Metabolomics study of blood vessels-on-chip model

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Chapter 1

General Introduction and Scope of the thesis

Microvascular diseases and endothelial dysfunction

For decades, cardiovascular diseases (CVDs) remain the leading causes of death globally and have a substantial impact on health decline and excessive expenses in the healthcare system. The leading risks of CVDs are environmental, metabolic, behavioral, and several others [1,2]. Multiple studies have established a link between CVDs and endothelial dysfunction (ED) [3,4], with the severity of the condition often linked to the availability of vascular nitric oxide (NO).

In order to understand ED mechanisms, it is especially relevant to study the structural components of microvasculature. Under healthy conditions, vascular endothelium is a major regulator of cardiovascular homeostasis through its modulation of fibrinolysis and blood viscosity, vascular constriction, angiogenesis, and leukocyte adhesion. In particular, endothelium-derived NO is essential for the regulation of vascular tone and blood pressure, prevention of platelet aggregation and the inhibition of vascular smooth muscle proliferation [5].

CVD is often caused by the long-term exposure of the vasculature to adverse hemodynamic or metabolic risk factors such as hypertension, smoking, hyperglycemia or dyslipidemia [6]. In time, these risk factors can lead to a chronic, low grade systemic inflammatory condition that, through oxidative stress, impairs the bioavailability of NO. This pathological condition contributes to platelet and leukocyte activation and adhesion, the activation of cytokines [7] and increased permeability to oxidized lipoproteins and inflammatory mediators. While these processes are the main drivers of atherosclerosis and stenosis in the large conduit arteries and veins, chronic inflammation of the microvasculature can lead to the loss of cell-cell contacts, pericyte attachment and, ultimately, microvascular rarefaction [6,8]. Therapies and life-style approaches to counteract CVD progression aim to reverse ED by enhancing the release of NO from the endothelium [9–11]. Therefore, a healthy vascular endothelium is considered a guardian of cardiovascular health, while any abnormality from its normal state significantly contributes to several cardiovascular disorders, including atherosclerosis, aging, hypertension, ischemic heart disease, and obesity [12]. ED, in addition to cardiovascular

diseases (CVDs), is a systemic disease that affects different systems in the body and causes serious impacts (**Fig. 1**).

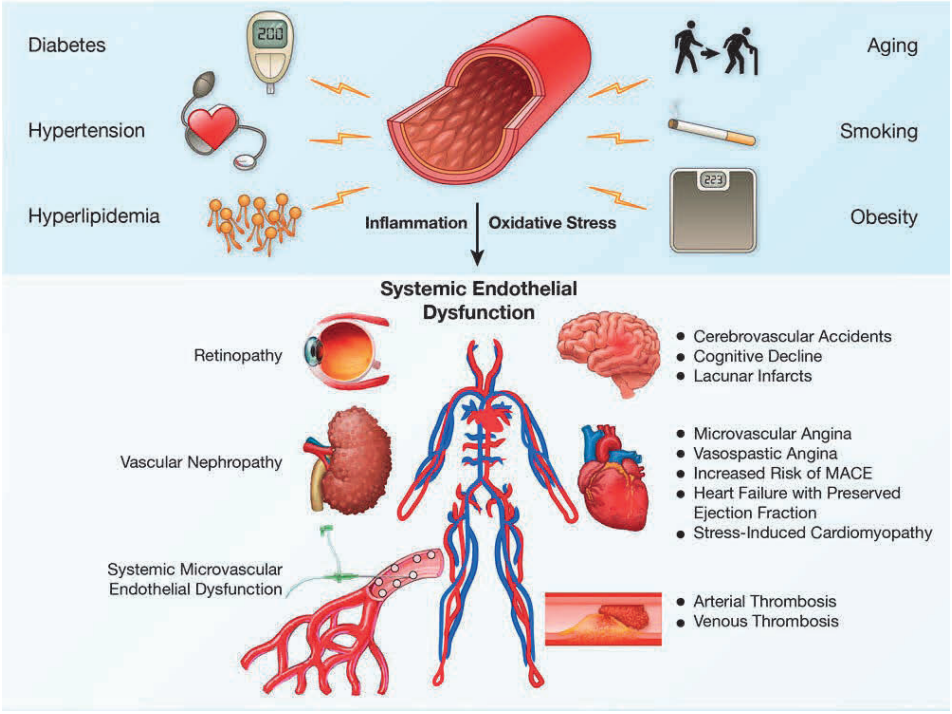

Figure 1. Endothelial dysfunction (ED)—a systemic disease. Figure represents risk factors and widespread systemic manifestations of vascular ED. MACE indicates major adverse cardiovascular events [13].

Vascular nitric oxide: mechanism and action

Nitric oxide (NO) is a highly reactive radical molecule specifically produced to function as a major messenger in the mammalian cardiovascular system. The innermost lining of microvessels is composed of endothelium, a monolayer of endothelial cells (ECs), and therefore is in direct contact with the blood/ circulating cells.

