OPEN

Perfusate Metabolomics Content and Expression of Tubular Transporters During Human Kidney Graft Preservation by Hypothermic Machine Perfusion

Quentin Faucher, PhD,[1] Hugo Alarcan, PharmD,[1,2] François-Ludovic Sauvage, MSc,[1] Lionel Forestier, MSc,[3] Elodie Miquelestorena-Standley, MD,[4,5] Lydie Nadal-Desbarats, PhD,[6] Hélène Arnion, MSc,[1] Jean-Christophe Venhard, MD,[7] Nicolas Brichart, MD,[8] Franck Bruyère, MD, PhD,[9] Pierre Marquet, MD, PhD,[1,10] and Chantal Barin-Le Guellec, PharmD, PhD[1,2]

Background. Ischemia-related injury during the preimplantation period impacts kidney graft outcome. Evaluating these lesions by a noninvasive approach before transplantation could help us to understand graft injury mechanisms and identify potential biomarkers predictive of graft outcomes. This study aims to determine the metabolomic content of graft perfusion fluids and its dependence on preservation time and to explore whether tubular transporters are possibly involved in metabolomics variations. **Methods.** Kidneys were stored on hypothermic perfusion machines. We evaluated the metabolomic profiles of perfusion fluids (n = 35) using liquid chromatography coupled with tandem mass spectrometry and studied the transcriptional expression of tubular transporters on preimplantation biopsies (n = 26), both collected at the end of graft perfusion. We used univariate and multivariate analyses to assess the impact of perfusion time on these parameters and their relationship with graft outcome. **Results.** Seventy-two metabolites were found in preservation fluids at the end of perfusion, of which 40% were already present in the native conservation solution. We observed an increase of 23 metabolites with a longer perfusion time and a decrease of 8. The predictive model for time-dependent variation of metabolomics content showed good performance (R^2 = 76%, Q^2 = 54%, accuracy = 41%, and permutation test significant). Perfusion time did not affect the mRNA expression of transporters. We found no correlation between metabolomics and transporters expression. Neither the metabolomics content nor transporter expression was predictive of graft outcome. **Conclusions.** Our results call for further studies, focusing on both intra- and extratissue metabolome, to investigate whether transporter alterations can explain the variations observed in the preimplantation period.

(*Transplantation* 2022;106: 1831–1843).

Received 6 October 2021. Revision received 21 January 2022.

Accepted 10 February 2022.

[1] *P&T UMR1248, Université de Limoges, institut national de la santé et de la recherche médicale (INSERM), Limoges, France.*

[2] *Service de biochimie et biologie moléculaire, CHRU de Tours, Tours, France.*

[3] *BISCEm, US 042 INSERM-UMS 2015 CNRS, Université de Limoges, Limoges, France.*

[4] *Service d'anatomie et cytologie pathologiques, Hôpital Trousseau, CHRU Tours, Tours, France.*

[5] *EA4245, Transplantation, Immunologie, Inflammation, Université de Tours, Tours, France.*

[6] *UMR 1253, iBrain, Université de Tours, INSERM, Tours, France.*

[7] *Coordination hospitalière des prélèvements d'organes et de tissus, CHRU de Tours, Tours, France.*

[8] *Service de chirurgie urologique, CHR Orléans, Orléans, France.*

[9] *Service d'urologie, hôpital Bretonneau, CHRU Tours, Tours, France.*

[10] *Service de pharmacologie, toxicologie et pharmacovigilance, CHRU Limoges, Limoges, France.*

Q.F. and H.Al. contributed equally.

Clinical Trial Notation: Metabolomics in assessing the quality of kidney transplants retained on a Lifeport Perfusion Machine (RENALIFE), ClinicalTrials.gov NCT03024229.

Q.F. and H.Al., participated in research design, data collection, analysis and interpretation, and the writing of the article. F.L.S. participated in data analysis and the writing of the article. L.F., L.N.S., and H.Ar. participated in data analysis and article review. E.M.S. participated in data collection and article review. J.C.V., N.B., and F.B. participated in research design and data collection. P.M. participated in research design, data analysis and interpretation, and article review. C.B.L.G. participated in research design, data collection, analysis and interpretation, and the writing of the article.

The authors declare no conflicts of interest.

This research was funded by Organ Recovery System.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

Correspondence: Chantal Barin-Le Guellec, PharmD, PhD, Service de Biochimie et Biologie Moléculaire, CHU Bretonneau, 2 Blvd, Tonnellé 37044 Tours, France. (chantal.barin-leguellec@univ-tours.fr).

ISSN: 0041-1337/20/1069-1831

DOI: 10.1097/TP.0000000000004129

INTRODUCTION

Kidney transplantation is the treatment of choice for patients having end-stage renal diseases. According to the World Health Organization, the death rate because of kidney diseases will continue to increase to reach 27 deaths per 100 000 people by 2060,[1] whereas worldwide use of renal replacement therapy (ie, dialysis or kidney transplantation) will reach ±5 million people by 2030.[2] With regard to this increase of renal diseases, the gap between the demand for organs and the limited group of donors will continue to widen. To overcome this issue, many centers are gradually accepting suboptimal donors, including donation after circulatory death and extended criteria donors (ECDs); however, kidneys retrieved from such donors are more prone to ischemia-reperfusion injury (IRI) and delayed graft function (DGF) in the posttransplant period.[3] IRI is a multifactorial pathophysiological process incumbent to the transplantation procedure and a major cause of DGF, which in turn increases the risk of short- and long-term poor graft outcomes.[4-7] For these suboptimal donors, hypothermic machine perfusion (HMP), an ex vivo circulating, hypoxic environment, is recommended for kidney preservation, compared with static cold storage (SCS), to reduce DGF rates and improve graft survival[8]; however, reliable tools are needed for the evaluation of graft quality during HMP preservation. Metabolomic analysis of the perfusion fluid provides the possibility not only to reveal potential biomarkers of graft quality or predictive of transplantation outcomes but also to inform about the cellular mechanisms occurring during organ perfusion.[9] The few studies previously conducted on this topic showed a variation of the metabolomic content according to the perfusion time: mainly an increase of lactate and amino acids and a decrease of glutathione.[10-12] These variations suggest that metabolites are taken up or released by the kidney during its preservation. Renal tubular membrane transporters (mainly of the solute carrier and ATP binding-cassette families) play a major role in cell and tissue homeostasis owing to the bidirectional, transcellular exchanges they are involved in. Alteration of their activity, previously demonstrated (mainly for the solute carrier) during ischemia or ischemia/reperfusion,[13,14] could be responsible for some metabolic variations observed during organ perfusion but could also have deleterious consequences for the graft outcomes.[15] Concerning the predictive value of the perfusion fluid metabolomic content, controversial results have been found, and a recent review suggests that further studies are needed.[16]

In this clinical study, we determined the metabolomic contents of perfusion fluids collected at the end of kidney graft perfusion on HMP and the transcriptional expression of renal tubular transporters on graft biopsies taken just before reimplantation. The study had 3 objectives: (1) explore the impact of ischemia duration on the metabolomic content of perfusion liquids and on renal tubular transporters expression; (2) explore the relationships between metabolomic profile and expression of renal tubular transporters during HPM; and (3) find new biomarkers of immediate graft function (IGF) by exploring the relationships between ischemia duration, ex situ metabolites, and expression of transporters on graft outcome. To the best of our knowledge, this is the first evaluation of the potential impact of membrane transporter alterations on the variations of the perfusion fluid metabolomic contents observed during graft preservation by HMP.

MATERIALS AND METHODS

A more detailed description is available in **Supplemental Material and Methods** (**SDC,** http://links.lww.com/TP/C397).

