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Metabolomics study of blood vessels-on-chip model

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Citation

Kallakkudi Pandian, K. (2025, March 26). *Metabolomics study of blood vessels-on-chip model*. Retrieved from <https://hdl.handle.net/1887/4209144>

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Metabolomics study of blood vessels-on-chip model

Kanchana K Pandian

காஞ்சனா பாண்டியன்

The publication of the thesis was financially supported by:

Leiden University Libraries

Cover design: Kanchana Pandian

Thesis Layout: Kanchana Pandian

Printing: PrintSupport4U

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ISBN: 978-94-93289-75-8

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Metabolomics study of blood vessels-on-chip model

Proefschrift

ter verkrijging van

de graad van doctor aan de Universiteit Leiden,
op gezag van rector magnificus prof.dr.ir. H. Bijl,
volgens besluit van het college voor promoties
te verdedigen op woensdag 26 maart 2025

klokke 16:00 uur

door

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geboren te Tamilnadu, India

in 1992

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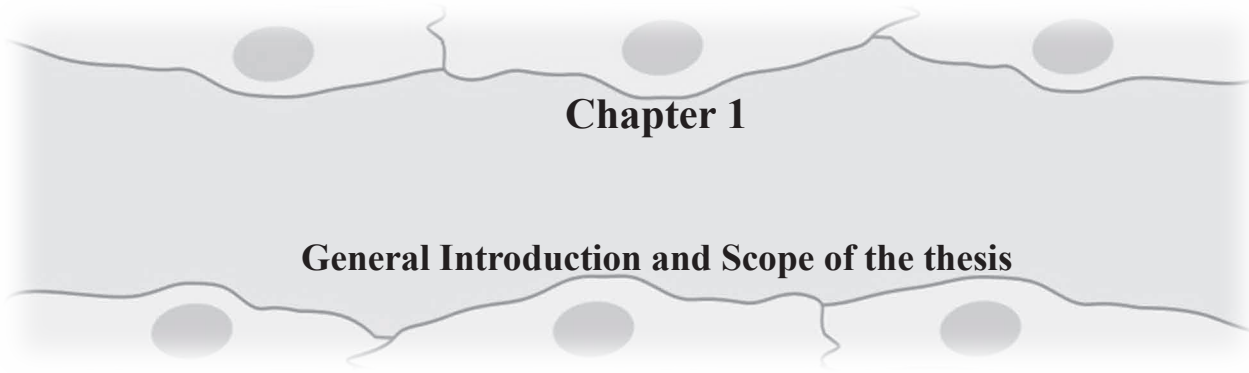
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The research described in this thesis was performed at Metabolomics and Analytics Center (MAC) of the Leiden Academic Centre for Drug Research (LACDR), and Leiden University of Medical Centre (LUMC), Leiden University (Leiden, The Netherlands). The research was financially supported as indicated in each chapter.

To my beloved family....

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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**).

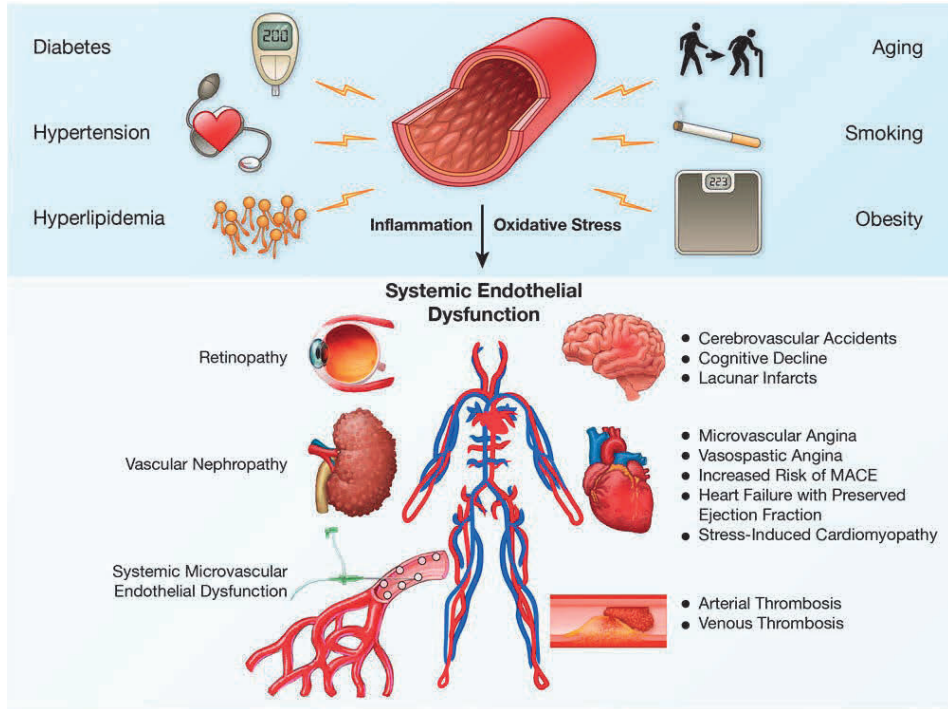


Figure 1. Endothelial dysfunction (ED)—a systemic disease. Figure represents risk factors and widespread systemic manifestations of vascular ED. MACE indicates major adverse cardiovascular events [13].

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- $^{15}\text{N}_2$ - 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.

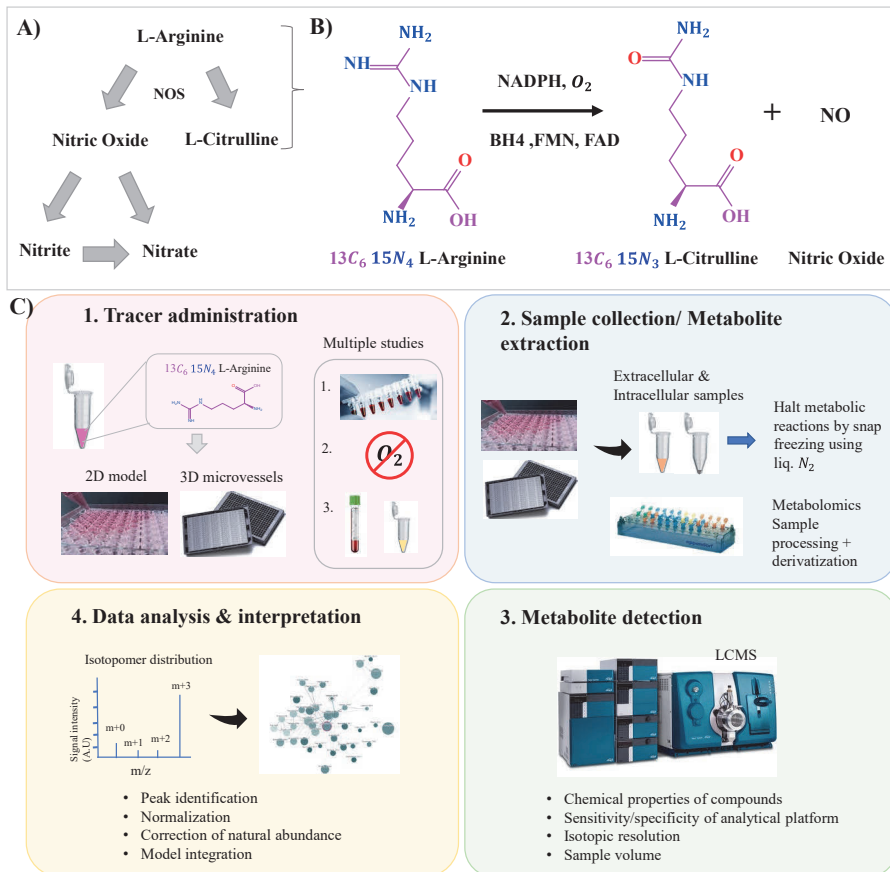


Figure 2. Endothelial nitric oxide synthase (eNOS)-catalyzed conversion of (A) L-arginine to L-citrulline and NO; NO and NO-derived nitrite is oxidized to nitrate. (B) Isotope labelled conversion of L-arginine ($^{13}\text{C}_6\ ^{15}\text{N}_4$ L-arginine) to L-citrulline ($^{13}\text{C}_6\ ^{15}\text{N}_3$ 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].

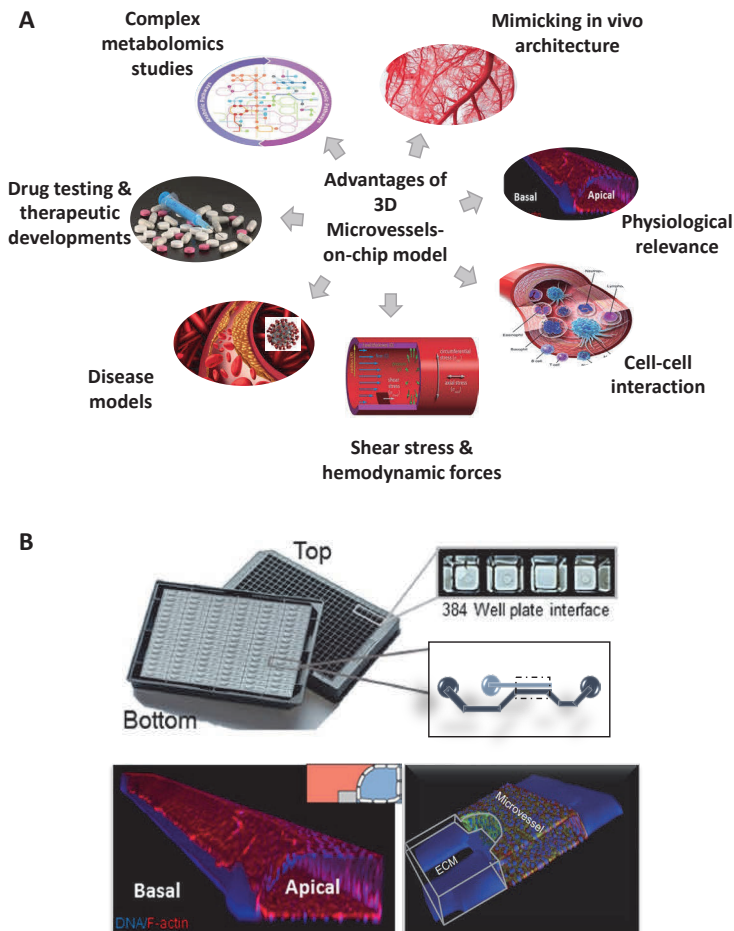


Figure 3. A) Some of the major advantages of the in vitro 3D microvessels-on-chip model. B) 3D microvessels-on-chip models with extra cellular matrix (ECM) [48,51].

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.

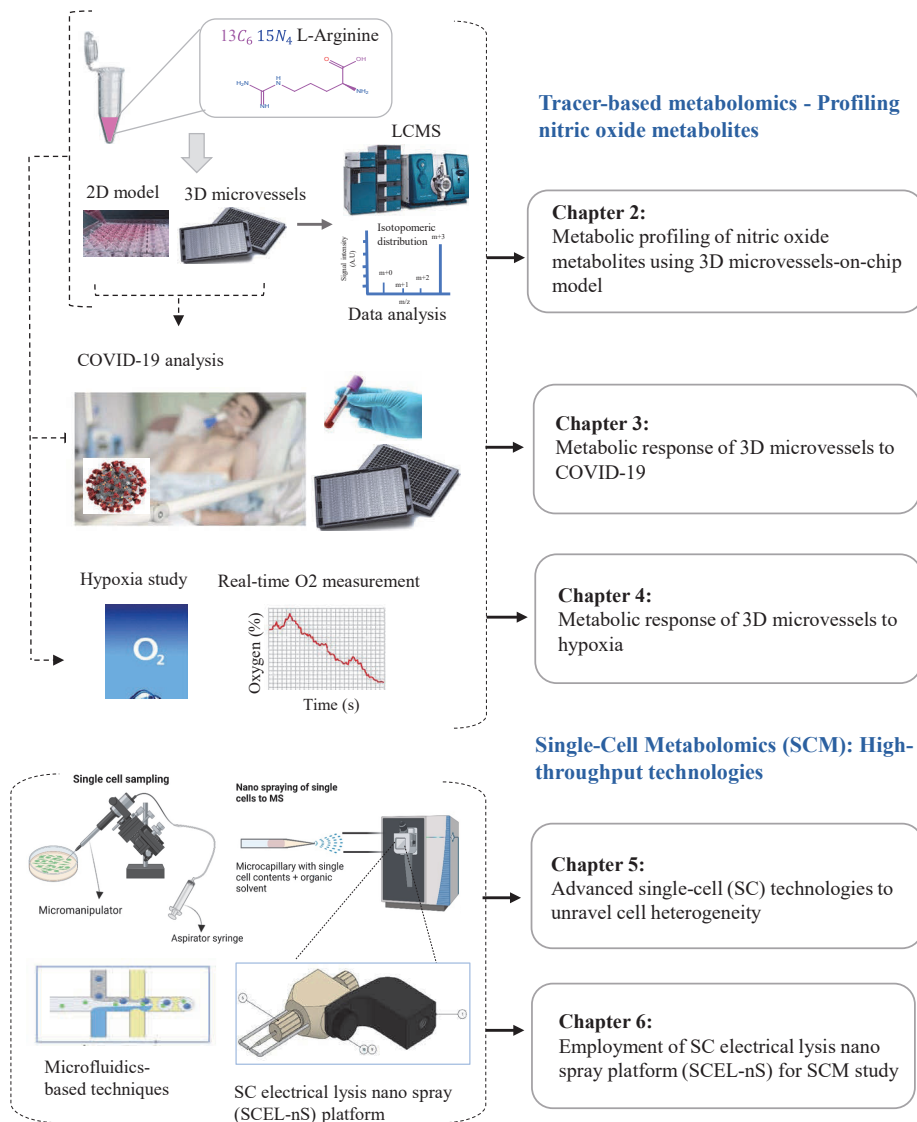


Figure 4. Overview of research chapters thesis structure and scope of each chapter.

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 HIF1 α 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.

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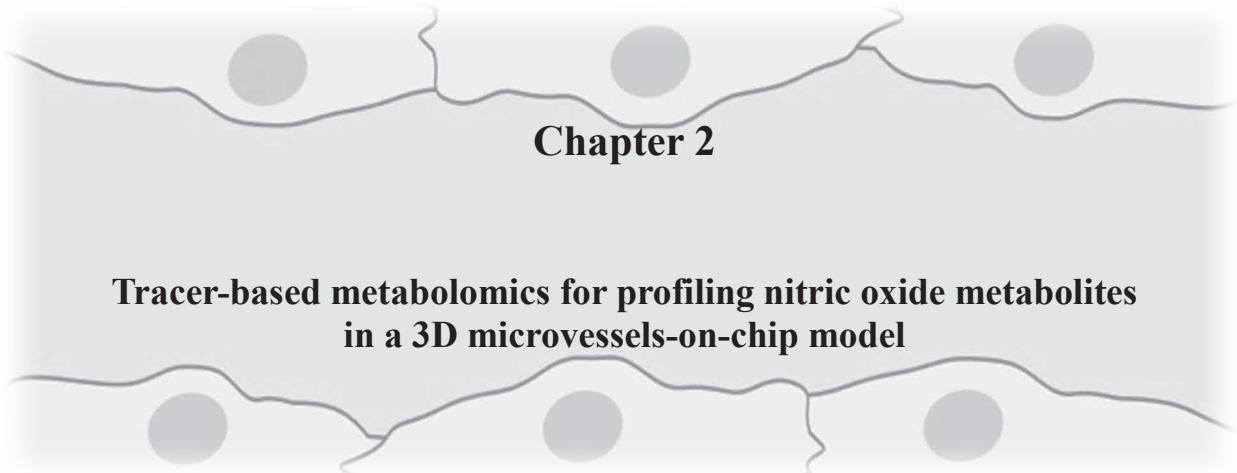
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Chapter 2

Tracer-based metabolomics for profiling nitric oxide metabolites in a 3D microvessels-on-chip model

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The FASEB Journal. 2024;38(16):370005. doi: 10.1096/fj.202400553R

Abstract

Endothelial dysfunction, prevalent in cardiovascular diseases (CVDs) and linked to conditions like diabetes, hypertension, obesity, renal failure, or hypercholesterolemia, is characterized by diminished nitric oxide (NO) bioavailability—a key signaling molecule for vascular homeostasis. Current two-dimensional (2D) in vitro studies on NO synthesis by endothelial cells (ECs) lack the crucial laminar shear stress, a vital factor in modulating the NO-generating enzyme, endothelial nitric oxide synthase (eNOS), under physiological conditions.

Here we developed a tracer-based metabolomics approach to measure NO-specific metabolites with mass spectrometry (MS) and show the impact of fluid flow on metabolic parameters associated with NO synthesis using 2D and 3D platforms. Specifically, we tracked the conversion of stable-isotope labeled NO substrate L-Arginine to L-Citrulline and L-Ornithine to determine eNOS activity.

We demonstrated clear responses in human coronary artery endothelial cells (HCAECs) cultured with $^{13}\text{C}_6, ^{15}\text{N}_4$ -L-Arginine, treated with eNOS stimulator, eNOS inhibitor, and arginase inhibitor. Analysis of downstream metabolites, $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline and $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine, revealed distinct outcomes. Additionally, we evaluated the NO metabolic status in static 2D culture and 3D micro vessel models with bidirectional and unidirectional fluid flow. Our 3D model exhibited significant effects, particularly in microvessels exposed to the eNOS stimulator, as indicated by the $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline / $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine ratio, compared to the 2D culture.

The obtained results indicate that the 2D static culture mimics an endothelial dysfunction status, while the 3D model with a unidirectional fluid flow provides a more representative physiological environment that provides a better model to study endothelial dysfunction.

Key words

Nitric Oxide metabolites, eNOS, endothelial dysfunction, 3D micro vessel model, shear stress

Introduction

Nitric Oxide is an essential diatomic molecule that is generated by eNOS/NOS3 and that exerts multiple key roles in vascular homeostasis, including vasodilation and inflammation. The eNOS enzyme can be activated by multiple physiological (oxygen and shear stress) [1] or humoral (bradykinin and insulin) cues that drive diverse phosphorylation events on multiple sites such as serine 114, 615, 633, 1177, 1179 and threonine 495 in the oxygenase and reductase domains of the protein [2]. When released from the endothelium, NO diffuses into vascular stromal cells such as pericytes or smooth muscle cells to promote vascular relaxation through the stimulation of cyclic guanosine monophosphate (cGMP synthesis by soluble guanylyl cyclase [3–5].

Dysregulation of NO signaling pathways is associated with pathophysiological conditions such as endothelial dysfunction, a main driver for atherogenesis and cardio-metabolic disorders [6–8]. In endothelial dysfunction, reduced availability of NO converts the endothelial cell phenotype to a pro-inflammatory state with increased oxidative stress and a loss of vasodilatory capacity [9,10]. At the molecular level, multiple factors have been reported to contribute to the loss of NO generation in endothelial dysfunction, including a deficiency in the substrate L-arginine or co-factors such as flow-mediated dilation (FMD), nicotinamide adenine dinucleotide phosphate (NADPH), or tetrahydrobiopterin (BH₄). For instance, when BH₄ is converted to BH₂ (7,8-dihydrobiopterin) in an oxidative environment, the eNOS enzyme gets uncoupled from the cofactor leading to the generation of superoxide rather than NO [11–16].

At the physiological level, it is well established that fluid shear stress enhances the activity of eNOS [17,18]. Endothelial cells detect changes in local hemodynamics through mechanosensors located on the luminal side of the membrane, in the focal adhesions in the abluminal side, and in the cell-cell junctional complexes. When exposed to laminar shear stress, endothelial cells activate and mobilize Ca²⁺ from intercellular stores. This mobilization leads to the formation of Ca²⁺/calmodulin complexes, which then bind and activate the eNOS enzyme [19]. Furthermore, fluid shear stress induces the eNOS activation through the tyrosine phosphorylation of PECAM-1 (platelet endothelial cell adhesion molecule-1). This process enhances the phosphorylation of eNOS at Ser¹¹⁷⁷ and

Akt Ser⁴⁷³ residues. In contrast, lower levels of activation are observed in static conditions in human umbilical cord vein endothelial cells (HUVECs) [20]. Under laminar flow conditions, endothelial cells exhibit an atheroprotective phenotype. However, when laminar flow is disturbed or absent, the cells shift to an inflamed phenotype characterized by the activation of NF- κ B [21]. This shift in phenotype can contribute to thermogenesis, inflammation, atherosclerosis [22,23] and obstructive pulmonary disease [24].

Given the crucial role of shear stress in accurately modeling physiological endothelial responses to risk factors, it is essential for *in vitro* models to incorporate environmental cues such as laminar shear. This necessity is underscored by studies indicating that static endothelial cell-culture models exhibit a proinflammatory phenotype [25], display features of complement-mediated injury [26] and show impaired NO production [27,28]. When it comes to developing 3D models, microfluidics emerges as an optimal technology for introducing flow into a 3D microvascular structure [25,29]. The accurate measurement of NO poses a challenge due to its strong reactivity, short half-life, and low physiological concentrations. Traditional techniques such as the Griess assay, chemiluminescence, and amperometric NO sensor readings faces limitations in accuracy and sensitivity [30–34]. However, measuring stable metabolites involved in NO production provides a viable approach to quantify NO, even at the low abundance in microfluidic cell models.

In particular, tracer-based metabolomics stands out as the gold standard method. This approach utilizes stable isotope-labelled precursors to trace complex pathways by following the labeled atom(s) to downstream metabolites [35,36]. This technique, applied to enzymatic reactions involved in NO production [37–42] enhances accuracy and sensitivity, overcoming the challenges associated with traditional NO measurement methods. To evaluate the influence of adverse metabolic and inflammatory plasma factors on eNOS activity in both 2D and 3D endothelial cell models, we aimed to employ our tracer-based metabolomics strategy. In our study, we used ¹³C₆, ¹⁵N₄ L-arginine as a substrate for eNOS, and traced its downstream metabolites ¹³C₆, ¹⁵N₃ L-citrulline (a co-

product of NO production produced at equal molar amounts) and $^{13}\text{C}_5$, $^{15}\text{N}_2$ L-ornithine (contributing to citrulline production).

To assess these L-arginine downstream metabolites, we employed an optimized AccQ-Tag amino acid derivatization sample preparation method coupled with a targeted LCMS (liquid chromatography and Mass spectrometry) approach. To validate the accuracy of our method in reflecting endothelial cell biochemistry, we measured changes in metabolite flux in cell models treated with stimulatory and inhibitory compounds for eNOS and arginase. Furthermore, we investigated the impact of unidirectional fluid flow on metabolic parameters associated with NO synthesis. This involved a comparison between a three-dimensional (3D) microvessels-on chip model with controlled fluid flow [29,43], and a 2D static cell culture model. The results obtained highlight the suitability of the LCMS method for measuring low-volume and low-abundance NO downstream metabolites. Notably, the 3D model reveals significant changes upon VEGF stimulation compared to the control situation, as evidenced by changes in Citrulline+9/Ornithine+7. In contrast, static 2D culture lacks significant changes upon VEGF stimulation, indicating a non-regulated eNOS and arginase pathway, reflecting an endothelial dysfunction condition. Furthermore, under flow conditions, there are elevated levels of mechanosensitive gene expression compared to static, low shear and bidirectional flow statuses. These results underscore the potential importance of shear stress and NO metabolite levels in influencing endothelial responses and gene expression patterns.

Material and Methods

Chemicals and reagents

Vascular Endothelial Growth Factor (VEGF) (PeproTech, Netherlands), L-NAME (Abcam, Netherlands), BEC (Sigma Aldrich, Netherlands), BH4 (Merck, Netherlands). RPMI SILAC - medium deficient in Arginine was obtained from (Thermofisher, Netherlands). Isotope labelled L-Arginine was obtained from (CORTECNET, Netherlands). Cells were grown under 5% CO₂, with 95% atmospheric air. Oxygen

experiments were performed in a Panasonic 97 oxygen incubator. DAF-2D was obtained from (Abcam, Netherlands).

Cell culture and experimental procedures

Human coronary artery endothelial cells (HCAECs) (PromoCell, Netherlands) were resuspended in 10 ml fresh EGM MV2 medium with supplements (PromoCell, Netherlands) and cultured in T75 flasks (Nunc Easyflask, Sigma, Netherlands). Cell cultures were maintained at 37 °C with 5% CO₂ and media was refreshed three times a week. Cells were detached at 85% confluence with 0.25 % Trypsin EDTA (Lonza, Netherlands) and cell pellets were collected by centrifugation at 300g for 5 minutes.

Commercially available RPMI SILAC medium was used, a formulation specifically designed for stable isotope labeling with amino acids in cell culture (SILAC). These medium lacks arginine, allowing for the incorporation of isotopically labeled L-arginine (¹³C₆ ¹⁵N₄-L-arginine) at a concentration of 150 μM into the cells. Following treatment, we measured the downstream labeled metabolites in the cells. For 2D culture experiments, the collected cell pellets were suspended in a fresh medium to a concentration of 7 x 10⁵ cells/ml and cultured in a 48 well plate for 48 hours. The following day, cells were serum starved with 1% FCS in basal EGM2 (Bioconnect, Netherlands) medium and incubated overnight. To synchronize or equilibrate the cell cycle, we treated cells with Krebs buffer solution, pH 7.4 (Thermo fisher Scientific, Netherlands) for 1 hour before the start of each experiment. Subsequently the cells were incubated for 12 hours with RPMI SILAC (ThermoFisher Scientific, Netherlands) supplemented with 150μM ¹³C₆, ¹⁵N₄-L-arginine (CORTECNET, Netherlands), 10 μM of (6R)-5,6,7,8,-Tetrahydrobiopterin dihydrochloride (BH₄) and 1% FCS (Thermofisher Scientific, Netherlands) in the presence of 100ng/ml VEGF, 1mM L-NAME or 100μM BEC separate or in the indicated combinations. The medium and cells were collected separately and snap frozen in liquid nitrogen and stored in -80°C for LCMS analysis.

For 3D cultures, we used a modified chip design 2-lane rerouted OrganoPlate (MIMETAS, Netherlands) that was adapted to attach a microfluidic pump to apply fluid flow and a normal 2-lane OrganoPlate to apply bidirectional flow. After seeding HCAECs

cells into the 3D microvessels, they were cultured for 3-4 days with media refreshed every other day. Similar to the 2D culture, once the cells formed a micro vessel, the following day, cells were serum starved with 1% FCS in basal EGM2 medium and incubated overnight. To synchronize or equilibrate the cell cycle, we treated cells with Krebs buffer solution, pH 7.4 for 1 hour before the start of each experiment. Subsequently, the cells were incubated for 12 hours with RPMI SILAC supplemented with 150 μ M 13C6, 15N4-L-arginine, 10 μ M of BH4 and 1% FCS in the presence of 100ng/ml VEGF, 1mM L-NAME or 100 μ M BEC separate or in the indicated combinations. The medium and cells were collected separately and snap frozen in liquid nitrogen and stored in -80°C for LCMS analysis

The approximate cell count in each 2D plate well is in the range of $7 - 8 \times 10^3$ and in the 3D OrganoPlate the approximate cell count is in the range $9 \times 10^3 - 1 \times 10^4$ cells/chip (with 96 chips per OrganoPlate).

Immunofluorescence microscopy

HCAECs were fixed using 4% paraformaldehyde (PFA) in HBSS+ for 10 min at room temperature. The fixative was aspirated, and the cells were rinsed once with HBSS+. The cells were permeabilized for 2 min with 0.2% Triton X-100 in HBSS+ and washed with HBSS+. Permeabilization was followed by blocking cells using 5% BSA in HBSS+ for 30 min and incubated with the primary antibody solution - Mouse anti-human CD144 (1:150) (BD Biosciences, USA) for overnight at 4°C. The cells were washed with HBSS+, followed by a one-hour incubation with Hoechst (1:2000) (Invitrogen, USA), rhodamine phalloidin (1:200) (Sigma-Aldrich, The Netherlands) and the secondary antibody solution, containing Alexa Fluor 488-conjugated goat anti-mouse (1:250) (ThermoFisher, USA). The cells were washed three times with HBSS+. High-quality images of the stained cells were acquired using a high-content confocal microscope (Molecular Devices, ImageXpress Micro Confocal).

Sample preparation for LC-MS

Samples were analyzed using a method based on an amine profiling platform that employed an AccQ-Tag derivatization strategy, adapted from the protocol provided by Waters [44]. The cell-treated media and cell lysates (10 μ L) were thawed on ice. For deproteination, water (5 μ L), Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (10 μ L), and absolute methanol (75 μ L) were added. Quality control (QC) samples were generated by pooling equal volumes of all cell media and cell lysate samples, and 10 μ L of this pool underwent the same process as individual samples. After centrifugation at 13,200 rpm for 10 minutes, the supernatant was transferred to a fresh Eppendorf tube and dried under a speed vacuum. The dried residue was reconstituted in borate buffer – pH 9 (10 μ L) vortexed for 10s and treated with 2.5 μ L of AccQ-TagAQC derivatization reagent (Waters, Waters B.V. Art. No. 186003836, The Netherlands). The samples were then kept at 55°C for 30 minutes in a shaker (Incubating microplate shaker, VWR, The Netherlands), 20% of formic acid (5 μ L) was added for neutralization. After a quick vortex, each sample was transferred to a deactivated auto sampler vial for LC-MS injection.

Instrumentation and LC-MS acquisition

Three μ L of sample solution was injected onto a UPLC Class I (Acquity, Waters Chromatography Europe BV, Etten-Leur, The Netherlands) system with an AccQ-Tag Ultra C18 Column (1.7 μ m, 100 x 2.1 mm, Waters, Ireland) coupled to a Sciex QTRAP® 6500 mass spectrometer [44]. For liquid chromatography (LC) separation, mobile phase A consisted of 0.1% formic acid in water. Mobile phase B consisted of 0.1% formic acid in acetonitrile. The flow rate used was 0.7 mL/min and the starting gradient condition was 99.8% A for 0.5 min, changing linearly to 90% A over the next 5.50 min, 80% A over 7.50 min, 40% A at 8.00 min, 5.0% A at 9.00 min, after which the solvent composition returned to 99.8% A over next 9.10 min and ends up with 99.8 % A and 0.2% B over in next 11.00 min. Mass spectrometry experiments were carried out on a SCIEX QTRAP 6500. The ESI source parameters were as follows (positive ion mode): Spray voltage $\pm$ 5.5kV, capillary temperature 350°C, sheath gas 60 psi, auxillary gas 70 psi, curtain gas 30 psi. Data acquisition was performed in scheduled multiple reaction monitoring mode

targeting compounds with different labeling statuses. Q1 and Q3 resolutions for all transitions were set at unit resolution (0.7 amu fwhm) after testing with labeled cell samples. This setting was selective enough to reduce interferences from adjacent ions peaks. The compound list with target m/z for parent and product ions is shown in **Supplementary Table 1**. Raw LC-MS/MS data was processed with AB Sciex PeakView™ 2.0 and MultiQuant™ 3.0.1 software for targeted metabolite peak identification and integration. Metabolite isotopologue levels were quantified using the corresponding peak areas.

Microfluidic pump setup

HCAECs were seeded into an OrganoPlate (similar protocol mentioned in the above section) that was specially designed for controlled perfusion using a pump attachment. The pump is made of stainless steel and the tube connections for the fluid transfer from the inlet and outlet of the chips are made of silicone. For controlled rotation, a small motor is operated with the help of LabVIEW solutions software. This software allows the control of fluid flow rate and related shear stress (dyne/cm^2), use of portal connections, motor position and value. Typically, shear stress was applied for 12 hours for metabolic readouts and 12 and 24 hours for gene expression studies to condition the cells.

Validation of eNOS activity using DAF-2DA

HCAECs were cultured in a 48 well plate with $7-8 \times 10^3$ cells/ well suspended in a fresh EGM MV2 medium with supplements. Upon 80% confluence, the cells were starved with 1% FCS in EGM basal medium for 17 hours at 37°C with 5% CO₂. Next the cells were treated with Krebs buffer solution for 1 hour at 37°C. After two washes of PBS, 5μM of DAF-2DA stain (Abcam, Netherlands) was added and the cells were incubated for 1 hour at 37°C and then washed twice with PBS. The cells were treated with VEGF, L-NAME, BEC and only the working solution (basal EGM2 media with 1% FCS and 10μM BH₄) separately and incubated for 10-12 hours as described before. The cells were washed

twice with PBS and imaged in EVOS CO₂ incubator build fluorescent microscope with maximum excitation: 491nm and maximum emission: 513nm. The fluorescence intensities were measured by choosing ten random individual cells and grey scale intensity was analyzed using imageJ software to correlate with citrulline level.

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

To perform RT-PCR, cells from 2D wells and OrganoPlate chips were collected by adding 100µL of RLT buffer (QIAGEN, Netherlands); after 60s of incubation at RT the lysates were collected, and RNA was extracted using Qiagen's RNeasy kit and Buffer RLT lysis buffer based on the manufacturer's recommendations. Next, to enable analysis of gene expression at low concentrations due to low cell counts, the lysate of eight microvessels from 3D cell culture models and eight well samples from 2D- 48 well plate was combined to make one sample. Reverse transcription-mediated cDNA synthesis was carried out using random and oligo(dT) primers (Bio-Rad, Netherlands) in accordance with the manufacturer's instructions using 200 ng of total RNA. For the qRT-PCR analysis, SYBR Select (Invitrogen) and a Biorad CFX384 were utilized. Krüppel-like transcription Factor2 (Klf2) (sense), CTACACCAAGAGTTCGCATCTG; Klf2 (antisense), AGCACGAACCTTGCCCATCA; 18S rRNA (sense), GGATGTAAAGGATGGAAAATACA; 18S rRNA (antisense), TCCAGGTCTTCACGGAGCTTGTT were the target genes whose primer sequences were employed. Expression levels were standardized to 18S rRNA and quantified using the comparative cycle threshold ($\Delta\Delta C_t$) method.

Statistical analysis

Bar plots and box plots were created with GraphPad Prism 9.3.1 software. Significance determined by ANOVA, Tukey's analysis and student's t-test.

Results

The method for measuring eNOS activity in 2D cell culture was first optimized and validated using isotope-labeled arginine, LC-MS/MS analysis, and staining using DAF. Next, the method was also optimized for the 3D microvessels-on-chip model, and finally, the eNOS activity measured for the 2D and 3D microvessels-on-chip model.

Tracer-based measurement of eNOS-dependent arginine to citrulline conversion - Marker metabolites that reflect eNOS activity

The targeted analysis of isotope-labeled L-arginine and its downstream metabolites, L-citrulline and L-ornithine, was conducted using the sample preparation method involving AccQ-Tag derivatization. The metabolic pathway of L-arginine metabolism and its conversion to downstream metabolites, as illustrated in (Fig. 1A), was the focal point of our analysis. We optimized the method by analyzing potential isotopologues of these marker metabolites in the cell system (Supplementary Table. 1).

After treating the HCAECs with isotope labelled L-arginine, we analyzed the resulting samples to identify peaks representing a series of isotopomeric citrulline, arginine and ornithine in the reconstructed ion chromatograms (Fig. 1B). Arginine was detected with M+10 (representing $^{13}\text{C}_6$, $^{15}\text{N}_4$ labeling) as a primary isotopologue, accounting for over 60% in all samples. Citrulline was detected with M+9 (representing $^{13}\text{C}_6$, $^{15}\text{N}_3$) as the primary isotopologue, produced by losing one nitrogen. Ornithine was detected with M+7 (representing $^{13}\text{C}_5$, $^{15}\text{N}_2$), resulting from the loss of one carbon and two nitrogen atoms from arginine. In the subsequent analysis, $^{13}\text{C},^{15}\text{N}$ -arginine (M+10), $^{13}\text{C},^{15}\text{N}$ -citrulline (M+9) and $^{13}\text{C},^{15}\text{N}$ -ornithine (M+7) were selected as biomarkers for further investigation. The measurements of blank samples (only media) showed no background interference in isotope-labeled citrulline or ornithine peaks (results not shown).

To optimize the incubation time, we considered the effects observed with stimulatory and inhibitory compounds. We utilized the detected isotopologues to verify the uptake of $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine and reconstruct the metabolic pathways controlled by eNOS and

Figure 1. Tracer-based Nitric Oxide (NO) metabolomics study. (A) Metabolic pathway of L-arginine and a few metabolites involved in other functions. Three marker metabolites (in bold) were analyzed based on stimulated and inhibited conditions of the respective enzymes eNOS and arginase. (B) Representative ion chromatograms of metabolite isotopologues detected in HCAECs sample obtained after cells were incubated with 150 μ M of, $^{13}\text{C}_6$ $^{15}\text{N}_4$ L-arginine.

The optimization process led us to select a 12-hour incubation time after the introduction of tracers. We observed lower labeling incorporation at 3 and 6 hours, as indicated by the ratio of citrulline+9/arginine+10 and ornithine+7/arginine+10 (**Supplementary Fig. 1**). This rate of label incorporation could be influenced by the exchange of intracellular and extracellular (in medium) metabolites. Thus, we opted for a 12-hour treatment to see a maximal transfer of ^{13}C and ^{15}N atoms. The calculated isotopologue fractions after 12 hours of incubation time show the utilization of labelled arginine converted into maximum transition state of labelled citrulline (M+9) and ornithine (M+7) in extracellular samples.

In intracellular samples, the degree of labelling (citrulline (M+9) and ornithine (M+7) compared to the unlabeled isotopologue) was lower than in the medium. (**Supplementary Fig. 2A & 2B**). While the precise reason for this remains unclear, the suppression of citrulline generation by L-NAME is evident in both media and cells. Additionally, the hindrance of ornithine production by BEC is also distinctly observed in both media and cells. Indeed, the collective impact (notably, the isotopologue fraction of labeled citrulline (M+9)) is more pronounced in the medium.

Extracellular and intracellular measurement of Isotomeric compounds

In order to comprehensively understand the effects of stimulatory and inhibitory compound treatments and to evaluate cellular metabolism, we conducted measurements of marker metabolites both intracellularly and extracellularly in a 2D cell culture platform. HCAECs were treated with eNOS stimulator (VEGF), eNOS inhibitor (L-NAME) and arginase inhibitor (BEC) in conditioned media containing $^{13}\text{C}_6$, $^{15}\text{N}_4$ L-arginine for 12 hours. Subsequently, both extracellular (media) and intracellular (cell)

samples were analyzed with LC-MS/MS. The targeted metabolic routes for individual treatments are illustrated in (Fig. 2 A-C).

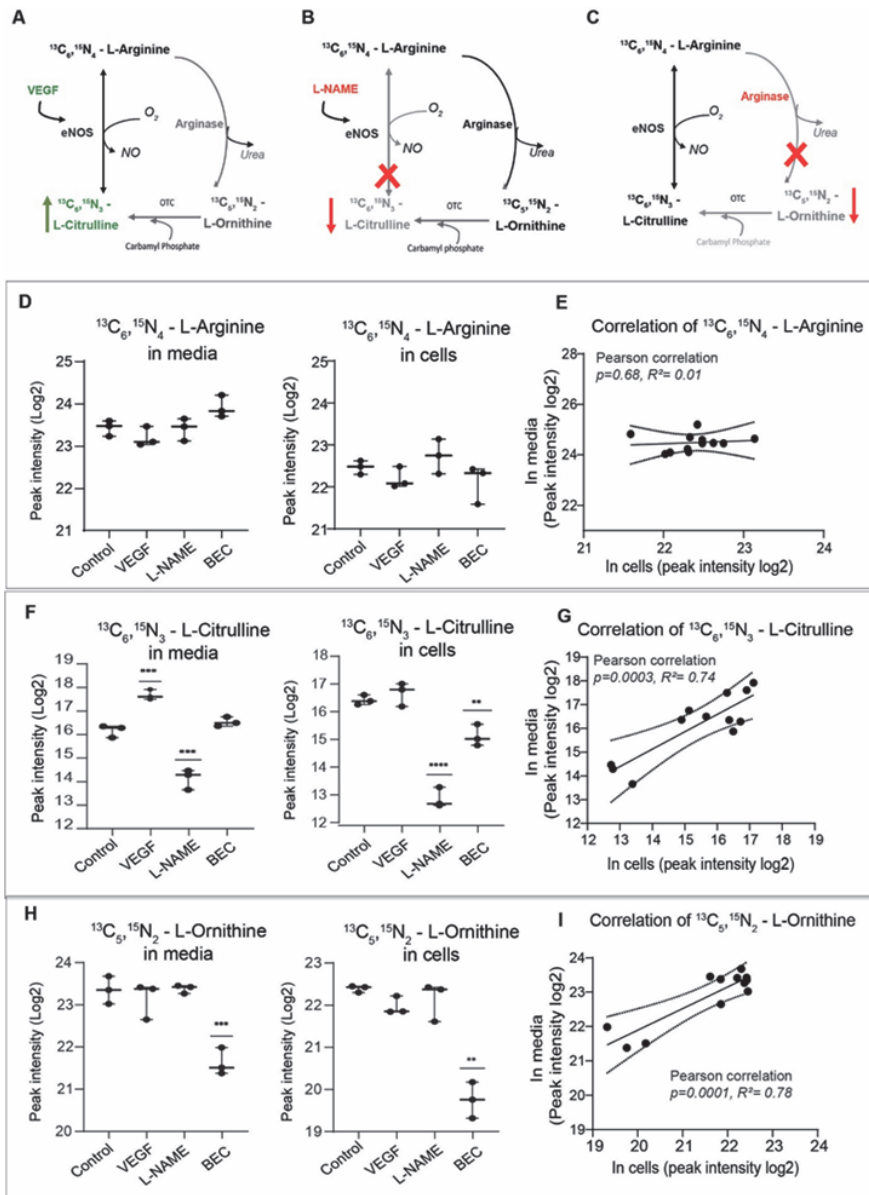


Figure 2. Measurement of isotope labelled extracellular (media) and intracellular (cells) marker metabolites involved in NO mechanism in 2D model. A) Schematic representation of

NO pathway upon treatment of A) VEGF – eNOS stimulation B) L-NAME - eNOS inhibition and C) BEC – arginase inhibition. Highlighted and non-highlighted paths represent activation and inhibition conditions. Measurement of metabolites in media and cells (D) $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine and its (E) Pearson correlation (F) $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline and its (G) Pearson correlation (H) $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine and its (I) Pearson correlation. All error bars represent SD and mean, each dot represents technical replicates, $n=3$. Significance determined by student t-test of treated group versus control group. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

$^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine was present in a high amount in the medium and was also high in the cell. VEGF, L-NAME and BEC did not change the labelled arginine level significantly in media or cells, and that is not unexpected due to the high level as substrate (**Fig. 2D**), and as the levels were rather constant in cell and medium for the various conditions, no significant correlation was found for values of $^{13}\text{C}_6, ^{15}\text{N}_4$ L-Arginine (Pearson correlation coefficient P value = 0.6, $R^2 = 0.01$) (**Fig. 2E**). The peak intensity of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline in the medium was increased in VEGF when compared to control but no statistical difference seen in cells (**Fig. 2F**).