These endothelial cells are responsible for the production of NO through endothelial nitric oxide synthase (eNOS, NOS3), an enzyme that utilizes a semi essential amino acid, L-

arginine as a substrate. The synthesis of NO by eNOS involves a series of enzymatic actions. Activation of this enzyme is tightly regulated by numerous factors, including Ca^{2+}, calmodulin and phosphatases such as Protein kinase B (Akt). In addition, this process requires cofactors and co-substrates. These include tetrahydrobiopterin (BH_4) [14], flavin adenine dinucleotide (FAD), Flavin mono nucleotide (FMN), nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (O_2). The electron transfer pathway begins with the transfer of electrons from the cofactor NADPH to FMN and FAD and subsequently, electrons are transferred to the heme group, leading to the activation of molecular
oxygen. The activated oxygen species, in combination with L-arginine, undergo a series of reactions within the catalytic domain of eNOS to produce NO and L-citrulline. The NO production can be initiated by several stimuli including shear stress [15,16], acetylcholine [17], bradykinin [18], vascular endothelial growth factor (VEGF) [19]. Following synthesis, NO can diffuse into the lumen and contributes to passivation of platelet aggregation and into the underlying smooth muscle cells to stimulate cyclic guanosine monophosphate-dependent relaxation/vasodilation. Along with its key role in vasodilation, NO exhibits anti-inflammatory properties by inhibiting the adhesion of white blood cells to the endothelium [20]. Outside of vascular system NO acts as a neurotransmitter [21], plays a role in immune responses when produced by macrophages (iNOS, NOS2) and contributes to the elimination of microbes [22].

Application of stable isotope technique to measure arginine-based NO metabolites

Due to its high reactivity and short lifespan [23], the quantification of NO production from the endothelial cells relies on indirect methods of measurement. The labelled L-arginine to L-citrulline conversion to measure of eNOS activity was introduced by Castillo et al group [24]. Isotopic approaches are considered indirect since they rely on precursor-end product paradigms to determine the pharmacokinetics of a certain metabolic pathway. This way tracer-metabolomics helps in understanding the flow through certain biochemical pathways representing the dynamic input and output of

chemical processes, enabling the evaluation of metabolite concentrations in different system states [25].

The measurement of NO synthesis can utilize various isotopic procedures that target distinct stages of the arginine–NO–nitrate pathway. These protocols can monitor the conversion of arginine into either citrulline or NO_3^- **(Fig. 2A)**. The production of L-citrulline can run through different routes. The majority of citrulline in humans is produced from L-glutamine [26–28] and from ADMA [29]. The production of L-citrulline occurs within the urea cycle, where the conversion of L-arginine to L-ornithine is followed by the conversion to L-citrulline [30]**.** Conversion of L-arginine to L-citrulline through the action of eNOS also produces equimolar molecules of NO (**Fig. 2B)**. Therefore, L-arginine can be used as a precursor compound to be labeled and the arginine-citrulline pathway is selected for most applied methods to measure NO indirectly [31,32]. This was validated in studies showing that citrulline is a coproduct of NO production and has strong effects in vascular relaxation [33–35].

However, citrulline alone cannot predict the whole NO production, in addition, ratio analysis of citrulline, ornithine and arginine levels have to be taken into account to understand the healthy or pathological statuses [30]. Also, less frequently applied assays such as arginine-nitrate and oxygen- nitrate protocols have been reported [36–38]. Using L-arginine as a tracer compound, serum and urine nitrate was examined in cytokine-induced renal carcinoma patients. The findings indicated elevated concentrations of labeled nitrates [39]. This work provided the initial evidence that L-arginine as a source of NO_3^- in humans and only a small fraction (<1%) of the labeled arginine was recovered in urine.

Stable isotope tracers have also been used in the past decade for the determination of *in vivo* whole-body NO production. The intravenous primed-constant infusion of labeled arginine (L- $15N_2$- arginine) has been the most common protocol used to investigate NO production [40]. The rate of conversion of L-arginine into NO was measured by determining the isotopic enrichment in plasma citrulline. Overall, stable isotopic tracers have made substantial contributions to the understanding of the arginine-citrulline and NO^- pathway's biochemistry and regulation.

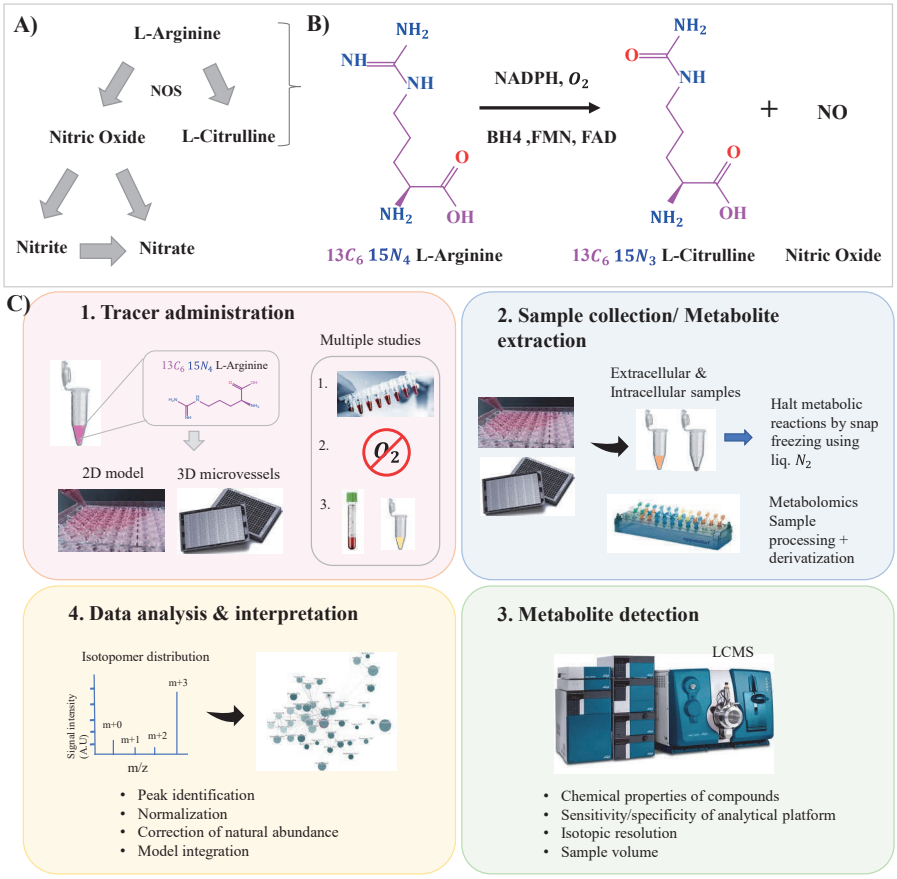

Figure 2. Endothelial nitric oxide synthase (eNOS)-catalyzed conversion of (A) L-arginine to L-citrulline and NO; NO and NO-derived nitrite is oxidized to nitrate. (B) Isotope labelled conversion of L-arginine ($^{13}C_6$ $^{15}N_4$ L-arginine) to L-citrulline ($^{13}C_6$,$^{15}N_3$ L-Citrulline) and NO and (C) Steps in stable-isotope metabolomics analysis.

