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REGULATION OF ENDOTHELIAL FATTY ACID UPTAKE BY VEGF-B: MECHANISMS AND THERAPEUTIC IMPLICATIONS

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Cover illustration: Artistic depiction of circulating fatty acid tracer in cerebral blood vessels.

Image acquired with epifluorescence using a two-photon microscope.

Regulation of endothelial fatty acid uptake by VEGF-B: mechanisms and therapeutic implications

Thesis for Doctoral Degree (Ph.D.)

By

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To the ones that matter and the ones that were always there.

To my family.

To Louisa.

Popular science summary of the thesis

Obesity, type 2 diabetes and their co-morbidities, including ischemic stroke and diabetic kidney disease, are increasing. Type 2 diabetes is characterized by insensitivity to insulin, a hormone known to stimulate sugar (glucose) uptake in cells. It is believed that insulin insensitivity is partially caused by accumulation of fatty acids in tissues outside of the specialized fat storing adipose tissue. For fatty acids to accumulate in tissues they first must be taken up from the blood. The endothelium lines the inner blood vessel wall and facilitates the passage of fatty acids and glucose from the blood to the underlying tissues. It is therefore especially interesting to study the endothelium in the context of obesity and type 2 diabetes. There are several factors identified that can increase endothelial fatty acid uptake and transport from the blood, including VEGF-B. Despite this, it remains largely unknown exactly how the process of endothelial fatty acid uptake works.

The content of this thesis demonstrates that fatty acid uptake by the endothelium is driven by a process known as lipolysis that mobilizes fatty acids from intracellular fat storage which are used to generate the required energy for fatty acid uptake. We discovered that the cells of the endothelium increase their rate of lipolysis when exposed to either the fatty acid oleic acid or when exposed to VEGF-B. This concept was studied both in ischemic stroke which occurs when a blood clot blocks a blood vessel in the brain, and in diabetic kidney disease characterized by the progressive failure of the kidney to filter and clean the blood. We observed that obese mice accumulated more fatty acids in blood vessels of the brain and in the cells of the kidney filter unit. These events were coupled with worse outcome after stroke and reduced kidney function. However, blocking VEGF-B activity reduced the degradation of fat stored in adipose tissue thus decreasing the amount of circulating fatty acids in the blood. Reducing blood fatty acids in turn reduces endothelial transport of fatty acids from the blood to the brain and the kidney. Blocking VEGF-B therefore prohibited fatty acids from accumulating in the brain resulting in lower incidence of brain swelling and bleedings, as well as protected against life-threatening side effects of a thrombolytic agent used in stroke treatment. Ameliorating fatty acid accumulation in the kidney filtration unit likewise improved kidney function in obese mice. In this thesis we therefore suggest targeting endothelial transport of fatty acids from the blood as a therapeutic strategy to ameliorate type 2 diabetes co-morbidities.

Abstract

The incidence of type 2 diabetes (T2D) and its co-morbidities including ischemic stroke and diabetic kidney disease (DKD) are increasing at an alarming rate. T2D patients typically present with high levels of circulating fatty acids (FAs) in the blood as well as increased tissue lipid droplet (LD) accumulation resulting in insulin resistance. The transport of FAs from blood to tissues therefore plays a key role in the development of insulin resistance and T2D. The endothelium facilitates FA and glucose uptake and transport from blood to the underlying tissues, but the contribution of dysregulated endothelial FA transport to ischemic stroke and DKD remain unclear. The metabolic pathways that govern endothelial FA uptake are also poorly understood since endothelial cells (ECs) rely almost exclusively on anaerobic glycolysis for energy consumption while mitochondrial ATP synthesis has been suggested to facilitate FA uptake.

In this thesis, we demonstrate that lipolysis drives endothelial FA uptake by promoting mitochondrial oxidation of FAs for ATP production. We provide evidence that both vascular endothelial growth factor-B (VEGF-B) stimulation and oleic acid exposure drive FA uptake by activating lipolysis. Conversely, VEGF-B and oleic acid decreased endothelial glucose uptake by modulating glucose transporter 1-dependent transport. *In vivo*, elevated circulating FA levels correlated with increased FA uptake and reduced glucose uptake to brain. We further discovered that high-fat diet feeding exacerbates cerebrovascular LD accumulation acutely after ischemic stroke, and that this was associated with increased vascular permeability, intracerebral hemorrhage incidence and infarct size. Intriguingly, we also discovered an unexpected pro-lipolytic activity of the thrombolytic agent tissue plasminogen activator in adipose tissue. Treatment with neutralizing antibodies against VEGF-B inhibited adipose lipolysis and the subsequent rise in circulating FAs, improving the outcome after ischemic stroke. Similarly, T2D animal models and transgenic animals that overexpress VEGF-B in podocytes accumulate LDs in the glomeruli of the kidney resulting in DKD-associated pathology, while systemic inhibition of VEGF-B reduced glomerular LD accumulation and ameliorated DKD progression in T2D animals.

Collectively, our data suggests that targeting endothelial FA transport both locally and systemically via VEGF-B inhibition reduces ectopic LD accumulation in brain and kidney correlating with improved outcome after ischemic stroke and ameliorated DKD-associated pathology.

List of scientific papers

- I. **Benjamin Heller Sahlgren**, Jil Protzmann, Ulf Eriksson, Linda Fredriksson and Ingrid Nilsson.
Endothelial fatty acid uptake is activated by lipolysis and depends on lipid droplet metabolism.
Manuscript, 2024
- II. Christine Moessinger, Ingrid Nilsson, Lars Muhl, Manuel Zeitelhofer, **Benjamin Heller Sahlgren**, Josefin Skogsberg, Ulf Eriksson.
VEGF-B signaling impairs endothelial glucose transcytosis by decreasing membrane cholesterol content.
EMBO Reports, 2020, 21, 7, e49343
- III. Ingrid Nilsson, Enming J. Su, Linda Fredriksson, **Benjamin Heller Sahlgren**, Zsuzsa Bagoly, Christine Moessinger, Christina Stefanitsch, Frank Chenfei Ning, Manuel Zeitelhofer, Lars Muhl, Anna-Lisa E. Lawrence, Pierre D. Scotney, Li Lu, Erik Samén, Heidi Ho, Richard F. Keep, Robert L. Medcalf, Daniel A. Lawrence and Ulf Eriksson.
Thrombolysis exacerbates cerebrovascular injury after ischemic stroke via a VEGF-B dependent effect on adipose lipolysis.
Manuscript, 2024
- IV. Annelie Falkevall, Annika Mehlem, Isolde Palombo, **Benjamin Heller Sahlgren**, Lwaki Ebarasi, Liqun He, A. Jimmy Ytterberg, Hannes Olauson, Jonas Axelsson, Birgitta Sundelin, Jaakko Patrakka, Pierre Scotney, Andrew Nash and Ulf Eriksson.
Reducing VEGF-B Signaling Ameliorates Renal Lipotoxicity and Protects against Diabetic Kidney Disease.
Cell Metabolism, 2017, 25, 713–726

