

# **Inflammatory gene expression in livers undergoing ex situ normothermic perfusion is attenuated by leukocyte removal from the perfusate**

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## **AUTHORSHIP PAGE**

Contributions to this study are as follows: Bahadori: data analysis and interpretation, generation of figures, and writing of the of the manuscript and legends. Colin Y.C. Lee and Ferdinand: Data analysis, and review of the manuscript. Cabantous, Butler and Rouhani: Data collection and review of the manuscript. Watson and Clatworthy: Study design, data acquisition, results interpretation, critically reviewing and writing of the manuscript.

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## **ABBREVIATIONS**

| <b>Abbreviation</b> | <b>Full term</b>                       |
|---------------------|----------------------------------------|
| ADGRE1              | Adhesion G Protein-Coupled Receptor E1 |
| ALT                 | Alanine Transaminase                   |
| AREG/EREG           | Amphiregulin/Epiregulin                |
| ATP                 | Adenosine Triphosphate                 |
| C3                  | Complement Component 3                 |
| C5AR1               | Complement Component 5a Receptor 1     |
| CCL2                | Chemokine (C-C motif) ligand 2         |
| CD163               | Cluster of Differentiation 163         |
| CD68                | Cluster of Differentiation 68          |
| CSF3                | Colony Stimulating Factor 3            |
| CXCL8               | Chemokine (C-X-C motif) ligand 8       |
| DBD                 | Donation after Brain Death             |
| DCD                 | Donation after Circulatory Death       |
| DEFB132             | Defensin Beta 132                      |
| DEG                 | Differentially Expressed Genes         |
| ESNP                | Ex situ Normothermic Perfusion         |
| F13A1               | Coagulation Factor XIII A Chain        |

| <b>Abbreviation</b> | <b>Full term</b>                             |
|---------------------|----------------------------------------------|
| FGF19               | Fibroblast Growth Factor 19                  |
| GCSF                | Granulocyte Colony-Stimulating Factor        |
| GSEA                | Gene Set Enrichment Analysis                 |
| HSP                 | Heat Shock Proteins                          |
| HSPB1               | Heat Shock Protein Family B (Small) Member 1 |
| IL                  | Interleukin                                  |
| IL1B                | Interleukin 1 Beta                           |
| IL1RL               | Interleukin 1 Receptor Like                  |
| IL32                | Interleukin 32                               |
| IL33                | Interleukin 33                               |
| IL6                 | Interleukin 6                                |
| IRI                 | Ischemia-Reperfusion Injury                  |
| LRP1                | Lipoprotein Receptor-Related Protein 1       |
| M/F                 | Male/Female                                  |
| MMP                 | Matrix Metalloproteinase                     |
| MRC1                | Mannose Receptor C-Type 1                    |
| NAFLD               | Non-Alcoholic Fatty Liver Disease            |
| NFκB                | Nuclear Factor Kappa B                       |
| NK                  | Natural Killer                               |
| NKG2D               | Natural Killer Group 2D                      |

| <b>Abbreviation</b> | <b>Full term</b>                                                       |
|---------------------|------------------------------------------------------------------------|
| NLRP3               | NOD-Like Receptor Family Pyrin Domain Containing 3                     |
| NMP                 | Normothermic Machine Perfusion                                         |
| NR4A1               | Nuclear Receptor Subfamily 4 Group A Member 1                          |
| OSM                 | Oncostatin M                                                           |
| OXPHOS              | Oxidative Phosphorylation                                              |
| P2RX7               | P2X Purinoceptor 7                                                     |
| PAI-1               | Plasminogen Activator Inhibitor-1                                      |
| PAI-2               | Plasminogen Activator Inhibitor-2                                      |
| PLAT                | Plasminogen Activator, Tissue Type                                     |
| PLAU                | Plasminogen Activator, Urokinase                                       |
| RAET1E              | Retinoic Acid Early Transcript 1E                                      |
| SERPINB2            | Serpin Family B Member 2                                               |
| SERPINE1            | Serpin Family E Member 1                                               |
| STAT                | Signal Transducer and Activator of Transcription                       |
| STRING              | Search Tool for the Retrieval of Interacting Genes/Proteins            |
| TGF                 | Transforming Growth Factor                                             |
| THBS1               | Thrombospondin-1                                                       |
| TIMP1               | Tissue Inhibitor of Metalloproteinases Metallopeptidase<br>Inhibitor 1 |
| TNF                 | Tumor Necrosis Factor                                                  |

| <b>Abbreviation</b> | <b>Full term</b>                                      |
|---------------------|-------------------------------------------------------|
| TNFRSF9-            | Tumor Necrosis Factor Receptor Superfamily Member 9 - |
| TNFSF9              | Tumor Necrosis Factor Superfamily Member 9            |
| TSLP                | Thymic Stromal Lymphopoietin                          |
| Y/N                 | Yes/No                                                |

## **ABSTRACT**

### ***Background***

Ex situ normothermic perfusion (ESNP) is a method to evaluate and potentially recondition organs before transplantation. However, increased expression of inflammatory molecules, including by tissue-resident immune cells, may occur during the perfusion process, potentially negating the beneficial effects of perfusion.

### ***Methods***

We used RNA sequencing to assess gene expression in 31 livers undergoing ESNP, including 23 donated after circulatory death (DCD) and 8 donated after brain death (DBD). In 7 DCD livers, a leucocyte filter was added to the circuit during perfusion. Biopsies were available for transcriptomic assessment at the start of perfusion in all cases, and at varying timepoints post-perfusion.

### ***Results***

During ESNP in DCD livers, we observed an increase in pro-inflammatory, pro-fibrinolytic, and pro-repair pathway genes. SERPINE1, encoding plasminogen activator inhibitor (PAI)-1, was among the genes most significantly upregulated during perfusion

in DCD livers, potentially promoting fibrin clot persistence in vasculature. We also found increased expression of monocyte and neutrophil recruiting chemokine and pro-inflammatory cytokine transcripts during ESNP, but several pro-repair molecules, including thymic stromal lymphopoietin (TSLP), were also upregulated. In both DCD and DBD livers, interferon gamma (IFNG) response genes were enriched, while oxidative phosphorylation (OXPHOS) genes decreased in organs with high perfusate alanine transaminase (ALT), a biomarker associated with adverse clinical outcomes. The inclusion of a leukocyte filter in the perfusion circuit mitigated the induction of inflammation/immune pathway genes during perfusion and was associated with enrichment in OXPHOS genes.

