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Challenging the Ex Vivo Lung Perfusion Procedure With Continuous Dialysis in a Pig Model

Julien De Wolf, MD, MSc,[1] Matthieu Glorion, MD, MSc,[1] Luc Jouneau, PhD,[2] Jérôme Estephan, MD,[2] Jean-Jacques Leplat,[3] Fany Blanc, PhD,[3] Christophe Richard, PhD,[4] Céline Urien, PhD,[2] Antoine Roux, MD, PhD,[2,5] Morgan Le Guen, MD, PhD,[2,6] Didier Journois, MD, PhD,[7] Isabelle Schwartz-Cornil, DVM, PhD,[2] and Edouard Sage, MD, PhD[1,2]

Background. Normothermic ex vivo lung perfusion (EVLP) increases the pool of donor lungs by requalifying marginal lungs refused for transplantation through the recovery of macroscopic and functional properties. However, the cell response and metabolism occurring during EVLP generate a nonphysiological accumulation of electrolytes, metabolites, cytokines, and other cellular byproducts which may have deleterious effects both at the organ and cell levels, with impact on transplantation outcomes. **Methods.** We analyzed the physiological, metabolic, and genome-wide response of lungs undergoing a 6-h EVLP procedure in a pig model in 4 experimental conditions: without perfusate modification, with partial replacement of fluid, and with adult or pediatric dialysis filters. **Results.** Adult and pediatric dialysis stabilized the electrolytic and metabolic profiles while maintaining acid-base and gas exchanges. Pediatric dialysis increased the level of IL-10 and IL-6 in the perfusate. Despite leading to modification of the perfusate composition, the 4 EVLP conditions did not affect the gene expression profiles, which were associated in all cases with increased cell survival, cell proliferation, inflammatory response and cell movement, and with inhibition of bleeding. **Conclusions.** Management of EVLP perfusate by periodic replacement and continuous dialysis has no significant effect on the lung function nor on the gene expression profiles ex vivo. These results suggest that the accumulation of dialyzable cell products does not significantly alter the lung cell response during EVLP, a finding that may have impact on EVLP management in the clinic.

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INTRODUCTION

Ex vivo lung perfusion (EVLP) has been developed as a method to reassess and improve the function of marginal lungs before transplantation and thereby EVLP has increased the pool of donor lungs with outcome results comparable to those of cold-preserved standard lungs.[1]

During the EVLP procedure, oxidative stress and inflammatory responses occur as the organ is reperfused after the anoxic steps of donor lung extraction and preservation, corresponding to an ischemia-reperfusion response. Indeed during EVLP, there is expression of reactive oxygen species (ROS),[2] Damage-associated molecular pattern (DAMPs) molecules including high-mobility group box 1[3] and heat-shock protein 70,[4] as well as synthesis and liberation of inflammatory cytokines.[5,6] Despite these innate responses, EVLP-preserved lungs present a better molecular cell survival profile as compared to cold-preserved

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[1] Department of Thoracic Surgery and Lung Transplantation, Foch Hospital, Suresnes, France.

[2] INRAE, UVSQ, VIM, Université Paris-Saclay, Jouy-en-Josas, France.

[3] INRAE, AgroParisTech, GABI, Université Paris-Saclay, Jouy-en-Josas, France.

[4] UVSQ, INRAE, BREED, Université Paris-Saclay, Jouy-en-Josas, France.

[5] Department of Pneumology, Foch Hospital, Suresnes, France.

[6] Department of Anesthesiology, Foch Hospital, Suresnes, France.

[7] Université de Paris, Paris, France.

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J.D.W. participated to the design of the study, performed the pig surgery and the ex vivo lung perfusion experiment, and participated in the writing of the article. M.G. participated in the design of the study. J.E. performed the lactate deshydrogenase measurements, performed the statistical analyses of the physiological data and of the perfusate data, and drew Figures 1 and 2. L.J. performed the bioinformatics and biostatistical analyses of the genomic data. J.-J.L. managed the animal experiment and wrote the ethic application. C.R. managed the surgery platform. F.B. performed the cytokine dosages. C.U. performed the RNA extractions and quality check. A.R. participated in the design of the study. M.L.G. participated in the design of the study. D.J. participated in the design of the study and provided his expertise in the dialysis procedure management. I.S.-C. participated in the design of the study, supervised the biological analyses, performed the functional genomics analyses, drew the functional genomic figures, and wrote the article. E.S. conceptualized, designed, and supervised the whole study, raised funding, supervised, and participated in the writing of the article. All named authors have agreed to its submission.

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Correspondence: Isabelle Schwartz-Cornil, PhD, Centre de Recherche INRAE, UMR 892 VIM "Virologie et Immunologie Moléculaires" Equipe Vaccins Immunopathologie Immunomodulation (V2I), Bâtiment 440, Etage 1, Bureau 703, 78352 Jouy-en-Josas Cedex, France. (isabelle.schwartz@inrae.fr).

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lungs after transplantation in a pig model, with reduced apoptosis-related gene expression.[4] Furthermore, prolonged EVLP up to 12 h in pigs also led to excellent graft performance after transplantation, with less reperfusion injuries than cold preservation.[7] Even in an exceptional clinical situation, an EVLP conducted during 17 h led to a successful outcome.[8]

However, EVLP has to be further improved to use this window of opportunity to optimally precondition the organ before transplantation and lead to better outcomes than with standard transplantation. For instance to avoid edema accumulation during the process, prone positioning of the graft[9] and negative pressure ventilation[10] provided promising results. To enrich the composition of the synthetic Steen perfusate, erythrocytes were added to the Steen solution and improved the early transplantation outcome in a 24-h-EVLP pig model.[11] Several targeted anti-inflammatory regimens were also evaluated to mitigate the inflammatory response during EVLP with vectorized IL-10,[12] drugs such as 1-antitrypsin,[13] A2AR agonist,[14] pyrrolidine dithiocarbamate (an inhibitor of NF-κB),[15] and poly(ADP-ribose) polymerase inhibitors.[2]

