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Plasma proteome of brain-dead organ donors predicts heart transplant outcome

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KEYWORDS:
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BACKGROUND: The pathophysiological changes related to brain death may affect the quality of the transplanted organs and expose the recipients to risks. We probed systemic changes reflected in donor plasma proteome and investigated their relationship to heart transplant outcomes.

METHODS: Plasma samples from brain-dead multi-organ donors were analyzed by label-free protein quantification using high-definition mass spectrometry. Unsupervised and supervised statistical models were used to determine proteome differences between brain-dead donors and healthy controls. Proteome variation and the corresponding biological pathways were analyzed and correlated with transplant outcomes.

RESULTS: Statistical models revealed that donors had a unique but heterogeneous plasma proteome with 237 of 463 proteins being changed compared to controls. Pathway analysis showed that coagulation, gluconeogenesis, and glycolysis pathways were upregulated in donors, while complement, LXR/RXR activation, and production of nitric oxide and reactive oxygen species in macrophages pathways were downregulated. In point-biserial correlation analysis, lysine-specific demethylase 3A was moderately correlated with any grade and severe PGD. In univariate and multivariate Cox regression analyses myosin Va and proteasome activator complex subunit 2 were significantly associated with the development of acute rejections with hemodynamic compromise within 30 days. Finally, we found that elevated levels of lysine-specific demethylase 3A and moesin were identified as predictors for graft-related 1-year mortality in univariate analysis.

CONCLUSIONS: We show that brain death significantly changed plasma proteome signature Donor plasma protein changes related to endothelial cell and cardiomyocyte function, inflammation, and vascular growth and arteriogenesis could predict transplant outcome suggesting a role in donor evaluation.
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Abbreviation: CRP, c-reactive protein; FDR, false discovery rate; HTx, heart transplantation; IPA, ingenuity pathway analysis; NO, nitric oxide; OPLS-DA, orthogonal projections to latent structure-discriminant analysis; PCA, principal component analysis; ROC, receiver operating characteristic; ROS, reactive oxygen species; S-Plot, variance vs correlation plot; SOM, self-organizing map; hsTnI, high-sensitivity troponin I; hsTnT, high-sensitivity troponin T

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Brain death is the result of irreversible injury of the central nervous system. The injury may lead to systemic inflammatory, hormonal, and metabolic changes, as well as affect peripheral organs and compromise cardiorespiratory function. This may increase the risk of primary graft dysfunction, acute rejection, and survival of the recipient.[1–4]

Most of our understanding of donor organ quality is based on studies investigating donor demographics, clinical parameters, and a limited number of cytokines or proteins.[5,6] In the last few years, high-throughput technologies have advanced the discovery of pathophysiological molecular signatures. The ultra-high-performance liquid chromatography, connected to tandem mass spectrometry (UPLC-MS/MS), has facilitated a detailed measurement of the plasma proteome. In systemic biology approach, uni- and multivariate statistical analyses identify proteins that distinguish a group from another. This allows the integration of proteomics data with existing knowledge of involved biological processes and detailed examination of potential.[7]

Our results show that brain death induced prominent protein expression and pathway alterations in the donor plasma proteome. Furthermore, changes in donor plasma protein related to endothelial cell and cardiomyocyte function, inflammation, and vascular growth and arteriogenesis could predict transplant outcome suggesting their role in donor evaluation. To conclude, our results enhance the understanding of the plasma proteome in brain-dead donors, and changes in their signature may be used to predict the heart transplant outcome.

Methods

Study design and study population

This study is a post hoc analysis of multi-organ donors participating in a prospective, randomized clinical trial on the effects of donor simvastatin treatment on ischemia-reperfusion injury after heart transplantation (Nykänen et al.).[8] We analyzed donor plasma protein samples by nano ultra-performance liquid chromatography and quantified them with UPLC-MS/MS and investigated their relationship to heart transplant outcome. Plasma samples were collected in lithium heparin tubes before heparinization and organ procurement. After cooling down, we used the "Top 12 Abundant Protein Depletion kit" (Pierce, Thermo Fisher) to deplete greater than 95% of the most abundant proteins from 10 μl of plasma. The list of 12 depleted proteins was alpha-1-1acid-glycoprotein, alpha-1-antitrypsin, alpha-2-macroglobulin, albumin, apolipoprotein A-I, apolipoprotein A-II, fibrinogen, haptoglobin, IgA, IgG, IgM, and transferrin and the remaining proteins were digested by trypsin. UPLC-MS/MS was performed as described.[9] Out of the original 84 trial donors and recipients, 54 donors were chosen for proteomics analysis as they had complete sets of all time points samples available of the donor and recipient pair. Label-free quantification failed on 1 donor sample due to batch effect, therefore this sample was excluded from the study. Control samples were collected from 24 healthy controls. One control sample failed normalization and was removed from the study. For details about donor inclusion and exclusion criteria, donor management, plasma sample processing, definitions of clinical outcomes, and bioinformatics and statistical analyses, see Methods in Supplemental Material.

Results

Brain-dead donors showed a unique but heterogenous proteomic profile

The final proteomic analysis consisted of 53 multi-organ donors for HTx, and 23 healthy controls (Figure 1). The median age of the organ donors was 44 years, and 10 were female (Table 1). We detected 1259 plasma proteins with a minimum of 1 unique peptide by UPLC-MS/MS. For sufficient stringency and confidence in proteomics data, we filtered to the proteins with 2 or more unique peptides and obtained 463 quantified proteins. To describe the changes in protein abundance between donors and healthy controls, the fold change was calculated by dividing the mean protein expression of a single protein in donors by mean expression in controls. The fold change ranged from 0.11 to 2584.

Of note is that donor treatment with simvastatin did not classify the treated and untreated donor groups, and therefore was not considered a confounding factor (Figure S1).

PCA was performed on all 463 quantified proteins (Figure 2A). The scatter plot (t1 versus t2) revealed that samples of donors and healthy controls were only partially separated. Four donors were outside of the 95% confidence ellipse of measurement. The unsupervised learning method of SOM displayed 2 main clusters of protein expression in donors (Donor A and Donor B), 1 of them having 2 subclusters (Donor B1 and Donor B2) (Figure 2B, red color for donor samples), and 2 clusters in healthy controls (Figure 2B, blue color for healthy control samples), confirming the findings of PCA. A subset of healthy controls and donors merged into the same cluster which was due to the similarity of few proteins in those samples and the use of the complete set of all 463 quantified proteins in SOM clustering.

