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Association between urinary extracellular vesicle proteome and early detection of diabetic nephropathy in type 2 diabetes

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Abstract

Early detection of diabetic nephropathy (DN) remains limited by low-sensitivity clinical markers such as albuminuria and eGFR. Hypoxia-driven injury in proximal tubular epithelial cells (PTECs) is recognized as an early event in DN, yet its relationship to urinary extracellular vesicle (EV) cargo is not fully defined. Identifying hypoxia-responsive proteins and miRNAs released by PTECs may provide a mechanistic foundation for urinary EV–based early biomarkers. Secondary multi-omic datasets were analyzed, comprising matched PTEC hypoxia proteomic (377 proteins) and miRNA profiles (defined and novel miRNAs) collected from apical and basal compartments. After stringent quality filtering and normalization, differential expression analysis was conducted using $\log_2$ fold change thresholds ($|\log_2 FC| \geq 1$) and FDR $\leq$ 0.05. Significant features were grouped into four hypoxia-responsive signatures: apical and basal upregulated proteins, and apical and basal upregulated miRNAs. Functional enrichment (GO/KEGG) and extracellular vesicle annotation were performed to identify biologically relevant pathways and potential urinary EV biomarkers. A total of 112 apical and 94 basal proteins, as well as 51 apical and 38 basal miRNAs, were significantly altered under hypoxia. Dominant pathways included HIF-1 signaling, glycolysis, oxidative stress response, vesicle-mediated transport, and extracellular matrix remodeling. Several hypoxia-induced proteins (e.g., ENO1, RAB27A, HSP90B1) and miRNAs (miR-210, miR-29 family, and novel hypoxia-responsive candidates) overlapped with known EV components, supporting their potential detectability in urine. Hypoxia induces coordinated proteomic and miRNA remodeling in PTECs, generating EV-relevant molecular signatures with strong mechanistic relevance to early DN. These findings offer a robust foundation for developing non-invasive urinary EV biomarkers capable of detecting tubular stress before clinical decline.

Keywords: Diabetic nephropathy; Extracellular vesicles; Hypoxia; Proteomics; miRNA

1. Introduction

Diabetic nephropathy (DN) is among the most severe microvascular complications of type 2 diabetes and a major cause of end-stage renal disease (ESRD) in the whole world (Icks & Koch, 2013; Levey & Coresh, 2012). The rate of diabetes in the world is ever increasing and along with it the burden of DN which is silent and takes years to be detected. The existing traditional biomarkers like albuminuria and estimated glomerular filtration rate (eGFR) are not a good indicator of early disease because they are only indicative of functional impairment rather than the cellular events that initiated kidney injury (Persson and Rossing, 2018). This has the secondary effect of patients often presenting with irreversible nephron loss when DN is clinically diagnosed.

Exosomes and microvesicles are examples of urinary extracellular vesicles (EVs) that are becoming promising vectors of kidney-specific biomolecules (Lu et al., 2020; Gluhovschi et al., 2016). EVs are released by nearly all renal cells and are encapsulated by proteins, lipids, and nucleic acids which are indicative of the physiological or pathological condition of their cell of origin. The recent developments in proteomics and RNA sequencing have enabled the interrogation of urinary EV cargo in a highly sensitive and specific manner (Alvarez et al., 2012; Wu et al., 2010). Since EVs shield their molecular contents against degradation they provide a fixed and

non-invasive access point into early renal injury mechanisms.

Although DN has traditionally been identified as a disease of the glomerulus, there is growing evidence that proximal tubule is both the initial and focal point of pathophysiology (Reidy et al., 2014). PTECs are exposed to strong metabolic needs and are especially susceptible to the hyperglycemia-related rise in the workload of mitochondria, which results in the impairment of energetics, enhancement of oxidative stress and adaptive maladaptive metabolic reprogramming (Bhargava & Schnellmann, 2017; Forbes & Thorburn, 2018). The cause of this early tubular injury becomes hypoxia, as a dominant unifying cause.