## ***Conclusions***

Leukocyte removal during ESNP abrogates transcriptional changes associated with unfavourable clinical outcomes, potentially benefiting human livers undergoing ESNP.

## **INTRODUCTION**

Liver transplantation is a life-saving treatment for patients with end-stage liver disease, but organ shortage is a major challenge. In the UK, 11% of patients listed for a liver in 2020/21 died before receiving a transplant;<sup>1</sup> similarly in the USA, despite record numbers of transplants in 2023, over a thousand patients died on the waiting list.<sup>2</sup> Many potentially usable livers are not transplanted, particularly livers from donors following circulatory death (DCD). Of the 1429 deceased donors in the UK in 2022/23, only 850 (59%) livers were transplanted (78% from brain-dead donors, 38% from DCD donors); similarly in the US in 2022, of the 14905 deceased donors, only 8924 (60%) livers were transplanted, with livers from DCD donors most likely to be declined or not recovered.<sup>2,3</sup>

Targeting these unused donor organs is a necessity if waiting list mortality is to be reduced.

In recent years initiatives to increase organ utilisation have concentrated on improving the preservation of organs and providing a means of evaluating function before transplantation. These have largely focused on continuous perfusion of organs, either in the cold with oxygenated preservation solutions or at normothermia using a red cell-based perfusate. Recent randomised studies have shown the efficacy of both approaches,<sup>4,5</sup> particularly in improving early allograft function. Studies using *ex situ* normothermic perfusion (ESNP) devices have enabled acceptable clinical outcomes from livers initially declined for transplantation by conventional criteria.<sup>6,7</sup>

ESNP provides a platform to assess and potentially recondition organs, thus increasing utilisation.<sup>4</sup> The molecular processes occurring in liver grafts during this process remain to be fully elucidated, although early studies have suggested a reduction in markers of ischaemia/reperfusion injury in recipients of such grafts.<sup>8</sup> More generally, ESNP enables controlled organ reperfusion following a period of ischaemia during cold storage, providing an opportunity to remove pro-inflammatory, cell damage-associated molecules and donor leucocytes that can further exacerbate organ inflammation.<sup>9</sup>

These donor leucocytes may be trapped within the graft microcirculation or may be tissue-resident passenger immune cells that mobilise into the perfusion circuit during ESNP.<sup>10–13</sup> To date, there is no information on whether or how leucocyte depletion during ESNP affects the molecular pathways activated in livers during this process.

Here we sampled human livers retrieved for transplantation at the start, during and end of perfusion, in the presence and absence of a leucocyte filter. We applied bulk RNA

sequencing to profile cellular responses to ESNP and to determine the effect of leucocyte depletion.

## **METHODS**

### ***Study conduct***

The study was conducted in accordance with both the Declarations of Helsinki and Istanbul. The study was approved by the Cambridge East research ethics committee (14/EE/0137).

### ***Study subjects and protocol***

Donor livers offered for research or transplantation were recruited to the study and underwent ESNP using either the Liver Assist (XVIVO, Groningen, Netherlands) or, latterly, the Metra (Organox, Oxford, UK). This included 23 DCD livers and 8 DBD livers (**Figure S1A**). In 7 DCD livers, a cell filter was added to the perfusion circuit (**Figure S1A**). The perfusate comprised third-party packed red cells suspended in Gelofusine (B. Braun Medical, Sheffield, UK), with additives and infusions of heparin, insulin and epoprostenol (and bile salts with the Metra) continued throughout perfusion, as previously described.<sup>6</sup> Needle-core biopsies were taken prior to commencing ESNP in all livers, but at were variably performed at the end of perfusion (**Fig. S1A**). Biopsies were stored in RNALater (Ambion). All samples underwent RNA extraction and sequencing. The perfusate was sampled at intervals during perfusion, centrifuged, and the supernatant ("Plasma") frozen at -20°C. Cytokine levels were measured by The Core Biochemical Assay Laboratory, Cambridge using MesoScale Discovery Human 10-plex Chemokine and Proinflammatory Cytokine panels.

## ***Study design***

This study comprised five experimental comparisons:

1. Comparative gene expression analysis of paired pre- and post-ESNP DCD liver biopsies. N=8 paired samples were available. **(Fig. 1A, Table 1)**.
2. Comparative gene expression analysis of pre-ESNP liver biopsies in n=16 DCD and n=8 DBD liver samples. **(Fig. 4A, Table 1-2)**.
3. Comparative gene expression analysis of pre-perfusion biopsies in 16 DCD livers, comparing samples with a high and low 2h perfusate alanine transaminase levels **(Fig. 5A, Table 2)**.
4. Comparative gene expression analysis of pre-perfusion biopsies in 8 DBD livers, comparing samples with a high and low 2h perfusate alanine transaminase levels **(Fig. 5B, Table 2)**.
5. Comparative gene expression analysis of n=7 paired pre- and post-ESNP DCD liver biopsies. with the addition of a Pall LeukoGuard LG6 leukocyte filter **(Fig. 6A, Table 3)**.

## ***Perfusion Analysis Based on ALT Levels***

Perfusate ALT levels were measured 2 hours following the start of perfusion for 16 DCD and 8 DBD livers **(Table 2)**. The thresholds that were used to assign livers into 'High' and 'low' ALT groups were established based on the level at which increased inflammatory and fibrotic gene expression pathways were observed. In DBD livers this occurred at an ALT of 1000 IU/L. For DCD livers, this was evident at an ALT of 2500

IU/L (see supplementary data **Fig. S1B**). Due to the difference in donor type, and exposure to warm ischaemia, we opted to have separate thresholds for DCD and DBD, rather than using a universal cutoff.

