Renal Microvascular Endothelial Heterogeneity In Sepsis
Luxen, Matthijs

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CHAPTER 8
Summary, Conclusions & Future Perspectives
SUMMARY

Endothelial cells (EC) inhabit the inner lining of blood vessels and exhibit remarkable heterogeneity regarding function, and gene and protein expression profiles dependent on their microvascular location, as discussed in chapter 1. While all EC are crucially involved in vital biological processes including leukocyte recruitment, coagulation, and microvascular barrier integrity, the contribution of EC to these processes is highly dependent on the microvascular compartment they reside in. During sepsis, these processes become dysregulated, thereby underscoring the central involvement of EC in sepsis pathophysiology. Consequently, EC represent promising therapeutic targets in sepsis-associated organ failure, an area currently lacking effective drug treatment regimens. The overarching research aim of this thesis is to investigate microvascular endothelial responses to sepsis-like insults in vitro and sepsis in vivo to shed light on the molecular intricacies of endothelial heterogeneity within the renal microvasculature. I employed omics-analyses, which are platforms that provide an unbiased assessment of various biological molecules, to study kinases, miRNAs, and mRNAs.

During sepsis, EC are exposed to disturbed blood flow and inflammatory mediators such as lipopolysaccharide (LPS), a bacteria-derived cell wall component, and tumor necrosis factor alpha (TNFa), a pro-inflammatory cytokine predominantly produced by monocytes and macrophages during infection. These stimuli trigger the activation of intracellular signaling pathways, in which kinases play pivotal roles as specialized proteins that relay signals by phosphorylating their substrates. In chapter 2, I reviewed the role of NF-κB, Rac1/ RhoA GTPases, AP-1, APC/S1P, Angpt/Tie2, and VEGF/VEGFR2 and their associated signal transduction cascades in endothelial responses to sepsis conditions. I integrated our knowledge regarding these pathways and revealed an intricate network with a high level of interconnectedness, which underscores the complexity of sepsis-associated signal transduction in EC. Strikingly, the majority of studies included in this review focused on a single gene or kinase, which only represent one piece of a much larger puzzle. Therefore, to gain a deeper understanding of the identity and kinetics of kinases involved in endothelial responses to sepsis-like stimuli in an unbiased manner, we conducted kinome analysis on in vitro EC exposed to LPS and TNFa in chapter 3 and chapter 4, respectively. In the first 4 h following LPS exposure, we identified 58 kinases with altered activity in EC. We then assessed which kinases had previously been implicated by literature to either partake in cellular responses to sepsis or in pro-inflammatory pathways, and selected Axl tyrosine kinase (Axl), focal adhesion kinase 1 (FAK1), and anaplastic lymphoma kinase (ALK) for further studies into their involvement in endothelial inflammatory activation. Through siRNA-based gene silencing and pharmacological inhibition, we discovered that Axl inhibition exacerbated endothelial inflammatory activation in response to LPS, whereas inhibition of FAK1 and ALK attenuated LPS-induced endothelial inflammatory activation. These findings suggest that FAK1 and ALK represent potential therapeutic targets to ameliorate endothelial responses in sepsis.
Kinome analysis of cultured EC following TNFα exposure revealed a total of 152 kinases with differential activity, as described in chapter 4. We next selected 3 kinases with the highest increases in activity in response to TNFα, namely Axl, Fyn, and Lck kinases. As endothelial expression of Lck expression could not be validated, we focused on the involvement of Axl and Fyn in TNFα-induced endothelial inflammatory activation. Pharmacological inhibition of both Axl and Fyn attenuated endothelial expression of adhesion molecules and cytokines. As the kidney is frequently affected in sepsis, we assessed whether Axl and Fyn were expressed by renal microvascular EC. Notably, Fyn was expressed in EC of arterioles and glomeruli, while Axl expression was exclusive to glomeruli. Together, these data indicate that these two kinases may represent suitable druggable targets in sepsis for these microvascular compartments.