As part of the clinical research project "Metabolomics in Assessing the Quality of Kidney Transplants Retained on a LifePort Perfusion Machine," 38 kidneys taken from ECDs were included. Organs were stored on HMP LifePort Kidney Transporter 1.0 (Organ Recovery Systems) with KPS-1 (Organ Recovery System) used as a preservation solution. Approval was obtained from the Tours University Hospital (IRB ID/CPP: 16-10-40), and this clinical trial was registered with the access number NCT03024229. All patients/participants gave consent, and the appropriate institutional forms were archived. HMP parameters (temperature, flow, resistance) were recorded during kidney conservation, and a perfusate sample was collected at the end of perfusion (storage at –20 °C after centrifugation: 3000*g*, 10 min). Preimplantation biopsy (targeting the renal cortex) was performed and split into 2, with one fragment embedded in paraffin after fixation for anatomopathological evaluation and the other stored at –20 °C. Serum creatinine and clinical events were recorded up to 7 d and 3 mo, respectively. IGF was characterized by a serum creatinine ≤250 μmol/L on day 7 without the need for dialysis and DGF by the necessity of dialysis within the first 7 d. Mass spectrometry chromatographic analysis was performed using an LCMS-8060 tandem mass spectrometer (Shimadzu) and the package "Method Package for Cell Culture Profiling Ver.2" (Shimadzu). Infusion of pure substances was performed to add some molecules to the package. Perfusates were analyzed in duplicate, and native KPS-1 was injected to determine its basal composition. A COBAS 6000 analyzer (Roche Diagnostics) was used to determine sodium, potassium, calcium, phosphate, chloride, bicarbonate, urea, creatinine, and glucose concentrations and to add them to the previous metabolites.

Graft RNA was extracted from frozen preimplantation biopsies. After quantification and integrity evaluation, retrotranscription was performed. TaqMan low-density array cards were used to determine the transcriptional expression of 35 membrane tubular transporters, 3 aquaporins, 2 Na/K-ATPase subunits, and 4 housekeeping gene candidates (*NME4, CHFR, C16ORF62,* and *NASP*) chosen according to the literature[17] (see Table 1 for probe sets and targeted genes). Undetermined or >35 Ct values were replaced by 35. Expression of transporters was analyzed by the comparative $2^{-\Delta Ct}$ method with ΔCt = Ct (target gene) – Ct (mean of the housekeeping genes finally retained).[18] Then $2^{-\Delta Ct}$ was normalized by log2 transformation. Three housekeeping genes (*NME4*, *CHFR,* and *C16ORF62*) were finally selected using Genorm[19] and Normfinder.[20]

To explore the impact of ischemia duration on the perfusate metabolomics contents and tubular transporters expression, grafts were allocated to 3 different perfusion duration groups: <12 h, between 12 and 20 h, and >20 h according to the distribution of patient perfusion durations (**Figure S1, SDC,** http://links.lww.com/TP/C397).

TABLE 1.

Custom-designed TaqMan low-density array card

Assay ID	Amplicon length	Gene symbol(s)	Protein	Family
Hs00537914_m1	68	*SLC22A6*	OAT1	SLC transporter
Hs00198527_m1	69	*SLC22A7*	OAT2	
Hs00188599_m1	144	*SLC22A8*	OAT3	
Hs01056646_m1	76	*SLC22A8*	OAT3	
Hs00945829_m1	82	*SLC22A11*	OAT4	
Hs00427552_m1	79	*SLC22A1*	OCT1	
Hs01010726_m1	70	*SLC22A2*	OCT2	
Hs00268200_m1	76	*SLC22A4*	OCTN1	
Hs00929869_m1	65	*SLC22A5*	OCTN2	
Hs00217320_m1	74	*SLC47A1*	MATE1	
Hs00945652_m1	63	*SLC47A2*	MATE2	
Hs01030727_m1	64	*SLC22A12*	URAT1	
Hs00192639_m1	76	*SLC15A1*	PEPT1	
Hs01113665_m1	69	*SLC15A2*	PEPT2	
Hs00903842_m1	77	*SLC9A3*	NHE3	
Hs00919316_g1	72	*SLC13A2*	NaDC1	
Hs00955744_m1	68	*SLC13A3*	NaDC3	
Hs01092910_m1	84	*SLC34A1*	NaPilla	
Hs02341453_g1	94	*SLC34A3*	NaPillc	
Hs00698884_m1	77	*SLCO4C1*	OATP4C1	
Hs01573793_m1	60	*SLC5A1*	SGLT1	
Hs00894642_m1	75	*SLC5A2*	SGLT2	
Hs00892681_m1	76	*SLC2A1*	GLUT1	
Hs01096908_m1	65	*SLC2A2*	GLUT2	
Hs01047033_m1	68	*SLC4A4*	NBCe1	
Hs01384157_m1	70	*SLC6A19*	B(0)AT1	
Hs00909948_m1	79	*SLC7A7*	y^+LAT1	
Hs00794796_m1	87	*SLC7A8*	LAT2	
Hs00374243_m1	77	*SLC3A2*	4F2hc	
Hs00942976_m1	66	*SLC3A1*	rBAT	
Hs00204638_m1	50	*SLC7A9*	BAT1	
Hs00188172_m1	76	*SLC1A1*	EAAT3	
Hs01028916_m1	96	*AQP1*		Aquaporins
Hs00292214_s1	87	*AQP2*		
Hs00185020_m1	63	*AQP3*		
Hs00933601_m1	76	*ATP1A1*		Na/K-ATPase subunits
Hs00426868_g1	89	*ATP1B1*		
Hs00960489_m1	62	*ABCC2*	MRP2	ABC transporter
Hs00988720_g1	86	*ABCC4*	MRP4	
Hs00988721_m1	141	*ABCC4*	MRP4	
Hs01053790_m1	83	*ABCG2*	BCRP	
Hs00184500_m1	67	*ABCB1*	P-gp	
Hs01561483_m1	65	*ABCC1*	MRP1	
Hs99999905_m1	-	*GAPDH*	GAPDH	Housekeeping genes
Hs00943494_m1	67	*CHFR*	E3 ubiquitin-protein ligase	
Hs00359037_m1	70	*NME4*	Nucleoside diphosphate kinase	
Hs01032748_g1	65	*NASP*	Nuclear autoantigenic sperm protein	
Hs00220422_m1	82	*C16ORF62*	UPF0472 protein	

ABC, ATP binding-cassette; OAT, Organic Anion Transporter; SGLT, Sodium Glucose Transporter; SLC, solute carrier.

For all association studies, we used a 2-steps approach with univariate analysis followed by multivariate analysis to assess a combination of potential biomarkers rather than each independently. Multivariate analysis was started with principal component analysis, an unsupervised method used to observe clustering patterns, trends, and outliers. Next, supervised methods like partial least square (PLS), PLS-discriminant analysis (PLS-DA), or random forest (RF), a tree-based machine-learning algorithm adapted to small-sized data sets, were used in standard manners[21]

to evaluate the discriminative power of the biomarker combination between perfusion duration groups. These methods can be used when the number of observations is less than that of the explanatory variables, which was the case here, and when the latter are correlated between them. With PLS-DA, a variable influence on projection (VIP) value was estimated for each variable, and features with VIP >0.8 were considered important to the model. The MetaboAnalyst 5.0 computational platform (www.metaboanalyst.ca/faces/home.xhtml) was used for all the analyses except for PLS, which was performed using the MixOmics package (version 1.6.3) in R (version 4.0.2).

RESULTS

Study Population

Thirty-eight donor–recipients couples were included (Table 2). All donors were brain dead and ECDs. The median perfusion time and cold ischemia time were 831.5 and 1020 min, respectively. Graft function postimplantation was defined as either DGF, characterized by the requirement for dialysis in the first 7 d postimplantation, or IGF, defined by the absence of DGF.

Perfusate Metabolomic Content and Transcriptional Expression of Tubular Transporters

Metabolomic Content of Graft Perfusion Fluid

In the 35 perfusion fluids available, 72 different metabolites were identified, 66 with liquid chromatography–tandem mass spectrometry (**Table S1, SDC,** http://links.lww.com/TP/C397) and 6 with COBAS analyzer. All of them were present in each sample, with a few exceptions (Table 3). Twenty-nine of them were already present in the native KPS-1. At the end of perfusion, 6 were increased (inosine, guanosine, xanthosine, 5-methyl adenosine, tryptophan, and riboflavin) and 10 were decreased (gluconic acid, methionine sulfoxide, glucosamine, oxidized glutathione, adenine, 2-ketoisovaleric acid, 3-methyl-2-oxovaleric acid, D-gluconic acid sodium salt, D-ribose, and deoxycytidine monophosphate) compared with native KPS-1. Forty-three metabolites were exclusively found in the graft perfusates. These metabolites belong mainly to the amino acid metabolism pathways (**Figure S2, SDC,** http://links.lww.com/TP/C397).