To further characterize the separation between donors and healthy controls, supervised multivariate OPLS-DA model and univariate S-Plot were performed. OPLS-DA showed a clear separation between the 2 groups, confirming the findings suggested by PCA and SOM (Figure 2C). S-Plot analysis revealed that 32 proteins were statistically significant in both univariate and multivariate analyses between donors and healthy controls, and thereby represent proteins mostly contributing to the differences between donors and healthy controls (Figure 2D). Three proteins were upregulated, while 29 proteins were downregulated. Of these proteins, apolipoprotein A-IV, complement C1q C chain, leucine-rich alpha-2-glycoprotein 1, and 14-3-3

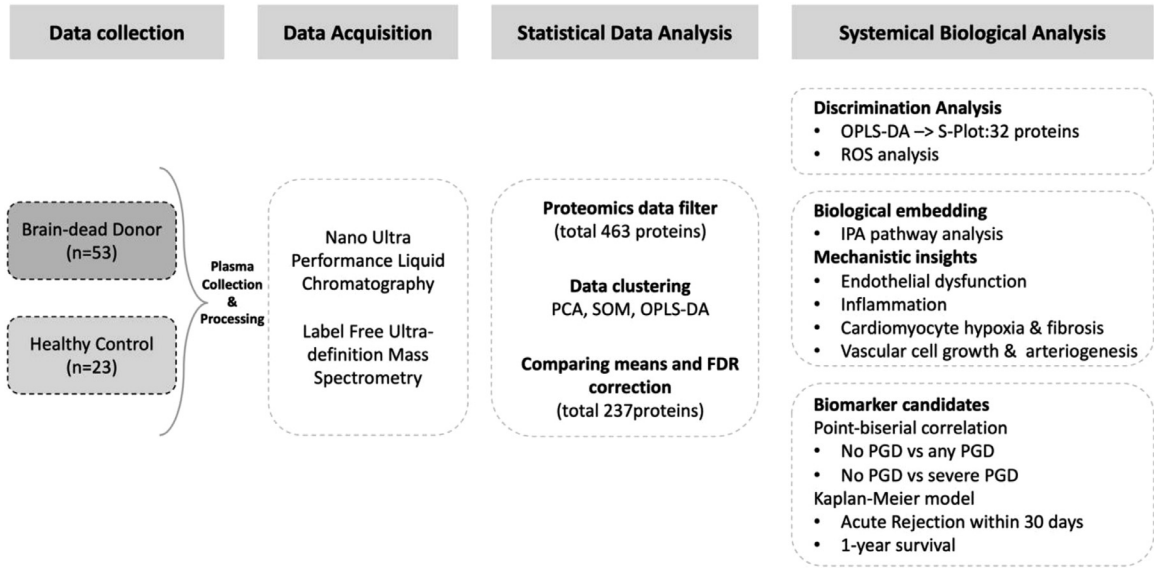

Figure 1 Flow chart of the study.

Data collection. One control sample failed normalization and was removed. One donor sample failed due to batch effect and was removed. Data acquisition. Label-free ultra-definition mass spectrometry presents the structural identity of the individual peptides based on the mass to charge ratio. Nano ultra performance liquid chromatography as the second part of this tandem method (UPLC-MS) separates peptides within the plasma sample. Statistical Data Analysis. 463 proteins were quantified which contained 2 or more unique peptides. Principal component analysis (PCA) was used as a clustering technique to determine if the protein expression separates organ donors and healthy controls as two classes and to find the expressed proteins that explain the majority of the variance noticed in a much bigger number of measured protein expressions. Self-organizing map (SOM) was used to visualize and analyze high-dimensional proteomics datasets by presenting them as lower-dimensional ones. Orthogonal projections to latent structure-discriminant analysis (OPLS-DA) is a regression model method to discriminate 2 or more classes using multivariate proteomics data. Benjamini-Hochberg FDR correction revealed 237 identified proteins with FDR-corrected p value <0.05, accounting for differences between the classes which are visualized in PCA, SOM, and OPLS-DA. Systemical Biological Analysis. S-Plot was created based on OPLS-DA loadings plot to extract 32 statistically most significant proteins between brain-dead donors and healthy controls. IPA pathway enrichment analysis was performed on 237 identified proteins. Point-biserial correlation analysis was applied to investigate the correlation between donor plasma proteome and any PGD or severe PGD. Kaplan-Meier model was applied on the clinical outcome endpoints 30 days rejection with hemodynamic compromise and 1-year survival to find biomarker candidates among 237 identified proteins in brain-dead donors.

protein beta/alpha showed a good area under the ROC curve (AUC) value of >0.8 (Table S1).

Next, we performed univariate analysis to calculate log2 (fold change) and p value using the Wilcoxon-Mann-Whitney test to find out which of 463 proteins were statistically significantly different between donors and healthy controls. Univariate analysis based on FDR-corrected p value of <0.05 revealed 237 differently expressed proteins between the donors and healthy controls of which 90 proteins were upregulated, while 147 proteins were downregulated (Table S2).

Brain-dead donor protein profile revealed significantly altered pathways

IPA pathway analysis of 237 differentially expressed proteins revealed 65 significant pathways with a p value of <0.05. Furthermore, using more stringent statistical criteria for protein data set in IPA pathway analysis, we found that 118 proteins with log2(fold change) ≥1 belonged to 58 significant pathways, while 66 proteins with log2(fold change) ≥1.5 showed 50 significant pathways (Table S3).

In IPA pathway analysis based on z-score orientation (absolute z-score greater than 1) and the most stringent FDR-corrected p value of <0.001, we observed that on the one hand coagulation, gluconeogenesis, and glycolysis were significantly enriched, and these pathways showed a trend towards upregulation. On the other hand, complement system, LXR/RXR activation, and production of NO and ROS in macrophages showed a trend towards downregulation (z-score ≤-1) (Table 2, Figure S2A-F). When considering log2 (fold change) ≥1, we found that only gluconeogenesis, glycolysis, and xenobiotic metabolism pathways were significant and that they were upregulated. No significant pathway was found with log2(fold change) ≥1.5 (Table 2).

Out of 32 S-Plot proteins, 10 S-Plot proteins belonged to the pathways with absolute z-score greater than 1 and p value of <0.001, while the remaining 22 S-Plot proteins were present in other significant pathways. We found that these 10 S-Plot proteins were mostly enriched in coagulation, complement, LXR/RXR activation, and production of NO and ROS in macrophages pathways (Table 2).

Proteome profile discriminated 3 subclusters within brain-dead donors

To exclude a methodological artifact of healthy controls to brain-dead donors, we carried out separate statistical

Table 1 Clinical Characteristics of Brain-Dead Heart Transplant Donors and Allocation of Other Solid Organs Based on Different Donor Plasma Proteome Profiles