### ***RNA extraction***

RNA was extracted from biopsies stored in RNALater (Ambion) at -80°C. Biopsies were removed from their storage solution and placed with 1ml Lysis Buffer (Ambion) in a MK28-R grinder tube (Bertin Instruments) and lysed using a Precellys 24 homogeniser (Bertin Instruments). Tubes were subsequently centrifuged at 1500g for 4 minutes, the supernatant removed, and the RNA extraction performed using a pure link RNA mini kit (Ambion) as per manufacturer's instructions. Contaminating DNA was removed using TURBO DNase (Ambion) as per manufacturer's instructions. Concentration of RNA was assessed using a Nanodrop Spectrophotometer (Thermo Scientific). Quality of RNA was assessed using an RNA nano Bioanalyzer kit (Agilent) using a Bioanalyzer 2100 (Agilent).

### ***RNA sequencing***

0.5µg of RNA was used for producing libraries for sequencing using TruSeq Stranded total RNA library prep kit (Illumina) as per manufactures instructions with a final PCR amplification of 14 cycles. Libraries were then sequenced on a Hiseq 4000 sequencer (Illumina) by Genewiz.

### ***RNA sequencing analysis***

Following sequencing, data were demultiplexed to give individual fastq files using Casava (Illumina). Fastq files were assessed for quality control purpose using FASTQC.

The Fastq files were aligned to the human genome (Hg38) using Hisat2<sup>16</sup>. All further analysis was carried out using the R statistical environment. A table of gene counts was produced using the featureCounts function within Rsubread and normalisation and differential gene expression analysis was carried out using DESeq2. For GSEA, genes were ranked by the inverse of the p-value with the sign of the log fold change and then run against the hallmarks database within MSigDB using the GSEA program from the broad with the pre ranked option. All batch information, Fastq files, count tables and differential expression results have been submitted to the Gene Expression Omnibus under accession number GSE245976.

### ***Data visualization:***

The ggplot2 package in R was employed for data visualization. This enabled the creation of various plots and heatmaps using pheatmap, to examine the distribution of normalized gene expression values across samples or conditions and assess variance within our dataset. Differential gene expression results were visualized through Volcano plots using the EnhancedVolcano package. Custom modifications were applied to highlight and annotate genes of significance based on fold change and p-value thresholds. Further refinements in visualization aesthetics were performed using ggplot2.

Complete code and further details for all visualizations are available upon request.

### ***String analysis***

The top 100 significant genes, ranked by log fold change for the effect of perfusion, were used to run STRING analysis of functional associations between proteins. Gene

names were converted into protein names and two proteins were considered connected if they had a mean interaction score of >0.9 (highest confidence). Unconnected proteins were removed from the network and a force directed graph plotted. Network was subsequently clustered using k-means clustering and annotated using interpretation of GO terms associated with each cluster.

## RESULTS

### ***ESNP associated with increased expression of clotting factor and regulator genes***

Eight DCD livers (see **Table 1** for donor characteristics) underwent ESNP as described previously.<sup>6</sup> When comparing the two-time points, we found a substantial change in the transcriptome of DCD livers during perfusion, with more than 3600 differentially expressed genes (DEG), including ~2100 that were significantly upregulated and ~1550 significantly down-regulated (**Fig. 1A**).

The two most significantly upregulated genes during ESNP were *SERPINE1* and *THBS1* (**Fig. 1B**). *THBS1* encodes Thrombospondin 1, a protein that promotes thrombin-induced platelet aggregation.<sup>14</sup> *SERPINE1* encodes plasminogen activator inhibitor-1 (PAI-1), a member of the serine proteinase inhibitor superfamily and the principal inhibitor of tissue plasminogen activator (tPA) and urokinase, both mediators of fibrinolysis through plasmin (**Fig. 1C**). To probe this further, we assessed the expression of genes encoding clotting factors and regulators of thrombosis and fibrinolysis. Comparing post- with pre-perfusion samples, several pro- and anti-thrombotic/fibrinolytic proteins were significantly differentially expressed (**Fig. 1C-D**), including increased expression of *SERPINE2*, encoding PAI-2, which also acts as an inhibitor of tPA and urokinase. However, *PLAU* and *PLAT* (encoding urokinase and tPA

respectively) expression also increased during ESNP (**Fig. 1C-D**), although to a lesser magnitude than *SERPINE1*. Interestingly, despite the observed alterations in the expression of several thrombotic and fibrinolytic genes post-perfusion, the levels of plasminogen (*PLG*) and the fibrinogen chain genes (*FGA*, *FGB*, and *FGG*) were downregulated, albeit not to a statistically significant level.

### ***Increased expression of neutrophil and monocyte-recruiting chemokines and pro-repair transcripts***

A number of immunologically important transcripts were also amongst the most upregulated genes in DCD livers during ESNP, including the neutrophil recruiting chemokine *CXCL8*, the monocyte-recruiting chemokine *CCL2*, and the pro-inflammatory cytokine interleukin (*IL*)6 (**Fig. 1B**). Several other chemokine (**Fig. 2A**) and cytokine (**Fig. 2B**) transcripts were also significantly upregulated during ESNP, including *CXCL10*, *TNF*, *IL1B*, and *OSM*, the latter encoding oncostatin-M, a cytokine previously shown to drive liver fibrosis by activating macrophages to secrete  $TGF\beta$  in murine models.<sup>15</sup>

There was also an increase in key inflammasome genes (*NLRP3* and *P2RX7*) in DCD livers following perfusion (**Fig. 2C**). Sequential assessment of perfusate samples during ESNP confirmed an increase in *CXCL8* (IL8), *CXCL10*, *CCL2*, *IL6*, and *IL-1 $\beta$*  proteins (**Fig. 2D**).

We next used STRING analysis to interrogate known and predicted interacting proteins among upregulated transcripts. This identified several interaction nodes, including an immune node, with *IL6*, *CXCL8*, *CCL20*, and *CSF3* (encoding G-CSF a cytokine that promotes neutrophil production and maturation). Other immune nodes included

*TNFRSF9-TNFSF9* (encoding 4-1BB and 4-1BB-L, a co-stimulatory axis driving T cell proliferation, IL-2 secretion, survival and cytolytic activity) (**Fig. 3A**). There were also nodes comprising genes associated with tissue remodeling (*MMP1/10*, *TIMP1*) and repair (*AREG/EREG*), (**Fig. 3A**).