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List of abbreviations

3-HIB	3-hydroxyisobutyrate
ANGPTL4	Angiopoietin-like protein 4
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BEC	Blood-brain barrier endothelial cell
Cav-1	Caveolin-1
CNS	Central nervous system
CoA	Coenzyme A
CPT1A	Carnitine palmitoyl transferase 1A
DGAT	Diacylglycerol O-acyltransferase
DKD	Diabetic kidney disease
EC	Endothelial cell
FA	Fatty acid
FABP	Fatty acid binding protein
FAO	Fatty acid β -oxidation
FATP	Fatty acid transporter protein
GLUT1	Glucose transporter 1
HFD	High-fat diet
HSL	Hormone-sensitive lipase
HT	Hemorrhagic transformation
HUVEC	Human umbilical vein endothelial cell
ICH	Intracerebral hemorrhage
LCFA	Long-chain fatty acid
LD	Lipid droplet
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor

MAFLD	Metabolic-associated fatty liver disease
MCAO	Middle cerebral artery occlusion
MMP	Matrix metalloproteinase
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NEFA	Non-esterified fatty acid
OXPHOS	Oxidative phosphorylation
PDGF-C	Platelet-derived growth factor-C
PDGFR α	Platelet-derived growth factor receptor- α
PEC	Proliferating endothelial cell
PFKB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3
PPAR γ	Peroxisome proliferator-activated receptor- γ
QEC	Quiescent endothelial cell
ROS	Reactive oxygen species
rtPA	Recombinant tissue plasminogen activator
siRNA	Small interfering RNA
T2D	Type 2 diabetes
TAG	Triglyceride
TCA	Tricarboxylic acid
TEER	Transendothelial electrical resistance
tPA	Tissue plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

The blood vasculature composes a vast interconnected circulatory network of arteries, arterioles, capillaries, venules, and veins, that allow for the continuous flow of blood to and away from tissues of the entire body. Oxygenated blood travels through arteries and arterioles into capillary beds where nutrient and gas exchange occur. De-oxygenated blood is then drained from venules into veins, and is subsequently transported to the pulmonary circulation for re-oxygenation (Potente and Makinen, 2017). In addition to its role in the transportation of blood, the vasculature also functions as an active metabolic and endocrine organ that aid in the regulation of metabolism, inflammation, coagulation, blood flow and fluid homeostasis (Kruger-Genge et al., 2019). This functional heterogeneity highlights the role of the vasculature as a major player in the regulation of general homeostasis both during development, physiology as well as disease.

1.1 The endothelium

Blood vessels are organized into three distinct layers (tunica intima, media and adventitia) where the endothelium constitutes the internal layer. The endothelium is composed of endothelial cells (ECs) that line the inner vessel wall with their luminal membrane facing the blood (Kruger-Genge et al., 2019). These cells play an integral part in the establishment of new vascular circuits, for instance in the context of wound healing or development. During development, arterial and venous ECs originate from mesoderm-derived angioblasts that assemble to form both the large axial and cranial vessels as well as the first major artery and vein. Further expansion of the vasculature occurs through the process of angiogenesis where migratory tip cells, followed by dividing stalk cells, guide new vessel sprouts that eventually fuse and establish new vessel circuits (Potente and Makinen, 2017). Angiogenesis is dependent on factors belonging to the family of vascular endothelial growth factors (VEGFs) that, in mammals, contain five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor. These together with additional splice variants mediate their effects via three separate vascular endothelial growth factor receptors (VEGFRs) known as VEGFR1, VEGFR2 and VEGFR3, and take part in multiple processes including angiogenesis and lymph angiogenesis (Ylä-Herttuala et al., 2007) as well as the transport of energy substrates from the blood to the underlying tissues (Hagberg et al., 2010). Establishing new vessel circuits is dependent on VEGF-A, expressed by tissues that respond to hypoxia owing to poor perfusion that occur during new tissue

development, wound healing, or high metabolic demand. VEGF-A expression, regulated by hypoxia-inducible factor 1 alpha, results in VEGF-A signaling via VEGFR2 expressed on ECs. The activation of the VEGFR2 signaling pathway stimulates the transition of stationary ECs into migratory tip cells who are then guided into the hypoxic tissue led by a VEGF-A based concentration gradient. Vessels that migrate into the tissue subsequently undergo lumen formation through chord hollowing, vacuolar fusion and inverse membrane blebbing, and consequently establish new vessel circuits that allow for functional perfusion (Potente and Makinen, 2017). Once a complete vascular network has been established, ECs in the adult display a low degree of proliferation and maintain a quiescent profile but remain responsive to events that require the mobilization and establishment of new vessels and vessel circuits (Kruger-Genge et al., 2019).

In the established vasculature, the localization of ECs puts them in direct contact with a large quantity of blood-borne molecules and cells including erythrocytes, immune cells, hormones, cytokines, coagulation factors and metabolic substrates (Kruger-Genge et al., 2019). They thus play a pivotal role in the regulation of both fluid and bidirectional molecular and cell passage from and to the tissues and hence act as a barrier between the blood and the underlying parenchyma (Kruger-Genge et al., 2019; Potente and Makinen, 2017).

1.1.1 The endothelial barrier and permeability

The organization of the endothelium differs depending on the vessel type. ECs in arteries and veins are organized into a continuous non-fenestrated monolayer, while capillary ECs can either be continuous, fenestrated or discontinuous. This organizational difference often reflects local function as the robustness of the endothelial barrier will differ dramatically between different types of endothelia (Potente and Makinen, 2017). For example, the more permeable non-continuous and continuous fenestrated endothelium without slit diaphragms are found in liver sinusoids and the glomeruli of the kidney respectively, reflecting their organotypic high degree of solute transportation from the blood. In contrast, the much less permeable continuous non-fenestrated endothelia are found in the brain where the molecular passage is highly restricted (Claesson-Welsh et al., 2021). In addition, permeability differences can also vary between endothelial beds (Kruger-Genge et al., 2019) as illustrated by the increase in leukocyte migration in post-capillary venules as compared to pre-capillary arterioles (Nourshargh and Alon, 2014).

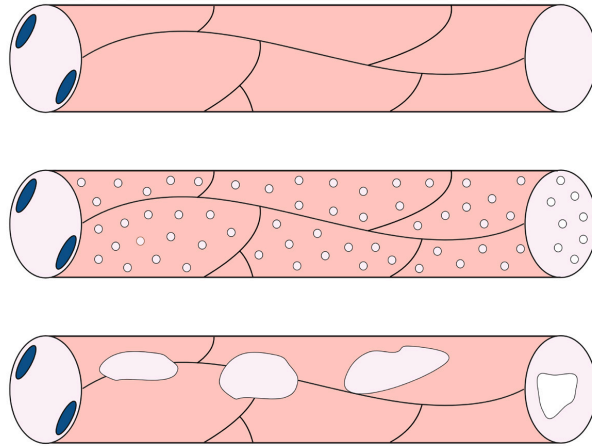


Figure 1: Illustration depicting the three types of capillary endothelium. Continuous endothelium (top), continuous fenestrated endothelium (middle) and discontinuous endothelium (bottom). The cells of the continuous endothelium are tightly connected via adherence and tight junctions and are found with or without fenestrae. The discontinuous endothelium is characterized by large gaps between adjacent cells.