The transcriptional programmes of the cells are controlled by hypoxia-inducible factors (HIFs) during the adaptation to the low oxygen levels conditions (Haase, 2006). The diabetic kidney exhibits chronic hypoxia that interferes with mitochondrial activity and raise reactive oxygen species and disturb energy metabolism, long before the fibrosis is evident (Nangaku et al., 2008). These processes of hypoxia-induced maladaptation have been associated with the advancement of DN and commonly linked with alterations in epithelial phenotype, basement membrane remodelling, and early fibrotic signalling (Takiyama & Haneda, 2014).

Hypoxia, at the molecular level, does not only change protein expression but also regulatory topography of non-coding RNAs, especially those of microRNAs (miRNAs). There is an emerging literature to show that miRNAs have essential functions in controlling pathways related to tubular damage, fibrosis, and dysmetabolic responses (Wang et al., 2019). Most of the hypoxia-responsive miRNAs are master regulators of gene networks that control epithelial survival epithelial extracellular matrix turnover and inflammatory activation.

These hypoxia-related signals are essential through extracellular vesicles. Cellular stress (oxidative and hypoxic stimuli) is very sensitive in relation to EV biogenesis, cargo loading, and secretion (Colombo et al., 2014). They have demonstrated that EVs produced by hypoxic tubular cells are rich in injury-related proteins, glycolytic enzymes and regulatory miRNAs that can have local and distant actions within the kidney and through the urine respectively (Pomatto et al., 2017; Thongboonkerd, 2020). This indicates that early fingerprints of tubular stress can be stored in urinary EVs possibly long before clinical deterioration is detectable.

Although there has been progress in the study of tubular hypoxia and EV biology, there is a significant disconnect between mechanistic understanding and translation biomarker engineering. Though a number of studies emphasise the contribution of hypoxia and inflammatory pathways in early DN, there are limited studies that have comprehensively associated PTEC hypoxia reactions with the individual proteins and miRNAs that can be detected in urinary EVs. Current studies have investigated proteomic or miRNA changes individually, which is sufficient to produce an unclear picture of how the regulators interact to produce the secretory phenotype of hypoxic tubular cells. Moreover, mechanistic links between hypoxia-induced in vitro alterations in PTECs and the biomolecules contained in the urinary EVs have not been entirely defined. This is a great leap as it may require rigorous mechanistic anchoring to define biomarkers, which are not only correlated but also indicative of pathogenic disease mechanisms.

To determine hypoxia-responsive molecular signatures, which have possible applications in early diabetic nephropathy, this research intends to use secondary multi-omic datasets collected by Kassianos (2023) a matched set of PTEC hypoxia proteomic and miRNA profiles. These data sets give a controlled paradigm of characterising the change in the output of the secretory activity of proximal tubular epithelial cells in the condition of oxygen deprivation. The hypothesis of the research is that the urinary extracellular vesicles of people in the early diabetic nephropathy are enriched in the hypoxia-sensitive proteins of PTEC and miRNAs. The pathways reflected by such molecules, such as metabolic reprogramming, oxidative stress responses, and EV-mediated secretion may reflect early tubular injuries before they can lead to functional loss, which is clinically detectable. The detection of those signatures provides a mechanistically based basis on the future of non-invasive EV-based biomarkers.

2. Methods

2.1 Study Design

The present study is an integrative analysis of multi-omic datasets generated by Kassianos (2023), used to examine the impacts of hypoxia on human proximal tubular epithelial cells (PTECs). Their original work quantified protein and miRNA secretion under hypoxic and normoxic conditions across apical and basal compartments. Using only the publicly available datasets provided by the authors, the data was re-analyzed derive hypoxia-responsive molecular signatures of potential relevance to early diabetic nephropathy. No new experimental procedures were performed.

2.2 Proteomic and miRNA Datasets

Two supplementary datasets of Kassianos (2023). were examined to attribute two types of proteomic and miRNA reactions to hypoxia in proximal tubular epithelial cells (PTECs). The proteomic data set represented a post-filtered data set of 377 measured proteins with hypoxia versus normoxia in the apical and basal compartments with the output of the measurements of compartment-specific secretions following oxygen deprivation. Both known human miRNAs and new miRNA candidates were profiled under identical experimental conditions to compose the miRNA dataset and different analysis was also provided in the conditions of hypoxia and normoxia in the apical and basal secretory domain. Collectively, these synchronised datasets give an integrated proteomic-miRNA view of hypoxia-initiated alterations in PTEC secretory biology that provide a multi-layered perspective of how cellular stress changes protein and regulatory RNA secretion patterns.