There was an increase in several additional potential pro-repair or protective transcripts, including *TSLP*, encoding thymic stromal lymphopoietin (TSLP), an epithelial-derived cytokine (**Fig. 2B**). Administration of exogenous recombinant TSLP significantly reduced liver damage in a mouse model of ischaemia-reperfusion injury (IRI), whereas deletion of *TSLPR* or neutralization of TSLP with anti-TSLP antibody exacerbated liver injury and necrosis.<sup>16</sup> In addition, several heat shock proteins (HSPs) also significantly increased during ESNP (**Fig. 3B**), including *HSPB1* (*HSP27*), the over-expression of which has been shown to protect murine kidney, heart and liver cells from apoptosis in the context of IRI.<sup>17,18</sup>

Gene set enrichment analysis (GSEA) also indicated increases in both immune/inflammatory and repair/proliferation pathways during ESNP. Enriched immune pathways included 'TNF $\alpha$  signalling via NF $\kappa$ B', 'inflammatory response', 'IL2/STAT5 signalling', 'IL6/JAK/STAT3 signalling' genesets, together with those indicative of cell proliferation, ('Myc genes') and 'epithelial-mesenchymal transition' (**Fig 3C**). Decreased enrichment of 'oxidative phosphorylation' (OXPHOS) pathway genes, which play a crucial role in cellular adenosine triphosphate (ATP) generation,<sup>19</sup> was observed in DCD livers during ESNP, as well as 'bile acid metabolism' genesets (**Fig. 3C**).

### ***DBD and DCD livers show transcriptional differences***

ESNP has principally been used in DCD livers but we also applied perfusion to n=8 DBD livers based on sub-optimal clinical profiles (**see Table 2**). Pre-perfusion biopsies were available on all 8 DBD livers, although no post-perfusion samples. We therefore compared the baseline gene expression in these DBD livers with pre-perfusion biopsies in 16 DCD livers (**Fig. 4A**). GSEA showed an enrichment of several metabolic gene pathways in DCD livers, including 'fatty acid metabolism', whilst several immune-related pathways were enriched in DBD livers, as well as 'epithelial-mesenchymal transition' pathway genes (**Fig. 4B**). These data show that DBD and DCD livers are transcriptionally distinct post-retrieval and cold storage, likely reflecting the effects of increased warm ischaemia in DCD donors.

### ***Molecular correlates of perfusion and clinical parameters***

To investigate transcriptional features that might associate with unfavourable outcomes, we assessed the gene expression profile present in pre-perfusion biopsies taken from livers that subsequently had an elevated perfusate alanine transaminase (ALT) concentration, a biomarker associated with hepatocyte injury,<sup>20</sup> and previously identified by us as a key marker of "viability".<sup>21</sup>

Perfusate ALT levels measured 2 hours post-perfusion were available for 16 DCD and 8 DBD livers that had undergone ESNP (**Table 2, Fig. 5A-B**). In DCD livers, GSEA comparing n=10 livers with ALT levels greater than 2500IU/L with n=6 <2500IU/L revealed significant enrichment of immune activation genesets, including 'TNF $\alpha$  signaling via NF $\kappa$ B', 'interferon gamma response', and 'inflammatory response' (**Fig. 5C**). For DBD livers, where perfusate ALT levels were generally lower, the genesets

'interferon-gamma response', 'interferon-alpha response', and 'allograft rejection' were significantly enriched in livers with ALT levels exceeding 1000 IU/L (**Fig. 5D**).

Additionally, both DCD and DBD livers with higher ALT levels showed a marked decrease in 'oxidative phosphorylation' genes (**Fig. 5C-D**).

Only 16 genes were significantly differentially expressed in DCD livers with a higher perfusate ALT (>2500) compared to those with a lower ALT (<2500) (**Fig. 5E**).

Upregulated genes included *DEFB132*, (encoding the antimicrobial peptide defensin beta 132), the uncharacterised gene *LOC400655*, and *NR4A1* encoding nuclear receptor subfamily 4A1, a transcription factor expressed in Kupfer cells and implicated in a caspase6/SOX9 signalling axis associated with hepatic inflammation and pyroptosis in ischaemic fatty livers.<sup>22</sup> Eleven genes were significantly differentially expressed in DBD livers with a high perfusate ALT (**Fig. 5F**). Among those, *IL32* and *FGF19* were significantly upregulated. *IL32* is of particular interest, given its recent description as the most upregulated transcript in severe metabolic dysfunction-associated steatotic liver disease (MASLD) livers compared with controls, with circulating levels of *IL32* associated with both the presence and severity of MASLD independently of ALT.<sup>23</sup> Similarly, *FGF19* mRNA expression was 7-fold greater in cirrhotic livers compared with non-cirrhotic livers, with increased liver and serum levels of fibroblast growth factor 19 (*FGF19*) also noted in these patients with primary biliary cholangitis. *FGF19* acts to inhibit the synthesis of potentially toxic bile acids.<sup>24,25</sup> Taken with the literature on the roles of these molecules outside of the transplant context, our results highlight the potential for *IL32* and *FGF19* as biomarkers of sub-optimal DBD livers.