Transcriptional Expression of Tubular Transporters in Preimplantation Biopsies

Thirty-four biopsies were available for RNA extraction. Eight were excluded: 2 because of RNA yield and 6 because they contained renal medulla. The mean RNA integrity number (RIN) value for the 26 biopsies retained was 5.4 ± 2.4. All the transporters of interest (Table 1) were identified in these biopsies. We found high expression correlations between them (**Figure S3, SDC,** http://links.lww.com/TP/C397).

Impact of Perfusion Duration on Metabolomic Profiles and Transporter Expression

Metabolomics Variations According to Perfusion Duration

Univariate analysis showed that 31 features were significantly (false discovery rate, <0.05) different between the perfusion duration groups (Figures 1 and 2). Twenty-three metabolites increased with longer perfusion durations (eg, lactate or leucine), but 8 decreased (eg, glutathione or inosine). Unsupervised principal component analysis showed good separation of the scores between the 2 extreme groups (<12 and >20 h), whereas group 2 overlapped with the others. Fifty-two percent of the variation was explained by the first and second components (Figure 3A). Similarly, the PLS-DA scores plot (first 2 components) showed complete separation between groups 1 and 3 but overlap with group 2 (Figure 3B). Cross-validation showed good performances: goodness-of-fit (R^2) = 76%, predictive cumulative variation (Q^2) = 54%, and accuracy = 41%. The model was significant according to the permutation test ($P < 0.05$), supporting the absence of overfitting. The most important metabolites (top 15) for the model are shown in Figure 3C. Significant pathways based on the most important metabolites (VIP >0.8) are listed in **Figure S4 (SDC,** http://links.lww.com/TP/C397), among others: glutamate, glutathione, glycine, serine and alanine metabolism, urea cycle, ammonium recycling, and purine metabolism.

Transporter Expression According to Perfusion Duration

According to the perfusion duration, the transcriptional expression of transporters was not significantly different in univariate analysis. The PLS-DA model performance was: R^2 = 34%, negative Q^2, and accuracy = 30%, and the permutation test was not significant (Figure 4).

Correlation Between Transporters Expression and Metabolomic Content

Correlation between transporters expression and metabolomic content was investigated by PLS regression. The clustered image maps of the model are displayed in Figure 5. It does not show patterns of correlation between metabolites and transporters. The maximal positive and negative correlations were weak: 0.54 and –0.54, respectively.

Predictive Biomarkers of Graft Outcome

We did not find significant differences between graft recipients with IGF (n = 33) or DGF (n = 5) regarding the clinical variables (even though all patients with DGF received a graft from a female donor and had a tendency to be older), machine parameters recorded (temperature, resistive index, flow rate), cold ischemia time, or biopsy characteristics (Table 2). In terms of outcomes, all DGF patients were dialyzed before transplantation, and all had received their first graft (Table 2).

Metabolomics in Assessing the Outcome of Kidney Grafts

Univariate analysis showed that 4-hydroxyproline (fold change: 0.28, raw *P* value: 0.04) and taurine (fold change: 0.61, raw *P* value: 0.04) tended to be lower in perfusion fluids of DGF patients (Figure 6A). PLS-DA displayed modest separation between IGF and DGF on the score plot of the first 2 components (Figure 6B) with good accuracy (87%) and R^2 (0.41) but a negative Q^2. The permutation test was not significant. With RF analysis, the out-of bag error (samples wrongly predicted) was 17% for the overall data set, but 100% of DGF samples were predicted as IGF, showing that the model was unable to predict graft recovery (Figure 6C).

TABLE 2.

Characteristics of kidney graft donors, recipients, and storage conditions

	Overall (N = 38)	IGF (n = 33)	DGF (n = 5)	*P*	Unknown, N (%)
Donor					
Age (y)	67.5 (61.75–75)	66 (61–73)	79 (68–82)	0.054	
Sex, N (%)					
F	21 (55.3)	16 (48.5)	5 (100)	0.053	
M	17 (44.8)	17 (51.5)	0 (0)		
Donation after brain death, N (%)	38 (100)	33 (100)	5 (100)	1	
Expanded criteria donors, N (%)	38 (100)	33 (100)	5 (100)	1	
Recipient					
Age (y)	65.5 (57–72)	65 (57–69)	72 (65–72)	0.27	
Sex, N (%)					
F	13 (34.2)	12 (36.4)	1 (20)		
M	25 (65.8)	21 (63.6)	4 (80)		
Ethnicity, N (%)					1 (2.6)
Caucasian	30 (78.9)	26 (78.8)	4 (80)		
Afro-American	2 (5.3)	1 (3)	1 (20)		
Other	4 (10.5)	5 (15.2)			
Renal disease, N (%)					
Diabetes	9 (23.7)	6 (18.2)	3 (60)		
Hypertension	14 (36.8)	11 (33)	3 (60)		
Glomerulopathy	2 (5.3)	1(3)	1 (20)		
Tubulointerstitial nephropathy	3 (7.9)	2 (6.1)	1 (20)		
Polycystic kidney disease	6 (15.8)	4 (12.1)	1 (20)		
Other	16 (42.1)	15 (45.5)	1 (20)		
No. of transplantation, N (%)				1	
1	35 (92.1)	30 (90.9)	5 (100)		
>1	3 (7.9)	3 (9.1)	0 (0)		
Dialysis before transplantation, N (%)	34 (89.5)	29 (87.9)	5 (100)	1	
Induction therapy, N (%)				0.3	
Thymoglobulin	11 (28.9)	11 (33.3)	0 (0)		
IL-2R antibody	27 (71.0)	22 (66.7)	5 (100)		
Maintenance therapy, N (%)				1	
MMF–corticosteroids–CNI	32 (84.2)	27 (81.8)	5 (100)		
MMF–corticosteroids	3 (7.9)	3 (9.1)	0 (0)		
Corticosteroids–CNI–imTOR	2 (5.3)	2 (6.1)	0 (0)		
Corticosteroids–CNI	1 (2.6)	1 (3)	0 (0)		
Death, N (%)	1 (2.6)	1 (3)	0 (0)	1	
Banff classification (preimplantation biopsies), N (%)				0.43	10 (26.3)
Normal	17 (44.7)	13 (39.4)	4 (80)		
Acute tubular injury	10 (26.3)	10 (30.3)			
Interstitial fibrosis/tubular atrophy	1 (2.6)	1 (3)			
Graft rejection, N (%)					
Total	2 (5.3)	2 (6.1)	0 (0)		
Antibody-mediated rejection	2	2	0		
T cell–mediated rejection	0	0	0		
Storage conditions, N (%)					
Perfusion duration (min)	831.5 (622.75–1031.25)	844 (625–1065)	654 (646–920)	0.60	
Cold ischemia duration (min)[b]	1020 (810–1231.5)	1020 (840–1260)	810 (810–1065)	0.34	
Initial temperature (°C)	2.8 (2.3–5.8)	2.8 (2.4–5.4)	2.8 (2.1–3.4)	0.91	3 (7.9)
End temperature (°C)	2.6 (2.2–4.2)	2.75 (2.3–4)	2.2 (2–2.5)	0.26	3 (7.9)
Decrease temperature (°C)	0.1 (–0.4 to 1.3)	0.00 (–0.37 to 1.3)	0.8 (–0.4 to 1.5)	0.6	3 (7.9)
Initial Resistance (mm Hg/mL/min)	0.45 (0.3–0.8)	0.5 (0.3–0.8)	0.4 (0.2–0.9)	0.84	2 (6.4)
End resistance (mm Hg/L/min)	0.2 (0.1–0.2)	0.2 (0.12–0.2)	0.2 (0.2–0.2)	0.84	3 (7.9)
Decrease resistance (mm Hg/mL/min)	0.2 (0.1–0.6)	0.15 (0.1–0.6)	0.20 (0.1–0.6)	0.91	3 (7.9)
Initial flow (mL/min)	60 (31–91)	57.5 (33.5–85.8)	60 (29–109)	0.94	3 (7.9)
End flow (mL/min)	111 (82–136)	113.5 (86–136)	94 (91–111)	0.47	3 (7.9)
Increase flow (mL/min)	43 (4–82)	49 (4.25– 81.25)	34.00 (31–82)	0.83	3 (7.9)

Data reported as median (quartiles) or N (%), with *P* value from Fisher exact tests or Wilcoxon tests, as appropriate.

[a]Significant at $P < 0.05$.

[b]Corresponds to the perfusion duration as well as all the periods of graft ischemia between procuring and HMP perfusion and between HMP perfusion and reimplantation.