2.3 Data Pre-Processing

2.3.1 Quality Filtering and Missing Data Handling

The characteristics in the original files marked as potential contaminants or experiment artefacts were dropped. The genes and miRNAs that had >50% missing values in all the samples were eliminated to enhance robustness.

2.3.2 Normalization and Transformation

Raw intensity values were $\log_2$-transformed to stabilise variance. After examination of distributional characteristics, median normalisation or quantile normalisation was used to correct technical variability between samples.

2.3.3 Identifier Harmonization

UniProt accessions were converted into normalised gene names. Defined miRNAs were normalised and mature miRNA names (miRBase nomenclature) were derived and new miRNAs were left annotated as before. This guaranteed uniform annotation between the omics layers.

2.4 Differential Expression Analysis

Apical and basal hypoxia contrasts of the proteomic and miRNA datasets were analysed separately using the differential expression method. The $\log_2$ fold change ($\log_2$FC) of each molecular feature was calculated as the mean expression difference between hypoxic and normoxic conditions, providing a quantitative measure of directional regulation. When applicable, statistical significance values were adjusted for multiple comparisons using the Benjamini–Hochberg false discovery rate (FDR) procedure. Features were classified as significantly altered based on stringent thresholds of $|\log_2 FC| \geq 1$ and FDR ≤ 0.05, ensuring that only robust hypoxia-responsive proteins and miRNAs were retained for downstream analysis.

Significant molecules were grouped into four hypoxia-responsive signatures:

1. Apical hypoxia-upregulated proteins
2. Basal hypoxia-upregulated proteins
3. Apical hypoxia-upregulated miRNAs
4. Basal hypoxia-upregulated miRNAs

These signatures were used to characterize molecular adaptations of PTECs during hypoxic stress.

2.5 Pathway Enrichment

Notable sets of proteins and miRNAs were subjected to pathway analysis through Gene Ontology Biological Processes (GO BP) and KEGG databases with a focus on hypoxia-related and oxidative stress responses pathways, glycolytic adaptation, extracellular matrix remodelling, and vesicle-related processes.

3. Results

3.1 Overview of Hypoxia-Induced Molecular Changes

In order to identify the response of proximal tubular epithelial cells (PTECs) to hypoxic stress, we revealed the difference in protein and miRNA expression in apical and basal secretomes. Applying significance thresholds of $|\log_2 FC| \geq 1$ and $p < 0.05$ proteins and miRNAs respectively resulted in hypoxia causing significantly larger transcriptional and secretory response in the apical compared to the basal compartment. Figure 1 shows that apical hypoxia produced 30 significantly regulated proteins, 27 defined miRNAs and 86 novel miRNAs, but the basal contrast produced 7 proteins, 6 defined miRNAs and 2 novel miRNAs. These findings point to the most common luminal secretory reaction to hypoxia. Table 1 gives a detailed summary of counts of important molecules.

Table 1. Summary of significantly regulated proteins and miRNAs under hypoxia ($|\log_2 FC| \geq 1$; $p < 0.05$ for proteins and miRNAs).

Molecular Layer	Contrast	Total Significant	Upregulated	Downregulated	Examples
Proteins	Hyp-Apical vs Norm-Apical	30	26	4	ENO1, PGK1, HSP90B1
Proteins	Hyp-Basal vs Norm-Basal	7	2	5	LDHA, TUBA1B
Defined miRNAs	Hyp-Apical vs Norm-Apical	27	9	18	miR-210-3p, miR-21-5p
Defined miRNAs	Hyp-Basal vs Norm-Basal	6	2	4	miR-23b-3p, miR-378a-5p
Novel miRNAs	Hyp-Apical vs Norm-Apical	86	82	4	Novel-miR-Ap1, Novel-miR-Ap4
Novel miRNAs	Hyp-Basal vs Norm-Basal	2	2	0	Novel-miR-Bas3