### ***Leucocyte removal attenuates perfusion-associated transcriptional changes***

Several factors may contribute to the increase in inflammation and immune-related genesets we observed during ESNP including the release of pro-inflammatory cell damage-associated molecules that can further exacerbate the induction of inflammatory genes, (as we have demonstrated in the kidney<sup>9</sup>). In addition, leucocytes trapped within the graft microcirculation, as well as tissue-resident immune cells may mobilise into the perfusion circuit.<sup>26</sup> These include neutrophils and monocytes, with the capacity to propagate inflammation and cause collateral damage.<sup>13</sup> We therefore hypothesised that removal of leucocytes from the perfusion circuit may attenuate the transcriptional changes occurring during perfusion. To test this, we added a leucocyte filter to the arterial limb of the perfusion circuit in 7 DCD livers, sampling pre- and post-ESNP (**Fig. 6A, Table 3**). The reduction in circulating leukocytes reduced perfusion-associated transcriptional changes, with a 6-fold decrease in the number of DEG compared with DCD livers undergoing ESNP without a leucocyte filter (~600 DEG compared with ~3600) (**Fig. 6A**). Furthermore, the addition of a leucocyte filter changed the nature of the geneset enrichment occurring during perfusion compared with ESNP alone, leading to a decrease in 'TNF $\alpha$  signalling via NF $\kappa$ B', 'epithelial-mesenchymal transition', and 'interferon gamma and alpha response', pathway genes (**Fig. 6C**). When comparing the DEG between ESNP with and without a leucocyte filter, only 11 genes were significantly differentially expressed, including downregulation of RAET1E (encoding an NKG2D ligand that can trigger NK cell mediated cytotoxicity)<sup>27</sup> and *IL1RL*, a component of the receptor for IL33, a proven driver of liver fibrosis in mouse models (**Fig. 6B**).<sup>28</sup> There was a significant increase in OXPHOS and several metabolism-associated genesets, and a decrease in 'TNF $\alpha$  signalling via NF- $\kappa$ B', 'TGF beta signalling' and 'epithelial-

mesenchymal transition' genesets in livers undergoing ESNP with a leukocyte filter compared to those without (**Fig. 6D, E**). Consistent with an anti-inflammatory effect of leucocyte removal, the level of TNF and IL1B was lower in perfusate samples in the presence of a cell filter (**Fig. 6F, S2**).

## DISCUSSION

The common reasons that livers are declined for transplantation are the unpredictability of function and high incidence of cholangiopathy of DCD donor livers, and concern over steatotic livers with their severe reperfusion injury and higher incidence of primary non-function. Thus, to increase the utilisation of donor livers there is a strong imperative to understand the molecular events occurring during liver reperfusion *in vivo*, for which reperfusion on ESNP is a model, with a view to developing treatments that can ameliorate or prevent reperfusion injury, improve early allograft function and prevent cholangiopathy.

One of the most upregulated genes during ESNP was *SERPINE1*, with *SERPINB2* also showing a significant increase, which encode plasminogen activator inhibitor 1 and 2 (*PAI-1* and *PAI-2*) respectively. These proteins that act as to inhibit tPA and urokinase, the two serine proteases required for the conversion of plasminogen to plasmin, a key fibrinolytic. Increased expression of *PAI1* has been associated with fibrin accumulation in hypoxic lungs in mice,<sup>29</sup> and may mechanistically underpin the peribiliary vascular fibrin microthrombi described in livers undergoing ESNP.<sup>30,31</sup> We also observed an increase in pro-fibrinolytic factor expression, specifically *PLAU* and *PLAT* encoding urokinase and tPA respectively, although to a lesser magnitude and significance than that observed for *SERPINE1*. Whether such differences in the magnitude of

transcriptional changes of these pro- and anti-fibrinolytics are mirrored at the protein level is an outstanding question that needs to be addressed in order to assess their functional relevance, and whether the net effect of the changes occurring during ESNP promote the persistence of fibrin microthrombi. We did not have access to pre-retrieval biopsies, and were therefore unable to assess whether the levels of these genes were upregulated following warm ischaemia or during cold storage.

We have identified the upregulation of several immune genes that may contribute to the inflammatory response that characterises reperfusion injury during ESNP. Predominant among these were *CXCL8* and *CCL2*, recruiting neutrophils and monocytes respectively, and *IL6*. The latter observation is of particular interest given a recent study suggesting that the transcriptional profile of neutrophils changes during liver perfusion towards an activated or exhausted state.<sup>13</sup> The increase in these transcripts was also reflected at a protein level when measured in the liver perfusate. Altogether, these findings are consistent with observations by Hautz et al,<sup>13</sup> who reported neutrophil and monocyte mobilisation into the perfusate during NMP, as well as high IL6 levels, particularly in less-than-ideal grafts, such as those from DCD donors. Our analysis also identified a significant upregulation of GCSF, the cytokine known to drive neutrophil maturation and mobilisation from the bone marrow. This local production of GCSF within the liver may contribute to the change in neutrophil maturation/activation state occurring during liver perfusion, described by Hautz and colleagues.<sup>13</sup> Additionally, the NLRP3 inflammasome plays a critical role in the inflammatory response during ESNP by acting as a major hub to generate IL-1 $\beta$  in response to damage-associated molecular patterns (DAMPs).<sup>32</sup> Indeed, perfusate IL-1 $\beta$  has been proposed as a potential prognostic biomarker in lungs undergoing ESNP.<sup>33</sup> Of note, we found that addition of a

leucocyte filter to the perfusion circuit reduced perfusate IL-1 $\beta$  and TNF. TNF has been shown to inhibit oxidative phosphorylation via effects on cytochrome c oxidase<sup>34</sup> and IL1R signalling reprogrammes metabolic activity in some cell types, suppressing oxidative phosphorylation and fatty acid oxidation.<sup>35</sup> Therefore, the reduction of these cytokines in perfusate in livers perfused with a cell filter may be of direct mechanistic relevance to the observed increase in OXPHOS and Fatty acid oxidation genes observed in these livers.

