Perfusate proteomes provide biological insight into oxygenated versus standard hypothermic machine perfusion in kidney transplantation

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Abstract

Objective

Mass spectrometry profiling of perfusate samples collected during a phase-3 randomized double-blind paired clinical trial of hypothermic machine perfusion (HMP) with and without oxygen (COMPARE) aimed to provide mechanistic insight into key biological alterations of DCD kidneys during continuous perfusion and inform about future interventions.

Summary Background Data

Despite the clinical benefits of novel perfusion technologies aiming to better preserve donor organs, biological processes that may be altered during perfusion have remained largely unexplored. Collection of serial perfusate samples during the COMPARE clinical trial provided a unique resource to study perfusate proteomic profiles, with the hypothesis that in-depth profiling may reveal biologically meaningful information on how donor kidneys benefit from this intervention.

Methods

Multiplexed liquid chromatography tandem mass spectrometry was used to obtain a proteome profile of 210 perfusate samples. Partial least squares discriminant analysis and multivariate analysis involving clinical and perfusion parameters were used to identify associations between profiles and clinical outcomes.

Results

Identification and quantitation of 1716 proteins indicated that proteins released during perfusion originate from the kidney tissue and blood, with blood-based proteins being the majority. Data show that overall HMP duration associate with increasing levels of a subgroup of proteins. Notably, high-density lipoprotein and complement cascade proteins are associated with 12-month outcomes and blood-derived proteins are enriched in the perfusate of kidneys that developed acute rejection.

Conclusions

Perfusate profiling by mass spectrometry was informative and revealed proteomic changes that are biologically meaningful and in-part explain the clinical observations of the COMPARE trial.

Introduction

Kidney transplantation is the gold standard treatment for patients with end-stage renal disease reducing morbidity and mortality whilst improving quality of life. The required need of kidney transplants continues to exceed the supply of suitable kidneys from deceased donors, especially when many organs are currently discarded as deemed untransplantable. To maximize organ utilisation without compromising transplant outcomes, machine perfusion technologies have been developed to better assess, preserve, and potentially improve donor organ quality prior to transplantation.[1] Previously, we demonstrated in a randomised controlled trial comparing static cold storage preservation of deceased donor kidneys with continuous hypothermic machine perfusion (HMP) that the latter improved short-term kidney function and survival.[2] Using HMP showed superior outcomes not only in donation after brain death (DBD) but also in extended criteria (ECD) and donation after circulatory death (DCD) donor kidneys.[3]

As pre-clinical studies repeatedly have suggested a clear benefit of oxygenation during cold preservation [4,6–10], we developed a novel HMP device that allowed continuous oxygenation of the perfusate during perfusion and tested this modality in a clinical trial [4]. The COMPARE trial was a phase-3 randomised double-blind paired trial performed by the Consortium for Organ Preservation in Europe (COPE), and has demonstrated clinical benefit of oxygenated ($HMPO_2$) compared to standard cold perfusion (HMP) of older DCD kidneys. The trial showed improved graft function of oxygenated donor kidneys at one year after transplantation when accounting for decreased graft failure. It also found fewer severe complications and a lower rate of acute graft rejection when $HMPO_2$ was used [4].

Whilst the clinical outcome of the COMPARE trial showed clear benefit for patients, it has remained unclear which biological changes take place during hypothermic machine

perfusion. In this study we have aimed to reduce the translational gap between the preclinical model and clinical scenario, contributing at least in part by analysing all perfusate samples collected from donor kidneys during both non-oxygenated and oxygenated HMP since we hypothesized that the perfusate contains the combination of signals released from the donor kidney tissue and donor blood. As we wanted to observe pre-existing as well as more definitive alterations and mechanistic changes of injury and/or repair, we focused on the profiling of the proteomes in perfusate samples reflecting the condition of the kidneys as well as potentially informing us about future beneficial interventions to enhance donor kidney quality during machine perfusion.

Methods

Perfusate samples

Perfusate samples were obtained as part of the Consortium for Organ Preservation in Europe (COPE) COMPARE clinical trial[4] (Figure 1) according to trial protocol using the Kidney Assist Transport device (Organ Assist BV, Groningen, The Netherlands). All participants provided written consent for the collection and storage of biological samples and use of follow-up clinical data. This study was approved by the relevant institutional review board and ethics committee. Perfusate samples were collected at three time points:15 minutes after the start of perfusion, immediately before the kidney left the donor hospital on the device (3.8±3.4 hours), and at the end of perfusion (7.8±5.4 hours). Perfusate samples were stored on ice until transfer to the processing lab in a central hub in the Netherlands, where they were centrifuged (1300 g for 15 min at room temperature), temporarily stored at -80 °C, and subsequently transferred for long-term storage at -80 °C in Oxford, UK, according to the

COMPARE protocol (ISRCTN32967929). All perfusate samples containing sufficient

biological material for proteomic analysis were included.

Clinical characteristics of donor and recipient populations (supplementary data file,

Supplemental Digital Content 1, http://links.lww.com/SLA/E779)

Untargeted Proteomics

Detailed methodology of proteomics analysis is described in Supplementary data,

Supplemental Digital Content 1, http://links.lww.com/SLA/E779. Samples were analysed by

liquid chromatography-tandem mass spectrometry (LCMS) using an isobaric tandem mass

tag (TMT; Thermo Fisher Scientific, Germany) labelling approach to allow sample

multiplexing and high-precision relative quantitation (Figure 1) as described in detail in

Supplementary methods, Supplemental Digital Content 1, http://links.lww.com/SLA/E779.