The eNOS inhibition with L-NAME treatment shows significantly lower $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline in cells and media, while with arginase inhibition, the levels were not significantly changed. Overall, our results show that there is a significant positive correlation between media and cells for $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline (Pearson correlation coefficient, $p = 0.0003$, $R^2 = 0.7$) (**Fig. 2G**). The $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine levels were not significantly affected by VEGF and L-NAME treatments, in contrast to the condition where a specific inhibition of the arginase enzyme with BEC treatment was added, resulting in decreased ornithine levels (**Fig. 2H**). When we measured $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine in media and cells, a significant positive correlation with p value = 0.0001, $R^2 = 0.7$ was observed (**Fig. 2I**).

Analysis of metabolic ratios to understand NO metabolic flux

Employing the tracer-based extracellular measurement method highlighted earlier, our emphasis was on ratios indicative of metabolic fluxes. We conducted inquiries involving

both individual and combined treatments of endothelial cells with compounds that stimulate and inhibit eNOS and arginase enzymes.

Under eNOS stimulation conditions (VEGF, Pathway **Fig. 2A**) the expected outcome is a maximized production of citrulline compared to conditions involving arginase and eNOS inhibition (Pathway **Fig. 2B & 2C**). The ratio $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline/ $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine was found to be significantly higher in the VEGF treatments, indicating a greater utilization of arginine for citrulline conversion (**Fig. 3A**). The $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline/ $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine ratio serves as an indicator of the balance in the metabolic pathway associated with arginase and eNOS activities. Our results show a significant increase is observed in arginase inhibition conditions (BEC, and VEGF+BEC) (**Fig. 3B**). The inhibition of eNOS by L-NAME treatments (L-NAME, and VEGF+L-NAME) (**Fig. 3B**), led to lower levels of citrulline and $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline/ $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine ratio indicating that the eNOS inhibition was selective. The $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine/ $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine ratio, analyzed to confirm the utilization of arginine to ornithine conversion, showed lower expression in all arginase inhibition (BEC) conditions compared to other treatments (**Fig. 3C**).

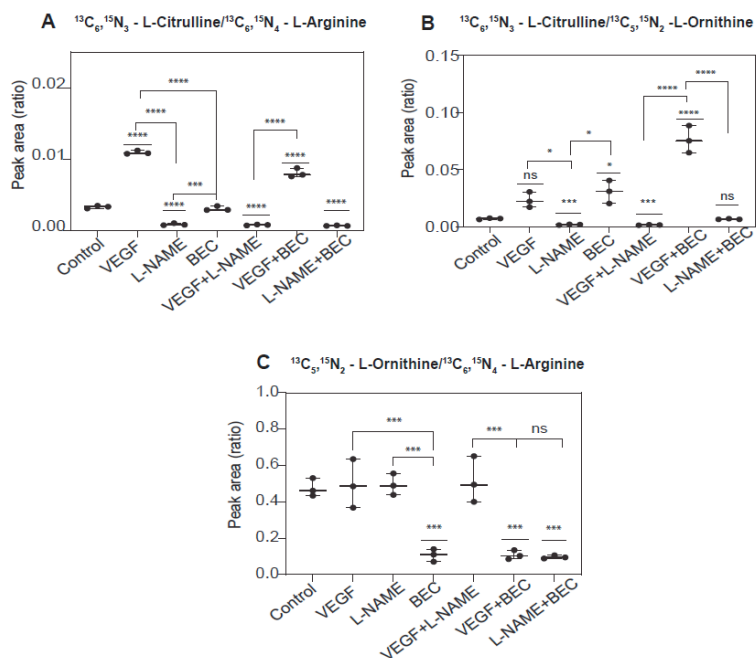


Figure 3. Measurement of extracellular labelled metabolites in 2D platform with the treatment of stimulators and inhibitors in an individual and combinational treatments. (A) Ratio of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine (B) ratio of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine and (C) ratio of $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine / $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine. All error bars represent SD and mean, each dot represents biological replicates. Significance determined by ANOVA and Tukey's multiple comparison test. ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Validation of eNOS activity using NO specific DAF-2DA staining

To determine if citrulline production can serve as an estimate of eNOS activity, we compared the peak intensities of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline with another measure of eNOS activity - Diaminofluorescein-2 diacetate (DAF-2DA) fluorescence staining for detecting intracellular NO. When DAF-2DA is taken up by cells, cytoplasmic esterase cleaves the acetate groups to generate DAF-2 which can readily react with dinitrogen trioxide (N_2O_3), an oxidation product of NO, to generate highly fluorescent triazolo fluorescein (DAF-2T) (Fig. 4).

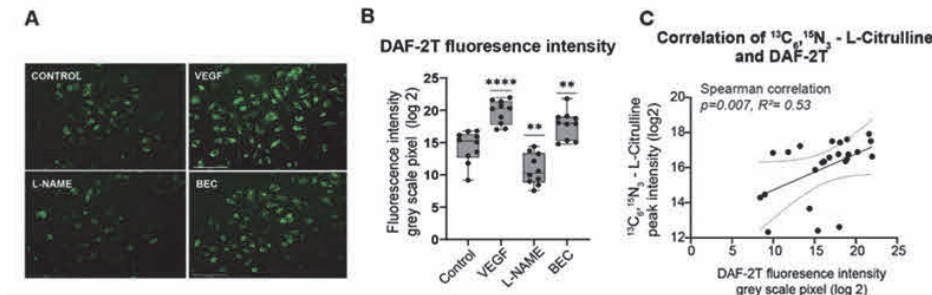


Figure 4. NO specific DAF- 2 DA fluorescent stain on HCAECs.

(A) Microscopic images of HCAECs using fluorescent NO stain DAF-2DA in all treatments; eNOS stimulator (VEGF), inhibitor (L-NAME) and arginase inhibitor (BEC). (B) Intensity of the fluorescent stain was calculated by grey scale measurement using ImageJ software; $n=10$. (C) Spearman correlation of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline and DAF-2T fluorescence intensity. Significance determined by student t-test. ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

This experiment was conducted in the 2D platform (48 wells cell culture plate) under the same conditions used for metabolic read out-as described in the preceding section. DAF-2T fluorescence intensity is significantly higher in VEGF and BEC conditions, and lower intensity was observed in the L-NAME condition (**Fig. 4A & 4B**). This outcome aligns with observations indicating that VEGF stimulation leads to a simultaneous increase in $^{13}\text{C}_6$ $^{15}\text{N}_3$ L-citrulline peak intensity (**Fig. 2F media**) and the augmentation of DAF-2T signals in HCAECs (**Fig. 4B**). To compare the relation between NO level and citrulline production, we performed a correlation analysis. Overall, the statistical analyses of the data indicate significant correlation between DAF-2T fluorescent intensity vs. $^{13}\text{C}_6$ $^{15}\text{N}_3$ L-citrulline peak intensity (Spearman correlation coefficient, $p = 0.007$, $R^2 = 0.53$) indicating that $^{13}\text{C}_6$ $^{15}\text{N}_3$ L-citrulline production and NO production from 2D model is significantly linked to the eNOS controlled treatments (**Fig. 4C**). Results from the DAF-2DA stain in the 3D model were inconclusive due to the presence of the collagen matrix. This matrix caused high background staining, making it difficult to distinguish the specific signal from the endothelial cells. Hence the results were not shown.

Comparison of mechanosensitive Klf2 gene in static (2D) and flow mediated (3D) cell culture models

To investigate the importance of shear stress on eNOS activation at the gene and metabolic level, we used a 3D culture model utilizing a novel setup – the MIMETAS 2-lane rerouted OrganoPlate (**Fig. 5A**). The commercially available MIMETAS 2-lane OrganoPlate's chip design was altered (rerouted) by connecting the route of windows 1 and 4 (**Fig. 5B**), extending the surface area for more cell growth. More important, this design is suitable to connect our microfluidic pump to create a flow, in contrast to the commercial MIMETAS OrganoPlate, where such a connected route is not present, as flow is applied using the rocker platform that generates bidirectional flow [29]. The microchannels in the OrganoPlate for gel and perfusion (blue -1 and pink-2 channel) are illustrated in **Fig. 5C**.

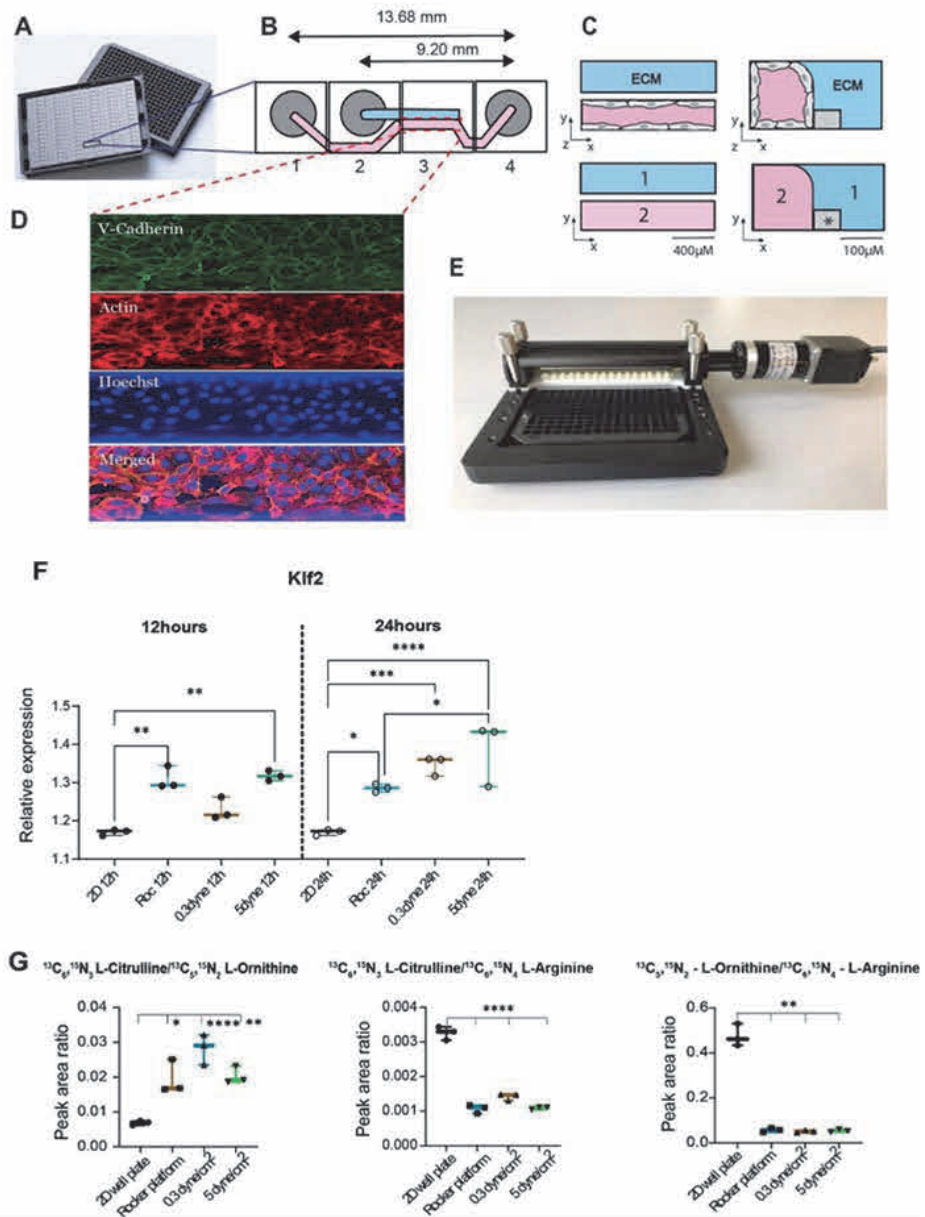


Figure 5. Representation of our 3D blood vessel model and comparison of NO marker metabolites in 2D and 3D platforms.

(A-C) Illustration of re-routed OrganoPlate's single chip design; Every microfluidic chip structure is positioned underneath 4 adjacent wells. Each microfluidic chip consists of two channels: The

blue color represents a ‘perfusion’ channel and pink color represents a ‘gel’ channel (ECM) separated by a phase guide (*). Every first well (1) and fourth well (4) is positioned on top of the inlet and outlet of the perfusion channel, while every second well (2) is for the gel channel and every third well (3) is used for imaging and observation of the experiment. (D) Immunostaining of HCAECs in a chip. After 48 hours of cell seeding, confluent vessels of HCAECs were immunostained with V-Cadherin, Actin and DAPI. (E) Image of a microfluidic pump with the two metal tubes attached to the inlet and outlet of the rerouted OrganoPlate to carry fluid and create unidirectional flow. (F) measurement of KLF2 gene expression of cell samples and (G) Measurement of extracellular metabolite’s ratio - $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine, $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_6,^{15}\text{N}_4$ L-arginine, $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine / $^{13}\text{C}_6,^{15}\text{N}_4$ L-arginine, all obtained from 2D static (black bars), 3D rocker platform (bidirectional flow model – blue bars) & the rerouted OrganoPlate with microfluidic perfusion pump operated with different shear stresses (unidirectional flow model) 0.3 dyne/cm² – (brown bars), 5 dyne/cm² - (green bars). Data for RT-PCR are presented as mean and s.e.m; n = 3. Metabolic data significance was determined by one way ANOVA multiple comparison test. *, P<0.05; **, P<0.01; ***, P <0.001; ****, P<0.0001.

The microchannels in the OrganoPlate were coated with gelatin, preventing HCAECs from growing on glass and enabling them to form stable microvessels which was checked by immunostaining (**Fig. 5D**). The rerouted OrganoPlate was connected to the microfluidic pump as shown in **Fig. 5E**.

For this study, we utilized three different cell culture models: a) the 2D model in which cells were grown in a static conventional culture, b) a bidirectional flow model – MIMETAS 2-lane OrganoPlate with normal chip design subjected to flow using an interval rocker device [29] and c) a unidirectional flow model - the rerouted plate with microfluidic pump set to two different dyne stresses, namely 0.3, and 5.0 dyne/cm². From these platforms, HCAECs were collected, and RNA was isolated at 12 and 24 hours. The isolated RNA was transcribed to cDNA with the respective primers and quantified using the RT-PCR technique. The results of the 12-hour incubation reveal a significant upregulation of the Klf2 gene in microvessels perfused with the interval rocker and with the microfluidic pump (5 dyne/cm²).

Furthermore, the results of the 24-hour incubation demonstrate that cells subjected to perfusion with interval rocker platform exhibit a significant upregulation of the Klf2 gene

compared to cells in the 2D well plate, and microvessels perfused with the pump may exhibit a further increase in Klf2 gene expression (**Fig. 5F**).

Comparison of NO marker metabolites in static (2D) and flow mediated (3D) cell culture models

Metabolic analysis was performed in the samples obtained from the above-mentioned models (in Section 3.2 and 3.3), and ratios of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline, $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine and $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine were evaluated. The ratio of $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline/ $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine and $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine / $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine are higher for 2D than 3D experiments, indicating that the total eNOS and arginase activity is higher in 2D than 3D. More relevant, the ratio of labelled citrulline and ornithine is higher for 3D than 2D indicating that the ratio of eNOS activity to arginase activity is higher for 3D than 2D, which is expected due to the flow present in 3D. (**Fig. 5G**).

The metabolic effects of VEGF, L-NAME and BEC treatments were comparable between 2D and 3D cultures for the above-mentioned ratios (**Supplementary Fig. 4 A- C**). However, the $^{13}\text{C}_6, ^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5, ^{15}\text{N}_2$ L-ornithine ratio showed a significant increase in all the 3D models when eNOS was stimulated by VEGF but was not significantly higher in 2D culture compared to control (**Supplementary Fig. 4A**).

Discussion

The purpose of this study was to understand endothelial NO production from a metabolic standpoint. We examined isotope-labeled arginine-based NO metabolic modifications by employing specific stimulant and inhibitory compounds targeting eNOS and arginase enzymes. Our focus was on measuring the impact of adverse metabolic and inflammatory factors on NO synthesis in endothelial cells, and for this purpose, we developed a highly sensitive tracer-based method to quantify eNOS-dependent conversion of arginine to citrulline and ornithine.

Upon introducing the arginine isotope to the HCAECs, we optimized the time required for the conversion of isotope-labeled citrulline and ornithine. Through the optimization process, we determined that a 12-hour incubation period after tracer introduction yielded the most favorable results. Notably, lower labeling incorporation was observed at 3 and 6 hours, as evidenced by the citrulline+9/arginine+10 and ornithine+7/arginine+10 ratios (**Supplementary Fig. 1**).

The incorporation rate of labels may be impacted by the exchange of metabolites between intracellular and extracellular (in medium) environments. Consequently, we chose a 12-hour treatment to observe the maximal transfer of ^{13}C and ^{15}N atoms. The calculated isotopologue fractions following a 12-hour incubation period reveal the utilization of labeled arginine, leading to the maximum transition state of labeled citrulline (M+9) and ornithine (M+7) in extracellular samples. In intracellular samples, the degree of labelling (citrulline (M+9) and ornithine (M+7) compared to the unlabeled isotopologue) was lower than in the medium (**Supplementary Fig. 2A & 2B**).

Upon introducing the isotope labeled arginine to the HCAECs and when treated with stimulatory and inhibitory compounds, it can be inferred that discernible (relative, not absolute) variations in eNOS and arginase activities can be identified through the assessment of the M+9 isotopologue of citrulline and M+7 ornithine in the medium. Additionally, it is imperative to emphasize that when evaluating the impact of conditions on eNOS activity, a control experiment must be incorporated as a reference. Moreover, a notable abundance of the M+6 isotopologue in arginine is observed, attributed to its conversion through an alternative pathway. It was concluded that the obtained labelled marker metabolites were relatively high after 12 hours in the medium, therefore, further analyses were performed on medium samples produced after 12 hours of incubation.

The response of eNOS stimulation using VEGF was to enhance the NO production through the activation of tyrosine kinase activity, resulting in the phosphorylation of intracellular domains. This activation increases calcium levels, facilitates binding with calmodulin (CaM), enhances eNOS phosphorylation, and ultimately leads to increase NO production [49–52]. The mechanism via receptor activation is depicted in

Supplementary Fig. 3. Our results indicate that eNOS activation leads to increased citrulline, consistent with its role as a co-product of NO production.

Earlier studies have reported BEC as a classical competitive inhibitor of arginase II at pH 7.5 with a dissociation constant (K_i) describing the binding affinity between the inhibitor and the enzyme as 0.25 and 0.31 μM [53]. Therefore, both citrulline and ornithine, upon stimulatory and inhibitory compound exposures, exhibited similar effects between media and cells. In general, most of the *in vitro* analyses measured NO metabolites only as intracellular levels [54,55]. In our study, we showed that the obtained promising results showed extracellular and intracellular expressions are positively correlated and this approach is helpful for fluxomics studies.

Ratios of NO metabolites can function as valuable indicators of diseased conditions and considered as useful markers in cardiovascular diseases, offering insights into the underlying biological mechanisms and disrupted pathways associated with the disease state. In particular, the tracer-based approach allowed us to gain a comprehensive understanding of how these compounds, both individually and in combination, influence the metabolic pathways associated with endothelial dysfunction. The ratio of unlabeled citrulline to arginine has been extensively examined in diabetes condition [56] and in children with chronic kidney disease (CKD) and abnormal cardiac blood pressure conditions, reporting a higher citrulline-to-arginine (cit-arg) ratio in plasma [57] but a lower ratio reported in urine [58]. In our study using stable isotope ratios instead of unlabeled ratios, we were able to overcome the difficulties resulting from the large volume of media used compared to the cell content and clearly link the arginine substrate to downstream products. The higher ratio resulting from the BEC inhibition suggests that arginase inhibition could be a potential target for recovering NO or citrulline production in diseased conditions. Our data supports the idea that the arginase inhibition recovers citrulline levels better than the stimulated condition with more arginine availability [59]. Furthermore, the combinational exposure experiments provide insights to the metabolic alterations in a controlled fashion. For instance, in VEGF+BEC condition, production of $^{13}\text{C}_6$, $^{15}\text{N}_3$ L-citrulline was directly from arginine rather than from ornithine as the pathway is blocked by the arginase inhibitor BEC. Such information is valuable for comparing the

underlying mechanisms of disease and identifying potential targets for therapeutic interventions.

Measurement of DAF-2T, the NO specific stain, revealed higher fluorescent intensity for both VEGF and BEC conditions which indicates an enhancement of NO production with VEGF addition, as previously demonstrated in HUVECs cells [60]. Similarly, increased DAF-2T intensity in arginase inhibition indicated the restoration of NO production, consistent with what was reported earlier in human aortic endothelial cells (HAECs) [61]. After successful use of the DAF-2T staining in the 2D HACEC model, we evaluated the staining in the 3D microvessels model. However direct staining was not successful, with the major challenge that the dye showed a too strong background signal when reacting to the collagen which was used as the extracellular matrix in the 3D microvessels model (results not shown). Therefore, to confirm the impact of flow that creates change in isotope labeled citrulline peak levels in 3D model, the flow-sensitive gene expression was measured.

Endothelial response to fluid flow is a crucial factor in vascular regulatory function. Flow-mediated eNOS activity in endothelium releases the endogenous nitro vasodilator, NO [17,62,63]. We validated the functionality of our microfluidic pump, using quantitative RT-PCR to measure the impact of shear stress on the shear-dependent transcription of the Klf2 gene [64] which also plays an important role in endothelial function, anti-inflammatory, anti-thrombotic and angiogenesis [63,65]. This indicates that our flow model induces substantial upregulation of the shear dependent gene – Klf2, highlighting the effectiveness of our 3D model in providing proper flow conditions to cells and allowing variations in shear stress compared to 2D.

The advantage of comparing ratios of 2D and 3D model rather than comparing individual metabolites is that ratios eliminate the need for additional normalization methods such as cell count or protein levels for some (but not all) comparisons. The ratio of $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline/ $^{13}\text{C}_6,^{15}\text{N}_4$ L-arginine and $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine / $^{13}\text{C}_6,^{15}\text{N}_4$ L-arginine are higher for 2D than 3D experiments, indicating that the total eNOS and arginase activity is higher in 2D than 3D, but this may be due to the higher number of cells in the 2D model. The

influence of flow is evident in the $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine ratio, indicating that flow “regulates” metabolic expression by enhancing eNOS activity when compared to the static 2D model. This might be because the cells in 2D model undergo stress due to the stiff base substrate and a static culture method, which mimics the status of endothelial dysfunction. These findings support the hypothesis of a relative arginase hyperactivity leading to endothelial dysfunction (ED) in mice [66] and in diabetes patients with ED condition [67]. Furthermore, the loss of fluid flow combined with a mTOR-based regulatory mechanism to balance eNOS and arginase II expression results in elevated $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine and decreased $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline levels, which indicates ED, consistent with what was reported in these studies [68,69].

Flow induced increases in NO production compared to static culture was previously reported [70]. Our results also show similar findings that in a 3D flow model, the fluid shear stress induces the signal of PECAM-1 that acts through adaptor molecules such as phosphatidylinositol-3-kinase (PI3K), which then activates eNOS, mTOR and the transcription factor Klf2 to regulate the functional genes [63]. Therefore, a lower $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine ratio in 2D static culture mimics more of endothelial dysfunction conditions. With the microfluidic pump, the flow can be controlled; for the $^{13}\text{C}_6,^{15}\text{N}_3$ L-citrulline / $^{13}\text{C}_5,^{15}\text{N}_2$ L-ornithine ratio, the data for the rocker platform and microfluidic platform using a shear stress of 5 dyne/cm² were not statistically significant.

Conclusion

We developed a stable isotope LC-MS/MS based assay to determine eNOS activity and verified this method by showing that indeed eNOS expression was present with reference staining (DAF-2D), and inhibitors of eNOS and arginase did show the expected effect. We then used this method to measure eNOS activity of HCAECs in 2D culture, and in a 3D microvessels model with flow through the microvessels. We used a bidirectional flow and used a set-up with a unidirectional flow system generated by a microfluidic pump. Compared to 2D culture, the 3D model has higher eNOS activity. 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. Actually, the method can be used to determine the effect of medium or plasma perfused through the microvessels on eNOS activity by measuring the conversion of labelled arginine to citrulline in medium (extracellular), which is much more straightforward than extracting the endothelial cell content. This eNOS activity assay has advantages over fluorescent probes which may have background from extracellular matrix and has the potential to be fast and high throughput as lysis of cells is not necessary and could be conducted at several time points throughout an experiment.

In future studies, this method could be applied to assess the effect of patient's plasma on eNOS activity by perfusing patient plasma samples in the 3D models and analyzing the degree of pathological conditions and drug responses. Combining flux data with other omics data in future studies would offer a more comprehensive understanding of NO's vasodilatory effects in studying endothelial dysfunctions. Furthermore, enhancing the pump system with higher shear forces could be explored to apply to various endothelial cell types, emulating different micro vessel models. In conclusion, our validation of shear stress's impact on eNOS using our 3D cell culture model from a metabolic perspective is a novel approach to study endothelial (dys)function.

Conflict of interest

T. Hankemeier is a shareholder in Mimetas B.V. All other authors declare no competing interests.

Data availability statement

The data that support the findings of this study are available in the Materials and Methods, Results, and Supplementary Material of this article. Additional data will be provided upon request.

Author contributions

K. Pandian, Conceptualization, Investigation, Methodology, Writing - original draft;
 L. Huang, Methodology – mass spectrometry method, Writing – review and editing;
 A. Junaid, Methodology – microfluidic pump designing, Writing – review and editing;
 A. Harms, Writing – review and editing;
 A. Jan van Zonneveld and T. Hankemeier, Conceptualization, Resources, Supervision,
 Funding acquisition, Writing – review and editing.

Acknowledgements

Parts of these studies were supported by the Dutch Heart Foundation (CVON RECONNECT) and ZonMW (MKMD: 114022501) grants to T.H and A.J.V.Z. Part was supported by METABODELTA (Medical Delta program). This project has received funding from the European Union's Horizon 2020 research and innovation Programme LogicLab under the Marie Skłodowska-Curie grant agreement No 813920, and RECONNECT funded by the Dutch Heart Foundation No 2020B008.

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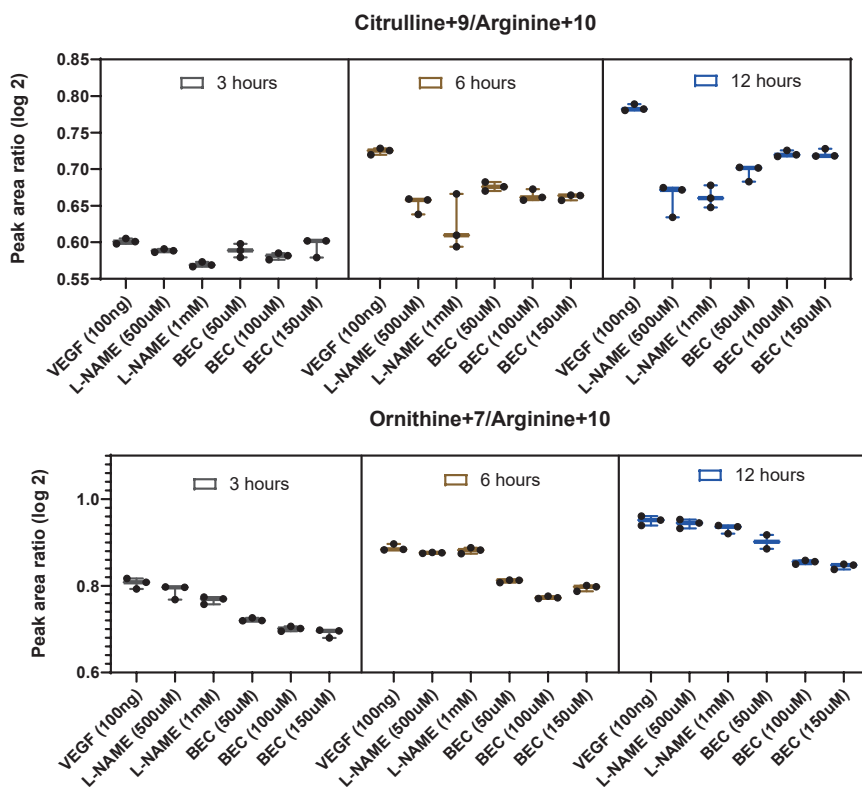
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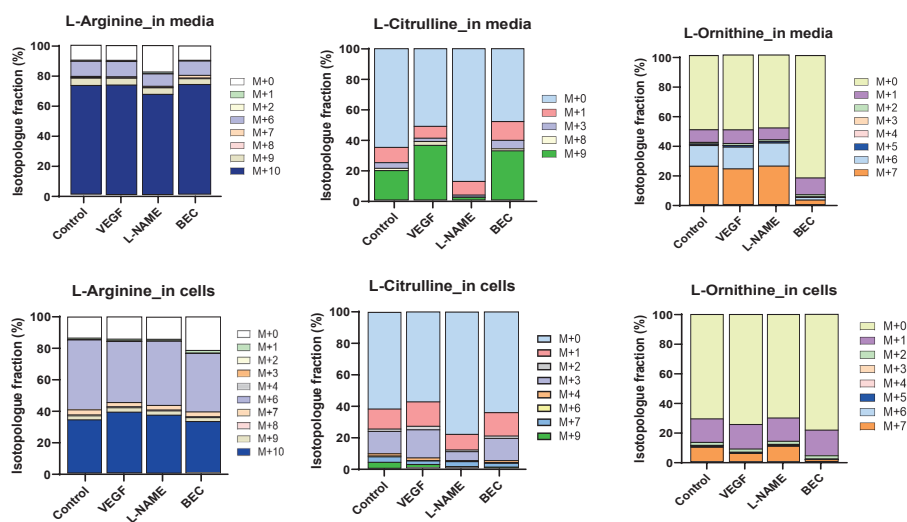
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Supplementary information

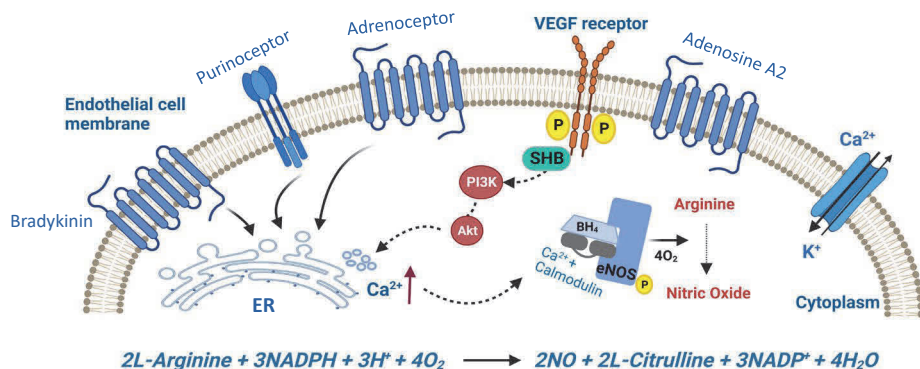


Supplementary Figure 1. Dosage study of stimulatory and inhibitory compounds and the extracellular measurement of marker metabolites.

(A & B) Ratios of $^{13}\text{C}_6$, $^{15}\text{N}_3$ L-Citrulline (Citrulline+9), $^{13}\text{C}_5$, $^{15}\text{N}_2$ L-Ornithine (L-Ornithine+7) and $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine (L-Arginine+10) after the different dosage treatment of stimulator and inhibitors of eNOS and arginase enzyme on HCAECs at different incubation hours. Incubation hours represented in different colors. 3 hours – black bars; 6 – hours – brown bars; and 12 hours – blue bars. Significance is determined by one way ANOVA multiple comparison test. For Citrulline+9/Arginine+10 and for Ornithine+7/Arginine+10, overall significant difference was observed between 3, 6 and 12 hours with p value <0.0001.

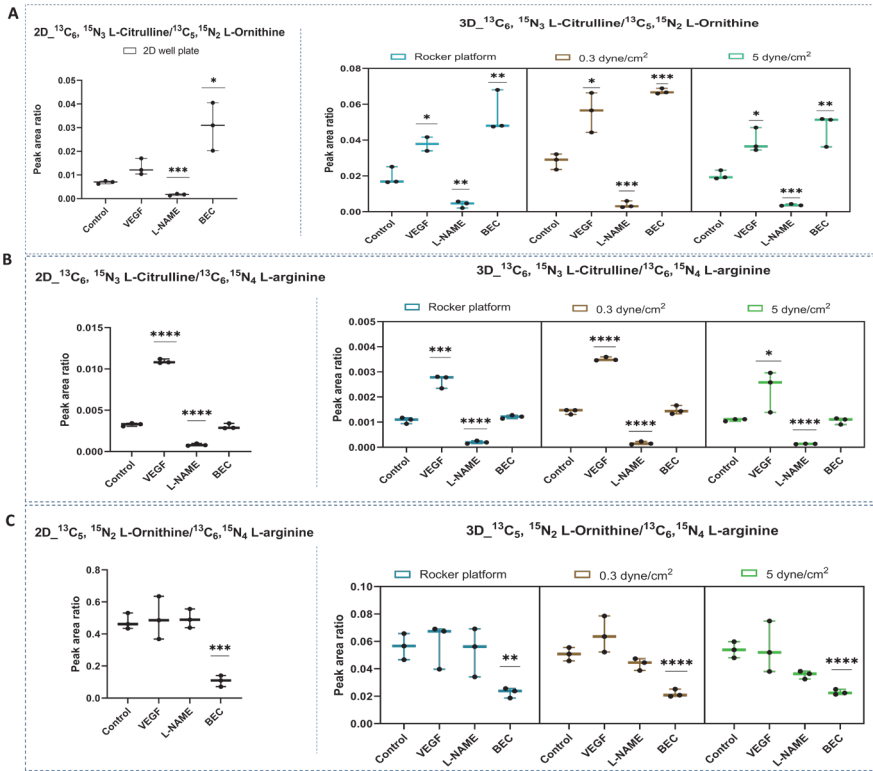


Supplementary Figure 2. Isotopologue fractions of A) extracellular and B) intracellular of L-Arginine, L-Citrulline and L-Ornithine in stimulated and inhibited treatments and in control.



Supplementary Figure 3. Schematic representation of Vascular Endothelial Growth Factor (VEGF) stimulation over eNOS enzyme and NO production.

The external signals – agonist of nitric Oxide (NO) such as acetylcholine, ATP & ADP (adenosine triphosphate and diphosphate), VEGF etc., have specific receptor sites on the endothelial cell surface (Blue). When VEGF binds to the muscarinic receptor, it releases Calcium²⁺, a major factor that leads to the chain of events cause NO production. Once the receptors are stimulated by the agonists (VEGF) they act upon the endoplasmic reticulum (ER) by transferring the signal via SHB adaptor protein, PI3K (Phosphoinositide3 kinase) and protein kinases (Akt), increase Ca²⁺ level, that binds to the calmodulin protein and cause some conformational changes. In the presence of tetrahydrobiopterin (BH₄) and oxygen, it phosphorylates the enhance the eNOS phosphorylation. The activated eNOS will convert L-Arginine to L-Citrulline and co-product as Nitric Oxide (NO). Calcium level is balanced by the calcium potassium channel from the circulation. Illustration created BioRender.com, accessed on HV24G0AUF2, September 24, 2022.

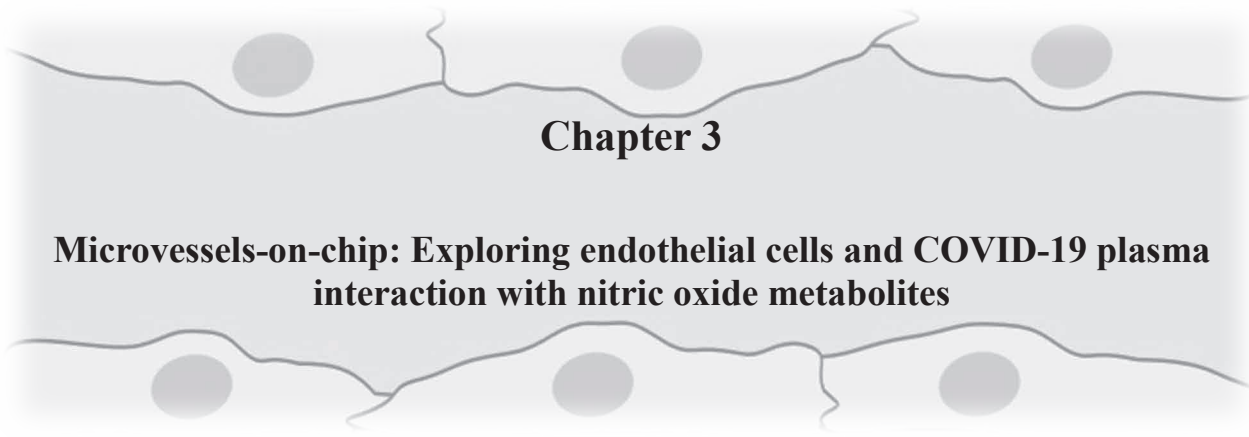


Supplementary Figure 4. Measurement of extracellular marker metabolites in all platforms.

A) Comparison of metabolites ratio between 2D and 3D models A) Ratio $^{13}C_6, ^{15}N_3$ L-Citrulline / $^{13}C_5, ^{15}N_2$ L-Ornithine B) Ratio of $^{13}C_6, ^{15}N_3$ L-Citrulline / $^{13}C_6, ^{15}N_4$ L-arginine C) Ratio of $^{13}C_5, ^{15}N_2$ L-Ornithine / $^{13}C_6, ^{15}N_4$ L-arginine in stimulatory and inhibitory compounds treated samples from 2D static, 3D rocker platform (bidirectional flow model) & the rerouted OrganoPlate with microfluidic perfusion pump operated with different shear stresses (unidirectional flow model). Metabolic data significance was determined by one way ANOVA multiple comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Supplementary table. 1. Modified MS acquisition method of 29 transition pairs of potential isotopologues of marker metabolites.

S.No	Metabolites name	Mass(Q1)	Mass(Q3)
1	L-Arginine [M+1]	346.170	171.000
2	L-Arginine [M+2]	347.170	171.000
3	L-Arginine [M+3]	348.170	171.000
4	L-Arginine [M+4]	349.170	171.000
5	L-Arginine [M+5]	350.170	171.000
6	L-Arginine [M+6]	351.170	171.000
7	L-Arginine [M+7]	352.170	171.000
8	L-Arginine [M+8]	353.170	171.000
9	L-Arginine [M+9]	354.170	171.000
10	L-Arginine [M+10]	355.170	171.000
11	L-Citrulline [M+1]	347.150	171.000
12	L-Citrulline [M+2]	348.150	171.000
13	L-Citrulline [M+3]	349.150	171.000
14	L-Citrulline [M+4]	350.150	171.000
15	L-Citrulline [M+5]	351.150	171.000
16	L-Citrulline [M+6]	352.150	171.000
17	L-Citrulline [M+7]	353.150	171.000
18	L-Citrulline [M+8]	354.150	171.000
19	L-Citrulline [M+9]	355.150	171.000
20	Ornithine [M+1]	237.600	171.000
21	Ornithine [M+2]	238.100	171.000
22	Ornithine [M+3]	238.600	171.000
23	Ornithine [M+4]	239.100	171.000
24	Ornithine [M+5]	239.600	171.000
25	Ornithine [M+6]	240.100	171.000
26	Ornithine [M+7]	240.600	171.000
27	L-Arginine	345.170	171.000
28	L-Citrulline	346.150	171.000
29	Ornithine	237.100	171.000



Chapter 3

Microvessels-on-chip: Exploring endothelial cells and COVID-19 plasma interaction with nitric oxide metabolites

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Nitric Oxide. 155, 12-19. Doi: [org/10.1016/j.niox.2025.01.002](https://doi.org/10.1016/j.niox.2025.01.002)

Abstract

COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), primarily manifests as a flu-like illness with lung injury, often necessitating supplemental oxygen. Elderly individuals and those with pre-existing cardiovascular diseases are at increased risk of mortality. The endothelial barrier disruption observed in patients indicates systemic viral invasion and widespread endotheliitis. Endothelial dysfunction, characterized by impaired nitric oxide (NO) production, contributes to vasoconstriction, inflammation, and coagulation abnormalities seen in COVID-19. In this study, we investigated the impact of COVID-19 patient-derived plasma on the endothelium through NO metabolite analysis using an *in vitro* 3D micro vessel model. Our experiments revealed alterations in NO metabolites in response to COVID-19 patient plasma perfusion, with BH4+BH2 supplementation improving citrulline levels in severe COVID-19 patient models. Positive correlation between arginase activity and eNOS activity was observed in the severe COVID-19 patient model but not in the mild COVID-19 patient model. These findings underscore the importance of endothelial dysfunction in COVID-19 pathogenesis and highlight potential therapeutic targets for mitigating vascular complications associated with severe infection.

Key words

Endothelial dysfunction, COVID-19, Nitric Oxide, Biopterin, Endothelial Nitric Oxide Synthase, Arginase, 3D microvessels-on-chip model

Introduction

COVID-19, an illness resulting from infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), manifests as a respiratory infection, with 24% to 54% of severe cases exhibiting lung injury, often requiring supplemental oxygen [1]. Individuals aged 60 and above, especially those with pre-existing cardiovascular diseases, experience higher mortality rates.