Microvascular EC exhibit heterogeneity in gene and protein expression patterns depending on their microvascular location, yet understanding of the underlying molecular mechanisms is lacking. While endothelial heterogeneity affects basic behavior of cells, it may also influence endothelial responses to diseases such as sepsis. As acute kidney injury is a frequent complication of sepsis associated with cortical changes, we zoomed in on the four main microvascular compartments of the kidney cortex, namely arterioles, glomeruli, peritubular capillaries, and postcapillary venules. In collaboration with Vivomicx BV and TAMiRNA GmbH, we combined laser microdissection (LMD) of microvascular compartments with miRNome and transcriptome analyses in healthy mice and mice subjected to cecal ligation and puncture (CLP) that leads to sepsis. Performing LMD prior to sequencing enables studying the behavior of specific microvascular compartments with minimal contamination with other renal structures. This approach enabled the characterization of microvascular endothelial profiles at miRNA and mRNA level in health, which may influence miRNA and mRNA changes in response to sepsis. In chapter 5, we conducted miRNome and transcriptome analyses of renal microvascular compartments in healthy mice. In this manner, we revealed distinct miRNome and transcriptome profiles per microvascular compartment, including individual miRNAs and mRNAs with enriched transcription in a particular microvascular compartment. These included mmu-miR-140-3p and vWF in arterioles, mmu-miR-322-3p and GABRB1 in glomeruli, mmu-miR-2137 in peritubular capillaries, and mmu-miR-486a/b-5p and IGF1 in postcapillary venules. To investigate putative functional implications of miRNAs with enrichment in particular microvascular compartments, miRNA-mRNA pair analysis unveiled more than 550 microvascular compartment-specific connections between miRNAs and their target genes. More than 500 of these miRNA-mRNA pairs were identified in arterioles, suggesting that miRNA-based gene repression may be more prevalent here compared with other microvascular compartments. Furthermore, functional pathway enrichment analysis of miRNA-targeted genes in arterioles revealed their hypothetical involvement in cell adhesion-related processes. This may underlie the absence of leukocyte recruitment from arterioles, which exemplifies a functional implication of heterogeneous microvascular miRNA profiles.

miRNAs exert functional effects by preventing translation of their target mRNAs into...
protein. Because comprehensive studies examining miRNA involvement in sepsis are scarce, in chapter 6, we investigated the identity and kinetics of miRNA transcription in different microvascular compartments in response to sepsis. miRNome analysis revealed 40 miRNAs with differential renal microvascular transcription in response to CLP-sepsis in mice, the majority of which were only altered in glomeruli. While most miRNAs exhibited microvascular compartment-exclusive changes in response to sepsis, mmu-miR-21a-5p transcription was increased in all microvascular compartments. To determine whether this change also occurred in sepsis in patients, we obtained microvascular compartments by LMD from renal biopsies taken post-mortem from patients with sepsis-associated acute kidney injury (sepsis-AKI), and confirmed that hsa-miR-21-5p levels also were increased in all microvascular compartments. In vitro studies next showed that hsa-miR-21-5p depletion exacerbated endothelial inflammatory activation elicited by LPS, suggesting a protective role of this miRNA in EC responses to inflammatory conditions.

Dysregulated coagulation and inflammation are frequent complications in sepsis, and while EC are involved in both processes, the contribution of different microvascular compartments to these processes in sepsis is poorly characterized. Therefore, in chapter 7 I employed transcriptome analysis to identify genes associated with coagulation and inflammation with altered microvascular expression in response to CLP-sepsis in the mouse kidney. A total of ~400 genes exhibited differential expression in microvascular compartments following sepsis, with the vast majority of genes changing exclusively in a single microvascular compartment. For instance, mRNA and protein levels of coagulation-associated cell surface protein thrombomodulin were induced in glomeruli in response to CLP-sepsis. In this analysis, two genes were found to be induced in the renal microvasculature following CLP-sepsis, namely inflammation-associated transcription factor signal transducer and activator of transcription (STAT3) and interferon induced transmembrane protein 3 (IFITM3). While STAT3 was induced at mRNA level in all microvascular compartments in response to sepsis, activated phospho-STAT3 was almost exclusively detected in glomeruli. The expression of IFITM3 was induced in glomeruli and peritubular capillaries at mRNA and protein level following sepsis. Since it was not known whether STAT3 and IFITM3 were involved in endothelial responses to sepsis, a functional role in endothelial coagulation and inflammatory activation was assessed in in vitro studies. These studies revealed attenuated responses of EC to LPS following STAT3 and IFITM3 knockdown. This suggests that STAT3 and IFITM3 partly control endothelial responses to inflammatory mediators, thereby implicating their potential as therapeutic targets in sepsis.

CONCLUSIONS

The endothelium is a multifaceted organ that is involved in leukocyte recruitment, coagulation, and microvascular leakage, and EC exhibit heterogeneity regarding their
contribution to these processes that is dependent on the microvascular compartments in which they reside. As these processes become dysregulated during sepsis, targeting the endothelium presents a promising avenue for therapeutic intervention in sepsis. In this thesis, I investigated the molecular nature of microvascular endothelial heterogeneity in health as well as in responses to sepsis-like insults in vitro and to sepsis in vivo. As loss of function of the kidney is a frequent complication in sepsis, I focused on the behavior of different microvascular compartments of the kidney cortex in health and in response to sepsis, and employed kinomics, miRNomics, and transcriptomics as high-throughput analytical tools to study microvascular behavior in an unbiased manner.

**Endothelial responses to sepsis-like stimuli are partly controlled by focal adhesion kinase 1, anaplastic lymphoma kinase, Axl receptor kinase, and Fyn kinase**

We identified dozens of kinases with altered activity following endothelial exposure to pro-inflammatory sepsis-like stimuli LPS and TNFα by performing kinome analyses in EC in vitro. FAK1 and ALK were shown to partly control endothelial inflammatory activation in response to LPS, whereas targeting Axl and Fyn kinases attenuated TNFα-induced responses in EC. These findings highlight the potential of these kinases as therapeutic targets to counteract endothelial dysfunction in sepsis, which will be discussed in more detail in the future perspectives.