Samples were allocated non-randomly across LCMS runs to minimize confounding between

the relevant clinical and technical variables (Table S1, Supplemental Digital Content 1,

http://links.lww.com/SLA/E779).

Statistical Analysis

Detailed description of the statistical analysis can be found on supplementary files,

Supplemental Digital Content 1, http://links.lww.com/SLA/E779. Data analysis was

performed using R. Unless otherwise specified, data are presented as the median $\pm$

interquartile range. $p < 0.05$, or where appropriate, a false discovery rate of 5% was

controlled using the method of Benjamini and Hochberg[12].

Results

Proteomic profiles were obtained for 210 perfusate samples that were longitudinally obtained

across oxygenated HMP (n = 101) and non-oxygenated HMP (n = 109) from the perfusion of

67 paired DCD kidneys (n=137 total kidneys) (Figure 1). The median perfusion duration was

7·40 h (4·8–9·9) for HMP and 6·85 h (4·5–9·1) for $HMPO_2$. The clinical characteristics of

the donors are shown in Table 1.

Perfusate contains proteins from both kidney tissue and blood

We identified and quantified 1716 proteins in the perfusate samples. Next, we searched for

the identified proteins across the atlas datasets and classified them according to their origin.

While many of these proteins originated from the kidney tissue, a large number of proteins

could also originate from blood components released in the vascular compartment of the

donor kidney. Therefore, plasma and red blood cell proteomes were used as a proxy; Figure

2A). The most abundant proteins identified were Complement C3, Apolipoprotein A1 and

Fibrinogen alpha chain. Taking abundance into account, blood proteins were quantitatively

dominant over those that originated unambiguously from the kidneys (Figure 2B).

A Reactome pathway enrichment analysis of proteins overrepresented in the perfusate

highlighted the enrichment of a number of biological functions (Figure 2C), including

complement cascade, platelet aggregation, and detoxification of reactive oxygen species

(Figure 2C).

Perfusion duration and effect on protein abundance

The perfusion duration had a major influence on protein abundance and identified a

relationship between high density lipoprotein during perfusion and recipient outcomes after

12-months. To quantitatively understand the abundance of proteins within the perfusate, we

used an unsupervised approach to identify relevant relationships between clinical variables

and the major sources of variation in the abundance of proteins in the perfusate (Figure S1A-

C, Supplemental Digital Content 1, http://links.lww.com/SLA/E779, Table S2, Supplemental

Digital Content 1, http://links.lww.com/SLA/E779). Significant correlations were observed

with perfusion duration (Pearson's $r = 0.34$, $p = 0.0002$, Fig 3A) and the total protein

concentration of samples as determined by the Bradford assay. Oxygenation of the perfusate

by itself during organ preservation appeared not important in determining protein abundance

in the perfusate (Figure S1A-C, Supplemental Digital Content 1,

http://links.lww.com/SLA/E779). We did not observe a relationship between the duration of

perfusion and 12-month eGFR (Figure S2A, Supplemental Digital Content 1,

http://links.lww.com/SLA/E779). At the level of individual proteins, 137 proteins correlated

significantly with perfusion duration. Notably, this includes both those that increase (e.g.,

Vimentin, Spearman's $\rho = 0.69$, $p < 2e-25$) as well as those that decrease (e.g., Cystatin C,

Spearman's $\rho = -0.41$, $p < 2e-7$) with the duration of perfusion (Figure 3C-D). Multivariate

methods were used to identify proteins that changed in a coordinated manner across perfusion

duration and in relation to 12-month eGFR (Figure S2B, Supplemental Digital Content 1,

http://links.lww.com/SLA/E779), and an enrichment test was then used to identify functional

gene sets (Figure 3E-F). We then examined the network of known protein-protein

interactions in the enriched protein networks. This analysis revealed a pertinent role for high-

density lipoprotein (HDL), as well as the complement cascade (Figure 3G).

Perfusate proteomes provide mechanistic insights into acute rejection of kidneys

To explore the biological process(es) associated with acute rejection, we tested for

differences in the composition of the perfusate proteome at the end of perfusion between

kidneys that developed acute rejection within the first year and those that did not using a

partial least squares discriminant analysis (PLS-DA) approach. The first latent variable

separated kidneys that developed acute rejection from those that did not (Figure 4A).

Performing a gene enrichment analysis, we found that pathways related to "regulation of

response to wound healing", "metabolic processes that contribute to ATP generation", and

"nucleotide phosphorylation', were all associated with acute rejection (Figure 4B).

Calculating the enrichment of proteins that might originate from the kidney tissue (Fisher's exact test, $p = 0.90$, one-tailed) or blood compartment (Fisher's exact test, $p = 0.025$, one-tailed) suggested that this signal originated primarily from the vascular compartment rather than the kidney tissue. Finally, we intersected our proteomics data with atlas single-cell transcriptomics datasets from both kidney and peripheral blood mononuclear cells (Figure S3A-B, Supplemental Digital Content 1, http://links.lww.com/SLA/E779). We tested for enrichment of cell types classified by their transcriptomic profile and found a significant enrichment of proteins originating from platelets (Figure 4C-D).

Discussion

In this study, we have focused on the alterations and mechanisms that take place in deceased donor kidneys from DCD donors during standard non-oxygenated and oxygenated hypothermic machine perfusion. We have analysed serial perfusate samples of donor kidneys obtained between organ retrieval and transplantation. We have performed an unbiased characterization of the proteome profiles in clinical perfusate samples from both non-oxygenated and oxygenated HMP. We quantified 1716 proteins across 210 samples, representing both the largest and most comprehensive measurements of proteins in the HMP perfusate to date.