CNI, calcineurin inhibitor; DGF, delayed graft function; HMP, hypothermic machine perfusion; IFG, immediate graft function; IL-2R, interleukin-2 receptor; imTOR, mammalian target of rapamycin inhibitor; MMF, mycophenolate mofetil.

TABLE 3.

Metabolomic contents of pure commercial KPS-1 and of perfusates at the end of machine perfusion

Detected in KPS-1 at baseline and at the end of the perfusion			
Increased	**Unchanged**	**Decreased**	**Not detected at baseline**
Inosine	Sodium[a]	Gluconic acid[a]	*O*-Phosphoethanolamine
Guanosine	Potassium[a]	Methionine sulfoxide	Cystine
Xanthosine	Calcium[a]	Glucosamine	Aspartic acid
Tryptophan	Phosphates	Oxidized glutathione	Serine
Riboflavin	Glucose[a]	Adenine[a]	4-Hydroxyproline
5-Methylthioadenosine	Glutathione[a]	2-Ketoisovaleric acid	Cystathionine
	Hexose[a]	3-Methyl-2-oxovaleric acid	Glycine
	Pipecolic acid	D-gluconic acid sodium salt[a]	Threonine
	Thymine	D-ribose[a]	Glutamic acid
	Thymidine	Deoxycytidine monophosphate	Alanine
	Biotin		Ornithine
	D-Mannitol		Proline
	4-Pyridoxic acid		2-Aminoethanol
			Lysine
			Histidine
			Lactic acid
			Arginine
			Uracil
			Uric acid
			Choline
			5-Glutamylcysteine
			Xanthine
			Hypoxanthine
			Valine
			Uridine
			Methionine
			Niacinamide
			Tyrosine
			Adenosine
			Pyridoxal[b]
			4-Aminobenzoic acid[c]
			Isoleucine
			Leucine
			Phenylalanine
			Kynurenine[c]
			Alpha-keto-glutarate
			Creatinine
			L-Carnitine
			PAH[c]
			Taurine
			1-Methylhistidine
			Anthranilic acid[c]
			Urea

[a]Listed as constituents of KPS-1.
[b]Present only in a few patients.
[c]Less than limit of quantification in a few patients.
PAH, para-aminohippuric acid.

Transcriptional Expression Extraction of Transporters According to Graft Outcome

Neither univariate nor multivariate analysis (PLS-DA and RF) revealed any discriminative transporter mRNA between IGF and DGF patients (**Figure S5, SDC,** http://links.lww.com/TP/C397).

DISCUSSION

This clinical study aimed to better characterize the metabolic variations occurring during organ preservation on HMP through metabolic profiling of the fluid collected at the end of perfusion. We observed marked modifications of the metabolomic content, with regard

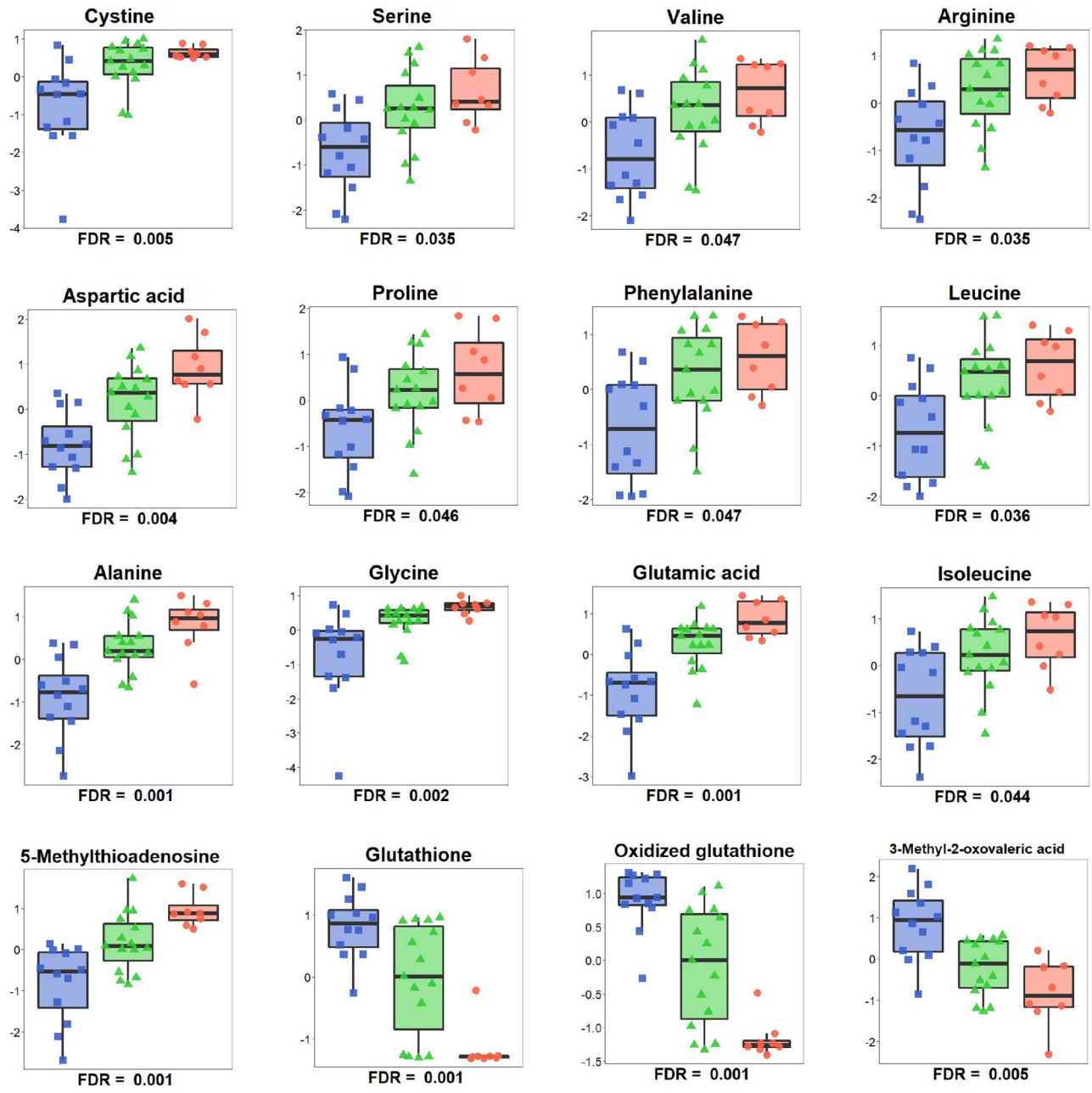


FIGURE 1. Amino acids and related compounds with significant variations according to perfusion duration (univariate analyses). Comparisons were made using the Kruskal-Wallis tests and adjusted for multiple testing by the FDR method. Blue box: perfusion duration <12 h, green box: perfusion duration between 12 and 20 h, and red box: perfusion duration >20 h. FDR, false discovery rate.

to the native preservation fluid (KPS-1), and also as a function of perfusion duration, which parallels the cold ischemia time duration. We also investigated the transcriptional expression of tubular transporters to explore whether variations in their activity could be linked with metabolic variations observed during machine perfusion; however, we did not observe any particular pattern between the expression of any transporter and the metabolic profiles or perfusion duration. Finally, we evaluated the predictive ability of perfusate metabolites and tubular transporters mRNA expression as potential biomarkers of graft function by comparing IGF and DGF patients and found none.