The integrity of the endothelial barrier is not static but actively stabilized as exemplified by the temporary increase in permeability and consequently fluid leakage upon certain stimuli such as acute inflammation (Kruger-Genge et al., 2019). Both the stability and the dynamic response of the barrier can, in part, be explained by its main constituents, the adherence- and tight junctional proteins. The main adherence junctional protein is vascular endothelial cadherin, a transmembrane protein with extracellular cadherin motifs that connect adjacent ECs together through Ca^{2+} -dependent homophilic interactions. The intracellular protein domain interacts with intracellular signaling proteins that can affect the cellular cytoskeleton and mediate both the formation and closure of endothelial gaps that take part in regulating molecular passage. Cadherin's role in barrier integrity can further be illustrated by factors that affect endothelial permeability. For example, VEGF-A can modulate cadherin expression by inducing phosphorylation at specific residues of the intracellular domain resulting in cadherin downregulation and consequently increased permeability (Claesson-Welsh et al., 2021). Similarly, tight junctions are composed of transmembrane proteins that facilitate the formation of focal contacts between cellular membranes. They include members of the claudin and junction-associated families as well as endothelial cell-selective adhesion molecule and occludin. Like

cadherin, the intracellular domain of tight junction proteins through their interaction with adaptor proteins such as zona occludens, can affect both cellular signaling and induce cytoskeletal changes (Zihni et al., 2016). Consequently, tight junction proteins also take part in regulating vascular permeability and endothelial barrier integrity is affected by both their composition and abundance (Claesson-Welsh et al., 2021).

1.1.2 Endothelial paracellular and transcellular transport

In the context of endothelial permeability and transport of proteins, fluids and solutes, there are two main routes: paracellular and transcellular. Paracellular transport (also known as paracellular permeability) involves the passive intercellular transport of fluids and solutes from blood to the underlying parenchyma and (as mentioned above) is dependent on the presence, formation and abundance of junctional proteins (Komarova and Malik, 2010). There are two modes of intercellular passage: the charge-selective and the size-selective pathway. Charge-selective permeation only allow the passive diffusion of ions and small uncharged molecules and is based on molecular charge and size, while in contrast the size-selective pathway allows for the diffusion of slightly larger molecules (Zihni et al., 2016). In contrast, the internalization and transport of macro-molecules such as albumin, hormones and lipids, are believed not to occur through paracellular transport but rather through receptor-mediated vesicular EC transcytosis with receptor-ligand binding occurring at the luminal surface. The endothelial transcytotic pathway is often attributed to the presence of the caveolin-1 (Cav-1) protein which together with cholesterol- and sphingolipid-enriched parts of the plasma membrane lipid bilayer form caveolae. Caveolae structures appear as invaginations of the plasma membrane allowing cargo encapsulation to occur through fission mediated by the protein dynamin. Subsequent scission and vectorial transport results in vesicular relocation to the cellular abluminal surface where they dock and fuse with the basolateral membrane resulting in vesicular cargo release into the interstitial space. This specific transport has been shown to play an important role in endothelial barrier integrity as the deletion of Cav-1 causes endothelial hyperpermeability in mice due to its role in maintaining fluid balance as well as albumin-mediated oncotic pressure (Komarova and Malik, 2010). In addition, Cav-1 also takes part in altering vascular permeability in the context of local energy substrate transport, a function of the endothelium that contributes to EC heterogeneity as will be discussed below.

1.1.3 Tissue metabolism and endothelial heterogeneity

The endothelium constitutes a barrier towards the blood and consequently all underlying tissues rely on functional EC transport to meet their metabolic needs. ECs are therefore actively engaged in energy substrate transportation and display a high degree of local metabolic adaptation. For example, ECs of the cardiac microvasculature have a higher expression of genes related to fatty acid (FA) uptake while ECs that constitute the blood–brain barrier (BBB) express high levels of glucose transporter 1 (GLUT1) hence reflecting the high degree of FA oxidation and glucose consumption of each organ respectively (Hasan and Fischer, 2021). However, ECs located at the BBB do not only express glucose transporters but also the major facilitator superfamily domain-containing 2a (MFSD2A), a sodium-dependent selective transporter of lysophosphatidylcholine-esterified FAs including oleate, palmitate and the omega-3 FA docosahexaenoic acid (Nguyen et al., 2014). Docosahexaenoic acid is a highly abundant lipid species in the brain and MFSD2A-mediated docosahexaenoic acid transport has been suggested to be involved in regulating BBB permeability by suppressing Cav-1 mediated transcytosis (Andreone et al., 2017). Local lipid or glucose uptake is also regulated by metabolic hormones as exemplified by the known effects of insulin signaling. The endothelium has been suggested to act as a mediator of functional insulin signaling by Kubota *et al.*, who demonstrated that EC-specific deletion of the insulin receptor substrate in mice impairs functional insulin signaling. These mice displayed reduced responsiveness to insulin as well as delayed uptake of glucose in skeletal muscle tissue, effects partially attributed to a reduction in EC-mediated transport of insulin to the underlying parenchyma (Kubota et al., 2011). However, ECs do not only act as logistic coordinators that simply transport energy substrates to the underlying parenchyma, but like all other cells engage in both anabolic and catabolic pathways and thus require metabolic substrates for their own adenosine triphosphate (ATP) production.

1.1.4 Endothelial cell metabolism

Although the endothelium for long was depicted as a metabolically inert cellular layer, in recent years it is becoming more and more evident that ECs themselves have distinct unique metabolic profiles that differ from most other cell types (Li et al., 2019b). The two ways in which cells produce ATP are anaerobic glycolysis and oxidative phosphorylation (OXPHOS). The process of anaerobic glycolysis involves a series of glycolytic enzymes that take part in catabolizing glucose for ATP generation in the cytosol. These enzymatic reactions catabolize glucose into

pyruvate that instead of being shunted into the mitochondria is converted to lactate thus allowing cells deprived of oxygen to generate ATP. In contrast, OXPHOS is the last ATP-generating step of a series of electron transfer reactions that occur in the mitochondria during oxygen-dependent cellular respiration. Surprisingly, despite having access to a large amount of oxygen, ECs are highly glycolytic cells that maintain their ATP production through anaerobic glycolysis (De Bock et al., 2013). Despite promoting a lower ATP yield, when compared to OXPHOS, glycolysis reduces the amount of OXPHOS-generated reactive oxygen species (ROS) hence protecting ECs from the oxidative milieu of the blood. In addition, the dedication to anaerobic ATP generation may reflect both the ability of ECs to oxidize substrates and their important role in rationing and distributing oxygen to underlying tissues with less availability (Eelen et al., 2018). Remarkably, despite being harmful, the resulting acidic metabolic substrate (lactate) can even act as a proangiogenic signal and its toxic properties is easily neutralized by EC expression of monocarboxylate transporter proteins that facilitate transport of lactate from the cytosol to the blood (Sonveaux et al., 2012). The rate of EC glycolysis differs depending on the cellular state as exemplified by angiogenesis. In response to VEGF-A signaling, tip cells upregulate the expression of both endothelial GLUT1 as well as important glycolysis enzymes including lactate dehydrogenase and 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKB3) hence becoming more glycolytic (Eelen et al., 2018). In contrast, notch signaling in the adjacent stalk cell results in reduced PFKB3 expression thus reducing glycolytic activity (De Bock et al., 2013). In coherence with the observations that alterations in metabolism facilitate angiogenesis, Schoors *et al.* showed that inhibition of FA transport into the mitochondria reduced vessel sprouting as a consequence of impaired EC proliferation. Further investigations using radioactively labeled FAs, revealed that FA-derived carbons incorporated into most tricarboxylic acid (TCA) cycle intermediates that are used as precursors for *de novo* synthesis of FAs, glucose, amino acids as well as nucleotides. Interestingly, a large quantity of radioactive FA-derived carbons incorporated into DNA and subsequent inhibition of FA oxidation reduced overall cellular deoxyribonucleotide levels. Since preventing FA oxidation only had modest effects on total ATP generation, the impaired proliferation was attributed to a reduction in deoxyribonucleotide synthesis rather than an imbalance in the energy production (Schoors et al., 2015). Thus, despite being highly glycolytic: ECs also rely on FAs for their metabolism.

