fibrinolysis early after graft reperfusion. While this is a self-limited event that resolves within 2 hours of reperfusion, it is associated with increased blood product utilisation. Future research is needed to determine whether these metabolites serve as biomarkers of increased sensitivity to t-PA-mediated fibrinolysis or have a mechanistic link.

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Authors' contributions

- Study conception and design: HBM, AD'A, TLN.
- Acquisition of data: HBM, AD'A, MW, PJL, BRH, RC.
- Analysis and interpretation of data: HBM, AD'A, EEM, MW, KH, TLN.
- Drafting of manuscript: HBM, AD'A, PJL, BRH, MW, RC.
- Critical revision: HBM, AD'A, EEM, MW, KH, TLN.

Disclosure of conflicts of interest

HBM and EEM both have shared intellectual property with Haemonetics and are both co-founders of Thrombo Therapeutics Incorporated. HBM has acted as a consultant for Instrument Laboratories. The other Authors declare no conflicts of interest.

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