**Renal microvascular compartments exhibit unique microRNA and mRNA expression patterns**

The microvascular compartments of the mouse kidney cortex – arterioles, glomeruli, peritubular capillaries, and postcapillary venules – exhibit unique miRNA and mRNA profiles. We identified miRNAs and mRNAs that were exclusively or highly enriched in one microvascular compartment. miRNA-mRNA pair analysis and subsequent pathway enrichment analysis provided a first insight into potential functional implications of heterogeneously expressed miRNAs by microvascular compartments. These differential miRNA and mRNA expression patterns likely underlie differential behavior of microvascular EC in health, and may affect endothelial responses to disease-related stimuli.

**Microvascular microRNA and mRNA responses to sepsis are spatiotemporally induced**

The vast majority of miRNAs and mRNAs identified by miRNome and transcriptome analyses were exclusively induced in specific microvascular compartments and at specific time points following sepsis, thereby emphasizing the spatiotemporal and heterogeneous character of microvascular responses. Taken together, we have demonstrated heterogeneous microvascular responses following sepsis by successfully establishing and applying a workflow that combines LMD with omics approaches to investigate compartmentalized structures of interest in health and disease.
FUTURE PERSPECTIVES

Microvascular biomarkers for identification of sepsis patient phenotypes

Signal transduction pathways that underlie diseases are intricately connected and exceptionally complex. Together with the fact that patients who suffer from the same disease can vary with regard to clinical presentation and involved molecular mechanisms, it seems improbable that a single therapeutic approach could ever benefit all patients. For this reason, the oncology field has started implementing personalized medicine based on the specific genetic mutations of cancer in patients in recent years, although this has been on the horizon since the early 2000s\textsuperscript{1,2}. In contrast, the treatment of sepsis patients still largely follows more conventional approaches despite variability in the clinical presentation of patients. Importantly, patient subphenotyping has now also started to emerge in sepsis, with a recent study by Seymour et al. identifying four distinct sepsis phenotypes\textsuperscript{3}. The overarching idea of subphenotyping in sepsis is to identify groups of patients that may benefit from specific treatments. While Seymour et al. investigated the extent of endothelial dysfunction in patients, represented by soluble E-selectin, P-selectin, VCAM1, and ICAM1, none of the identified phenotypes exhibited distinct alterations in the expression of these molecules\textsuperscript{3}, although another study reported increased plasma levels of soluble E-selectin and ICAM1 in sepsis-AKI\textsuperscript{4}. These results indicate a distinct lack of sensitive and specific biomarkers reflecting function and activation status of the microvasculature in sepsis. Based on the results of miRNAome and transcriptome analyses as presented in chapters 6 and 7, we developed a biomarker panel comprising eight genes and four miRNAs with altered transcription in renal microvascular compartments in response to sepsis in mice. Our objective is to establish sensitive indicators of microvascular engagement in sepsis, as the microvasculature is not only functionally involved in sepsis, but also has a significantly larger surface area than the macrovasculature, and therefore represents an important source of soluble biomarkers. While our current focus is on the renal microvasculature, it is likely that some of these biomarkers are also produced in the microvasculature of other organs, which may cause the biomarker panel to not solely reflect the microvascular involvement of the kidney in sepsis. Although we are at present prohibited from disclosing the identity of the biomarkers due to patent-related constraints, we demonstrated their capability to discern between healthy and sepsis conditions in the renal microvasculature in mice (Fig. 1). The biomarker panel exhibited its highest discriminatory potential in distinguishing between healthy and sepsis conditions in glomeruli. This could stem from the endothelial origin of the biomarkers, as glomeruli display a relatively high enrichment of EC. Alternatively, it is possible that the biomarkers exhibit enhanced sensitivity in detecting dysfunction in glomerular EC compared with other microvascular compartments. Additional investigations are necessary to elucidate this aspect definitively.
A biomarker panel consisting of eight mRNAs and four miRNAs was based on the differentially expressed miRNAs following CLP-sepsis as identified in chapters 6 and 7. Principal component analysis (PCA) shows clustering of samples based on their expression pattern of the biomarker panel. Included microvascular compartments are arterioles, glomeruli, peritubular capillaries (PTC), and postcapillary venules (PCV) laser microdissected from healthy control mice, and sham-operated and cecal ligation and puncture (CLP) sepsis mice sacrificed 4, 24, or 72 h post-surgery \( (n = 4/\text{group}) \). Prediction ellipses are shown per group to indicate with 95% probability that new observations of the same group will fall inside the ellipse. PC1 (x-axis) and PC2 (y-axis) represent the components with the highest variance projected in two dimensions. Protein and miRNA levels of these biomarkers are currently being measured in blood samples of sepsis patients.