As EVLP occurs at normothermia, it allows a full metabolic activity associated with the recovery of organ functions without clearance of byproducts as done in physiological conditions by kidney and liver. This metabolic activity inadvertently results in aberrant electrolytic and metabolite concentrations in the tissue and in the perfusates, with risk of cell death. Concentration of lactate and glutamate above physiological levels can affect the cellular responses.[16,17] Furthermore, elevated concentrations of lactate and glutamate as well as reduction of glucose concentration during EVLP have been correlated with unfavorable lung outcomes.[18,19]

To normalize the perfusate composition modified by several hours of EVLP, we assessed herein 2 dialysis procedures on the ex vivo functions of lungs in a 6-h pig preclinical EVLP model, and we evaluated their impact at the physiological, metabolic, and gene expression levels in comparison to partial or no replacement of perfusion fluid.

MATERIALS AND METHODS

Ethics

The animal experiments were conducted in accordance with the EU guidelines and the French regulations (DIRECTIVE 2010/63/EU, 2010; Code rural, 2018; Décret n°2013-118, 2013). The procedures were evaluated and approved by the Ministry of Higher Education and Research. The experiments were approved by the COMETHEA ethic committee under the APAFIS notification number authorization number APAFIS#14942-2018020720215374 v3).

Animals and Lung Extraction Procedures

Sixteen large white pigs (50 ± 5 kg) were hosted in the Animal Genetics and Integrative Biology unit (GABI-INRAE, France). The lung extraction was performed on the Animal Surgery and Medical Imaging Platform (MIMA2-CIMA-BREED-INRAE, France). Heart-lung monobloc retrievals were performed using a swine nonheart-beating donor model. Anesthesia was induced by a combination of 1 mg/kg Rompun 2% (Elanco, Heinz-Lohmann-Strasse 4, Cuxhaven, Allemagne) and 15 mg/kg Imalgene 1000 (Boehringer Ingelheim Animal Health, Lyon, France) and pursued with 6% isofluorane. Pigs were euthanized with an overdose of Dolethal (Vetoquinol, Magny-Vernois, France, 50 mg/kg). After cardiorespiratory arrest, ventilation was stopped and a period of 10 minutes of no touch was applied. Lungs were flushed with Perfadex (XVIVO Perfusion, Göteborg, Sweden) and the heart-lung block was removed with inflated lungs.

EVLP and Dialysis Procedures

After 1 h of cold ischemia, a 6-h EVLP procedure was performed according to the Toronto protocol.[7] The circuit was filled with 1.5 L of Steen solution supplemented with 1 mg of methylprednisolone, 1.5 mg of cefuroxime, and 15000 UI of heparin. A flow rate at 40% of the theoretical cardiac output was applied at normothermia. The pig lungs were allocated into 4 EVLP groups (4 lungs/group). The size of the pig lung groups was decided using a power calculation based on our preliminary data of IL-6 cytokine and lactate concentration in the perfusion fluid of a Gold Standard procedure (mean and SD). This power calculation indicated that 4 pig lungs per group would allow the detection of a presupposed 40% decrease or increase of IL-6 and lactate concentration in the test groups versus their values in the Gold Standard group (error rate alpha set to 0.05 and power to 0.80). In the No Steen change group, the perfusate was kept without any change during the 6-h procedure. In the Gold standard group, 500 mL of Steen solution was replaced every 2 hours like in the clinic practice. In the Pediatric dialysis and Adult dialysis groups, a continuous dialysis was applied to the perfusate using a Continuous Veno-Venous Hemodialysis mode. We created a homemade dialysis bath from Phoxilium (Baxter, Deerfiled) by adjusting electrolytes to be neutral on the acid-base balance and to maintain the strong ion difference over time. Dialysate and blood flows were chosen to stay in the best coupling range of the selected membrane and to be sufficiently high to allow electrolytes and small substances concentrations control in <2 hours. In the Pediatric dialysis group, we used an AVpaed pediatric membrane (Fresenius, Bad Homburg vor der Höhe, Germany) with an effective surface area of 0.2 m^2 and a cutoff at 30 kDa, and we applied a flow rate of 800 mL/h and 50 mL/min. In the adult dialysis group, we used an Emic 2 membrane (Fresenius, Bad Homburg vor der Höhe, Germany) with an effective surface area of 1.8 m^2 and a cutoff at 40 kDa and we applied a flow rate of 800 mL/h and 80 mL/min.

EVLP Monitoring, Sample Collections, and Biological Dosages

During EVLP, pulmonary artery pressure, graft weight, temperature, ventilation peak pressure, ventilation plateau pressure, and compliance were recorded every hour. Perfusates were collected hourly and frozen at –80 °C for lactate deshydrogenase (LDH) and cytokine detection. Blood gases, electrolytes, and proteins were detected on fresh perfusates. The LDH was measured with the CytoTox 96 Non-Radio Cytotoxicity Assay (Promega, Madison). Cytokines concentrations were assessed by

cytometric beads assay for simultaneous detection of 10 swine cytokines (IL-4, IFNα, TNFα, IL-2, IL-8, IL-12p40, IL-6, IL-10, IL-17, IL-1β) as described.[20] Lung biopsies were frozen in liquid nitrogen right after the cold ischemia period (control lungs) and 6 h after EVLP initiation for RNA extraction and deep sequencing.