Donor characteristics	All donors (N=53)	Donor A (N=20)	Donor B (N=33)	Donor B1 (N=26)	Donor B2 (N=7)
Age, y	44 (33-51)	44 (35-52)	43 (33-50)	44 (34.5-50)	43 (27-49.5)
Female sex, No. (%)	10 (18.9)	2 (10)	8 (24.2)	5 (19.2)	3 (42.9)
Body mass Index, kg/m^2	25.2±4.8	24.3±6.2	25.7±3.7	25.7±3.9	25.8±3.2
Simvastatin treatment, No. (%)	27 (51)	13 (65)	14 (42.4)	13 (50)	1 (14.3)
Previous medical history[a], No. (%)					
Hypertension	6 (11)	0 (0)*	6 (18.2)	6 (23.1)	0 (0)
Smokin, No. (%)					
Current	23 (43)	8 (40)	15 (45.5)	10 (38.5)	5 (71.4)
Former	4 (8)	3 (15)	1 (3)	1 (3.8)	0 (0.0)
Never	15 (28)	4 (20)	11 (33.3)	10 (38.5)	1 (14.3)
Unknown	11 (21)	5 (25)	6 (18.2)	5 (19.2)	1 (14.3)
CMV-positive, No. (%)	44 (83)	16 (80)	28 (84.8)	21 (80.8)	7 (100)
Cause of brain death, No. (%)					
Intracranial hemorrhage	26 (49.1)	10 (50)	16 (48.5)	11 (42.3)	5 (71.4)
Traumatic brain injury	19 (35.8)	5 (25)	14 (42.4)	12 (46.2)	2 (28.6)
Cerebral infarction	6 (11.3)	5 (25)*	1 (3)	1 (3.8)	0 (0.0)
Other	2 (3.8)	0 (0.0)	2 (6.1)	2 (7.7)	0 (0.0)
P-troponin I, ng/l	47 (9-207)	38 (8-88)	76 (14-293)	78 (14-286)	27 (6-250)
P-troponin T, ng/l	21 (9-55)	16 (9-33)	25 (11-67)	27 (10-90)	20 (14-60)
Hemoglobin, g/L	121±23	117±26	124±20	126±22	116±14
CRP, mg/L	43 (12-122)	95 (27.8-177)	31 (9-89)	34 (9.8-89.8)	21 (10-43.5)
Thrombocytes, E9/L	186±80	171±62	196±89	208±89	111±13***
Total P-cholesterol, mmol/l	2.72±0.94	2.76±0.89	2.68±0.98	2.93±0.93	1.87±0.7
P-HDL, mmol/l	1±0.37	0.97±0.38	0.91±0.37	0.95±0.37	0.76±0.37
P-LDL, mmol/l	1.23±0.72	1.20±0.73	1.24±0.73	1.40±0.73	0.71±0.41*
P-triglycerides, mmol/l	0.86±0.5 1	1.02±0.58	0.79±0.44	0.82±0.47	0.59±0.27
Echocardiogram					
Left ventricle ejection fraction, %	62 (59-65)	61 (60-65)	62 (58-66)	63 (58-66)	61 (60-65)
Presence of regional wall motion abnormality, No. (%)	6 (11)	2 (10)	4 (12.1)	2 (7.7)	2 (28.6)
Diastolic posterior wall thickness, mm	11 (9-12)	10.5 (10-11)	11 (10-13)	11 (10-13)	9.7 (9-10)
Diastolic septum thickness, mm	11 (10-12)	10.75 (10-11)	11 (10-12)	11 (10-12)	10.75 (11-11)
Coronary angiography[b]					
Performed, No.(%)	30 (57)	13 (65)	17 (51.5)	14 (53.8)	3 (42.9)
Abnormal finding angiography, No. (%)	6 (11)	3 (15)	3 (9.1)	3 (11.5)	0 (0.0)
Inotropic support, No. (%)	37 (70)	12 (60)	25 (75.8)	18 (69.2)	7 (100)
Resuscitation, No. (%)	9 (17)	2 (10)	7 (21.2)	7 (26.9)	0 (0.0)
Time of ROSC for resuscitated donors, min	17±13	30±0	14±12	14±12	0.0
The time between the declaration of brain death and organ procurement, h	14.86±4	14.58±3.7	14.76±4	14.86±4	14.39±3
Organs transplanted from donors, No. (%)					
Heart	53 (100)	20 (100)	33 (100)	26 (100)	7 (100)
Lung	17 (32)	6 (30)	11 (33.3)	8 (30.8)	3 (42.9)
Liver	36 (68)	12 (60)	24 (72.7)	20 (76.9)	4 (57.1)
Kidneys	86 (90.6)	29 (85)	57 (93.9)	46 (96.1)	11 (85.7)
Pancreas	31 (58)	10 (50)	21 (63.6)	16 (61.5)	5 (71.4)

Plus-minus values are mean ±SD; values with the range in parentheses are median (interquartile range). P values are marked as asterisks (*p<0.05. **p<0.01. ***p<0.001). CMV, indicates cytomegalovirus; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ROSC, return of spontaneous circulation; and Tx, transplantation.

[a]In the previous medical history of the donors there was no coronary artery disease, chronic obstructive pulmonary disease, peripheral vascular disease, previous malignancy, prior stroke, and no history of sternotomy.

[b]Donor coronary angiography was performed for donors with >40 years of age, strong family history of coronary disease, or smoking.

analyses including only brain-dead donors and found 3 subclusters within donors with only minor changes in their demographics (Figure S3, Table 1). When comparing the recipient outcomes between Donor A and Donor B groups, we could not see any statistically significant difference in PGD, acute rejection, or graft-related survival (Table 3). Detailed information, stratified by the Donor subgroups, on the donor demographics and recipient outcomes is given in Tables 1 and 3, and on enriched pathways in Tables S4 and S5, and Supplement.

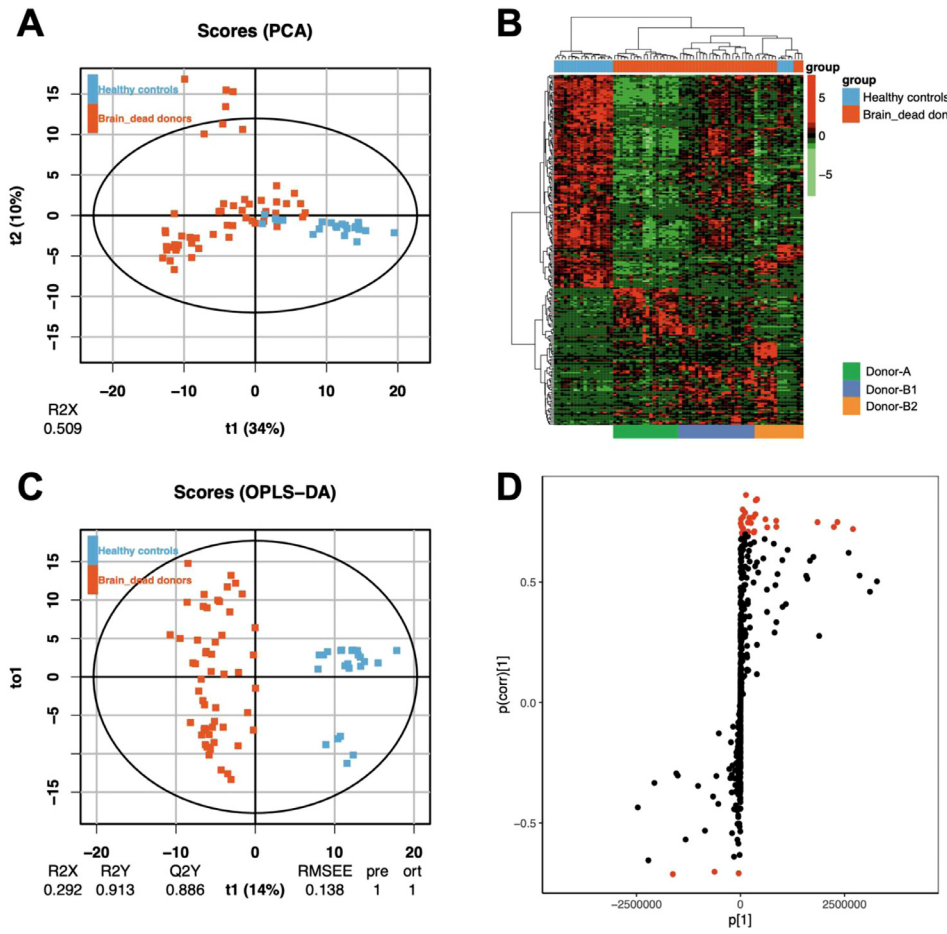

Figure 2 Comparison of differentially expressed plasma proteins between brain-dead donors and healthy controls.