Any discussion of biomarkers necessitates the acknowledgement of ongoing debates regarding their overall utility. Recently, there has been a surge of publications focusing on novel biomarkers across various diseases. Evaluating biomarker performance is problematic in conditions where the precise timing of the insult is unclear, as is prevalent in patients who present in the clinic with sepsis\(^5,6\). The biomarker panel we developed successfully differentiated between early (4 h) and later (24-72 h) stages of sepsis in the renal microvasculature of mice (Fig. 1). Therefore, this panel holds the potential to aid in assessing the time of disease onset in the clinic, which is crucial for future determination of optimal treatment strategies. These treatment strategies can consist of both faster administration of conventional therapies already available, as well as yet to be developed treatment strategies. For instance, these biomarkers may in the future contribute to sepsis subphenotyping and aid the identification of specific groups of patients who would benefit from tailored drug treatments targeting microvascular EC. To further evaluate the performance and relevance of these biomarkers, we are currently quantifying protein and
miRNA levels in the blood of patients with sepsis. These analyses will shed light on whether these biomarkers can identify or predict development of AKI. Furthermore, exploring biomarker presence in the urine of patients with sepsis would be of interest. Given that urine is produced in the kidney, biomarkers derived from the renal microvasculature may exhibit relatively higher levels in urine compared with blood, which also contains miRNAs and proteins from other organs. miRNAs are relatively stable in urine\textsuperscript{7}, and urinary miRNAs have been described as biomarkers in different types of cancer\textsuperscript{8–10}.

**Exploring novel therapeutic targets in endothelial cells in sepsis**

We revealed in chapter 6 that miR-21(a)-5p was induced across all renal microvascular compartments in response to sepsis in both mice and patients. *In vitro* experiments demonstrated that suppressing miR-21(a)-5p exacerbated LPS-induced endothelial inflammatory activation, suggesting a protective role of this miRNA by counteracting EC responses to inflammatory mediators. To further investigate the *in vivo* impact of miR-21(a)-5p on EC during sepsis, I propose to subject inducible endothelial-specific miR-21(a)-5p knockout (KO) mice to CLP-sepsis. Previous studies have successfully generated such transgenic mice for other miRNAs\textsuperscript{11}, which could also be applied to miR-21(a)-5p. Based on our *in vitro* findings, I hypothesize that endothelial miR-21(a)-5p KO will exacerbate endothelial responses in sepsis, leading to reduced survival following CLP-sepsis. To explore the therapeutic potential of miR-21(a)-5p, it would be of interest to investigate whether augmenting endothelial levels of miR-21(a)-5p after induction of sepsis could ameliorate sepsis pathophysiology. A possible approach could be employing endothelial-targeted lipid nanoparticles (LNPs) that contain miR-21(a)-5p mimics\textsuperscript{12}. It will have to be established per organ and per microvascular compartment whether EC benefit from these treatments based on functional consequences such as microvascular leakage and leukocyte recruitment in response to sepsis.

FAK1 and ALK were involved in the activation of EC in response to LPS, whereas the inhibition of Axl and Fyn kinases attenuated endothelial responses to TNFα, as shown in chapters 3 and 4. Additionally, in chapter 7 we provided evidence for the participation of the transcription factor STAT3 and the transmembrane molecule IFITM3 in endothelial coagulation and inflammatory activation. These insights were derived from *in vitro* studies, and the subsequent research phase should aim to employ targeted pharmacological inhibition of kinases specifically in EC or to conduct endothelial-specific KO of these target genes in animal models of sepsis. It will be crucial to provide proof that these treatments have effectively inhibited the targeted signal transduction pathways in EC. Since harvesting EC from organs results in gene drift\textsuperscript{13}, validation of successful endothelial targeting will need to be provided without removing EC from their *in vivo* niche. This could be achieved through immunohistochemistry to assess the presence and/or location of target proteins, or by establishing the phosphorylation status of target kinases and their downstream targets with phospho-specific antibodies. Confirming successful endothelial targeting is a
prerequisite for therapeutic intervention studies that aim to pharmacologically investigate the involvement of EC in sepsis-associated pathophysiology.

Importantly, this thesis has focused on microvascular endothelial heterogeneity in the kidney, and distinct microvascular expression patterns were found for several of the potential therapeutic targets we revealed. For instance, Fyn kinase was found to be expressed in both arteriolar and glomerular EC in the kidney. Yet, it is conceivable that pharmacological inhibition of Fyn may elicit differential effects in arterioles compared with glomeruli. Therefore, while the development of endothelial-targeted drug delivery systems is still in progress\textsuperscript{12}, I anticipate that we eventually need to advance towards microvascular compartment-specific inducible gene KO models and delivery of small molecule inhibitors and (non-coding) RNAs to elucidate the contribution of individual microvascular compartments to biological processes in health and disease. These developments will be guided by and dependent on the discovery of microvascular EC compartment-specific genes that can function as drivers of e.g. Cre recombinase\textsuperscript{14}, and compartment-specific cell surface receptors that may serve as epitopes for targeted delivery systems. Microvascular endothelial heterogeneity hence not only presents a fascinating biological phenomenon, but also carries important implications for the design of future therapeutic interventions in EC and associated challenges.