The adult vasculature is mostly quiescent rendering the idea that in contrast to proliferating ECs (PECs), non-proliferating quiescent ECs (QECs) display a hypometabolic phenotype. Counteracting this assumption is evidence that suggest QECs to be quite metabolically active, albeit they are not dedicated to anabolic metabolic pathways such as the case with PECs. Using unbiased transcriptomics to investigate differences in metabolic gene expression, Kalucka *et al.* were able to group PECs and QECs into distinct metabolic clusters. Genes related to fatty acid β -oxidation (FAO) were upregulated, while the expression of anabolic genes important for glycolysis, serine biosynthesis, TCA cycle, OXPHOS, nucleotide- and FA synthesis were downregulated in QECs compared to PECs (Kalucka *et al.*, 2018). Carnitine palmitoyl transferase 1A (CPT1A), located in the outer mitochondrial membrane, is the main transporter of FAs ≥ 12 carbons into the mitochondria and consequently: act as a rate-limiting enzyme of FAO (Schoors *et al.*, 2015). The transition of PECs into QECs increased the levels of CPT1A while simultaneously decreasing the expression of PFKFB3. Consequently, this transition resulted in increased FAO with a simultaneous reduction in glucose consumption. The increase in FAO could further be explained by the induction of Notch signaling in the QEC state, as activation of Notch increased CPT1A expression while inhibition of Notch reduced CPT1A expression respectively. In line with the findings of Schoors *et al.*, Kalucka *et al.* further demonstrated that FA-derived carbons incorporated into TCA cycle intermediates in QECs but in contrast to PECs, TCA cycle intermediates were used for substrate oxidation rather than being shunted into anabolic pathways. As QECs additionally showed higher expression of glutathione reductase, they hypothesized that the higher rate of FA flux may contribute to maintenance of redox homeostasis by increasing the generation of reduced nicotinamide adenine dinucleotide phosphate (NADPH). Indeed, both increased levels of NADPH and increased expression of glutathione reductase rendered QECs more resistant to ROS-induced cellular stress demonstrating that FAs are vital for QEC redox homeostasis (Kalucka *et al.*, 2018).

These findings contradict the idea that the endothelium is metabolically mute. In fact, they demonstrate that ECs are metabolically active, maintain distinct metabolic phenotypes reflective of cellular state (PECs vs QECs) and that metabolic processes dictate fundamental EC functions including angiogenesis, ROS defense and consequently vascular homeostasis.

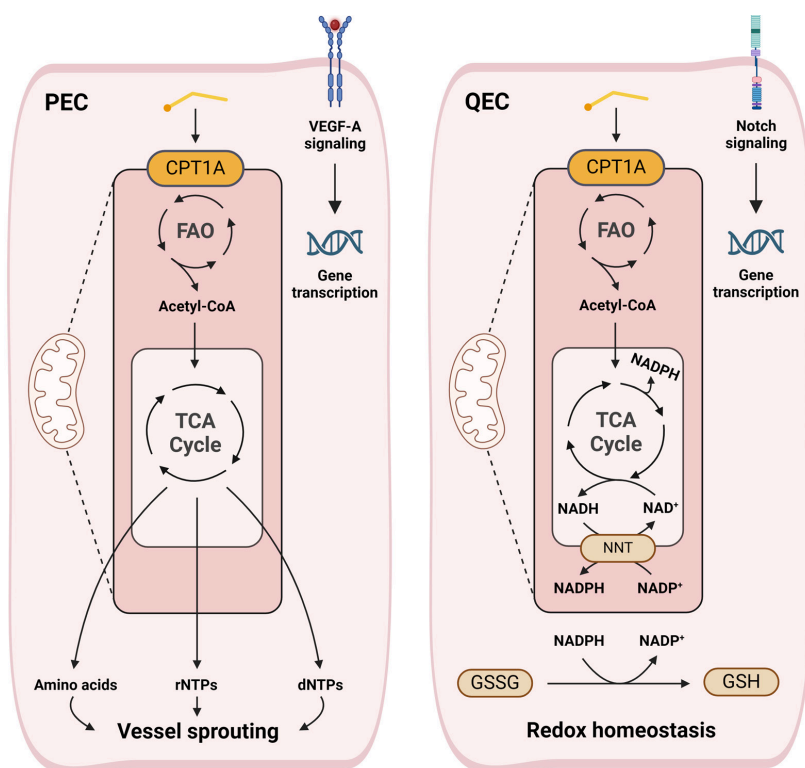


Figure 2: Schematic overview of the metabolic pathways of FA handling in PECs vs QECs. Left image: PECs incorporate FA-derived carbons into TCA intermediaries that can subsequently be used for anabolic pathways including amino acid, ribonucleotide and deoxynucleotide synthesis during VEGF-A induced vessel sprouting. Right image: Notch-induced quiescence increases CPT1A expression in ECs thus inducing mitochondrial FA transport and FAO. Carbons derived from β -oxidation of FAs contribute to the generation of NADPH and reduced nicotinamide adenine dinucleotide (NADH) in the TCA cycle. NADH can be converted to NADPH via the Nicotinamide Nucleotide Transhydrogenase protein (NNT) and mitochondrial-generated NADPH can subsequently be utilized for redox homeostasis facilitated by glutathione reductase (GSH) during ROS neutralization.

1.1.5 Endothelial cell energy storage and mobilization

It is important to note that being metabolically active does not equate with immediate substrate consumption as this would render cells unable to mobilize energy in situations of low availability. Most cell types can therefore shunt excess energy in the form of glucose and lipids into diverse biosynthesis pathways that

promote energy storage. Storing glucose in the form of glycogen is achieved by the shunting of excess glucose into the glycogen synthesis pathway, while storage of FAs requires their incorporation into lipid droplets (LDs). The LD particle consists of a hydrophobic neutral lipid core covered by a phospholipid monolayer with their hydrophilic portion facing the cytosol. These structures allow cells to store and maintain hydrophobic compartments in the hydrophilic environment of the cytosol without interfering with vital cellular processes (Gluchowski et al., 2017). During the construction of LDs, FAs are first incorporated into triglycerides (TAGs) that consist of three acyl groups conjugated to a glycerol backbone. This process occurs in the bilayer of the endoplasmic reticulum, and is mediated by the group of enzymes known as glycerol-3-phosphate acyltransferases, 1-acylglycerol-3-phosphate acyltransferases, lipins and diacylglycerol O-acyltransferases (DGATs) (Buhman et al., 2001). The synthesis and the subsequent accumulation of TAGs, in conjunction with other neutral lipids, result in the deformation of the endoplasmic reticulum membrane consequently forming structures known as lipid lenses. Continued accumulation of lipids further expands these structures until the deformation of the bilayer becomes critical thus triggering budding of the LD into the cytosol (Gluchowski et al., 2017). During LD formation, a specific group of LD-associated proteins are recruited to the intermediary particle. These proteins include the family of perilipins, CGI-58, adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoacylglycerol lipase (MGL) and are involved in both the stabilization as well as the degradation of LDs (Gluchowski et al., 2017; Ikura and Caldwell, 2015). LD degradation can either occur through the process known as lipolysis or through the process known as lipophagy. Lipolysis is mediated by ATGL, HSL and MGL, that hydrolyze acyl groups from the glycerol backbone of the TAG molecule thus liberating FAs that can be utilized for diverse cellular processes. Lipophagy on the other hand involves the direct engulfment of the LD by a phagophore, thus subsequently forming the autophagosome that upon fusing with the lysosome results in acidic LD degradation mediated by the lysosomal acid lipase (Filali-Mouncef et al., 2022).