Patients with rapid clinical deterioration and multi-organ failure often show evidence of systemic endotheliitis [2,3], indicating a break in the endothelial barrier that allows systemic viral invasion. In particular, the impaired vascular homeostasis secondary to the structural and functional damage of the endothelium and its main component, the endothelial cells, contributes to the systemic proinflammatory state and the multiorgan involvement observed in COVID-19 patients. [4]. In previous studies, COVID-19 was related to endothelial dysfunction, due to direct (endothelial cells are infected by the virus) and indirect damage (systemic inflammation) factors [5,6]. Vasoconstrictive, proinflammatory, and pro-coagulant states, along with subsequent organ ischemia and tissue edema, result from endothelial dysfunction observed in COVID-19, possibly resulting from direct viral infection or an excessive inflammatory response triggered by neighboring epithelial cell infection and high levels of pro-inflammatory cytokines [7].

A characteristic feature of endothelial dysfunction is the inhibition of endothelial nitric oxide synthase (eNOS), leading to a concurrent deficiency in nitric oxide (NO) production [8], explaining the elevated risk of adverse outcomes in COVID-19 for individuals with pre-existing endothelial dysfunction. The human aging process leads to vascular endothelial dysfunction, characterized by an imbalance between vasodilators and vasoconstrictors produced by the endothelium [9] and lower NO production in the elderly, likely making them more susceptible for COVID-19 related complications [10,11]. Akaberi et al. established a correlation between NO levels and COVID severity, demonstrating that NO derived from S-nitroso-N-acetyl penicillamine (SNAP) can effectively delay or entirely prevent the development of the SARS-CoV-2 viral cytopathic effect in treated cells and inhibit viral replication [12]. Inhaled NO gas has emerged as a

potential therapeutic process to enhance patient oxygenation and potentially improve clinical outcomes [13]. However, the complications associated with direct NO inhalation result in the conversion of NO to nitrogen dioxide, a toxic gas with no therapeutic value [14]. Potential therapeutic mechanisms encompass vasodilation by improving the NO levels, antiviral, anti-inflammatory, and antithrombotic properties.

Healthy ECs utilize L-arginine as a substrate for endothelial nitric oxide synthase (eNOS) to produce NO and L-citrulline, in conjugation with NADPH, molecular oxygen, and tetrahydrobiopterin (BH4) [15]. Endothelial dysfunction can be caused by several factors, including inflammation, hypoxia, metabolic and behavioral factors, as well as mechanical issues such as reduced blood flow. These factors contribute to decreased production of nitric oxide (NO) due to the inadequate availability of the substrate (L-arginine) and cofactor (BH4), resulting in the uncoupling of endothelial nitric oxide synthase (eNOS) from its substrate—a phenomenon also observed in COVID-19. A previous study by Gomez et.al., showed that increase in arginine levels and decrease in citrulline levels may be indicative of a deficiency in the cofactor, leading to eNOS uncoupling from arginine and consequently reduced conversion to citrulline or NO [16]. Infusion of BH4 in coronary artery disease patients showed improved effect of vasodilation [17] and adding dietary sources of nitrates and nitrites containing food to the regular diet improves immunity and NO was considered as a potential targeted therapy by improving NO production [18]. However, there have been no studies conducted in COVID-19 patients demonstrating that BH4 supplementation improves vasodilation or reverses endothelial dysfunction. It is important to consider that enhancing NO production is viewed as one of the key treatment strategies due to its antiviral and anti-inflammatory properties [19]. Microvascular complications and endothelial dysfunction are frequently seen in deceased COVID-19 patients.

The precise role of endothelial dysfunction, whether triggered by inflammation or caused by SARS-CoV-2 pathogenesis, in disrupting nitric oxide metabolism remains a topic of active investigation. A key limitation of current in vitro models of COVID-19 is the incomplete understanding of how specific components within COVID-19 patient plasma impact the eNOS pathway, particularly the conversion of arginine to citrulline and

ornithine. A critical gap exists in translating previous work on models for endothelial dysfunction to the specific context of COVID-19. A study utilizing a 3D endothelial model on a microfluidic chip has provided insights into Ebola virus-hemorrhagic fever syndrome [20] and another study employed an effective 3D microvascular model to investigate the influence of blood plasma components on microvascular integrity [21].

Given the importance of 3D model architecture, our study focusses on the impact of COVID-19 plasma components using a 3D endothelial system through the analysis of metabolites involved in the production of NO. We conducted *in vitro* perfusion experiments using an endothelial 3D microvessels cell model developed in our previous work [22] to study the impact of COVID-19 patient-derived plasma from severe and mild cases, enriched with $^{13}\text{C}_6, ^{15}\text{N}_4$ L-arginine (arginine+10), on endothelial cells. Our 3D microvessels model utilizes MIMETAS rerouted 2-lane OrganoPlate which incorporates collagen as the extracellular matrix, with a specifically designed tubular channel mimicking a microvessels lumen.. We conducted an observational pilot-case study using two COVID-19 patient's blood samples. Following sample collection, analysis was conducted to identify downstream metabolites indicative of NO production. The eNOS enzyme activity was assessed by measuring the conversion of the NO substrate, arginine+10, to $^{13}\text{C}_6, ^{15}\text{N}_3$ L-Citrulline (citrulline+9). Additionally, we examined the generation of $^{13}\text{C}_5, ^{15}\text{N}_2$ L-Ornithine (ornithine+7) by the arginase enzyme, which also utilizes L-arginine as a substrate. We supplemented the perfused plasma within the microvessels with the eNOS enzyme stimulator VEGF, the inhibitor L-NAME, and the arginase enzyme inhibitor BEC. Significantly, we observed the impact of BH4+BH2 in the interaction between COVID-19 plasma and endothelial cells by monitoring changes in citrulline levels after supplementing the plasma with the cofactor BH4+BH2. The obtained results confirm that supplementation of BH4+BH2 could improve the citrulline level in severe COVID-19 patients. Furthermore, the significant correlation observed between arginase activity and eNOS activity after BH4+BH2 supplementation in COVID patients suggests a potential diagnostic marker, whereby enhanced citrulline levels and reduced ornithine levels could be indicative of healthy status.

Material and Methods

Patient study

We conducted an observational case pilot study of two patients with a diagnosis of COVID-19, whose blood samples were collected at the time they were admitted to the intensive care unit with a COVID-19 diagnosis. Enrolled subjects were classified as severe (with high viral load) or mild (minimal viral load) COVID-19 with their clinical blood reports shown in **Table. 1**. Plasma from healthy individuals (Female 20-30 years old) were used as controls for each COVID-19 patient. The approximate range of CRP level in age-based COVID-19 patient's CRP levels were evaluated in earlier studies [23]. According to that, elevated CRP levels correlate with the severity of COVID-19. In our study, the severely ill COVID-19 patient (a 65-year-old female) who was deceased exhibited significantly higher CRP levels compared to the mildly ill COVID-19 patient (a 45-year-old female) who survived. The use of human samples in this study was approved by the METC Leiden-Den-Haag. The study code was P20.051 and the CCMO code was NL73762.058.20. Informed consent was obtained from all participants. All experiments were conducted in accordance with ethical guidelines and relevant regulations.

Patient details & clinical report	COVID-19 - severe (patient 1 - PT1)	COVID-19 – mild (patient 2 - PT2)	Healthy control - Normal range	References
Gender and age	Female - 65	Female - 45		
Creatinine (μMol/L)	152	66	44 - 97	[24]
Urea (mMol/L)	9.2	2.3	1.8 to 7.1	[24]
Albumin (g/L)	32	41	34 - 54	[25]
Estimated glomerular filtration rate (EGFR) (mL/min)	31	90	90 - 120	[26]
Viral load in throat (copies/ml)	59630	887		
CRP (mg/L)	66.9	3.0	Less than 10.0	[27]

Table 1. Clinical characteristics of severely (PT1) and mildly (PT2) infected COVID-19 patients. CRP – C-reactive protein. Highlighted in red indicates deviation from the normal range.

Cell culture

Human coronary artery endothelial cells (HCAECs) (PromoCell, Netherlands) were resuspended in 10 ml fresh EGM MV2 medium with supplements (PromoCell, Netherlands) and cultured in T75 flasks (Nunc Easyflask, Sigma, Netherlands). Cell cultures were maintained at 37°C with 5% CO₂ and media was refreshed three times a week. Cells were detached at 85% confluence with 0.25 % Trypsin EDTA (Lonza, Netherlands) and cell pellets were collected by centrifugation at 300g for 5 minutes.

For the microvessel model, HCAECs were seeded in the re-routed OrganoPlate (MIMETAS, Netherlands), following the protocol described in the referenced work [22]. The microvessels-on-chip model is shown in the **Supplementary Fig. 1**. Once the cells were confluent, media in microvessels were replaced with fresh EGM MV2 media and the OrganoPlate was attached to the microfluidic pump with set 5 dyne/cm² shear stress using LabView software. The setup was kept in the incubator and the flow of media through the chips was maintained for 6 hours with unidirectional flow followed by perfusion of plasma through the microvessels is described in next section.

Plasma assay and perfusing human blood plasma in microvessels in vitro

The collected EDTA blood samples were centrifuged to isolate the plasma fractions (2 x 15 minutes at 2500 g) and stored in -80°C for further experiments.

EDTA plasma samples were initially treated with 1 µM hirudin (94581 – 1EA, Sigma-Aldrich), 50 µg/ml CTI (CTI -01, Haematologic Technologies), and 25 µM compstatin. Subsequently, the samples were recalcified with 3.1 mM CaCl₂. These treated samples were then combined with 50% SILAC media containing 150µM of ¹³C₆, ¹⁵N₄-L-

arginine. After 6 hours of unidirectional flow with the cell growth media (EGM MV2), microvessels were exposed to the cocktail mix of either healthy or COVID-19 plasma supplemented with 150µM of $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine (CORTECNET, Netherlands) and different stimulatory and inhibitory compounds and cofactor BH4+BH2. The oxidation of the BH4 was seen leading to BH2, therefore we represented cofactor treatment as BH4+BH2 to indicate the presence of both BH4 and BH2. This treatment was conducted at 37°C with 5% CO₂ for 12 hours by placing the OrganoPlate on the rocker platform that generates bidirectional fluid flow. The experimental conditions are listed below in **Table. 2**.

Compound exposure and conditions	Concentrations
Blank	Working solution
Control	Working solution perfused into the microvessels
BH4+BH2	10µM
VEGF	20ng/ml, 50ng/ml and 100ng/ml
L-NAME	1 mM
VEGF + L-NAME	100ng/ml + 1mM
VEGF + BEC	100ng/ml + 100µM
L-NAME + BEC	1mM + 100µM
iArg (isotope labeled arginine)	500 µM

Table 2. Experimental conditions and concentrations of stimulatory and inhibitory compounds of eNOS and arginase enzyme was prepared in working solution. Note: Working solution composition - Healthy or COVID-19 plasma (50%) + 150µM of $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine in 50% SILAC media.

iArg composition, 50% of healthy or COVID plasma (50%) + 500 μM of $^{13}\text{C}_6$, $^{15}\text{N}_4$ -L-arginine in 50% SILAC media.

Following the application of shear stress and compound treatments, samples were collected, from the outlet of each microvessels, snap frozen with liquid nitrogen and stored at -80°C for LCMS analysis.

Metabolic profiling using ultra-high performance liquid chromatography - mass spectrometry (UPLC- MS/MS)

Samples were analyzed using a method based on previous work [22] using an amine profiling platform that employed an AccQ-Tag derivatization strategy, adapted from the protocol provided by Waters [28]. The microvessels-treated plasma samples (10 μL) were thawed on ice. Quality control (QC) samples were generated by pooling equal volumes of all media + plasma samples, and 10 μL of this pool underwent the same process as individual samples. For reduction and deproteination, water (5 μL), Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) (10 μL), and absolute methanol (75 μL) were added. After centrifugation at 12000g for 10 minutes, the supernatant was transferred to a fresh Eppendorf tube and dried under vacuum. The dried residue was reconstituted in borate buffer – pH 9 (10 μL) vortexed for 10s and treated with 2.5 μL of AccQ-TagAQC derivatization reagent (Waters, Waters B.V. Art. No. 186003836, The Netherlands). The samples were then kept at 55°C for 30 minutes in a shaker (Incubating microplate shaker, VWR, The Netherlands), 20% of formic acid (5 μL) was added for neutralization. After a quick vortex, each sample was transferred to the autosampler vial for LC-MS injection.

Three μL of sample solution was injected onto a UPLC Class I (Acquity, Waters Chromatography Europe BV, Etten-Leur, The Netherlands) system with an AccQ-Tag Ultra C18 Column (1.7 μm , 100 x 2.1 mm, Waters, Ireland) coupled to a Sciex QTRAP® 6500 mass spectrometer [28]. Liquid chromatography (LC) separation and mass spectrometry parameters were the same from the work [22].

The compound list with target m/z for precursor and product ions is shown in Supplementary Table 1. Raw LC-MS/MS data was processed with AB Sciex PeakView™

2.0 and MultiQuant™ 3.0.1 software for targeted metabolite peak identification and integration.

Statistical analysis

Box plots were created with GraphPad Prism 9.3.1 software. Significance is determined by a two-way ANOVA- multiple comparison test and student's t-test. In this study, correlation analysis was performed between the three treatment groups (healthy, mild COVID-19, and severe COVID-19) using a Pearson Correlation coefficient with a significance threshold of $p > 0.05$. All biological and technical replicates were included in the analysis.

Results

This study utilized a microfluidic chip model to investigate how COVID-19 infection affects eNOS-derived nitric oxide metabolite production. We cultured microvessels in the rerouted OrganoPlate using HCAECs and exposed them to plasma from COVID-19 patients (mild and severe) and healthy controls. By measuring metabolites like citrulline+9 and ornithine+7, we compared metabolite levels across groups to understand the impact of COVID-19.

Measurement of nitric oxide metabolites from severe COVID-19 patient plasma perfused 3D microvessels.

Differences in NO metabolites assessed using UPLC-MS/MS in a severely infected COVID-19 patient compared with a healthy control are shown in **Fig. 1 A-C**. Before the cells were exposed to the plasma samples, the direct measurement of plasma levels of unlabeled arginine, citrulline, and ornithine showed no difference between healthy and COVID-19 (**Supplementary Fig. 2 A-C**).

In the context of COVID-19, the addition of the eNOS stimulator VEGF at varying concentrations in the presence of BH4+BH2 resulted in an elevated ratio of L-citrulline+9/L-arginine+10. However, when BH4+BH2 was not added to VEGF (50ng), no significant difference was observed. Arginase inhibition using BEC treatments resulted in only slightly elevated levels of citrulline+9/arginine+10 ratio in both healthy and COVID-19 models (**Fig. 1A**). The ratio of citrulline+9/ornithine+7 (**Fig. 1B**) showed no significant difference in any of the conditions in COVID-19, compared to the healthy plasma control, suggesting that there are no changes in the relative activity of eNOS to arginase. However, when evaluating the ratio of ornithine+7/arginine+10 (**Fig. 1C**), the arginase inhibition by BEC showed reduced levels with a non-significant difference and a higher ratio was observed in VEGF (100ng) in the presence of BH4+BH2. This suggests that at higher concentration of VEGF, the utilization of arginine by arginase in COVID conditions was significantly higher.

Numerous research efforts have delved into enhancing NO production by administering higher L-arginine concentrations to address substrate deficiency in endothelial dysfunction, our study did not reveal any significant difference between healthy and COVID-19. To understand the impact of COVID on substrate availability, arginine bioavailability was assessed but no significant difference was seen between healthy and COVID-19 conditions (result not shown). Correlation analysis was conducted to assess the relationship between eNOS and arginase enzyme activity. In the healthy cohort (**Fig. 1D**), the correlation analysis revealed an association between eNOS and arginase enzyme activity ($r = -0.01$, p value = 0.9) and in severe COVID-19 patient, the correlation analysis revealed a significant association between eNOS and arginase enzyme activity ($r = 0.3$, $P = 0.02$), indicating a positive relationship between the two variables. (**Fig. 1E**).

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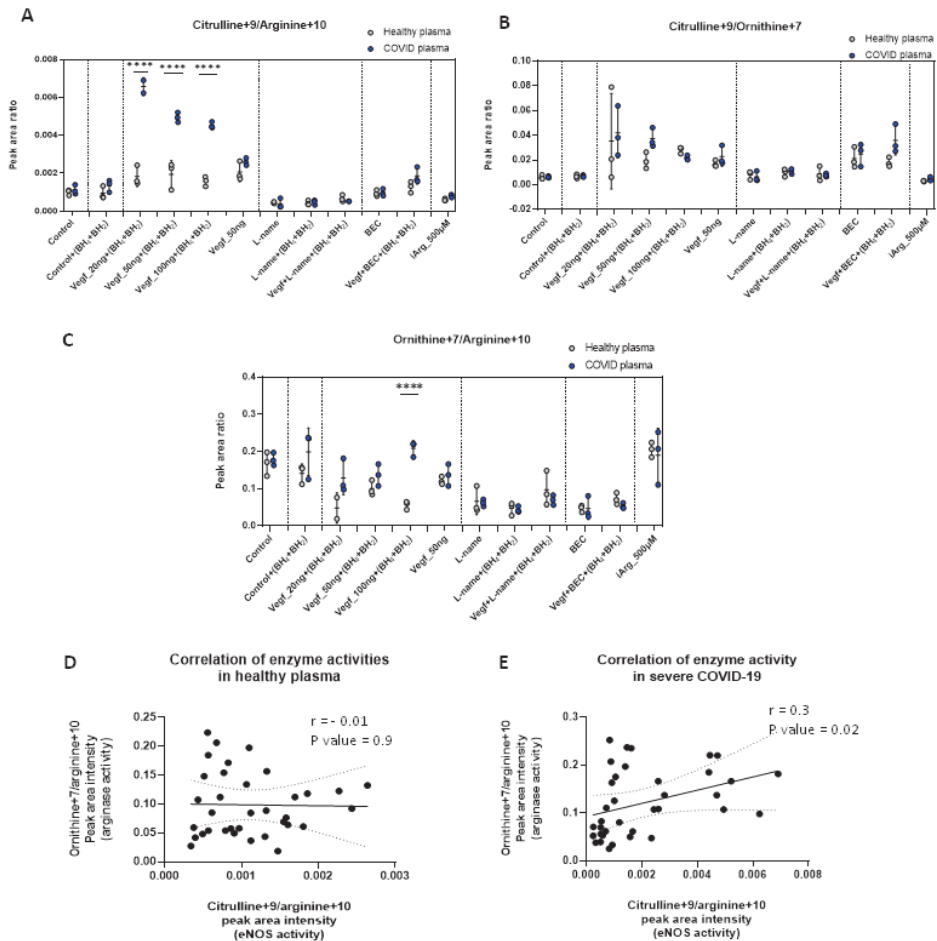


Figure 1. Measurement of arginine+10, citrulline+9 and ornithine+7 ratios from healthy and PT1 - COVID-19 plasma under stimulatory or inhibitory compound exposure after perfusion through 3D microvessels. A) The ratio of citrulline+9/arginine+10 B) the ratio of citrulline+9/ornithine+7 and C) the ratio of ornithine+7/arginine+10. Pearson correlation of ornithine+7/arginine+10 and citrulline+9/arginine+10 represents the arginase and eNOS activities D) in healthy plasma ($r = -0.01$, $p \text{ value} = 0.9$) and E) in severe COVID-19 plasma ($r = 0.3$, $p \text{ value} = 0.02$). All error bars represent SD and mean, each dot represents technical replicates, $n=3$. Significance determined by Two-way ANOVA –multiple comparison test of COVID group versus healthy control group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

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Measurement of nitric oxide metabolites from mild COVID-19 patient plasma perfused 3D microvessels

The levels of eNOS-based NO metabolites were measured following the perfusion of plasma samples from mildly infected COVID-19 patient and healthy plasma (control) into the 3D microvessels. Significant differences in NO metabolites assessed using UPLC-MS/MS in a mildly infected COVID-19 patient compared with a healthy control is shown in **Fig. 2 A-C**. The mild condition of COVID-19 was confirmed with the lower viral load, and normal ranges of urea, CRP, albumin, GFR.

The direct measurement of mild COVID-19 plasma shows similar levels of unlabeled citrulline and ornithine in healthy plasma with no statistical difference (**Supplementary Fig. 3A-C**), except arginine levels were higher in COVID-19 plasma.

When analyzing the Citrulline+9/arginine+10 ratio (**Fig. 2A**), no significant changes were observed between healthy and COVID conditions, implying that the utilization of arginine by eNOS for NO production remained consistent in both scenarios. However, in the case of the citrulline+9/ornithine+7 ratio, a significantly lower level was noted in COVID-19 when VEGF+BEC+BH2 was introduced (**Fig. 2B**). This suggests that the relative activity of eNOS to arginase appeared to be notably lower in COVID.

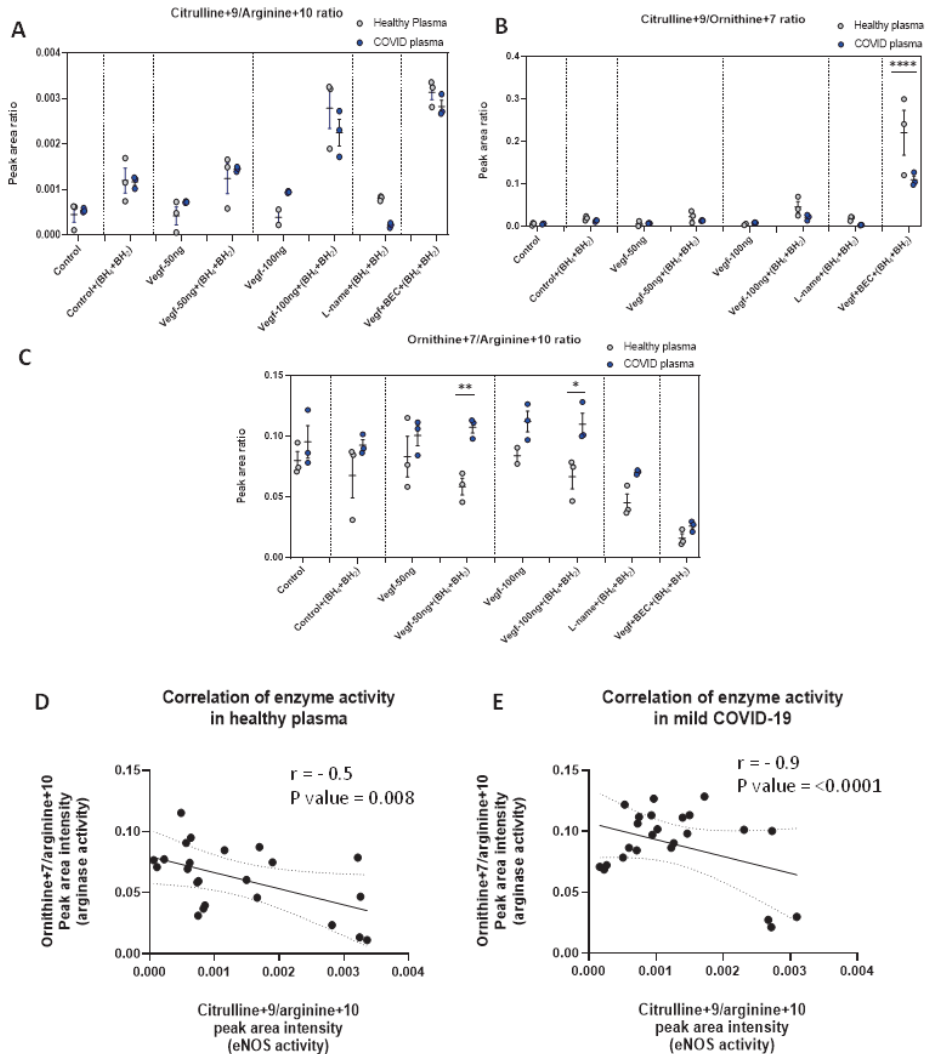


Figure 2. Measurement of arginine+10, citrulline+9 and ornithine+7 ratios from healthy and mild COVID-19 plasma under stimulatory or inhibitory compound exposure after perfused into 3D microvessels. A) The ratio of citrulline+9/arginine+10 B) the ratio of citrulline+9/ornithine+7 and C) the ratio of ornithine+7/arginine+10. Pearson correlation of ornithine+7/arginine+10 and citrulline+9/arginine+10 represents the arginase and eNOS activities D) in healthy plasma ($r = -0.5$, $p \text{ value} = 0.008$) and E) in mild COVID-19 plasma ($r = -0.9$, $p \text{ value} = <0.0001$). All error bars represent SD and mean, each dot represents technical replicates, $n=3$.

Significance determined by Two-way ANOVA –multiple comparison test of COVID group versus healthy control group. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

In the COVID-19 context, the introduction of the eNOS stimulator VEGF in the presence of BH4+BH2 resulted in increased levels of the L-ornithine+7/L-arginine+10 ratio (**Fig. 2C**). This indicates that the utilization of arginine by arginase was higher during eNOS stimulation in the presence of the cofactor BH4+BH2, but not in its absence. This suggests that the utilization of arginine+10 to citrulline+9 and ornithine+7 conversion was higher in mild COVID-19. Correlation analysis to assess the relationship between eNOS and arginase activity shows a significant negative correlation in healthy ($r = -0.5$, $P = 0.008$) (**Fig. 2D**) and in COVID-19 ($r = -0.9$, $P = < 0.0001$) (**Fig. 2E**).

Discussion

Given the ongoing discoveries regarding the enduring effects of COVID-19, it is evident that there is a pressing requirement to create in vitro models for a more profound comprehension of the pathology and consequences of the plasma components. Our 3D microvessels-on-chip model, utilizing collagen as the extracellular matrix and incorporating a microfluidic pump to induce flow, was previously demonstrated to be the optimal experimental setup to study endothelial nitric oxide mechanism [22]. The cell densities and area-to-volume ratios in this model closely mimic physiological conditions [22,29], surpassing those in conventional 2D monolayer cultures. Our study highlights the specific pathways utilizing arginine involved in NO production using our tracer-based method [22]. This study explored how components in COVID-19 plasma might influence eNOS-generated NO and citrulline levels.

In relation to the NO mechanism, our results from severe COVID-19 patient plasma (**Fig. 1A**) support the notion that the BH4+BH2 supplementation with VEGF may improve eNOS coupling in COVID-19, where the NO mechanism may be compromised due to insufficient BH4. Although we did not observe statistically significant changes

with BH4+BH2 supplementation alone when we found a response comparable to that of the healthy condition. The non-significant levels of unlabeled plasma arginine, citrulline and ornithine (**Supplementary Fig. 2A – C**) in clinically confirmed COVID-19 case could reflect that the downstream metabolites (unlabeled ornithine and citrulline) can be produced through other pathways such as glutamine [30,31] or ADMA [32]. However, in mild COVID-19 the significantly higher arginine level indicates the excess of substrate (**Supplementary Fig. 3A – C**).

Through our experimental procedure, it is hypothesized that supplementing BH4+BH2 could elevate BH4 levels by activating the GTP cyclohydrolase, the first step of BH4 synthesis along with the presence of shear stress [33]. The significance of citrulline levels in COVID-19 under VEGF+BH4+BH2 treatments was attributed to the previously studied concentration-dependent VEGF increase in NO production [34], leading to higher citrulline+9 levels (**Supplementary Fig. 2D & 3D**) in both severe and mild patients but not such increase was observed in VEGF alone treatment. Other treatment groups showed no significant difference between healthy controls and COVID-19 patients. This could be due to two possible reasons: 1) The lack of a specific stimulus like VEGF, or 2) The inherent presence of BH4 in the cells or shear stress induced endogenous BH4 production [33], which might already be sufficient to regulate citrulline levels in these treatment groups in COVID-19 cases.

Under same VEGF+BH4+BH2 treatments, the observed increase in the ornithine+7/arginine+10 ratio (**Fig. 1C & 2C**) in COVID-19 patients receiving VEGF supplementation might be due to the potential feedback mechanism. VEGF stimulates eNOS activity, leading to more L-arginine conversion to citrulline, leaving less L-arginine for arginase, potentially reducing its activity, and causing a slight rise in ornithine levels. Additionally, some studies suggest high ornithine might even feedback and further inhibit arginase, indirectly boosting arginine availability for eNOS [35].

Interestingly, the citrulline+9/ornithine+7 ratio was not indicative in severe COVID-19 (**Fig. 1B**), suggesting similar citrulline and ornithine production across treatments. In mild cases, VEGF+BEC+BH4+BH2 treatment lowered the ratio compared to healthy controls

(**Fig. 2B**). However, the same treatment in mild COVID-19 showed the highest citrulline (**Supplementary Fig. 2D**) levels despite the unclear effect on the ratio.

Increase in ornithine level serves as an indicator of SARS-CoV-2 infection where upregulation of ornithine was reported in earlier studies [36]. In our study, elevated ornithine+7 levels were observed in severe COVID (**Supplementary Fig. 2E**) in higher concentration ($\leq 500\mu\text{M}$) of arginine+10 supplementation, while no significant increase was seen in citrulline+9 (**Supplementary Fig. 2D**). Numerous research efforts have delved into enhancing NO production by administering higher L-arginine concentrations to address substrate deficiency in endothelial dysfunction [37–39], our findings suggest that L-arginine supplementation alone might not be sufficient to significantly increase citrulline levels. While adequate substrate (arginine) was provided, the lack of a sufficient crucial cofactor (BH₄) seems to be a limiting factor for eNOS activity. This is further supported by the presence of shear stress, a known activator of eNOS, which was already incorporated into the study design.

Maintaining a balanced enzyme activity (eNOS and arginase) and ratio of citrulline to ornithine is essential for optimal nitric oxide (NO) production. Our study suggests that interventions promoting this balance, achieved through BH₄+BH₂ external supplementation or shear stress-induced production of BH₄, VEGF treatment in minimal concentrations, and arginase inhibition in both severe and mild COVID-19, show promising therapeutic strategy (**Supplementary Fig. 2D & 3D**). These interventions led to a significant decrease in ornithine levels with BEC/VEGF+BEC+BH₄+BH₂ treatment. While the effect on the ratio in severe COVID-19 was less pronounced (**Fig. 1B & 2B**), the observed increase in citrulline suggests potential benefits. This approach aims to suppress ornithine production, indirectly promoting citrulline conversion to NO and potentially improving NO production, a crucial molecule for vascular health.

Arginase competes with eNOS for the same substrate, L-arginine. High arginase activity can deplete arginine levels, limiting its availability for eNOS to generate NO. This has led to the exploration of arginase inhibition as a potential therapeutic strategy to address arginine depletion observed in COVID-19 patients [40]. Overall correlation of our treatment strategy revealed a distinct inverse correlation between eNOS and arginase

activity for a mild COVID-19 case when compared to the healthy plasma enzyme activity (**Fig. 2D & 2E**), where eNOS activity increased as arginase activity decreased. Conversely, severe COVID-19 displayed a positive correlation (**Fig. 1E**), potentially reflecting the inflammatory state observed in these patients (**Table 1**).

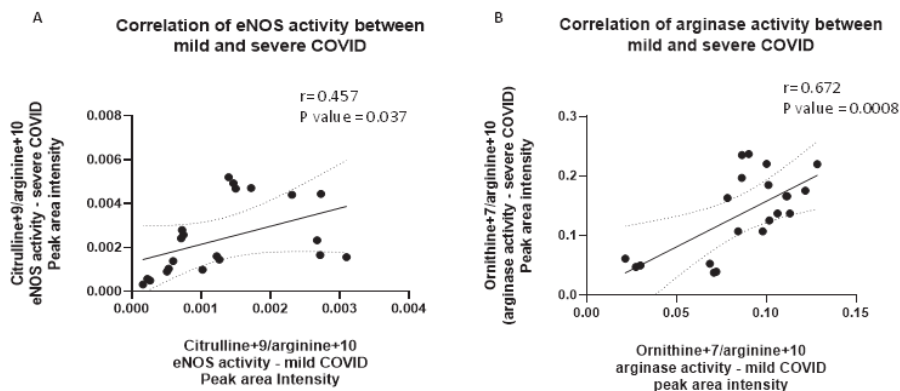


Figure 3. Measurement of eNOS and arginase enzyme activities in the mild COVID-19 case and the severe COVID-19 case. Pearson correlation of A) eNOS activity (citruilline+9/arginine+10) ($r = 0.457$; $p \text{ value} = 0.03$) and B) arginase activity (ornithine+7/arginine+10) ($r = 0.672$; $p \text{ value} = 0.0008$).

In healthy individuals, arginase and eNOS compete for L-arginine, with eNOS using it for beneficial nitric oxide (NO) production. The positive correlation between these enzymes in severe COVID-19 suggests endothelial dysfunction where the dysregulation of ornithine cycle in COVID-19 was shown in a previous study [41]. eNOS might be upregulated to boost NO production, but arginase activity is not suppressed effectively. This could be due to disrupted arginase regulation or the inflammatory environment of COVID-19 favoring arginase. Ideally, therapies would target both enzymes: upregulating eNOS and suppressing arginase to ensure sufficient L-arginine for NO production. In severe cases, high arginase activity excessively consumes L-arginine, converting it to ornithine. This limits the availability of L-arginine for eNOS to produce NO and

citrulline. Insufficient levels of bioavailable NO are a key factor contributing to endothelial dysfunction [8,42]. Previous studies have reported a strong correlation between hypertension and COVID-19, suggesting an underlying vascular dysfunction [43]. Interestingly, supplementation of BH4 showed to mitigate endothelial dysfunction and oxidative stress in hypercholesterolemic conditions [44] and improved vascular function in heart failure subjects [45]. However, there is limited research on the impact of cofactor supplementation on endothelial dysfunction and NO levels, particularly in the context of COVID-19 recovery.

A correlation analysis was performed to investigate the relationship between eNOS and arginase activity in mild versus severe COVID-19, as shown in **Fig. 3**.

Significantly different and the moderate positive correlation of Arginase and eNOS activities indicates that under various stimuli and inhibitors, both severe and mild COVID-19 cases exhibited similar response patterns, however, the magnitude of the response differed based on the viral load and the disease severity.

Conclusion

The necessity for the best in vitro endothelial model to study COVID-19 arises from the complexity of the disease's effects on the vascular system. In our study we employed a 3D microvessels model, perfusing COVID-19 plasma along with eNOS and arginase enzyme stimulator or inhibitors. This model enabled us to investigate the intricate interactions between endothelial cells and patient plasma components resulting from viral infection, providing insights into the mechanisms underlying COVID-19 pathogenesis. Using a tracer-based UPLC-MS/MS method, perfusing patient plasma samples through the 3D microvessels revealed significant effects on NO metabolites, especially with VEGF and BH4+BH2 supplementation which induced eNOS activity, as seen in higher labeled citrulline levels. Similarly, the interplay between arginase activity and eNOS activity provides insights into the regulatory pathways governing citrulline and ornithine metabolism. This new understanding could serve as a valuable tool for identifying the pathological status associated with vascular diseases. Further investigation into

therapeutic strategies utilizing eNOS stimulation alongside arginase inhibition is warranted based on these results. Finally, our study stands out as we observe a distinct response of NO metabolites based on the clinical data and disease severity of each patient. This model is highly recommended for investigating the impact of plasma components on endothelial cells, especially given our understanding of the detailed eNOS mechanism. Furthermore, such models facilitate the evaluation of potential therapeutics and interventions targeting endothelial dysfunction, inflammation, thrombosis, and other vascular complications associated with COVID-19.

Limitations of the study

This preliminary study underscores the need for additional patient samples to establish a broader conclusion. Moreover, insights from patients' pathological history, medication practices, and clinical reports pertaining to endothelial dysfunction would be valuable. Furthermore, measuring downstream metabolites of BH₄, such as BH₂ and neopterin, could provide additional validation to our study and also provide insights into the importance of BH₄ in the eNOS mechanism and potentially serve as a more effective therapeutic target.

Acknowledgements

Parts of these studies were supported by the Dutch Heart Foundation (CVON RECONNECT) and ZonMW (MKMD: 114022501) grants to T.H and A.J.V.Z. Part was supported by METABODELTA (Medical Delta program). This project has received funding from the European Union's Horizon 2020 research and innovation Programme LogicLab under the Marie Skłodowska-Curie grant agreement No 813920, and RECONNECT funded by the Dutch Heart Foundation No 2020B008.

We would like to acknowledge the contributions of Sam Heijjer, Sabine Bos, and Mengle Zhu to the initial analysis of biopterin compounds. Additionally, we would like

to express our sincere gratitude to Faiza Guled for her help in developing the mass spectrometry method for measuring these compounds.

Author Contributions

Kanchana Pandian, Conceptualization, Investigation, Methodology, Writing - original draft;

Rudmer Postma, Writing – review and editing;

Anton Jan van Zonneveld, Amy Harms, and Thomas Hankemeier, Conceptualization, Resources, Supervision, Funding acquisition, Writing – review and editing.

Declaration of interest

T. Hankemeier is a shareholder in Mimetas B.V. All other authors declare no competing interests.

Data availability statement

The data that support the findings of this study are available in the Materials and Methods, Results, and Supplementary Material of this article. Additional data will be provided upon request.

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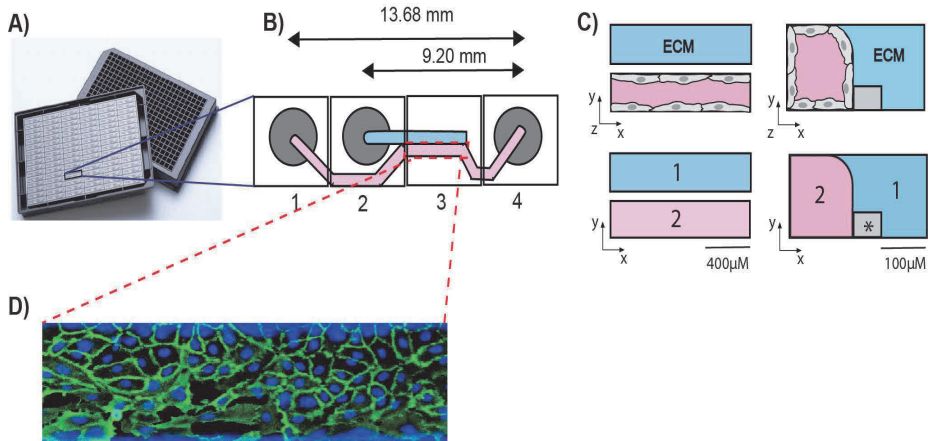
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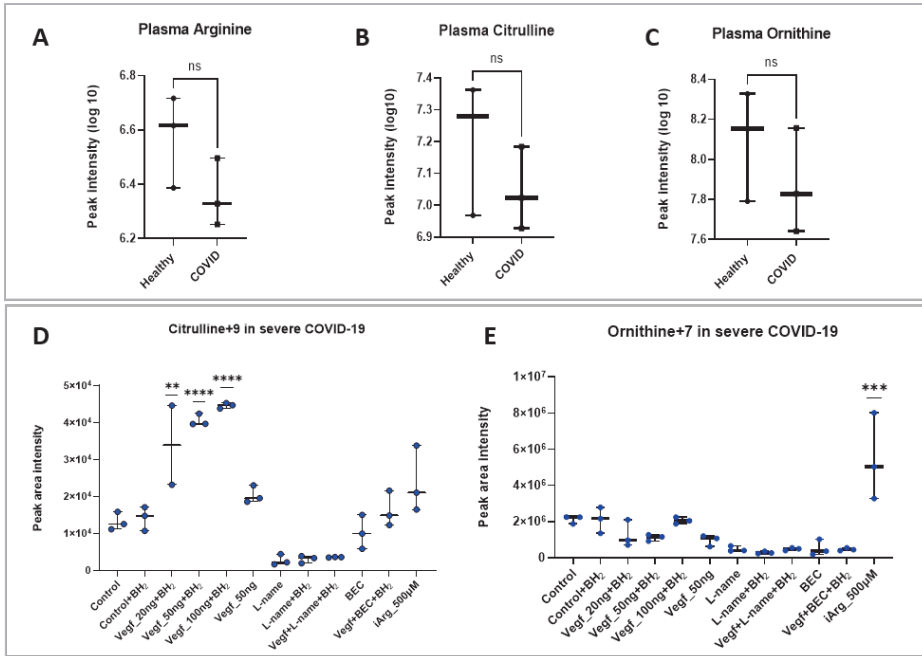
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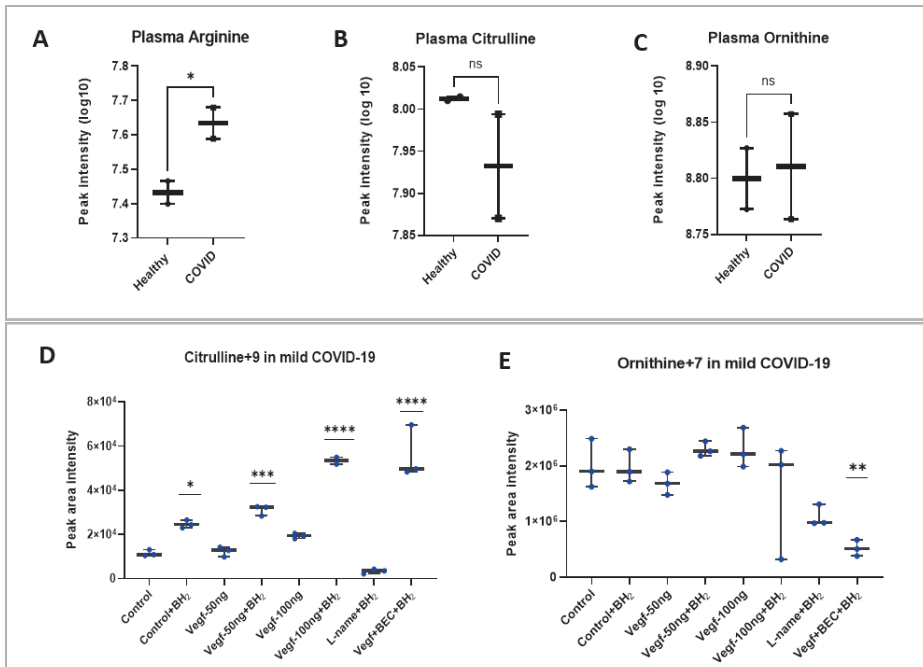
Supplementary Information



Supplementary Figure 1. Illustration of (A) re-routed OrganoPlate and its (B) Single chip design was explained below. Every microfluidic chip structure is positioned underneath 4 adjacent wells and it consists of two channels: 1) an ‘perfusion’ channel (pink color) and 2) a ‘gel’ channel (ECM) (blue color). Every first well (1) and fourth well (4) is positioned on top of the inlet and outlet of the perfusion channel, while every second well (2) are for the gel channel. And every third well (3) is used for imaging and observation of the experiment. (C) Perfusion and gel channel was shown vertical and horizontal view and its separated by a phase guide (*). (D) Immunostaining of HCAECs in a chip. After 48 hours of cell seeding, a confluent vessel of HCAECs were immunostained with V-Cadherin and DAPI. After treatment with plasma and other compounds, the perfusate was collected from the outlet of each micro vessel for subsequent metabolic analysis.



