

## **Investigating metabolic and protease dysregulation associated with ischaemia in kidney cells**

### **Background**

Kidney transplantation remains the gold-standard treatment for end-stage kidney disease, offering substantial benefits in patient survival, quality of life, and healthcare costs. Unfortunately, the demand for donor kidneys far exceeds availability, due to a shortage of suitable organs for transplantation.

In the UK, the utilisation of kidneys from donors after circulatory death (DCD) has increased substantially since the 1990s and now accounts for nearly half of all deceased-donor kidney transplants annually. Despite these gains, acceptance of DCD kidneys remains inconsistent, largely due to concerns regarding organ quality and both short- and long-term transplant outcomes. Warm ischaemia, defined as the period between withdrawal of life-sustaining treatment and the commencement of cold perfusion of the organ in situ, is an inherent feature of the DCD donation pathway. The duration of this period varies considerably between donors and is thought to contribute to variability in transplant outcomes and increased kidney discard rates.

Prolonged warm ischaemia, specifically functional warm ischaemia (fWIT; time when donor blood pressure drops below 50mmHg until cold perfusion), has consistently been linked to tissue injury, increased incidences of delayed graft function and extended posttransplant recipient hospitalisation. During this period, the donor's blood pressure drops, resulting in organ hypoperfusion and a state of hypoxia, causing a cellular energy crisis which is particularly detrimental to the kidney due to its high metabolic demand.

### **Preliminary work**

Previously, we have shown that kidney injury associated with posttransplant outcomes is donor-type specific. To investigate the mechanisms of injury specific to the DCD pathway, we previously profiled the degradome of 42 DCD kidney biopsies with prolonged fWIT. This work characterised changes in endogenous peptide abundance, biological pathways and protease activity associated with increasing ischaemic injury.

Using degradomics, we demonstrated that prolonged fWIT induces significant perturbations in protein degradation and metabolic pathways. The most notable findings involved disruption of key metabolic processes, particularly glucose metabolism, including increased degradation of fructose-1,6-bisphosphatase (FBP1), the rate-limiting enzyme in gluconeogenesis. As the kidney is a highly metabolically active organ, dependent on ATP-driven processes to maintain normal function, these

findings provided strong evidence that prolonged ischaemia results in substantial energy depletion and metabolic dysfunction. Pathway analysis further supported widespread disruption of metabolic pathways within DCD kidneys.

The significance of these metabolic findings prompted further investigation using a hypoxic *in vitro* model designed to mimic ischaemic stress and explore the mechanisms linking hypoxia to metabolic dysfunction. In parallel, degradomic analyses identified legumain (LGMN) as a protease whose activity increased progressively with prolonged fWIT. This was a particularly important finding, as it suggested that prolonged ischaemia not only alters metabolic pathways but also drives specific proteolytic processes that may contribute directly to kidney injury.

Building on these observations, subsequent work characterised LGMN within the kidney as well as our established *in vitro* model, demonstrating significant alterations in LGMN expression and abundance under ischaemic conditions. Importantly, these studies identified a previously uncharacterised link between LGMN dysregulation and glucose availability within proximal tubular epithelial cells (PTECs).

## Aims

This study aimed to investigate the impact of varying concentrations of glucose on kidney cells and evaluate changes under both normoxic and hypoxic conditions. Specifically, this study aimed to:

1. **Assess the effects of varying glucose concentrations on kidney cells cultured under normoxic and hypoxic conditions**, with samples collected at defined intervals over a 48-hour period.
2. **Measure metabolic changes specific to the culture conditions**, focusing on key metabolic pathways including glycolysis, gluconeogenesis, and mitochondrial function.
3. **Characterise changes in the expression, abundance, and activity of key proteases**, including Legumain and CATB. These proteases are known to play critical roles in cellular stress responses, and their regulation may be influenced by metabolic shifts in the kidney cells.
4. **Untargeted metabolomic analysis of DCD kidneys**, to identify metabolic alterations in DCD kidneys associated with prolonged fWIT. Specifically, profile differentially abundant metabolites and enriched metabolic pathways associated with increasing ischaemic stress.