RNA Extraction, Quality Check, and Deep Sequencing

Frozen lung biopsies were embedded in Tissue-Tek O.C.T. Compound (Sakura), cut in five 60 µm slices from which total RNA was extracted using the Arcturus PicoPure RNA Isolation Kit (Arcturus Life Technologies). RNA was checked for quality with an Agilent 2100 Bioanalyzer using RNA 6000 Nano Kits (Agilent Technologies). Directional RNA-Seq Libraries were constructed from 500 ng of total RNA using the *TruSeq mRNA Stranded library prep kit* (Illumina), following the manufacturer's instructions. The final libraries' quality was assessed with an Agilent Bioanalyzer 2100, using an Agilent High Sensitivity DNA Kit. Libraries were pooled in equimolar proportions and sequenced in paired-end runs (50 nt forward-34 nt reverse) on an Illumina NextSeq500 instrument, using NextSeq 500 High Output 75 cycles kits. Demultiplexing has been done with bcl2fastq2 V2.2.18.12. Adapters were trimmed with Cutadapt1.15 and only read longer than 10pb were kept.

Bioinformatic Analyses

The Illumina sequencing produced from 22 to 39 million reads per sample. Sequences were aligned with tophat2 (v2.0.14) on the porcine transcriptome (reference Ensembl Release 101, Sscrofa11.1). Gene counts were transformed using RLOG function of DESeq2 package (v1.18.1). The mixOmics (v6.3.1) multilevel principal component analysis (PCA) algorithm was applied on the data in each group to reveal a partition of the samples.

To study the functional signatures of the gene expression changes between controls and the EVLP groups at the 6-h time point, we performed a differential analysis using the DESeq2 R package (v1.18.1) for unpaired data sets. Differentially expressed genes (DEGs) with a Benjamini-Hochberg adjusted *P* below 0.01 were selected and used as input into the ingenuity pathway analysis (IPA; QIAGEN Silicon Valley, Redwood City, CA, edition 2019) to perform pathways and functional analyses. The association likelihood of the DEG sets with a given pathway/function is given by *P* calculated with the Right-tailed Fisher's Exact Test and the prediction of their activation or inhibition is given by z scores (>2 or <–2, respectively). The z scores are calculated with an algorithm that integrates (i) the expression orientation of the genes in the data set that contribute to the pathway/function and (ii) the knowledge on these genes expression from experimental data published in peer-reviewed journals.

Statistical Analyses

The physiological, electrolytic, metabolic, and cytokine data were analyzed with the GraphPad Prism 7.0c software. An unpaired parametric 2-way ANOVA was used to compare data across groups when the data followed a normal distribution as assessed with the D'Agostino-Pearson omnibus normality test (lung compliance, pulmonary artery pressure, Po_2, glucose). In other cases, the non-parametric Mann-Whitney *U* test was used to compare the data between 2 groups (Na^+, Cl^-, Ca^{2+}, lactate, LDH, cytokines).

RESULTS

Organ function, electrolyte, and metabolic profiles ex vivo

We analyzed pig lungs allocated in 4 experimental EVLP groups described in *Materials and Methods* (4 lungs per group): No steen change, gold standard (partial fluid replacement), adult dialysis, and pediatric dialysis. Lung compliance, pulmonary artery pressure, and gas exchanges showed no significant differences between groups (Figure 1A). Edema and weight gain remained very limited in all groups. Whereas the Na^+ and Cl^- levels increased overtime in the Gold Standard and No Steen change groups, their levels were maintained at statistically significant lower levels in the 2 dialysis groups (Figure 1B, $P < 0.05$ from 4 to 6 h). Conversely, the Ca^{2+} levels reached higher levels in the dialysis groups (Figure 1B) and the K^+ levels were similar across groups (not shown). The glucose levels were kept at higher levels in the 2 dialysis groups ($P < 0.05$ at 6 h) whereas lactate levels strongly increased in the Gold Standard and No Steen change groups and were maintained at low levels in the dialysis groups (Figure 1C, $P < 0.05$ from 2 to 6 h). LDH was detected after 1-h procedure in all groups, and there was no difference in its concentrations between groups over the 6-h procedure. Altogether, the dialysis procedures did not alter the measured hemodynamic and respiratory functions and they rebalanced electrolytic and metabolic profiles during the ex vivo procedure.

Cytokine Detection in the Perfusates

To evaluate the impact of the EVLP procedures on the inflammatory cytokine levels, perfusion fluids were assayed for detection of pig cytokines using a multiplex cytometric bead assay. Among the 10 targeted cytokines (IL-4, IFNα, TNFα, IL-2, IL-8, IL-12p40, IL-6, IL-10, IL-17, IL-1β), only TNFα, IL-6, IL-8, and IL-10 were detected. Their levels increased at around 3 h after the start of the procedure in a variable proportion of animals in the Gold standard, No Steen change, and Pediatric dialysis groups, but almost not in the adult dialysis group (Figure 2). There was no clear differences in the cytokine levels between groups, except at 6 h where the levels of the anti-inflammatory IL-10 and of the proinflammatory IL-6 cytokines were higher in the Pediatric dialysis than in the Gold standard group ($P < 0.05$, Figure 2). These results show that several inflammatory cytokines accumulate in the perfusates, with higher levels of IL-10 and IL-6 in the Pediatric dialysis group.