**Heterogeneous microvascular expression of pan endothelial markers**

In the context of endothelial heterogeneity, it is of interest to explore the relative expression of pan endothelial markers platelet/EC adhesion molecule 1 (Pecam1) and cadherin 5 (Cdh5) across microvascular compartments. Both genes are widely employed to normalize gene expression relative to endothelial input, which implies the assumption that every EC uniformly expresses Pecam1 or Cdh5 irrespective of their microvascular location. Our findings in the microvasculature of the renal cortex challenge this notion. Pecam1 expression in arterioles is higher compared with the other microvascular compartments, whereas Cdh5 levels are similar in all compartments, resulting in a significantly higher Pecam1:Cdh5 ratio in arterioles than in the other microvascular compartments (Fig. 2). The abundance of Pecam1 in arteriolar EC is puzzling from a functional perspective, as PECAM1 is involved in cell survival\textsuperscript{15} and acts as adhesion molecule involved in leukocyte migration\textsuperscript{16}, yet this latter process does not take place in arterioles\textsuperscript{17–19}. In EC scRNA-seq data sets, no differences in Pecam1 or Cdh5 expression were found between different renal endothelial subsets\textsuperscript{20,21}, thereby indicating that increased arteriolar Pecam1 expression may be lost following enzymatic release from its \textit{in vivo} environment\textsuperscript{13}. The heterogeneous distribution of pan endothelial marker expression across microvascular compartments represents yet another example of microvascular endothelial heterogeneity, the biological significance of which remaining enigmatic to this day.
Figure 2 | Expression of pan endothelial gene Pecam1 relative to Cdh5 in microvascular compartments of the mouse kidney

RT-qPCR data of laser microdissected arterioles (A), glomeruli (G), peritubular capillaries (PTC), and postcapillary venules (PCV) from healthy mouse kidney. Whole kidney (WK) was included as reference. mRNA expression of pan endothelial gene platelet/endothelial cell adhesion molecule 1 (Pecam1), relative to the pan endothelial gene cadherin 5 (Cdh5) (n = 8/group). Statistical testing was performed using a one-way ANOVA comparing every column with every other column, applying Tukey’s multiple comparisons test. Only comparisons with p ≤ 0.05 are shown. Graphs display column means ± SD.

Novel applications of laser microdissection-based analyses

In this thesis, we have successfully developed a workflow to investigate microvascular EC within their pathophysiological niche by combining laser microdissection with miRNome and transcriptome analyses. While our primary focus has been on studying renal microvascular signatures in health and in response to sepsis, there are three additional applications that deserve attention, namely the study of microvascular compartments in different organs, applying LMD to non-microvascular compartments, and the combination of LMD with kinome and proteome analysis.

Firstly, I conducted preliminary experiments in mouse lungs to collect arterioles, alveolar capillaries, and postcapillary venules using LMD. The RNA yield of these compartments was similar to that of renal microvascular compartments. As expected based on our previous studies, compartment-specific gene expression patterns were preserved, including enriched expression of Pecam1 in all microvascular compartments, and lower Emcn levels in arterioles compared with alveolar capillaries and postcapillary venules (data not shown). This opens up novel avenues for omics-based investigations into the behavior of...
microvascular compartments in the lung and likely other organs. Studying the pulmonary microvasculature in the context of sepsis-associated acute respiratory distress syndrome (ARDS) in mouse CLP-sepsis using this workflow is of major interest, as ARDS is a frequent complication in sepsis. Hence, detailed descriptions on local microvascular responses in the lung during onset and development of sepsis could elucidate the molecular signature of pulmonary microvascular responses.

Secondly, LMD is also well-equipped to study non-microvascular structures with a high level of precision. For instance, we have successfully collected cortical tubules from the mouse kidney and bronchioles from the lung using LMD. By capitalizing on the capability of LMD to compartmentalize distinct anatomical structures based on their histological characteristics, it provides a viable approach to molecularly characterize various structures, including different tubular subsets in the kidney or infiltrating leukocyte clusters. Currently, the workflow is optimized for visualization using hematoxylin or fluorescently labeled lectins. Although alternative staining methods or antibody-based detection may also be viable options for identifying target structures, it is important to note that these approaches often require longer incubation periods or more washing steps. These all affect the RNA content and integrity within the tissue. Hence, it is crucial to limit the frequency and length of washing steps to a minimum when setting up an alternative staining method in combination with LMD, and to assess RNA concentration and integrity in the sample afterwards.