## Results

Preliminary *in vitro* optimisation experiments showed that cell viability declined markedly by 48 hours when cells were cultured under glucose-free or hypoxic conditions. As this loss of viability compromised the reliability of the experimental model, all subsequent experiments were restricted to a maximum culture duration of 24 hours.

To further investigate the relationship between glucose availability and LGMN regulation, and to support previous findings relating to changes in LGMN expression and abundance, LGMN enzymatic activity was measured. Prior to experimental application, the assay underwent method validation using established protocols adapted for use within this model system.

For these experiments, proximal tubule epithelial cells (PTECs) and podocytes were cultured under normoxic (Norm) or hypoxic (Hypo) conditions in the presence or absence of glucose (+/-Gluc) for up to 24 hours. Samples were collected at 30 minutes, 1 hour, 3 hours, 6 hours, and 24 hours. LGMN activity was quantified by measuring fluorescence every 10 minutes over a 160-minute assay period.

In PTECs, LGMN activity showed a trend towards increased activity during the early stages of culture under hypoxic conditions, although these changes did not reach statistical significance. After 1 hour of culture, LGMN activity was significantly reduced in Hypo-Gluc cells compared with baseline Norm+Gluc controls ( $p = 0.006$ ). Reduced activity was also observed in glucose-deprived conditions through to 6 hours, indicating a strong early dependence of LGMN activity on glucose availability.

By 24 hours, however, hypoxia became the dominant factor influencing LGMN activity. At this time point, LGMN activity was significantly increased under both hypoxic conditions regardless of glucose availability. Two-factor analysis supported this temporal shift, demonstrating that glucose availability exerted the primary influence on LGMN activity during the early culture period, whereas hypoxia became the predominant driver following prolonged exposure.

These findings are consistent with our previous analyses of LGMN abundance and processing. Together, the data demonstrate that glucose deprivation suppresses LGMN abundance and activity in PTECs despite evidence of transcriptional compensation, highlighting a previously unrecognised metabolic dependence of LGMN regulation within the proximal tubule. Furthermore, the enhanced activity observed following prolonged hypoxic exposure suggests that oxygen tension may act as a secondary regulator of LGMN function during sustained cellular stress.

In contrast, LGMN activity in podocytes remained largely stable across all culture conditions during the early and intermediate time points. Significant changes were only observed following prolonged culture, with a marked reduction in LGMN activity detected in Hypo-Gluc podocytes at 24 hours ( $p < 0.001$ ). This decrease mirrored the reduction in LGMN protein abundance previously identified by Western blot analysis, further indicating that prolonged metabolic stress adversely affects LGMN regulation in podocytes.

Factor analysis supported this pattern, demonstrating that the influence of hypoxia on LGMN activity diminished over time, while glucose availability remained a key determinant of activity at the 24-hour time point. These findings suggest that podocytes exhibit a slower and more moderated response to metabolic perturbation compared with PTECs, with changes in LGMN activity emerging only after sustained exposure to

stress. Collectively, these data indicate cell type-specific regulation of LGMN, whereby PTECs respond rapidly to alterations in nutrient availability and oxygen tension, while podocytes appear more resistant to short-term metabolic challenge but are susceptible to functional decline during prolonged glucose deprivation under hypoxic conditions.