Supplementary Figure 2. Measurement of A) unlabeled arginine, B) unlabeled citrulline, and C) unlabeled ornithine from healthy and severe COVID-PT1 plasma before perfusion through the 3D microvessels. D) Measurement of citrulline+9 and E) ornithine+7 from healthy and severe - COVID-19 plasma under stimulatory or inhibitory compound exposure after perfusion through 3D microvessels. All error bars represent SD and mean, each dot represents a technical replicate, n=3-4. Significance was determined by student t-test and two-way ANOVA –multiple comparison test of healthy control versus COVID group. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, ns = non-significant.



Supplementary Figure 3. Measurement of A) unlabeled arginine, B) unlabeled citrulline, and C) unlabeled ornithine from healthy and mild COVID-19 (PT2) plasma before perfusion through the 3D microvessels. D) Measurement of citrulline+9 and E) ornithine+7 from healthy and mild - COVID-19 plasma under stimulatory or inhibitory compound exposure after perfusion through 3D microvessels. All error bars represent SD and mean, each dot represents a technical replicate, n=3-4. Significance was determined by student t-test and two-way ANOVA –multiple comparison test of healthy control versus COVID group. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001, ns = non-significant.

Targeted LC-MS/MS method for BH4 and BH2

Targeted LC-MS/MS analysis was performed for BH4 and BH2 measurement using an ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system Class I (Acquity, Waters Chromatography Europe BV, Etten-Leur, The Netherlands) system with an SeQuant® ZIC®-cHILIC column (2.1 mm × 100 mm, 3.0

μ m- Merck, Darmstadt, Germany) coupled to a Sciex QTRAP® 6500 mass spectrometer. Detailed descriptions of the measurement methods can be found in the supplementary material.

The LCMS method was adapted from the previous work [28] with some modifications. Briefly, mobile phase A consisted of 90% acetonitrile and 10% 5 mM ammonium formate. Mobile phase B consisted of 10% acetonitrile, 90% 5 mM ammonium formate. The aqueous 5 mM ammonium formate was adjusted to pH 7.4 using formic acid or ammonium hydroxide. The ZIC-c column is composed of silica-based particles (1:1 charge-balanced phosphorylcholine functional group) which lose stability in pH above 8. Therefore, the ZIC-c was operated only at acidic and neutral conditions.

The flow rate was 0.50 mL/min with the following mobile phase gradient: 0.0–1.0 min (0% B), 1.0–2.0 min (0–15% B), 2.0–5.0 min (15–21% B), 5.0–6.0 min (21–40% B), 6.0–7.0 min (40% B), 7.0–7.5 min (40–0% B), 7.5–12.5 min (0% B).

BH4 and BH2 standards were obtained from Sigma-Aldrich (The Netherlands) and the details were shown in **Suppl. Table 2**. Both the standards were accurately weighed and prepared in an appropriate solution to obtain the individual stock solution at a concentration of 100 μ M to prepare calibration solutions of concentrations 2 μ M, 5 μ M, 10 μ M, 25 μ M, 50 μ M and 100 μ M.

Mass spectrometry experiments were carried out on a Sciex QTRAP® 6500 mass spectrometer. Electrospray ionization (ESI) was operating in positive ion mode. The ESI source parameters were as follows (positive ion mode): spray voltage ± 4.5 kV, capillary temperature 250 °C, sheath gas 40, auxiliary gas 45, curtain gas 30.

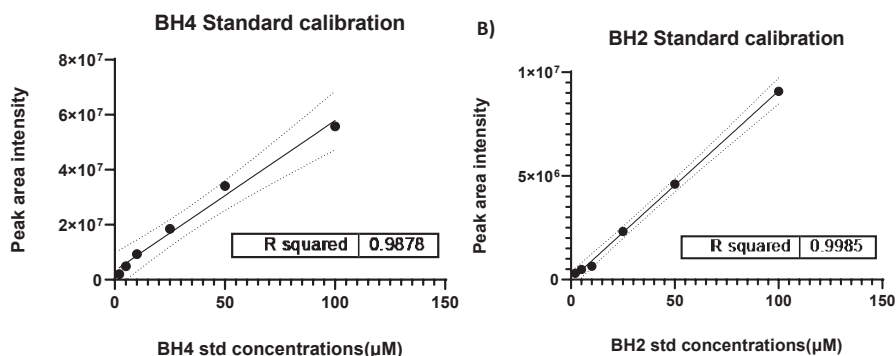
Measurement of BH4 and BH2

Mass spectrometry analysis revealed a significantly higher abundance of fragment ions at m/z 241.11/166 for BH4 and 239.10/164 m/z for BH2 compared to other fragmentation products. The compound list with observed mass adducts and m/z ratios is shown in **Supplementary Table 2**. Raw LC-MS/MS data was processed with AB Sciex PeakView™ 2.0 and MultiQuant™ 3.0.1 software for targeted metabolite peak

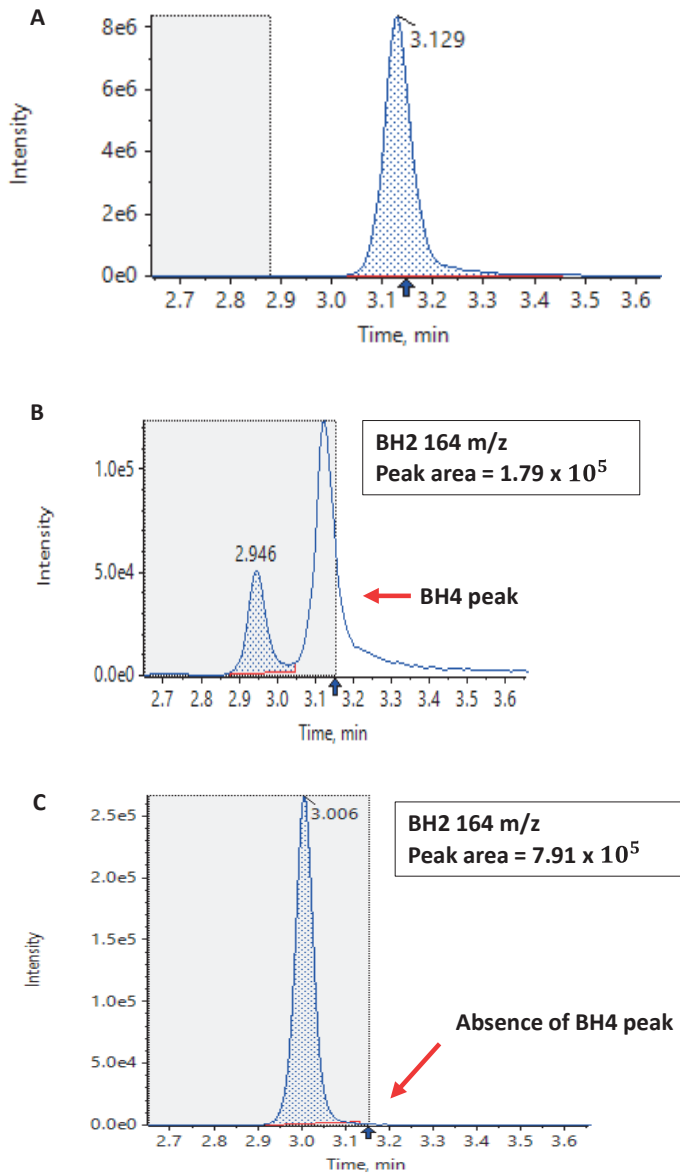
identification and integration. We included calibration curves for each standard in **Supplementary Fig. 4.**

We assessed BH4's susceptibility to oxidation at room temperature by comparing its behavior in the presence and absence of 1 mM dithiothreitol (DTT, Sigma Aldrich, Netherlands), a common antioxidant. In both scenarios, BH4 displayed limited stability, readily converting to BH2 (**Supplementary Fig. 5A & 5B**). Even BH4 standards stored at -80°C exhibited similar instability when measured the following day, with complete conversion to BH2 observed (**Supplementary Fig. 5C**).

Due to the inherent instability of BH4 during direct measurement, the BH4 standard likely oxidized to BH2. Consequently, we report BH4+BH2 in the results to reflect the actual form detected by mass spectrometry. Independent verification of these metabolites in extracts is crucial for definitive identification.



Supplementary Figure 4. Calibration curves of BH4 and BH2 with concentrations - 2 μM, 5 μM, 10 μM, 25 μM, 50 μM, and 100 μM. Note: Higher calibration concentrations was used due to the high susceptibility of the analyte to oxidation.



Supplementary Figure 5. Measurement of BH4 standard (50 μ M) by direct injection and the stability check. A) Peak of BH4 standard (241.11/166 m/z) on day 1, B) Oxidation of BH4 to BH2(239.10/164 m/z) an hour after standard solution preparation, and C) Peak intensity comparison showing complete oxidation of BH4 to BH2 after 12 hours at room temperature. Note: BH4 standard was prepared using 90% acetonitrile and 10% 5mM ammonium formate adjusted to

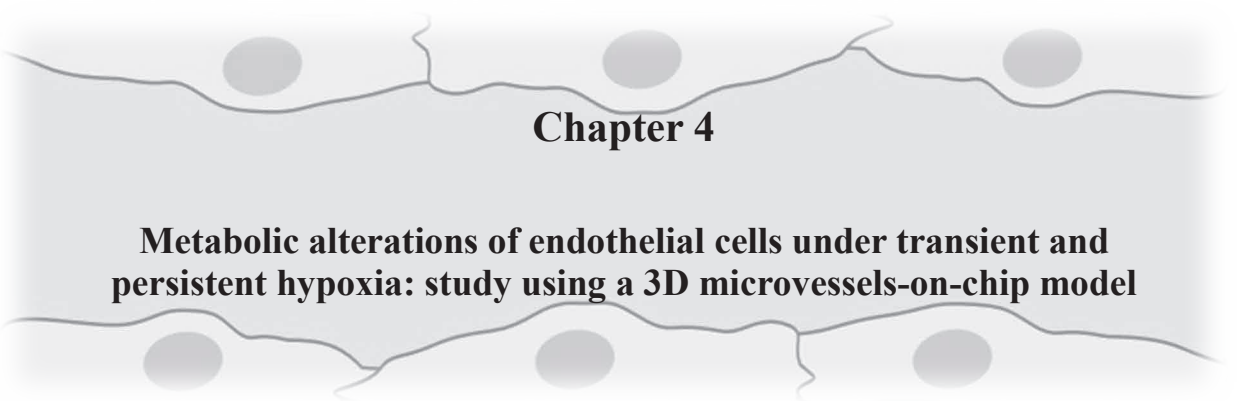
pH 7.4. BH2 standard was prepared using 10% DMSO in 90% acetonitrile and 10% 5mM ammonium formate (pH 7.4).

Supplementary Table 1. MRM method and the compound list with target m/z for precursor and product ions for tracer metabolites measurement.

Metabolites name	Mass (Q1)	Mass (Q3)
L-arginine	345.170	171.00
L-arginine [M+10]	355.170	171.00
L-citrulline	346.150	171.00
L-citrulline [M+9]	355.150	171.00
L-ornithine	237.100	171.00
L-ornithine [M+7]	240.600	171.00

Supplementary Table 2. Details of BH4 and BH2 standards used in the study.

Compound	CHEBI ID	Supplier	Class	Formula	Mass adducts	ESI m/z	Stock concentration (mM)	Stock solvent
BH4	15372	Sigma-Aldrich	Pteridines	C ₉ H ₁₅ N ₅ O ₃ · 2HCl	[M+H] ⁺	241.1175 166, 107,149	1	90% acetonitrile and 10% 5 mM ammonium formate
BH2	64277	Sigma-Aldrich	Pteridines	C ₉ H ₁₃ N ₅ O ₃	[M+H] ⁺	239.1018 164, 168, 196	1	10% DMSO in 90% acetonitrile and 10% 5 mM ammonium formate



Chapter 4

Metabolic alterations of endothelial cells under transient and persistent hypoxia: study using a 3D microvessels-on-chip model

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Tissue Barriers (2024) Nov 25:2431416. doi: 10.1080/21688370.2024.2431416

Abstract

Numerous signaling pathways are activated during hypoxia to facilitate angiogenesis, promoting interactions among endothelial cells and initiating downstream signaling cascades. Although the pivotal role of the nitric oxide (NO) response pathway is well-established, the involvement of arginine-specific metabolism and bioactive lipid mechanisms in 3D flow-activated in vitro models remains less understood. In this study, we explored the levels of arginine-specific metabolites and bioactive lipids in HCAECs under both transient and persistent hypoxia. We compared targeted metabolite levels between a 2D static culture model and a 3D microvessels-on-chip model. Notably, we observed robust regulation of NO metabolites in both transient and persistent hypoxic conditions. In the 2D model under transient hypoxia, metabolic readouts of bioactive lipids revealed increased oxidative stress markers, a phenomenon not observed in the 3D microvessels. Furthermore, we made a novel discovery that the responses of bioactive lipids were regulated by hypoxia inducible factor-1 α (HIF-1 α) in the 2D cell culture model and partially by HIF-1 α and flow-induced shear stress in the 3D microvessels. Immunostaining confirmed the HIF-1 α -induced regulation under both hypoxic conditions. Real-time oxygen measurements in the 3D microvessels using an oxygen probe validated that oxygen levels were maintained in the 3D model. Overall, our findings underscore the critical regulatory roles of HIF-1 α and shear stress in NO metabolites and bioactive lipids in both 2D and 3D cell culture models.

Key words

Hypoxia, endothelial dysfunction, 3D microvessels-on-chip model, nitric oxide metabolites, signaling lipids, hypoxia inducible factor – 1 alpha

Introduction

The vascular endothelium, the first cellular layer in contact with blood, continuously adapts to a variety of physiological and pathological environmental cues to maintain homeostasis. One challenging stimulus is an oxygen tension deficit [1]. While the response of the endothelial cells (ECs) to hypoxic stress can differ depending on the degree and duration of the hypoxia and organ specific vascular bed, it mostly entails the modulation of vasodilation, constriction, and cell proliferation through transcriptional responses driven by HIFs [7-9]. Additional physiological factors, like blood flow and shear stress may further modulate the hypoxic endothelium-based homeostatic mechanisms. Two central signaling pathways involved in the endothelial response to hypoxia involve the endothelial nitric oxide synthase (eNOS) pathway [3–6] and mechanisms involving the signaling lipids [7].

In the presence of sufficient co-factors, eNOS can catalyze the formation of the potent vasodilator nitric oxide (NO) from oxygen and L-arginine [8–10]. However, in patients at risk for cardiovascular disease, systemic low grade inflammatory conditions and tissue ischemia, this enzyme can become ‘uncoupled’ leading to the leak of electron from the transport chain in the reductase domain to molecular oxygen. As a consequence, the superoxide anion ($O^{\cdot -}$) is formed that can react with residual NO to yield peroxynitrite ($ONOO^{\cdot -}$) [11–13]. Recently, NO was found to attenuate accumulation of HIF-1 α under hypoxic conditions because of several mechanisms: redistribution of oxygen toward non-respiratory oxygen-dependent targets (like HIF-1 α proline hydroxylases—PHDs, which perform hydroxylation of Pro402/564 of HIF-1 α leading to its proteasomal degradation); in addition, peroxynitrite formed during interactions between NO and mitochondria derived superoxide leads to an increase in cytosolic iron/2-oxoglutarate (2-OG), which required for PHD activation. Here, we propose a hypothesis that peroxynitrite, formed in the cells upon exposure to NO under low oxygen availability, serves as an alternative donor of oxygen for activated PHDs so they can perform HIF-1 α proline hydroxylation to de-accumulate the protein [14]. In addition, any disturbance of this complex regulatory

mechanism causes changes to NO mechanisms, leading to increased cellular oxidative stress and decreased NO bioavailability. Hypoxic conditions and the downregulation of eNOS have been studied widely in different cell models under hypoxic conditions such as in HUVECs [15,16], in HCAECs [17], in PAECs [18], as well as in vivo conditions such as pulmonary hypertension patients [19] and aortic and mesenteric arteries of mice exposed to chronic intermittent hypoxia [20]. In contrast, some studies have reported the upregulation of eNOS in hypoxia induced persistent pulmonary hypertension of the newborn rat [21], and in vitro cultured porcine aortic endothelial cells (PAECs) also doubled the nitrite level after exposed to hypoxia [22].

Similarly, endothelial adaptations to hypoxia involve alterations in phospholipase A2 (PLA2) metabolism, a system that generates bioactive lipids that can mediate in a variety of signaling cascades including vascular homeostasis and inflammation [23] [24,25]. Hypoxia is associated with higher expression of lipoxygenases (LOX) [26] and cyclooxygenase-2 (COX-2), the inducible cyclooxygenase in most tissues [27,28] [29,30]. Typical signaling lipid classes from arachidonic acid (AA) metabolism involve oxylipins and (nitro) free fatty acids, and lysophospholipids. In the vascular endothelium, hypoxia elicits an inflammatory response [31,32] characterized by an elevated production of ROS leading to stabilization of HIF-1 α [33]. These ROS effects in ECs were investigated with assessment of stable ROS marker metabolites in lipid peroxidation and went hand in hand with reduced NO production [17,34–38].

In this study, we demonstrated the transient and persistent hypoxic response of endothelial cells in 2D steady culture and 3D microvessels model in terms of metabolic standpoint. Using stable isotopes, we tracked the eNOS pathway by using isotope labeled L-arginine, the NO substrate, to track its downstream metabolites L-Citrulline and L-Ornithine. The findings reveal no statistically significant differences in the levels of L-citrulline and L-ornithine when comparing normoxia to hypoxia in both 2D and 3D culture models. In addition, we analyzed the signaling cascades of bioactive lipids under transient and persistent hypoxia. In the 2D model under transient hypoxia, the metabolic readout of bioactive lipids demonstrated an increase in oxidative stress markers, a trend not observed in 3D microvessels. Under persistent hypoxia, both 2D and 3D models exhibited

downregulation of bioactive lipids associated with inflammation. Furthermore, immunostaining confirmed the higher nuclear localization of the hypoxia marker HIF-1 α in persistent hypoxia.

Materials and methods

Cell culture models and oxygen treatment

Human coronary artery endothelial cells (HCAECs) (PromoCell, C-12221) were resuspended in 10 ml fresh EGM MV2 medium with supplements (PromoCell, C-22022, C-39216) and cultured in T75 flasks (Nunc Easyflask, Sigma, F7552). Cell cultures were maintained at 37 °C with 5% CO₂ and media was refreshed three times a week. Cells were detached at 85% confluence with 0.25 % Trypsin EDTA (Lonza, CC-5012) and cell pellets were collected by centrifugation at 300g for 5 minutes.

For 2D culture experiments, the collected cell pellets were suspended in a fresh medium to a concentration of 7×10^5 cells/ml and cultured in a 48 well plate for 48 hours. To synchronize or equilibrate the cell cycle we treated cells with Krebs buffer solution, pH 7.4 (Thermo fisher Scientific, J67795.AP) for 1 hour before the start of each experiment. Subsequently the cells were incubated for 12 and 48 hours with 100 μ L of RPMI SILAC (ThermoFisher Scientific, 88365) supplemented with 150 μ M ¹³C₆, ¹⁵N₄-L-arginine (CORTECNET, 130541), and 2% FBS (ThermoFisher Scientific, A4736301) under 21% and 1% oxygen, balanced to 5% CO₂ + 74% N₂ and 5% CO₂ + 94% N₂ (Panasonic oxygen incubator).

For 3D cultures, we used a modified chip design 2-lane rerouted OrganoPlate (MIMETAS, Netherlands) that was designed to attach a microfluidic pump to apply fluid flow and was adapted from the work described in [39]. The protocol for the 3D model is as follows: HCAECs were seeded into an OrganoPlate that was specially designed for controlled perfusion using a pump attachment. The cells were cultured for 3-4 days with media refreshed every other day. For controlled flow of the media in the plate, a small motor is operated with the help of LabVIEW solutions software. This software allows the control of fluid flow rate and related shear stress (dyne/cm²), use of portal connections,

motor position and value. Using the pump, the flow was operated to create shear stress of 5 dyne/cm² and the cells were incubated under normoxia or hypoxia for 12 or 48 hours. The incubation protocols, and oxygen exposure were similar to that of the 2D assay described above.

After the incubation hours, the medium samples were collected separately in 1.5 mL Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C for LCMS analysis.

Metabolic profiling

For eNOS-based NO marker metabolite's measurement, 20 µL of media were transferred to fresh Eppendorf tubes to equalize the sample volumes for further processes. Sample preparation procedure and LC-MS acquisition method were adapted from the previous work [39]. Briefly, samples were analyzed using a targeted UPLC-MS/MS method that employed an AccQ-Tag derivatization strategy (Waters, Waters B.V. Art. No. 186003836, The Netherlands). Quality control (QC) samples were generated by pooling equal volumes of all cell media and cell lysate samples, and 10 µL of this pool underwent the same process as individual samples and QCs were injected every 10 study samples. For the MS measurements, 3 µL of sample solution was injected onto a UPLC Class I (Acquity, Waters Chromatography Europe BV, Etten-Leur, The Netherlands) system with an AccQ-Tag Ultra C18 Column (1.7 µm, 100 x 2.1 mm, Waters, Ireland) coupled to a Sciex QTRAP® 6500 mass spectrometer. For liquid chromatography (LC) separation, mobile phase A consisted of 0.1% formic acid in water. Mobile phase B consisted of 0.1% formic acid in acetonitrile. The final flow rate used was 0.7 mL/min and the end gradient were 99.8% A and 0.2% B mobile phase. The ESI source parameters were same as previous work. Data acquisition was performed in multiple reaction monitoring mode targeting compounds with different labeling statuses. The compound list with target m/z for parent and product ions is shown in **Supplementary Table 1**. Raw LC-MS/MS data was processed with AB Sciex PeakView™ 2.0 and MultiQuant™ 3.0.1 software for targeted metabolite peak identification and integration. Metabolite isotopologue levels were quantified using the corresponding peak areas.

For signaling lipids measurements, metabolite extraction, sample preparation procedure was according to the in-house experimental protocol which has been standardized and published [40]. Briefly, the cell media samples were thawed on ice, and randomized prior to analysis. The sample media volume of 50 μL for each condition was aliquoted in fresh tubes and the remains were pooled and used for quality control (QC) samples. The thawed samples underwent the addition of 2.5 μL of antioxidant solution and 5 μL of internal standards (ISTDs). Acidification with citric acid/phosphate buffer (pH 4.5) followed, and all samples underwent liquid-liquid extraction (LLE) using 1 mL of butanol and ethyl acetate (1:1 v/v). Following vortexing and centrifugation, the organic phase was collected and subjected to drying. After reconstitution with an ice-cold 70% MeOH injection solution, each sample underwent additional vortexing and centrifugation, and the resulting supernatant was transferred to the insert in a glass vial.

The method makes use of two optimized chromatographic separations with different pH gradients. The first one is the High-pH runs, for that the injection volume of 5 μL was measured by a LCMS-8060 system (Shimadzu, Japan) with a Kinetex® Core-Shell EVO 100 Å C18 Column (50 x 2.1 mm, 1.7 μm) maintained at 40 °C. The MS system used was a Shimadzu LCMS-8060 triple-quadrupole mass spectrometer with an electrospray ionization source.

The chromatographic separations were achieved using two sample injections into two complimentary separations with different stationary phases and different gradients. We tailored a high pH run and low pH run for the classes of signaling lipids [40]. For the low-pH runs, 5 μL was injected and analyzed using a LCMS system of Shimadzu LC-30AD hyphenated to a SCIEX Triple-Quad 7500 system with an electrospray ionization source (USA) with a Waters Acquity® BEH C18 column (50mm x 2.1mm, 1.7 μm) maintained at 40 °C. QC samples and blank samples were injected together with study samples to ensure data quality.

Data Pre-Processing

LabSolutions (Shimadzu, Version 5.91) was used for peak determination and integration. The response ratio for each metabolite was calculated as the ratio of the target compound's

peak area to that of the assigned internal standard. Metabolites mean with RSDs below 30% were selected for further statistical analysis following QC evaluation.

HIF- α measurement

Cells from 2D culture model and 3D microvessels were fixed with 4% paraformaldehyde and 0.3% Triton X-100, washed with PBS, blocked with 0.5% Bovine Serum Albumin (BSA) for 30 min at room temperature, and stained with HIF-1 α antibody (#610,959; BD Biosciences, Franklin Lakes, NJ, USA) and Rhodamine Phalloidin (R415; Thermo Fisher Scientific, Waltham, MA, USA) overnight at 4 °C and 2h at RT, respectively. Cells were rinsed with 0.5% BSA every 10 min 3 times and stained with secondary antibody and Hoechst 33,258 (#610959; Thermo Fisher) for 1 h at room temperature (RT) in the dark. After washing with PBS, plates were stored at 4 °C. Images were taken with a Nikon Eclipse Ti microscope with a 20 X objective. Scale bars shown in the images represent 50 μ m.

Measurement of real time oxygen in a 3D micro vessels-on-chip model

The cells were seeded into the 3D microvessels - OrganoPlate (similar protocol mentioned in the above section) which is specially designed for the pump attachment for unidirectional flow and in-line oxygen measurement [39] & [41]. The pump is made of stainless steel and the tube connections for the fluid transfer from the inlet and outlet of the chips are made of silicone. For controlled rotation of the pump, a small motor is operated with the help of LabVIEW software.

For oxygen measurement in the 3D rerouted plate fluid flow conditions, we used the optical fiber-based detection system. The oxnano probe (Pyroscience, OXNANO oxygen nanoprobes) in cell growth media was infused onto a chip and calibrated the initial oxygen value in the oxygen incubator. We performed a two-point calibration with oxnano probe prepared in cell media and set to 21% oxygen and with sodium nitrite solution, which acts as an oxygen scavenger, and calibrated to 0% oxygen. The probe's fluorescence intensities

at various time points were used to determine the continuous measurement of oxygen within the integrated microvessels-on-chips (**Supplementary Fig 2**).

Statistical analysis

Bar plots were created with GraphPad Prism 9.3.1 software. Significance was determined by ANOVA, Tukey's analysis, and student t-test. Lipid profiles – Volcano plots were plotted using R Studio version 2023.06.1.

Results

HCAECs respond to persistent oxygen exposure by increasing HIF1- α expression and nuclear relocation

To validate the impact of hypoxia (1% oxygen) in our models (**Supplementary Fig. 2**) we analyzed the expression of the key hypoxic biomarker HIF-1 α by immunostaining. Compared to normoxic conditions, or short term 18-hour hypoxia (not shown), 48hr persistent hypoxia led to a marked accumulation of HIF-1 α in the nuclei of HCAECs in static 2D cultures (**Fig. 1A & 1B**). Likewise, in the 3D HCAECs cultures, increased expression, and accumulation of HIF-1 α was observed in 50% of cell populations. Quantification of the fluorescent intensity of HIF-1 α staining in both models showed a slightly higher HIF-1 α level in the nuclei of the 2D cultured cells compared to those cultured in the 3D microvessels (**Fig. 1A & 1B**).

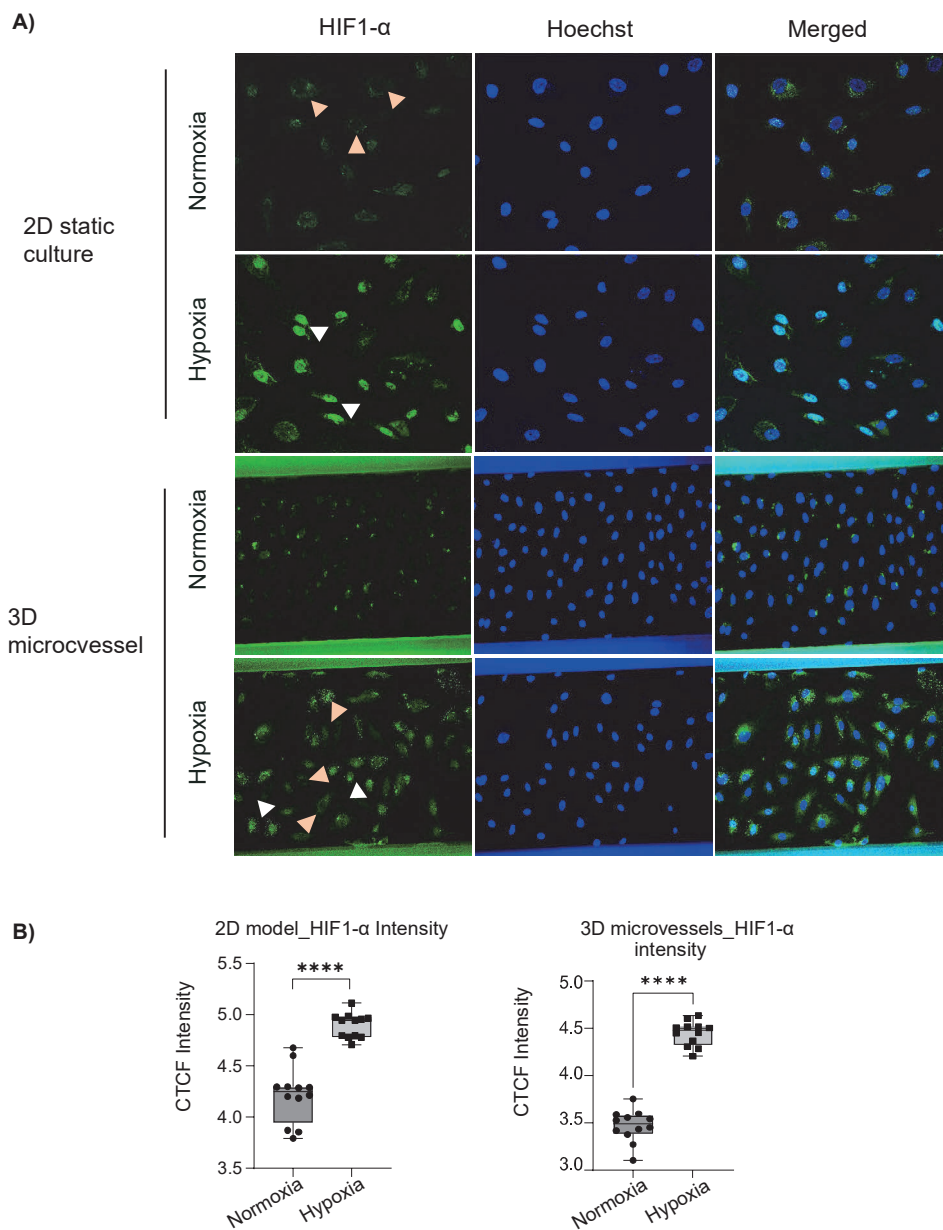


Figure 1. Immunofluorescence staining of HIF1- α . A) HIF1- α localization by confocal microscopy after 48hrs (persistent) incubation under normoxia and hypoxia in 2D culture well plate and 3D microvessels-on-chip model. Green, HIF1- α Ab; Blue, Hoechst; (Biological experiments n=2). White mark – Localization of HIF1- α in the nucleus and Brown arrow – No localization of

HIF1- α in the nucleus. Magnification - 20X. B) Fluorescence intensity of immunofluorescent cells calculated by corrected total cell fluorescence (CTCF) in 2D culture and 3D microvessels using ImageJ. Significance was calculated by a student T-test. ****, $P < 0.0001$.

Measurement of arginine-based NO metabolites in response to transient and persistent hypoxia exposure in HCAECs

In the presence of sufficient co-factors such as NADPH, FMAD, BH₄, Ca²⁺, eNOS utilizes L-arginine and molecular oxygen to generate NO [42–44]. In the process, L-arginine is converted to L-citrulline and therefore the rate of this conversion is widely used as a measure for eNOS activity (**Fig. 2A**).

Hence, to compare the impact of transient (12 hr) and persistent (48 hr) hypoxia on the activity of eNOS in our static 2D and 3D model systems, we measured the conversion of the NO substrate ¹³C₆, ¹⁵N₄ L-arginine to ¹³C₆, ¹⁵N₃ L-Citrulline by the enzyme. In addition, we assessed the generation of ¹³C₅, ¹⁵N₂ L-Ornithine by the arginase enzyme that also uses L-arginine as a substrate. HCAECs were cultured in 48-well cell culture plate and in our 3D microvessels-on-chip model that includes perfusion facilitated by a microfluidic pump [39]. **Fig. 2B & 2C** shows that while the L-arginine+10 levels are maintained, eNOS and arginase are active in both models, showing markedly elevated levels of L-citrulline+9 and L-ornithine+7 in conditioned media of both models after 48 hrs of hypoxia compared to the levels of these compounds measured after 12 hrs. However, no significant differences in citrulline+9 and ornithine+7 levels were observed between normoxia and hypoxia in the models.

When we compared the ratios of these target metabolites, the 2D static cultures exhibit no statistical significance in ratios such as citrulline+9/ornithine+7, reflecting relative eNOS to arginase activity and ornithine+7/arginine+10, indicating unchanged arginine utilization by arginase in both transient and persistent hypoxic conditions (**Supplementary Fig. 3**). A minor lowering of the citrulline+9/arginine+10 ratio was observed in transient 12 hr hypoxia when compared to normoxia in 2D static culture

which indicated a decrease in the utilization of arginine by eNOS for NO production, but this difference was lost in the 48 hr in persistent hypoxia cultures.

In the 3D models, no significant differences between normoxia and hypoxia in the ratios could be observed. Interestingly, unlike the 2D model, there is a significantly lower level of citrulline+9/ornithine+7 in both normoxia and hypoxia in microvessels exposed to persistent hypoxia, indicating that relative eNOS to arginase activity is lower and suggesting that the flow-mediated mechanical response may impact the hypoxic response of the HCAECs.

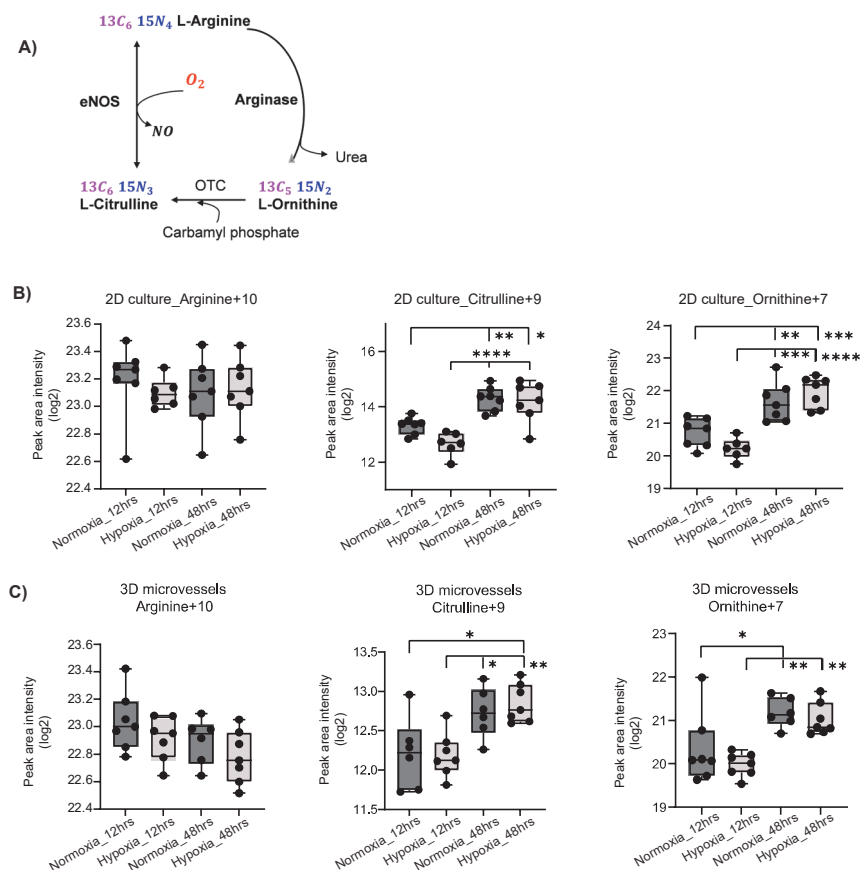


Figure 2. Measurement of isotope-labeled nitric oxide (NO) metabolites. (A) Metabolic pathway of $^{13}\text{C}_6$ $^{15}\text{N}_4$ L-arginine to $^{13}\text{C}_6$, $^{15}\text{N}_3$ L-Citrulline and $^{13}\text{C}_5$, $^{15}\text{N}_2$ L-Ornithine. Measurement of Arginine+10 ($^{13}\text{C}_6$ $^{15}\text{N}_4$ L-arginine), Citrulline+9 ($^{13}\text{C}_6$, $^{15}\text{N}_3$ L-Citrulline), and Ornithine+7 ($^{13}\text{C}_5$, $^{15}\text{N}_2$ L-Ornithine), (B) in 2D static culture, and (C) 3D microvessels-on-chip model under transient and persistent hypoxic conditions. Significance is determined by the one-way ANOVA multiple comparison test. ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Generation of signaling lipids in transient and persistent hypoxic conditions in 2D static and 3D microvessels-on-chip model

Next, we compared the impact of hypoxia on the generation of a selected set of signaling lipids by the HCAECs between our static 2D and perfused 3D model. Conditioned media samples were collected for UPLCMS/MS analysis.

In particular, we assessed prostaglandins, lysosphingolipids, eicosanoids, and oxylipins. The control samples are the cells exposed to normoxia (21% oxygen) for 12 hours and 48 hours. We conducted statistical analysis on metabolites that passed the QC threshold during the assessment of the bioactive lipid composition. As shown in **Figure 3**, transient hypoxia exposure to HCAECs in a static 2D culture displayed increased levels of oxylipins from CYP450 pathway such as trihydroxyoctadecenoic acid (TriHOME) & hydroperoxyeicosatetranoic acid (HpETE)., Also increased oxylipins from the LOX pathway (hydroxydocosahexanoic acid (HDoHE) & hydroxy eicosapentanoic acid (HEPE) and from the COX-2 metabolite Prostaglandin K2 (PGK2) (**Fig. 3A**).

In the perfused 3D vasculature model (prostaglandin F2a) PGF2a and lysophosphatidylethanolamine (LPE) were downregulated (**Fig. 3B**). The upregulation of oxylipins indicates that the hypoxic exposure increased the generation of reactive oxygen species by the HCAECs and the upregulation of all these enzymes in hypoxic conditions were shown in earlier studies [27,45,46].

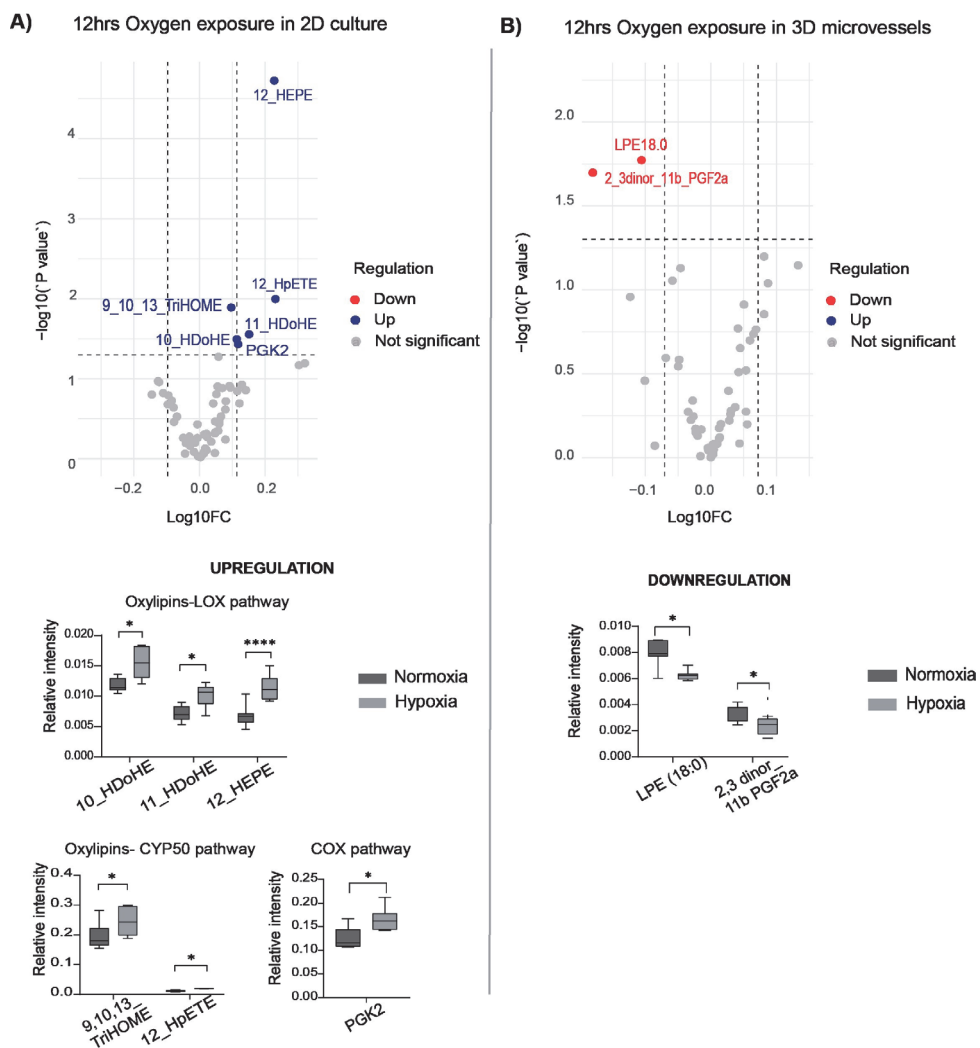


Figure.3. Measurement of signaling lipids in (A) 2D static culture and (B) 3D microvessels after transient oxygen exposure (12hours). Significance determined by the students T- test. ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

48 hrs of persistent hypoxic exposure of the HCAECs in the static 2D cultures revealed the upregulation of Sphingosine1P (S1P) and lysophosphatidylglycerol (LPG) and the downregulation of LPE and lysophosphatidic acid (LPA) (**Fig. 4A**). The increased

generation of Sphingosine1P and fatty acids was also seen in the perfused 3D model. Persistent 48 hr hypoxia decreased the levels of cyclic lysophosphatidic acid (cLPA), lipopolysaccharide (LPS), prostaglandin D2 (PGD2) and PGK2 in the conditioned media in this model (**Fig. 4B**).