Shear stress: A crucial stimulus for nitric oxide synthesis

Under healthy conditions, the endothelium tends to maintain the blood vessels in a state that promotes dilation rather than contraction. Regarding this matter, the endothelium can respond to different internal physical stimuli, including shear stress, temperature,

and transmural pressure, as well as external stimuli like temperature, mental stress, neurohumoral responses, and drugs.

Functionally, the mechanical stimulus (shear stress) generated on endothelial cells by the fluid flow is sensed by mechanosensors located in the endothelial cell membrane and trigger signaling cascades which are then transduced to the nucleus to activate transcription factors and gene expression. Similarly, shear stress acts as the prime regulator of the endothelium-dependent vasodilation response, by triggering the activation of eNOS. This, in turn, through the synthesis of intracellular cyclic cGMP by the neighboring VSMC, lowers vasomotor tone to accommodate the increased blood flow to the relevant tissues [41,42].

To model such conditions *in vitro*, 3D fluid flow-based models are instrumental for a better understanding of the physiologic regulation of eNOS [43]. This is further confirmed by observations that 2D steady state culture models often adopt a proinflammatory status with structural and functional changes similar to those of EC in contact with stiff substrates such as plastic or glass [44–46]. When shear stress is applied to endothelial cells in 3D *in vitro* models the cells display more physiological directional structural morphologies with improved cell-cell junction as when compared to steady state cultures [47]. In addition, in 3D microvessels-on-a-chip models that use collagen as extracellular matrix (ECM), stiff substrate related inflammatory phenotypes diminished (**Fig. 3B**) [48,49]. The critical role of extracellular matrix is to support the vascular endothelium by interacting with integrins on the surface of endothelial cells. It also offers a necessary scaffold for organizing vascular endothelial cells into microvessels [50].

Several *in vitro* 3D models have been reported that emphasized the significance of fluid-shear stress in vascular cell integrity and alignment. In order to investigate the biomechanical wall stress/strain conditions, the fluid-structure interaction (FSI) was simulated and investigated in a blood vessel on-a-chip model [52].

This model was used to optimize the culture conditions and investigate mechanical factors that contribute to cardiovascular disease development. Another small blood vessel model that uses mouse artery segments onto a microfluidic chip eliminated the need for tissue sectioning and processing outside the chip for immunohistochemical analysis. This model helps in understanding the intact blood vessels from different vascular beds, vascular

constructs and vascularized microtissues [53]. This study examines the end-user experience with organ-on-chip technology, focusing on assay development and usability for both healthy and diseased models [54]. Other organ-on-a-chip models investigated the vessel leakage or permeability to evaluate the endothelial barrier function [48], to study the pathophysiological changes in disease conditions by perfusing whole blood samples [55], 3D microvascular models enabled studies on the effect of oxygen conditions on endothelial sprouting [56], angiogenesis [57], co-culture responses [58–61] and drug-testing studies [62].

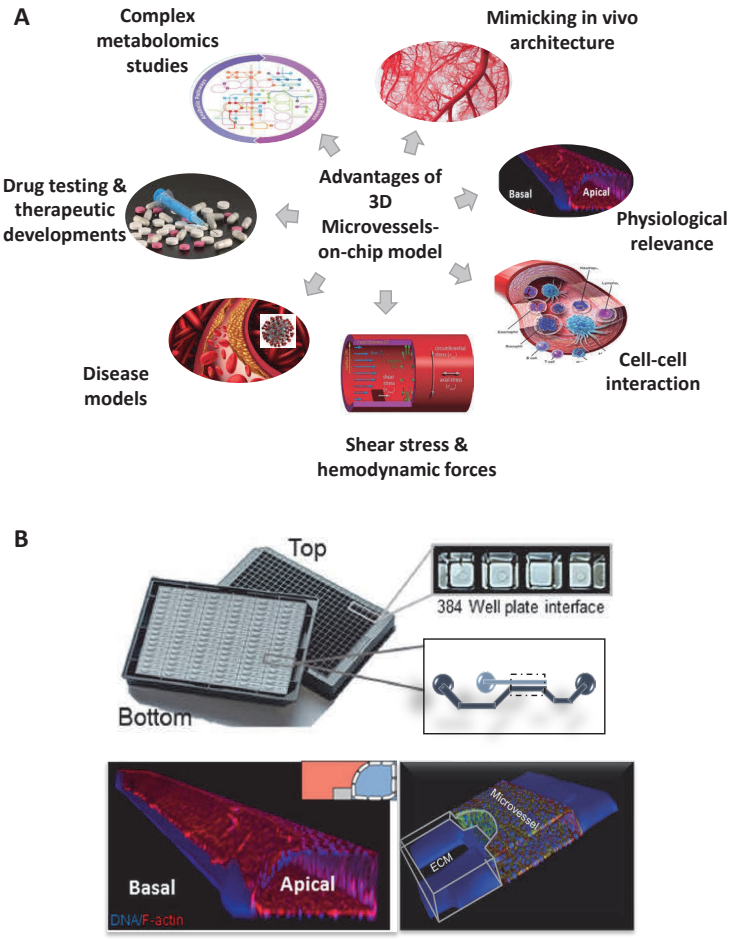

Figure 3. A) Some of the major advantages of the in vitro 3D microvessels-on-chip model. B) 3D microvessels-on-chip models with extra cellular matrix (ECM) [48,51].