In addition to LGMN, further investigations were undertaken to evaluate proteins identified through our degradomic analysis. Cathepsin B (CATB) was prioritised and profiled in both DCD kidneys and our *in vitro* model prior to the commencement of this grant. In DCD kidneys, pro-CATB expression decreased with increasing fWIT, consistent with the reduction in CATB activity observed in the degradomic dataset. However, analysis of CATB in the *in vitro* model did not demonstrate clear changes in either protein abundance or gene expression in response to increasing ischaemia or glucose deprivation. These findings suggest that the reduction in CATB activity associated with prolonged fWIT may arise from alternative cellular mechanisms that are not reflected by changes in expression, or may indicate alterations in protease activation, processing, or regulation. More specifically, the observed pattern was more consistent with a general cellular response to nutrient deprivation and hypoxic stress than with disruption of a specific metabolic pathway. Consequently, CATB was deprioritised within the scope of the current project. Nevertheless, future studies will seek to develop methodologies capable of directly assessing CATB activity in both DCD kidneys and *in vitro* models, with the aim of elucidating the mechanisms underlying the changes identified in the degradomic analysis, specific to DCD donation.

Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor-1 (NHERF1) abundance and expression were also evaluated. Degradomic analysis indicated that NHERF1 degradation increased with increasing fWIT in DCD kidneys. Although primarily recognised as a cytoskeletal scaffolding protein, NHERF1 has also been implicated in the regulation of cellular architecture, inflammatory signalling, injury responses, and metabolic processes, including pathways linked to pentose phosphate metabolism. Consequently, considerable effort was directed towards further characterising NHERF1 alterations in both DCD kidneys and our *in vitro* cell models; however, these investigations were hindered by technical challenges. Western blot analysis failed to consistently detect NHERF1 at the expected molecular weight and did not produce reproducible results across biological replicates. Despite these limitations, no clear changes in NHERF1 expression were observed in podocytes. In contrast, NHERF1 expression in PTECs appeared to decline over time under hypoxic conditions, suggesting that prolonged oxygen deprivation may contribute to disruption of NHERF1-associated pathways and consequently impair cytoskeletal stability. While these findings provide preliminary evidence that hypoxia may influence NHERF1 regulation, further optimisation of analytical methods will be required to fully define its role in DCD-associated injury and metabolic dysregulation.

To further explore the metabolic consequences of glucose deprivation and hypoxia, a panel of metabolic markers related to glucose utilisation and energy metabolism was selected for analysis by qPCR and Western blotting. However, progress was limited by the substantial time required for assay optimisation and validation, coupled with the

large number of candidate pathways and markers that could potentially contribute to the observed cellular responses.

Given the breadth and complexity of the metabolic alterations associated with hypoxic and ischaemic injury, it became apparent that a targeted approach risked overlooking important biological changes outside predefined pathways. As such, and with the support of OTF, the focus of the project shifted towards an untargeted metabolomic analysis of DCD kidney biopsies spanning a range of functional warm ischaemia times (fWIT), which is currently being undertaken. This approach substantially broadens the scope of investigation, enabling a comprehensive assessment of metabolic changes associated with progressive ischaemic injury without being constrained by prior assumptions regarding candidate pathways or biomarkers. The analysis is being conducted in collaboration with the FORTH Institute in Patras, Greece, whose extensive expertise in metabolomics and advanced analytical platforms uniquely positions them to undertake high-quality untargeted metabolic profiling. This collaborative work is expected to identify novel metabolic signatures associated with increasing fWIT and provide a more comprehensive understanding of the biochemical consequences of DCD kidney injury to identify targets for intervention and injury prevention.

The untargeted metabolomic analysis currently underway will generate a rich multidimensional dataset that will form the basis of a dedicated manuscript and provide important preliminary data for future studies. The findings will help prioritise key metabolic pathways for further mechanistic investigation and are expected to provide the rationale for future spatial omics analyses. These approaches will enable the exploration of metabolic heterogeneity within the kidney and how these changes in response to increasing warm ischaemia, thereby extending our understanding of the biological processes underlying DCD kidney injury.

## **Outputs**

### *Publications*

Vaughan RH, Fawaz S, van Leeuwen LL, Vendrell I, Ploeg RJ, Kessler BM, Charles PD, Kaiser M. *Prolonged functional warm ischaemia drives degradome remodelling and legumain proteolytic processing in DCD kidneys*. Unpublished manuscript.



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