Unsupervised PCA analysis was performed on 463 quantified proteins. In PCA analysis, the t1 axis showed the variation of plasma proteins between the brain-dead donors (orange dots) and healthy controls (blue dots), whereas the t2 axis showed the variation of protein profile within the same group. The 95% confidence ellipse showed that 4 out of 53 heart donors had increased protein expression heterogeneity. Within 95% confidence ellipse, brain-dead donor samples, as well as healthy control samples, were grouped into two major clusters. (B) SOM clustering of all 463 quantified plasma proteins in the heatmap showed separate clusters in brain-dead donors (orange) and healthy controls (blue), confirming the observation of the PCA plot. Red color represents upregulated, and green color downregulated protein expression in samples. Donor samples grouped into Donor A (dark green), Donor B1 (pink), and Donor B2 (orange). Subsets of healthy controls (blue, right side) and Donor B2 (orange, right side) grouped into the same cluster. (C) Supervised OPLS-DA analysis on 463 quantified plasma proteins showed a clear separation between the 2 groups, confirming the findings suggested by PCA and SOM. (D) S-Plot generated from the OPLS-DA analysis revealed 32 significant differentially expressed proteins between brain dead donors and healthy controls. The right upper corner of the Figure shows that 29 proteins were downregulated, whereas the left lower corner shows that 3 proteins were upregulated in brain dead donors. Proteins with the cut-offs, ± 0.1 for p[1] and $> +0.7$ or < -0.7 for p(corr) [1] were considered as significant proteins. (Color version of figure is available online)

Donor plasma lysine-specific demethylase 3A was moderately associated with PGD

Next, we investigated whether donor plasma proteins could predict and any PGD grade or severe PGD after transplantation. 17 of 53 recipients (32%) recipients developed PGD and only 6 (11%) had severe PGD. The characteristics of respective donors of recipients with any PGD grade or severe PGD were not statistically different (Table S6). However, the recipients with any or severe PGD had longer intubation time, longer stay at ICU and index hospitalization, and higher levels of proBNP, hsTnI, hsTnT, and lactate (Table S7).

The point-biserial correlation analysis revealed that only 5 proteins correlated with any PGD, while 6 proteins correlated with severe PGD. Only lysine-specific demethylase 3A showed a moderate correlation with any PGD and severe PGD (Table S8).

High donor plasma myosin Va and proteasome activator subunit 2 predicted acute rejection episodes with hemodynamic compromise

Next, we investigated whether donor plasma proteins could predict episodes of acute rejections with hemodynamic compromise. The prerequisite to establish the diagnosis of acute rejection with hemodynamic compromise was that the patient was treated by high dose of intravenous pulse steroids and/or anti-thymocyte globulin. Three patients

were excluded from the analysis as they expired due to graft-related reasons within 30 days (Table S9). Sixteen patients received treatment for acute rejection with hemodynamic compromise. The characteristics of respective donors were not different (Table S10). However, the recipients with rejection episodes had significantly higher plasma levels of troponins and lactate during the first 24 hours, higher ProBNP and lower left-ventricle ejection fraction at 1 month, and longer ICU and hospital stay after transplantation (Table S11).

Univariate Cox regression analysis of differentially expressed 237 proteins revealed that 7 donor plasma proteins were significantly associated with acute rejections with hemodynamic compromise within 30 days. These proteins included CD163, CRP, keratin 76, myosin Va, proteasome subunit alpha type 6, proteasome activator subunit 2, and transaldolase 1. We further explored the possibility of an association between hemodynamically compromised acute rejection rejections and concentration thresholds for these proteins in univariate analysis. After stratification of patients based on each protein expression level, we found that higher donor plasma levels of all these proteins were associated with a significantly increased number of acute rejections with hemodynamic compromise. In Kaplan-Meier analysis, all the 7 donor plasma proteins passed the log-rank test with a p value less than 0.05 (Figure 3A-G, Table 4). Higher expression of these 7 proteins was linked to higher hazard/risk (Table 4).

Table 2 Effect of log2 Fold Change on Ingenuity Pathway Analysis of Identified Proteins in Heart Transplant Donors

Pathway (z-score =>1)	Donor vs Controls No fold change (237 proteins)	-log(p value)	Donor vs Controls Fold change ≥1 (118 proteins)	-log(p value)	Donor vs Controls Fold change ≥1.5 (66 proteins)	-log(p value)	Donor A vs. B No fold change (164 proteins)	Donor B1 vs B2 No fold change (107 proteins)	S-Plot proteins
Coagulation System	1,732	15,7	-	-	-	-	-	-	plasma kallkrein, kininogen 1, plasminogen, protein C,antithrombin-III
Complement System	-1,265	15,2	-	-	-	-	-1,265	1	complement C1q C chain, mannan binding lectin serine peptidase 1
Gluconeogenesis I	1,633	5,33	1,342	5,62	-	-	-2,236	-	
Glycolysis I	1,633	5,62	1,342	5,87	-	-	-2,236	-	
LXR/RXR Activation	-4,536	29,6	-0,816	4,52	-	-	-3,13	3	alpha 2-HS glycoprotein, apolipoprotein A4, kininogen 1, paraoxonase1

(*continued on next page*)

Pathway (z-score =>1)	Donor vs Controls No fold change (237 proteins)	-log(p value)	Donor vs Controls Fold change ≥1 (118 proteins)	-log(p value)	Donor vs Controls Fold change ≥1.5 (66 proteins)	-log(p value)	Donor A vs. B No fold change (164 proteins)	Donor B1 vs B2 No fold change (107 proteins)	S-Plot proteins
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	-2,111	6,46	-	-	-	-	-2,121	-	apolipoprotein A4, paraoxonase 1
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	-	-	-	-	-	-	-1,342	-	complement C1q C chain
Xenobiotic Metabolism CAR Signaling Pathway	-	-	1,633	3,63	-	-	-	-	glutathione S-transferase mu 2

In IPA pathway analysis, we considered pathways with a -log(*p* value) of >3.0 (*p* value <0.001) and a z-score of ±1 as significant. Upregulated pathways are highlighted in red and downregulated in green. S-Plot proteins enriched into specific pathways are presented.

Additionally, a donor plasma proteomic predictive risk score was calculated based on the concentration levels of these proteins and corresponding regression coefficients. This predictive risk score was calculated by giving 1 point for each of the 7 proteins that were within their respective high-risk levels, therefore yielding a score of 0 to 7 for each donor. In risk score calculation, 18 patients had a score of 0, 16 patients a score of 1, 6 patients a score of 2, 5 patients a score of 3, and 5 patients had a score greater than 3. Based on the donor proteomics risk score, we found that a higher score significantly predicted acute rejection with hemodynamic compromise (Figure 3H). In addition, we observed that donors with high-risk score (score ≥3) had an 80% probability of acute rejection with hemodynamic compromise within 30 days (Figure S4).

In multivariate Cox regression analysis, myosin Va and proteasome activator subunit 2 remained significant suggesting that these 2 proteins are key candidates for prediction of acute rejection with hemodynamic compromise within 30 days after transplantation (Figure S5).

High levels of moesin and lysine-specific demethylase 3A were associated with worse graft-related 1-year survival

Next, we investigated whether donor proteome could predict graft-related mortality. 7 of 53 recipients died due to graft-related reasons, and 6 of them during the first year, and 1 patient died 730 days after transplantation. PGD was the cause of death in 4 patients, acute rejection in 2 patients, and chronic rejection in 1 patient (Table S9). Therefore, we tested whether donor proteome could predict 1-year graft-related mortality.