Genome-wide Gene Expression Analyses

EVLP, by modifying the electrolytic, metabolic, and cytokine levels in the perfusates, may affect the lung cell response at the gene expression level. Biopsies were collected in the 4 groups at the end of the 4 EVLP procedures (6 h) for RNA isolation and deep sequencing. In parallel, we

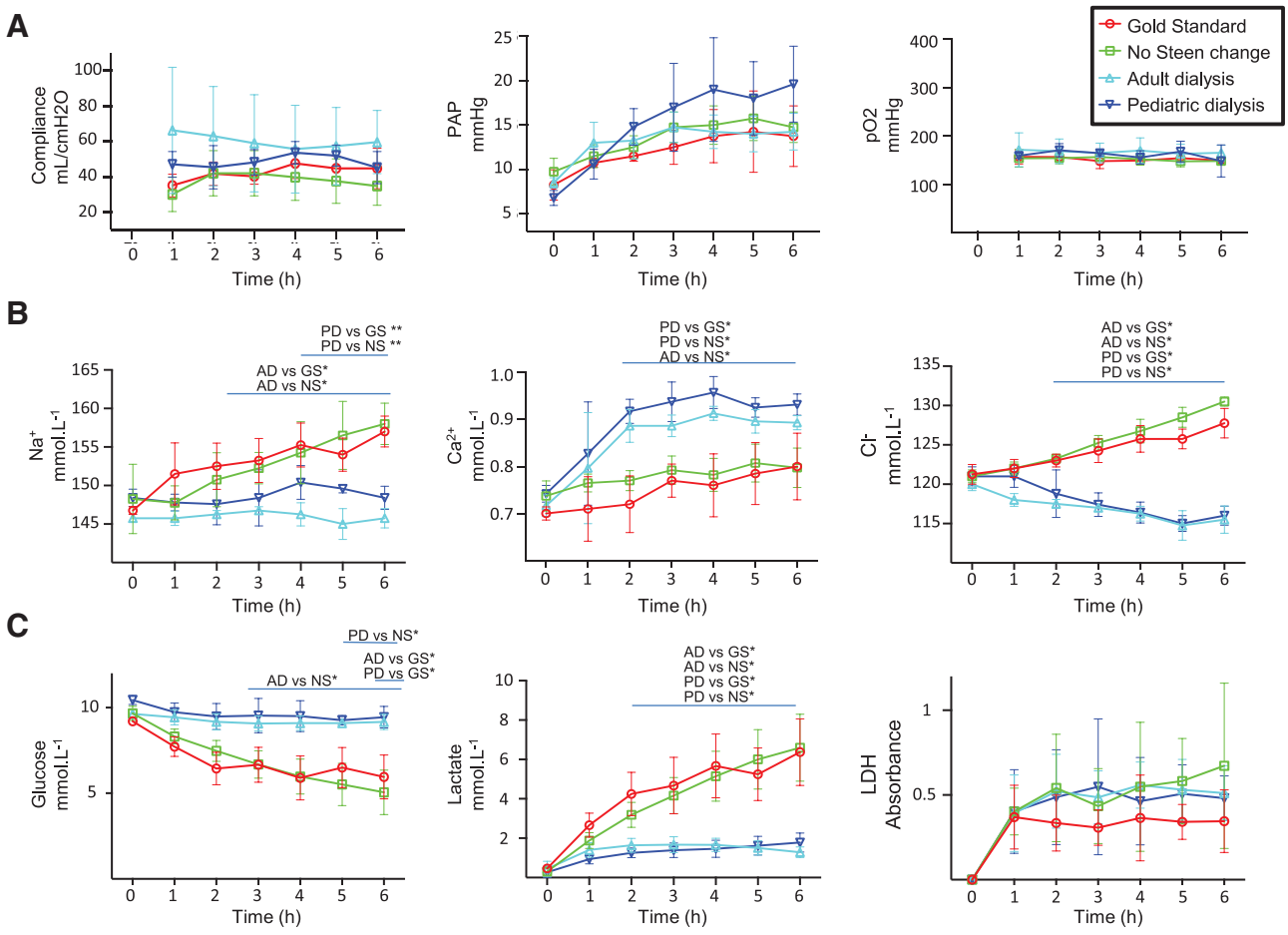


FIGURE 1. Monitoring of the physiological parameters (A), electrolyte (B), and metabolite (C) concentrations in the perfusates during the 4 EVLP procedures. A, From left to right: lung compliance, pulmonary artery pressure, Po_2 on FiO_2 30%. B, From left to right: Na^{2+}, Ca^{2}+, Cl^{-}. C, From left to right: glucose, lactate production, LDH. Based on normality test results, an unpaired parametric 2-way ANOVA was used to compare data across groups in the case of lung compliance, pulmonary artery pressure, Po_2 and glucose. A nonparametric Mann-Whitney *U* test was used to compare the data between 2 groups in the cases of Na^{+}, Cl^{-}, Ca^{22+}, lactate, LDH, cytokine concentrations. $^{*}P<0.05$; $^{**}P<0.01$. The error bars represented standard deviations. AD, adult dialysis; ANOVA, analysis of variance; FIO_2, fraction of inspired oxygen; GS, gold standard; LDH, lactate dehydrogenase; NS, no steen change; PAP, pulmonary artery pressure; PD, pediatric dialysis; pO_2, partial pressure of oxygen.

used biopsies from 10 pig lungs collected before the start of EVLP, as common controls to the 4 groups. Common controls and nonpaired controls were used for cost limitations. The first 2 axes of a principal component analysis shows that the RNA-seq data of the control group separate from the data of the EVLP groups, whereas the data of the 4 EVLP groups overlap (Figure 3). DEGs ($P<0.01$ with unpaired data sets and Benjamini-Hochberg correction) were found between the controls and the 4 EVLP groups: as shown Table S1, **SDC,** http://links.lww.com/TP/C282, between 914 and 1126 genes were upregulated and 468 and 980 genes were down-modulated at 6 h in the 4 groups ($P<0.01$).

The DEG gene list of each group was loaded in the IPA software for investigating whether modification of the EVLP procedure would be associated with enriched pathways and biological functions. IPA has developed algorithms for prediction of activation or inhibition of pathways/functions based on the integration of the gene expression orientation and knowledge from the literature. In a comparative IPA analysis of all groups, 10 top canonical pathways were found significantly enriched in most instances ($P<0.05$, Figure 4; Table S2, **SDC,** http://links.lww.com/TP/C282 for details). Eight pathways were predicted to be upregulated (z scores > 2, Figure 4). These pathways, that is, ERK5, IL-6, Th17, Toll-like receptor, NF-kB, HMGB1, iNOS, TNFR2, are related to activation of the inflammatory response. No meaningful pathway was enriched in a specific group.