The ability of ECs to store energy substrates has only recently been dissected with more evidence emerging that energy is stored in a similar manner to most other cells. For example, ECs can shunt excess glucose into the glycogenesis pathway allowing them to mobilize glucose from glycogen through glycogenolysis that occurs during hypoglycemic conditions (Vizan et al., 2009). The capacity to

store and mobilize energy is not exclusive to glycogen as ECs additionally can store and mobilize FAs. In fact, ECs possess the entire LD synthesis machinery and upon exposure incorporate FAs into LDs as demonstrated by Kuo *et al.* When investigating the response of ECs to exogenous FAs, they observed that cultured ECs exposed to oleic acid increase their expression of enzymes coupled to both synthesis and degradation of LDs. Oleic acid treatment of cultured ECs was further coupled to an increased expression of LD-associated proteins including ATGL, HSL and monoacylglycerol lipase, and subcellular fractionation experiments revealed fractions enriched with both LD synthesis as well as lipolytic enzymes. Visualization of lipids revealed a significant accumulation of LDs both in cultured ECs post oleic acid treatment and in the aorta of mice post oral gavage with oleic acid, and inhibition of DGAT1 and ATGL decreased and increased the presence of LDs respectively (Kuo *et al.*, 2017). These findings illustrate that ECs do not only utilize FAs for anabolic pathways but in fact store and degrade LDs in response to a FA-rich environment. However, they do not answer whether ECs are able to mobilize energy from said storage.

To address this question, Kuo *et al.* further investigated whether ECs could utilize LD-derived FA carbons for ATP generation. Although the mitochondrial respiration as well as the total ATP production remained unchanged, ECs reduced their lactate production in response to oleic acid supplementation. This reduction was partially rescued by CPT1A inhibition which in addition prompted a reduction in the baseline OXPHOS, hence suggesting that in response to FAs, ECs partially switch from anaerobic glycolysis to FA oxidation for their ATP generation. Although it may appear that these findings partially contradict the observations made by Kalucka *et al.* (Kalucka *et al.*, 2018), it is important to note that Kuo *et al.*, in contrast to Kalucka *et al.*, did not induce EC quiescence using complementary delta-like protein 4 and it is therefore unclear whether the observations of Kuo *et al.* are more applicable to partially quiescent ECs. Nonetheless, that ECs would switch to FAO upon exposure to large quantities of FAs are supported by the Randle cycle theorem which postulates that metabolic substrate selection is determined by substrate availability (Hue and Taegtmeyer, 2009). In addition to metabolic adaptation, Kuo *et al.* could further demonstrate that activation of lipolysis in lipid-laden ECs increased the release of LD-derived FAs into the media and consequently promoted LD accumulation in an adjacent monolayer of non-FA treated cultured myocytes (Kuo *et al.*, 2017). The process of releasing FAs from LDs appears to be regulated by Cav-1 as removal of Cav-1 reduces lipolysis in ECs

(Kuo et al., 2018), suggesting that ECs depend on vesicular transport when releasing FAs from intracellular compartments to the extracellular environment. The observation that EC-derived FAs are not only utilized for energy production but can in fact be taken up by neighboring cells, hints to that EC degradation of LDs may reflect a specific cellular mechanism which can supply the underlying tissues with FAs in situations of low glucose availability. This latter finding thus highlights the role of EC metabolism in the transportation of metabolic substrates to the underlying parenchyma as will be discussed later.

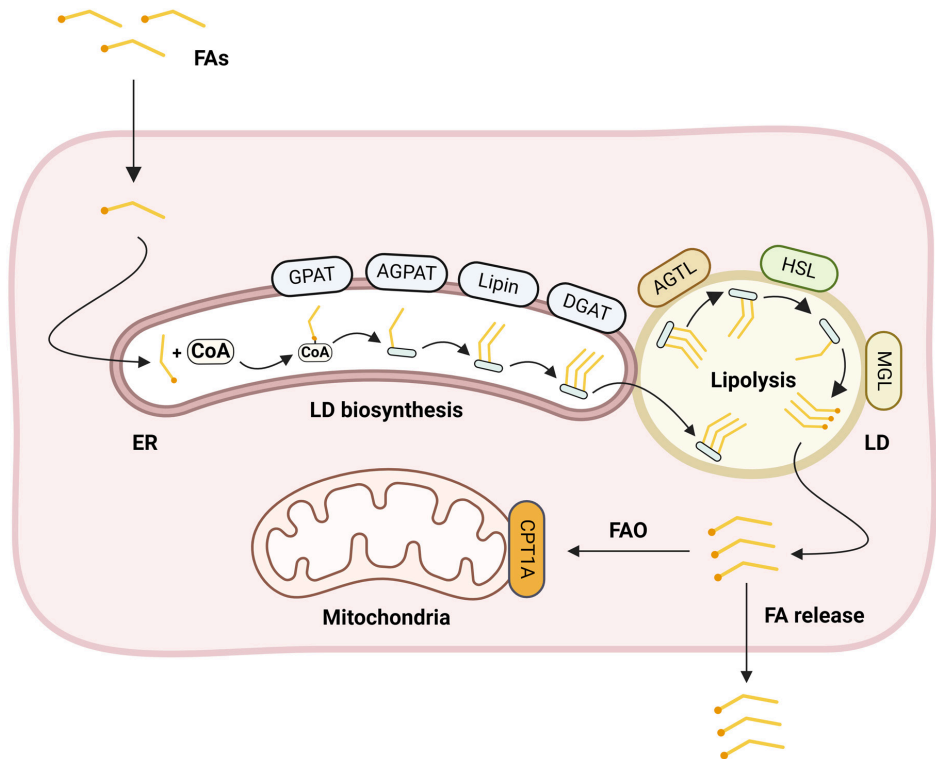


Figure 3: Schematic overview of FA storage and mobilization in ECs. Upon FA exposure, ECs shunt FAs into the LD synthesis pathway where they are esterified to glycerol via the glycerol-3-phosphate acyltransferase (GPAT), 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT), lipin and DGAT in the endoplasmic reticulum (ER) thus generating detectable LDs. Formation of LDs allows for energy storage and the mobilization of FAs that are either used for energy production by the mitochondria or are released to the underlying tissues.