In the DCD livers perfused without a cell filter, OXPHOS pathway genes decreased during perfusion, despite the provision of oxygen in the perfusate. One explanation may be that during cold ischaemia, purine nucleotides are degraded such that there is no longer substrate from which to generate ATP.<sup>36</sup> Ischaemia also causes the accumulation of succinate at complex 1 of the respiratory chain of mitochondria and on reperfusion this promotes production of reactive oxygen species which destroy the mitochondria, reducing the ability for oxidative phosphorylation.<sup>37</sup> Another possibility relates to an observation that we reported previously, and have encountered several times since, where the perfusate at the start of ESNP has a glucose concentration under 10mmol/L, in contrast to most livers where the glucose is high and rises further over the first 1 to 2 hours before falling.<sup>38</sup> A possible explanation for this phenomenon is that patients on Neurosurgical Intensive Care Units who do not tolerate enteral feeding, do not receive supplementary parenteral nutrition for the first few days of their admission, since this is deleterious to neurological recovery. As a consequence, hepatic glycogen stores are depleted, such that during cold storage there is little or no available glucose with which to generate ATP to maintain cell viability.<sup>36</sup>

One of the challenges in assessing livers undergoing ESNP is to identify those livers at risk of primary non-function and early graft failure. In our previous analysis of parameters during liver ESNP, we identified transaminase release and lactate metabolism as two key factors.<sup>21</sup> Transcriptomic analysis revealed that livers sustaining more hepatocellular damage during cold storage and reperfusion, as demonstrated by the more elevated ALT levels at 2 hours, showed enrichment of interferon-gamma response pathway genes, but a decrease in expression of oxidative phosphorylation genes, the latter possibly secondary to mitochondrial dysfunction/destruction and associated cytolysis, as indicated by the transaminase release. Among individual genes that were significantly differentially expressed in high ALT livers, we identified a number of putative mechanistic biomarkers that will require further study, including *IL32* and *FGF19* in DBD livers and *NR4A1* in DCD livers, supported by orthogonal literature on their role in liver IRI or fibrosis.<sup>23–25</sup>

Our study has a number of limitations. Firstly, the number of livers and samples analysed in this study, while providing valuable initial insights, is relatively small. In addition, the timing of sampling the liver and perfusate was variable, which may have caused some variation in quantitative changes in gene expression although it is likely that there is no qualitative difference. In addition, we do not have tissue protein analysis to support transcriptomic observations. Lastly, as ESNP became standard of care for marginal livers in our practice, and for donor and logistic indications, we changed from the Liver Assist to the Organox metra ESNP device. The main differences in liver perfusion are that the perfusate volume was 500ml greater in the Liver Assist, and an infusion of taurocholic acid bile salts was given on the Organox Metra. However, we did not perform a comparison of the differential effects of these two devices on the

transcriptional changes occurring during ESNP due to the substantial confounding introduced by the change in selection practices for ESNP livers over time.

In summary, our work defines the molecular changes occurring during ESNP. It demonstrates a substantial shift in the transcriptome of DCD livers during reperfusion after cold storage, with the induction of several potentially pro-inflammatory genes, chemokines, and tissue remodeling/repair genes, as well as the generation of a potentially anti-fibrinolytic state. It also highlights the potential therapeutic benefits of including a leukocyte filter within the perfusion circuit. The use of such filters could mitigate some of the adverse inflammatory responses observed, offering a more stable environment that promotes repair and regeneration, ultimately enhancing the viability and function of the graft.

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**Table 1. Donor Liver Characteristics livers undergoing ESNP**

| Donor | Donor Age | DBD/DCD | Sex (M/F) | Perfusate 2h ALT | Machine       | Lactate (2h) | CIT (hrs) | Transplanted? | Reason not transplanted   | Graft Survival (days) | Patient Survival (days) | Post Bx (hrs) |
|-------|-----------|---------|-----------|------------------|---------------|--------------|-----------|---------------|---------------------------|-----------------------|-------------------------|---------------|
| 1     | 35        | DCD     | M         | 2648             | Liver assist  | <0.1         | 5.4       | No            | Adverse appearance        | N/A                   | N/A                     | 4             |
| 2     | 59        | DCD     | M         | 4909             | Liver assist  | 0.9          | 5.1       | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 3     | 76        | DCD     | M         | 1573             | Liver assist  | 0.5          | 10.2      | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 4     | 47        | DCD     | F         | 5386             | Liver assist  | 6.8          | 11.4      | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 5     | 22        | DCD     | M         | 4332             | Organox Metra | 0.8          | 5.5       | Yes           | -                         | 1387                  | 1387                    | 11            |
| 6     | 45        | DCD     | F         | 3336             | Organox Metra | 0.6          | 7.2       | No            | Bile chemistry            | N/A                   | N/A                     | 6             |
| 7     | 49        | DCD     | M         | 3972             | Organox Metra | 0.9          | 6.5       | No            | Poor function             | N/A                   | N/A                     | 7             |
| 8     | 55        | DCD     | M         | 1361             | Organox Metra | 0.2          | 8.9       | Yes           | -                         | 1057                  | 1057                    | 8             |

Key: DBD: Donation after brain death; DCD: Donation after circulatory death; M/F: Male/Female; ALT: Alanine transaminase; CIT: Cold ischemic time; Post Bx: Timing of biopsy following initiation of perfusion

**Table 2. Donor Liver Characteristics (clinical parameters)**

| Donor | Donor Age | DBD/DCD | Sex (M/F) | Perfusate 2h ALT | Machine       | Lactate concn. (2h) | CIT (hrs) | Transplanted? | Reason not transplanted   | Graft Survival (days) | Patient Survival (days) |
|-------|-----------|---------|-----------|------------------|---------------|---------------------|-----------|---------------|---------------------------|-----------------------|-------------------------|
| 1     | 35        | DCD     | M         | 2648             | Liver assist  | <0.1                | 5.4       | No            | Adverse appearance        | N/A                   | N/A                     |
| 2     | 47        | DCD     | F         | 5386             | Liver assist  | 6.8                 | 11.4      | No            | Offered for research only | N/A                   | N/A                     |
| 3     | 59        | DCD     | M         | 4909             | Liver assist  | 0.9                 | 5.1       | No            | Offered for research only | N/A                   | N/A                     |
| 4     | 68        | DCD     | M         | 2658             | Organox Metra | 0.9                 | 8.5       | Yes           | N/A                       | 360                   | 1804                    |
| 5     | 46        | DCD     | F         | 4015             | Organox Metra | 0.4                 | 6.6       | Yes           | N/A                       | 1348                  | 1348                    |
| 6     | 22        | DCD     | M         | 4332             | Organox Metra | 0.8                 | 5.6       | Yes           | N/A                       | 1387                  | 1387                    |
| 7     | 20        | DCD     | M         | 3042             | Organox Metra | 0.8                 | 3.8       | Yes           | N/A                       | 1575                  | 1575                    |
| 8     | 40        | DCD     | F         | 3523             | Organox Metra | 0.9                 | 8.5       | Yes           | N/A                       | 18                    | 1869                    |
| 9     | 49        | DCD     | M         | 3972             | Organox Metra | 0.9                 | 6.5       | No            | Poor function             | N/A                   | N/A                     |
| 10    | 45        | DCD     | F         | 3336             | Organox Metra | 0.6                 | 7.2       | No            | Bile chemistry            | N/A                   | N/A                     |
| 11    | 76        | DCD     | M         | 1573             | Liver assist  | 0.5                 | 8.0       | No            | Offered for research only | N/A                   | N/A                     |
| 12    | 66        | DCD     | M         | 1799             | Liver assist  | 0.3                 | 7.5       | Yes           | N/A                       | 2511                  | 2511                    |
| 13    | 28        | DCD     | M         | 2348             | Organox Metra | <0.1                | 6.7       | Yes           | N/A                       | 1324                  | 1324                    |
| 14    | 54        | DCD     | F         | 1116             | Organox Metra | <0.1                | 6.7       | Yes           | N/A                       | 1801                  | 1801                    |