Increased flow-induced nitric oxide production and eNOS activity was also demonstrated in a perfusable 3D microvessels model using the DAF-FM DA fluorescence dye [60,63]. [64]. These platforms may serve as prominent tools to study ED that measures the NO availability. However, issues with disturbed or irregular flow leads to unfavorable responses similar to those causing atherogenesis in vascular diseases [65] [66].

Over the years there has been a continued interest in the design of 3D micro vessel models to study NO metabolism. The integration of 3D models with single-cell analysis offers a potent approach to unraveling the complexities of vascular biology [67], advancing our comprehension of vascular disease, and facilitating the development of precision medicine approaches for patient care. While single-cell proteomics [68] and transcriptomics [69] have been extensively explored in endothelial cells, the exploration of in vitro single-cell metabolomics of vascular cells remains limited. The imperative need for single-cell metabolomics (SCM) arises from the substantial heterogeneity observed in vascular cells across different vascular beds, organotypic structures, and non-organotypic/vascular beds [70–72]. SCM becomes essential for unveiling the diversity in cellular responses, identifying biomarkers, understanding drug responses, and advancing precision medicine in the realm of vascular physiology and pathology.

To address this, an efficient live single-cell sampling technique in 2D culture [73] utilizing fabricated glass micropipettes, coupled with electrical lysis and the subsequent spray of cell content to nanoESI MS, has been developed. This method enables the effective detection of drugs at the single-cell level, marking a significant advancement toward high-throughput detection of single-cell metabolites. Furthermore, this highly sensitive approach holds potential for sampling single cells from complex 3D micro vessel models or co-culture models, providing enhanced insights into cell-cell interactions.

Scope and outline of this thesis

The central idea of this thesis is that endothelial dysfunction plays a critical role in vascular, particularly microvascular, diseases. This research aims to investigate endothelial dysfunction using in-vitro model systems, with the hypothesis that measuring eNOS activity is key to understanding this dysfunction. Tracer-based mass spectrometry, focused on arginine-specific NO metabolism, is highlighted as an effective tool for this analysis. The thesis ultimately seeks to develop complex in-vitro models, incorporating various cell types, to advance our understanding of vascular biology and support disease and drug research. Developing an in-vitro model of endothelial dysfunction involves creating a complex system that incorporates various cell types and cellular heterogeneity. This complexity necessitates the metabolic analysis of individual cells within vascular models, providing a powerful method to explore the nuances of vascular biology and improve our understanding of vascular diseases. The ultimate goal of the tools developed in this thesis is to enhance research in disease mechanisms and drug development.

We hypothesized that flow-mediated shear stress significantly impacts eNOS activity within a high-throughput 3D microvessels-on-chip platform equipped with a unidirectional fluid flow system. This microvessels-on-chip model, combined with metabolic profiling, is applied to study endothelial dysfunction in conditions such as acute hypoxia and vascular pathologies associated with COVID-19. Moreover, we also explored the significance of single-cell metabolomics in vascular models, offering a potent approach to unraveling the intricacies of vascular biology and advancing our comprehension of vascular disease. The ultimate aim is to offer tools for drug research and development of novel medications that are accessible to academia and industry.