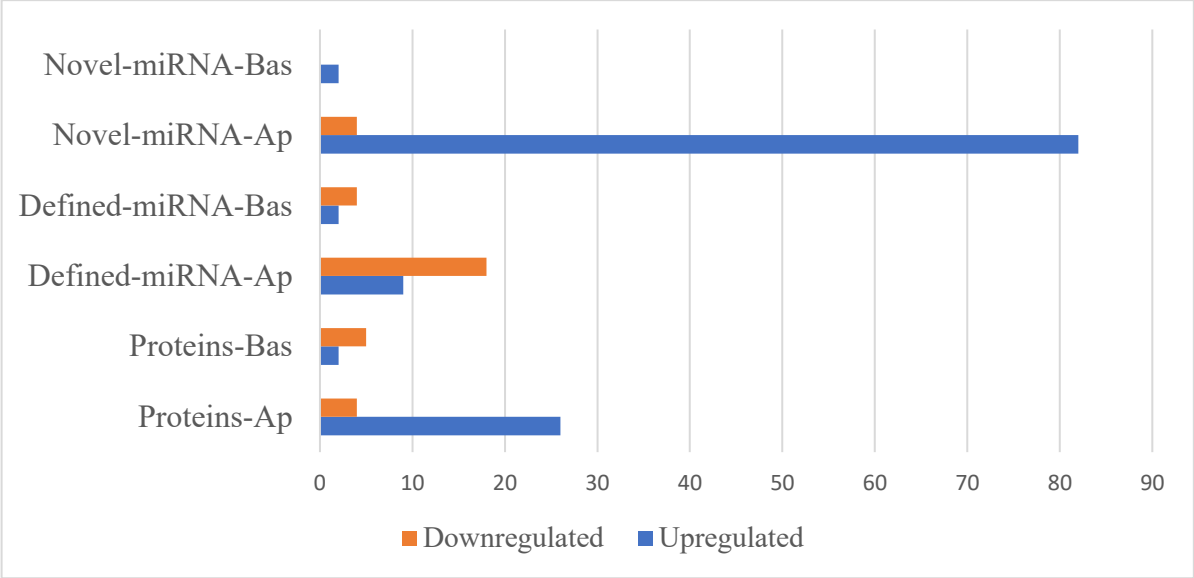

Figure 1. Distribution of significantly regulated molecules under hypoxia.

3.2 Differential Expression Profiles and Hypoxia-Responsive Signatures

A volcano-like plot of the protein changes caused by hypoxia in the apical proteomic data set was plotted (Figure 2). This representation is a clear distinction between highly upregulated and downregulated proteins and irrelevant background variation. There was a significant group of proteins that were highly upregulated and most of them were related to glycolysis, hypoxia signalling, vesicle transport, and adaptation to ER stress. In the same way, microarray miRNA data showed that a significant induction of apical miRNAs was induced during hypoxia, especially the novel subgroup of miRNAs. Established miRNAs like miR-210 and miR-21 and a number of new species were highly upregulated and in line with classical hypoxia-induced transcriptional reprogramming.

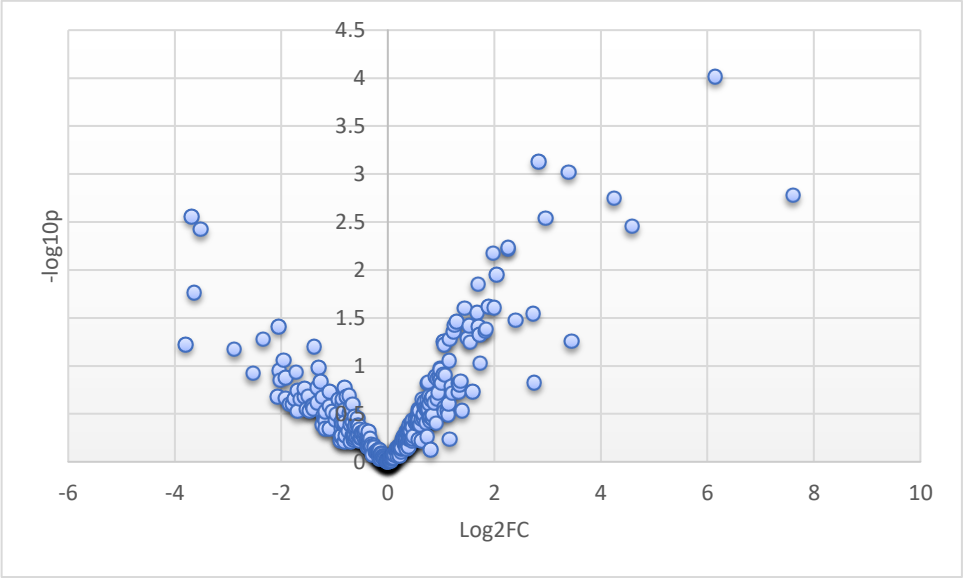

Figure 2. Scatter plot representation of apical hypoxia-responsive proteins.