We identified many proteins originating from kidney tissue and blood in the vascular compartment of the donor kidney with a high overlap between enriched pathways and blood-centric mechanisms. This was despite the fact that all kidneys had been flushed according to clinical standards prior to initiation of perfusion. This observation is consistent with previous work that has performed proteomics on perfusate during HMP, i.e. the enrichment of complement and coagulation cascades in samples both early and late in perfusion as reported

by van Leeuwen et al[13]. Coskun and colleagues similarly observed blood proteins in their proteomic dataset but suggested that they are artefactual[14]. In contrast, the data presented here indicate that the blood in the vascular compartment contributes to pathophysiological mechanisms and should be considered as part of the biologically relevant signals. In our dataset, for most proteins, it was difficult to unambiguously determine their origin between kidney tissue and blood, which necessitates careful inference about the tissue or cell types of origin.

Based on our data and those of others, we suggest that these blood proteins may originate from the occult non-perfused vasculature of the kidney. Histologically detectable fibrin and/or fibrinogen have previously been reported in biopsies taken after static cold storage[15,16]. We are aware of reports that cold storage of kidneys may also result in the accumulation of fibrinogen within the tubular epithelium [15], but note that the two mechanisms are not mutually exclusive.

Non-perfused vasculature results in regions of the tissue undergoing warm ischemia upon reperfusion. This may be of particular importance in the kidney in comparison to other organs: the kidney has a high metabolic rate[17] but does not regulate its own blood flow to match metabolic requirements[18]. Furthermore, certain regions also operate at low oxygen tensions to enable the concentration of urine[19]. Therefore, we hypothesised that pathophysiological signals related to organ damage and transplant outcomes may originate from both the kidney tissue and blood.

Our unbiased analysis of perfusate samples detected proteomic changes longitudinally over the duration of perfusion. Notably these changes show strong associations with transplant

outcomes. Specifically, we identified that the HDL complex decreases in abundance over perfusion duration and these changes over time related to a higher recipient eGFR at 12-months post-transplant. Previous work has highlighted the release of lipids into the perfusate of the kidneys during HMP[20]. Further studies are required to confirm the extent to which HDL complexes remain intact during perfusion.

We also observed that proteins from the complement cascade decreased during more extended durations of perfusion and in relation to a higher 12-month eGFR. Complement has already been shown to be activated within the plasma of circulatory death donors[21], but equally preclinical research indicates that local activation within the graft can be detrimental[22]. These findings suggest that a better understanding of the proximal consequences of changes during HMP and the relationship to systemic activation in the donor might allow better experimental designs in future HMP trials.

At the end of perfusion, in kidneys that developed acute rejection, we found an increased abundance of a subset of proteins originating specifically from platelets. We speculate that this may be at least partially responsible for (micro) microvascular obstructions. HMP demonstrably cause at least partial clearance of obstructed vasculature, as per our own findings regarding the detection of blood proteins within the perfusate. This speculation is consistent with our own practical experiences in the laboratory, where a period of HMP before subsequent warm reperfusion improves the perfusion characteristics of porcine kidneys[23]. Partial clearance of obstructed vessels is also supported by preclinical studies in human kidneys, where marked heterogeneity in initial perfusion was observed during reperfusion (either in the recipient or during normothermic machine perfusion), even after HMP[24–26]. We note that there is also mechanistic evidence for non-enzymatic fibrinolysis

from in vitro assays assessing thrombolysis, where small amounts of lytic activity are observed, even in the absence of fibrinolytic enzymes[27–29]. Following preservation in cold conditions, there is an emerging consensus that these obstructions are aggravated upon warm reperfusion, either by normothermic machine perfusion[15,30] or by reperfusion at time of transplantation in the recipient[31].

Among our mechanistic findings, we observed no difference between the perfusate proteomes of oxygenated and non-oxygenated HMP. However, in preclinical models, oxygenated HMP has been shown to result in higher ATP levels[10] and an increased rate of aerobic metabolism[8]. It is logical to anticipate that such metabolic differences are also reflected in the perfusate proteome, but our results do not support this conclusion. A lack of difference suggests that the energy required for biological processes during HMP is limited by the temperature irrespective of oxygenation, that there is enough alternative energy supplied through nonaerobic processes such as glycolysis, or that in our study, the metabolic-associated signal was potentially obscured by biological variance. Whether perfusate metabolomic profiling will be more informative on detecting metabolic changes, it is to be determined.

We acknowledge the limitations of this study. We report an association between the presence of complement proteins and recipient eGFR at 12-months, indicative of immune-related processes. However, this does not provide proof of actual complement activation, which would require either immunostaining on pre-transplant biopsies, or specific immunoassays for complement activation fragments. Proteomic profiling typically has poor coverage of small, low-abundance proteins and peptides, which include many likely relevant species, including inflammatory regulators, such as cytokines. While reporting mechanistic associations between the perfusate proteome and clinical outcomes, we must stress that,

despite using an experimental design that minimised the effects of confounding experimental

effects, practicality limited the cohort size and, therefore, its power to identify individual

biomarkers. Finally, a parallel proteomic profiling of perfusate and tissue biopsies of DBD,

DCD kidneys to include HMP and a cohort of cold stored kidneys will answer many

additional questions regarding associations of kidney tissue molecular mechanisms and

released proteins during perfusion, although such an analysis would require considerable

analytical resources to perform.