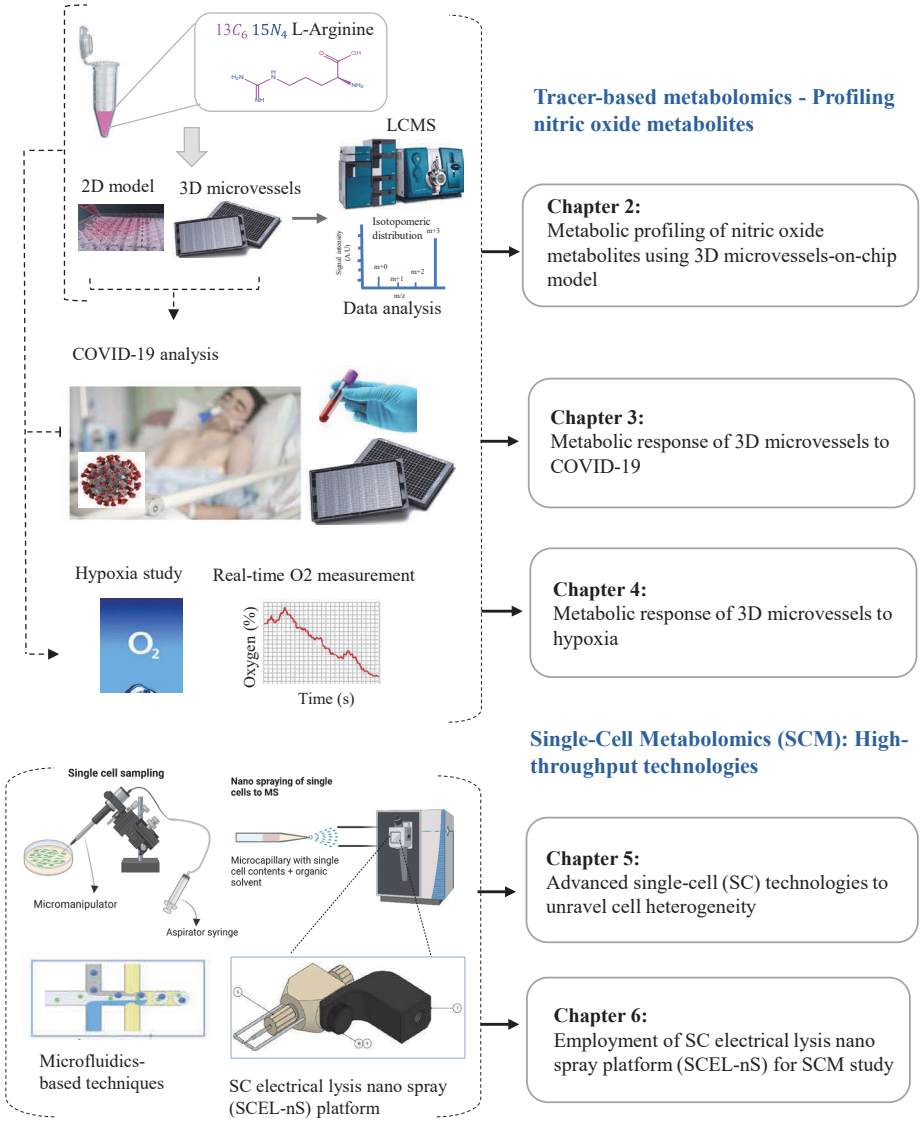

Figure 4. Overview of research chapters thesis structure and scope of each chapter.

Chapter 2 aims to develop a tracer-based metabolomics method using a liquid chromatography–tandem mass spectrometry-based assay to measure arginine-specific marker metabolites that are associated with eNOS activity and NO production. The developed assay is verified by showing that indeed eNOS expression is present, as indicated by an independent reference assay NO staining (DAF-2DA), and inhibitors of eNOS and arginase show the expected effect. Additionally, our optimized MS method proves to be ideal for measuring NO marker metabolites at both extracellular and intracellular levels, providing valuable insights into metabolic flux. Further, the impact of flow-mediated and shear-stress induced eNOS activity is studied by utilizing a three-dimensional perfusable high-throughput microvessels-on-chip model with a unidirectional fluid flow system. The result of shear stress- induced marker gene is observed high in expression under flow conditions. With an optimized sample preparation procedure, this sensitive method allows the detection of L-arginine and its downstream metabolites even at low-volume and low-abundance. The results of these tracer measurements in flow-mediated microvessels-on-chip models exhibit enhanced eNOS activity when compared to the conventional static culture.

Utilizing the microvessels and optimized tracer-based nitric oxide metabolite measurement platform developed in Chapter 2, the objective of **Chapter 3** is to investigate the roles of nitric oxide metabolites as potential diagnostic and disease progression markers in COVID-19. This chapter studies whether ED is associated with factors in COVID-19 plasma and eNOS uncoupling due to decreased BH4 levels. Perfusion of COVID-19 patient plasma (both severe and mild conditions) along with BH2 (a precursor of eNOS enzyme cofactor, BH4), eNOS enzyme stimulators, and inhibitors into the 3D microvessels is used to elucidate the impact of BH4 on eNOS activity with different COVID-19 conditions. The study investigates the mechanism by which ED in COVID-19 may be reversed through enhanced eNOS activity using stimulants, external BH2 introduction, and arginase enzyme inhibition.

In **Chapter 4**, we employ the optimized tracer-based metabolomics method developed in Chapter 2 to quantify NO metabolites under hypoxic conditions. Molecular oxygen's involvement in nitric oxide production and vasodilation is increasingly recognized as a

key factor in endothelial cell metabolism. Under hypoxia, the cells trigger adaptive responses to regulate the metabolic pathways and modulate angiogenesis. In addition to our NO metabolite analysis, we simultaneously measure signaling lipids using liquid chromatography-tandem mass spectrometry [74], a method that quantifies a panel of oxidative stress and anti-inflammatory bioactive lipids. These are used to generate expression profiles using the microvessels-on-chip model under transient and persistent hypoxic conditions to assess whether hypoxia influences eNOS activity and bioactive lipid metabolism. Furthermore, to evaluate the impact of shear stress on the HIF1a mechanism, the metabolic profiles of 3D microvessels and a 2D cell culture model are examined under hypoxic conditions. Advancing vascular metabolomics necessitates developing in vitro models that accurately replicate the complex vascular environment, including diverse cell types and cellular heterogeneity. Precise metabolic analysis of individual cells within these models is crucial for unraveling the intricacies of vascular biology. Hence, the following chapters will discuss potential advanced methods for single-cell metabolite measurement.

The objective of **Chapter 5** is to provide a concise overview of the latest techniques in single-cell sampling and detection methods in metabolomics, with a particular focus on achieving efficient analysis by mass spectrometry. We summarize various microfluidic chip models for performing active single-cell separation or sorting based on size, biophysical, acoustic properties, and fluorescent-based sorting. This analysis offers a comprehensive range of techniques that can be utilized to evaluate biological properties and responses and address biological, clinical, or pharmacological inquiries at the individual cell level. This chapter demonstrates that these technologies can be utilized for any cell type. However, only a limited number of systems have achieved both efficient and high-throughput analysis, which is necessary for the development of potential detection tools.

Chapter 6 aims to demonstrate the application of the single-cell electrical lysis and nano spray (SCEL-nS) platform, building upon the advanced technologies for single-cell metabolomics discussed in the previous chapter (Chapter 5). This study involves fabricating a glass microcapillary with a precise tip diameter by optimizing the fabrication

parameters and then applying metal deposition to enhance its electrical conductivity. The constructed micropipettes are employed to extract single-cell samples from cells treated with drugs. Subsequently, the cells are lysed and introduced into a mass spectrometry (MS) system using an SCEL-nS platform. Furthermore, the effectiveness of cell lysis is compared between the non-lysed cells, which were processed using a traditional platform, and the electrical lysed cells, which were processed using the SCEL-nS platform.