Maintained Metabolic Activity of Grafts Stored on Perfusion Machine

We determined the relative concentrations of 72 metabolites, some of which, to our knowledge, had never been studied in this context. We observed a marked difference in metabolomic profiles between the perfusate collected at the end of perfusion and the native fluid KPS-1 (Table 2). We found 29 metabolites in KPS-1, which is more than its theoretical composition. Among these, some showed decreased quantities at the end of perfusion, suggesting that they were reabsorbed or consumed by the kidney, whereas others increased (Table 2). The 43 other metabolites, mainly amino acids (Figure S2, SDC,

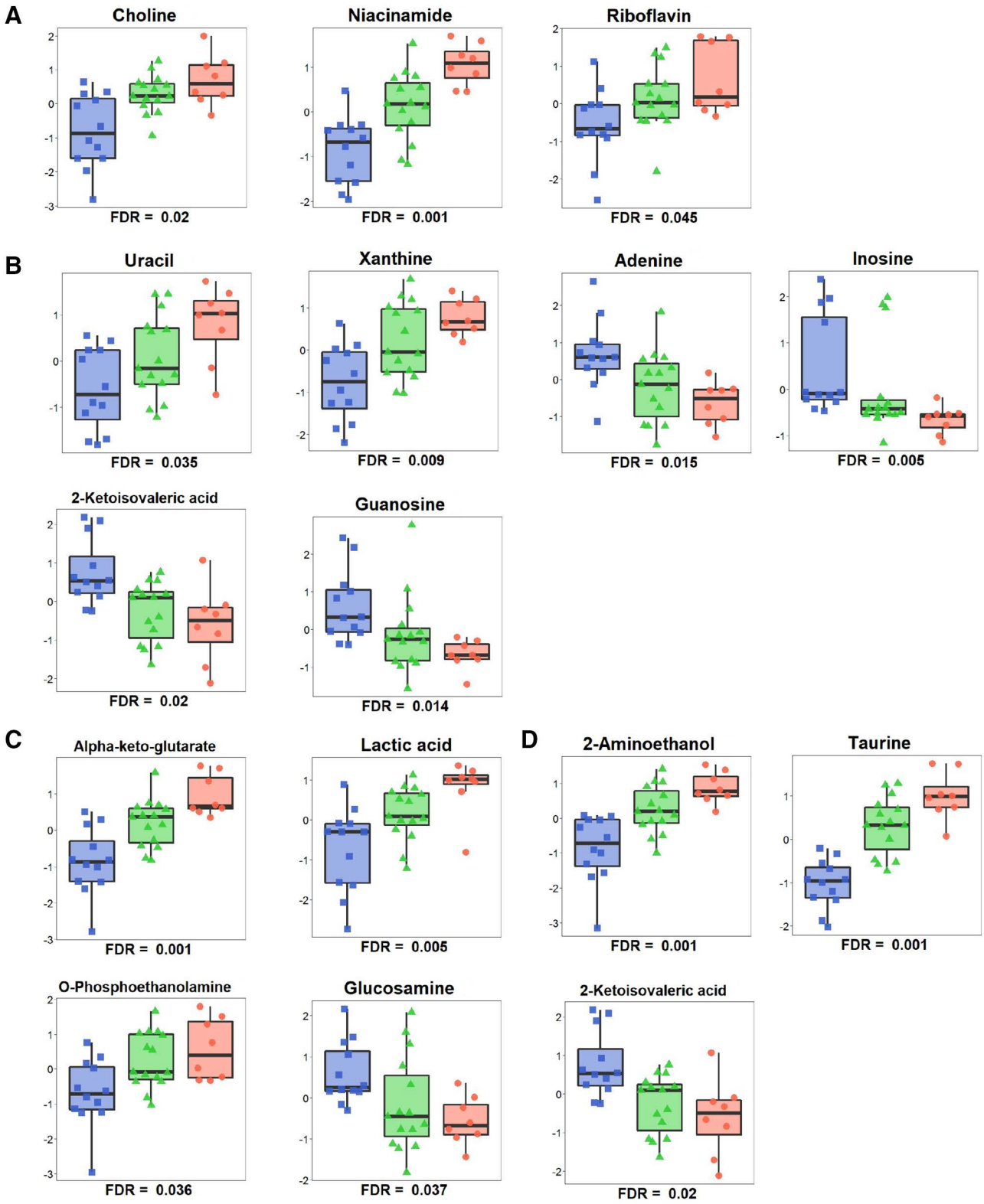


FIGURE 2. Other metabolites with significant variations according to perfusion duration (univariate analyses). Comparisons were made using the Kruskal-Wallis tests and adjusted for multiple testing by the FDR method. Blue box: perfusion duration <12 h, green box: perfusion duration between 12 and 20 h, and red box: perfusion duration >20 h. Box plots are grouped according to compound classes: (A) vitamins, (B) nucleic acids and their metabolites, (C) TCA Cycle and lactate, and (D) others. FDR, false discovery rate; TCA, trichloroacetic acid.

http://links.lww.com/TP/C397), were exclusively detected in the fluid at the end of perfusion. Metabolites that appeared, or increased from basal value, were thus released by the graft, which can be related to sustained metabolic activity, ischemic damages, or both. Guy et al[11] previously observed marked differences in the metabolic profiles of perfusates from human kidneys stored on HMP compared with the theoretical constituents of commercial KPS-1, but they did

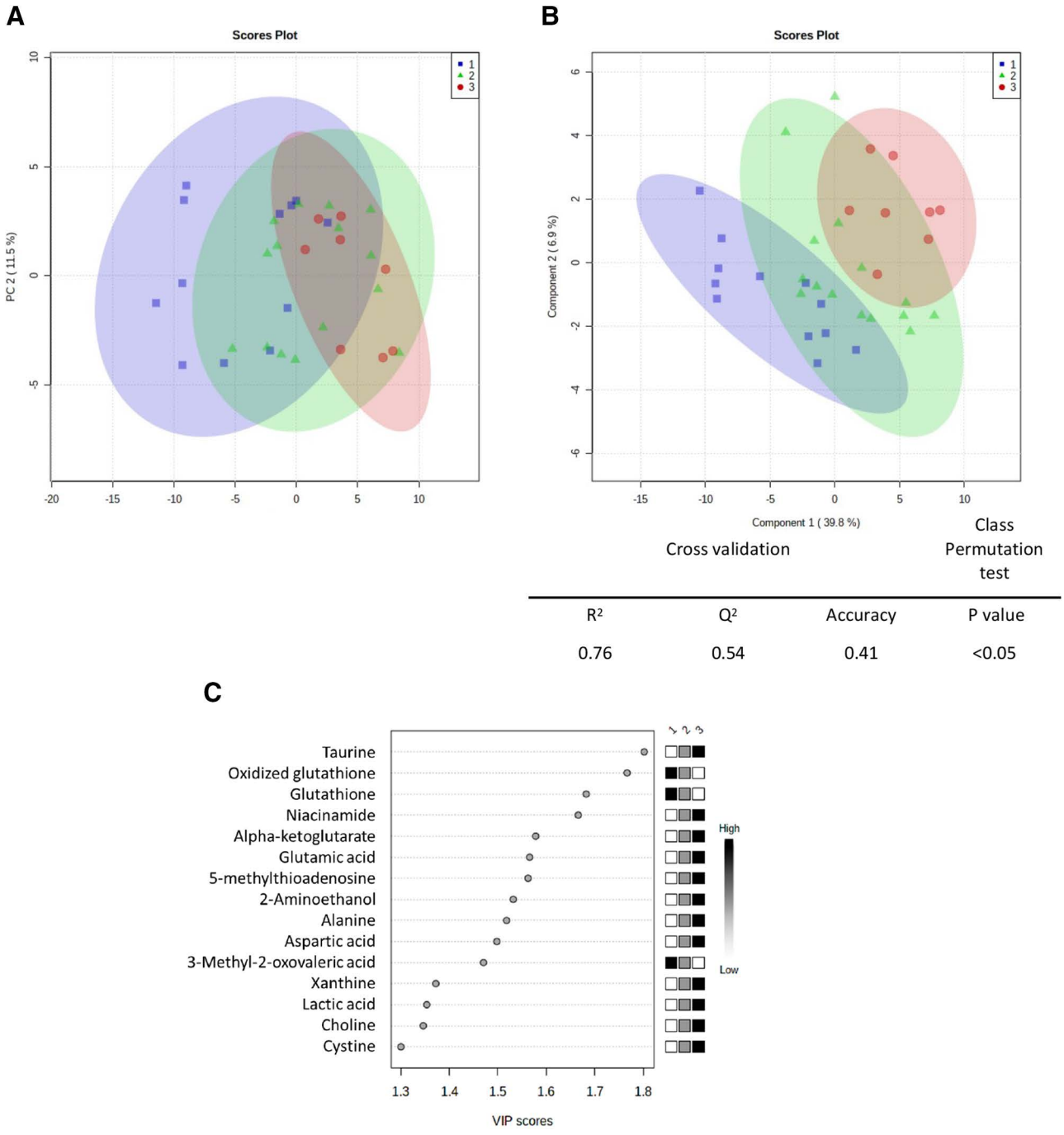
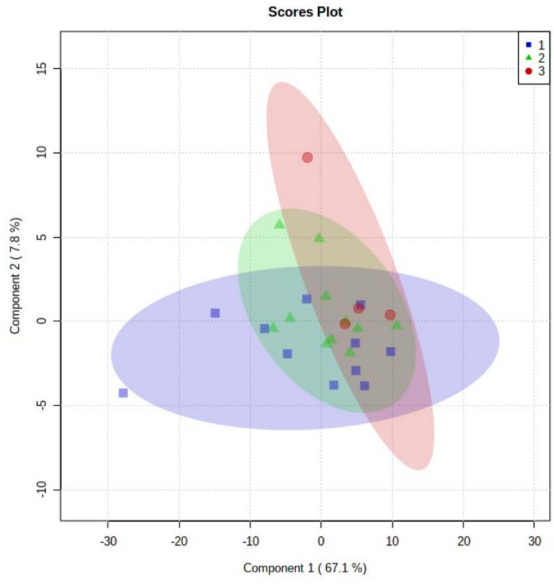