In conclusion, in this study we have identified associations between the perfusate proteome

and clinical outcomes, also highlighting the underappreciated role of proteins originating

from the blood compartment versus those from the kidney tissue. These associations suggest

that dismissal of blood proteins as artifacts risks to ignore pathologically relevant signals. On

the other hand, the large overlap that we observed between kidney and blood proteins

necessitates further experimental investigations to trace the origin of identified proteins and

describe their biological significance. Future work on this will enrich our understanding of

key determinants of pre-transplant kidney assessment. Our findings reinforce previous

suggestions that transplant outcomes are by no means predetermined at retrieval, and

highlight the importance of further studies on new interventions during kidney preservation to

resuscitate organs.

Acknowledgements

Conflict of Interest declaration

IJ reports speaker fees from XVIVO Perfusion paid to her institution, RP has been advising Bridge to Life Ltd in UK on organ preservation. All other authors declare no competing interests.

Data and code availability

Raw files from the proteomic analysis along with all metadata were deposited in PRIDE with the project accession number PXD041078. The code required to reproduce our analysis, as well as all the underlying figure data, will be made available at 10.5281/zenodo.7638684.

Abbreviations

COPE – Consortium for Organ Preservation in Europe

DCD – Donation after circulatory death

eGFR – Estimated glomerular filtration rate

HDL – High density lipoprotein

HMP – Hypothermic machine perfusion

$HMPO_2$ – Hypothermic machine perfusion with oxygen

PLS-DA – Partial least squares discriminant analysis

TMT – Tandem mass tag

LC MS – Liquid Chromatography mass spectrometry

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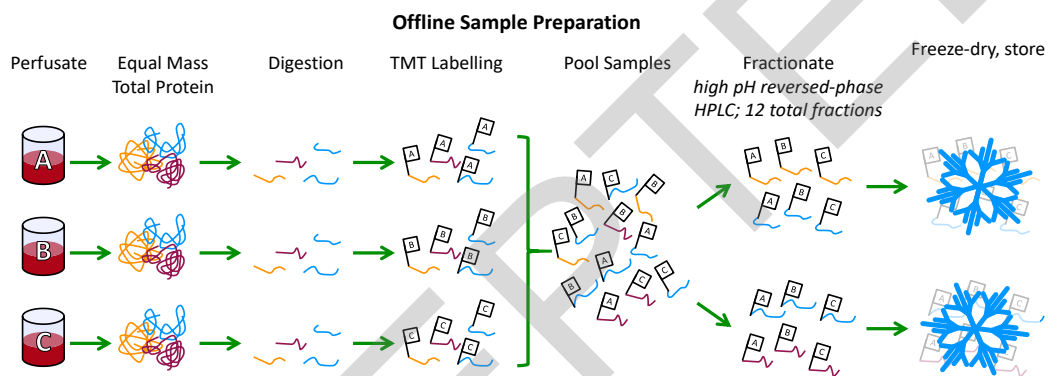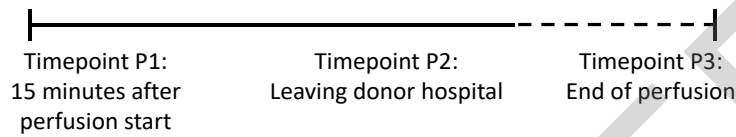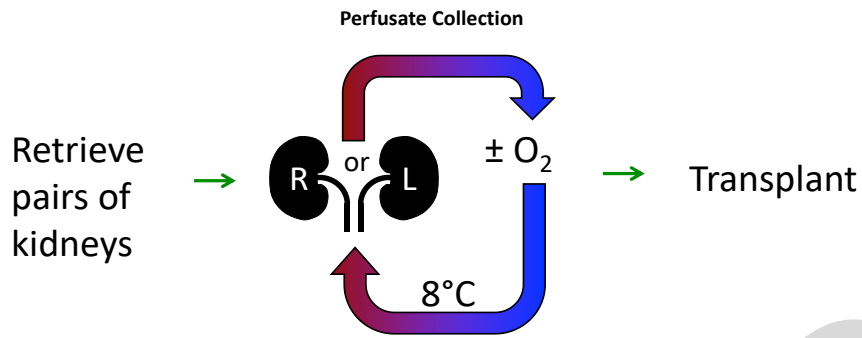
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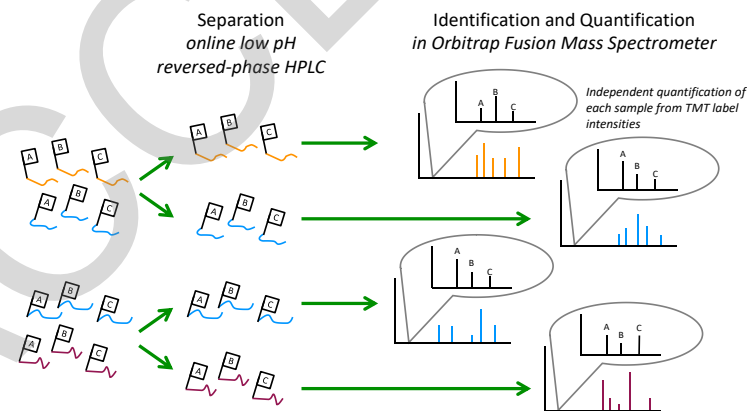**Figure 1: Experimental workflow of perfusate proteomic analysis**

In the COMPARE trial, pairs of kidneys donated after circulatory death were randomized into hypothermic machine perfusion with ($HMPO_2$) or without oxygen (HMP). Perfusate samples were collected during the perfusion. Perfusate samples were collected 15 min after the start of perfusion, just before the departure of the kidney from the retrieving hospital, and at the end of perfusion prior to transplantation.