Finally, **Chapter 7** provides discussions and conclusions of this thesis. A critical evaluation of the research is revealed together with a discussion about the future perspective and directions of the field of vascular biology and organ-on-chips to study microvascular diseases and high-throughput single-cell metabolomics.

References

[1] Lindstrom, M. *et al.* (2022). Global Burden of Cardiovascular Diseases and Risks Collaboration, 1990-2021. *Journal of the American College of Cardiology*. https://doi.org/10.1016/j.jacc.2022.11.001.

[2] Vaduganathan, M. *et al.* (2022). The Global Burden of Cardiovascular Diseases and Risk: A Compass for Future Health. *Journal of the American College of Cardiology*. https://doi.org/10.1016/j.jacc.2022.11.005.

[3] Little, P.J. *et al.* (2021). Endothelial Dysfunction and Cardiovascular Disease: History and Analysis of the Clinical Utility of the Relationship. *Biomedicines*. https://doi.org/10.3390/biomedicines9060699.

[4] Widmer, R.J. and Lerman, A. (2014). Endothelial dysfunction and cardiovascular disease. *Global Cardiology Science & Practice*. https://doi.org/10.5339/gcsp.2014.43.

[5] Moncada, S. and Higgs, E.A. (2006). Nitric oxide and the vascular endothelium. *Handbook of Experimental Pharmacology*. https://doi.org/10.1007/3-540-32967-6_7.

[6] Yang, Z. *et al.* (2013). Impairment of vascular endothelial function following reperfusion therapy in patients with acute myocardial infarction. *The Journal of International Medical Research*. https://doi.org/10.1177/0300060513487650.

[7] Zhang, C. (2008). The role of inflammatory cytokines in endothelial dysfunction. *Basic research in cardiology*. https://doi.org/10.1007/s00395-008-0733-0.

[8] Goligorsky, M.S. (2010). Microvascular rarefaction: The decline and fall of blood vessels. *Organogenesis*. https://doi.org/10.4161/org.6.1.10427.

[9] Tousoulis, D. *et al.* (2012). The role of nitric oxide on endothelial function. *Current Vascular Pharmacology*. https://doi.org/10.2174/157016112798829760.

[10] Prasad, A. *et al.* (1999). Glutathione reverses endothelial dysfunction and improves nitric oxide bioavailability. *Journal of the American College of Cardiology*. https://doi.org/10.1016/S0735-1097(99)00216-8.

[11] Janaszak-Jasiecka, A. *et al.* (2023). Endothelial dysfunction due to eNOS uncoupling: molecular mechanisms as potential therapeutic targets. *Cellular & Molecular Biology Letters*. https://doi.org/10.1186/s11658-023-00423-2.

[12] Sun, H.-J. *et al.* (2020). Role of Endothelial Dysfunction in Cardiovascular Diseases: The Link Between Inflammation and Hydrogen Sulfide. *Frontiers in Pharmacology*. https://doi.org/10.3389/fphar.2019.01568.

[13] Corban, M.T. *et al.* (2019). Endothelial Dysfunction. *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/ATVBAHA.119.312836.

[14] Rhodes, C.J. *et al.* (2009). Therapeutic targets in pulmonary arterial hypertension. *Pharmacology & Therapeutics*. https://doi.org/10.1016/j.pharmthera.2008.10.002.

[15] Corson, M.A. *et al.* (1996). Phosphorylation of Endothelial Nitric Oxide Synthase in Response to Fluid Shear Stress. *Circulation Research*. https://doi.org/10.1161/01.RES.79.5.984.

[16] Ngai, C. and Yao, X. (2010). Vascular Responses to Shear Stress: The Involvement of Mechanosensors in Endothelial Cells~!2009-12-23~!2010-04-27~!2010-08-06~! https://doi.org/10.2174/1874382601003010085.

[17] Kellogg, D.L. *et al.* (2005). Acetylcholine-induced vasodilation is mediated by nitric oxide and prostaglandins in human skin. *Journal of Applied Physiology*. https://doi.org/10.1152/japplphysiol.00728.2004.

[18] Bae, S.W. *et al.* (2003). Rapid increase in endothelial nitric oxide production by bradykinin is mediated by protein kinase A signaling pathway. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/S0006-291X(03)01086-6.

[19] Feliers, D. *et al.* (2005). VEGF regulation of endothelial nitric oxide synthase in glomerular endothelial cells. *Kidney International*. https://doi.org/10.1111/j.1523-1755.2005.00575.x.

[20] Sharma, J.N. *et al.* (2007). Role of nitric oxide in inflammatory diseases. *Inflammopharmacology*. https://doi.org/10.1007/s10787-007-0013-x.

[21] Picón-Pagès, P. *et al.* (2019). Functions and dysfunctions of nitric oxide in brain. *Biochimica Et Biophysica Acta. Molecular Basis of Disease*. https://doi.org/10.1016/j.bbadis.2018.11.007.

[22] Coleman, J.W. (2001). Nitric oxide in immunity and inflammation. *International Immunopharmacology*. https://doi.org/10.1016/s1567-5769(01)00086-8.

[23] Kelm, M. (1999). Nitric oxide metabolism and breakdown. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*. https://doi.org/10.1016/S0005-2728(99)00020-1.

[24] Castillo, L. *et al.* (1996). Whole body nitric oxide synthesis in healthy men determined from [15N] arginine-to-[15N]citrulline labeling. *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.93.21.11460.

[25] El-Hattab, A.W. and Jahoor, F. (2017). Assessment of Nitric Oxide Production in Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-Like Episodes Syndrome with the Use of a Stable Isotope Tracer Infusion Technique. *The Journal of Nutrition*. https://doi.org/10.3945/jn/117.248435.