1.1.6 Transendothelial fatty acid transporters

FAs in the blood cannot move freely throughout the vascular system due to their hydrophobic properties. Circulating FAs are therefore either bound to albumin or re-esterified into TAGs that are packaged into lipoprotein particles, including chylomicrons or very-low density lipoproteins (Niot et al., 2009). For transendothelial lipid transport to occur, lipoprotein-derived FAs first need to be hydrolyzed from TAGs, mediated by the lipoprotein lipase (Davies et al., 2010). The uptake and transport across the endothelium of both TAG-derived and albumin-bound FAs, is largely facilitated by receptor-mediated transport. FAs are transported in a vectorial manner across the cellular membrane consequently resulting in FA conjugation with FA-binding proteins in the cytosol. CD36, a member of the class B scavenger receptor family, is perhaps the best described endothelial FA transporter protein (Abumrad et al., 2021). It carries both an extracellular ligand binding domain and an internal lipid transport tunnel that empties in the proximity of the membrane bilayer (Abumrad et al., 2021; Hasan and Fischer, 2021). Ligands include FAs and lipoproteins (Abumrad et al., 2021), that upon binding to CD36 triggers Cav-1 mediated FA endocytosis and the formation of FA-containing intracellular vesicles that are released as small extracellular vesicles at the abluminal side (Peché et al., 2023). Although Cav-1 appears to be important for the function of CD36, ECs lacking Cav-1 do not display altered FA uptake (Kuo et al., 2018) and hence the exact role of Cav-1 in endothelial FA uptake is unclear and remains to be determined. The fatty acid transporter protein (FATP) family have also been extensively studied in the context of endothelial FA uptake. The FATPs belong to the SLC27 protein family known to possess ATP-dependent acyl-coenzyme A synthase activity where activation of the incoming FA is achieved through conjugation of the carboxyl group to coenzyme A (CoA). This esterification reaction allows for cellular FA trapping and subsequent binding to fatty acid binding proteins (FABPs) that constantly remove CoA-activated FAs from the cytosol post FATP-mediated esterification (Doege and Stahl, 2006). There are six described members of the FATP family that display different tissue expression as exemplified by high expression of FATP1 and FATP3 in cardiac and skeletal muscle respectively. ECs, on the other hand, selectively express FATP3 and FATP4 (Abumrad et al., 2021), both demonstrated to be important for functional transendothelial long-chain fatty acid (LCFA) transport (Hagberg et al., 2010; Ibrahim et al., 2020; Mehlem et al., 2016). Although their exact role remains unclear, their acyl-CoA-synthase activity appears to be vital for endothelial FA uptake and transport as demonstrated by Ibrahim *et al.* By using a

small molecule screening assay with endothelial FA uptake as readout, they discovered that the most potent compounds able to inhibit FA uptake and transport target the ability of the mitochondria to generate ATP. Consequently, boosting mitochondrial ATP production, by increasing cellular ATP demand or mitochondrial capacity, increased FA uptake in ECs while induction or inhibition of glycolysis had no effect. Since selective inhibition of mitochondrial ATP transport to the cytosol was sufficient to inhibit FA uptake, they speculated that the locally produced ATP was utilized by FATPs to carry out their acyl-CoA synthase activity. FATP4 is localized to the endoplasmic reticulum and hence adjacent to the mitochondria, and indeed knockdown of FATP4 or the introduction of loss of function point mutations in the acyl-CoA-synthase domain reduced FA uptake in ECs (Ibrahim et al., 2020).

1.1.7 Regulators of transendothelial fatty acid transport

ECs have permanent access to blood and consequently energy in the form of glucose. Since endothelial glucose uptake is GLUT1 and not GLUT4 dependent, their uptake of glucose is not directly regulated by insulin. Hence, ECs are less sensitive to changes in glucose availability when compared to underlying tissues, despite acting as a barrier for FA transport. To solve this dilemma, the underlying parenchyma has developed ways to communicate to the endothelium to increase transport of FAs when glucose levels are low (Hasan and Fischer, 2021). There are several factors reported to affect transendothelial FA transport including peroxisome proliferator-activated receptor- γ (PPAR γ) (Goto et al., 2013; Kanda et al., 2009), notch signaling (Jabs et al., 2018), angiopoietin-like protein 4 (ANGPTL4) (Chaube et al., 2023), apelin (Hwangbo et al., 2017), 3-hydroxyisobutyrate (3-HIB) (Jang et al., 2016), angiopoietin-2 (Bae et al., 2020), lactate (Ibrahim et al., 2022) and VEGF-B (Hagberg et al., 2010). Many of these function by increasing expression of genes important for FA uptake (Hasan and Fischer, 2021). For example, EC specific deletion of PPAR γ in mice result in decreased FA uptake in heart, skeletal muscle and adipose tissue due to reduced EC expression of *Cd36* and *Fabp4* (Goto et al., 2013), and EC specific inhibition of notch signaling impair transendothelial transport of LCFAs to muscle cells largely attributed to the lack of notch-induced CD36 and FABP4 expression (Jabs et al., 2018). Likewise, injection of ANGPTL4 increases plasma lipid levels by inhibiting LPL activity in mice (Yoshida et al., 2002), and knockdown of *ANGPTL4* increases endothelial FA uptake and transport via increased endothelial lipase activity and surface expression of CD36 *in vitro* (Chaube et al., 2023). Apelin, on the other hand, has

been shown to reduce expression of FABP4 resulting in decreased FA uptake and increased glucose uptake respectively (Hwangbo et al., 2017), while angiopoietin-2 modulate CD36 and FATP through $\alpha_5\beta_1$ integrin signaling thus increasing endothelial FA uptake specifically in subcutaneous adipose tissue (Bae et al., 2020).

1.1.7.1 3-hydroxyisobutyrate and lactate

Unlike the aforementioned factors, lactate and 3-HIB are both formed during metabolism but originate from different metabolic substrates. 3-HIB is a metabolite formed for example during peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) induced catabolism of valine in muscle tissue. During branched-chain amino acid catabolism, 3-HIB act as an intermediate and is degraded into propionyl-CoA that upon further degradation into succinyl-CoA can enter the TCA cycle. Like the previously mentioned factors, 3-HIB has been shown (both *in vitro* and *in vivo*) to increase transendothelial FA transport specifically through FATP3- and FATP4-mediated mechanisms as for example small interfering RNA (siRNA) knockdown of these transporters diminishes 3-HIB's effect on endothelial FA uptake. Although 3-HIB appears to require both FATP3 and FATP4 to stimulate endothelial FA uptake, their expression is not altered post 3-HIB treatment (Jang et al., 2016). These counterintuitive observations may be reconciled when considering the role of FATP4 in the esterification of incoming FAs. Since the ATP-requiring acyl-CoA-synthase activity of FATP4 appears to be vital for its function (Ibrahim et al., 2020), 3-HIB might thus mediate FA uptake by increasing mitochondrial ATP generation which can thus be used for FA-CoA esterification. In ECs however, siRNA knockdown of enzymes that take part in valine catabolism downstream of 3-HIB does not affect its ability to induce the uptake of FAs (Jang et al., 2016), and it is therefore not fully understood exactly how this metabolite increases endothelial FA uptake. Lactate on the other hand is formed via the conversion of pyruvate during anaerobic metabolism of glucose and has been demonstrated to take part in post-prandial transport of FAs from the endothelium to the adipose tissue (Ibrahim et al., 2022). However, like 3-HIB, it remains poorly understood exactly how lactate induces FA uptake in ECs.