Equal amounts of protein that had previously precipitated from perfusate samples (n=210) were digested to tryptic peptides, isolated by HPLC, and subsequently each sample individually labelled by isobaric mass tagging of every peptide, using TMT. LC-MS/MS analysis of TMT labelled tryptic peptides was performed using LC-MS^n. Each sample has an individual tag that allows the analysis of individual samples and comparison across samples.

Perfusate Collection

Retrieve
pairs of
kidneys

R or L

± O₂

Transplant

8°C

Timepoint P1:
15 minutes after
perfusion start

Timepoint P2:
Leaving donor hospital

Timepoint P3:
End of perfusion

Offline Sample Preparation

| Perfusate | Equal Mass Total Protein | Digestion | TMT Labelling | Pool Samples | Fractionate *high pH reversed-phase HPLC; 12 total fractions* | Freeze-dry, store |

LC-MSMS Analysis

Separation
*online low pH
reversed-phase HPLC*

Identification and Quantification
in Orbitrap Fusion Mass Spectrometer

*Independent quantification of
each sample from TMT label
intensities*

Figure 2: Analysis of perfusate by Tandem Mass Tag (TMT) labelled LC MS-MS

A. Proteins identified by mass spectrometry were searched using atlas and consensus datasets and proteins were classified according to their origin. 532 proteins were classified as only blood derived proteins, 35 were classified as only kidney tissue derived proteins and 579 proteins were common in blood and kidney tissue and could have originated from either.

B. The proportion of proteins identified in perfusate samples plotted according to tissue of origin with 37% median proportion of total samples signal from blood, 2% from kidney, 58% from blood or kidney and 3% unknown origin.

C. Pathway overrepresentation analysis identifies main biological functions within the perfusate: 1-3 = unassigned, 4 = Platelet aggregation, 5 = Extracellular matrix organisation, 6 = Signalling by ALK, 7 = Detoxification of Reactive Oxygen Species, 8 = Phagocytosis, 9 = Semaphorin interactions, 10 = Complement cascade, 11 = Metabolism of carbohydrates.
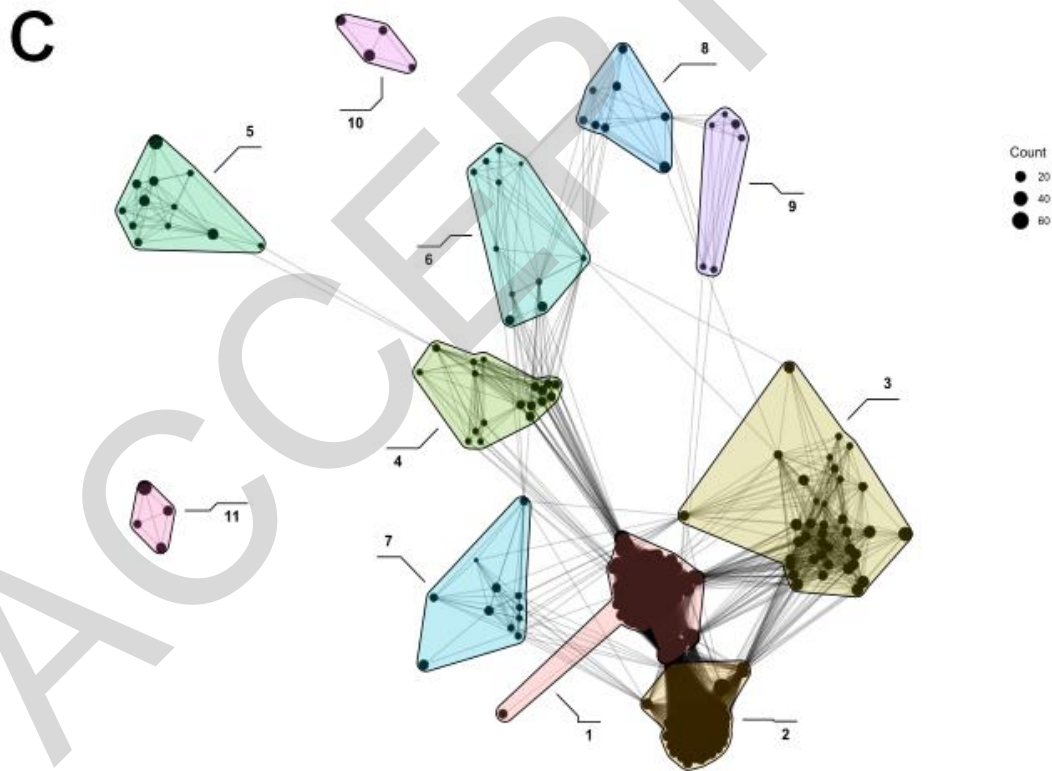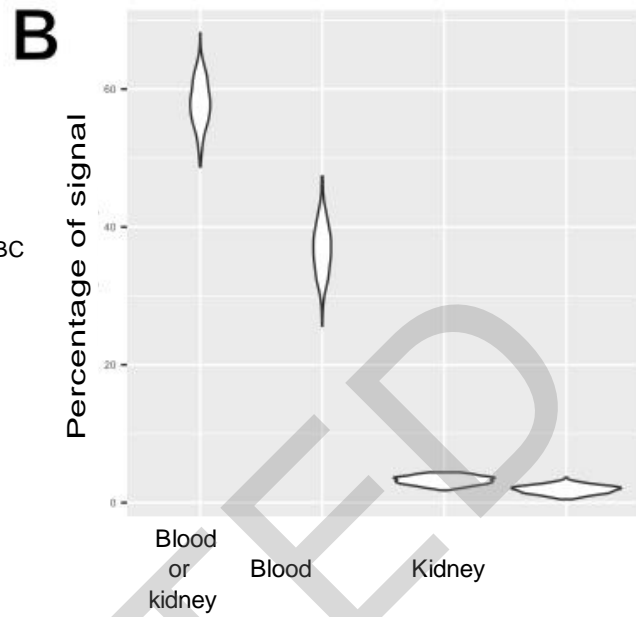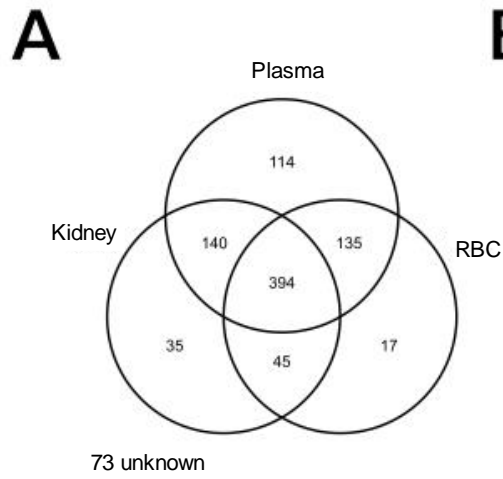
Figure 3: Perfusion duration has a major influence of the abundance of proteins in the perfusate, and identifies biological processes related to recipient outcomes 12-months after transplant.