The comparison of the disease and function categories retrieved by the IPA Downstream Effect Analysis (Figure 5; Table S3, **SDC,** http://links.lww.com/TP/C282) pointed to similarly modulated functions in the different groups, with activation of cell survival, proliferation of connective tissue cells, inflammatory responses, and cell movement (z scores > 2) and with inhibition of bleeding (z score < –2). No function was found to be differentially modulated in a specific group. The expression fold changes of a selection of genes contributing to the modulated functions are shown in Figure 5B to E. The upregulated genes involved in cell survival include the *BCL3*, *BCL2L1*, and *BCL10* whose encoded proteins are reported to antagonize apoptosis[21-23] supporting that the 4 EVLP procedures similarly promote cell survival (Figure 5B). Many genes associated with proliferation of connective tissue were also upregulated by the 4 procedures suggesting cell regeneration and tissue repair processes (Figure 5C). In the inflammatory response function which is well known to be activated by

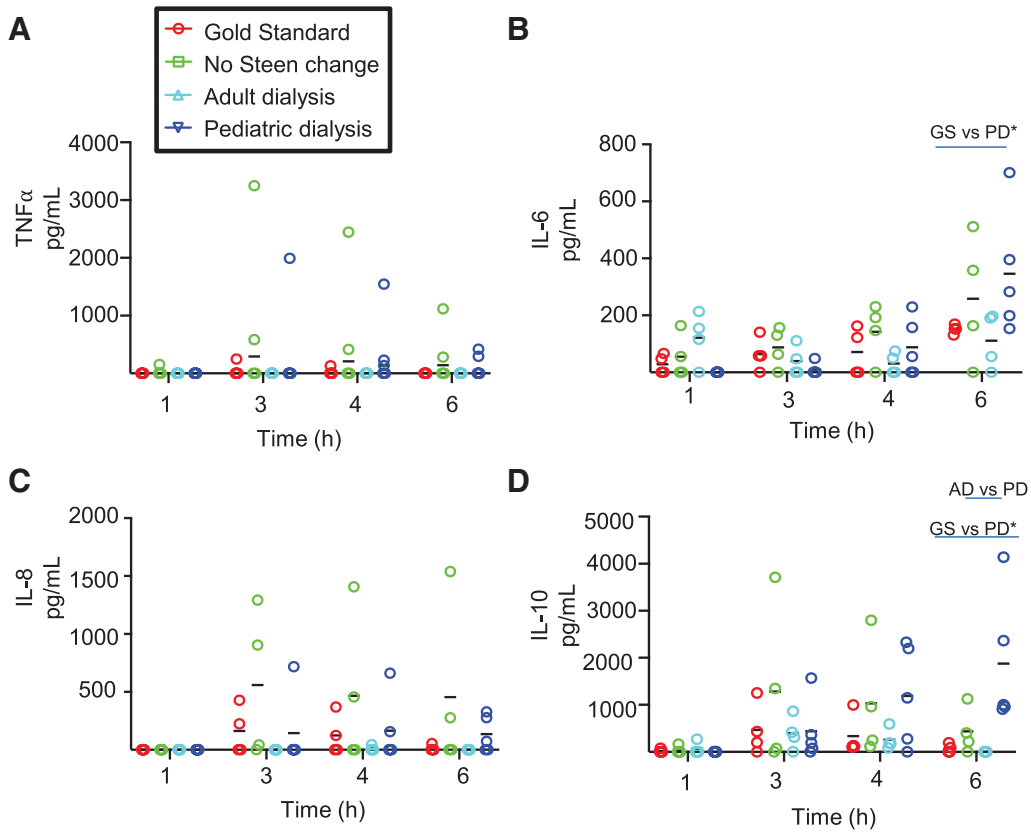


FIGURE 2. Cytokine level detection in the perfusates. A, TNFα; B, IL-6; C, IL-8; D, IL-10. A nonparametric Mann-Whitney *U* test was used to compare the data between 2 groups. *$P<0.05$. AD, adult dialysis; GS, gold standard; IL, interleukin; NS, no steen change; PD, pediatric dialysis; TNFα, tumor necrosis factor α.

EVLP procedures,[1] many genes encoding inflammatory cytokines were similarly upregulated in the 4 groups, such as *IL17F*, *IL22*, *IL1B*, *CSF3*, *IL6*, *CCL3L1*, *CCL4*, *TNF* in link with increased expression of *REL* and *NFKB2*, that encode transcription factors activating many inflammatory genes[24] (Figure 5D). Notably, IPA could not attribute a z score to the inflammatory response prediction in the adult dialysis group, due to an unclear balance between the significantly modulated genes contributing to the function, impairing the z score calculation by the IPA algorithm. However, Figure 5C shows that proinflammatory genes were increased in the adult dialysis group like in the other groups but reached either lower fold change or lower significance in some cases, probably due to variability between

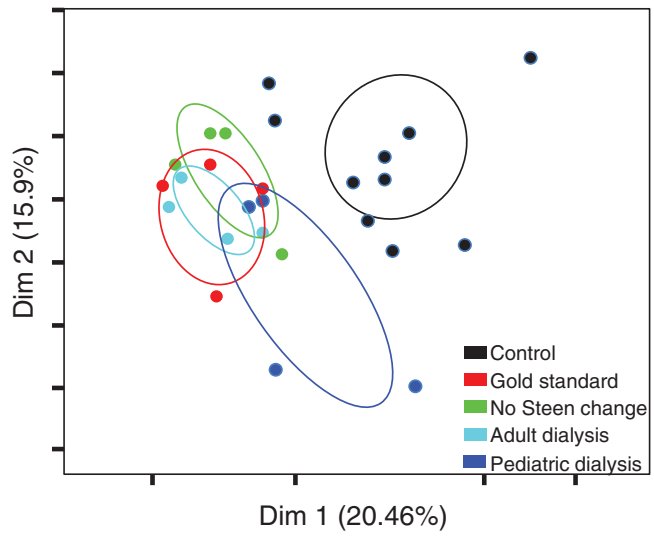


FIGURE 3. PCA of the lung transcriptomic data from the different EVLP procedures (6 h) and control groups. The read counts (RLOG transformed—package DESeq2) for each mapped gene of the pig lung RNA-seq data obtained from 4 EVLP treatment groups (gold standard, no steen change, adult dialysis, pediatric dialysis, 4 pigs per group) and from controls (10 pigs) were used for the PCA analysis. The expression values for the 2 first components (Dim 1, 2) of the principal component analysis are plotted. EVLP, ex vivo lung perfusion; PCA, principal component analysis.