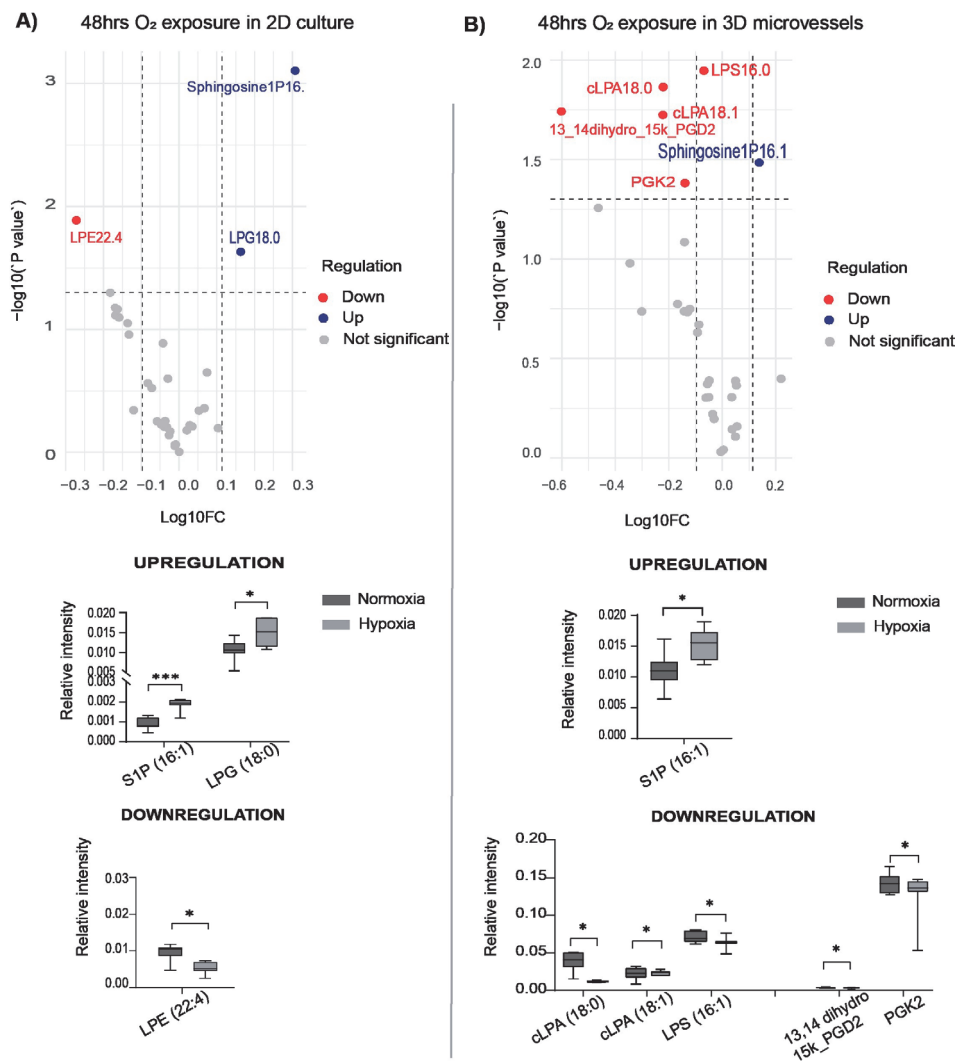


Figure.4. Measurement of signaling lipids in (A) 2D static culture and (B) 3D microvessels after persistent oxygen exposure (48hours). Significance is determined by the students T-test. ns = not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Discussion

Perfused, in vitro 3D microfluidic vascular models are increasingly used to study human microvasculature. Compared to the 2D monolayer cultures, these microfluidic models are thought to provide more physiological responses due to their configuration and exposure to various degrees of shear stress. Here, we set out to compare both models in their response to hypoxia, focusing on two main homeostatic signaling pathways. Both models responded to 48 hrs of hypoxia as evidenced by the accumulation of HIF-1 α in the nuclei of the HCAECs, consistent with an earlier study demonstration a time- and ROS dependent stabilization of HIF-1 α in response to an environment of 1% oxygen. However, in the perfused 3D microvessels only 50% of the nuclei stained positive for HIF-1 α . A likely explanation could be that the increased expression of the shear stress induced kruppel-like factor 2 in the HCAECs in the 3D model could inhibit stabilization of HIF-1 α [47].

While most previous studies reported hypoxia-mediated downregulation of eNOS activity [17,34–37], opposite results were also reported, and the differences may well be explained by the different models used [48]. We recently developed a tracer-based methodology that is sufficiently sensitive and reproducible to assess eNOS activity in our small-scale microfluidic 3D microvascular model [39]. Clearly, we observed no differences between normoxia and hypoxia both in the static 2D model and the 3D perfused model. However, when assessing the ratios of L-citrulline over L-ornithine in the 3D model, unlike in the 2D model we observed a lowering of the relative eNOS activity over that of arginase after 48 hrs of hypoxia (supplementary figure 2). A potential explanation would be a hypoxia-induced increase of arginase II activity as has been reported in a study with human lung endothelial cells [49]. However, in the absence of an elevation of the absolute level of L-

ornithine (Figure 2B), reduced eNOS activity due to the 48 hs of hypoxia would be the most likely explanation for this change in the L-citrulline/L-ornithine ratio.

Numerous signaling lipids can facilitate the endothelial response to changing environmental cues or inflammation [49–53]. In a previous study we optimized the methodology for the profiling of bioactive lipids in our 3D micro vessel model. We demonstrated that, in response to the pro-inflammatory cytokine TNF- α , static 2D cultures release a more inflammatory repertoire of bioactive lipids compared to perfused 3D-microvascular models [54]. Also, the activating, anti-inflammatory impact of shear stress on membrane phospholipid metabolism [55], and the vasodilatory compound prostacyclin (PGI₂) were reported previously [7,56]. Consistent with these studies, in the static 2D culture model we observed a short-term hypoxia related increase in the levels of oxidative stress markers such as HDoHE, HEPE, TriHome, and HpETE potentially resulting from the peroxidation of their substrates [57]. Given the fact that we observed that the translocation of HIF-1 α is not established after the 12 hrs of hypoxia in this model, the increased release of these markers of oxidative stress is most likely unrelated to the action of HIF-1 α .

The modulation of endothelial functions in hypoxic conditions is influenced by arachidonic acid-derived prostaglandin [58]. Under shear stress conditions, a significant downregulation of prostaglandins (PGF2 α) and (PGD2) is observed, partially associated with hypoxia, although not statistically significant in 2D culture hypoxic conditions. The reduced levels of PGF2 α are linked to anti-inflammatory properties, and contrasting its higher levels reported in inflammation and atherosclerosis [59–61]. Research indicates that higher shear stress increases PGD2 synthesis, while lower shear stress (below 5 dyne/cm²) shows no effects [62]. Our observations suggest that the lower levels of PGF2 α may be associated with shear stress, as HIF-1 α was not observed after 12 hours of hypoxia. . Similarly, the lower level of PGD2 in persistent hypoxic conditions could be attributed to a combination of shear stress and hypoxia, consistent with the 50% HIF-1 α translocation. However, the combined impact of hypoxia and shear stress on prostaglandin synthesis remains unclear.

The significance of lysophospholipids as a crucial lipid mediator in inflammatory conditions has been acknowledged [63]. Earlier investigations involved studying the impact of LPAs on endothelial dysfunction in HCAECs through dosage treatment. This resulted in a decrease in eNOS activation alongside elevated levels of superoxide anion and ROS [64]. In contrast, a few other studies have highlighted the association between LPAs and their modulation of endothelial cell functions, including cell proliferation, migration, barrier function, and capillary-like tube formation [52,65–68]. Our study, conducted in a 3D model under prolonged hypoxia, revealed diminished levels of LPAs (cLPA 18:0, 18:1), possibly influenced by shear stress-mediated regulation. The reduced levels in 3D microvessels align with the observation that laminar flow decreases toll-like receptor 2 (TLR2) expression, a gene crucial in endothelial inflammation triggered by LPS induction [69,70]. This suggests that, in our model, flow plays a role in downregulating inflammatory compounds.

S1P, a potent and versatile phospholipid, plays a crucial role in eliciting diverse vascular cell responses such as migration, proliferation, and survival. Additionally, it is implicated in pathological events related to endothelial dysfunction, including atherosclerosis, thrombosis, and inflammation [71–74]. The signaling mechanism of S1P under hypoxic conditions has been previously elucidated [75], where the accumulation of S1P in endothelial cells, facilitated by HIF-1 α and HIF-2 α , results in an increased level of sphingosine kinase (SK) protein [76]. In our study, exposure of microvessels to short-term hypoxia showed no significant changes in S1P, while prolonged exposure led to a significant upregulation of S1P levels. This suggests a regulatory mechanism through HIF α that induces S1P levels. Notably, the impact of hypoxia on S1P levels influences the vascular model, as very brief hypoxia (30 minutes) increases S1P protein expression in coronary artery endothelial cells and induces vasodilation [77]. Given that S1P upregulation was observed in both 2D and 3D culture platforms, it strongly indicates a HIF-1 α -mediated regulatory mechanism.

Conclusion

Examining hypoxia in endothelial cells is vital for understanding the adaptive mechanisms and its substantial involvement in various physiological and pathological processes. Importantly, oxygen-dependent pathways such as NO and signaling lipid pathways play a crucial role in vascular homeostasis and resolving inflammation in specific contexts. Our research highlights the significant impact of low oxygen levels on the metabolism of nitric oxide and signaling lipids, particularly in a 3D microvessels model. The exploration of the combined effects of hypoxia and shear stress in an in vitro culture setup is an underexplored area. Our study suggests that our model may offer relevance and appropriateness for analyzing metabolite levels under both normoxic and hypoxic conditions.

Collectively, our findings indicate that exposure to hypoxia in a 2D static culture environment leads to an increase in oxidative stress metabolites, a response mediated by the HIF-1 α mechanism. Conversely, the hypoxic 3D platform demonstrates flow-mediated regulation of eNOS-based NO metabolites (L-citrulline+ and L-ornithine+7) and bioactive lipids. These changes contribute to anti-inflammatory effects. Immunostaining analysis of HIF-1 α was consistent with our findings. Our study unveils a novel mechanism governing HIF-1 α and flow-mediated regulatory metabolisms, proposing new therapeutic strategies for addressing arterial endothelial dysfunctions in prolonged hypoxic conditions.

Acknowledgements

We would like to express our sincere gratitude to the BMFL technicians at LACDR, Leiden University, for their invaluable assistance in measuring samples using the signaling lipids platform. Additionally, we extend our thanks to Master students Parjanya Kocherla and Adel Saaidy for their contributions to the development of initial

experimental protocols. We would like to express our sincere gratitude to Sylvia Le Dévédec for providing us with the HIF-1 α antibody for immunostaining.

Author contributions

Kanchana Pandian, Conceptualization, Investigation, Methodology, Writing - original draft;

Anton Jan van Zonneveld, Amy Harms, and Thomas Hankemeier, Conceptualization, Resources, Supervision, Funding acquisition, Writing – review and editing.

Declaration of competing interest

T. Hankemeier is a shareholder in Mimetas B.V. All other authors declare no competing interests.

Funding

Part of these studies we supported by the Dutch Heart Foundation (CVON RECONNECT) and ZonMW (MKMD: 114022501) grants to T.H and A.J.V.Z and part was supported by METABODELTA (Medical Delta Program). This project has received funding from the European Union's Horizon 2020 research and innovation program (LogicLab network) under the Marie Skłodowska-Curie grant agreement No 813920, and RECONNEXT funded by the Dutch Heart Foundation No 2020B008.

Data availability statement

The data that support the findings of this study are available in the Materials and Methods, Results, and Supplementary Material of this article. Additional data will be provided upon request.

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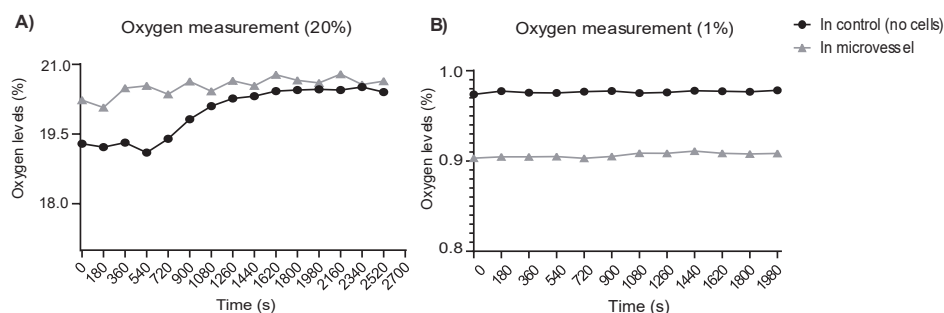
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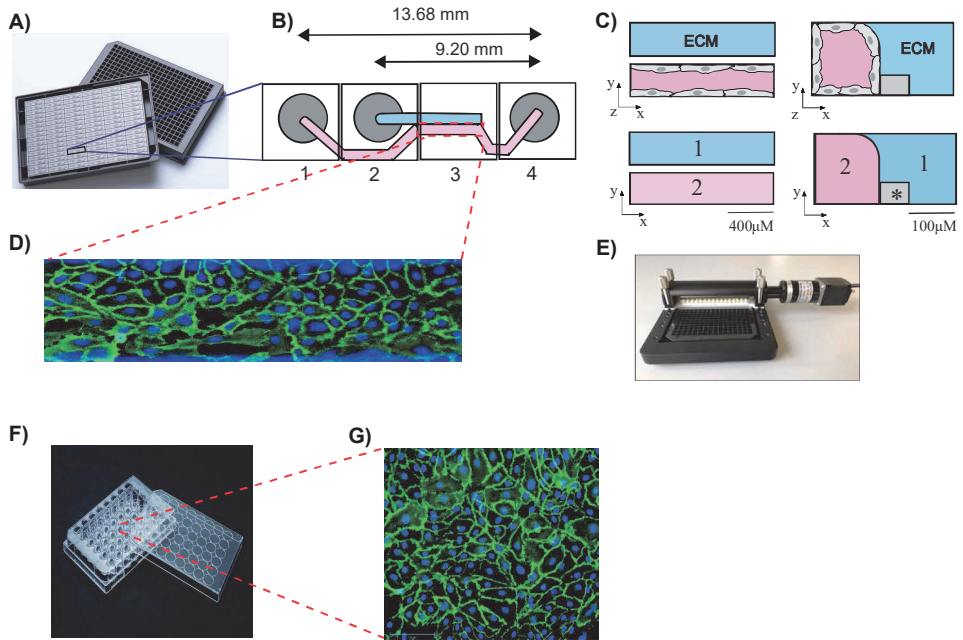
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Supplementary information

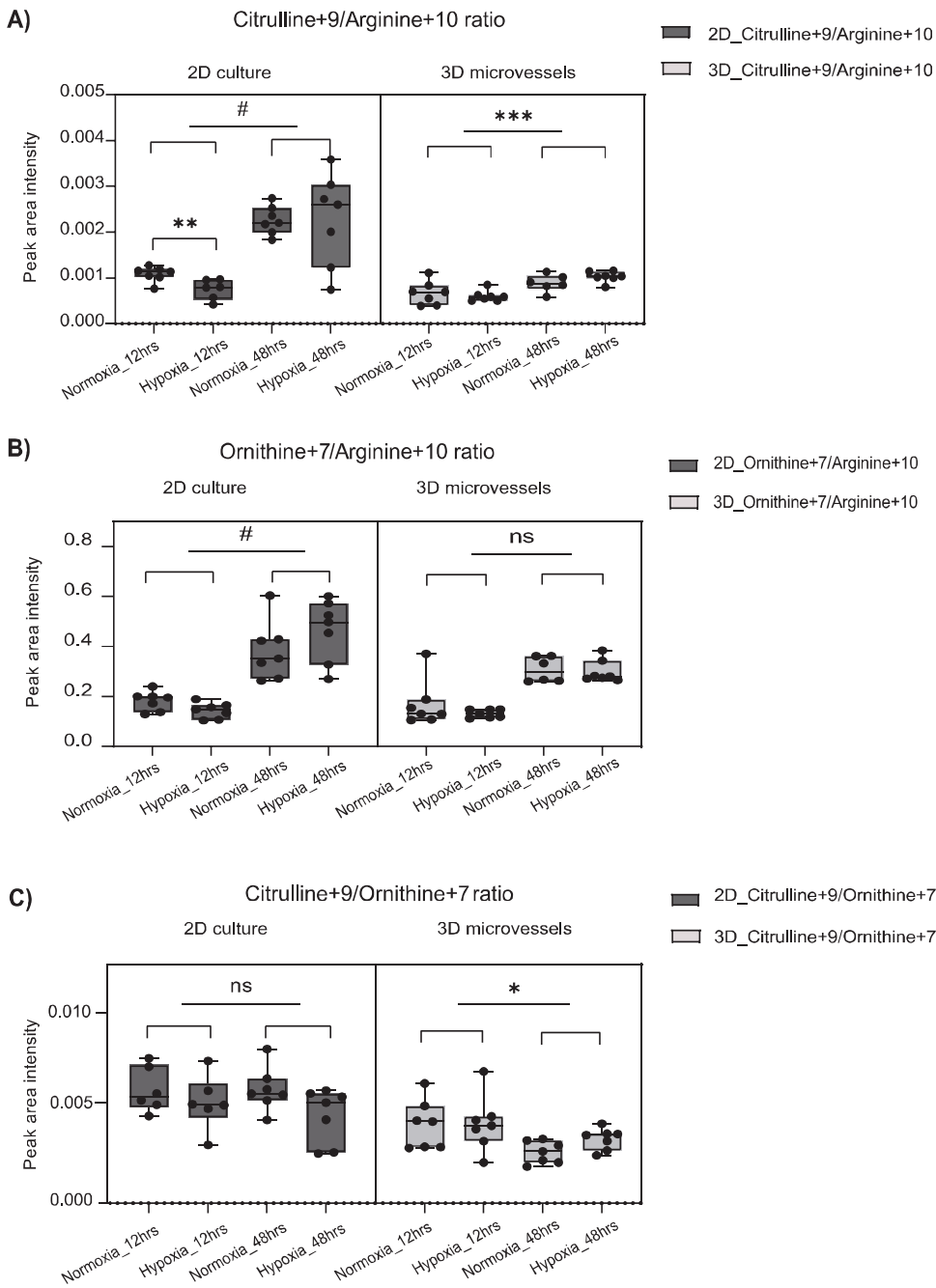


Supplementary Figure 1. Measurement of real time oxygen in a 3D micro vessel-on-a-chip model. (A) Percentage of oxygen in normoxic condition (21% oxygen) and (B) Percentage of oxygen in hypoxia (1% oxygen). Control – Chip with no cells seeded, Microvessels – HCAECs seeded. Measurement was performed in LABVIEW software.



Supplementary Figure 2. Representation of our 3D micro vessel-on-chip model and the 2D model.

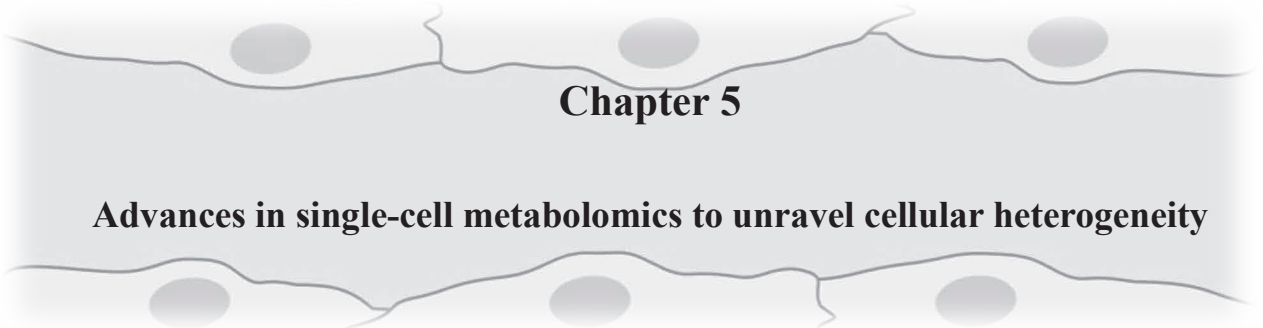
Illustration of re-routed OrganoPlate and its (B) Single chip design was explained below. Every microfluidic chip structure is positioned underneath 4 adjacent wells, and it consists of two channels: 1) an ‘perfusion’ channel (pink color) and 2) a ‘gel’ channel (ECM) (blue color). Every first well (1) and fourth well (4) is positioned on top of the inlet and outlet of the perfusion channel, while every second well (2) is for the gel channel. And every third well (3) is used for imaging and observation of the experiment. (C) Perfusion and gel channel was shown vertical and horizontal view and it's separated by a phase guide (*). (D) Immunostaining of HCAECs in a chip. After 48 hours of cell seeding, a confluent vessel of HCAECs were immunostained with V-Cadherin and DAPI. (E) Image of a microfluidic pump with the two metal tubes attached to the inlet and outlet of rerouted OrganoPlate to carry fluid and create unidirectional flow. (F) Representation of 2D steady state culture model and (G) Immunostaining of HCAECs in a 2D platform. Green – F-actin; Blue- Hoechst. Scale bar – 50 μm .



Supplementary Figure 3. Measurement of isotope-labeled nitric oxide (NO) metabolites ratio Citrulline+9/Arginine+10, Ornithine+7/Arginine+10, and Citrulline+9/Ornithine+7 in 2D static culture and in 3D microvessels-on-chip model under transient and persistent hypoxic conditions. Significance is determined by the one-way ANOVA multiple comparison test. ns = not significant; *, P<0.05; **, P<0.01; ***, P <0.001; ****, P<0.0001; #, P<0.0001.

Supplementary Table 1. MRM method and the compound list with target m/z for parent and product ions for tracer metabolites measurement.

Metabolites name	Mass (Q1)	Mass (Q3)
L-arginine	345.170	171.000
L-arginine [M+10]	355.170	171.000
L-citrulline	346.150	171.000
L-citrulline [M+9]	355.150	171.000
L-ornithine	237.100	171.000
L-ornithine [M+7]	240.600	171.000



Chapter 5

Advances in single-cell metabolomics to unravel cellular heterogeneity

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Based on: Advances in single-cell metabolomics to unravel cellular heterogeneity in
plant biology

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Plant Physiology (2023)1 -17. Doi: 10.1093/plphys/kiad357

Abstract

Single-cell metabolomics is a powerful tool that can reveal cellular heterogeneity and can elucidate the mechanisms of biological phenomena in detail. It is a promising approach in studying humans or animals, especially when cellular heterogeneity has an impact on different biological processes and diseased conditions. In addition, metabolomics, which can be regarded as a detailed phenotypic analysis, is expected to answer previously unanswered questions which will lead to understand the disease mechanism, early disease diagnosis, personalized medicine, drug discovery and development, and cell-cell interactions. By providing a detailed view of metabolic heterogeneity, this technology has the potential to revolutionize various fields of biomedical research. In this review, we will introduce the flow of sample acquisition, and single-cell analytical techniques to facilitate the adoption of single-cell metabolomics.

Key words

Single cell metabolomics, Mass spectrometry, microfluidic techniques, live single cell sampling, single cell imaging, high-throughput analysis.

Introduction

Each cell is unique in its behavior and reacts distinctively to its environment. This heterogeneity impacts biological and pathological processes yet to be fully explored. To address this, a myriad of single-cell analytical techniques has emerged that aim to gain deeper insights into cell characteristics and their response to perturbations by analyzing their genome, transcriptome, proteome, and metabolome [1].

Single-cell metabolomics (SCM) has a wide range of applications across various research areas. Some of the key applications involved in different areas such as stem cell research, developmental biology, microbiology, pharmacology, and cancer biology. Such analysis allows a deeper understanding of cellular metabolism in humans and provides insights into metabolic heterogeneity and dynamic changes in response to various stimuli. This information will deliver metabolic differences between individual cells which aids a better understanding of the underlying mechanisms of biological and disease condition [2,3]. SCM is a technique that enables the measurement and analysis of metabolites at the level of individual cells, whereas bulk analysis measures the average metabolite levels across a population of cells. There are several important reasons why single-cell metabolomics is considered a more informative approach compared to bulk analysis. Recent studies have shown that even in populations that are relatively homogenous, differences in the metabolism on the single cell level due to heterogeneity or changes in microenvironment have several biological implications [4]. Furthermore, SCM enables three-dimensional metabolite mapping within single cells, offering unprecedented insights into intracellular molecular processes. By visualizing metabolite distribution, researchers can elucidate complex cellular behaviors, such as cell division, and their implications in cancer development and progression [5]. Metabolite production and accumulation can vary significantly across different cell types, potentially rendering traditional bulk metabolomics data misleading. Furthermore, the dynamic nature of cellular metabolism, influenced by microenvironmental changes or external stimuli, necessitates the use of single-cell metabolomics. Unlike bulk analysis, which provides a static overview, single-cell analysis captures the rapid fluctuations in metabolite levels, offering a more accurate representation of cellular function [6–9].

Studying the metabolome, i.e., metabolomics, has become the field of choice for the comprehensive understanding of cell cycles, cell perturbations, and general cell functions due to its ability to translate current cell behavior into biochemical terms. Not only that, it is also a useful tool for the mystery of the synthesis of several organ-specific components that are produced in specific cells.

While single-cell metabolomics offers immense potential, it presents unique challenges due to the extremely small sample size (Pico-liter volume). Unlike genomics and transcriptomics, which amplify signals, metabolomics grapples with minute metabolite quantities. Single-cell metabolomics offers a dynamic snapshot of an organism's physiological state. While transcriptomics and proteomics provide blueprints (gene and protein expression), metabolomics reveals the functional consequences, demonstrating how these blueprints are executed in terms of cellular metabolism and physiology. This allows us to understand the downstream effects of genetic and protein-level changes. Furthermore, metabolomics can pinpoint metabolites directly impacted by interventions, such as drug treatments, offering insights beyond transcriptomics and proteomics. Additionally, the latest research using tracer-based approaches with stable isotopes sheds light on active pathways within the metabolic network. By analyzing the flux of metabolites, researchers can determine the direction and extent of metabolic changes induced by the intervention [10,11]. This unique ability of metabolomics, to reveal which parts of the network are functionally utilized, provides valuable insights not obtainable through transcriptomics or proteomics alone.

Recent advancements in mass spectrometry, coupled with innovative miniaturized sampling and processing techniques, have revolutionized the field of metabolomics. This powerful combination now allows us to study the metabolome at the single-cell level, offering unprecedented insights into cellular heterogeneity. This review delves into the critical steps involved in SCM using mass spectrometry. We will meticulously examine the workflow, from cell isolation and sample preparation techniques to advanced measurement methods. Furthermore, we will highlight the utility and applications of SCM with a specific focus on cutting-edge technologies in single-cell sampling and mass spectrometry detection.

Single-cell sampling

Sampling is a crucial step for single-cell metabolomics experiment compared to other single-cell omics techniques. Challenges in single-cell sampling are often centered around the small cell size and volume available for analysis. This is exacerbated further in the case of metabolomics where the inability to amplify the metabolite signal, unlike DNA or RNA, and the sensitivity of the metabolome to changes in the microenvironment pose significant hurdles in single-cell sampling. These challenges and the method of sampling used impact downstream metabolic analysis and the resulting biological interpretation of said analysis. Therefore, careful consideration is needed when choosing the correct sampling method. Several techniques have been developed or adapted to sample and isolate single cells with these challenges in mind. They can be broadly classified into capillary sampling techniques, and microfluidics-based methods. The following sections will delve into a critical review of these techniques, discussing their specific uses, advantages, and limitations.

Capillary sampling methods

Microcapillary sampling methods, where a single cell is sampled using a fabricated micro glass capillary with a fine tip of inner diameter based on the cell size are well established. These methods require a microscope and a manipulator to localize and aspirate a single cell. The prominent advantage of this method is that sampling will be achieved in the cell's native environment. However, automation is a major problem resulting in low throughput sampling. Additional strategies have been applied to sample single cells for metabolic analysis as detailed below.

Single-cell sampling and analysis (SiCSA) were performed by inserting a fine oil-filled glass microcapillary into the cell. The capillary was mounted on to a micromanipulator and operations were viewed under a microscope. The cell sample was expelled from the capillary by increasing the pressure in the oil using a full cell-pressure probe setup. This sampling setup was coupled with X-ray microanalysis to determine the significant inorganic cellular constituents in individual cells [12].

Another live cell-sampling approach for mass spectrometry using a video-microscopic method has been developed. In this method, the cell, or its contents, are sucked out by a glass capillary and directly sprayed into the mass spectrometer using electrospray ionization. However, this method lacks sensitivity and, additionally, single-cell molecular detection was not achieved [13]. After further development, a live single-cell mass spectrometry (LSC-MS) technique for untargeted metabolic analysis was extensively explained by Fujii *et al.* in which the sampled plant cell was sprayed directly on to the mass spectrometer. The initial procedure involved sampling a single cell with a 3D micromanipulator fixed to a microscope. The sample dish was placed under the microscope to choose single cells for sampling. Whole cells or the cell contents were drawn out using a nano spray tip or a microcapillary tip (with a diameter based on the cell size) using a 3D manipulator attached to a syringe that was used to apply positive and negative pressure to pull out the samples. Ionization solvent was injected using a pipette into the other end of the microcapillary. Finally, this nano spray tip was placed against the mass spectrometer inlet, and a high voltage was applied to introduce the contents into the mass spectrometer. As a result, many metabolites' signals were obtained for further analysis highlighting its sensitivity for metabolomics applications [14]. Using the same sampling technique, the microbial cells were transferred into a culture media, amplified its DNA and analyzed the genes. Not only for single cell genetic analysis, but the sensitivity was also proven to be good in another study which involves sampling a single plant cell for direct metabolomics analysis. The versatility of this sampling technique extends beyond single-cell studies. One study demonstrated its effectiveness in transferring cells or cellular contents to culture media for subsequent DNA amplification and gene analysis [15–17].

The major advantage of these capillary techniques on single-cell sampling is that sampling occurs with minimal disruption to the cells' microenvironment, and therefore reduces internal biochemical changes in the cell during sampling. However, there are major drawbacks, including a longer sampling time and getting the correct microcapillary diameter. The latter depends on the cell type and cannot be more than six microns or the spray to the mass spectrometer will be ineffective when the voltage is applied. In addition,

there is a chance that the cells will stick to the capillary edge if the capillary is not positioned correctly and there may be sample evaporation within the capillary if there is a delay in measurement. A further major complication with this technique is that it is not guaranteed to retrieve the sampled cells from the capillary for culture. To circumvent these low throughput and cell integrity issues, microfluidics-based sampling procedures have been developed as described in the following methods.

Microfluidics-based single cell sampling/sorting

Microfluidics-based approaches are uniquely suited to single-cell metabolomics due to their ability to handle small volume samples, have operations with limited dilution and high throughput. This review will explore various microfluidics techniques used for this purpose, categorized as either label-free or labeled methods. Label-free methods offer the advantage of not requiring prior modifications of the cells. These techniques include:

- Droplet-based microfluidics: This approach utilizes microfluidic chips to create droplets containing single cells, enabling precise isolation and analysis.
- Cell sorting techniques: These methods leverage various physical properties to separate single cells. Some examples include:
 - Acoustofluidics: Sound waves are used to manipulate and sort cells based on their size and compressibility.
 - Cell size-based sorting: Cells are physically separated based on their size through microfluidic channels or filtration techniques.
 - Cell deformability-based sorting: This method sorts of cells based on their stiffness or elasticity, which can be indicative of their physiological state.

In contrast, labeled methods rely on attaching fluorescent tags to specific cell types for identification and sorting. The most common approach is:

- Fluorescence sorting: Cells are stained with fluorescent antibodies that bind to specific surface markers. Laser light then excites the fluorescent tags, allowing for identification and sorting of the desired cell population.

This review will delve deeper into the specific applications and limitations of each technique within the context of single-cell metabolomics.

Droplets-based cell sorting

Droplet microfluidics is a promising technique that is suitable for single-cell sampling and sorting from a bulk cell population [18–21](**Fig.1**). An advantage of it is the rapid mixing inside the droplets, which aids rapid reactions if cells are treated with a reagent [22]. Single-cell sorting using droplet microfluidics has been performed mostly in eukaryotic [23,24] and bacterial cells [25,26].

Successful droplet generation in microfluidic systems hinges on careful optimization of several key parameters. The choice of chip material plays a crucial role, as different materials possess varying surface properties that can influence droplet formation and stability. Additionally, the size of the orifices within the microfluidic chip significantly impacts droplet size and uniformity. Furthermore, the fluid properties of both the dispersed and continuous phases must be considered. Factors like viscosity and density influence how these fluids interact and ultimately affect droplet formation. Finally, the capillary number, a dimensionless parameter that represents the ratio of viscous forces to interfacial forces, needs to be taken into account. By carefully balancing these parameters, researchers can achieve precise control over droplet size, uniformity, and generation frequency within their microfluidic experiments.

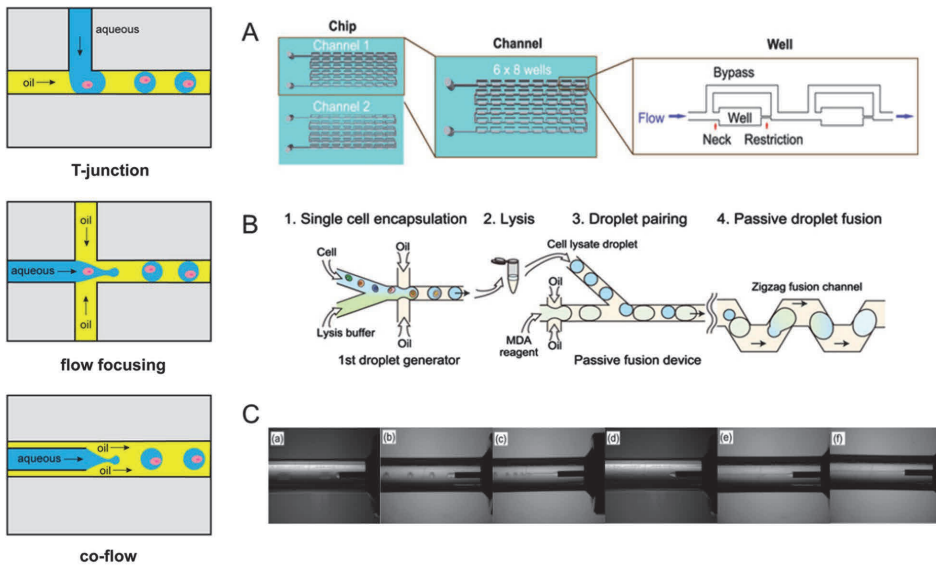


Figure 1. Common methods and applications for droplet generation, including T-junctions, flow focusing, and co-flow [27]. A) Droplets generated using T-junctions. The channel facilitated fluid flow in one direction, and droplets were formed in the well because of restricted flow. B) Droplets generated using flow focusing. In the droplet generator, single cells were mixed with lysis buffer and encapsulated into aqueous droplets in an oil-based emulsion. C) Droplets generated using co-flow. Cell-enclosing droplets can be obtained from a high viscous aqueous solution under ambient co-flowing liquid.

The generated droplets can be sorted based on their fluorescence and cell specificity using microfluidic-based fluorescence-activated droplet sorting (FADS). Benefits of this technique include minimal reagent volumes (12 μ L of aqueous phase generates 10^6 droplets), short setup time at below 10 minutes per sample, and high enrichment. Furthermore, additional development involving the integration of the sorting device with modules for droplet splitting will generate high-speed manipulations [28,29]. Droplet sorting can not only be achieved based on fluorescent properties, but also on droplet size [30–32], deformation [33], density [34] and directly using non-inertial lift-induced forces [35], sheath fluid properties with acoustics [36], and mechanical properties [37].

Fluorescent tags may change the metabolome and hence sorting by a cell's physiological and mechanical properties is highly efficient, especially for further downstream analysis [38]. As the technology has advanced further, droplets have been sorted using an automated system that performs real-time dual-camera imaging. The objective of this system is to replace manual cell handling techniques with automation via machine learning algorithms. Major advantages of droplet-based single cell sorting are sample handling with minimal dilution, low carry over, label-free sorting, and high throughput. Major disadvantages are that the device fabrication is complicated depending on the different sample treatment steps and the droplet varies with cell size. However, electrospray ionization produces charged ions directly from the charged liquid, so it is ideal for coupling with the microfluidic platform.

Acoustics-based cell sorting

To achieve an oil-free sorting mechanism, acoustic-based sorting of cells and detection in mass spectrometry is an ideal procedure. Acoustic-based single-cell sampling is an interesting pipeline for metabolic analysis due to its well-known advantages. Acoustic or sound energy is nothing but kinetic mechanical energy, a result of when sound or pressure makes a substance or cells vibrate. Through these vibrations, particles tend to move according to their mechanical and sound characteristics and at a different range of wavelengths. Acoustic energy distribution in microfluidics technology enables the use of minute volumes of fluid for analysis and hence it is ideally suitable for single cell sorting from bulk cell numbers [39]. This method also highlights that sorting can be achieved label free without destroying the cells which can then be grown on in culture after the isolation [36,40]. An advanced acoustofluidics development is acoustofluidic fluorescence-activated cell sorting (FACS) that has high-throughput, high-resolution cell detection and sorting in a single chip. This device has sorted labeled cervical cancer cells and polystyrene beads from non-labeled ones [41]. Major advantages of acoustic-based cell sorting are high throughput, ease of incorporation into microfluidics, high purity, biocompatibility, maintaining high integrity of the cells and label-free sorting. Acoustic

cell sorting offers a promising label-free approach, but it faces some limitations. Fabrication procedures can be complex, often requiring the incorporation of transducers (devices that convert electrical energy into sound waves) within the microfluidic chip. Additionally, these systems can be sensitive to the properties of the fluid being used, requiring multiple instruments for optimal operation and to dispense individual cells on a plate using automated system. This complexity can hinder the widespread adoption of acoustic cell sorting techniques. To overcome these, cells can be tagged with specific antibodies and sorted in a high-throughput manner. This fluorescence –activated cell sorting technique is highly effective and is discussed in the section below in combination with MS imaging techniques.

Single-cell mass spectrometry

Single-cell analysis is an evolving approach to analyze more details of cellular genomics, proteomics, and metabolomics in a quantitative manner. The development of such new tools aids the understanding of the intercellular differences within large-cell populations. Especially, mass spectrometry is one of the powerful techniques can enable detection and quantification of many thousands of metabolites that helps in understanding the metabolic fate of each individual metabolites. Many methods have been developed such as electrospray/nano-electrospray ionization (ESI/nano-ESI), laser ablation electrospray ionization/laser desorption ionization (LAESI [42] /LDI) and secondary ionization mass spectrometry (SIMS), matrix assisted laser desorption/Ionization (MALDI) [43], single probe MS [44], and induced nESIMS [45]. These methods have been used in different metabolic studies including those of organic acids [46], lipids [47], and microbial metabolism [48]. Typically, single cell metabolomic techniques can be classified to micorsampling, microfluidics, and mass spectrometry imaging methods. In this section, the advances of a few methodologies will be critically reviewed in terms of their advantages and limitations which will dictate their compatibility with different single-cell experiments.

Single cell microcapillary sampling and mass spectrometry integration

Micro sampling techniques aim to combine microscopy data with mass spectrometry measurements with minimal disruption to the cell's microenvironment. This is often achieved by means of a pulled glass capillary or probe that is manipulated using a 3D micromanipulator attached to a microscope. The probe is then used to sample a whole cell, or in some cases, parts of the cell. Afterwards, the sample is then transferred to the MS instrument of choice for analysis.

Live single-cell mass spectrometry (LSC-MS) is a micro sampling technique that utilizes a metal coated glass capillary to sample cells and then introduces them to the mass spectrometry via nanospray ESI. The method is already described in the previous sampling section. By using the same capillary for sampling and measurements, sub-attomolar sensitivities can be achieved [14]. LSC-MS method is unique as it links information from microscopy with mass spectrometry data. This facilitates a cell specific analysis in a label free condition and unlocks more insights about the cell behavior.

To achieve more precision in sampling volume of single cells, electromigration can be used to sample and introduce cells to the mass spectrometer. In this method single yeast cells were sampled using a microcapillary and migrated to the capillary tip using controllable electro migration by a direct current (DC) current of 1.2kV, lysed with a brief time pulses and then driven to nESI-MS. However, this technique requires optimization of electro-migration and electroporation voltages for each cell type because the arrangement of the cell wall layers that comprise cellulose, microfibrils, hemicellulose, pectin, lignin, and soluble proteins varies between cells. Therefore, achieving electrical lysis or to migrate a cell to the capillary tip different potential is needed depending on the cell wall composition [49,50]. One key limitations of these methods are it cannot be used for on-line and dynamic monitoring of the metabolome of single cells since in both techniques, the sampling of the cells is done either offline (LSC-MS) or whole cells are sampled which prohibits repeated measurements.

Recent advancements have led to the development of high-throughput direct infusion probes capable of handling low-volume samples. These probes are positioned directly in

front of a mass spectrometer and utilize electrospray ionization at a flow rate of 1 $\mu\text{L}/\text{min}$. While this method offers advantages in terms of throughput, it is essential to note a key limitation: sample collection was performed at the bulk level rather than at the single-cell level. To achieve lower cell numbers per microliter, cell suspensions were subjected to serial dilutions, which could potentially introduce variability and bias into the analysis [51]. Low throughput limitation in LSC-MS was addressed by Yang's group and the development of the T-probe. The T-probe can continuously sample the cell of interest through a glass pipette with simultaneous injection of an extraction and ionization solvent which introduces the sample to the MS using nESI-MS via a T-piece connection [52,53]. This multiscale multifunctional device was employed for single-cell mass spectrometry (SCMS). This device integrated single-cell sampling, solvent mixing, and direct spray ionization into a single platform. The T-probe's sensitivity was evaluated by comparing it to a standard nano-electrospray ionization (nano-ESI) source, demonstrating comparable limits of detection for certain compounds. However, this method requires computational simulations for individual probes due to the variance in volume mixing i.e. sample and solvent volumes. A limitation that's also shared with other micro sampling methods is the lack of separation techniques such as capillary electrophoresis, ion mobility separations which can enhance the identification and maximum coverage of metabolites.