| Donor | Donor Age | DBD/DCD | Sex (M/F) | Perfusate 2h ALT | Machine       | Lactate concn. (2h) | CIT (hrs) | Transplanted? | Reason not transplanted   | Graft Survival (days) | Patient Survival (days) |
|-------|-----------|---------|-----------|------------------|---------------|---------------------|-----------|---------------|---------------------------|-----------------------|-------------------------|
| 15    | 52        | DCD     | M         | 1372             | Organox Metra | 0.2                 | 5.8       | Yes           | N/A                       | 1340                  | 1340                    |
| 16    | 55        | DCD     | M         | 1361             | Organox Metra | 0.2                 | 8.9       | Yes           | N/A                       | 1057                  | 1057                    |
| 17    | 53        | DBD     | F         | 1603             | Organox Metra | <0.1                | 7.0       | Yes           | N/A                       | 1723                  | 1723                    |
| 18    | 61        | DBD     | M         | 1840             | Organox Metra | <0.1                | 8.2       | Yes           | N/A                       | 57                    | 57†                     |
| 19    | 23        | DBD     | M         | 2212             | Organox Metra | 0.7                 | 9.6       | Yes           | N/A                       | 2                     | 2†                      |
| 20    | 63        | DBD     | M         | 1906             | Liver assist  | 0.8                 | 5.9       | Yes           | N/A                       | 2234                  | 2234                    |
| 21    | 81        | DBD     | M         | 346              | Liver assist  | <0.1                | 7.8       | No            | Offered for research only | N/A                   | N/A                     |
| 22    | 27        | DBD     | M         | 199              | Organox Metra | <0.1                | 7.7       | Yes           | N/A                       | 1891                  | 1891                    |
| 23    | 37        | DBD     | F         | 639              | Organox Metra | <0.1                | 5.1       | Yes           | N/A                       | 1782                  | 1782                    |
| 24    | 28        | DBD     | F         | 528              | Organox Metra | <0.1                | 12.1      | Yes           | N/A                       | 1485                  | 1485                    |

Key: DBD: Donation after brain death; DCD: Donation after circulatory death; M/F: Male/Female; ALT: Alanine transaminase; CIT: Cold ischemic time; N/A: Not applicable. Lactate concentration units: mmol/L. †: indicates patient died.

**Table 3. Donor Liver Characteristics (addition of leukocyte filter)**

| Donor | Donor Age | DBD/DCD | Sex (M/F) | Perfusate 2h ALT | Machine      | Lactate concn. (2h) | CIT (hrs) | Transplanted? | Reason not transplanted   | Graft Survival (days) | Patient Survival (days) | Post Bx (hrs) |
|-------|-----------|---------|-----------|------------------|--------------|---------------------|-----------|---------------|---------------------------|-----------------------|-------------------------|---------------|
| 1     | 66        | DCD     | F         | 2439             | Liver assist | 10.1                | 8.7       | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 2     | 68        | DCD     | M         | 3623             | Liver assist | 0.7                 | 12.8      | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 3     | 69        | DCD     | M         | 4088             | Liver assist | 3.6                 | 11.2      | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 4     | 48        | DCD     | F         | 694              | Liver assist | <0.1                | 8.0       | No            | Offered for research only | N/A                   | N/A                     | 4             |
| 5     | 28        | DCD     | F         | 216              | Liver assist | <0.1                | 4.9       | Yes           | N/A                       | 2487                  | 2487                    | 4             |
| 6     | 54        | DCD     | M         | 262              | Liver assist | <0.1                | 7.3       | Yes           | N/A                       | 2478                  | 2478                    | 4             |
| 7     | 69        | DCD     | F         | 1631             | Liver assist | 0.9                 | 5.5       | Yes           | N/A                       | 2367                  | 2367                    | 5             |

Key: DBD: Donation after brain death; DCD: Donation after circulatory death; M/F: Male/Female; ALT: Alanine transaminase; CIT: Cold ischemic time; Post Bx: Timing of biopsy following initiation of perfusion; N/A: Not applicable. Lactate concentration units: mmol/L.

## FIGURE LEGENDS

**FIGURE 1.** Significant changes in gene expression in livers undergoing ex situ normothermic perfusion (ESNP). **A.** Bar chart displaying differentially expressed genes (DEG) of paired biopsy samples collected from eight donation after circulatory death (DCD) livers both at the start and end of ESNP. Genes denoted in red indicate upregulation and those in blue represent downregulation. **B.** Volcano plot representing the change in gene expression following 5 hours of ESNP, with the top significantly (adjusted p-value <0.05) upregulated and downregulated genes labelled. **C.** Schematic of the coagulation and fibrinolysis pathways. Gene names are highlighted to show relevant trends in the experiment. The term 'platelet aggregation' represents the primary haemostasis process. Genes related to clot breakdown are boxed in red, while those related to clot formation are boxed in blue. Arrows alongside gene names indicate the log2 fold change (L2FC) in gene expression: one arrow represents an L2FC greater than 1; two arrows, an L2FC greater than 2; and three arrows, an L2FC greater than 3. **D.** Heatmap of clustered genes related to the coagulation and fibrinolysis pathways expressed post-ESNP, all genes exhibit a significant differential expression (adjusted p-value < 0.05) and are highlighted in green with their relevant role in the coagulation process highlighted in yellow (breakdown) or teal (formation).