1.1.7.2 Vascular endothelial growth factor B

The first regulator of transendothelial FA transport to be identified was VEGF-B, originally described by Hagberg *et al* (Hagberg et al., 2010). The VEGF-B protein

exists as two variants formed upon alternative splicing of the VEGF-B gene: the VEGF-B₁₆₇ and the VEGF-B₁₈₆ isoforms. Unlike VEGF-A, VEGF-B does not appear to play a major role in angiogenesis (Li et al., 2009). Instead, VEGF-B expression is coordinated with nuclear-encoded mitochondrial genes and is highly expressed in tissues largely engaged in FAO such as cardiac and skeletal muscle, as well as brown adipose tissue (Hagberg et al., 2010). Similar to 3-HIB, VEGF-B facilitates endothelial FA uptake in a paracrine fashion and its expression in muscle tissue is under the control of peroxisome proliferator-activated receptor gamma coactivator 1- α (PGC-1 α) (Mehlem et al., 2016). In addition, VEGF-B's effect on FA uptake has been demonstrated to be mediated by increased gene expression of both FATP3 and FATP4. This is attributed to signaling events that occur downstream of the VEGFR1 and its co-receptor neuropilin-1 (Hagberg et al., 2010). In addition, VEGF-B can modulate tissue glucose availability by reducing endothelial glucose uptake and transport both *in vivo* and *in vitro*. The effect on glucose uptake has been coupled with a VEGF-B mediated reduction in membrane cholesterol content, thus affecting the ability of GLUT1 to efficiently transport glucose over the endothelium. As VEGF-B was demonstrated to reduce low-density lipoprotein receptor (LDLR) recycling, the effect on glucose transcytosis was linked to a reduction in low-density lipoprotein (LDL) uptake (Moessinger et al., 2020). Interestingly, VEGF-B has also been suggested to induce lipolysis in adipose tissue. When studying the contribution of VEGF-B to the development of metabolic-associated fatty liver disease (MAFLD), Falkevall et al. observed increased plasma levels of non-esterified fatty acids (NEFAs) when overexpressing VEGF-B specifically in the adipose tissue of mice. The changes in plasma NEFAs were connected to an increased activity of HSL in adipocytes. In contrast, systemic VEGF-B inhibition via anti-VEGF-B antibody treatment reduced HSL phosphorylation and increased glucose uptake in the adipose tissue of diabetic mice when compared to isotype IgG control. As a result, plasma NEFA levels decreased significantly (Falkevall et al., 2023). In addition to promoting HSL activity in adipose tissue, VEGF-B has also been demonstrated to promote lipolysis in myocytes. Indeed, VEGF-B treatment increases the expression of both *Pnpla2* (ATGL) and *Lipe* (HSL) and induces FAO in *in vitro* cultured myocytes (Li et al., 2019a), although the *in vivo* relevance is questionable since myocyte expression of VEGFR1 in muscle tissue is low (Moessinger et al., 2020). Nevertheless, these observations collectively suggest that in addition to inducing endothelial FA uptake, VEGF-B promotes lipolysis therefore strengthening the link between endothelial metabolism and endothelial nutrient transport.

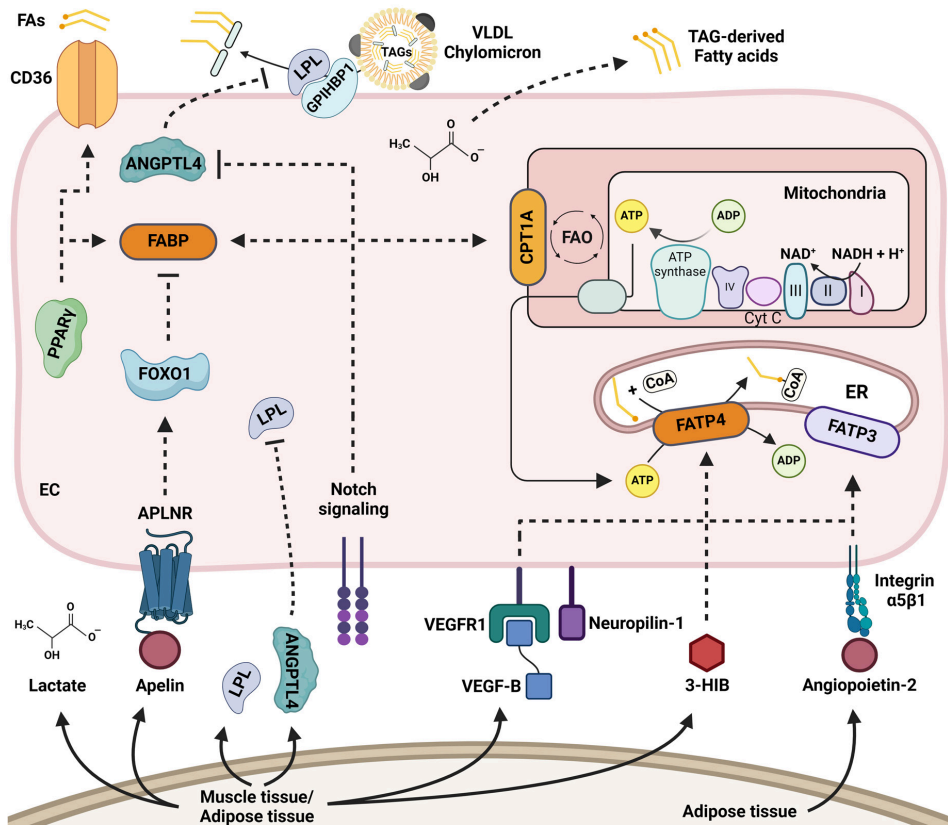


Figure 4: Schematic overview of factors that regulate endothelial FA uptake and transport. Most regulators act in a paracrine fashion. Upon secretion from the underlying parenchyma, these factors act on ECs to induce transendothelial FA transport consequently increasing FA availability to the underlying tissues.

1.2 Type 2 diabetes and diabetic co-morbidities

The global incidence of type 2 diabetes (T2D) mellitus is exponentially increasing with an expected incidence of 592 million by 2035. T2D is a metabolic disorder and hence it is not surprising that obesity is by far the strongest risk factor for disease development. The disease is characterized by the systemic resistance to insulin, believed to be mediated by an excess in tissue lipid accumulation that can impair insulin receptor signaling thus resulting in reduced insulin-dependent glucose uptake. Patients diagnosed with T2D often present with the paradoxical combination of hyperinsulinemia together with hyperglycemia and display both increased fasting glucose levels as well as increased postprandial circulating

NEFAs. Chronic disease progression often leads to the manifestation of T2D co-morbidities ultimately contributing to disease mortality. These include diabetic retinopathy, neuropathy, MAFLD, nephropathy, congestive heart failure and stroke (DeFronzo et al., 2015). Since hyperglycemia has been proposed to be a major risk factor, therapeutic strategies aiming to prevent or limit the development of diabetes-associated co-morbidities have largely focused on maintaining blood glucose homeostasis. However, studies evaluating the efficacy of intensive glucose management have failed to show any major effect (Gerstein et al., 2008; Patel et al., 2008), suggesting that hyperglycemia may serve better as a predictor for disease development. Targeting hyperlipidemia and excessive lipid accumulation to prevent disease progression is therefore gaining more attention and attempts to elucidate the mechanisms that underlie the development of T2D co-morbidities are ongoing. Especially the association between T2D-related dyslipidemia and the incidence of ischemic stroke and nephropathy is of great interest as will be discussed in detail below.