Overall, micro sampling techniques are among the most sensitive single cell metabolomic methods, especially when little to no dilution is applied to the sample prior to analysis. They also sample the cell with minimal perturbations to the microenvironment, and therefore the metabolome. However, they also share the common limitation of having low throughput, due to the often manual and labor-intensive sampling process and complicated to integrate to MS. Microfluidic-based mass spectrometry methods attempt to address this limitation through high-throughput and automatable single cell isolating and sorting followed by MS measurements.

Microfluidics-based sampling mass spectrometry integration

Microfluidic chips facilitate efficient single-cell isolation, sorting, lysis, and protein digestion. Coupled with LC-MS/MS, they enable comprehensive protein identification and quantification. Droplet-based microfluidic chips encapsulate individual cells within droplets, allowing for subsequent sorting and analysis. These droplets can be labeled and tracked using biochemical reactions and fluorescence microscopy. While single-cell metabolomics and lipidomics present unique challenges, single-cell proteomics [54,55] has witnessed significant progress, particularly in sampling and analysis techniques. To achieve high-throughput sampling, microfluidics platforms, such as droplet microfluidics, array of microwells, acoustofluidics, and microfluidic-based flow cytometry, can be coupled to mass spectrometry for high throughput detection of single cell metabolites [56,57].

Using segmented flow condition, cells from suspension were sampled using the fabricated narrow bore capillary. The sampled cells were sprayed to the self-manufactured MS source and gave a potential high throughput single cell metabolic analysis [58]. Combining droplet microfluidics and integrating to MS was achieved in the method, where droplets generated from egg white solution were segmented within the microfluidic chip and transported to a stainless-steel emitter. This emitter was integrated into a mass spectrometer equipped with various ion sources (DTIMS-Q-TOF, TWIMS-Q-TOF, Orbitrap). Droplet frequency and diameter were optically analyzed [59]. However, electrospray instabilities were identified with the increased salt concentrations at the emitter outlet in droplet mode but not in direct infusion. Similarly, the study analyzed phytase enzyme activity from individual yeast cells within nanoliter droplets. Cells were transferred to a glass slide with hydrophilic/hydrophobic patterns to prevent droplet spreading. The secreted phytase was characterized using MALDI-MS to monitor enzymatic reactions and distinguish inositol phosphates. To enhance this method, future work should focus on reducing droplet wetting to increase enzyme accumulation [60]. To standardize the generated droplet volume, Smith's group achieved droplet stabilization using surfactants and used it in the analysis of cocktail droplets that contain cytochrome C, alpha-chymotrypsinogen A, carbonic anhydrase, and chicken lysozyme. However,

cross-contamination was between residual proteins and the surfactant ions due to incomplete spraying of the sample before the arrival of the next droplet remains a challenge [61].

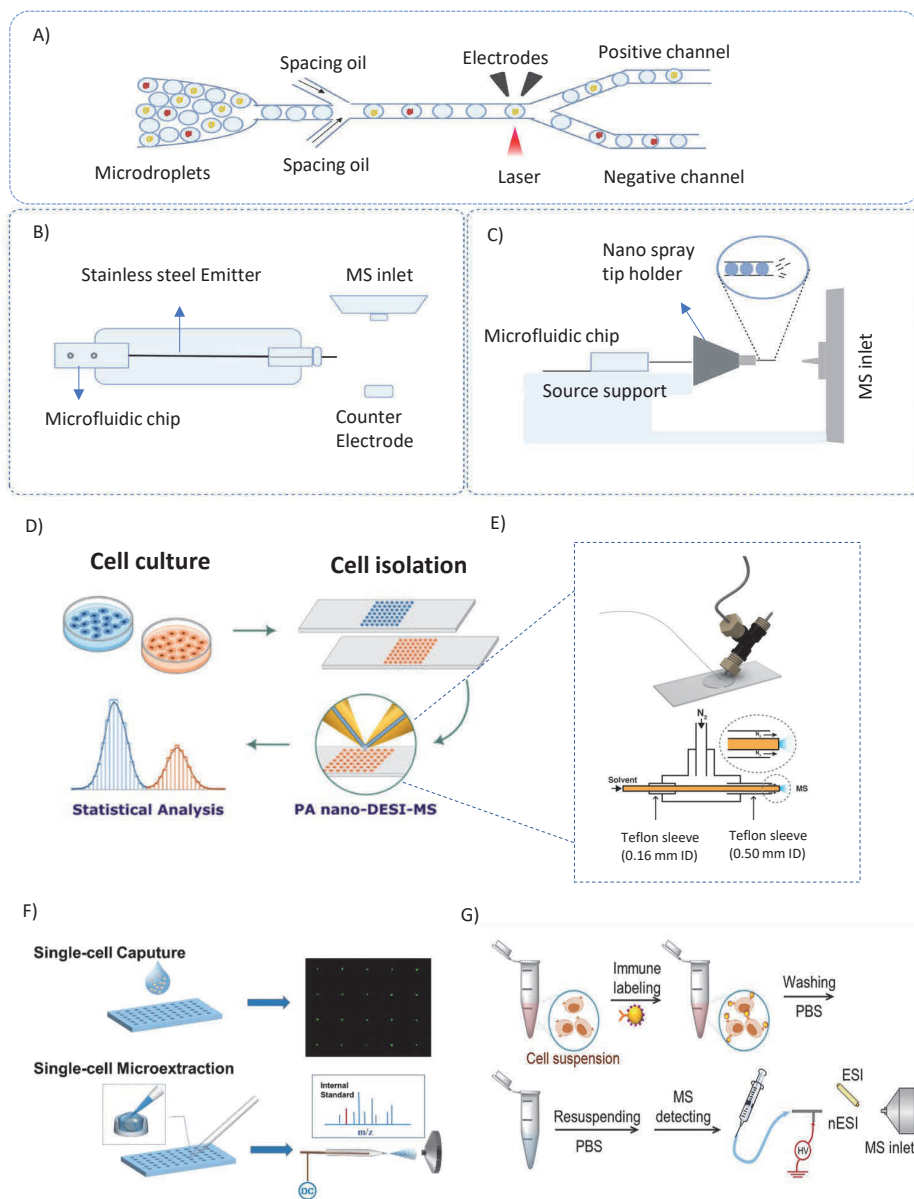


Figure 2. Single-cell isolation techniques for metabolomics study. A) Illustration of integrating a microcapillary single cell sampling method to droplet microfluidics for single cell (SC) metabolomics studies. Illustration of a platform for droplet microfluidic sorting of encapsulated protoplasts based on the genetic expression of a fluorescent protein. B) & C). Top and side view of the Nanospray ESI source to incorporate microfluidic chip. (Figures are adapted from Yu et.al., and Kempa et al., and created with BioRender.com, accessed on FX2509SD00, February 13th, 2023). D) Schematic of the single-cell sampling and integration of pneumatically assisted (PA) nano-DESI for metabolomics study [65]. E) Representation of the PA nano-DESI nebulizer and schematic of the secondary capillary detailing solvent and gas flow [66]. F) Workflow of droplet microextraction of single cell in microwells [67]. G) Schematic of a single-cell immunoassay: immune labeling of cell surface proteins using mass tag RMT443 and antibody-modified gold nanoparticles, cell washing and resuspending in PBS buffer, and MS detection by ESI-nESI MS [55].

The aforementioned technologies can be used for SCM in principle as it described the successful integration of microfluidics chip to MS ion source (**Fig. 2**). The proof of that, Zhang et al., analyzed single cancer cell metabolites by combining the droplet extraction and pico-ESI-MS [62,63]. Plant protoplasts encapsulated individually in aqueous microdroplets, based on the genetic expression of a fluorescent reporter protein (**Fig. 2A**) [64]. These sorted individual protoplasts could be directed to the MS ion source to analyze its metabolites in a high-throughput manner with an idea of Kempa et al., model (**Fig. 2B & 2C**) and other SC-MS techniques were shown in **Fig. 2D-G**.

The control on droplet size and stability can be easily managed due to the broader range of cell sizes. However, considering the general limitations of droplet microfluidics like droplet coalescence, break down, and chip surface wetting, furthermore developments should be achieved in designing a chip with controllable and consistent droplet generation, high throughput, and the possibility for automation. Furthermore, microfluidic techniques, and micro sampling techniques cannot provide spatial information such as the distribution of a certain metabolite in cellular space. One of the techniques that can provide such information on the single-cell level is mass spectrometry imaging, which will be discussed in the upcoming section.

Single-cell Mass Spectrometry Imaging

Mass spectrometry imaging methods were developed to provide biochemical information of biological models in 2D/3D. Furthermore, additional modalities such as dual microscopy/MS instruments can be incorporated to obtain both structural and chemical information of individual cells in 3D.

To achieve the highest sub cellular resolution, secondary ion mass spectrometry (SIMS) is often used. SIMS is the prevailing technique used to map the distribution of endogenous biomolecules with sub-cellular resolution. It allows two- and three- dimensional localization of analytes in a variety of cells. SIMS is a label-free, matrix-free technique that utilizes a focused primary ion beam to desorb and ionize analyte molecules [68]. The benefits of using SIMS are high spatial resolution and the ability to analyze molecules on the surface of biological cells like cell membrane components and it is an ideal technique for single-cell analysis due to its high-resolution sensitivity in 2D and 3D level and sub-micron resolution [69–71]. Although, with a low throughput and sample ionization under a high vacuum may cause some fragmentation of some cellular components and affect structural analysis and detection sensitivity. Such limitations have been partially addressed by methods such as cryogenic orbiSIMS. This approach is operated under – 100°C to lower the vapor pressures of semi volatile organic compounds. This was used successfully to analyze semi-volatile organic compounds and it limited vaporization prior to ionization [72].

Matrix-assisted laser desorption ionization mass spectrometer (MALDI-MS) is one of the main techniques used when higher throughput is needed in single-cell MS-imaging studies. MALDI is one of the main label-free techniques to visualize and study large molecules, e.g. drugs, lipids, and proteins, in single cells with minimal fragmentation and multiple-compound analysis in two-dimensional (2D) and 3D molecular distributions [73,74]. It allows the samples to be mixed uniformly in a large quantity of matrix. The matrix absorbs ultraviolet light (nitrogen laser light – 337nm) and converts it to heat energy. Cellular heterogeneity was explored using MALDI-MS by combining imaging, MALDI-compatible staining, and optical microscopy. This approach enabled analysis of

individual unperturbed cells. By correlating mass spectral data with microscopic images, we identified subpopulations based on molecular profiles. This method allowed for the investigation of heterogeneous lipid expression within the cell culture [75]. A similar study, SpaceM, combined microscopy and MALDI-MS to analyze diverse metabolites in individual cells. This open-source method detected over 100 metabolites from more than 1,000 cells per hour, preserving spatial information and enabling fluorescent readouts. SpaceM demonstrated potential for broad applications in various cellular models, advancing single-cell metabolomics accessibility [76]. In MALDI-MS, higher spatial resolution was achieved by modifying the laser spot shape are often necessary. Takahashi et al, succeeded in mapping the 2D distribution of small metabolites in plant cells with high spatial resolution. This was achieved by the modifications of laser spot's shape and position and the combination of the shorter pulse width laser. With this new design, non-targeted analysis of small metabolites was achieved in high resolution and high mass accuracy in 2D [77]. Another instrument modified technique was identified by Korte *et al.* in which high mass resolution (five-micron level) was achieved by modifying beam-delivery optics of a commercial MALDI-laser ion trap-Orbitrap instrument, incorporating an external Nd:YAG laser, beam-shaping optics, and an aspheric focusing lens to reduce minimum laser spot size [78].

Despite their utility, typical MSI methods often lack separation steps or structure elucidation mechanisms due to their inherent design limitations. Integrating and combining other MS-based platforms can alleviate this issue. An example of this is an integrated experiment for comprehensive analysis of gangliosides in transgenic mice brain, where MALDI-MSI and ESI MS were employed to elucidate the lipid profiles and the data was correlated for qualitative, semi-quantitative and *in situ* analysis [79]. A major drawback of MALDI-based methods is that the matrix used often interferes with measurements of low molecular weight ions. This drastically reduces metabolic coverage since most metabolites have low molecular weight. One such effort to address this was led by Brown et al., where new matrix enhanced surfaces were developed that were specifically optimized for low molecular weight compounds [80].

LAESI MS has been shown that lipids and small metabolites can be imaged in various samples such as plant material [81], tissue sections [82] or bacterial colonies without any sample pretreatment. Further, LAESI has been shown to produce multiplied charged protein ions from liquids or solid surfaces. And a study focused on analyzing intact tissue proteins with a LAESI ion source combined with a hybrid ion-trap FT-ICR mass spectrometer [83,84]. While LAESI-MS excels in providing spatial information for studying cellular heterogeneity, it faces several limitations. These include suboptimal spatial resolution, potential ion suppression effects in complex biological matrices, suitable for higher molecular weight samples, specific sample preparation requirements, and challenges with analyzing samples with low water content. Desorption ionization electrospray mass spectrometry (DESI) is another technique that can be analyzed in low molecular weight compounds with the added benefit of on-line coupling with HPLC for metabolite profiling. DESI-MSI produces less fragmentation and is ideal for imaging low-quality regions with non-destructive and high throughput capacity [85,86]. A further developmental approach of nano-DESI [87] was pneumatically assisted nano-DESI MS where the device was constructed to assist solvent flow through the secondary capillary in nano-DESI. This prime setup enhanced S/N for metabolite species by upto 1000 times, and more detailed ion images [66]. In addition, the sensitivity of nano-DESI analysis could be enhanced by combining nano-DESI with ion mobility spectrometry that can provide additional separation based on ion size, shape, enhancing sensitivity and selectivity.

Single-cell mass spectrometry imaging methods are mainly used to visualize the spatial distribution of biomolecules in 3D space with varying resolution. However, it also introduces the most perturbations to the cells' microenvironment due to the often-extensive sample preparation involved prior to analysis. In addition, one of the major technical hurdles reported in single-cell metabolomics is the broad dynamic range of concentrations that can have a considerable impact on the observable metabolome, something that MSI methods are poorly equipped to handle, due to the lack of a separation or enrichment steps. Despite these challenges, MSI, and the other techniques discussed before are already being used to uncover previously unknown insights about cell behavior

on the single-cell level. The summary of all available methods on single cell sampling and their detection techniques are compiled below in **Table.1**.

Topics	Method	Advantages	Limitations	Ref.
<i>Single cell sample acquisition</i>	Live single-cell mass spectrometry using capillary micro sampling	Minimal disruptions to the cell's microenvironment	Lack of high throughput No possibility of sample preparation	[12], [13], [14], [15–17]
	Droplet microfluidics	High throughput Automation Controlled volume Free of contamination	Relatively low sensitivity and robustness Complicated device fabrication techniques Large disruption to the cell content due to extensive manipulation	[18–21] [22] [23,24] [25,26] [27], [28,29] [30–32] [39] [36,40]
	Acoustic-based sorting And mechanical sorting	Label-free isolation, High throughput	Complicated device fabrication No sample preparation Sensitive to fluid properties	[41]
<i>Mass spectrometry for single cell analysis</i>	Ion-mobility triple/MS, nanospray ESI, and pico-ESI MS	Increased selectivity	Complicated data analysis Requirement of additional detection method.	[12], [13], [14], [15–17], [45], [54,55] [62,63]
	Single probe/ T- probe MS	Real-time analysis High spatial resolution, Applied to any biological sample Rapid analysis	Ion suppression effects Limited sensitivity	44] [51] [52,53]
	DTIMS Q-TOF, TWIMS Q-TOF and orbitrap	High quality spectra obtained from cell encapsulated droplets	Cross-contamination with some residual proteins to the surfactant ions due to incomplete spraying.	[58], [59]
<i>Mass spectrometry imaging</i>	LAESI-MS with ion mobility separation	Less disruptions to cells microenvironment Higher selectivity than direct infusion-based methods	Non-sensitive method for protein analysis.	81], [82], [83,84]
	SIMS	High spatial resolution	Fragmentization of cellular components due to monoatomic	[68], [69–71]
	MALDI-MSI DESI-MS, Laser-induced acoustic desorption	Label-free technique, Minimal fragmentation, multiple compound analysis in 2D and 3D molecular distributions	Lack of sensitivity Require additional derivatization reactions.	8], [60], [61], [73,74], [5], [76], [77], [78], [79]

Table 1. Summary of references on advanced single cell sampling methods and metabolites detection using different mass spectrometers.

Conclusion and future perspectives

Single-cell analysis is a powerful tool that can be used to unravel cellular heterogeneity and reveal hidden cell subpopulations. Analysing single-cell data can sometimes be challenging, as it may be difficult to distinguish true cellular signals from background noise. However, this can often be overcome by aggregating data from large numbers of individual cells and looking at the data in the context of other omics techniques.

Combining single-cell isolation techniques with complementary methods like imaging mass spectrometry enables efficient and comprehensive single-cell analysis. The combined use of these approaches can provide rich insights into the native state of biological systems. However, to overcome the biological noise, high throughput single cell technologies must be developed. Because advances in technology and analytical methods have allowed the comprehensive analysis of cellular metabolism, enabling the discovery of novel metabolic pathways and metabolic diseases. Furthermore, developments in automated high throughput microfluid-based technologies must be integrated with mass spectrometry aiming for high throughput, and quantitative studies of larger cell populations in a shorter time without significantly altering the cells' native environment.

Funding

This work was supported by JSPS KAKENHI Grant number 22K19883.E.O.K

Declaration of competing interest

T. Hankemeier is a shareholder in Mimetas B.V. All other authors declare no competing interests.

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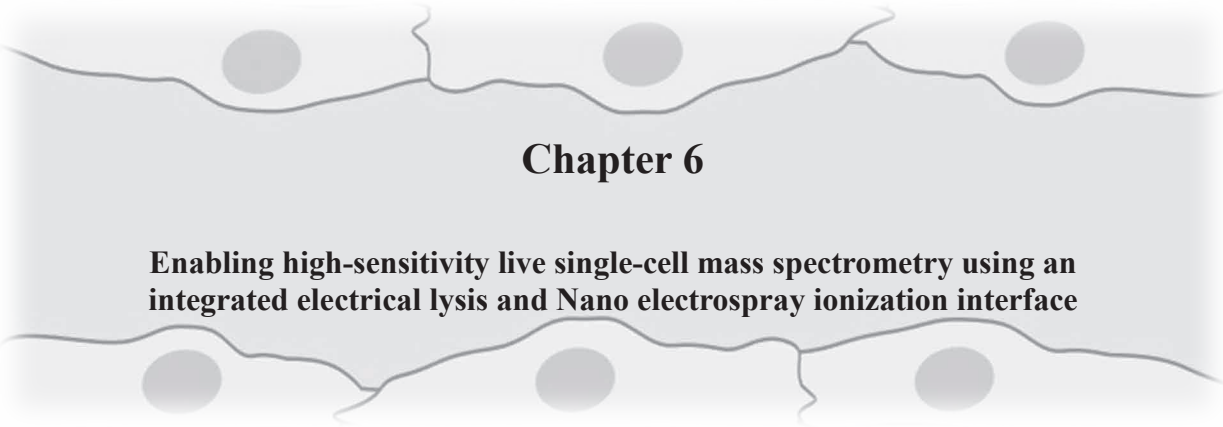
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Chapter 6

Enabling high-sensitivity live single-cell mass spectrometry using an integrated electrical lysis and Nano electrospray ionization interface

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Analytica chimica acta. 2024; 1324: 343068

doi: 10.1016/j.aca.2024.343068

Abstract

Background: Live single-cell metabolomic studies encounter inherent difficulties attributed to the limited sample volume, minimal compound quantity, and insufficient sensitivity in the Mass Spectrometry (MS) method used to obtain single-cell data. However, understanding cellular heterogeneity, functional diversity, and metabolic processes within individual cells is essential. Exploring how individual cells respond to stimuli, including drugs, environmental changes, or signaling molecules, offers insights into biology, oncology, and drug discovery. Efficient release of cell contents (lysis) is vital for accurate metabolite detection at the single-cell level. Despite this, traditional approaches in live single cell metabolomics methods often avoid efficient lysis to prevent sample dilution. Instead, current live single cell metabolomics methods use direct infusion to introduce the cell into the mass spectrometry without prior chromatographic separation or a lysis step, which adversely affects sensitivity and metabolic coverage.

Results: To address this, we developed an integrated single-cell electrical lysis and nano spray (SCEL-nS) platform coupled to an Orbitrap MS capable of efficiently lysing a single cell after being sampled with specially manufactured micropipettes. Lysis efficiency was validated by comparing live cell stain fluorescent intensities of intact and electrically lysed cells through microscopy imaging. The SCEL-nS platform successfully induced the breakdown of a single cell, significantly reducing the live cell stain's fluorescent intensity indicating cell membrane breakdown. Additionally, SCEL-nS was validated by measuring single cells spiked with the anti-cancer drug tamoxifen by MS. SCEL-nS use resulted in a statistically significant increase of the peak measured method compared to the traditional non-lysis method.

Significance: Overall, our results demonstrate that the newly incorporated SCEL-nS platform achieved higher sensitivities compared to traditional live single cell analysis methods.

Key words

Single-cell metabolomics, single-cell electrical lysis, Nano spray, mass spectrometry

Introduction

Single-cell metabolomics (SCM) explores metabolites present in individual cells, providing a high-resolution snapshot of the metabolic processes taking place within a cell. Thus, it provides novel insights obtained from individual cells, which offers advantages such as characterization of changes in tumor heterogeneity, quantification of sub populations, and prioritization of the biomarkers of each subpopulation [1–3]. Mass spectrometry (MS) has risen as the method of choice for SCM coupled with a variety of sampling and ionization techniques [4]. Single-cell (SC) MS-based methods can be classified into mass spectrometry imaging techniques, ESI techniques such as laser ablation electrospray ionization mass spectrometry [5], and capillary electrophoresis MS [6]. Despite their utility, these techniques often require extensive sample preparation steps which could cause disruptions to the cell's microenvironment [7].

An alternative technique, termed Live Single-Cell MS (LSC-MS) [8,9] uses a glass capillary to sample a living cell from its microenvironment and directly spray the entire cell into the MS. This method enables the sampling and analysis of individual cells with minimal disruption to the cell or its microenvironment. This technique [10,11], offers a distinct advantage by eliminating the need for elaborate sample preparation steps. Unlike traditional methods, LSC-MS bypasses procedures like matrix removal and derivatization. This allows for the direct infusion of single-cell content into the mass spectrometer, streamlining the analysis and potentially minimizing analyte loss or modification. Effective cell lysis techniques are crucial in LSC-MS for several reasons. Firstly, thorough exposure of cellular contents ensures a more comprehensive characterization of the intracellular metabolite profile. This completeness allows for a more accurate representation of the cell's metabolic state. Secondly, efficient cell lysis improves the sensitivity, robustness, and quantitative accuracy of nano-spray ionization. This translates to more reliable and reproducible data acquisition for single-cell metabolomics studies [12–14]. It is therefore necessary to address the existing technological gap in live single-cell mass spectrometry (MS) methods by incorporating a cell lysis step into the current methodology.

Conventional cell lysis techniques such as chemical, thermal, acoustic, mechanical, and optical lysis are widely used in SC analysis [15,16]. However, these come with drawbacks making them incompatible with LSC-MS. Ionic detergents are unsuitable for metabolomic analysis [17] due to their interference with MS measurements. While MS-compatible detergents are often utilized for proteomics research [18], using them necessitates dilution of the sample, which limits the detection of low abundance metabolites in single cells. On the other hand, uncontrollable lysis based on osmosis can change the microenvironment and the biochemistry of all cells [19], which adversely affects downstream analysis of said cells. Electrical lysis (EL) is a promising technique that can be seamlessly integrated into live single-cell MS. EL works by subjecting the cell to electric fields, inducing irreversible changes in the permeability of the cell membrane. It does not damage internal cellular components, is highly controlled, does not cause dilution, and is MS compatible [20–22]. However, no current techniques exist that successfully incorporate EL into LSC-MS.

To address this challenge, we have successfully developed and demonstrated the potential of an integrated electrical lysis setup. This single cell electrical lysis and nano spray (SCEL-nS) platform is fully compatible with LSC-MS and similar micro sampling SCM techniques. First, specially designed sampling capillaries were manufactured that can be used for sampling single cells, single cell lysis, and introduction into the MS via nano spray ionization. Then, steady-state numerical simulations were conducted using multi physics software to model the electric potential and field across a cell as it traverses the electrodes. The simulation outcomes were used to design an innovative modified EL and nano spray MS interface. The efficacy of this novel SCEL-nS platform was validated through both fluorescence imaging and MS measurements of single cells spiked with tamoxifen to evaluate the lysis process and the increase in MS signal, respectively.

Materials and methods

Micropipette fabrication for single cell sampling

Capillary pulling

Special micropipettes needed to be fabricated for single-cell sampling and lysis. For this, the following procedures were performed with GD1.2 capillaries (Narishige, Japan).

The capillaries were shortened to a length of 70mm with a ceramic cutter stone as shown in the workflow of micropipette fabrication in **Fig. 1**. A P-97 flaming/brown micropipette puller (P-2000, Sutter Instrument, USA) was used to pull the capillaries into pipettes with long tapers for single cell sampling with the optimized settings shown in **Table 1**.

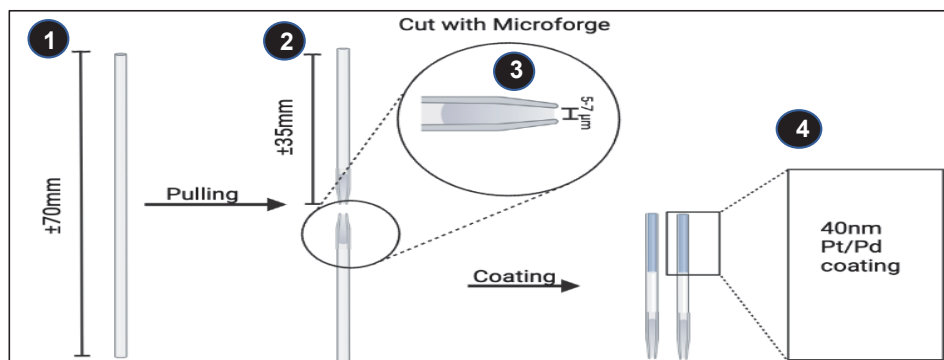


Figure 1. Micropipette fabrication workflow and process. 1) Adjusting optimal length from commercial capillaries. 2) Capillary pulling using Sutter instrument. 3) Capillary cleaving with micro-forge and 4) Micropipette metal (Pt/Pd) coating to obtain electrical conductivity.

Capillary cleaving

A micro forge (MF-900, Narishige, Japan) was used to cleave the capillaries further to obtain the target diameter that is suitable for single-cell sampling ($5-7\ \mu\text{m}$) after pulling (**Supplementary Table.1**). Micropipettes with a consistent inner diameter (ID) of

approximately 2 μm were achieved with good reproducibility, as evidenced by a low standard deviation of ± 0.65 ($n=10$). Therefore, the capillaries pulled with ~ 2 μm ID were cleaved using a micro forge to obtain 6.0 ± 1.3 μm ID **Supplementary Fig. 1A-D**.

Micropipette measurements

The cut capillaries were imaged using a brightfield microscope (EVOS™ Auto 2, Invitrogen, Thermo Fisher Scientific, USA) using 60X magnification with a set scale bar. The capillary images were imported to ImageJ software using the scaling option, and the capillary inner tip diameters were measured by comparing the scale bar length with the length of the inner diameter of the capillary tip (**Supplementary Fig. 1D**). Only the capillaries with a diameter of 6.0 ± 1.3 μm were used in further experiments.

Capillary coating

To enable capillaries to serve both in the lysis process and as nano spray sources, they must feature a non-conductive section for lysis and a conductive segment for effective nano spray functionality. Before sputtering, the tapered ends of the pipette tips were covered using a metal plate to produce the non-conductive region that can be used in the lysis process (**Supplementary Fig. 1E**). Therefore, capillaries were coated onto the surface, with tapered tip closed with the metal plate. To achieve the metal coating, the cut glass capillaries were coated with a thin film of conductive material (Platinum/palladium; Pt/Pd sputter target, Ø57 x 0.2mm Disc - 70-PP5708, EM-Tec, Micro to Nano, Germany).

The deposition of a thin, uniform film was carried out using a spin coater instrument (Model 208HR, No. C5989, Cressington Sputter Coater, UK) under specified parameters: conductivity set to 9.4×10^6 S/m, sputter coater thickness set to 40 nm with a tooling factor of 1.6, and a density of 19.52 gm/cm^3 .

The result of the micropipette fabrication process features a tip diameter measuring 6.0 ± 1.3 μm , with half of it coated with Pt/Pd as shown in **Supplementary Fig. 1F**.

Electric field simulation and constructing the SCEL-nS platform

Steady-state numerical simulations were performed with COMSOL Multiphysics 5.4 software to simulate the electric potential and electric field in the electrode zone across a cell as it passes the electrodes. To compute the electric potential and electric field, the software is configured with the electrostatic module for simulation. A three-dimensional (3D) geometrical model is generated, and three simulations were carried out: i) a cell positioned near the tip of the micropipette with a cell membrane thickness of 1 μm , ii) a cell membrane thickness of 200nm, and iii) without a cell. The cell diameter was set to 14 μm and G extra fine meshing was set to the minimum mesh size of 100nm. The dielectric constant values used in the simulation are as follows, the two L-shaped stainless-steel electrodes = 1.1×10^6 , the capillary (borosilicate glass) = 4, the fluid surrounding the cell = 17.5 (HBSS buffer), the cell membrane = 5 [23,24], and cytoplasm = 100 [25,26].

Accurate capillary dimensions were measured using a profile projector (Mitutoyo, The Netherlands) and the dimensions used for simulation. For the electrode and capillary holder design, 3D CAD (Autodesk Inventor Professional) software was used.

Cell culture and sample preparation for electrical lysis validation

Human coronary artery endothelial cells (HCAECs) were used to test the lysis setup. HCAECs (PromoCell, C-12221) were resuspended in 10 ml fresh EGM MV2 medium with supplements (PromoCell, C-22022, C-39216) and cultured in T75 flasks (Nunc Easy flask, Sigma, F7552). Cell cultures were maintained at 37°C with 5% CO₂ and media was refreshed three times a week. Cells were detached at 85% confluence with 0.25 % Trypsin EDTA (Lonza, CC-5012) and cell pellets were collected by centrifugation at 300g for 5 minutes. The collected cell pellets were suspended in fresh medium to a concentration of 1×10^6 cells/ml and cultured in 35mm Petri dishes (60mm, Sarstedt AG, Germany).

For imaging, cells were stained with the live cell stain – calcein AM (Thermofisher, The Netherlands) of dilution 1:1000 in HBSS and incubated at 37 °C for an hour. If the cells are alive, the nonfluorescent calcein AM is converted to green, fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterase. After incubation, the stain solution was removed and washed thrice with HBSS and replaced with 1mL fresh HBSS for imaging. Before single cell sampling, the cells were imaged to check for the fluorescent intensity using EVOS fluorescent microscope with maximum excitation: 491nm and maximum emission: 513nm. For intact cells, either a stained cell or tamoxifen treated cell was first aspirated into a micropipette, followed by the addition of 3 µL of organic solvent. The cell was then imaged using a fluorescence microscope or sprayed to MS.

For MS analysis, cells in 35mm petri dishes were treated with 3µM of tamoxifen (T5648, Sigma Aldrich, The Netherlands) for 24 hours at 37°C. After the incubation, cells were washed thrice with HBSS buffer (14025, Thermofisher Scientific, The Netherlands) and cells were ready for single cell sampling.

Single cell sampling was carried out using a micromanipulator attached to a syringe to sample a single cell into the micropipette [8]. The single cell sampling setup is shown in **Supplementary Fig. 2**.

Electrical lysis and MS method

For cell lysis, a single, fluorescently stained cell or tamoxifen treated cell was aspirated into a micropipette. Next, the SCEL-nS platform was used to lyse the cell electrically. To lyse the cells, a switch system was integrated to the SCEL-nS platform, which directs the electric current to either lyse a single cell or to spray the cell's content to the MS. After cell lysis 3µL of organic solvent containing 0.1% formic acid in 80% methanol was pipetted into the capillary and the cell was ready either to image using microscope or for the electrospray process.

An orbitrap Exploris 480 (ThermoFisher Scientific, USA) was used for MS measurements. Parameters for the acquisition were as follows: mode = positive, spray voltage = 1500 V, Ion Transfer Tube Temp = 400°C, Resolution = 120,000 FWHM. MS/MS with normalized collision energy of 30% and an isolation width of 1 Da. The tamoxifen peak was measured based on the 372/72.0807 m/z transition.

Data processing and statistical analysis

After MS measurements, the raw spectra were exported from the manufacturer's raw format to .csv format using Qual browser (ThermoFisher Scientific, USA). Each .csv file contained the extracted ion chromatogram (XIC) of the peaks of interest. Average peak intensities were calculated by dividing each peak's total signal by the time a stable spray was observed in each sample. Data analysis and plotting were done using GraphPad Prism (Version 9.3.1).

Results and discussion

Micropipette fabrication and optimization

Single-cell electrical lysis involves a multi-step process typically encompassing micropipette fabrication, single-cell isolation, pre- and post-lysis imaging using a suitable microscopy technique (SC imaging), and mass spectrometry (MS) analysis. The fabrication process of micropipettes consisted of capillary pulling, cleaving, and metal coating. The dimensions of the micropipette tips were optimized according to the cell size of endothelial cells, which typically have dimensions of approximately 50 μm $\times$ 10 μm in culture and $25.8 \pm 8.5 \mu\text{m}$ $\times$ $13.2 \pm 4.1 \mu\text{m}$ when in suspension [27]. Our fabrication method was aimed to produce a tip of 5-8 μm ID, which is suitable for both single-cell sampling and nano spray generation. In earlier studies, using similar micropipette fabrication techniques [28,29], single cells were sampled, and the membrane integrity proved to be undamaged [30]. To obtain the optimal tip size of a

capillary to sample a single cell, the capillaries were pulled by applying parameters given in the Sutter instrument protocol book such as heat, velocity, pull range, and time/delay. To produce longer tapered and narrowed capillaries, a higher heat setting of 542 units was applied. A pull value of 20 units was established, wherein both filament ends are compressed as the glass softens; the smaller the tip, the higher the pull achieved. Utilizing a higher velocity of 42 units in delayed mode aids in controlling the air-cooling duration and lower pressure units facilitate the creation of long tapered capillaries.

Using different combinations of pulling methods and different parameter settings yielded different IDs and SDs of the micropipette presented in the **Supplementary Table 1**. The optimized parameters can consistently produce micropipettes of mean ID $1.96\ \mu\text{m} \pm 0.65$ (**Supplementary Fig. 2A & 2C**). Consistent manufacturing of the micropipettes was achieved with the optimized values shown in the **Table.1**

Parameters	Values
Heat	Ramp values -12 units
pull	20 units
Velocity	42 units
Time	200 ms
Delay	Nil
Pressure	200 pounds

Table1. Optimized parameters of micropipette pulling protocol.

To obtain the desired ID $6.0\ \mu\text{m}$, the pulled capillaries were cleaved at approximately $24\ \mu\text{m}$ of length from the tip of the micropipette with SD of $\pm 2.1\ \mu\text{m}$. The mean distance and SD required to cleave the capillary from the tip to achieve an inner diameter of $6.0 \pm 1.3\ \mu\text{m}$ are presented in **Supplementary Table 2**. The micro-forge was employed to produce micropipettes with long taper ends and an inner tip diameter of $6.0\ \mu\text{m} \pm 1.3$ (**Fig. 2**). To confirm the reliability of these procedures and capillary diameter, the cut capillaries were measured using the microscope under brightfield.

With the optimized protocol, these capillary ($6.0 \mu\text{m} \pm 1.3$) dimensions were suitable for efficient single cell sampling, subsequent lysis, and measurement (**Fig. 2**).

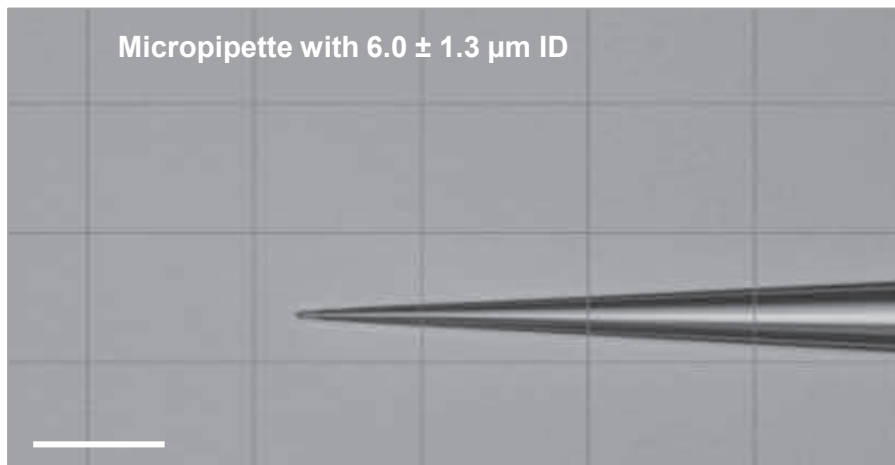


Figure 2. Measurement of capillaries after cleaving step with ideal inner diameter $6.0 \pm 1.3 \mu\text{m}$ using EVOS microscope. Scale bar – $125 \mu\text{m}$.

In addition, the capillary coating with Pt/Pd of 40nm thickness was effective at transmitting the voltages required for cell lysis and electrospray initiation. In previous studies, Pt/Pd alloy coating on glass substrate has been demonstrated as a suitable choice due to its high electrical conductivity, and resistance to corrosion [31]. Typically, micropipettes for single cell manipulation are crafted with a shorter taper [32,33]. However, in our study, longer taper micropipettes were necessary to achieve the desired size of $6.0 \mu\text{m}$ with a lower SD (± 1.3). This led to the effective sampling of single cells and reduced media pipetting in the capillary due to the small tip diameter while preserving favorable nano spray characteristics.

Electric field simulation for single cell lysis

Prior to the SCEL-nS platform development, numerical simulations were conducted to accomplish efficient single-cell lysis by optimizing the design, and comprehending the

electric field experienced by the cell. The simulation result aids in estimating the electric field that helped to predict the lysis efficiency based on the dielectric properties of the cell and the surrounding environment. The simulation results also helped in determining the transmembrane potential (TMP) across a cell when it is exposed to an electric field [21,34] and designing electrodes to generate efficient electric fields to enable efficient lysis.

The simulation was performed by giving a positive potential of 500 V to the outer surface of one of the L-shaped electrodes and the other electrode surface is grounded. Three simulations were performed: i) with no cell, and by constructing a pseudo spherical cell with membrane thickness of ii) 1 μm (**Fig. 3C**) and iii) 200nm. Electric field simulation and graphical representation of the electric field, and a schematic of the simulation domain were displayed in **Supplementary Fig. 3** without the inclusion of a single cell within a micropipette.

The simulation of the electric field and an SC electrical lysis was illustrated in **Fig. 3C**. The electric field generated by applying 500V across the cell membrane along the axis of the electrode and the capillary exceeds the required range of electric field for SC lysis. The graph of the electric field generated near the wall of the capillary without a single cell showed a maximum of 5×10^5 V/m. This dropped to 2×10^5 V/m (**Fig. 3C**) as a direct consequence of the presence of a cell as it caused a change in the dielectric constant across the capillary. However, the generated EF with the maximum boundary values is sufficient to pass across the TMP of a single cell which agrees with earlier studies showing that 6×10^4 to 2×10^5 V/m of electric field strength is required to lyse a single cell [21,34,35]. When the cell membrane thickness was reduced to 200nm there were no changes in the peak electric field obtained (results are not shown).

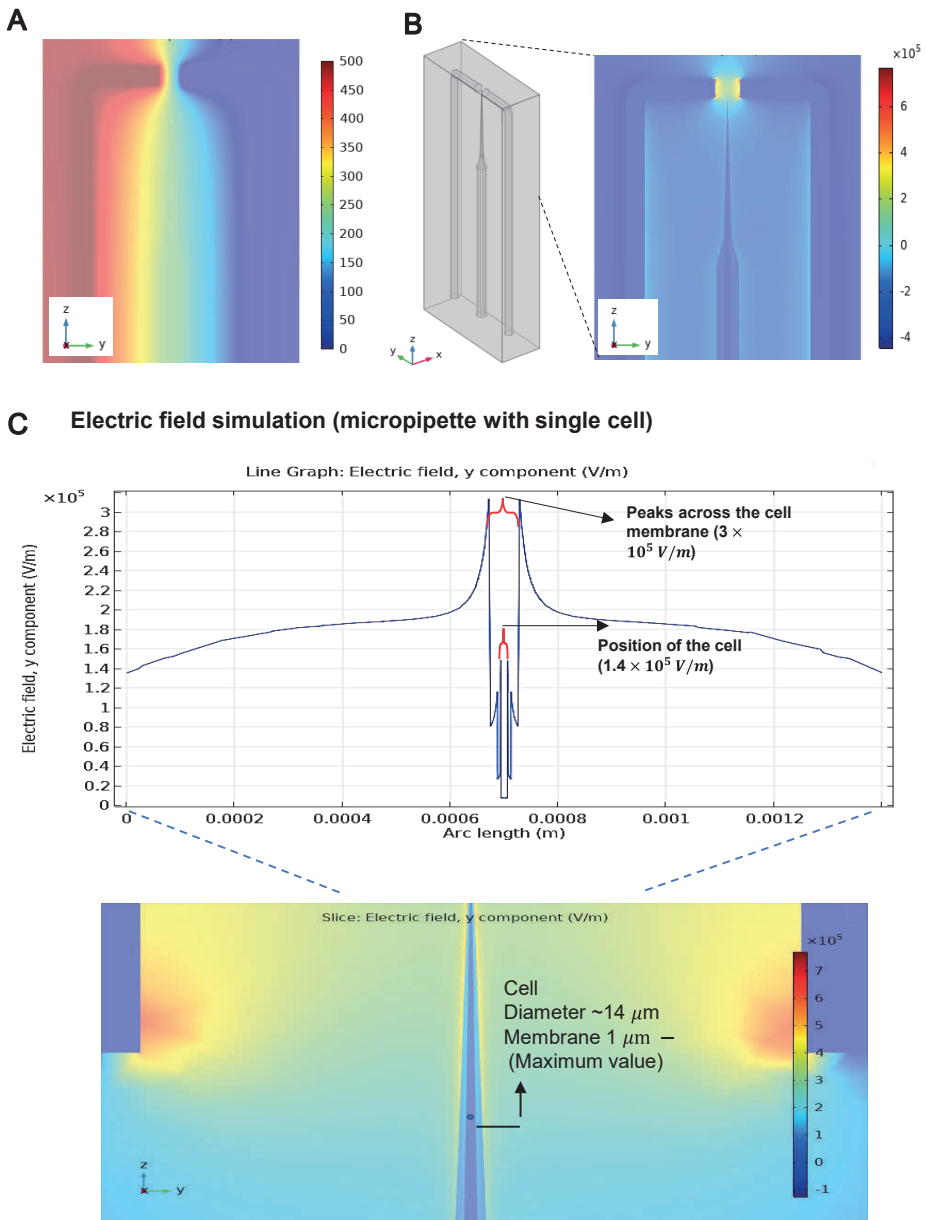


Figure 3. Electric field simulation using COMSOL multi physics software. A) Electric potential across the electrodes with applied voltage (500V). B) The computational field generated with the 3D mesh for the steady-state simulation and electric field (EF) in V/m across the electrodes in the presence of the micropipette. C) Graphical representation of electric field graph and simulation domain schematic, featuring a $14\mu\text{m}$ spherical cell positioned within the micropipette. The

micropipette is then situated inside the air domain, centrally positioned between a pair of adjacent electrodes. The EF was simulated with the maximum boundary values to obtain optimal field energy to lyse a single cell. EF is represented in V/m.