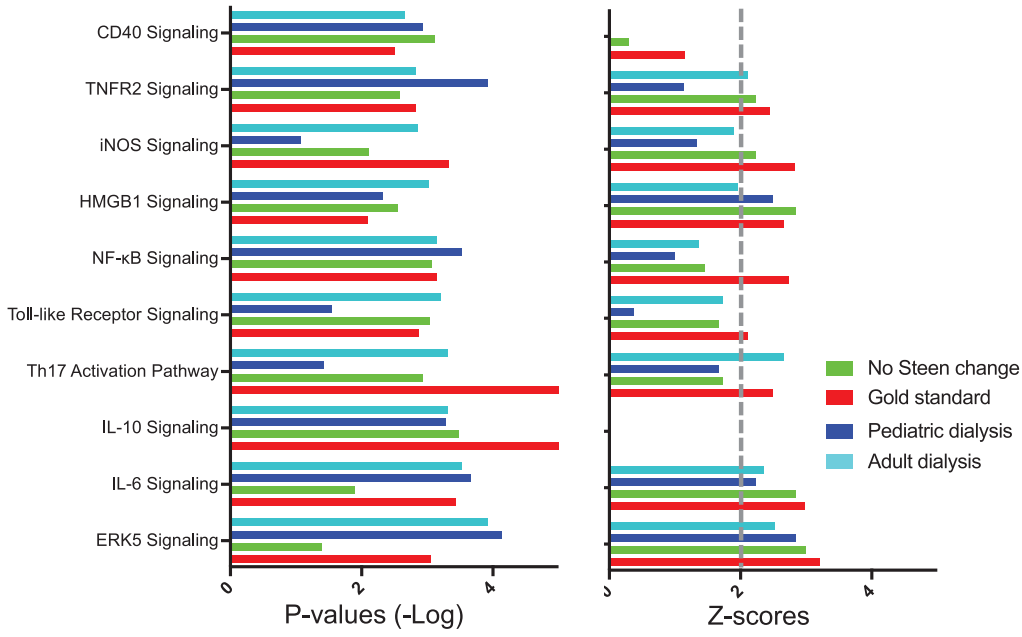


FIGURE 4. Canonical pathways modulated by the different EVLP procedures. The mean fold changes (log 2 transformed) and corrected *P* of the DEGs at 6 h vs controls from the 4 EVLP treatments (gold standard, no steen change, adult dialysis, pediatric dialysis) were calculated. The DEGs with corrected $P<0.01$ (Benjamini-Hochberg) were selected and the mean fold changes were loaded and processed through the IPA core analysis for identification of dominant canonical pathways. A comparative analysis of the 10 top canonical pathways sorted by their –Log *P* (right-tailed Fisher's Exact test) across the 4 groups is represented in parallel to their z scores. –Log $P>1.3$ are considered as significant; z scores <–2 and >2 correspond to prediction of inhibited and activated pathways, respectively. The details of the pathways' analysis are shown in **Table S2, SDC,** http://links.lww.com/TP/C282. DEG, differentially expressed gene; ERK, extracellular signal-regulated kinase; EVLP, ex vivo lung perfusion; HMGB1, high mobility group box protein 1; IL, interleukin; iNOS, inducible nitric oxide synthase; IPA, ingenuity pathways analysis; NF-κB, Nuclear factor kappa B; TNFR2, tumor necrosis factor-alpha receptor type II.

pigs. Many of the genes contributing to the inflammatory response are included in the cell movement function which is also predicted to be activated in the 4 groups (Figure 5A). The increase of the cell movement function may be related to the decrease of the myeloid cell representation in the perfused lung, attested by the decreased expression of *CCR2*, *CCR4*, and *CX3CR1* genes (Figure 5C), as also reported in the case of human lungs undergoing EVLP.[25] Finally, the inhibition of the bleeding function in all groups corresponds to activation of genes involved in coagulation, that is, the tissue platelet activator *PLAT*, thrombospondin *THBS1*, and prostaglandin E receptor *PTGER4*, and to regulation of genes controlling vascular endothelial permeability, that is, upregulation of the metallopeptidase inhibitor *TIMP1* and downregulation of the metallopeptidase *MMP8*,[26] in agreement with the maintenance of the alveolar blood barrier function.[27]

Finally to validate the IPA results, we used the open-source Reactome algorithm. The top retrieved biological pathways were related cytokine signaling in the 4 groups (Table S4, **SDC,** http://links.lww.com/TP/C282), thus confirming the IPA findings.

Overall the functional genomic analysis shows that canonical pathways and functions are similarly modulated by the 4 different EVLP procedures, indicating that maintenance of the perfusate, periodic replacement, or permanent dialysis do not impact on the cellular functions and signaling of the ex vivo organ in a 6-h procedure.

DISCUSSION

We observed that the perfusate management by dialysis corrected the electrolyte and metabolite concentrations but had no significant effect on the gene expression profiles in perfused lung tissues in a 6-h duration process. Surprisingly omission of Steen replacement, a standardized approach included in clinical protocols, did not impact on gene expression.