A. Pearson correlation was calculated between all relevant clinical variables and the most important principal components explaining protein abundance. After correcting for multiple testing, the perfusion duration is significantly related to dimension 1, and the total protein concentration in the perfusate to dimension 2.

B. Scatterplot of perfusion duration against sample coordinates along dimension 1, illustrating the correlation reported in panel A.

C. At the level of individual proteins, we identified proteins that had significant positive or negative monotonic correlation with the perfusion duration (x-axis Spearman rank correlation coefficient; y-axis negative log significance)

D. Individual samples show substantial variation in the abundance of proteins whose aggregate abundance significantly correlates with perfusion duration.

E. Gene set enrichment analysis identifies that coordinated changes across perfusion duration relate to recipient kidney function 12-month posttransplant using multivariate analysis.

F. Heatmap of GO terms found to be statistically significant in the GSEA as shown in panel E.

G. Protein-protein interaction network of proteins which decrease over perfusion duration and correlate positively with 12-month eGFR. High density lipoprotein complex (red), as well as early (purple) and late (mustard green) complement proteins were identified.
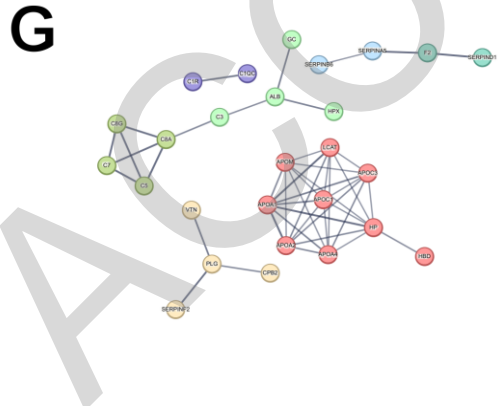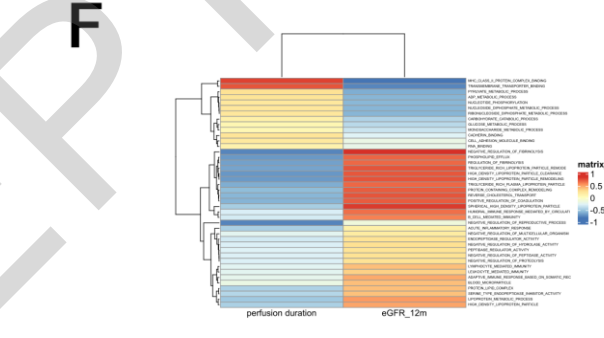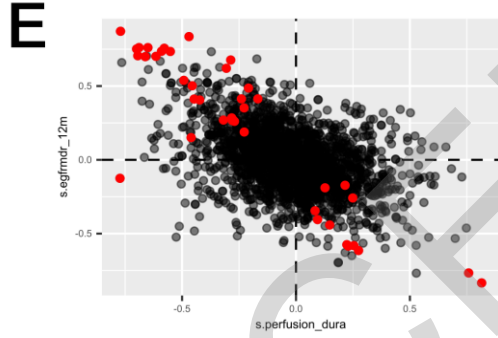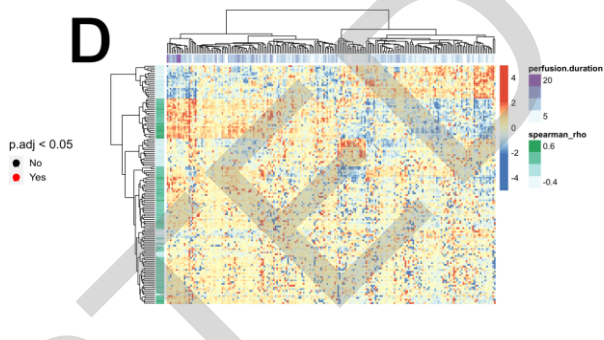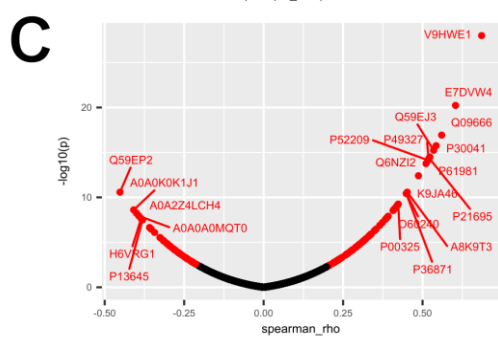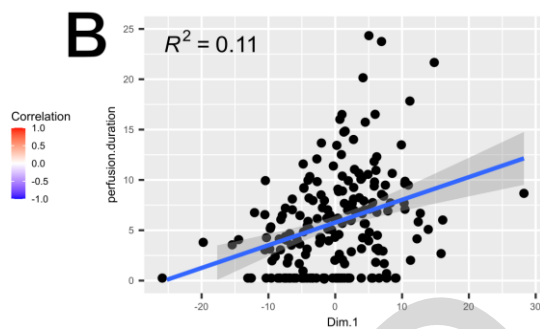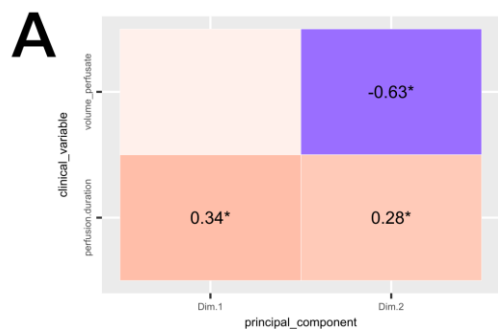
Figure 4: Machine learning identifies proteins associated with incidence of acute rejection posttransplant.