FIGURE 3. Multivariate analysis showing variations of metabolomic profiles according to perfusion duration. Group 1 (blue squares): perfusion duration <12 h, group 2 (green triangles): perfusion duration between 12 and 20 h, and group 3 (red dots): perfusion duration >20 h. A, PCA scores plot showing separation between groups 1 and 3 but not with group 2. PC1 described 40% of the variation, and PC2 described 11.5% of the variation. B, PLS-DA scores plot showing good separation between the 3 groups. C, The 15 most important features based on the VIP values derived from PLS-DA (a total of 40 metabolites had VIP >0.8); boxes on the right indicate the way of variation according to the different groups. PC, principal component; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis; VIP, variable influence on projection.

not analyze pure KPS-1, which hampers direct comparison with our results. The metabolomics variations that we observed suggest that the grafts maintained a high level of exchange with their environment during machine perfusion.

Modifications Occur During Preservation on HMP

Understanding ischemic phenomena occurring during graft preservation remains a leading issue in renal transplantation for the improvement of graft management; however, our understanding of the metabolic activity occurring during kidney preservation on HMP is still partial. Only a few studies have analyzed kidney graft in situ or perfusate metabolomics.[10-12,22] They aimed to identify early predictive biomarkers of graft function and investigate the underlying mechanisms. Our work attempted to consolidate and complement previous studies by (1) the use of liquid chromatography–tandem mass spectrometry instead of nuclear magnetic resonance,[10-12] (2) the



Cross validation			Class Permutation test
R^2	Q^2	Accuracy	P value
0.34	-0,39	0.30	0.95

FIGURE 4. Variations of mRNA expression of tubular transporters according to perfusion duration. PLS-DA scores plot showed no separation between the 3 groups. The PLS-DA model yielded poor performance with cross-validation (R^2 = 34%, Q^2 = –39%, and accuracy = 30%) and the permutation test (P = 0.95). Group 1 (blue squares): perfusion duration <12 h, group 2 (green triangles): perfusion duration between 12 and 20 h, and group 3 (red dots): perfusion duration >20 h. PLS-DA, partial least squares-discriminant analysis.

exploration of human kidneys instead of pig[10] or rabbit models,[22] and (3) the exploration of longer perfusion times.[11,12] Among the 72 metabolites analyzed, the concentration of 8 significantly decreased when the perfusion time increased (Figures 1 and 2), including glutathione and oxidized glutathione. This decrease had already been described by ^{1}H-nuclear magnetic resonance analyses of perfusates in both humans and pig.[10-12] Glutathione is involved in free radicals scavenging, and its decrease sensitizes the graft to IRI. We also found an increased level of lactic acid, choline, and some amino acids (eg, valine, alanine, glycine, and glutamic acid) because of longer perfusion. These observations are consistent with previous results obtained by Bon et al,[10] who reported an increase in concentrations of these metabolites in porcine kidney perfusates between 2- and 22-h perfusion. Increased levels of lactic acid simply reflected anaerobic glycolysis occurring in a hypoxic environment, and increased levels of amino acids reflected intracellular release. Most of our findings are consistent with those previously published, except for some. For example, in the study by Guy et al, the level of glucose and inosine increased with perfusion time, whereas it was unchanged and decreased in ours; however, Guy et al explored 2 early time points, 45 min versus 4 h, whereas the first quartile of our perfusion duration was already about 10 h. The usefulness of following the kinetics of metabolites has been demonstrated recently, using solid-phase microextraction and liquid chromatography–mass spectrometryfor in situ kidney metabolomics analysis at 5 sequential time points in a rabbit model: immediately after removal of the donor's kidneys and after 2, 4, 6, and 21 h of SCS.[22] The authors found that metabolites related to various metabolic pathways, including amino acids and purine metabolism, significantly increased during the first hours of kidney preservation, whereas a decrease occurred with longer perfusion durations; however, their methodology (in situ, limited number of samples, SCS conservation) hampers the direct comparison with our results. Nevertheless, we also observed at the extratissue level that the amounts of some intermediates of purine metabolism such as adenine, inosine, and guanosine decreased, whereas that of xanthine increased, with perfusion time. The results observed here most likely illustrate that kidneys with longer ischemia consume more adenine and inosine and produce more xanthine in response to ATP deprivation. To the best of our knowledge, other metabolites (eg, taurine, niacinamide, glucosamine) and their association with perfusion duration had never been studied in this context.

Weak Correlation Between Metabolites in the Perfusate and Tubular Transporters

The metabolomic signatures observed during perfusion time reflect sustained metabolic activities by kidneys stored on HMP, but the underlying mechanisms are currently poorly understood. Some metabolites are physiologically reabsorbed or secreted through specific membrane tubular transporters.[15] Their sensitivities to ischemia[15,23] and potentially altered activity could explain a part of the ex situ metabolomic variations observed. A decrease at the mRNA and protein levels of *SLC22A6* (Organic Anion Transporter 1 [OAT1]) and *SLC22A8* (OAT3), involved in the uptake of organic anions, was observed after 30 min of ischemia in the rat,[13] and similar results were found for *SLC22A1* (OCT1) and *SLC22A2* (OCT2), involved in organic cation uptake, also in a rat model.[14] Considering the limited quantity and quality of RNA in our samples, we chose the TaqMan low-density array technique for the determination of transcriptional expression because it only requires small quantities of RNA and is able to amplify small cDNA fragments (ie, compatible with some RNA degradation). RIN values were modest, as it sometimes happens with RNA extracted from clinical samples (here, half kidney biopsies). Moreover, the RIN was not associated with storage duration in our cohort. We successfully determined the transcriptional expression of all targeted genes; however, no association was observed between transporter expression and perfusion duration (Figure 3). Previous works suggest that the downregulation of membrane transporters occurs only after 30 min of warm ischemia.[23] In our study, some transporter alterations may have occurred before the biopsy, which was performed after the warm ischemia period and after at least 176 min of perfusion. We might have confirmed this hypothesis with nonischemic biopsy controls, but we could not obtain any. It is also possible that some transporters are not affected by ischemia during preservation by HMP.

We did not find any particular correlation with the metabolomic profile in the perfusion fluid, even for well-known transporter/substrate couples (eg, Sodium Glucose Transporter 2/glucose or OAT1/para-aminohippuric acid) and amino acid transport systems (Figure 4). In HMP, the