Our results contrast with the effect of dialysis in a rat model of liver ex vivo perfusion, where the levels of the TNFα transcripts were reduced after a 6-h process.[28] The expression of TNFα (Figure 3) was not decreased by our 2 types of dialysis procedures. The effect of dialysis may differ depending on the targeted organ or on the model species. In agreement with our findings, Buchko et al found in a pig EVLP model that continuous hemodialysis did not improve graft function up to 24 h despite improved maintenance of perfusate composition, but at the difference with our study, they did not investigate the dialysis impact on cytokine and gene expression responses.[29] The effect of dialysis has also been recently assessed in the EVLP of human lungs refused for transplantation.[30] Gene expression was not evaluated in this study, but the authors observed decreased apoptosis levels in the lung tissue after a 12-h perfusion with dialysis versus regular perfusate replacement. Our gene expression analysis at the 6-h time point did not predict improved cell survival in the dialysis groups. In addition, we did not observe significant change of LDH accumulation—which reflects cell damage and death—in the perfusates of the dialysis groups. It is nevertheless possible that the benefit of dialysis on cell survival occurs beyond a 6-h procedure at later time points.

Few studies have investigated the effect of EVLP using genome-wide expression analyses. The present study provides a genomic analysis of the lung tissue responses to

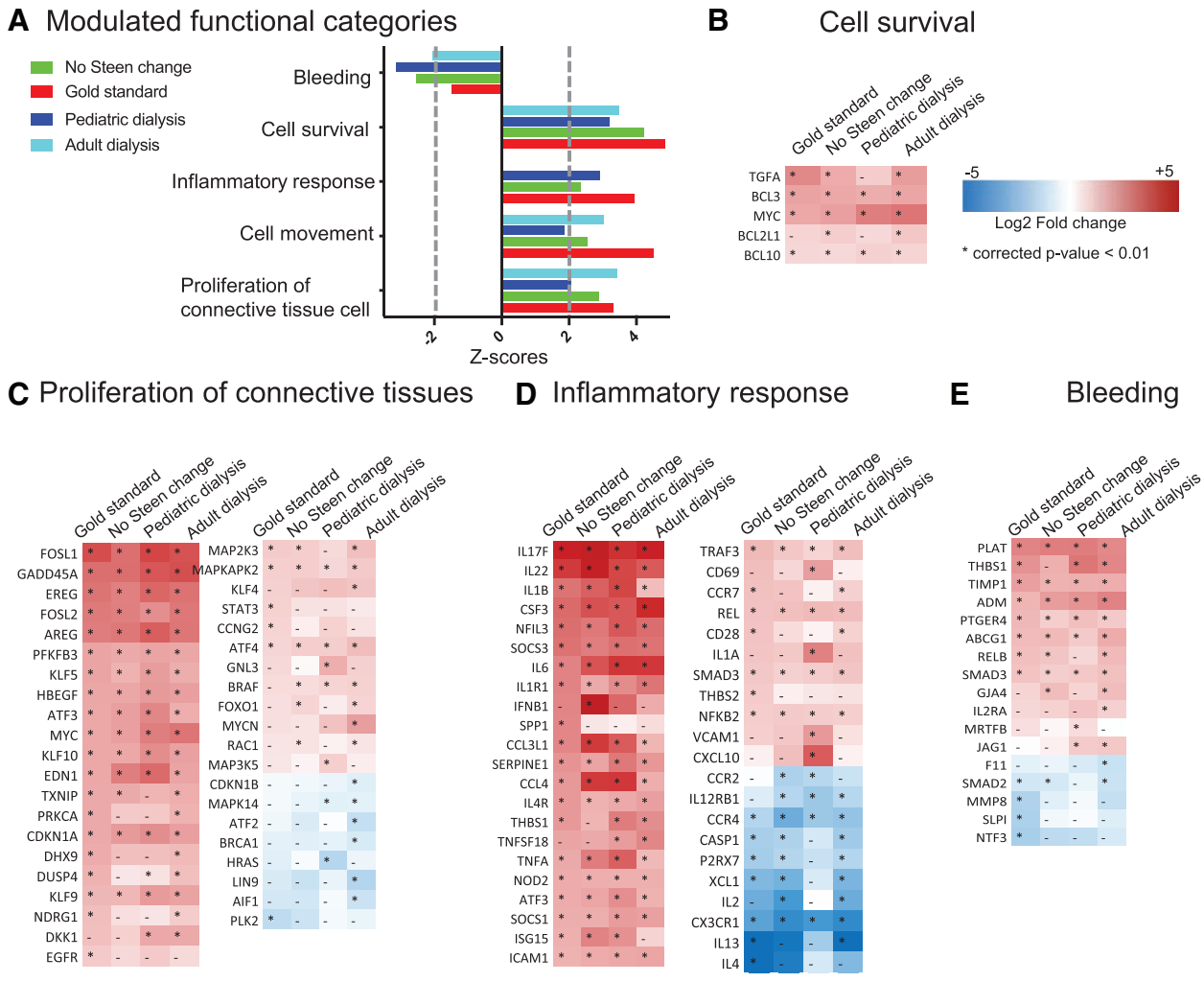


FIGURE 5. Biological functions predicted to be activated and inhibited by the different EVLP procedures. The mean fold changes (Log 2 transformed) and corrected *P* of the DEGs at 6 h vs controls from the 4 EVLP treatments were calculated. The DEGs with corrected $P < 0.01$ were selected and the mean fold changes and corrected *P* were processed through the IPA downstream effect algorithm, which integrates the gene expression orientations. Z scores < -2 and > 2 correspond to prediction of inhibited and activated functions, respectively. A, The z scores of functional categories are represented for each of the 4 EVLP groups. B–E, The mean fold changes (Log 2, from –5 to +5) of a selection of genes contributing to the cell survival (B), proliferation of connective tissue (C), inflammatory response (D), bleeding (E) functions are represented with a color map. The genes contributing to cell movement overlap with the genes contributing to inflammatory response and are therefore not represented. The corrected $P < 0.01$ is represented with "*," and lack of significance is represented by "–." The selected genes were obtained from the union of the contributing genes to the function in all EVLP groups, explaining why the modulated gene expression is not found to be significant in some instances. Further details of the functional analysis are presented in **Table S3, SDC,** http://links.lww.com/TP/C282. DEG, differentially expressed gene; EVLP, ex vivo lung perfusion; IPA, ingenuity pathways analysis.