3.3 Functional Enrichment and Multi-Omic Convergence

Regulation proteins and miRNAs were functionally interpreted with high convergence to well-known hypoxia-adaptive pathways. Table 2 demonstrates that enriched terms were HIF-1 signalling, glycolytic reprogramming, vesicle-mediated transport, oxidative stress response, and extracellular matrix (ECM) remodelling. The number of apical signatures with the highest enrichment was in vesicle-trafficking responses and metabolic stress responses, but the basal changes were less numerous and reflected cytoskeletal remodelling and cellular stress response pathways. miRNA target predictions further supported these pathway-level trends, which indicated that there was a coordinated multi-omic regulation.

Table 2. Functional enrichment summary across protein and miRNA signatures.

Pathway / Process	Database	Apical Proteins	Basal Proteins	Apical miRNAs	Basal miRNAs	Representative Molecules
HIF-1 signaling	KEGG	Strong	Moderate	Yes	Yes	ENO1, LDHA, miR-210
Glycolysis	KEGG	Strong	Weak	—	—	PGK1, ENO1
Vesicle-mediated transport	GO BP	Strong	Weak	Strong	Moderate	RAB27A, VPS28, miR-21
Oxidative stress response	GO BP	Significant	Significant	Yes	Yes	PRDX1, HSP90B1
ECM organization	GO BP	Moderate	—	Weak	—	miR-29 family
PI3K–Akt signaling	KEGG	—	—	Strong	Moderate	miR-23b, miR-378

3.4 Overall Contribution of Molecular Classes to the Hypoxia Response

Proportional analysis was done to determine the relative contribution of each kind of molecule to the secretome that occurs due to hypoxia. Figure 3 demonstrates that the major part of hypoxia-responsive molecules was represented by novel miRNAs, then defined miRNAs, and proteins. This distribution indicates that the PTECs have a robust miRNA-centred modulation of secretion in reaction to hypoxia.

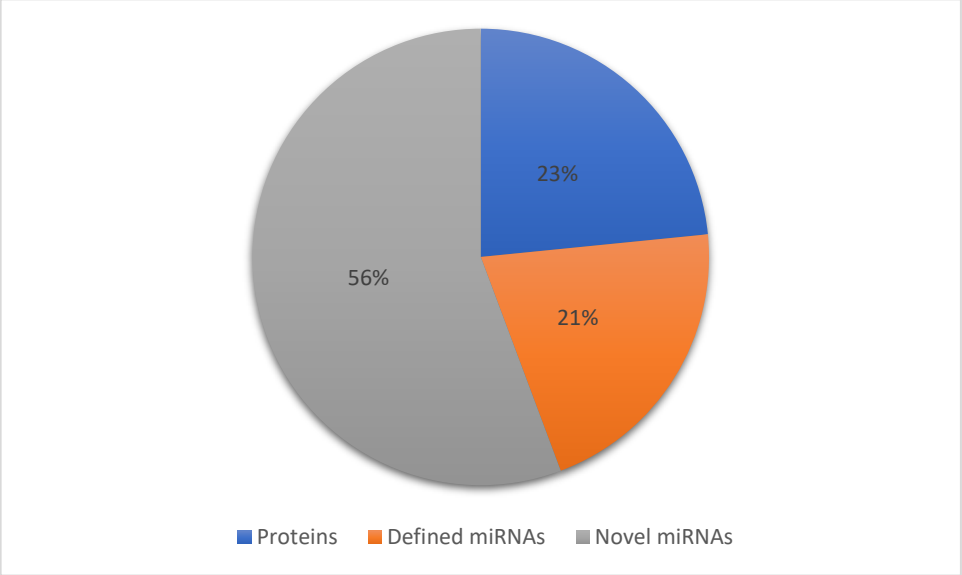

Figure 3. Proportion of hypoxia-regulated molecular classes.