[26] Luiking, Y.C. *et al.* (2004). In Vivo Whole Body and Organ Arginine Metabolism During Endotoxemia (Sepsis) Is Dependent on Mouse Strain and Gender. *The Journal of Nutrition*. https://doi.org/10.1093/jn/134.10.2768S.

[27] Windmueller, H.G. and Spaeth, A.E. (1981). Source and fate of circulating citrulline. *American Journal of Physiology-Endocrinology and Metabolism*. https://doi.org/10.1152/ajpendo.1981.241.6.E473.

[28] Windmueller, H.G. and Spaeth, A.E. (1974). Uptake and Metabolism of Plasma Glutamine by the Small Intestine. *Journal of Biological Chemistry*. https://doi.org/10.1016/S0021-9258(19)42329-6.

[29] Pai, J.K. (2008). Asymmetric dimethylarginine as a marker of metabolic dysfunction and cardiovascular disease. *Current Cardiovascular Risk Reports*. https://doi.org/10.1007/s12170-008-0028-x.

[30] Marini, J.C. (2012). Arginine and ornithine are the main precursors for citrulline synthesis in mice. *The Journal of Nutrition*. https://doi.org/10.3945/jn.111.153825.

[31] Soeters, P.B. *et al.* (2002). Quantitative in vivo assessment of arginine utilization and nitric oxide production in endotoxemia. *American Journal of Surgery*. https://doi.org/10.1016/s0002-9610(02)00847-4.

[32] Luiking, Y.C. *et al.* (2008). Reduced citrulline availability by OTC deficiency in mice is related to reduced nitric oxide production. *American Journal of Physiology. Endocrinology and Metabolism*. https://doi.org/10.1152/ajpendo.00055.2008.

[33] Raghavan, S.A. and Dikshit, M. (2001). L-citrulline mediated relaxation in the control and lipopolysaccharide-treated rat aortic rings. *European Journal of Pharmacology*. https://doi.org/10.1016/s0014-2999(01)01407-8.

[34] Wileman, S.M. *et al.* (2003). Role of L-citrulline transport in nitric oxide synthesis in rat aortic smooth muscle cells activated with LPS and interferon-γ. *British Journal of Pharmacology*. https://doi.org/10.1038/sj.bjp.0705407.

[35] Theodorou, A.A. *et al.* (2021). Acute L-Citrulline Supplementation Increases Nitric Oxide Bioavailability but Not Inspiratory Muscle Oxygenation and Respiratory Performance. *Nutrients*. https://doi.org/10.3390/nu13103311.

[36] Sakinis, A. and Wennmalm, A. (1998). Estimation of total rate of formation of nitric oxide in the rat. *Biochemical Journal*.

[37] Böger, R.H. *et al.* (2004). Hypercholesterolemia impairs basal nitric oxide synthase turnover rate: a study investigating the conversion of L-[guanidino-(15)N(2)]-arginine to (15)N-

labeled nitrate by gas chromatography--mass spectrometry. *Nitric Oxide: Biology and Chemistry*. https://doi.org/10.1016/j.niox.2004.07.008.

[38] Wickman, A. *et al.* (2003). A technique to estimate the rate of whole body nitric oxide formation in conscious mice. *Nitric Oxide: Biology and Chemistry*. https://doi.org/10.1016/j.niox.2003.09.001.

[39] Leaf, C.D. *et al.* (1989). L-arginine is a precursor for nitrate biosynthesis in humans. *Biochemical and Biophysical Research Communications*. https://doi.org/10.1016/0006-291x(89)92325-5.

[40] van Eijk, H.M.H. *et al.* (2007). Methods using stable isotopes to measure nitric oxide (NO) synthesis in the L-arginine/NO pathway in health and disease. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*. https://doi.org/10.1016/j.jchromb.2006.08.054.

[41] Boo, Y.C. *et al.* (2002). Shear Stress Stimulates Phosphorylation of Endothelial Nitric-oxide Synthase at Ser1179 by Akt-independent Mechanisms: ROLE OF PROTEIN KINASE A*. *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M108789200.

[42] Chiu, J.J. *et al.* (1999). Nitric Oxide Regulates Shear Stress–Induced Early Growth Response-1. *Circulation Research*. https://doi.org/10.1161/01.RES.85.3.238.

[43] Meng, F. *et al.* (2022). In vitro fluidic systems: Applying shear stress on endothelial cells. *Medicine in Novel Technology and Devices*. https://doi.org/10.1016/j.medntd.2022.100143.

[44] Sack, K.D. *et al.* (2016). Extracellular Matrix Stiffness Controls VEGF Signaling and Processing in Endothelial Cells. *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.25312.

[45] Baeyens, N. *et al.*Endothelial fluid shear stress sensing in vascular health and disease. *The Journal of Clinical Investigation*. https://doi.org/10.1172/JCI83083.

[46] Hu, M. *et al.* (2020). Substrate stiffness differentially impacts autophagy of endothelial cells and smooth muscle cells. *Bioactive Materials*. https://doi.org/10.1016/j.bioactmat.2020.10.013.

[47] Noria, S. *et al.* (1999). Transient and Steady-State Effects of Shear Stress on Endothelial Cell Adherens Junctions. *Circulation Research*. https://doi.org/10.1161/01.RES.85.6.504.

[48] van Duinen, V. *et al.* (2017). 96 perfusable blood vessels to study vascular permeability in vitro. *Scientific Reports*. https://doi.org/10.1038/s41598-017-14716-y.

[49] Campisi, M. *et al.* (2018). 3D self-organized microvascular model of the human blood-brain barrier with endothelial cells, pericytes and astrocytes. *Biomaterials*. https://doi.org/10.1016/j.biomaterials.2018.07.014.