EVLP using the power of next generation sequencing technology whereas previous studies used Affymetrix microarrays in human[25,31] and targeted methods of gene expression detection in pig *EVLP*[4,32] and rat EVLP.[33] In particular, 1 study was conducted using Affymetrix microarrays on human lungs refused for transplantation and treated with EVLP for different period of times.[25] Strikingly, among the top 15 genes modulated by EVLP in human lungs, 11 were also found similarly modulated in our present work and include *CSF3, SERPIN1/2, THBS1, IL1B, EREG, CYP1A1, FOSL1, CCR2, CX3CR1, PPP1R3C, HBB,* and *OSGEPL1* (Table S5, **SDC,** http://links.lww.com/TP/C282). This observation supports the pertinence of the pig model to study the effects of EVLP. In addition, we found that survival and recovery was a major predicted genomic profile induced by EVLP, with the upregulation of antiapoptotic genes *BCL3, BCL2L1,* and *BCL10* and activation of many genes involved in proliferation, like in another study.[25] Notably, we observed that the cell survival and proliferation profiles were not altered by our different perfusion procedures. Interestingly, upregulation of the antiapoptotic BCL2 and downregulation of the proapoptotic Bax proteins were found 24 h posttransplantation in pig lungs treated with EVLP as compared to in classically transplanted lungs,[4] indicating that the survival pathways induced upon EVLP have a prolonged effect even after transplantation.

An important inflammatory response has been reported in animal models of EVLP such as rat[33] and pig[32] as well as in human patients.[5] Like in this studies, we detected IL-6, IL-8, TNFα, and IL-10 in the perfusates. However, these cytokines were not detected in the adult dialysis group, possibly due to a more potent purification process than in the pediatric dialysis group. Surprisingly, the levels of IL-10 and IL-6 were significantly higher in the pediatric group versus in the gold standard group, as also observed

for these cytokines in the human study.[30] It is possible that these cytokines of about 18.5 and 21 kDa are not well filtered out due to the relatively low pressure and the filtering membrane properties of the pediatric dialysis, while they are partially removed along with the Steen replacement. Nevertheless, the differences in IL-10 and IL-6 were not associated with higher level of gene transcription for these cytokines in the pediatric group at 6 h (data not shown). We could not detect a clear effect of our perfusate management procedure on the expression of genes related to inflammatory response. This innate response is likely part of the ischemia-reperfusion response inevitably occurring upon the reoxygenation, and it may lead to activation of the graft's antigen-presenting cells. Anti-inflammatory pretreatments during EVLP could reduce this activation and thereby may inhibit the priming of adverse allogeneic T-cell responses. Interestingly in all 4 procedures, we also observed a decreased expression of the *CX3CR1, CCR2,* and *CCR4* genes, which probably reflects the egress of myeloid cells out of the perfused lung, as also observed in human lungs.[25] Removal of lung leukocytes during EVLP has been proposed to be beneficial to transplantation outcome.[34]

The metabolic and electrolytic balances were corrected by the 2 dialysis protocols that led to stabilization of lactate and glucose concentrations. Whereas these imbalances were initially suspected to have deleterious effects, we could not see any significant effects on the cell transcriptomes at 6 h. It is even possible that such accumulated molecules during EVLP have positive effects. For instance, elevated levels of lactate could be beneficial in the context of transplantation: indeed lactate can be taken up by T-cells through a MCT1 transporter and thereby induces tolerogenic signaling.[35] Therefore, the different perfusate management protocols may generate complex effects on cell signaling with potential impact beyond the 6 h time point, possibly at posttranscriptional levels, and in discrete cell subsets. Indeed, the whole tissue transcriptomic approach that we undertook does not allow to detect changes in specific cell subsets of the lungs, that is, in the epithelial, vascular, fibroblastic, and immune cell compartments. The new technique of single-cell RNA-seq (scRNA-seq) offers the possibility to analyze transcriptome data on individual cells, thus permitting to reveal changes in gene expression programs in specific cell subsets identified by their identity profiles. Therefore, the next steps of this research program is to assess the effects of Steen fluid change and dialysis in longer duration procedures using scRNA-seq approaches and to perform correlation analyses between transcriptomic responses at the level of cell subset with posttransplant outcomes. Monitoring the graft response upon experimental transplantation will be essential for bringing definitive conclusions on the effects of perfusate management.

Our results have potential clinical implications. Indeed, our data reveal that the costly periodic replacement of the Steen fluid, which is currently used in the clinic may not be useful to preserve the macroscopic, functional, and cellular qualities of EVLP lungs. It opens the way to reconsider the effects of Steen exchange included in the clinical protocol and to develop alternatives. In this way, our dialysis protocol was capable to maintain electrolytes balance with less handling but unfortunately without reducing the acute inflammatory response, which could have negative consequences. In that respect, recent promising preclinical results indicate that progresses in EVLP management could come from negative pressure ventilation that reduced the inflammatory response[10] and from adsorption of cytokines during the perfusion that improved early posttransplant lung function and induced less intense inflammatory response to reperfusion with blood.[36] Anti-inflammatory drug or cell-therapy treatments delivered during the EVLP window of opportunity still await to show their benefit on lung transplantation outcomes.

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