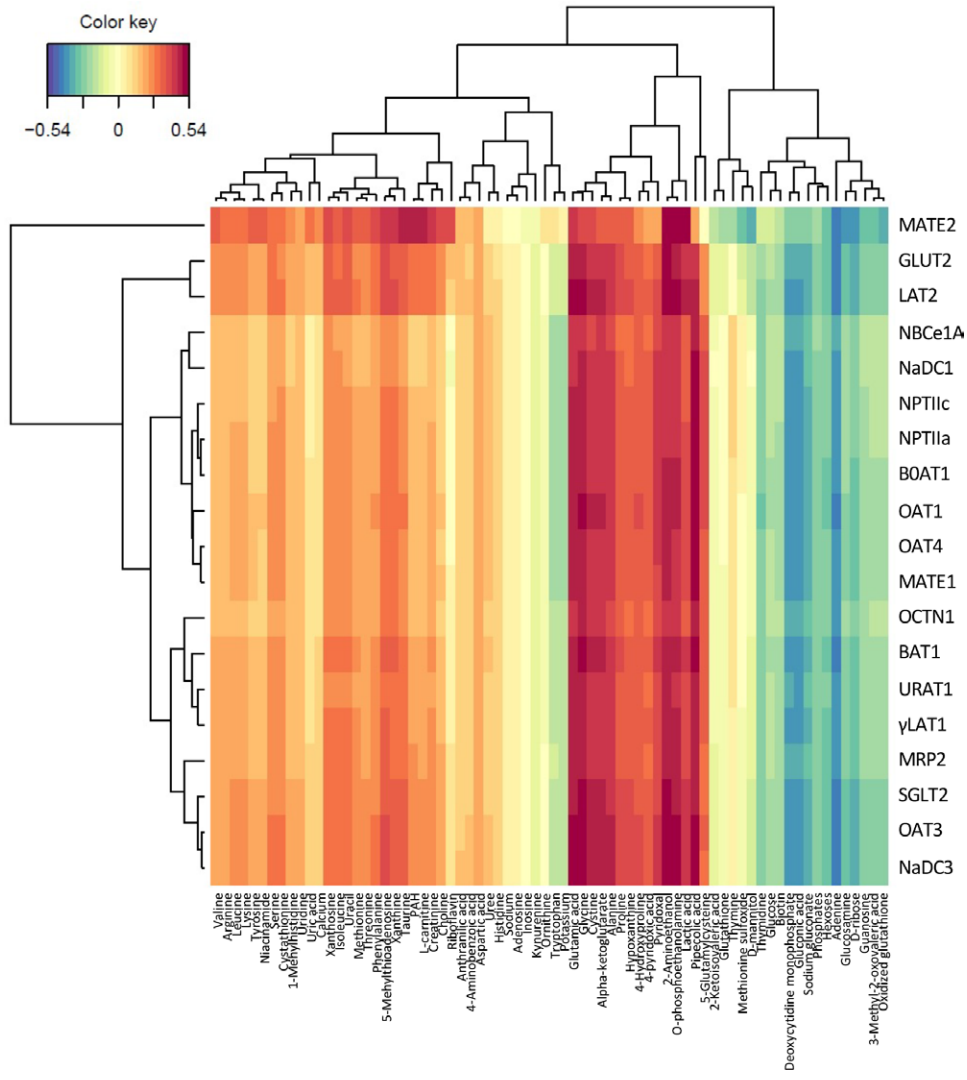
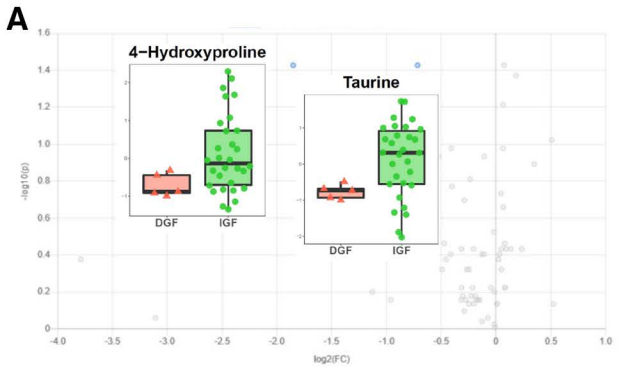


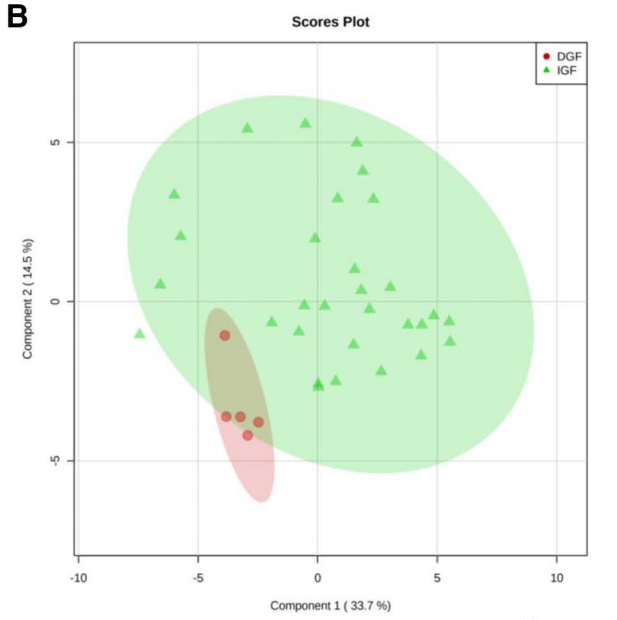
FIGURE 5. CIMs of the relationships between transporters and metabolites (sPLS method). The red and blue colors indicate positive and negative correlations, respectively, whereas the yellow color indicates no significant correlation. CIM, clustered image map; sPLS, partial least squares.

perfusion fluid irrigates both the basolateral and apical poles of the tubule, whereas the function of transporters and thus the identification of variations reflecting their activity are highly dependent on a polarized environment. Moreover, the transcriptional expression of transporters alone cannot perfectly reflect their real activity, but we could not evaluate their protein expression (because of too low amounts of proteins) or cellular localization (because of histological fixation). Globally, these results call for further studies, evaluating both the intra- and extratissue metabolome in relation to the expression, localization, and functions of transporters (eg, based on transporter-specific labeled substrates) as a function of ischemia time, to uncover potential relationships between transporters and metabolites during kidney graft perfusion; however, it is worth mentioning that such studies may still be confounded by injury-induced cellular release of metabolites.

Metabolomic Signatures and Expression of Tubular Transporters Do Not Predict IGF

The present study also aimed to identify noninvasive biomarkers predictive of graft outcomes that could be measured in the preimplantation period. Reliable biomarkers would be instrumental to optimize patient management and graft outcome. Assessment of graft quality in the transplantation period is currently based on perfusion parameters, such as the restrictive index or the flow rate, and pathological evaluation of a preimplantation biopsy; however, these indicators are not sufficiently reliable to safely discard a graft. Accordingly, in our study, no indicators predicted posttransplant graft function (data not shown). Rapid determination of the perfusate metabolomic profiles in the preimplantation period would be convenient and adapted to clinical routine. The proof of concept was brought by Bon et al[10] in a porcine renal autotransplantation model, in which valine, alanine, glutathione, and glutamate concentrations in the perfusion fluid were found to be correlated with serum creatinine at 3 mo. Guy et al[11] also found in a human cohort that glucose and inosine concentrations were lower in the perfusate of DGF kidneys, whereas leucine was higher. In our study, we did not identify any metabolite or multivariate model predictive of graft recovery (Figure 5). We did not replicate previous results, maybe because of the





Cross validation			Class Permutation test
R^2	Q^2	Accuracy	P value
0.41	-0.28	0.87	0.5

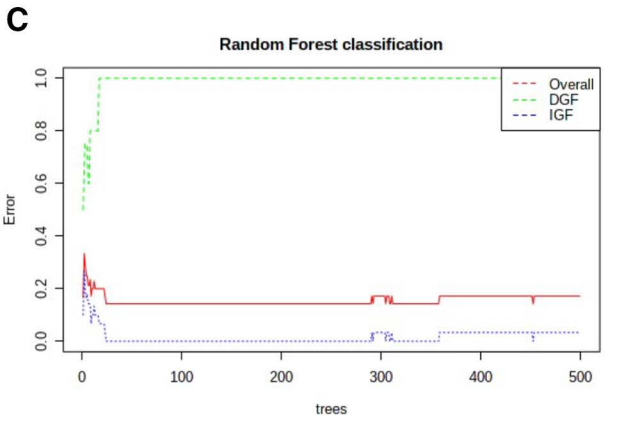


FIGURE 6. Prediction of DGF based on metabolomic profiles. A, Univariate analyses: The volcano plot shows a decreased tendency for 4-hydroxyproline and taurine in the DGF (red boxes) vs IGF (green boxes) groups (FC threshold of 1.2 and raw *P* value of 0.05). B, Multivariate analysis: The PLS-DA scores plot shows incomplete separation between groups with correct accuracy (87%) and R^2 (0.41) but poor predictability (Q^2=−0.28). The permutation test was not significant (*P*=0.5). C, RF classification showing the error rate for the overall data set and for each class. DGF, delayed graft function; FC, fold change; IGF, immediate graft function; PLS-DA, partial least squares-discriminant analysis; RF, random forest.

differences of perfusion durations explored and given that in the study by Guy et al, cadaveric kidneys arrived at their unit on SCS before being transferred to HMP, whereas in our study, the kidneys retrieved from each donor were put directly on HMP. Moreover, the metabolites identified previously were not the same between the 2 studies.