Thirdly, while I focused on miRNome and transcriptome analyses of microvascular compartments, we have also explored the implementation of other omics analyses. The EBVDT team combined LMD-based collection of glomeruli from healthy and LPS-challenged mice and control and sepsis-AKI human kidney biopsies with kinome analysis of serine-threonine kinases (STKs) in collaboration with Vivomicx BV and PamGene International BV. The protein input from glomeruli proved sufficient to detect phosphorylation of target peptides (Fig. 3). Notably, a decrease in peptide phosphorylation levels was found in samples of patients with sepsis-AKI compared with healthy controls, suggesting reduced kinase activity in glomeruli in the course of sepsis-AKI development. These preliminary findings require additional biological and technical repeats, yet demonstrate the potential of integrating kinome analysis with LMD.

We encountered two limitations when combining LMD with this kinome analysis. First, at $2.5 \times 10^6 \mu m^2$, a relatively large amount of tissue was required as input to obtain a signal from the kinase arrays. Secondly, we encountered considerable inter-run variation between replicates, which will be discussed in more detail later. To circumvent these issues, phosphoproteomics represent an alternative method to study kinase activity by identifying and quantifying phosphorylated peptides. As sample compatibility with regular proteomics is a prerequisite for performing phosphoproteomics, we next investigated the combination of LMD with proteomics in collaboration with Vivomicx BV, Biogenity ApS, and the Technical University of Denmark. We determined the optimal input of LMD glomeruli, and based on the number of identified proteins and intragroup variation, we achieved an optimal balance
between LMD workload and sensitivity of analysis at 1x10⁶ µm² input (Fig. 4A). Another study reported proteomics analysis of 8,000 alveolar cells collected by LMD, which resulted in the identification of ~2,200 unique proteins. If we assume that on average we collected ~50 cells per glomerulus with LMD, and one glomerulus is approximately 160 µm² in size, then ~8,000 cells were collected in 1x10⁶ µm² surface area, which is comparable to the alveolar cell study. At the Technical University of Denmark, in the group headed by Dr. Erwin Schoof, we were able to identify 2,800 unique proteins in the glomerular samples, which is almost 30% higher. Our preliminary results surpassed the published study in terms of the number of identified proteins, showcasing the potential of combining LMD with proteomics. Moreover, we are currently exploring the possibility of extracting proteins from the “waste products” generated during sample processing for RNA isolation. Preliminary data indicated successful protein extraction, with an average of more than 4,000 detected proteins (Fig. 4B). It is of note that while increased tissue input for these samples is not associated with increased proteins numbers, it did result in decreased intragroup variation at the level of individual proteins (data not shown). These findings represent an important step towards the development of a workflow in which miRNome, transcriptome, and proteome profiles are generated from the same LMD sample. This integrated approach will not only reduce resource requirements, but also provides the major advantage of generating a multiomics molecular profile, thereby maximizing the information obtained from scarce samples.

Emergence of digital spatial profiling to study microvascular endothelial cells in vivo

An important limitation of using LMD to study microvascular EC is the unavoidable sample contamination with non-endothelial vascular support cells that dilute and contaminate endothelial signatures. Although alternative techniques such as single-cell RNA sequencing (scRNA-seq) can provide relatively pure EC populations, gene expression profiles are known to change following enzymatic cell dissociation. To address these challenges, Digital Spatial Profiling (DSP) represents a novel technology to study the transcriptome or proteome of regions of interest while preserving spatial information and eliminating the need for enzymatic digestion. Given that microvascular EC typically form a thin cell layer closely associated with surrounding vascular support cells, it remains to be determined whether DSP can yield pure microvascular endothelial gene expression signatures. It is important to note that currently available approaches possess clear strengths as well as limitations. Considering the rapid advancements in the field of spatial profiling, any aforementioned obstacles hindering the acquisition of microvascular endothelial transcriptome and proteome signatures are likely to be overcome by further technical improvements of spatial profilers.
Figure 3 | Kinome analysis by Serine-Threonine Kinase activity arrays of glomeruli following sepsis
PamChip® Serine-Threonine Kinase (STK) arrays were performed on laser microdissected glomeruli from (A) mouse and (B) human kidney. Mouse samples included kidneys from control and lipopolysaccharide-treated (LPS, 4 h) mice, and human samples included control kidney and kidney of patients with sepsis-associated acute kidney injury (n = 3/group). Heatmaps display peptide phosphorylation levels with averaged results per group, with peptides with highest phosphorylation levels indicated at the top (red) and peptides with lowest phosphorylation levels at the bottom (blue). Data were obtained in collaboration with Vivomicx BV and PamGene International.

Figure 4 | Proteomic analysis of mouse glomeruli
(A) Glomeruli were laser microdissected (LMD) from the kidney of healthy mice in different quantities prior to LC-MS (liquid chromatography-mass spectrometry) proteome analysis (n = 6/group). LC-MS was performed on samples that were either (A) directly analyzed following LMD (unprocessed) or (B) obtained from “waste
products” generated during RNA isolation of LMD samples (processed). Graphs indicate the unique number of proteins identified by proteomic analysis in \(1 \times 10^5\), \(5 \times 10^5\), \(1 \times 10^6\), and \(3 \times 10^6\) µm\(^2\) laser microdissected glomeruli. Statistical testing was performed using a one-way ANOVA comparing every column with every other column, applying Tukey’s multiple comparisons test. Only comparisons with \(p \leq 0.05\) are shown. Graphs display column means ± SD. Data were obtained in collaboration with Vivomeix BV, Biogenity ApS, and the Technical University of Denmark.