In univariate analysis, we found that 5 proteins were significantly associated with 1-year graft-related mortality (Figure 4A-E). After stratification of donors using the Maxstat method, we found that high donor plasma levels of moesin and lysine-specific demethylase 3A were associated with increased graft-related 1-year mortality, while low plasma levels of D-dopachrome decarboxylase, leucine-rich alpha-2-glycoprotein, and keratin 79 were associated

Table 3 Clinical Characteristics and Outcomes of the Heart Transplant Recipients Based on Different Donor Plasma Proteome Profiles

	All donors (N=53)	Donor A (N=20)	Donor B (N=33)	Donor B1 (N=26)	Donor B2 (N=7)
Recipient characteristics					
Age, y	58 (46.5-61)	55 (46-59)	59 (49-62)	61 (49-63)	58 (48-60)
Female sex, No. (%)	13 (24.5)	3 (15)	10 (30.3)	7 (26.9)	3 (42.9)
Body mass index, kg/m^2	26±4.4	26±4.6	25.6±4.5	25.9±4.7	24.4±3.3
Previous medical history† No. (%)					
Hypertension	8 (15.1)	1 (5)	7 (21.2)	6 (23.1)	1 (14.3)
Coronary artery disease	11 (20.8)	5 (25)	6 (18.2)	4 (15.4)	2 (28.6)
Chronic obstructive pulmonary disease	2 (3.8)	0 (0.0)	2 (6.1)	2 (7.7)	0 (0.0)
Diabetes	7 (13.2)	1 (20)	6 (18.2)	5 (19.2)	1 (14.3)
Previous malignancy	5 (9.4)	1 (5)	4 (12.1)	3 (11.5)	1 (14.3)
Prior stroke	7 (13.2)	2 (10)	5 (15.2)	4 (15.4)	1 (14.3)
Amiodarone <6 months prior transplantation, No. (%)	14 (26.4)	4 (20)	10 (30.3)	9 (34.6)	1 (14.3)
History of sternotomy	15 (28.3)	5 (25)	10 (30.3)	7 (26.9)	3 (42.9)
Primary disease, No. (%)					
Endstage coronary disease	12 (22.6)	4 (20)	8 (24.2)	6 (23.1)	2 (28.6)
Dilatative cardiomyopathy	26 (49)	11 (55)	15 (45.5)	12 (46.2)	3 (42.9)
Congenital	4 (7.6)	2 (10)	2 (6.1)	1 (3.8)	1 (14.3)
Myocarditis	3 (5.7)	0 (0.0)	3 (9.1)	3 (11.5)	0 (0.0)
Other	8 (15.1)	3 (15)	5 (15.2)	4 (15.4)	1 (4.3)
Donor-recipient sex mismatch, No. (%)	6 (11.3)	4 (20)	2 (6.1)	2 (7.7)	0 (0.0)
Mechanical circulatory support prior to HTx, No. (%)	13 (24.5)	2 (10)	11 (33.3)	9 (34.6)	2 (28.6)
ECMO, No. (%)	6 (11.3)	2 (10)	4 (12.1)	4 (15.4)	0 (0.0)
LVAD, No. (%)	7 (13.2)	0 (0.0)*	7 (21.2)	5 (19.2)	2 (28.6)
Days on waiting list	190 (41.8-352.5)	203 (49-360)	180 (28.5-330)	157 (29.3-335)	200 (68-221)
Graft ischemia, min					
Cold	97±50.1	94±50.2	98±50.8	96±47.1	106±65.8
Warm	80±20.2	86±21.4	77±19.2	77±21.4	78±8.9
Total	173±54.1	170±59.3	175±51.5	172±49.1	184±63
Organ functions before heart transplantation					
PVR, Wood units	3±1.3	2.7±1.1	3.2±1.5	3.1±1.4	3.8±1.9
TPG, mmHg	10 (7-12)	11 (10-12)	8 (7-13)	8 (7-11)	10 (6.3-13.5)
SPAP, mmHg	43±12.8	43±10.9	43±14.3	40±12.6	55.5±17.1
P-bilirubin, μmol/L	13 (10-19)	14 (10-22.5)	11 (9-15)	11 (9-15.8)	10 (9.5-14)
Glomerular filtration rate, mL/min per 1.73 square meters	55.7 (45-73)	51.7 (44-65.3)	57 (48-73)	56.4 (45.7-72.5)	58.3 (55.5-69.5)
NT-proBNP, ng/L	3171 (1075-5686)	3304 (2263-5208)	3100 (852-5942)	3132 (934-6146)	1982 (756-4619)
Immunosuppressive therapy, No. (%)					
Induction Therapy					
Anti-thyomocyte	21 (39.6)	10 (50)	11 (33.3)	11 (42.3)	0 (0.0)
Maintenance therapy					
Cyclosporine A	10 (18.9)	4 (20)	6 (18.2)	4 (15.4)	2 (28.6)
Tacrolismus	39 (73.6)	14 (70)	25 (75.8)	21 (80.8)	4 (57.1)
Azathioprine	2 (3.8)	1 (5)	1 (3.3)	1 (3.8)	0 (0.0)
Mycophenlic acid	46 (86.8)	16 (80)	30 (90.1)	24 (92.3)	6 (85.7)
Prednisolone	53 (100)	20 (100)	33 (100)	26 (100)	7 (100)
Recipient Outcome					
Intubation time, h	42 (20-125)	60 (24-111)	42 (18-180)	23 (18-183)	19 (16-63)
Time on ICU, h	216 (144-480)	204 (168-372)	216 (120-492)	264 (120-552)	168 (90-378)
Hospital length of stay, d	44±29	48±37	42±24	45±24	29±18
Inotropic support, No. (%)	47 (88.7)	19 (95)	28 (84.8)	23 (88.46)	5 (71.4)
30-day survival. No. (%)	50 (94.3)	19 (95)	31 (93.9)	25 (96.2)	6 (85.7)
1-year survival, No. (%)	46 (86.8)	18 (90)	28 (84.8)	22 (84.6)	6 (85.7)
LV-EF at 7 days	59±9.4	58±7	59±10.9	57±10.6	69±6.3*
Primary graft dysfunction, No. (%)					
Any PGD	17 (32.1)	6 (20)	11 (33.3)	9 (34.6)	2 (28.6)
Severe PGD	6 (11.3)	2 (10)	4 (12.1)	4 (15.4)	0 (0.0)
30-day acute rejection with hemodynamic compromise, No. (%)*	16 (30.2)	5 (25)	11 (33.3)	10 (38.5)	1 (14.3)
30-day acute rejection with myocyte damage, No. (%)*	3 (5.7)	1 (5)	2 (6.1)	2 (7.7)	0 (0.0)
1-year acute rejection with hemodynamic compromise, No. (%)	20 (37.7)	7 (35)	13 (39.4)	11 (42.3)	2 (28.6)
1-year acute rejection with myocyte damage, No. (%)	8 (15.1)	3 (15)	5 (15.2)	4 (16.7)	1 (14.3)
P-troponin I, ng/l					
6h	86310 (40324- 149706)	88155 (45683- 162006.25)	79373 (39820 −149187)	86957(44060-215360)	43188 (21286-75966)**
12h	95187.5(42482-195580)	101563 (48698-181267)	91186 (41185- 186515)	115680.5(4492 −267527)	50133 (23255-67453)**
24h	57679 (32912- 106589)	69300 (36760-124360)	49437 (31803 −103140)	60123 (33374-110863)	34828 (21565-57794)*

(*continued on next page*)

Table 3 (*Continued*)

	All donors (N=53)	Donor A (N=20)	Donor B (N=33)	Donor B1 (N=26)	Donor B2 (N=7)
P-troponin T, ng/l					
6h	8940 (4637-17150)	8896 (5712-14588)	8940 (4217-17150)	11665 (4830-18535)	4153 (2818-9120) **
12h	8460 (4399- 14453)	7947 (4531-14555)	9593 (4421-14220)	12080 (5505- 16310)	4421 (2345- 6115) **
24h	5918 (3262-9269)	5713 (3847-9458)	6055 (2706- 8425)	7563.5 (3288-9202)	2313 (2079- 4829)*
hsCRP, Mg/L					
1h	2.8 (1.9-7)	3.22 (2-7)	2.8 (1.5-6.5)	2.2 (1.6-6.7)	4.7 (1.9-5.7)
6h	5.6 (3.8-12.6)	6.8 (5-11.8)	5.5 (3.2-12.6)	5 (3.2-12.4)	6.5 (4-13.2)
12h	26.2 (16.2-44.8)	25.3 (15-48.6)	26.9 (19.6-43.2)	28.5 (21.6-44.6)	20 (15.5-32.8)
24h	87.1 (61.4-123.1)	99.3 (66.3-119.1)	85.5 (63.6-122.2)	96.5 (77.8-126.6)	63.6 (45.4-74.5)*