The relative heterogeneity and the small size of our cohort probably limited the predictive ability of the metabolome. Also, the low DGF rate (13.2%) of kidneys of ECDs preserved with the LifePort Kidney Transporter in our cohort (as compared with a DGF rate of 30% for SCS in France in 2017[24]) supported the utility of HMP for such donors but limited the statistical power of our study; however, our negative result is in line with a recent systematic review that highlighted the lack of accuracy and hindsight of metabolomics in human kidney graft perfusates.[16] The evaluation of perfusate metabolomics variations might still be relevant[25] because it recently suggested a higher de novo metabolic activity of kidneys preserved on machine versus SCS[26] or it could highlight the metabolic variations occurring with oxygen supplementation[27,28] or pharmacological agents.[29,30]

Finally, we aimed to determine whether the expression of tubular transporters in our cohort could predict early posttransplant graft outcomes. To the best of our knowledge, no study has yet been conducted in this regard. Our results do not support evaluating transcriptional expression of transporters at the end of kidney graft HMP, with the aim of predicting graft function.

CONCLUSION

In summary, we did not find any predictive biomarkers of graft function in the perfusate metabolome or among tubular transporter mRNAs of human kidneys stored on HMP; however, we observed marked differences between the metabolomics profiles collected at the end of perfusion and the perfusion liquid initial composition, which reflects persistence of metabolic activity during HMP. Moreover, the concentration of many metabolites was modified after the longer perfusions, mostly in agreement with the literature and also for some metabolites that have never been studied in this context to date. The transcriptional expression of 40 membrane transporters determined at the same time was not correlated with the variations of these metabolites or with perfusion time. We suggest conducting further translational studies to evaluate the ratio of tissue-to-perfusion fluid concentrations of metabolites and tubular transporter activity to decipher the deleterious mechanisms associated with ischemia in the preimplantation period.

ACKNOWLEDGMENTS

The authors thank the Nouvelle Aquitaine Region, INSERM, and the Organ Recovery System for their financial support. The authors are grateful to Karen Poole for correcting English and Etienne Broggi for his valuable input during the initiation of the project.

REFERENCES

1. World Health Organization. *Projections of mortality and causes of death, 2016 to 2060*. [No longer available] https://www.who.int/healthinfo/global_burden_disease/projections/en. Accessed May 13, 2021.

2. Liyanage T, Ninomiya T, Jha V, et al. Worldwide access to treatment for end-stage kidney disease: a systematic review. *Lancet*. 2015;385:1975–1982.
3. Wong G, Teixeira-Pinto A, Chapman JR, et al. The impact of total ischemic time, donor age and the pathway of donor death on graft outcomes after deceased donor kidney transplantation. *Transplantation.* 2017;101:1152–1158.
4. Chen CC, Chapman WC, Hanto DW. Ischemia-reperfusion injury in kidney transplantation. *Front Biosci (Elite Ed).* 2015;7:117–134.
5. Gill J, Dong J, Rose C, et al. The risk of allograft failure and the survival benefit of kidney transplantation are complicated by delayed graft function. *Kidney Int.* 2016;89:1331–1336.
6. Nieuwenhuijs-Moeke GJ, Pischke SE, Berger SP, et al. Ischemia and reperfusion injury in kidney transplantation: relevant mechanisms in injury and repair. *J Clin Med.* 2020;9:E253.
7. Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: pathogenesis and treatment. *World J Transplant.* 2015;5:52–67.
8. Peng P, Ding Z, He Y, et al. Hypothermic machine perfusion versus static cold storage in deceased donor kidney transplantation: a systematic review and meta-analysis of randomized controlled trials. *Artif Organs.* 2019;43:478–489.
9. Barin-Le Guellec C, Largeau B, Bon D, et al. Ischemia/reperfusion-associated tubular cells injury in renal transplantation: can metabolomics inform about mechanisms and help identify new therapeutic targets? *Pharmacol Res*. 2018;129:34–43.
10. Bon D, Billault C, Claire B, et al. Analysis of perfusates during hypothermic machine perfusion by NMR spectroscopy: a potential tool for predicting kidney graft outcome. *Transplantation.* 2014;97:810–816.
11. Guy AJ, Nath J, Cobbold M, et al. Metabolomic analysis of perfusate during hypothermic machine perfusion of human cadaveric kidneys. *Transplantation.* 2015;99:754–759.
12. Nath J, Guy A, Smith TB, et al. Metabolomic perfusate analysis during kidney machine perfusion: the pig provides an appropriate model for human studies. *PloS One.* 2014;9:e114818.
13. Matsuzaki T, Watanabe H, Yoshitome K, et al. Downregulation of organic anion transporters in rat kidney under ischemia/reperfusion-induced acute [corrected] renal failure. *Kidney Int.* 2007;71:539–547.
14. Matsuzaki T, Morisaki T, Sugimoto W, et al. Altered pharmacokinetics of cationic drugs caused by down-regulation of renal rat organic cation transporter 2 (Slc22a2) and rat multidrug and toxin extrusion 1 (Slc47a1) in ischemia/reperfusion-induced acute kidney injury. *Drug Metab Dispos.* 2008;36:649–654.
15. Faucher Q, Alarcan H, Marquet P, et al. Effects of ischemia-reperfusion on tubular cell membrane transporters and consequences in kidney transplantation. *J Clin Med.* 2020;9:E2610.
16. Guzzi F, Knight SR, Ploeg RJ, et al. A systematic review to identify whether perfusate biomarkers produced during hypothermic machine perfusion can predict graft outcomes in kidney transplantation. *Transpl Int.* 2020;33:590–602.
17. Wang Z, Lyu Z, Pan L, et al. Defining housekeeping genes suitable for RNA-seq analysis of the human allograft kidney biopsy tissue. *BMC Med Genomics.* 2019;12:86.
18. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. *Nat Protoc.* 2008;3:1101–1108.
19. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol.* 2002;3:RESEARCH0034.
20. Andersen CL, Jensen JL, Ørntoft TF. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res.* 2004;64:5245–5250.
21. Chong J, Wishart DS, Xia J. Using MetaboAnalyst 4.0 for comprehensive and integrative metabolomics data analysis. *Curr Protoc Bioinformatics.* 2019;68:e86.
22. Stryjak I, Warmuzińska N, Bogusiewicz J, et al. Monitoring of the influence of long-term oxidative stress and ischemia on the condition of kidneys using solid-phase microextraction chemical biopsy coupled with liquid chromatography-high-resolution mass spectrometry. *J Sep Sci.* 2020;43:1867–1878.
23. Kwon O, Wang WW, Miller S. Renal organic anion transporter 1 is maldistributed and diminishes in proximal tubule cells but increases in vasculature after ischemia and reperfusion. *Am J Physiol Renal Physiol.* 2008;295:F1807–F1816.
24. Agence de la Biomédecine. *Agence de la biomédecine – Le rapport annuel médical et scientifique 2017*. Available at https://www.agence-biomedecine.fr/annexes/bilan2017/accueil.htm. Accessed October 15, 2019.
25. Kvietkauskas M, Zitkute V, Leber B, et al. The role of metabolomics in current concepts of organ preservation. *Int J Mol Sci.* 2020;21:E6607.
26. Nath J, Smith TB, Patel K, et al. Metabolic differences between cold stored and machine perfused porcine kidneys: a 1H NMR based study. *Cryobiology.* 2017;74:115–120.
27. Darius T, Vergauwen M, Smith TB, et al. Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model. *Transplantation.* 2020;104:731–743.
28. Patel K, Smith TB, Neil DAH, et al. The effects of oxygenation on ex vivo kidneys undergoing hypothermic machine perfusion. *Transplantation.* 2019;103:314–322.
29. Hosgood SA, Hoff M, Nicholson ML. Treatment of transplant kidneys during machine perfusion. *Transpl Int.* 2021;34:224–232.
30. Franzin R, Stasi A, Fiorentino M, et al. Renal delivery of pharmacologic agents during machine perfusion to prevent ischaemia-reperfusion injury: from murine model to clinical trials. *Front Immunol.* 2021;12:673562.