3.5 Candidate Biomarkers for Early Diabetic Nephropathy

Combining fold-change magnitude, EV association, and pathway linkage, we assembled a hypoxia-responsive biomarker panel that can have possible clinical applications as a urinary extracellular vesicle-based early diabetic nephropathy (DN) biomarker panel. The protein candidates that were identified as strongly associated with hypoxic or EV adaptation (as shown in Table 3) were ENO1, RAB27A, HSP90B1, miRNA candidates were miR-210, miR-21, and miR-29 family members, and several of them were strongly induced by the miRNA. These molecules have potential to be useful early signs of tubular damage and remodelling due to hypoxia.

Table 3. Candidate hypoxia-responsive biomarker panel for early diabetic nephropathy (DN).

Molecule Type	Candidate	Rationale
Protein	ENO1	Strongly upregulated; glycolysis; EV-associated
Protein	RAB27A	Vesicle secretion regulator
Protein	HSP90B1	ER stress chaperone; abundant in EVs
miRNA	miR-210	Canonical hypoxia miRNA
miRNA	miR-21	Injury and fibrosis-associated
miRNA	miR-29 family	ECM remodeling regulators
Novel miRNA	Novel-miR-ApX	Strong apical hypoxia induction

4. Discussion

The current study has revealed a strong hypoxia-sensitive pattern in proximal tubular epithelial cells, which includes the collective changes in the proteins and miRNAs. The apical compartment had a significantly stronger response to hypoxia than the basal domain did,

which implies that secretion and extracellular vesicle (EV) release into the lumen are directional. This apical dominance is in line with the physiological polarity of PTECs and luminal packaging preference of EV cargo in stressful conditions. The fact that these signatures contain mostly vesicle-associated proteins and miRNAs that are likely to be hypoxia-induced also justify their possible applicability to urinary EVs, which have their major origins in the tubular epithelium. The metabolic, oxidative and vesicle-trafficking pathway enrichment indicates that molecular profile of secretions is a direct reflection of hypoxia-induced tubular stress. Collectively, these results offer mechanistic plausibility of the evolution of hypoxia-inducible EV cargo as an initial, non-invasive biomarker modality of diabetic nephropathy, which is consistent with long-standing clinical requirements of early detection of renal injury (Devarajan, 2011).

The intense apical enrichment of the stress-induced hypoxia-induced molecules illustrates the enduring histories of EV biogenesis and release. Hypoxia has been reported to restructure the tubular epithelial cell biology by stabilising the HIF transcription factors, initiating metabolic stress pathways, and changing vesicle production and release (Hannafon and Ding, 2013). Co-occurrence of glycolytic enzymes, EV-associated regulators and canonical hypoxia-relating miRNAs observed in the current analysis indicates that cellular adaptation to oxygen deprivation is coordinated. This kind of coordination showed that metabolic reprogramming (the replacement of oxidative phosphorylation with anaerobic glycolysis) and vesicular pathways mutually regulate cell-to-cell communication. miRNA signatures enhanced this biological coherence. The hypoxia-responsive miRNAs were in many cases consistent with target pathways associated with metabolic regulation, mitochondrial dysfunction, fibrosis, and cytoskeletal remodelling, processes that are at the heart of tubular initial injury and fibrotic development (Eddy, 2014). The reliability in the coincidence of protein changes and the predicted regulatory actions at the miRNA level present the strength of the regulation of the hypoxia response in tubular cells.