Plus-minus values are mean ±SD; values with range in parentheses are median (interquartile range). *p* values are *$p<0.05$. **$p<0.01$. ***$p<0.001$. During the first 24 hours, there was no difference in CKMB, lactate, and leukocytes between the donor groups. In addition, there was no difference in the function of heart transplants measured by ProBNP, and LV-EF between the donor groups after 7 days. †In the previous medical history of the heart transplant recipients, there was no peripheral vascular disease. *Acute rejection with hemodynamic compromise was diagnosed based on clinical decisions such as clinically significant decrease in left-ventricular function, increase in left-ventricular wall thickness and/or arrhythmias. The diagnose of acute rejection with hemodynamic compromise always required that the patient was treated by high dose of intravenous pulse steroids and/or antithymocyte globulin. *Acute rejection with myocyte damage is equal or more than G1Rb rejection. In this study cohort, we did not see any cases of antibody-mediated rejection within 30-days or 1-year after HTx.

with decreased graft-related 1-year mortality. In multivariate analysis of 1-year graft-related survival analyses, none of the proteins were significant (Table 4).

A summary of the possible biological role of key proteins predicting heart transplant outcome discussed further below, can be found in Table S12.

Discussion

In this study, we observed that brain-dead donors had a unique but heterogeneous proteomic signature. The changes were related to coagulation system, gluconeogenesis, and glycolysis pathways, complement, LXR/RXR activation, and production of NO and ROS in macrophages pathways. Furthermore, changes in donor plasma protein related to endothelial cell and cardiomyocyte function, inflammation, and vascular growth and arteriogenesis could predict transplant outcome suggesting their role in donor evaluation.

Despite our protein set enrichment analysis approach, making sound biological conclusions from high-dimensional MS data is still challenging.[10] Therefore, data filtering is crucially important for stringent statistical analysis. However, the relevant pathophysiology of the disease process must also be considered. In this study, FDR-corrected p value without further filtering by log2 fold change reproduced the results of the pathophysiology of donors when compared to earlier results and our recent observations in plasma extracellular vesicle transcriptomics (SeoJeong et al., unpublished).[11] Based on this, we found that donor plasma showed a distinct protein profile from healthy controls, with 237 differentially expressed proteins and 6 significantly altered pathways. Out of these, 32 proteins were identified by S-Plot as the most distinguishing proteins of donors, and 10 of these proteins were enriched into 6 significantly changed pathways.

Complement and coagulation are evolutionary-related proteolytic cascades that are critical in the innate immune response to injury.[12,13] In preclinical studies, brain death enhances complement activation and ischemia-reperfusion injury in heart transplants.[14] In our study, brain death was associated with the downregulation of complement and the upregulation of coagulation. The most significantly differentially expressed S-Plot proteins of complement and coagulation were downregulated in donors. In the comparison of donor subgroups, the complement pathway was upregulated in the Donor B group which showed more traumatic brain injury and hypertension, and in the B1 subgroup which had higher troponin and CRP within 24h and reduced cardiac function 7 days after HTx. However, lack of natural anticoagulants such as plasminogen, protein C, and antithrombin-III may lead to microvascular blood clot formation and thereby no-reflow during reperfusion of the transplant in the recipient. In addition, loss of vascular antithrombin has been linked to cardiac allograft vasculopathy and heart failure after HTx.[15]

Brain-dead donors showed significant downregulation of the LXR/RXR pathway. LXR/RXR are cholesterol-sensing nuclear receptors and key regulators of lipid metabolism. They may also control the innate immune response and reduce myocardial ischemia-reperfusion injury.[16,17] The downregulated proteins of the LXR/RXR pathway were alpha-2-HS-glycoprotein, apolipoprotein A4, plasma kallikrein, kininogen 1, and paraoxonase 1. Apolipoprotein A4 attenuates platelet aggregation, thrombosis, and platelet hyperactivity, and therefore the decreased levels of apolipoprotein A4 may reflect the aggravation of prothrombotic state in brain-dead donors.[18] In the kinin−kallikrein system, kininogen-1 is the precursor protein of high- and low-molecular kininogen, and bradykinin. The kinin-kallikrein system promotes blood coagulation, vasodilatation, and vascular inflammation.[19,20] Recently, decreased levels of pre-transplant kallikrein have been shown to predict PGD after HTx.[21] Paraoxonase-1 inhibits oxidation and apoptosis in endothelial cells and low serum levels may predispose donors to increased endothelial cell damage.[22] The downregulation of LXR/RXR suggests that this pathway was possibly depleted due to inappropriate activation of inflammatory and coagulation responses in brain-dead donors.

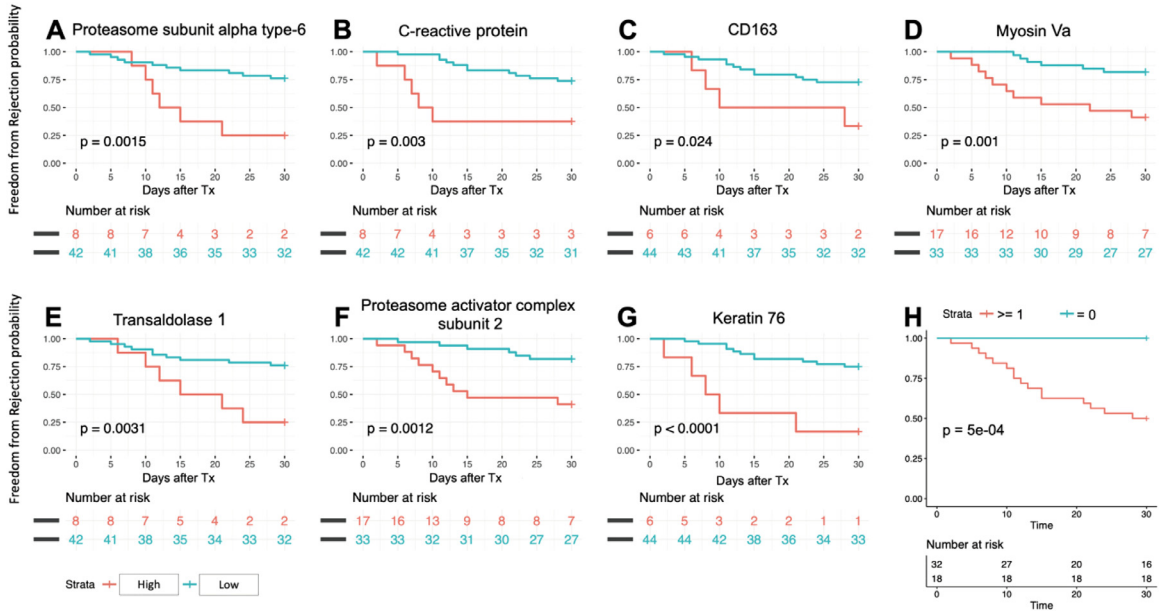

Figure 3 Impact of donor plasma protein levels on the development of acute rejection with hemodynamic compromise within the first 30 days after heart transplantation.