A. PLS-DA of perfusate proteomes separated kidneys that developed acute rejection posttransplant from those that did not.

B. Gene set enrichment analysis of proteins associated with acute rejection identified upregulation of biological processes related to cellular respiration and oxidative phosphorylation, as well as downregulation of tissue injury response and wound healing.

C. Intersection with single cell RNAseq data from both kidney tissue and peripheral blood mononucleocytes shows a significant enrichment of platelet derived proteins but with no other cell type reaching significance.
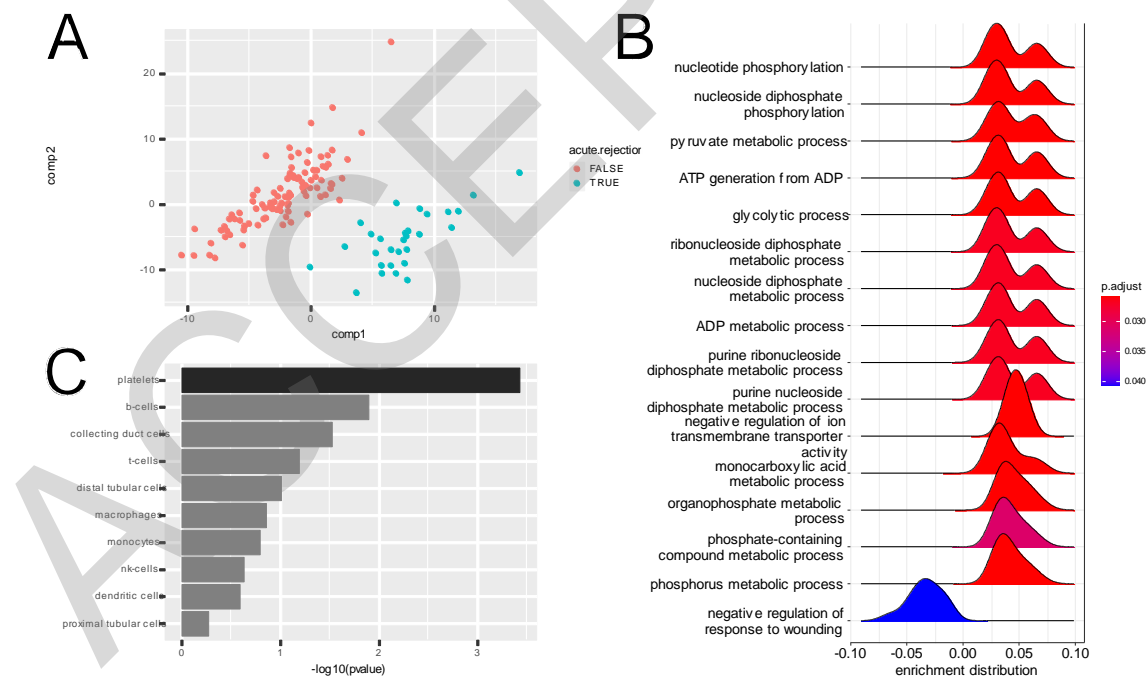
Table 1: Clinical metadata that associates to the perfusate samples analysed, grouped according to the standard and oxygenated perfusion