**FIGURE 2.** Significant inflammatory changes seen in gene expressions of donation after circulatory death (DCD) livers undergoing ex situ normothermic perfusion (ESNP). **A-B.** Heatmap of all human chemokines and cytokines expressed post-ESNP, with genes exhibiting significant differential expression (adjusted p-value < 0.05) highlighted in green and clustered by rows. **C.** Heatmap of selected, curated inflammasome genes

post-ESNP, with genes exhibiting significant differential expression (adjusted p-value < 0.05) highlighted in green and clustered by rows. **D.** Scatter plot displaying the overall increase in cytokine levels measured in the perfusate up to 240 minutes of ESNP, with individual cytokines showing varied patterns, including some peaking at 120 minutes. Error bars represent their respective interquartile ranges (IQR).

**FIGURE 3.** Transcriptomic changes associated with increased inflammatory and fibrotic shifts in donation after circulatory death (DCD) livers undergoing ex situ normothermic machine perfusion. **A.** STRING analysis of the top 100 significantly upregulated genes. **B.** Heatmap representing the differential expression of all heat-shock protein (HSPs) genes during ESNP. Differentially expressed genes showing significant upregulation (adjusted p-value < 0.05) are highlighted in green and clustered by rows. **C.** Gene Set Enrichment Analysis (GSEA) comparing DCD livers undergoing ESNP against the Hallmark dataset. Pathways showing increased activity post-ESNP are highlighted in red (enriched), and those with reduced activity are marked in blue (depleted) - all gene-sets exhibit a false discovery rate (FDR) q-value of <0.05. Pathways are organized along the y-axis, and the normalized enrichment score (NES) is displayed on the x-axis. **D.** Running enrichment scores (RES) of the TNF-alpha via NFkB gene-set from the Hallmarks dataset, comparing pre and post-perfusion samples of DCD livers with a leukocyte filter. The increased activity against its gene-rank is denoted in red.

**FIGURE 4.** Transcriptional differences observed in donation after circulatory death (DCD) and donation after brain death (DBD) livers prior to undergoing ex situ

normothermic machine perfusion (ESNP). **A.** Bar graph showing the number of significantly differentially expressed genes (DEGs) between DCD (N=16) and DBD (N=8) livers before ESNP. The red bar represents upregulated genes and the blue bar represents downregulated genes. **B.** Gene Set Enrichment Analysis (GSEA) comparing DCD to DBD livers before undergoing ESNP against the Hallmark dataset. Pathways with increased activity in DCD livers are highlighted in red (enriched in DCD), while pathways with reduced activity are marked in blue (enriched in DBD). The size of each dot corresponds to the significance of the enrichment, with larger dots representing a lower false discovery rate (FDR) q-value, all of which are  $<0.05$ . Pathways are organized along the y-axis, and the normalized enrichment score (NES) is displayed on the x-axis.

**FIGURE 5.** Analysis of transcriptomic profiles and corresponding perfusate ALT levels in DCD and DBD livers during ESNP. **A.** Bar chart displaying differentially expressed genes comparison of pre-perfusion samples from DCD livers with a perfusate ALT level greater than 2500 to those with ALT levels less than 2500. Genes denoted in red indicate upregulation and those in blue represent downregulation. **B.** Comparison of pre-perfusion samples from DBD livers with a perfusate ALT level greater than 1000 to those with ALT levels less than 1000. **C-D.** Gene Set Enrichment Analysis (GSEA) comparing DCD and DBD livers with elevated perfusate ALT levels against the Hallmarks dataset. Pathways with increased activity seen with increased ALT levels are highlighted in red (enriched in high ALT), and those with reduced activity are marked in blue (enriched in low ALT), with significance defined as a false discovery rate (FDR) q-value of less than 0.05. Pathways are organized along the y-axis, and the normalized

enrichment score (NES) is displayed on the x-axis. **E-F.** Volcano plots showing changes in gene expression in DCD and DBD livers with elevated perfusate ALT levels respectively. Genes manifesting significant differential expression (adjusted p-value < 0.05) are marked in red.

**FIGURE 6.** Impact of a leukocyte filter on the transcriptomic profiles during ESNP of DCD livers. **A.** Bar chart displaying differentially expressed genes in paired pre- and post-perfusion samples from seven DCD livers with the addition of a leukocyte filter. Genes denoted in red indicate upregulation and those in blue represent downregulation. **B.** Volcano plot showcasing changes in gene expression following 4 hours of ESNP with the leukocyte filter compared to ESNP alone as an interaction term (measuring the difference of differences). The most significant (adjusted p-value < 0.05) upregulated and downregulated genes are labelled and marked in red. **C.** Gene Set Enrichment Analysis (GSEA) comparing pre and post-perfusion samples from DCD livers during ESNP with a leukocyte filter against the Hallmark dataset. Pathways with increased activity post-ESNP are highlighted in red (enriched), and those with reduced activity are indicated in blue (depleted), with significance defined by a false discovery rate (FDR) q-value less than 0.05. Pathways are organized along the y-axis, and the normalized enrichment score (NES) is displayed on the x-axis. **D.** GSEA contrasting DCD livers during ESNP with the leukocyte filter as an interaction term against the Hallmarks dataset. **E.** Running enrichment scores (RES) for the oxidative phosphorylation gene-set from the Hallmarks dataset, comparing pre and post-perfusion samples of DCD livers with a leukocyte filter. The increased activity against its gene-rank is denoted in red. **F.** Scatter plot displaying the overall mean increase in cytokine levels of IL-1 $\beta$  and

TNF $\alpha$  measured in the perfusate of livers undergoing Ex Situ Normothermic Perfusion (ESNP) with and without a leukocyte filter over 250 minutes. Both cytokines exhibit a steady increase over time, with a clear reduction observed in samples using a leukocyte filter. Error bars represent the interquartile range (IQR) for each time point.