(A-G) Kaplan-Meier analysis on 50 heart transplant recipients showing the curves of high (red) and low (blue) protein levels of 7 donor proteins that were significantly associated with acute rejection with hemodynamic compromise episodes within 30 days after HTx. *p* value was calculated by log-rank test and revealed that rejection-free curves were significantly different between the high and low protein level groups. (A) Proteasome subunit alpha type-6 (PSMA6): *p* value = 0.0015, (B) CRP: *p* value = 0.003, (C) CD163: *p* value = 0.024, (D) myosin Va (MYO5A): *p* value = 0.001, (E) transaldolase 1 (TALDO1): *p* value = 0.0031, (F) proteasome activator subunit 2 (PSME2): *p* value = 0.0012, (G) keratin 76 (KRT76): *p* value < 0.0001. (H) Donor plasma proteomic immunological risk score was calculated based on the expression values of the 7 proteins. For high-risk level 1 point and for low-risk level zero points were given. A donor score of ≥ 1 was able to predict the risk of rejection. (Color version of figure is available online)

Under normal conditions, cardiac ATP is mainly derived from fatty acid oxidation. However, under stress conditions carbohydrates are predominantly used as an energy substrate.[23] This shift in glucose metabolism is reflected by the upregulation of the glycolysis pathway in brain-dead donors. The increased glycolysis is pivotal for anaerobic ATP production, but at the same time increased uncoupling of glycolysis and glucose oxidation may contribute to myocardial injury.[24,25] In aerobic glucose metabolism, accumulating lipid peroxidation products are metabolized by aldose reductase via the polyol pathway protecting the heart against oxidative injury. The protective activity of aldose reductase is dependent on the generation of NO.[26] In our donors, we observed a downregulation of the production of NO and ROS in macrophages pathway which may result in reduced NO bioavailability, and therefore increased aldose reductase activity and less myocardial oxidative stress. Moreover, brain-dead donors showed a substantial increase in the gluconeogenesis pathway resulting in hyperglycemia and worsening of systemic inflammation.[27–29]

Finally, we investigated whether donor plasma proteins may predict heart transplant outcomes. Interestingly, most of the proteins being correlated with any PGD or severe PGD were significantly associated with other recipient outcomes as well. Lysine-specific demethylase 3A was associated with any and severe PGD, and 1- survival, proteasome 20s subunit alpha 6 with severe PGD and acute rejection with hemodynamic compromise, moesin with severe PGD and 1-year survival, and keratin 76 with severe PGD and acute rejection with hemodynamic compromise.

In univariate Cox regression analysis, we found that higher donor plasma levels of CD163, CRP, keratin 76, myosin Va, proteasome subunit alpha type-6, proteasome activator subunit 2, and transaldolase 1 were associated with the development of acute rejection episodes with hemodynamic compromise during the first month after transplantation. Interestingly, multivariate analysis showed that the 2 proteins myosin Va and proteasome activator subunit 2 were the best predicting proteins for acute rejection with hemodynamic compromise episodes. Myosin Va is an intracellular motor protein that plays a role in channel trafficking in cardiomyocyte membrane and has been suggested as a novel therapeutic target in cardiovascular disease.[30] The circulating 20s proteasome is modulated by proteasome activator (PA28) subunits such as proteasome activator subunit 2. Abnormalities of this modulation contribute to increased intimal hyperplasia and atherosclerosis.[31]

Of note is that 7 proteins found in univariate analysis on acute rejection with hemodynamic compromise were not clearly related to the top pathways observed in brain-dead donors. However, they were related to inflammation, endothelial dysfunction, and cardiovascular protein trafficking. Therefore, we hypothesize that these proteins may reflect donors' cardiovascular morbidity or endothelial and cardiomyocyte injury induced during brain death. Based on the donor plasma proteomic immunological risk score, we

Table 4 Donor Plasma Proteins as Prognostic Biomarkers for acute Rejection with Hemodynamic Compromise Within 30 Days and Graft-Related 1-Year Survival After Heart Transplantation

Clinical endpoint	Protein	Level	Maxstat Cut-off	Number	Event	Percentage	HR (CPH univariable)	HR (CPH multivariable)
Acute rejection with hemodynamic compromise within 30d	CD163	Low	41407,8	44	12	72,70 %	reference	reference
	CD163	High	41407,8	6	4	33,30 %	3.41 (1.09-10.64, p=0.034)	0.15 (0.02-1.44, p=0.101)
	C-reactive protein	Low	490182,7	42	11	73,00 %	reference	reference
	C-reactive protein	High	490182,7	8	5	14,30 %	4.38 (1.50-12.76, p=0.007)	3.19 (0.62-16.30, p=0.164)
	Keratin 76	Low	16211,1	44	11	75,00 %	reference	reference
	Keratin 76	High	16211,1	6	5	16,70 %	7.31 (2.47-21.60, p<0.001)	2.18 (0.30-15.62, p=0.439)
	Myosin Va	Low	2143,2	33	6	81,80 %	reference	reference
	Myosin Va	High	2143,2	17	10	41,20 %	4.70 (1.70-12.97, p=0.003)	5.18 (1.17-22.91, p=0.030)
	Proteasome subunit alpha type-6	Low	798,6	42	10	76,20 %	reference	reference
	Proteasome subunit alpha type-6	High	798,6	8	6	25,00 %	4.64 (1.65-13.06, p=0.004)	3.80 (0.62-23.15, p=0.147)
	Proteasome activatorsubunit 2	Low	81,6	33	6	81,80 %	reference	reference
	Proteasome activatorsubunit 2	High	81,6	17	10	41,20 %	4.65 (1.68-12.87, p=0.003)	4.19 (1.16-15.14, p=0.029)
	Transaldolase1	Low	11926,2	42	10	76,20 %	reference	reference
	Transaldolase 1	High	11926,2	8	6	25,00 %	4.16 (1.50-11.58, p=0.006)	3.68 (0.70-19.44, p=0.124)
1-year survival	D-dopachrome decarboxylase	high	105,9	48	4	91,70 %	reference	reference
	D-dopachrome decarboxylase	low	105,9	5	2	60,00 %	5.77 (1.05-31.74, p=0.044)	2.09 (0.34-12.98, p=0.428)
	Moesin	low	6807,7	45	3	93,30 %	reference	reference
	Moesin	high	6807,7	8	3	62,50 %	6.94 (1.40-34.51, p=0.018)	1.14 (0.11-11.40, p=0.909)
	Leucine Rich Alpha-2-Glycoprotein 1	high	508587,7	34	1	97,10 %	reference	reference
	Leucine Rich Alpha-2-Glycoprotein 1	low	508587,7	19	5	73,70 %	10.40 (1.21-89.13, p=0.033)	6.78 (0.56-81.50, p=0.131)
	Lysine-specific demethylase 3A	low	2434,8	40	2	95,00 %	reference	reference
	Lysine-specific demethylase 3A	high	2434,8	13	4	69,20 %	6.87 (1.26-37.55, p=0.026)	5.50 (0.61-49.65, p=0.129)
	Keratin 79	high	4271,7	34	1	97,10 %	reference	-
	Keratin 79	low	4271,7	19	5	73,70 %	10.40 (1.21-89.13, p=0.033)	-

Number, the group size of donors with higher protein expression and of donors with lower protein expression. Event, number of acute rejections, or number of graft-related deaths. Percentage, freedom from rejection. HR, hazard ratio; CPH, cox proportion hazard. Significant proteins in univariate COX regression analyses of acute rejection: CD163, HR 3.41, p value 0.034; C-reactive protein, HR 4.38, p value 0.007; KRT76, HR 7.31, p value <0.001; Myosin Va5, HR 4.7, p value 0.003; Proteasome subunit alpha type-6, HR 4.64, p value 0.004; Proteasome activator subunit 2, HR 4.65, p value 0.003; and Transaldolase 1, HR 4.16, p value 0.006. Significant proteins in multivariate COX regression analyses of acute rejection: MYOA5, HR 5.18, p value 0.030; and PSME2 HR 4.19, p value 0.029. Significant proteins in univariate COX regression analysis of survival: D-dopachrome decarboxylase, HR 5.77, p value 0.044; Moesin, HR 6.94, p value 0.018; Leucine rich alpha-2-glycoprotein 1, HR 10.4, p value 0.033; Lysine-specific demethylase 3A, HR 6.87, p value 0.026; Keratin 79, HR 10.4, p value 0.033. There were no significant proteins in multivariate COX regression analyses of survival.