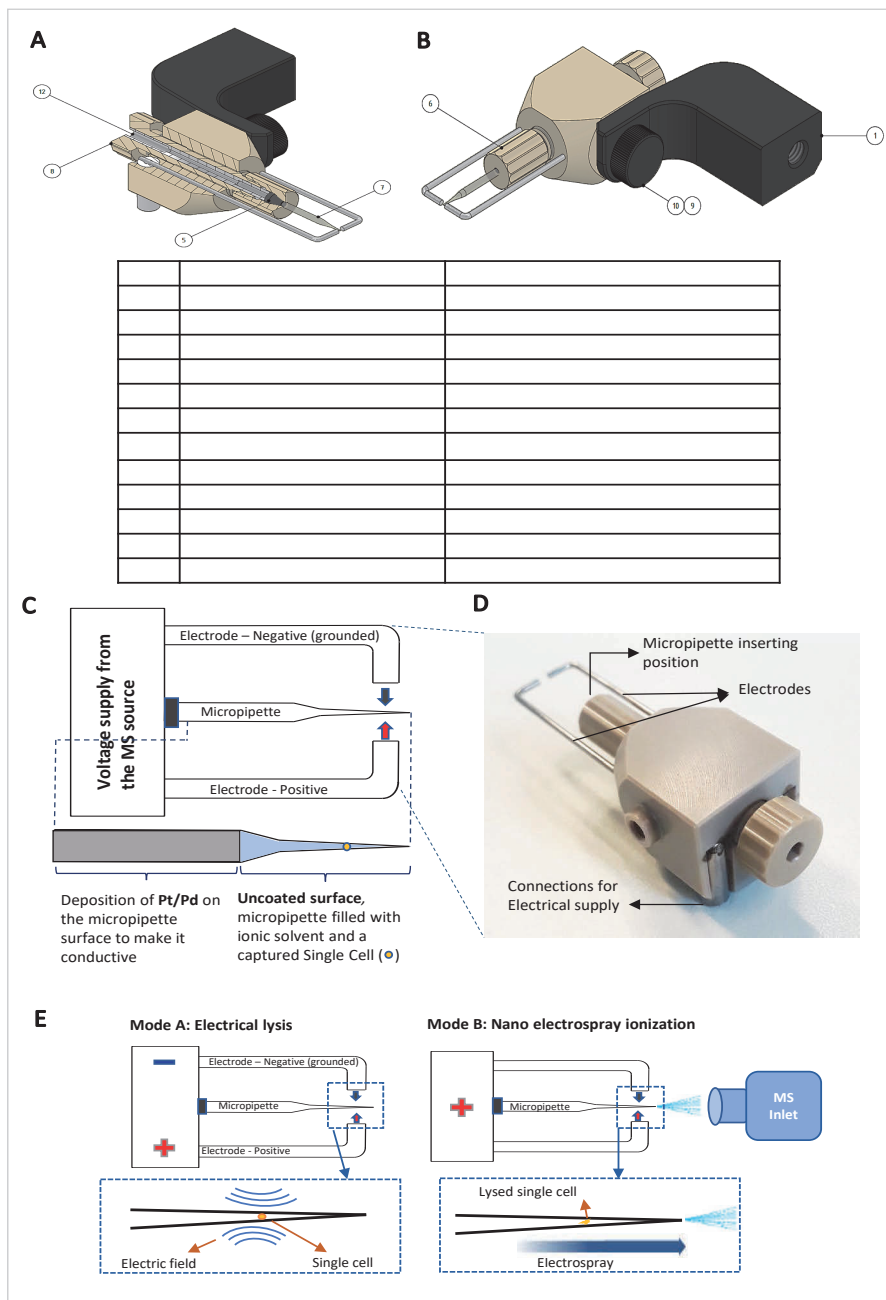


Figure 4. A & B) Schematic representation of electrical lysis setup with labelled parts and C) Illustration of the SCEL-nS platform showing the connection to the voltage supply from MS source and the micropipette placement and its conductive region D) Original single-cell electrical lysis and nano spray (SCEL-nS) device and E) Operating the SCEL-nS platform involves employing mode A of the switch system to direct electric current for lysing a single cell. In mode B, the platform electro-sprays the content of a single cell into the MS inlet.

With the simulation results, the SCEL-nS platform's electrodes were constructed with the following parameters:

- Electrode length (44.5mm): Tailored to accommodate the micropipette (**Fig. 4A-7**) length and maintain proximity to the MS inlet.
- Electrode distance (1.3mm): Set to achieve adequate electric field strength for the lysis process.
- Adjustment screw (**Fig. 4A-8**): Utilized to secure the needle, the adjustable screw compensated for slight variations in micropipette length. The design included an adjustment range of approximately 4.5mm, with a fine thread chosen for the screw to fit snugly into the housing with minimal clearance.

Such parameters were modified based on the simulation result. The newly adapted modified nano spray source is shown in **Fig. 4A-E**. Within the housing, the electrode tips were fully integrated and equipped with connections for the high-voltage plugs. The simulation results play a crucial role in predicting the lysis efficiency for a single cell electrical lysis/electroporation at an appropriate voltage, thus, achieving efficient lysis instead of allowing the cell membrane to reseal. These predictions are primarily based on the dielectric properties of the cell and the surrounding environment. In general, the simulation results differ depending on whether the cell was in static condition [36] or in movement when it is placed in a suspension liquid [21,37–39]. In our model, when a cell is sampled using a micropipette in a low media volume, it is not possible to restrict the cell's movement. Therefore, maximum boundary values were established to get an

appropriate electric field (EF) for cell lysis. The electroporation technique employs electrical stimulation to generate enhanced permeability in cell membranes [40]. The conventional EL or electroporation operates with an electric field of a few kilovolts per centimeter in bulk lysis and it perforates the entire population [41], whereas our method works at a single cell level, and the requisite EF can be achieved in less than a kilovolt operation. Furthermore, our SCEL-nS platform eliminates the need for an additional power supply, as it derives the voltage supply from the MS source, simplifying the design and instrumentation requirements.

Electrical lysis evaluation using microscopic imaging

To evaluate the SCEL-nS platform, microscopic imaging was used to compare individual single cells before and after lysis.

Cells were stained with a live cell cytoplasmic dye (**Fig. 5A**), a single cell was collected in the micropipette, and the fluorescence intensity was assessed before and after lysis using microscopy imaging (**Fig. 5B**). Results indicated that the cell subjected to EL showed significantly lower fluorescent intensity compared to the intact cell (p value = <0.0001 , **Fig. 5C**).

These results indicated that when the electric field was activated, the cell boundary was weakened due to cell membrane breakdown which led to leakage of the cell cytoplasm to the medium as made evident by the lower fluorescent intensity (**Fig. 5B & 5C**). Before lysis, the same cells showed adequate membrane integrity as is shown by the higher fluorescent intensity of the cytoplasm-specific stain. This is consistent with previous studies that attempted to use electroporation as a lysis technique [42,43]. In this study, we opted for further confirmation by using mass spectrometry to investigate the effect of the cell lysis on the MS signal.

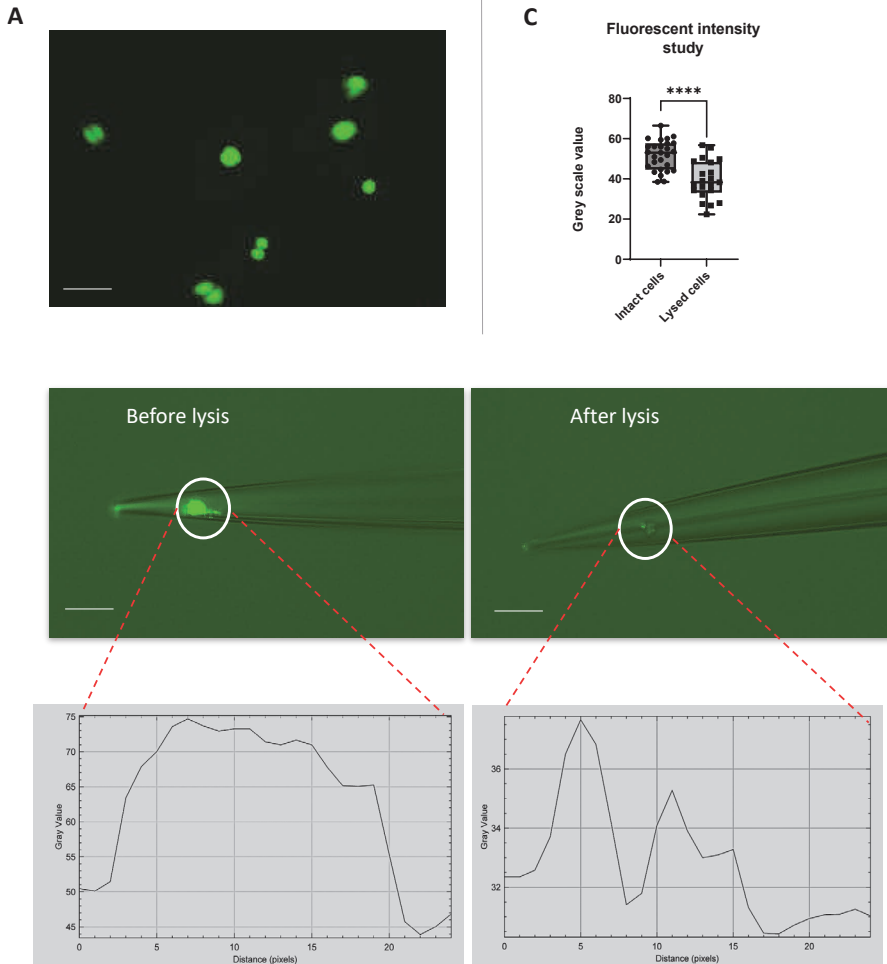


Figure 5. Fluorescent live stain images for lysis validation. A) Calcein AM stain images of intact cells before sampling, B) Reference image of a single cell within a micropipette i) before and ii) after lysis and the graphical representation of fluorescent intensity is highlighted. Gray values were measured using imageJ software, (gray scale values in pixel units; lysed cell = 38 pixels and for intact cells = 75 pixel units) and C) Gray scale intensity calculation of multiple single-cells – intact and lysed cells. A P value was obtained from the student T test. ****, $P < 0.0001$ ($n = 20$).

Evaluation of single-cell electrical lysis using mass spectrometry

To evaluate SC lysis using MS, tamoxifen (3 μ M) treated SCs were sampled in micropipettes using a micromanipulator, the EL was performed using SCEL-nS platform and cell contents were infused to the orbitrap. To investigate the lysis efficiency, the tamoxifen signal for each cell was measured using the transition 372/72.0807 m/z [11]. Average peak intensities were determined by dividing the total signal of each peak by the elution time.

Results showed higher peak intensity of tamoxifen in lysed cells compared to the non-lysed cells after elution time correction with P value = 0.002 obtained from student T-test; Log10FC = 0.2 is shown in **Fig. 6**.

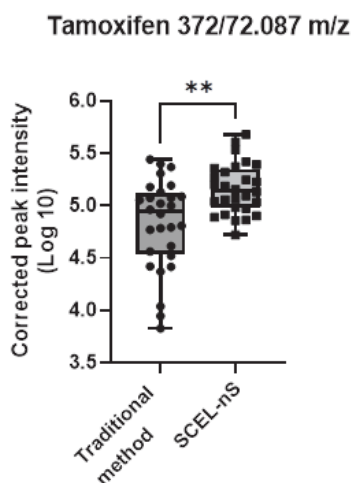


Figure 6. Comparison of single cell tamoxifen signal before (using traditional method) and after lysis (using SCEL-nS method). The peak intensity was corrected with elution time. A p value was obtained from the student T test and Log10FC =0.2. *, $P < 0.05$; **, $p < 0.001$. $n = 25-35$.

This outcome suggested that the content of lysed cells dispersed more effectively before detection compared to non-lysed cells that were directly infused.

Conclusion

Overcoming challenges in metabolomics, sensitivity, and dynamic range is crucial, especially at the single-cell level, where metabolite concentrations can vary from extremely low (nM) to remarkably highly abundant metabolites (mM), metabolite intermediates, signaling compounds, etc. The integrated SCEL-nS platform resulted in higher peak area intensities compared to the traditional LSC-MS methods. In addition, our novel SCEL-nS platform achieves efficient cell lysis with minimal dilution of the cell. In our study, we observed more peak variation when using traditional non-lysis methods compared to the consistent peak quality achieved with the SCEL-nS approach. Moreover, SCEL-nS was shown to be a more controllable approach with better sensitivity for single-cell lysis compared to harsh solvents. While the SCEL-nS platform offers advantages, its current throughput is mainly limited by labor-intensive steps like cell selection and sampling. Implementing an advanced, software-controlled robotic system for cell sampling and integrating the lysis setup could significantly address these bottlenecks and improve the platform's overall throughput. Future studies could explore reducing organic solvent volume through capillary advancements (shorter lengths) while expanding platform application to validate robustness across diverse analytes. Overall, this MS-compatible SCEL-nS platform stands out as an optimal choice for automation and high-throughput single-cell methods. Future applications may extend this approach to various cell types and drug treatments to identify sensitive/low-abundant metabolic biomarkers within heterogeneous subpopulations.

Limitations of this study

Further improvements could be achieved by developing more precise and automated methods for single-cell sampling which would improve the efficiency and accuracy of our platform. This could involve microfluidic technologies or robotic manipulation

systems capable of isolating individual cells with minimal disruption. Seamless integration of our single-cell sampling methods with microfluidic platforms would enable streamlined analysis workflows. This could involve miniaturized, automated systems that handle cell isolation, lysis (if needed), and downstream analysis steps on a single chip. To broaden the applicability of our platform, testing it with a wider range of drugs and cell types would be crucial. This would involve systematically evaluating the platform's performance across different pharmacological agents and cell lines/tissues. By demonstrating its effectiveness with various drugs and cell types, we can establish the platform's versatility and robustness.

Authors contribution/ Acknowledgements

KP, Conceptualization, Investigation, Methodology, Writing - original draft; LA, Methodology – Micropipette fabrication and mass spectrometry; LH, Methodology – Micropipette fabrication; RZ, Methodology – Designing the electrode setup; PV, Methodology – Electrical connections; CS, Methodology – Parameters optimization and micropipette fabrication; JK, Methodology – Electric field simulation; AH, Writing - review and editing; AA, Conceptualization, Investigation, Methodology, Writing – review and editing; TH, Resources, Supervision, Funding acquisition, Writing – review and editing. This project has received funding from the European Union's Horizon 2020 research and innovation program (LogicLab network) under the Marie Skłodowska-Curie grant agreement No 813920 and the NWO XS grant number OCENW.XS23.1.070.

Conflicts of interest statement

The authors declare that they have no conflict of interest.

Data availability statement

The data that support the findings of this study are available in the Materials and

Methods, Results, and Supplementary Material of this article. Additional data will be provided upon request.

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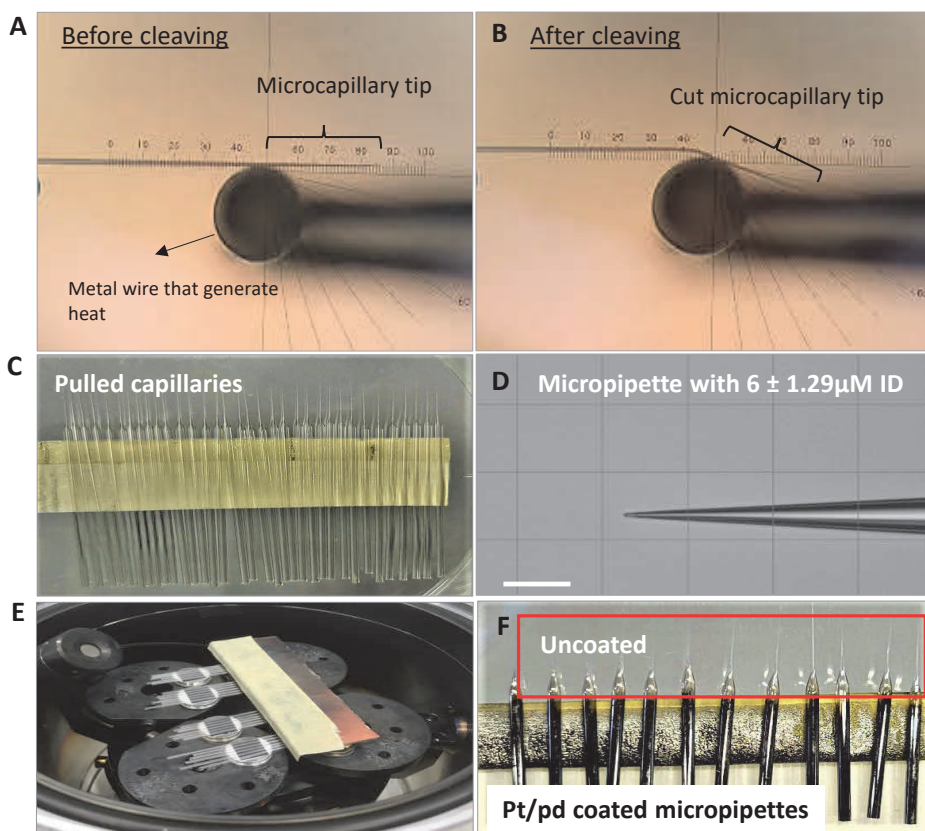
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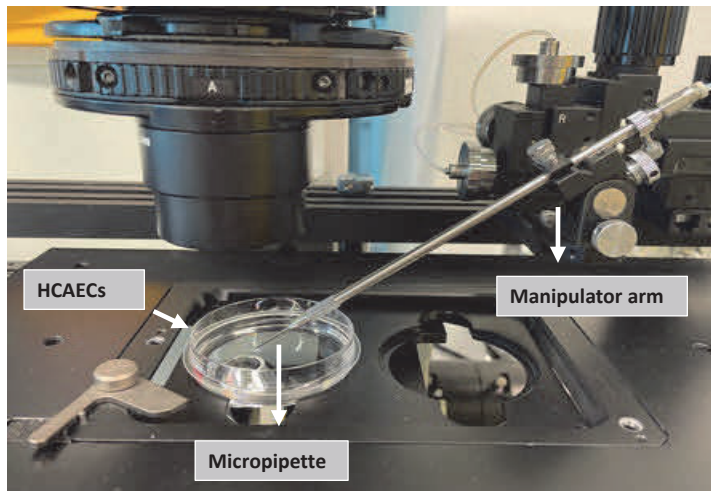
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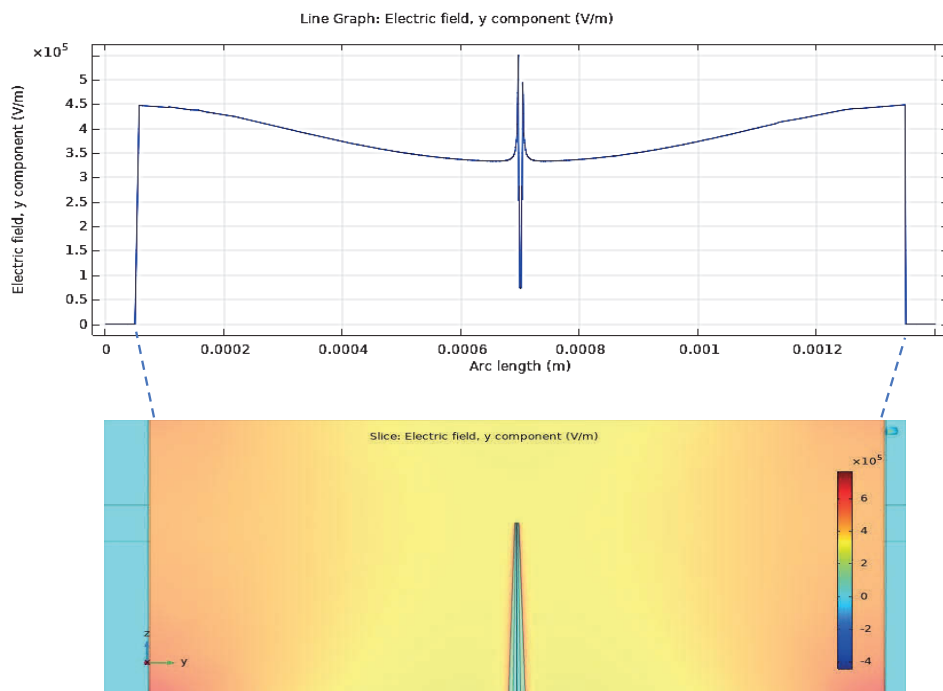
Supplementary information



Supplementary Figure 1. Micropipette fabrication processes. A) Micro-forge procedure prior to micropipette cleavage. B) Micro-forge operation following micropipette cleavage. C) Capillaries after pulling with 1-2 μm outer diameter D) Measurement of capillaries after cleaving step with ideal inner diameter $6 \pm 1.29 \mu\text{m}$ E) Capillaries were arranged for coating by safeguarding the tips with a metal plate, and F) Micropipettes subsequent to coating, showcasing the outcome of the fabrication process. Red box – uncoated region of micropipettes. Scale bar – 125 μm .



Supplementary Figure 2. Single cell sampling procedure using a micromanipulator and the fabricated micropipette under the microscope.

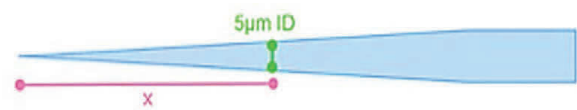


Supplementary Figure 3. Electric field simulation using multi physics software and the graphical representation of electric field and schematic of the simulation domain were displayed without the inclusion of a single cell within a micropipette.

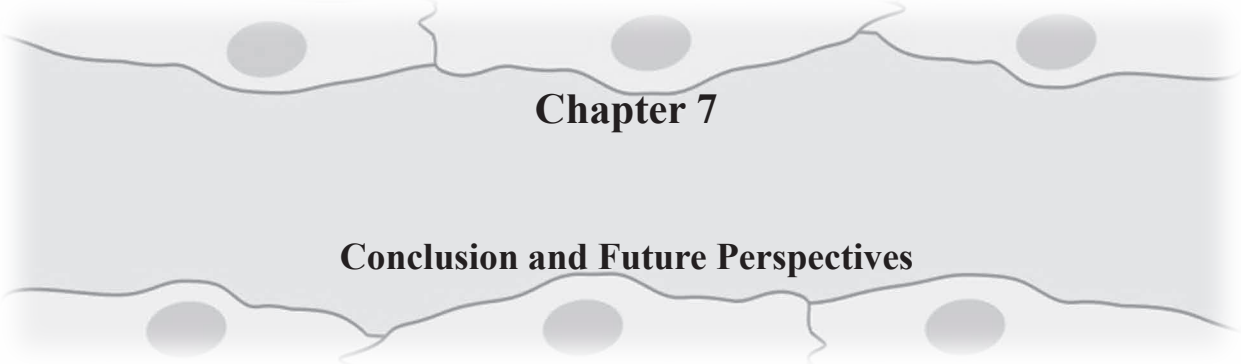
Supplementary Table 1. The microcapillary pulling method using different parameter settings and the resultant inner diameters and standard deviations (SD) of the micropipettes are listed in the table.

Adjusted parameters on different settings	Settings parameters	Mean ID	STDEV
Settings 1	H=542 units; pull=0 units; v=45 units; t=250ms	0.616	0.89
Settings 2: Heat ↓	H=540 units; pull=0 units; v=45 units; t=250ms	0.582	0.062
Settings 3: Heat ↓	H=537 units; pull=0 units; v=45 units; t=250ms	0.576	0.106
Settings 4: Heat ↓	H=535 units; pull=0 units; v=45 units; t=250ms	1.670	1.233
Settings 5: Heat ↓	H=532 units; pull=0 units; v=45 units; t=250ms	0.599	0.111
Settings 6: Heat ↑ + time ↑	H=542 units; pull=0 units; v=45 units; t=255ms	0.546	0.077
Settings 7: velocity ↓	H=542 units; pull=0 units; v=35 units; t=255ms	1.964	3.649
Settings 8: velocity ↓	H=542 units; pull=0 units; v=32 units; t=255ms	0.803	0.129
Settings 9: velocity ↓	H=542 units; pull=0 units; v=30 units; t=255ms	0.752	0.08
Settings 10: velocity ↓	H=542 units; pull=0 units; v=28 units; t=255ms	0.663	0.105
Settings 11: velocity ↓	H=542 units; pull=0 units; v=18 units; t=255ms	0.151	0.341
Settings 12: velocity ↓	H=542 units; pull=0 units; v=8 units; t=255ms	24.448	2.084
Settings 13: velocity ↓ + delay ↑	H=542 units; pull=0 units; v=28 units; delay= 1	0.686	0.095
Settings 14: velocity ↓ + delay	H=542 units; pull=0 units; v=15 units; delay= 1	0.100	0.102
Settings 15: Heat ↓	H=512 units; pull=0 units; v=28 units; t=255ms	0.998	0.103
Settings 16: Heat ↓	H=502 units; pull=0 units; v=45 units; t=250ms	0.928	0.08
Settings 17: Heat ↓	H=492 units; pull=0 units; v=28 units; t=255ms	1.023	0.185
Settings 18: Heat ↓ + velocity ↓	H=512 units; pull=0 units; v=17 units; t=255ms	1.310	0.233
Settings 19: Heat ↓	H=492 units; pull=0 units; v=17 units; t=255ms	22.056	9.946
Settings 20: Heat ↓	H=487 units; pull=0 units; v=17 units; t=255ms	25.067	4.471
Settings 21: Heat ↑	H=502 units; pull=0 units; v=17 units; t=255ms	1.709	0.14
Settings 22: velocity ↑	H=542 units; pull=0 units; v=28 units; t=255ms	1.097	0.111
Settings 23: time ↓	H=542 units; pull=0 units; v=45 units; t=230ms	1.150	0.099
Settings 24: pull ↑	H=542 units; pull=10 units; v=28 units; t=230ms	1.228	0.122
Settings 25: Heat ↓ + pull ↓ + velocity ↓ + time ↑	H=497 units; pull=0 units; v=17 units; t=255ms	12.280	13.785
Settings 26: velocity ↓	H=497 units; pull=0 units; v=17 units; t=255ms	17.422	3.001

Supplementary Table 2. Representation of mean distance x (μm) and standard deviation to cleave capillary from the tip to obtain 5-7 μm of ID. The setting No.15 is used due to the lower SD, and it is highlighted in red.



Settings	Mean distance x (μm)	Standard deviation (SD)
1	103.788	22.482
2	125.341	18.832
3	108.204	14.102
4	95.168	21.357
5	153.536	46.528
6	99.253	18.421
7	65.934	15.738
8	73.308	16.084
9	140.845	106.256
10	62.197	15.298
11	76.300	41.273
12	314.715	203.945
13	460.423	73.386
14	34.140	10.608
15	24.405	2.159



Chapter 7

Conclusion and Future Perspectives

Conclusion

Endothelial dysfunction and microvascular diseases are the leading causes of cardiovascular diseases, which account for the highest number of fatalities globally. While studies on endothelial dysfunction have advanced significantly in recent decades, the lack of widely used human-specific in vitro models has been a limitation in conventional setups [1]. These setups lack the complexity, blood flow, cell–cell interactions, or proper extracellular matrix (ECM) environment that are typical of living tissues and are needed to assess the functionality of the microvascular system. Accurate micro vessel models are essential for replacing animal models, particularly in cardiovascular diseases (CVDs), as animal models often fail to accurately replicate human pathophysiology.

Microfabricated vessels, also known as "vessels-on-a-chip," are engineered to mimic physiological blood vessels. These hold promises for overcoming limitations of traditional in vitro and in vivo models. The rapid development of vessel-on-a-chip models in the past decade is likely due to the convergence of several key disciplines: blood vessel physiology, transport phenomena, cell and matrix biology, and tissue microfabrication [2]. As an advanced cell culture technology, microfabricated vessels-on-a-chip have successfully recapitulated the endothelial cells, 3D ECM, and structural components of blood vessels of the microcirculation under well-controlled conditions. Thus, as a collective, vessels-on-a-chip have proven to be a reliable and highly useful complement to in vivo studies of microvascular physiology and function [3]. This standing has been propelled by tremendous advances in microfabrication and microfluidic techniques combined with a deep understanding of vascular biology and physiology. Further strengthening the integration between microscale engineering technology and methods from cell and molecular biology is imperative for the continued advancement of vessel-on-a-chip models.

Microfluidic models incorporating a flow condition are particularly valuable for studying vasodilatory compounds like nitric oxide (NO) because physiological flow is a key

regulator of NO production [4]. NO plays a crucial role in various diseases and even possesses antiviral properties [5,6]. However, direct measurement of NO is challenging due to its short half-life, the lack of reserve storage in the cells, and limitations of existing techniques. Metabolomics offers a valuable alternative by providing a snapshot of NO metabolism through measurement of its metabolites. In place of direct measurement, NO biochemistry can be studied by analyzing the metabolites involved in NO mechanisms. Stable isotope labelling coupled with metabolomics offers an even more specific view of the NO pathways and can track a specific metabolic pathway without the interference of other complicated pathways. In addition, personalized medicine and targeted therapies will greatly benefit from considering the unique characteristics of individual cells. Single-cell technologies represent a rapidly evolving field poised to bridge the gap between traditional in vitro analysis and a more nuanced understanding of cellular heterogeneity.

Therefore, this thesis aimed to investigate endothelial dysfunction using in-vitro model systems, with the hypothesis that measuring eNOS activity is key to understanding this dysfunction. For this, we develop a mass spectrometry method for analyzing metabolites involved in NO production using a 3D microvessels-on-chip model. The ultimate goal was to leverage this method and the 3D model to investigate disease conditions and the impact of hypoxia on blood vessels. **Chapter 3** focused on facilitating the use of this method for studying disease by incorporating COVID-19 patient plasma samples into the microfluidic system. **Chapter 4** explored the effects of hypoxia on vessels using the established model. To further refine our understanding, **Chapter 5** delved into advancements in single-cell techniques to unravel cellular heterogeneity within the microvessels/tissues. Finally, **Chapter 6** explored the application of high-throughput screening for efficient analysis of potential therapeutic targets.

Tracer-based LC-MS method and 3D microvessels-on-chip model for the measurement of nitric oxide metabolites

The ultimate aim of tracer-based metabolomics is to precisely identify and track the marker metabolites involved in a specific pathway. **Chapter 2** investigated the development of a mass spectrometry method to measure the metabolites that are involved in NO production using a 3D microvessels-on-chip model. Therefore, we established a stable isotope Ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS)/MS method that measured NO substrate isotope labeled L-arginine and its downstream metabolites L-citrulline and L-ornithine. The exact isotopologue peaks were identified based on the m/z of each calculated transition state of these metabolites. Even more precisely, the level of metabolites was understood better by using an endothelial nitric oxide synthase (eNOS) enzyme stimulator and inhibitor and ornithine (arginase enzyme) production inhibitor. This assay helped us to track the NO metabolites during stimulation and inhibition conditions. To validate this method in 2D well plate cell culture, we confirmed eNOS expression using a reference staining technique (DAF-2DA). Additionally, we demonstrated the specificity of the assay by producing expected results with eNOS stimulator and inhibitors of eNOS and arginase. Next, the same method was employed to measure eNOS activity in two different culture conditions including 2D Culture, which is the standard cell culture format using 2D well plates for the initial studies, and 3D Microvessels model with Flow, which incorporates a microfluidic system to simulate physiological flow conditions. We explored two flow configurations: 1) Bidirectional Flow, which mimics the back-and-forth movement of blood observed in some vessels, and 2) Unidirectional Flow generated by microfluidic pumps producing a more constant flow pattern found in other vessels. This was confirmed through the mechanosensitive gene expressions measured in cells from all these platforms. Importantly, our study demonstrated two key advancements. Firstly, compared to traditional 2D cultures, the 3D microvessels model exhibits higher eNOS activity, potentially reflecting a more physiological environment for endothelial cells. Secondly, the optimized MS method offers a valuable tool for measuring nitric oxide (NO) marker metabolites. This method allowed us to quantify both extracellular and intracellular levels, providing a comprehensive picture of NO metabolism (metabolic flux). Furthermore, the MS assay offers distinct advantages: 1) Medium/Plasma Perfusion: By measuring the conversion of labelled arginine to citrulline in the perfused medium

(extracellular), we can assess the impact of the perfused solution (medium or plasma) on eNOS activity. This approach is significantly simpler than extracting the content from endothelial cells. 2) Reduced Background: Unlike fluorescent probes, which suffered from background interference from the extracellular matrix, the MS method offers greater specificity for NO metabolites. Future investigations could explore higher shear forces for the pump system to simulate diverse microvascular environments with different endothelial cell types. Furthermore, we could optimize the current model to different types of organ-specific endothelial cells with higher shear stress. Additionally, validating the system by comparing its NO metabolism data to in vivo data would strengthen its credibility. This comparison would highlight how well the platform recapitulates the complex environment of a living organism.

Investigation of microvascular diseases

Microvascular dysfunction and COVID-19

Emerging evidence suggests microvascular disease plays a critical role in driving complications and death in severe COVID-19 patients [7–9]. Therapeutic potential of NO for the treatment of deadly COVID-19 is emerging research, already many studies showed the significance of NO in mitigating COVID-19-associated symptomatic complications [10,11]. However, in vitro human micro vessel models were not explored much to understand the NO metabolism in COVID-19. To explore the relationship between COVID-19 and NO, in **Chapter 3**, we mimicked the COVID-19 conditions by perfusing the patient plasma samples into the 3D microvessels-on-chip model. This study aimed to investigate the impact of COVID-19 plasma components on the 3D endothelial system, focusing on metabolites involved in NO production. We employed the 3D microvessels cell model described in Chapter 2, incorporating a collagen-based extracellular matrix and a microfluidic channel mimicking a micro vessel lumen, which served as an optimal in vitro platform to study the complex effects of COVID-19 on the vascular system. An observational pilot case-control study was conducted with plasma samples from two

patients diagnosed with mild COVID-19 (low viral load) and severe COVID-19 (high viral load). We perfused these plasmas through the 3D microvessels with NO enzyme stimulators and inhibitors and arginase inhibitors, allowing us to analyze the intricate interactions between endothelial cells and patient plasma components. This approach provided valuable insights into the mechanisms underlying COVID-19 pathogenesis and NO metabolites levels. Using a tracer-based UPLC-MS/MS method, we observed significant effects on NO metabolites in response to patient plasma perfusion. Supplementation with eNOS stimulators, the eNOS cofactor compound biopterin, and arginase inhibition resulted in higher labelled citrulline levels, indicating increased eNOS activity. Additionally, the interplay between arginase activity and eNOS activity provided insights into the regulatory pathways governing citrulline and ornithine metabolism depending on disease severity. A positive correlation between arginase activity and eNOS activity was observed in the severe COVID-19 patient model but not in the mild COVID-19 patient. These findings underscore the importance of endothelial dysfunction in COVID-19 pathogenesis and highlight potential therapeutic targets for mitigating vascular complications associated with the disease. Notably, our study observed distinct NO metabolite responses based on the patients' clinical data and disease severity. This highlights the model's exceptional capacity to differentiate between disease states. This understanding holds promise for identifying pathological markers associated with vascular diseases. This 3D microvessels model offered a valuable tool for investigating the impact of plasma components on endothelial cells, particularly considering its ability to capture detailed eNOS mechanisms. Furthermore, such models facilitate the evaluation of potential therapeutics and interventions targeting endothelial dysfunction, inflammation, thrombosis, and other vascular complications associated with COVID-19. This preliminary study underscored the need for additional patient samples to establish a broader conclusion. Moreover, insights from patients' pathological history, medication practices, and clinical reports pertaining to endothelial dysfunction would be valuable. Future studies would benefit from measuring downstream metabolites of BH₄, such as BH₂ and neopterin [12,13] in patient plasma before and after treatment with the 3D micro vessel model. This could provide additional validation to our study and also provide

insights into the importance of BH4 in the eNOS mechanism and potentially serve as a more effective therapeutic target.

Metabolic alterations in microvessels under hypoxic condition

Cardiovascular injury results from ischemia, a condition characterized by insufficient blood flow to tissues. This ischemia is often caused by a blockage within the artery walls, typically due to atherosclerosis (plaque buildup). Additionally, inflammation and damage to the endothelium (the inner lining of the artery) can further contribute to impaired blood flow. Hypoxia, a condition characterized by insufficient oxygen supply to tissues, disrupts vascular homeostasis and could lead to endothelial dysfunction. To understand the role of oxygen on NO metabolism in endothelial cells, **Chapter 4** investigated the response of endothelial cells to transient and persistent hypoxia, focusing on metabolic changes in both 2D static cultures and our established 3D microvessels model. Using stable isotope-labeled L-arginine, we tracked the eNOS pathway by monitoring downstream metabolites L-citrulline and L-ornithine and performed additional measurements of inflammation as it is reflected in signaling lipids. Understanding how endothelial cells adapt to hypoxia is vital for elucidating their role in various physiological and pathological processes.

Oxygen-dependent pathways, particularly those involving NO and signaling lipids, are crucial for maintaining vascular homeostasis and resolving inflammation. Our research revealed significant effects of low oxygen levels on the metabolism of NO and signaling lipids, especially within the 3D microvessels model. This model offered a promising tool for analyzing metabolite levels under both normoxic and hypoxic conditions, including the combined effects of hypoxia and shear stress, an understudied area in in vitro culture setups. Our findings suggested a distinct response between the culture models. Exposure to short term hypoxia in the 2D static environment led to increased oxidative stress metabolites such as HDoHE, HEPE, TriHome, and HpETE potentially resulting from the peroxidation of their substrates. Conversely, the 3D platform, incorporating flow, demonstrated regulation of eNOS-derived NO metabolites (L-citrulline⁺ and L-

ornithine+7) and lower levels of bioactive lipids such as 2,3, dinor-11b-PGF2 α and LPE:18.0, promoting an anti-inflammatory response. These observations were further supported by HIF1 α immunostaining analysis. Overall, this study unveiled a novel mechanism governing HIF1 α and flow-mediated metabolic regulation, potentially leading to new therapeutic strategies for addressing arterial endothelial dysfunction in prolonged hypoxic conditions. This study has certain limitations that warrant further investigation. Here, we focused on exploring the effects of hypoxia at two extremes: low oxygen and maximum hypoxia in short- and long-term exposure. Future studies could benefit from examining a wider range of oxygen concentrations and time of exposure to create a more comprehensive picture of how oxygen availability influences NO and signaling lipid metabolism. Additionally, this study focused on HIF-1 α and NO and signaling lipid metabolites. Investigating a wider range of hypoxia-responsive genes and metabolite expressions would offer a more comprehensive view of the cellular response to oxygen deprivation. Including tissue-specific markers would be particularly informative, as these markers can provide insights into the specific adaptation processes occurring within different tissues. Finally, while this study explored some aspects of the hypoxic response, a more in-depth analysis of metabolic pathways, such as energy metabolism, could provide a clearer understanding of how hypoxia regulates cellular function. Future studies could delve deeper into these pathways to elucidate the full extent of the hypoxic regulatory mechanism.

Advanced technologies for single-cell metabolism

Advancements in single-cell metabolomics techniques over recent decades have revealed a significant heterogeneity within cell populations. This newfound awareness presents a crucial challenge for personalized medicine: understanding how these variations impact individual health. To address this challenge, researchers utilized microfluidic technologies, offering a diverse range of approaches with varying levels of throughput and resolution for studying single-cell activity. Especially challenges at single-cell level are real, requiring efficient ways to sample a single cell and match this with a sensitive mass spectrometry method. **Chapter 5** provided a concise overview of the latest

techniques in single-cell sampling and detection methods for metabolomics, particularly focusing on efficient analysis by mass spectrometry. It summarized various microfluidic chip models used for active single-cell separation or sorting based on size, biophysical, acoustic properties, and fluorescent markers. These techniques offer researchers a comprehensive toolbox for evaluating biological properties and responses at the individual cell level, addressing biological, clinical, and pharmacological questions. The chapter demonstrated the applicability of these technologies to diverse cell types. This chapter also highlighted the importance of single-cell metabolomics. Unlike bulk tissue analysis, which might dilute important metabolites present only in a few cell types, single-cell analysis reliably extracts these crucial molecules. Additionally, it serves as a powerful tool for predicting phenotypes and other information with high precision. The combination with imaging mass spectrometry offers the potential to extract spatial information. Advancements in technology and analytical methods have enabled researchers to comprehensively analyze cellular metabolism, leading to the discovery of novel metabolic pathways and diseases.