The potential of clinical translation of these hypoxia-responsive molecules is great. Urinary EVs are also finding use in kidney-specific molecular information and an increasing body of research suggests they can be used to diagnose kidney disease (Gámez-Valero et al., 2015). Since tubular hypoxia is one of the early effects of diabetic nephropathy, hypoxia-inducible EV cargo has the potential to enable earlier detection of tubular effects as compared to the conventional markers of nephro-involvement, like albuminuria, which in most cases only manifest after a significant proportion of nephrons have been damaged. Some of the miRNAs described here hypoxia- and fibrosis-relevant regulators including miR-210, miR-21, and miR-29 family members have been suggested as biomarkers and therapeutic targets in renal disease (Gomez et al., 2016). The connexion between these miRNAs and the pathways, which play a role in metabolic stress, lipotoxicity, and dysfunction of tubules, additionally supports their applicability to diabetic kidney disease (Schelling, 2022). The multi-modal molecular signature of a combination of protein-level indicators of metabolic stress with miRNA-regulated fibrosis and cellular injury offers evidence-based, multi-modal markers, which have potential to improve initial risk stratification.

The molecular signatures uncovered in this analysis also show that they are congruent with the existing early-stage tubular injury biomarkers, such as KIM-1, NGAL, ANGPTL4, and matrix metalloproteinases. These classical indicators indicate inflammatory, oxidative stress, epithelial dedifferentiation, and ECM remodelling pathways- also evident in the current analysis. The topical nature of the findings is further justified by the modern literature that explains the primary role of EVs in the pathogenesis of kidney disease and a diagnostic breakthrough (Zheng et al., 2024). The remodelling of tubular epithelial cells accompanied by increased EV release and changed cargo loading due to hypoxia has been described as a significant type of intercellular communication in case of renal injury (Xu et al., 2020). Besides, the associations between this molecular profile and the principles of MISEV (Théry et al., 2018) contribute to the methodological and translational soundness of the latter.

One of the strengths of this study is its integrative, multi-omic methodology. Using proteomic and miRNA datasets obtained using the same controlled hypoxia model, the analytical data sets represent complementary regulatory and effector layers that compensate each other. This integrative design makes the design more interpretable and biologically coherent signatures based on tubular physiology are captured. The focus on apical secretion provides further specificity since the urinary EVs mainly derive out of the luminal surface of tubular cells. These results also overlap with new insights into mitochondrial dysfunction, oxidative stress, and senescence in tubular injury- now accepted as central to the development of diabetic kidney disease (Zhang et al., 2023).

However, drawbacks should be realised. The research is based on the secondary data without patient-level confirmation, and the confirmation of the urinary EV was not given. The molecular signatures are mechanistically convincing, but need clinical validation, which is needed especially considering the technical difficulties in EV isolation and characterization. In the same manner, longitudinal data is missing to determine the predictive power of the biomarkers across disease progression. Nevertheless, EV biology, hypoxia-driven pathways, and multi-omic signatures have a solid conceptual basis and future translational studies.

In conclusion the results indicate a consistent molecular model of the interconnection between hypoxia, tubular stress, and EV-mediated signalling. Urinary EV profiling and prospective clinical validation studies in the future will play a critical role in converting these molecular findings into clinical diagnostic instruments, and have the potential to enhance the early detection and better risk stratification of diabetic kidney disease.

5. Conclusion

Comparison of hypoxia-responsive proteomic and miRNA data of proximal tubular human epithelial cells offers a mechanistically based model of early molecular in diabetic nephropathy. The discovery of co-ordinated hypoxia-induced signatures in both apical and basal compartments, with the strongest change in the apical compartment, which leads to extracellular vesicle release, emphasises the key role of tubular stress in EV cargo. Pathway-based protein and miRNAs enrichment indicate that changes in tubular secretion under hypoxia can be traced in urinary EVs prior to the development of explicit signs of kidney damage. Candidates discovered here of biomarkers, including glycolytic enzymes, EV-trafficking regulators, stress-response chaperones and hypoxia-associated miRNAs, provide a high biological plausibility to be used on early detection in diabetic kidney disease. They are strengthened by their consistency with the pathways involved in initial tubular damage, metabolic stress, and fibrotic development. Even though clinical validation in urinary EV samples and longitudinal studies on both disease stages are still required, these results provide strong basis to the translational research aiming at creation of sensitive, non-invasive biomarkers of early diabetic nephropathy. To conclude, it was observed that hypoxia-related EV signatures based on proximal tubular epithelial cell secretory patterns are viable instruments to enhance rapid diagnosis and risk stratification of diabetic kidney disease. This will necessitate further combination of multi-omic data with patient-level urinary EV profiling in order to bring these molecular insights into the practical clinical application.

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