Table 1

Characteristics	Treatment Received			
	Overall, N = 210[1]	**HMP -02**, N = 107[1]	**HMP +02**, N = 101[1]	**Unknown**, N = 2[1]
Donor Gender				
Female	86 (41%)	45 (42%)	41 (41%)	0 (0%)
Male	124 (59%)	62 (58%)	60 (59%)	2 (100%)
Donor BMI (kg/m²)	25.1 (22.8, 27.7)	24.8 (22.6, 27.5)	25.1 (22.9, 27.7)	38.7 (38.7, 38.7)
Donor Age (years)	59 (54, 62)	59 (54, 62)	59 (56, 62)	72 (72, 72)
Donor Cause of death				
Cerebrovascular Accident	79 (38%)	43 (40%)	34 (34%)	2 (100%)
Hypoxia	79 (38%)	38 (36%)	41 (41%)	0 (0%)
Other	20 (9.5%)	9 (8.4%)	11 (11%)	0 (0%)
Trauma	32 (15%)	17 (16%)	15 (15%)	0 (0%)
Donor Last creatinine (umol/L)	60 (41, 71)	59 (41, 70)	60 (41, 69)	105 (105, 105)
Unknown	2	1	1	0
Total warm ischemia (min)	27 (17, 35)	27 (22, 35)	26 (16, 35)	28 (28, 28)
Recipient eGFR at 12 months (mL/min/1.73m2)	41 (29, 49)	40 (25, 52)	41 (30, 46)	34 (31, 36)
Biopsy confirmed acute rejection				
FALSE	164 (78%)	84 (79%)	80 (79%)	0 (0%)
TRUE	46 (22%)	23 (21%)	21 (21%)	2 (100%)

	Treatment Received			
Characteristics	**Overall**, N = 210[1]	**HMP -02**, N = 107[1]	**HMP +02**, N = 101[1]	**Unknown**, N = 2[1]

[1]n (%); Median (IQR)

#29

| ANNSURG-D- Rutger 23-00366 Ploeg | Perfusate proteomes provide biological insight into oxygenated versus standard hypothermic machine perfusion in kidney transplantation | ESA Paper |

First Discussant: Wolf O. Bechstein (Frankfurt Am Main, Germany)

Thank you for your interesting presentation, and to the Society for the privilege of the floor. I congratulate you and your co-workers on this tremendous translational work. During the latter half of the 20th century, the cold storage of kidneys following organ retrieval, during transport, and prior to transplantation has been the gold standard. More than a decade ago, randomized controlled trials (RCTs) have demonstrated that continuous machine perfusion may improve the function of donor kidneys retrieved from donors after circulatory death (DCD).

The paper presented here is a translational sub-project of an RCT which compares oxygenated vs. standard cold machine perfusion in kidney transplantation. The results of the main trial, the COMPARE study, were published more than two years ago. While mean eGFR was not significantly different between the groups, the incidence of severe complications and graft failure was significantly lower in the group of kidneys that underwent continuous oxygenated machine perfusion.

The strength of the paper presented today is that, as a translational study, the authors were able to match extensive laboratory analyses with well-documented clinical data from a large international, multicenter RCT. One of the weaknesses of the study is that it was limited to older donors whose kidneys were retrieved after circulatory death (DCD); in other words, the results may not be easily extrapolated to brain dead donors (DBD) or younger kidneys.

Some questions remain: First, in the COMPARE study, which had graft function as a primary endpoint, as measured by estimated glomerular filtration (eGFR), 23 kidney pairs had been excluded from primary analysis because of kidney failure or patient death. Were the perfusates of these kidney pairs analyzed in the study presented today or were they excluded? Second, could the analysis of the perfusate proteomes help to identify kidneys that never function after transplantation, thus sparing these often-multi-morbid recipients the risk of an – in the end – futile procedure?

Finally, having identified perfusate proteomes predicting an increased risk of rejection, could you envisage tailored immunosuppression for those grafts and recipients with the respective proteosome markers?

Response From Maria Kaisar (Oxford, United Kingdom)

Thank you for your insightful comments. I would like to thank you for highlighting the strength of our study, which is that perfusate proteomes can convey meaningful biological data. Let me take a closer look at the point you raised, regarding whether the findings of this DCD study can be extrapolated to DBD or younger donors. I agree that our observations on biological mechanisms and pathways may not extrapolate to DBD or younger donors; our group has demonstrated the differences in injury mode between DBD and DCD, as have

others. Also, we think that younger donors may have a higher metabolic resilience, and for this reason, they might be less affected by the challenges of ischemia or other injuries during donor management. However, as you mentioned, the study does link to the COMPARE trial, which showed a significant clinical benefit of oxygenated hypothermic machine perfusion for 1-year GFR and graft survival in older DCD donors, which is the fastest growing source of deceased organs in transplantation. Undoubtedly, the ideal situation would have been to compare both older and younger DBD and DCD. Even if we wanted to repeat this analysis, the perfusate cohorts are not currently available from these groups; however, we agree that this is an important follow-up analysis.

I will now move on to answer your specific questions. First, I can confirm that there was no exclusion of kidney pairs from the selection of samples. There were only two criteria for sample exclusion: (1) if perfusate samples had not been collected due to logistical challenges; (2) if the protein levels in the perfusate were below detection level.

Second, our study indicates that perfusate proteomes provide biologically meaningful information related to perfused kidneys. Additionally, our current research on pre-transplant biopsies that were obtained from the UK-wide Quality in Organ Donation (QUOD) biobank reveal associations between tissue protein profiles and primary non-function, and short- and long-term transplant outcomes. To this end, when we combine pre-transplant proteomic data and data from the perfusate proteomes, we think that we could improve the prediction of post-transplant function.

Finally, understanding how the identified proteomes associate with kidney immunophenotypes could lead to the development of immunomodulation strategies during perfusion and personalized immunosuppression, with the potential to extend graft longevity and improve transplant outcomes.