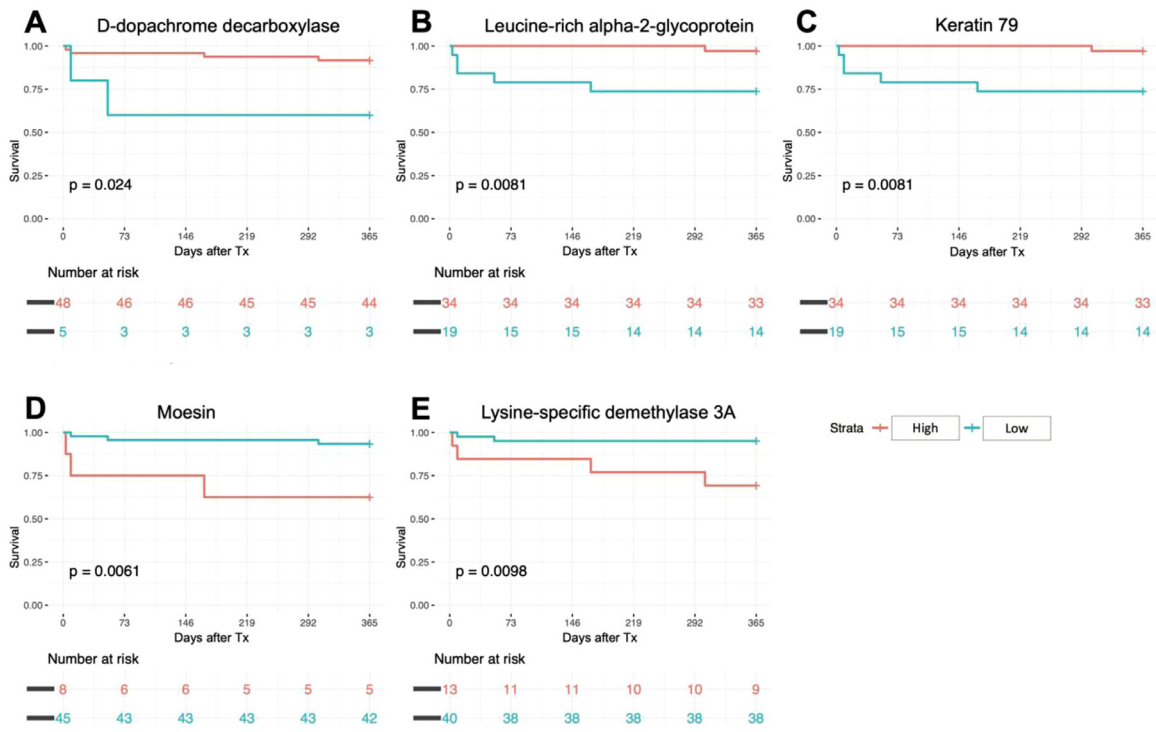

Figure 4 Impact of donor plasma protein levels on graft-related 1-year survival after heart transplantation.

Kaplan-Meier survival analysis on 50 heart transplant recipients showed that on the one hand, (A) donors with low levels of d-dopachrome decarboxylase, (B) leucine rich alpha-2-glycoprotein 1, and (C) keratin 79 had worse overall 1-year survival than donors with high levels of d-dopachrome decarboxylase (60% in low vs. 91.70% in high), leucine-rich alpha-2-glycoprotein (73.70% in low vs. 97.10% in high) and keratin 79 (73.70% in low vs. 97.10% in high). On the other hand, (D) donors with high levels of moesin and (E) lysine-specific demethylase 3A had worse overall 1-year survival than donors with low levels of moesin (93.30% in low vs. 62.50% in high) and lysine-specific demethylase 3A (95% in low vs. 69.20% in high).

showed that if any of the 7 donor proteins were upregulated, there was an increased risk of acute rejections with hemodynamic compromise within the first 30 days and that this risk increases depending on how many proteins were upregulated. These results suggest that the risk score based on these 7 proteins may be used to stratify the brain-dead organ donors.

In univariate Cox regression analysis, higher donor plasma levels of moesin and lysine-specific demethylase 3A were associated with an increased risk of graft-related 1-year mortality. Moesin, a member of the ezrin-radixin-moesin family, is expressed by vascular endothelium and has a pivotal role in vascular permeability and inflammatory responses. A recent study shows that increased serum moesin contributes to the sepsis-related endothelium damages by activating the Rock1/myosin light chain and NF-κB signaling.[32] Lysine-specific demethylase 3A promotes fibrosis in cardiomyocytes and, therefore, it has been suggested as a potential pharmacological target for cardiac hypertrophy and fibrosis.[33] Donor microvascular injury may lead to inappropriate and uncontrolled activation of the coagulation cascade and thrombin formation which may lead to the early development of tissue fibrosis in transplanted organs.[34] Based on these results we suggest that the high expression of these proteins may reflect the worse overall clinical status of these donors or donor hearts, which may be partly due to increased microvascular dysfunction and cardiomyocyte damage induced by events leading to brain death.

In conclusion, we demonstrate for the first time that brain-dead donors had a unique but heterogeneous proteomic profile. We also show those donor proteins involved in endothelial dysfunction, cardiomyocyte hypoxia, and fibrosis, and vascular cell growth and arteriogenesis may play a pivotal role in graft-related outcomes. Therefore, our results suggest that systematic characterization of circulating proteins may provide a deeper understanding of the effects of donor morbidity and brain death on donor organs and identify the transplants at increased risk.

Limitations of this post-hoc analysis of a prospective, single-center study are related to the nature of the analyses and the relatively small sample size which may have an impact on data quality. The patient cohort consisted of only clinically stable multi-organ donors that were accepted for HTx. However, the median age of donors was 44 years, which is equal to the median age of heart transplant donors in Europe, compared to 31 years in North America. Depletion of the top 12 high-abundance proteins enhances the sensitivity to detect lower-abundance proteins in plasma, but it could also lead to some bias as some of the depleted proteins may have a role in the pathophysiology of brain death or prediction of the outcomes. Further mechanistic studies with a larger patient population are needed to find any biomarker or therapeutic potential of these proteins and pathways.

Author contributions

AN, KL, JL, and RR: conceptualization and research funding; AN, KL, JL, KD, and RR: research design; AN, SS, KL, SJ, MS, EJH, JL, and RK: data collection; SJ, MS, KD, JL, and RR: contributed analytic tools; KD, JL, SJ, MS: data analysis; AN, JL, KD, MS, SJ, SS, and KL: data interpretation; AN, JL, KD, SS, MS, SJ, RK, and KL: writing of the paper

Disclosure Statement

The authors have no conflicts of interest to disclose.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at https://doi.org/10.1016/j.healun.2021.11.011.

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