[50] Davis, G.E. and Senger, D.R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. *Circulation Research*. https://doi.org/10.1161/01.RES.0000191547.64391.e3.

[51] Junaid, A. *et al.* (2020). Ebola Hemorrhagic Shock Syndrome-on-a-Chip. *iScience*. https://doi.org/10.1016/j.isci.2019.100765.

[52] Wang, L. *et al.* (2023). A new approach of using organ-on-a-chip and fluid–structure interaction modeling to investigate biomechanical characteristics in tissue-engineered blood vessels. *Frontiers in Physiology*.

[53] Yasotharan, S. *et al.* (2015). Artery-on-a-chip platform for automated, multimodal assessment of cerebral blood vessel structure and function. *Lab on a Chip*. https://doi.org/10.1039/C5LC00021A.

[54] Junaid, A. *et al.* (2017). An end-user perspective on Organ-on-a-Chip: Assays and usability aspects. *Current Opinion in Biomedical Engineering*. https://doi.org/10.1016/j.cobme.2017.02.002.

[55] Dupuy, A. *et al.* (2021). Thromboinflammation Model-on-A-Chip by Whole Blood Microfluidics on Fixed Human Endothelium. *Diagnostics*. https://doi.org/10.3390/diagnostics11020203.

[56] Lam, S.F. *et al.* (2018). Microfluidic device to attain high spatial and temporal control of oxygen. *PLOS ONE*. https://doi.org/10.1371/journal.pone.0209574.

[57] van Duinen, V. *et al.* (2019). Perfused 3D angiogenic sprouting in a high-throughput in vitro platform. *Angiogenesis*. https://doi.org/10.1007/s10456-018-9647-0.

[58] Tobe, Y. *et al.* (2022). Perfusable vascular tree like construction in 3D cell-dense tissues using artificial vascular bed. *Microvascular Research*. https://doi.org/10.1016/j.mvr.2022.104321.

[59] Lee, E. *et al.* (2018). A 3D in vitro pericyte-supported microvessel model: visualisation and quantitative characterisation of multistep angiogenesis. *Journal of Materials Chemistry B*. https://doi.org/10.1039/C7TB03239K.

[60] Kim, S. *et al.* (2013). Engineering of functional, perfusable 3D microvascular networks on a chip. *Lab on a Chip*. https://doi.org/10.1039/C3LC41320A.

[61] Paek, J. *et al.* (2019). Microphysiological Engineering of Self-Assembled and Perfusable Microvascular Beds for the Production of Vascularized Three-Dimensional Human Microtissues. *ACS nano*. https://doi.org/10.1021/acsnano.9b00686.

[62] Dávila, S. *et al.* (2021). Microvessel-on-Chip Fabrication for the In Vitro Modeling of Nanomedicine Transport. *ACS Omega*. https://doi.org/10.1021/acsomega.1c00735.

[63] Xu, S. *et al.* (2016). Development and Characterization of In Vitro Microvessel Network and Quantitative Measurements of Endothelial [Ca2+]i and Nitric Oxide Production. *Journal of Visualized Experiments : JoVE*. https://doi.org/10.3791/54014.

[64] Hsieh, H.-J. *et al.* (2014). Shear-induced endothelial mechanotransduction: the interplay between reactive oxygen species (ROS) and nitric oxide (NO) and the pathophysiological implications. *Journal of Biomedical Science*. https://doi.org/10.1186/1423-0127-21-3.

[65] Chiu, J.-J. and Chien, S. (2011). Effects of Disturbed Flow on Vascular Endothelium: Pathophysiological Basis and Clinical Perspectives. *Physiological Reviews*. https://doi.org/10.1152/physrev.00047.2009.

[66] VanderLaan, P.A. *et al.* (2004). Site specificity of atherosclerosis: site-selective responses to atherosclerotic modulators. *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/01.ATV.0000105054.43931.f0.

[67] Rosowski, S. *et al.* (2023). Single-cell characterization of neovascularization using hiPSC-derived endothelial cells in a 3D microenvironment. *Stem Cell Reports*. https://doi.org/10.1016/j.stemcr.2023.08.008.

[68] Feng, W. *et al.* (2019). Single Cell Analysis of Endothelial Cells Identified Organ-Specific Molecular Signatures and Heart-Specific Cell Populations and Molecular Features. *Frontiers in Cardiovascular Medicine*.

[69] Liu, Z. *et al.* (2021). Single-Cell RNA Sequencing Reveals Endothelial Cell Transcriptome Heterogeneity Under Homeostatic Laminar Flow. *Arteriosclerosis, Thrombosis, and Vascular Biology*. https://doi.org/10.1161/ATVBAHA.121.316797.

[70] Aird, W.C. (2007). Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. *Circulation Research*. https://doi.org/10.1161/01.RES.0000255690.03436.ae.

[71] Becker, L.M. *et al.* (2023). Deciphering endothelial heterogeneity in health and disease at single-cell resolution: progress and perspectives. *Cardiovascular Research*. https://doi.org/10.1093/cvr/cvac018.

[72] Pusztaszeri, M.P. *et al.* (2006). Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and Fli-1 in normal human tissues. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society*. https://doi.org/10.1369/jhc.4A6514.2005.

[73] Ali, A. *et al.* (2019). Single-Cell Screening of Tamoxifen Abundance and Effect Using Mass Spectrometry and Raman-Spectroscopy. *Analytical Chemistry*. https://doi.org/10.1021/acs.analchem.8b04393.

[74] Yang, W. *et al.* (2024). A comprehensive UHPLC-MS/MS method for metabolomics profiling of signaling lipids: Markers of oxidative stress, immunity and inflammation. *Analytica Chimica Acta*. https://doi.org/10.1016/j.aca.2024.342348.