To advance needed technologies for single-cell metabolomics, in **Chapter 6**, we successfully developed and demonstrated the potential of an integrated electrical lysis setup, the single cell electrical lysis and nanospray (SCEL-nS) platform to the Orbitrap mass spectrometry. This platform seamlessly integrated live single-cell mass spectrometry (LSC-MS) and similar micro sampling techniques for single-cell metabolomics. The development process involved two key steps. First, we designed and manufactured specialized sampling capillaries that facilitated single-cell collection, lysis via electrical stimulation, and subsequent introduction into the mass spectrometer through nanospray ionization. Second, we employed multi-physics software to conduct detailed simulations of the electric field and potential distribution across a cell as it traversed the electrodes within the capillary. These simulation results guided the design of a novel, modified interface specifically tailored for electrical lysis and nanospray MS. To validate the efficacy of the SCEL-nS platform, we employed a two-pronged approach. Firstly, we utilized fluorescence imaging to confirm complete cell lysis. Secondly, we performed MS measurements on single cells spiked with tamoxifen. This allowed us to assess the

efficiency of the lysis process and the subsequent increase in MS signal intensity. Our research addressed the significant challenges associated with single-cell metabolomics, particularly sensitivity and dynamic range. Within a single cell, metabolite concentrations can vary dramatically, ranging from extremely low levels (nanomolar) to highly abundant metabolites (millimolar) alongside intermediates and signaling compounds. The SCEN platform successfully addressed these challenges by delivering significantly higher peak area intensities compared to traditional LSC-MS methods. Notably, this platform achieved efficient cell lysis with minimal dilution, a crucial advantage for preserving precious cellular metabolites. Overall, the MS-compatible SCEN platform emerges as a highly promising solution for future single-cell studies due to its automation potential and high-throughput capabilities. Future improvements should focus on developing more precise and automated methods for single-cell sampling which would improve the efficiency and accuracy of our platform. This could involve microfluidic technologies or robotic manipulation systems capable of isolating individual cells with minimal disruption. Seamless integration of our single-cell sampling methods with microfluidic platforms would enable streamlined analysis workflows. This could involve miniaturized, automated systems that handle cell isolation, lysis (if needed), and downstream analysis steps on a single chip. To broaden the applicability of our platform, testing it with a wider range of drugs and cell types would be crucial. This would involve systematically evaluating the platform's performance across different pharmacological agents and cell lines/tissues. By demonstrating its effectiveness with various drugs and cell types, we can establish the platform's versatility and robustness.

Future Perspectives

Metabolomics technologies play a crucial role in discovering metabolic biomarkers, which aid in clinical diagnosis, prognosis, and therapy evaluation. Notably, tracer-based metabolomics offers a powerful tool for dissecting precise metabolic pathways and identifying dysfunctional pathways in disease states.

Additionally, a complex in vitro model incorporating endothelial cells, smooth muscle cells, and pericytes would provide deeper insights into NO function by mimicking the physiological microvasculature. Future research should explore NO levels in specific disease contexts, correlating them with disease markers and patient history. This could reveal how NO levels change during inflammation (potentially higher) and vasoconstriction (potentially lower). Furthermore, an extensive molecular network integrating eNOS regulatory mechanism and other metabolites as well as associated proteins and genes are crucial for drug discovery and targeted therapy.

Future method development could focus on absolute metabolite quantification with chemically similar analog internal standards to allow to compare studies better. In addition, the method could be further extended in applicability by incorporating enzyme controls and profiling diverse biospecimens. With this methodology we would aim for having real impact in assisting drug development, clinical diagnosis and treatment. Close collaboration with clinical experts is crucial to ensure research relevance and facilitate follow-up studies for validating our preliminary findings. Collaboration with clinicians could facilitate the translation of our in vitro model to clinical practice. This would involve testing promising interventions, such as angiotensin-converting enzyme inhibitors [14], angiotensin AT1 receptors blockers [15], angiotensin-(1-7), antioxidants, sphingosine-1-phosphate and statins on the model and comparing the results with improved endothelial function/ NO production in patients undergoing these treatments. This approach would allow us to assess whether the model accurately reflects NO production changes observed in clinical settings. Moving forward, we should refine our strategy by integrating clinical and mechanistic studies, while combining metabolomics data with multi-omics data. This comprehensive approach using systems biology will pave the way for personalized medicine.

Additionally, this approach could be readily adapted to analyze various cell types and responses to treatments. Such advancements hold promise for identifying sensitive and low-abundant metabolic biomarkers within the complex landscape of heterogeneous cell populations. 3D microvessels-on-chip coupled with isotope-labeled tracers and single-

cell methods hold immense promise for future NO metabolite research. This powerful combination offers a physiologically relevant, controlled, and high-throughput platform. It allows us to delve deeper into the complexities of NO biology, paving the way for novel therapeutic strategies. The integration of single-cell metabolomics with microfluidic techniques further strengthens this approach. This enables high-throughput, precise analysis of cellular responses at the single-cell level, accelerating the development of targeted and personalized therapeutic strategies.

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Nederlandse Samenvatting

De dodelijkste ziekten van de wereld zijn hart- en vaatziekten. De belangrijkste oorzaken van hiervan zijn een disfunctie van de enkelvoudige cellaag aan de binnenkant van bloedvaatjes (het endotheel) en aandoeningen van de allerkleinste bloedvaatjes van het lichaam (microvasculair). Ondanks dat er vooruitgang is geboekt in het onderzoek naar endotheeldisfunctie, ontbreken de complexiteit van menselijke weefsels bij traditionele *in vitro* modellen. Deze modellen missen vaak kenmerken zoals doorbloeding, interacties tussen cellen en een juiste extracellulaire matrix (ECM). Het beschikken over nauwkeurige modellen van de allerkleinste bloedvaatjes is cruciaal, met name voor cardiovasculaire ziekten, omdat diermodellen vaak tekortschieten in het nabootsen van de menselijke situatie. Vaatjes geplaatst op een microchip, oftewel vaatjes-op-een-chip, bootsen bloedvaatjes na en bieden daarmee een mogelijkheid om de beperkingen van traditionele modellen te overwinnen. De ontwikkeling van deze vaatjes verloopt erg vlot, waarschijnlijk omdat er kennis van verschillende disciplines, zoals bloedvatfysiologie, transportverschijnselen, cel- en matrixbiologie, en weefsel microfabricage samen gebruikt worden. Deze geavanceerde celkweektechnieken reproduceren onder gecontroleerde omstandigheden met succes endotheelcellen, 3D-ECM en structurele onderdelen van bloedvaatjes uit de microcirculatie. Hierdoor vormen ze een waardevolle aanvulling op *in vivo* studies van microvasculaire functie. Vooruitgang op het gebied van microfabricage en microfluidica, in combinatie met een beter begrip van vasculaire biologie, hebben de ontwikkeling van de vaatjes-op-een-chip sterk bevorderd. Al is verdere integratie van technieken op microschaal met cel- en moleculaire biologische methoden cruciaal, voor hun toekomstige optimalisatie.

Microfluidische modellen met vloeistofcirculatie zijn bijzonder waardevol voor het bestuderen van vaatverwijdende verbindingen zoals stikstofmonoxide (NO, *nitric oxide*), omdat mate van stroming de productie van NO reguleert. NO heeft antivirale eigenschappen en speelt een cruciale rol bij verschillende ziekten. Directe meting

van NO in het bloed is echter lastig vanwege zijn korte halfwaardetijd en beperkingen van bestaande meettechnieken. Metabolomics (onderzoek naar stofwisselingsproducten) biedt een alternatief door een momentopname van het NO-metabolisme te geven via zijn metabolieten. Het bestuderen van NO-metabolieten is een opkomende techniek om metabole routes te analyseren die betrokken zijn bij NO-mechanismen. Belangrijk is dat stabiel gelabelde isotopen, in combinatie met metabolomics, een specifiek beeld geven van de metabole route van NO, waarbij deze zonder verstoring van andere routes gevolgd kan worden. Bovendien verbeteren gepersonaliseerde geneeskunde (behandeling op maat) en doelgerichte therapieën substantieel als er rekening gehouden wordt met de eigenschappen van individuele cellen. Technologie waarmee losse cellen gemeten kunnen worden (*Single-cell-technologieën*) vertegenwoordigen een snelgroeiend onderzoeksveld dat klaar staat om de kloof tussen traditionele *in vitro* analyses en het begrip van cel heterogeniteit te overbruggen. Aldus is het doel van dit proefschrift de ontwikkeling van een massaspectrometriemethode (MS-methode) voor het analyseren van metabolieten die betrokken zijn bij NO-productie aan de hand van een fysiologisch relevant 3D-microbloedvatjes-op-een-chipmodel. Het uiteindelijke doel is om deze MS-methode en het 3D-model te gebruiken om ziekteverschijnselen en de invloed van hypoxie op bloedvaten te onderzoeken. Daarnaast onderzoekt dit proefschrift geavanceerde technieken voor de analyse van metabolieten op het niveau van de losse cel.

Om metabole routes te onderzoeken kunnen er zogenoemde tracers gebruikt worden, die gevolgd kunnen worden door een 3D-model. Het kerndoel van op tracer gebaseerde metabolomics is het identificeren en volgen van specifieke metabolieten binnen een bepaalde route. **Hoofdstuk 2** richtte zich op de ontwikkeling van een MS-methode voor het meten van metabolieten die betrokken zijn bij NO-productie met behulp van een 3D-microbloedvatjes-op-een-chipmodel.

We ontwikkelden een ultra-performance vloeistofchromatografie (UPLC)- MS methode voor het kwantificeren van isotoop-gelabeld L-arginine (NO-substraat) en zijn downstream metabolieten, L-citrulline en L-ornithine. Om ons begrip verder te

verfijnen, gebruikten we een stimulator en remmer van het enzym endotheliale stikstofmonooxidesynthase (eNOS), naast een remmer van de ornithine productie (arginase enzym). Deze test volgde NO-metaboliëten effectief onder stimulatie- en remmingsomstandigheden.

Om de activiteit van eNOS in cellen te bevestigen werd een test met een referentiekleuringsstechniek in een 2D-multititerplaat uitgevoerd. De test gaf de verwachte resultaten van eNOS onder invloed van arginase stimulators en -remmers, wat zijn specificiteit aantoonde. Vervolgens werd dezelfde methode ingezet om eNOS-activiteit te meten bij twee verschillende kweekomstandigheden. Eén met 2D-cultuur, die gebruikmaakte van standaard multititerplaten voor celkweek zonder vloeistofstroming, diende als het initiële onderzoeksplatform. De andere met 3D-cultuur waarbij er twee stroomrichtingsinstellingen getest werden. Het platform met de eenrichtingsstroom maakte gebruik van een microfluidische pomp en de andere opstelling gebruikte een tuimelplatform dat vloeistofbeweging in twee richtingen nabootst. De stroombeweging en het gevolg ervan werden gevalideerd aan de hand van de genexpressie. Het onderzoek beschreven in hoofdstuk 2, heeft twee belangrijke verbeteringen opgeleverd voor het onderzoek naar NO-metabolisme. Ten eerste, vertoonde het 3D-microbloedvatmodel een hogere eNOS-activiteit ten opzichte van de traditionele 2D-celkweek, wat mogelijk een meer fysiologische omgeving voor endotheelcellen weerspiegelt. Ten tweede, bood de geoptimaliseerde MS-methode een waardevol gereedschap voor het meten van markermetaboliëten van NO. Hiermee kunnen van zowel intra- als extracellulaire niveaus worden gekwantificeerd, wat een uitgebreid beeld van NO-metabolisme (metabole flux) weergeeft. De MS-metingen bieden significante voordelen ten opzichte van traditionele methoden. Ten eerste, kunnen door middel van celkweekmedium/bloedplasma-perfusie de eNOS-activiteit beoordelen worden door de omzetting van gelabeld arginine tot -citrulline in het extracellulaire perfusiemedium te meten. Deze benadering is aanzienlijk eenvoudiger dan het extraheren van de inhoud uit endotheelcellen. Ten tweede biedt de MS-methode, in tegenstelling tot fluorescerende markers die last hebben van achtergrondstoring door

de extracellulaire matrix, een hoogwaardige specificiteit voor NO-metabolieten. Deze voordelen zorgen voor accuratere en betrouwbaardere metingen.

Het therapeutische potentieel van NO voor de behandeling van COVID-19 krijgt steeds meer aandacht en studies benadrukken het belang van NO bij het verminderen van de symptomatische complicaties. Echter, is er beperkt onderzoek gedaan bij *in vitro* modellen van menselijke microbloedvaten om NO-metabolisme bij COVID-19 te begrijpen. Om dit doel te bereiken, werd in **Hoofdstuk 3** de mogelijkheid van NO-metabolieten als diagnostische- en ziekteverloopmarkers in COVID-19 onderzocht, door gebruik te maken van de in **Hoofdstuk 2** ontwikkelde microbloedvaatjes en het geoptimaliseerde, op tracers gebaseerde meetplatform. We bereikten dit door een COVID-19 situatie na te bootsen door bloedplasma van zowel mild als ernstig zieke patiënten te perfuseren in de 3D-microbloedvaatjes. Door gebruik te maken van verscheidene stimulators en remmers, konden we interacties tussen endotheelcellen en plasmacomponenten analyseren. Suppletie met eNOS stimulators, de eNOS-cofactor biotine en een arginaseremmer resulteerden in hogere niveaus van gelabeld citrulline, wat duidt op verhoogde eNOS activiteit. Daarnaast gaf de wisselwerking tussen arginase- en eNOS-activiteit, afhankelijk van de ernst van de ziekte, inzicht in de regulatorroutes die het metabolisme van citrulline en ornithine aansturen. Een positieve correlatie werd alleen gevonden tussen de arginase- en eNOS-activiteit in het model van ernstige COVID-19 patiënten, wat het potentieel van het model benadrukt om onderscheid te maken tussen ziekte toestanden. Deze bevindingen ondersteunen het belang van endotheel disfunctie in de pathogenese van COVID-19 en het stellen potentiële therapeutische doelwitten om vasculaire complicaties te verminderen. In onze studie werden duidelijke reacties van NO-metabolieten waargenomen op basis van de klinische gegevens en de ernst van de ziekte. Dit benadrukt de uitzonderlijke capaciteit van het huidige model om onderscheid te maken tussen ziekte toestanden. Dit inzicht biedt veelbelovende mogelijkheden voor het identificeren van pathologische markers die in verband gebracht worden met de ernst van vaatziekten. In het algemeen biedt dit 3D-microbloedvatmodel een waardevol hulpmiddel voor

onderzoek naar de invloed van componenten uit het bloedplasma op endotheelcellen, vooral door hun mogelijkheid om eNOS-mechanismen gedetailleerd in beeld te brengen. Bovendien zijn dergelijke modellen veelbelovend voor het evalueren van potentiële geneesmiddelen en interventies gericht op het verhelpen endotheeldisfunctie, ontsteking, trombose en andere vasculaire complicaties, die samenhangen met COVID-19.

Hoofdstuk 4 onderzocht hoe hypoxie het metabolisme van het endotheel en de productie van NO beïnvloedt, waarbij de geoptimaliseerde methoden van Hoofdstuk 2 zijn gebruikt. Er zijn zowel NO-metabolieten als een set signaallipiden gemeten in de 3D-microbloedvatjes. De 3D microbloedvatjes zijn zowel kort als langdurig gekweekt onder hypoxische omstandigheden en vergeleken met traditionele 2D-celkweek. Het doel hiervan was inzicht te verkrijgen, in hoe hypoxie de activiteit van eNOS en het metabolisme van bioactieve lipiden en de rol van schuifspanning (*shearing stress*) beïnvloedt. Deze studie onthulde duidelijk verschillende reacties tussen de modellen. Blootstelling aan kortdurende hypoxie in de statische 2D-omgeving leidde tot een toename van oxidatieve stressmetabolieten mogelijk als gevolg van peroxidatie van hun substraten. Daarentegen demonstreerde het 3D-platform, wat gecombineerd was met vloeistofstroom, de regulatie van eNOS-afgeleide NO-metabolieten (L-citrulline en L-ornithine) en lagere spiegels van bioactieve lipiden die een ontstekingsremmende reactie bevorderen. Dit toont een mogelijke ontstekingsremmende respons aan in het 3D-model. Deze bevindingen werden verder ondersteund door de analyse van HIF1 α , een belangrijke regulator van hypoxische reacties. Over het geheel genomen toonde ons onderzoek een nieuw mechanisme aan, waarbij HIF1 α en metabole regulatie onder invloed van vloeistofstroom in endotheelcellen betrokken zijn. Deze kennis zou kunnen leiden tot nieuwe therapeutische strategieën voor het behandelen van endotheeldisfunctie veroorzaakt door langdurig lage zuurstofniveaus. Bovendien, onderstreept deze gecombineerde benadering het belang van onderzoek naar zowel het NO-metabolisme als de bredere metabole respons op hypoxie, voor een vollediger begrip van vaatgezondheid en -ziekten.

Variaties binnen celpopulaties vormen een uitdaging voor gepersonaliseerde geneeskunde. *Single-cell*-metabolomics onthult deze verborgen heterogeniteit binnen celpopulaties om te kunnen onderzoeken wat de impact hiervan is op individuele gezondheid. Microfluïdische technologieën bieden diverse benaderingen voor het bestuderen van activiteit op eencellig niveau; echter, het isoleren van losse cellen en ze direct meten met een MS blijft moeilijk. **Hoofdstuk 5** gaat in op de nieuwste bemonsterings- en detectiemethoden voor metabolomics op eencellig niveau met behulp van de MS. Het behandelt microfluïdische chips voor scheiding van losse cellen, gebaseerd op grootte, fluorescente markers, biofysische- en akoestische kenmerken. Met deze technieken kunnen onderzoekers biologische reacties op eencellig niveau evalueren in verschillende celtypen. *Single-cell* metabolomics biedt duidelijke voordelen ten opzichte van bulkweefselanalyse. In tegenstelling tot bulkanalyse, waarbij cruciale metabolieten van specifieke celtypen verdund kunnen worden door de hoeveelheid cellen, meten *single-cell* methoden deze moleculen op een betrouwbare manier in één cel. Dit maakt zeer nauwkeurige voorspelling van fenotypes mogelijk. De combinatie hiervan met beeldvormende MS biedt bovendien de mogelijkheid om ruimtelijke informatie te verkrijgen over de cellen. Vooruitgang in technologie en deze methoden heeft de analyse van cellulair metabolisme revolutionair veranderd en heeft geleid tot de ontdekking van nieuwe metaboleroutes en ziekten.

Hoofdstuk 6 introduceert het *Single cell electrical lysis-nano Spray* (SCEL-nS) platform, een geïntegreerde elektrische lysis opstelling voor *single-cell* metabolomics gekoppeld aan Orbitrap MS. Dit platform integreert naadloos met live *single-cell* MS (LSC-MS) en vergelijkbare technieken. De ontwikkeling omvatte twee belangrijke stappen; ten eerste vergemakkelijkten gespecialiseerde capillairen de opname van losse cellen, elektrische lysis en de invoer door middel van *nanospray* ionisatie in de MS. Ten tweede, leidden multi-fysische software simulaties tot het ontwerp van een nieuwe interface die geoptimaliseerd is voor elektrische lysis en *nanospray* MS. Wij valideerden het SCEL-nS platform door

middel van fluorescentiemicroscopie om volledige cellysis te bevestigen en MS-metingen uit te voeren op tamoxifen-behandelde cellen. Dit toetste de lysis efficiëntie en de toename van de tamoxifen signaal.

Single-cell metabolomics staat voor een uitdaging door de enorme variaties in de concentraties van metabolieten tussen individuele cellen. Het SCEL-nS platform pakt dit aan door aanzienlijk hogere piekwaarden te geven in vergelijking met traditionele LSC-MS methoden. Daarbij zorgt het voor efficiënte lysis met minimale verdunning, waardoor kostbare metabolieten behouden blijven. Over het algemeen komt het SCEL-nS platform naar voren als een veelbelovend hulpmiddel voor toekomstig onderzoek naar individuele cellen, dankzij het potentieel voor automatisering en hoge doorvoersnelheid, wat het goed compatibel maakt met werkstromen voor MS.

Hoofdstuk 7 verzamelt de belangrijkste bevindingen van elk voorgaand hoofdstuk, wat een uitgebreid overzicht van het onderzoek naar NO-metabolisme en *single-cell* analysetechnieken biedt. Het gaat dieper in op hoofdstuk-specifieke inzichten en het schetst veelbelovende toekomstbeeld voor het onderzoeken naar de invloed van NO en de potentie van geavanceerde *single-cell* methoden.

Summary

Endothelial dysfunction and microvascular diseases are leading causes of cardiovascular disease, the world's deadliest illness. While research on endothelial dysfunction has progressed, traditional in vitro models lack the complexity of human tissues. These models often miss features like blood flow, cell interactions, and a proper extracellular matrix (ECM). Accurate micro vessel models are crucial, especially for cardiovascular diseases (CVDs), as animal models often fail to replicate human conditions. Microfabricated vessels, or "vessels-on-a-chip," mimic blood vessels and offer potential to overcome limitations of traditional models. Their rapid development likely stems from the merging of several disciplines: blood vessel physiology, transport phenomena, cell and matrix biology, and tissue microfabrication. These advanced cell culture tools successfully recreate endothelial cells, 3D ECM, and structural components of microcirculation vessels under controlled conditions. This makes them a valuable complement to in vivo studies of microvascular function. Advancements in microfabrication and microfluidics, combined with a deeper understanding of vascular biology, have fuelled their development. Further integration of microscale engineering with cell and molecular biology methods is crucial for their continued improvement.

Microfluidic models with flow are particularly valuable for studying vasodilatory compounds like nitric oxide (NO), as flow regulates NO production. NO plays a crucial role in various diseases and has antiviral properties. However, direct NO measurement is difficult due to its short half-life and limitations of existing techniques. Metabolomics offers an alternative by providing a snapshot of NO metabolism through its metabolites. Studying NO metabolites is a trending technique to analyze pathways involved in NO mechanisms. Importantly, stable isotope labelling coupled with metabolomics offers a more specific view of the NO pathway, tracing it without interference from other pathways. Furthermore, personalized medicine and targeted therapies benefit greatly from considering individual cellular characteristics. For that, single-cell technologies represent a

rapidly evolving field poised to bridge the gap between traditional in vitro analysis and understanding cellular heterogeneity. Therefore the scope of this thesis is to develop a mass spectrometry method to analyze metabolites involved in NO production using a physiologically relevant 3D microvessels-on-chip model. The ultimate goal was to use this method and the 3D model to investigate disease conditions and the impact of hypoxia on blood vessels. Furthermore, the thesis explored advanced techniques for analyzing metabolites at the single-cell level.

The core goal of tracer-based metabolomics is pinpointing and tracking specific marker metabolites within a particular pathway. **Chapter 2** focused on developing a mass spectrometry method to measure metabolites involved in NO production using a 3D microvessels-on-a-chip model.

We established a stable isotope Ultra-performance liquid chromatography (UPLC)-mass spectrometry (MS)/MS method to quantify isotope-labeled L-arginine (NO substrate) and its downstream metabolites, L-citrulline and L-ornithine. Exact identification of these isotopologue peaks relied on the m/z ratio of each calculated metabolite transition state. To further refine our understanding, we utilized endothelial nitric oxide synthase (eNOS) enzyme stimulator and inhibitor, alongside an ornithine (arginase enzyme) production inhibitor. This assay effectively tracked NO metabolites under stimulation and inhibition conditions.

For method validation in a 2D well plate format, we confirmed eNOS expression using a reference staining technique (DAF-2DA). Additionally, the assay yielded expected results with eNOS and arginase stimulators and inhibitors, demonstrating its specificity. Subsequently, the same method was employed to measure eNOS activity in two distinct culture conditions. One with 2D culture, that uses standard cell culture format using 2D well plates served as the initial study platform. Another with 3D culture, we explored two flow configurations. The unidirectional flow platform uses microfluidic pump and another configuration uses rocker platform that mimics bidirectional fluid movement. The flow movement and its impact was

validated with the gene expression. Mechanosensitive gene expressions measured in cells from all platforms confirmed these flow configurations. Notably, our study achieved two key advancements. First, compared to traditional 2D cultures, the 3D micro vessel model exhibited higher eNOS activity, potentially reflecting a more physiological environment for endothelial cells. Second, the optimized MS method provides a valuable tool for measuring nitric oxide (NO) marker metabolites. It allows quantification of both extracellular and intracellular levels, offering a comprehensive picture of NO metabolism (metabolic flux). The MS assay offers significant advantages over traditional methods. Firstly, through medium/plasma perfusion, we can assess the impact of the perfused solution (medium or plasma) on eNOS activity by simply measuring the conversion of labeled arginine to citrulline in the perfused medium (extracellular). This approach is considerably simpler than the complex process of extracting content from endothelial cells. Secondly, unlike fluorescent probes that suffer from background interference caused by the extracellular matrix, the MS method offers superior specificity for NO metabolites. This advantage ensures more accurate and reliable measurements.

While the therapeutic potential of NO for COVID-19 treatment is gaining traction, with studies highlighting its significance in mitigating symptomatic complications, limited research has explored in vitro human micro vessel models to understand NO metabolism in COVID-19. To address this, **Chapter 3** aimed to investigate the potential of nitric oxide metabolites as diagnostic and disease progression markers in COVID-19 using the microvessels and optimized tracer-based nitric oxide metabolite measurement platform developed in Chapter 2. We achieved this by mimicking a COVID-19 environment through perfusion of patient plasma samples (both severe and mild conditions) into the 3D microvessels with various inhibitors and stimulators allowed us to analyze interactions between endothelial cells and plasma components. Supplementation with eNOS stimulators, the eNOS cofactor compound biopterin, and arginase inhibition resulted in higher labelled citrulline levels, indicating increased eNOS activity. Additionally, the interplay between arginase activity and eNOS activity provided insights into the regulatory pathways

governing citrulline and ornithine metabolism depending on disease severity. A positive correlation between arginase activity and eNOS activity was observed only in the severe COVID-19 patient model, highlighting the potential for the model to differentiate between disease states. These findings underscore the importance of endothelial dysfunction in COVID-19 pathogenesis and propose potential therapeutic targets for mitigating vascular complications. Notably, our study observed distinct NO metabolite responses based on the patients' clinical data and disease severity, showcasing the model's exceptional capacity to differentiate between disease states. This understanding holds promise for identifying pathological markers associated with vascular diseases. Overall, this 3D microvessels model offers a valuable tool for investigating the impact of plasma components on endothelial cells, particularly considering its ability to capture detailed eNOS mechanisms. Furthermore, such models hold promise for evaluating potential therapeutics and interventions targeting endothelial dysfunction, inflammation, thrombosis, and other vascular complications associated with COVID-19.

Chapter 4 investigated how hypoxia affects endothelial cell metabolism and NO production. We used the optimized methods from Chapter 2 to measure both NO metabolites and a panel of signaling lipids under transient and persistent hypoxic conditions in our 3D microvessels and compared them to traditional 2D cell cultures. This approach aimed to understand how hypoxia influences eNOS activity, bioactive lipid metabolism, and the role of shear stress. The study revealed distinct responses between the models. Exposure to short term hypoxia in the 2D static environment led to increased oxidative stress metabolites such as HDoHE, HEPE, TriHome, and HpETE potentially resulting from the peroxidation of their substrates. Conversely, the 3D platform, incorporating flow, demonstrated regulation of eNOS-derived NO metabolites (L-citrulline⁺ and L-ornithine⁺) and lower level of bioactive lipids such as 2,3, dinor-11b-PGF2 α and LPE:18.0, promoting an anti-inflammatory response. This suggests a potentially anti-inflammatory response in the 3D model. These findings were further supported by analysis of HIF1 α , a key

regulator of hypoxic responses. Overall, our research highlights a novel mechanism involving HIF1 α and flow-mediated metabolic regulation in endothelial cells. This knowledge could lead to new therapeutic strategies for addressing endothelial dysfunction caused by prolonged low oxygen conditions. This combined approach underscores the importance of studying both NO metabolism and the broader metabolic response to hypoxia for a more complete understanding of vascular health and disease.

Single-cell metabolomics unveils hidden heterogeneity within cell populations, posing a challenge for personalized medicine. How these variations influence individual health remains unclear. Microfluidic technologies offer diverse approaches for studying single-cell activity, but isolating single cells and coupling them with mass spectrometry remains difficult. **Chapter 5** explores the latest single-cell sampling and detection methods for metabolomics, focusing on mass spectrometry analysis. It reviews microfluidic chips for single-cell separation based on size, biophysical properties, acoustics, and fluorescent markers. These techniques empower researchers to evaluate biological responses at the individual cell level across diverse cell types. Single-cell metabolomics offers distinct advantages over bulk tissue analysis. Unlike bulk analysis, which can dilute crucial metabolites from specific cell types, single-cell methods reliably capture these molecules. This allows for high-precision prediction of phenotypes and other information. Furthermore, combining this with imaging mass spectrometry holds the potential to extract spatial information. Advancements in technology and methods have revolutionized cellular metabolism analysis, leading to the discovery of novel pathways and diseases.

Chapter 6 introduces the SCEL-nS platform, an integrated electrical lysis setup for single-cell metabolomics coupled with Orbitrap mass spectrometry. This platform seamlessly integrates with live single-cell MS (LSC-MS) and similar techniques. Development involved two key steps. First, specialized capillaries facilitated single-cell collection, electrical lysis, and nano spray introduction into the mass spectrometer. Second, multi-physics software simulations guided the design of a

novel interface optimized for electrical lysis and nano spray MS. We validated the SCEL-nS platform through fluorescence imaging for complete cell lysis and MS measurements on tamoxifen-spiked cells. This assessed lysis efficiency and signal intensity increase. Single-cell metabolomics faces challenges due to vast variations in metabolite concentrations within a single cell. The SCEL-nS platform addresses this by delivering significantly higher peak intensities compared to traditional LSC-MS methods. Additionally, it achieves efficient lysis with minimal dilution, preserving precious metabolites. Overall, the SCEL-nS platform emerges as a promising tool for future single-cell studies due to its automation potential and high-throughput capabilities, making it compatible with mass spectrometry workflows.

Chapter 7 consolidates the key findings from each chapter, providing a comprehensive understanding of the research on NO metabolism and single-cell analysis techniques. It delves deeper into chapter-specific insights and outlines exciting future directions for exploring the significance of NO and the potential of advanced single-cell methods.

Curriculum Vitae

Kanchana Pandian was born on March 6, 1992, coinciding with the year the scientific community named Nitric Oxide as "Molecule of the Year."

She earned her Bachelor's degree at 2011, from Holy Cross College, Trichy, India, excelling with distinction and ranking second. During this time, she also completed a Diploma in Management and Laboratory Techniques (DMLT), earning first-class certification in clinical analysis and laboratory management. Her research journey began with a project on "Study of cardiac marker - heart-specific fatty acid binding protein (FABP)" at Doctors Diagnostic Canter, Trichy, sparking her enduring interest in the field.

In 2011, Kanchana Pandian began her Master's in Advanced Biochemistry at the University of Madras, with a joint research opportunity at the Indian Institute of Technology, Bombay (IITB). She interned at IITM (IIT Madras), and worked on "Isolation and Purification of Folate Receptors from Human Placenta," provided her with valuable experience in protein chemistry and analysis. For her Master's thesis at the Neurobiology Laboratory, IIT Bombay, under Prof. Samir Maji, she worked on "Study of Secretory Granule Biogenesis Using Growth and Prolactin Hormone". This experience allowed her to gain expertise in cell and molecular techniques. She was awarded an honorarium fellowship for her active contribution during this project. Encouraged by her growing interest, Kanchana applied for an international summer program (2015) at Leiden University, The Netherlands. During the program, she gained exposure to three major clusters: drug and target discovery, biotherapeutics, and systems biomedicine. She was awarded €500 for her motivation and active participation in research. Following the summer program, Kanchana sought an interdisciplinary scientist role and secured a Project Associate position (2017) in the Mechanical Engineering Department at IIT Madras, India focusing on microfluidics. This role expanded her perspective on bridging conventional techniques with physiologically relevant in vitro and ex vivo models for cancer research.

With a strong background in biology and microfluidics, Kanchana secured a PhD position in July 2019 at Leiden University under Prof. Thomas Hankemeier (MAC, LACDR) and Prof. Anton Jan van Zonneveld (Eindhoven Laboratory, LUMC). As an early-stage researcher in the LogicLab consortium (funded by the EU's Horizon 2020 program through the Marie Skłodowska-Curie grant), she pioneered an HPLC-MS tracer-based metabolomics method for precise detection of nitric oxide metabolites and bipterin compounds. Kanchana also developed a 3D blood-vessels-on-chip model and bioassays to study metabolic changes in vascular dysfunctions, including aging, COVID-19, and hypoxia. Additionally, she was involved in Single Cell research and contributed to high-sensitivity live single-cell mass spectrometry using integrated off-chip and on-chip electrical lysis techniques. She collaborated effectively with physicists, electrical and mechanical engineers, and biologists, actively participating in national and international conferences, delivering both oral and poster presentations. Kanchana presented her research at LogicLab consortium biannual meetings

and other notable events, such as the European Society for Microcirculation Conference in Berlin (2022), where she received a travel award for her outstanding poster. She also participated in the Basic Vascular Science Conference (2022), Euroanalysis (2023), and LACDR Spring Symposiums (2020, 2021). After her PhD, she plans to pursue a career as a scientist in a company that focuses on areas such as drug analysis, 3D organ-on-chip model studies, and biotherapeutics.

List of Publications

Part of this thesis

1. Tracer-based metabolomics for profiling nitric oxide metabolites in a 3D microvessels-on-chip model (*Chapter 2*)
Kanchana Pandian, LuoJiao Huang, Abidemi Junaid, Amy Harms, Anton Jan van Zonneveld*, Thomas Hankemeier*. *The FASEB Journal*. (2024);38(16):370005. doi: [10.1096/fj.202400553R](https://doi.org/10.1096/fj.202400553R)
2. Microvessels-on-chip: Exploring endothelial cells and COVID-19 plasma interaction with nitric oxide metabolites (*Chapter 3*)
Kanchana Pandian, Rudmer Postma, Anton Jan van Zonneveld, Amy Harms* and Thomas Hankemeier*. *Nitric Oxide*. (2024) 12-19. doi: [10.1016/j.niox.2025.01.002](https://doi.org/10.1016/j.niox.2025.01.002)
3. Metabolic alterations of endothelial cells under transient and persistent hypoxia: study using a 3D microvessels-on-chip model (*Chapter 4*)
Kanchana Pandian, Anton Jan van Zonneveld, Amy Harms* and Thomas Hankemeier*. *Tissue Barriers*. (2024):2431416. doi: [10.1080/21688370.2024.2431416](https://doi.org/10.1080/21688370.2024.2431416)
4. Advances in single-cell metabolomics to unravel cellular heterogeneity in plant biology (*Chapter 5*)
Kanchana Pandian, Minami Matsui, Thomas Hankemeier, Ahmed Ali*, Emiko Okubo-Kurihara*. *Plant Physiology*. (2023) 1 -17. doi: [10.1093/plphys/kiad357](https://doi.org/10.1093/plphys/kiad357)
5. Enabling high-sensitivity live single-cell mass spectrometry using an integrated electrical lysis and Nano electrospray ionization interface (*Chapter 6*).
Kanchana Pandian, Luís Daniel de Aguiar Homem e Almeida de Matos, Laura A. Hetzel, Raphaël Zwier, Peter van Veldhuizen, Charelle Schubert, Jayaprakash Karuppusamy, Amy C. Harms, Ahmed Ali*, and Thomas Hankemeier*. *Analytica Chimica Acta*. 1324 (2024) 343068. doi: [10.1016/j.aca.2024.343068](https://doi.org/10.1016/j.aca.2024.343068)

Not part of this thesis

- Functional deterioration of vascular mitochondrial and glycolytic capacity in the aortic rings of aged mice. Agnieszka Karaś, Anna Bar, **Kanchana Pandian**, Agnieszka Jaształ, Zuzanna Kuryłowicz, Barbara Kutryb-Zajac, Elżbieta Buczek, Stefano Rocchetti, Tasnim Mohaissen, Agata Jędrzejewska, Amy C Harms, Patrycja Kaczara, Stefan Chłopicki. *Geroscience*. 2024 Aug;46(4):3831-3844. doi: [10.1007/s11357-024-01091-6](https://doi.org/10.1007/s11357-024-01091-6)

- Continuous electrical lysis of cancer cells in a microfluidic device with passivated interdigitated electrodes. **Kanchana Pandian**, Ajanth Praveen, Sazid Hoque, Sudeepthi Aremenda, Ashis sen.
Biomicrofluidics. 2020 Nov; 14(6): 064101. doi: [10.1063/5.0026046](https://doi.org/10.1063/5.0026046)
- Non-inertial lift induced migration for label-free sorting of cells in a co-flowing aqueous two-phase system. Shamik Hazra, Jayaprakash Karuppusamy, **Kanchana Pandian**, Abishek Raj, S Mitra, and Ashis Sen.
Analyst. 2019,144, 2574-2583. doi: [10.1039/C8AN02267D](https://doi.org/10.1039/C8AN02267D)
- Acoustic impedance-based size-independent isolation of circulating tumour cells from blood using acoustophoresis. Subramaniam Karthick, P Pradeep, **Kanchana Pandian** and Ashis Sen.
Lab Chip. 2018,18, 3802-3813. doi: [10.1039/C8LC00921J](https://doi.org/10.1039/C8LC00921J)

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Acknowledgement

I am deeply grateful to everyone who supported me during my PhD journey, helping me complete it successfully and creating cherished memories along the way.

I would like to express my heartfelt gratitude to **Prof. Dr. T. Hankemeier**. Dear Thomas, I am grateful for this opportunity, inspiring guidance, valuable discussions, and unwavering commitment to this research. Your encouragement and thoughtful suggestions allowed me to grow scientifically and stay focused on my goals, culminating in this dissertation.

Dr. A. J. van Zonneveld, dear Anton, from our first conversation, your enthusiasm for vascular biology, particularly the nitric oxide mechanism and immune cell interactions, was truly inspiring. Your guidance and our endless project discussions boosted my confidence during challenging times. I enjoyed your PhD stories, passion, insightful explanations, scientific drawings, and unwavering support throughout this journey.

Dr. A. C. Harms, dear Amy, I am deeply grateful for your indispensable support throughout this process and your constant suggestions for improving my scientific writing. You consistently inspired me to strive for perfection in every aspect of my work, emphasizing data management and providing timely feedback on all my articles. Without your guidance, this completion might have been delayed.

Dr. A. Ali, dear Ahmed, I'm appreciative for your valuable time spent discussing the single-cell project, and for the enjoyable moments during fun activities and dinner with the group, which I will always cherish. Your ongoing support was truly invaluable.

Dear **Nicolas, Charlie, Aga, Bert, Edinson, Alida, Leike, Anne-Charlotte**, I appreciate all your constant support and valuable discussions during focus-group meetings. I enjoyed thoroughly those scientific discussions.

Prof. Dr. Stefan Chlopcki, dear Stefan, I'm inspired by your enthusiasm for this research, your excellent collaboration, and your continued support for the publication. I also extend my gratitude to my consortium members at LogicLab ITN for the many insightful discussions and collaborations. I will always cherish the memorable experiences from our meetings across Europe.

Special thanks to dear **Cathy, Ina, & Ariadne** – You always assisting with scheduling meetings with supervisors, and for their helpful and friendly nature, which I will always remember.

I sincerely thank my master's students—**Laurens, Parjanya, Judith, Glenn, Charelle, Luis, & Adel**—for your amazing contributions and constant support. We collaborated to overcome

challenges, refine solutions, and shared enjoyable moments and friendly conversations that I will always cherish.

Dear **Raphael, Peter, & Douwe**, I sincerely thank you for your continuous support and collaboration with the opportunity to engage in multidisciplinary collaborations.

Dear **Abidemi**, thank you for providing the microfluidic pump for the 3D model and offering insightful suggestions for my work. Dear **Rudmer**, I am deeply grateful to you for your kindness, constant support and being a good friend.

Dear **Hetty, Wendy, Vincent, Zhan, Kaley**, thank you for your support and valuable discussions on my research. I also extend my gratitude to all my wonderful LUMC colleagues for their encouragement and engaging scientific conversations.

Dear **Tim, Gerwin, & Faisa**, my sincere thanks for your constant support in ordering supplies, assisting with fixing the MS instruments & helping me understanding MS method development.

Also, my sincere thanks to you all dear friendly BMFL employees – **Sabine, Esperanza, Zeinab, Charlotte, Jelte** - thanks a lot for your assistance and help in measuring samples. Thank you so much, dear **Marielle & Hyung** for your invaluable time spent writing the Dutch version of the summary.

Dear **Michael and Pascal**, thank you so much for your assistance with MZ quality and other data-related support. You were both so friendly, and I truly appreciated your help.

My dear officemate friends – **Joyee** (special thanks to you for assisting me with the tracer-based method development and lots of fun moments), **Cornelius, Wei Zhang, Yupeng, Congrou, Grace** – I can't forget all those fun filled and wonderful moments with lots of laughter.

Dear friends **Mariyana, Madhu, Manchu, Ashok, Aswin**, Thanks to you all for being such good friends and as part of my NL life. The memories with you all are special. Also, my special thanks to all my friends in India.

Dear **ZZZ, Farideh, Wei Yang, Bingshu, Pingping, Simon, Sam, Barbara, Isabelle, Marlien, Sabrina, Mengle, Laura, Ischa, Vincent, Yu, Lu, Chunyuan, Guus, Xinyu**. The time spent with all of you was truly special—comfortable, inclusive, and unforgettable. I cherished our fun conversations, laughter, lunch chats, dance parties, borrel talks and games, barbecues, karaoke nights, table tennis, & more. You all hold a special place in the pages of my memories. I hope we all stay in touch forever.

My dear family—**Mr. Pandian, Mrs. Selvi, Shanthi, Sangeetha, Kalpana, Hari, Ezhil, Chittu, Pattu, & Madhav**—your unwavering support & happiness has been my strength since the beginning of my PhD. Your encouragement has shaped who I am, and I couldn't have made it without you. I am truly blessed to have each of you in my life.