The future of kinome and phosphatome analyses

To determine the feasibility of using kinome analysis to identify kinases with altered activation statuses following mouse CLP-sepsis, we conducted kinome profiling of protein tyrosine kinases (PTKs) and serine-threonine kinases (STKs) in kidney protein homogenates. Preliminary data indicated that predicted PTK activity was reduced at 4 h post-CLP, followed by increased activity at subsequent time points (Fig. 5A). Also predicted STK activity was reduced in response to CLP at 4 h, with increased activity observed at 7 and 72 h (Fig. 5B). It is of interest that the 4 h time point, characterized by the activation of various inflammation-related pathways as described in chapter 7, is associated with reduced PTK and STK activity. Although initially surprising, this observation might be explained by considering the close involvement of kinases in constitutively activated pathways required to maintain basic cellular functions. I hypothesize that in sepsis, while pro-inflammatory pathways and their associated kinases become activated, the net change in kinase activity is negative as a result of diminished activation of otherwise constitutively active pathways, such as cell structure and cell cycle related processes\(^{27,28}\). To validate the overall changes in kinase activity predicted by kinome analysis, techniques such as Western blotting with phosphotyrosine (p-Tyr) or phospho-serine/threonine (p-Ser/Thr) antibodies can be employed to assess total protein phosphorylation levels. Utilizing different molecular analysis techniques that reach similar conclusions can increase confidence in the robustness of these findings. Moreover, additional experiments can be performed to validate the kinases with altered activity in sepsis, e.g. c-Kit, platelet-derived growth factor receptor β (PDGFRβ), and the protein kinase C (PKC) family, as predicted by the upstream kinase analysis (Fig. 5, C and D). Western blotting for total and phosphorylated forms of these kinases or their known substrates will shed light on their presence in the target tissue and their activation status. In addition, immunohistochemistry can be employed to determine the localization of kinases in the target tissue, although the availability of phospho-specific antibodies compatible with this application remains a serious limiting factor.
Figure 5 | Kinome analysis in mouse kidney following sepsis

PamChip® Protein Tyrosine Kinase (PTK) and Serine-Threonine Kinase (STK) arrays were performed on kidney protein homogenates of sham-operated and cecal ligation and puncture (CLP)-sepsis mice sacrificed 4, 24, or 72 h post-surgery (n = 6/group) to identify kinases with altered activity following sepsis-associated acute kidney injury. Based on peptide phosphorylation patterns, the predicted activity of individual kinases is determined by upstream kinase analysis (UKA), which is expressed as the mean kinase statistic. Mean kinase statistics was shown for all kinases per time point for (A) PTK and (B) STK analysis. Graphs display column means ± SD. The top 25 kinases with the largest magnitude in predicted altered activity in response to CLP-sepsis are indicated per time point as determined by UKA for (C) PTK and (D) STK. Box color represents mean kinase statistic, with positive (red) values indicating an increase in predicted activity following CLP and negative (blue) values indicating the opposite. Black cells represent absence of kinases from the top 25 kinases with altered activity at the indicated time point.
An important limitation of kinome analyses based on PamChip® kinase activity arrays lies in the considerable inter-run variation observed in both biological and technical replicates. While this issue can be addressed during data analysis, it warrants cautious interpretations of the array data. Moreover, the specific settings of the kinase prediction algorithm significantly impact the identity and number of predicted kinases, as well as the level of certainty associated with these predictions. This characteristic makes this particular type of kinome analysis suboptimal for target identification studies. Other kinase activity arrays employ different approaches to assess kinase activity and may therefore not face similar technical issues, although we have not yet investigated this.

At the moment of writing, a search in PubMed for the term “kinome” yields 2,002 articles, whereas “phosphatome” returns only 61 publications. Given the pivotal role of kinases in cancer, this dichotomy is understandable. Perhaps due to the heightened focus on kinases, elevated peptide phosphorylation is often attributed to increased kinase activity, although such changes could similarly arise from reduced phosphatase activity. While commercial activity assays are available for specific phosphatases, the field lacks high-throughput platforms for this purpose, which require further technological developments. The emergence of phosphoproteomics, a powerful tool for identifying proteins with phosphate modifications, has allowed in-depth characterizations of the preferred substrates of numerous kinases, yet similar characterizations for phosphatases are still awaited. I anticipate that similar technological advancements will facilitate high-throughput phosphatase activity profiling and identification of substrate motifs in upcoming years. These developments will provide valuable insights into the biological roles of specific phosphatases, thereby supporting future developments of therapeutics targeting phosphatases.

High throughput analyses of non-coding RNAs
In chapter 5, we identified “marker miRNAs” with enriched transcription in renal microvascular compartments. A notable example is the glomerular-enriched transcription of mmu-miR-322-3p which was confirmed by ISH, although its presence in glomerular EC still needs co-localization experiments with endothelial-specific markers. At present this is not possible due to lack of fluorescent ISH probes for miRNAs. This technical issue could also be overcome by the introduction of single-cell small RNA sequencing, which represents a future avenue to investigate heterogeneous miRNA transcription patterns in EC. However, the reliable detection of small RNAs at single-cell level poses a technical obstacle that needs to be addressed first. Once these challenges are overcome, I expect that the ability to profile miRNAs at the single-cell level will further increase interest in miRNAs, and provide valuable insights into miRNA transcription patterns of both endothelial and non-EC, similar to the impact of scRNA-seq on mRNAs. As cell dissociation protocols required for single-cell analyses introduced gene expression artifacts at mRNA level, similar problems may occur at miRNA level. In chapter 5, we compared scRNA-seq data of renal microvascular EC from three different studies, and showed that there is substantial overlap in microvascular-
enriched genes between scRNA-seq studies, but also revealed that many genes were not shared (Fig. 6). While the genes that overlapped in these studies likely exhibit enriched microvascular EC expression patterns, the differences between these studies also highlight that results obtained with scRNA-seq differ. This may stem from differences in sample preparation or bioinformatics approaches. As small RNA-seq at the single-cell level may experience similar issues, it is crucial that results are partly validated using techniques such as RT-qPCR and (F)ISH, while also comparing them with other technologies, including the combination of LMD and small RNA-seq.

![Figure 6](image)

**Figure 6 | Comparison of LMD/RNA-seq and scRNA-seq in determining enriched microvascular endothelial genes in mouse kidney**

Venn diagrams show enriched genes per microvascular compartment as established via laser microdissection/RNA sequencing (RNA-seq) (Luxen et al.26) or as identified in endothelial cell (EC) single-cell RNA-seq studies (Barry et al.21, Kalucka et al.20, and Karaiskos et al.31) for arterioles, glomeruli, and postcapillary venules (PCV). The Karaiskos et al. data set only included glomerular ECs. Figure was taken from chapter 5 where it was included as Figure 8E, the identity of mRNAs included in the Venn diagrams is provided in Supplemental Table S4 in chapter 5.

While this thesis focused on miRNAs, other non-coding RNA categories are also of interest with regard to their microvascular transcription patterns and their effects on cellular behavior in health and disease. Circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) have been identified as potential miRNA sponges, thereby preventing miRNA-mediated repression of target mRNAs32,33. For instance, in EC, circRNA circGNAQ has been implicated in modulating senescence34, whereas lncRNA CASC7 was shown to ameliorate endothelial inflammatory activation35. Comprehensive research efforts are required to establish cell type-specific profiles of circRNA and lncRNA, and to identify the processes influenced by the sequestration of target miRNAs. Our microvascular sequencing data included sequences corresponding to other non-coding RNA classes, and in this manner, we identified significantly enriched transcription of lncRNAs in peritubular capillaries. In addition, small nucleolar RNAs (snoRNAs) and transfer RNAs (tRNAs) were enriched in glomeruli compared...
with the other microvascular compartments (Fig. 7). Further investigations are required to determine whether these patterns are associated with differences in identity of individual non-coding RNAs, and to subsequently elucidate the functional implications of observed differences. In this manner, by simultaneously profiling various classes of non-coding RNAs alongside transcriptome analysis, we can unveil a comprehensive overview of all known players involved in the inhibition of mRNA translation into proteins. Similar to the miRNA-mRNA pair analysis conducted in chapter 5, I expect this information to reveal intricate connections between mRNAs and non-coding RNAs, as well as between different classes of non-coding RNAs. These insights will contribute to unraveling elaborate networks of molecules involved in functional gene repression.

Figure 7 | Transcription patterns of non-coding RNAs in renal microvascular compartments
Small RNA sequencing was performed on laser microdissected arterioles (A), glomeruli (G), peritubular capillaries (PTC), postcapillary venules (PCV) from healthy mouse kidney (n = 4/group). Relative read counts of long non-coding RNA (lncRNA), small nucleolar RNA (snoRNA), and transfer RNA (tRNA) are shown. Statistical testing was performed using a one-way ANOVA comparing every column with every other column, applying Tukey's multiple comparisons test. Only comparisons with p ≤ 0.05 are shown. Graphs display column means ± SD.

CONCLUDING REMARKS

In this thesis, I investigated microvascular endothelial behavior in health, and in response to sepsis-like stimuli in vitro and sepsis-AKI in vivo, while emphasizing the heterogeneity of endothelial cells and their responses in different renal microvascular compartments. The uncovered changes in kinome, miRNome, and transcriptome form a single piece of an extraordinarily complex puzzle that we are gradually piecing together. Anticipating the outcomes of the experiments discussed in the future perspectives, I am convinced that as our understanding on endothelial heterogeneity expands further, the development of effective therapeutics in sepsis-AKI that target the endothelium will become within reach.