1.3 Ischemic Stroke

Ischemic stroke, defined by infarction of the brain, spinal cord or retina, is the most common form of stroke representing roughly 71% of all global cases (Campbell et al., 2019). The cause of ischemic stroke is thromboembolic occlusion of a large or small cerebral vessel resulting in disrupted blood flow, low perfusion and ultimately ischemic infarct (Campbell et al., 2019; Deb et al., 2010). Risk factors for ischemic stroke include obesity-related hyperlipidemia, hyperglycemia, insulin resistance and other cardiovascular complications (Ninomiya et al., 2004) that are part of the metabolic syndrome (Eckel et al., 2005). Diabetes and hyperglycemia have further been identified as predictors of increased mortality risk as well as poorer cognitive performance post stroke. However, despite novel studies suggesting that administration of blood glucose-lowering drugs (PPAR γ or glucagon-like peptide-1 receptor agonists) reduces stroke incidence in T2D patients, they fail to demonstrate any beneficial effect in patients with stroke (Gerstein et al., 2019; Malhotra et al., 2020; Yaghi et al., 2018). Likewise, strict glycemic control at stroke onset does not improve stroke outcome (Mosenson et al., 2023), and the relation between factors of the metabolic syndrome and stroke thus remain poorly understood.

1.3.1 Ischemic stroke pathology

In ischemic stroke, the most common etiology of the emboli includes atrial fibrillation or atherosclerosis, the latter describing the formation of an arterial atherosclerotic plaque composed of a lipid rich necrotic core thus narrowing the artery. With time the plaque may rupture and intraplaque components are thus exposed to the blood. This results in activation of the coagulation cascade and the formation of a thrombi that, via the bloodstream, can enter the brain and occlude smaller cerebral vessels. The occlusion of a cerebral artery reduces the delivery of oxygen and glucose to the affected brain region thus diminishing the neuron's ability to produce ATP (Campbell et al., 2019; Deb et al., 2010). Neurons rely on a high ATP production to continuously transport ions against their concentration gradient thus maintaining their resting membrane potential. This specific neuronal transport of ions is facilitated by the ATP-dependent sodium/potassium-ATPase, and the lack of ATP therefore results in neuronal influx of sodium ions and liquid. In turn, this will result in both neuronal swelling and the release of abnormally high levels of excitatory neurotransmitters including glutamate while simultaneously reducing the ability of glial cells to clear the neurotransmitters away from the synaptic cleft. The abnormally high concentration of neurotransmitters increases cellular sodium and calcium influx thus activating various enzymes such as nitric oxide synthase, proteases, lipases and nucleases that mediate cellular damage via ROS production and degradation of critical cellular components (Deb et al., 2010). The attempt to maintain metabolic activity without access to ATP result in lactic acidosis that contribute to fluid retention in ECs, astrocytes as well as neurons thereby causing them to swell (Khatri et al., 2012). Collectively, these processes are referred to as the ischemic cascade and result in the death of brain parenchymal cells including neurons and astrocytes that together make up the necrotic core of the ischemic tissue (Campbell et al., 2019).

Despite the hypoperfusion of a large portion of the tissue located downstream of the occluded vessel, blood flow is not completely absent as loss of perfusion due to large arterial occlusion is partially compensated for by collateral vessels such as leptomeningeal collaterals (Deb et al., 2010; Maguida and Shuaib, 2023). The compensation in blood flow allow for some influx of oxygen and glucose to the tissue surrounding the ischemic core therefore protecting it from immediate death (Maguida and Shuaib, 2023). However, due to the lack of adequate perfusion, the ischemic but salvageable tissue, referred to as the ischemic

penumbra, becomes necrotic with time thereby increasing the size of the infarct (Campbell et al., 2019; Deb et al., 2010; Maguida and Shuaib, 2023). Since larger loss of neuronal tissue is associated with poorer outcome, current therapeutic strategies attempt to rescue the cells of the penumbra thereby improving both survival and functional outcome post ischemic stroke (Campbell et al., 2019).

1.3.2 Therapeutic management of ischemic stroke

The only two therapies approved for the treatment of acute ischemic stroke are intravenous thrombolysis and thrombectomy, both focusing on re-establishing perfusion to the ischemic region of the brain by removal of the blood clot (Xiong et al., 2022). Intravenous thrombolysis is administered as an initial bolus of the thrombolytic agent or as an initial bolus followed by one-hour intravenous infusion (Campbell et al., 2019), while thrombectomy on the other hand is the mechanical removal of the thrombus using stent retrievers which are granted access to the cerebral vasculature through the use of catheters. However, patients eligible for thrombectomy require transport to comprehensive stroke centers capable of carrying out this procedure thus further extending the time until treatment (Xiong et al., 2022). Since less resources are needed, intravenous thrombolysis therefore remains the gold standard as it can be provided at primary stroke centers allowing for earlier administration after stroke symptom onset (Campbell et al., 2019; Xiong et al., 2022).

1.3.2.1 Tissue plasminogen activator

The most common thrombolytic used in ischemic stroke is alteplase, a recombinant form of tissue plasminogen activator (rtPA) which mediates cleavage of plasminogen to plasmin resulting in degradation of fibrin and consequently clot lysis (Campbell et al., 2019). The benefit of using rtPA as treatment for ischemic stroke was first determined in 1995 when successful clinical trials demonstrated improved outcome for patients given alteplase within three hours after symptom onset (National Institute of Neurological and Stroke rt, 1995). Since then, the therapeutic time window of thrombolysis has increased from three to four and a half hours after stroke onset (Campbell et al., 2019; Lees et al., 2010; Xiong et al., 2022). Unfortunately, delayed rtPA administration (after four and a half hours) has been associated with reduced treatment efficacy owing partially to a time-dependent loss of the penumbra as well as a reduced ability of rtPA to lyse the clot (Campbell et al., 2019). Time until reperfusion post ischemic stroke onset is also considered an important factor since the risk of developing intracerebral

hemorrhage (ICH) increases with time (Jickling et al., 2014; Molina et al., 2001). The risk of ICH is further increased by rtPA and delayed rtPA administration increases the risk of death (Lees et al., 2010). Therefore, providing thrombolytic treatment with rtPA outside the established therapeutic window establishes an unfavorable risk-benefit ratio that account for the rtPA-mediated increase in risk of ICH and death as well as the reduction in treatment efficacy (Jickling et al., 2014).

1.3.3 The blood brain barrier, cerebral edema and hemorrhagic transformation

The development of cerebral edema and ICH after stroke result from BBB disruptions and are both associated with poor prognosis (Wan et al., 2023). The BBB is localized at the interface between the neuronal tissues and the blood and is characterized by the highly restrictive bi-directional transport of fluid, molecules, ions and metabolic substrates. It is composed of specialized blood-brain barrier ECs (BECs) that are tightly coupled via adherence, GAP and tight junctions. Surrounding the BECs is the basement membrane consisting of extracellular matrix proteins that anchor BECs, perivascular pericytes and astrocyte end feet together thus facilitating cellular communication. Collectively, these cells and components form a highly dynamic structure that takes part in regulating several processes in the brain including cerebral blood flow and synaptic activity.

The unique structure of the BBB protects the brain parenchyma from exposure to toxic blood elements and maintains central nervous system (CNS) homeostasis (Bernardo-Castro et al., 2020; Khatri et al., 2012). A pathological increase in BBB permeability therefore results in transcellular and paracellular leakage of plasma proteins, ions and ultimately fluids from the blood into the brain interstitium. The extravasation of fluids into the cerebral tissues, referred to as vasogenic edema, occurs due to a larger influx of molecules into the parenchyma therefore altering the osmotic gradient in the brain (Han et al., 2023; Hong et al., 2021). It is important to distinguish vasogenic and cytotoxic edema since the latter describes the increased influx and retention of cerebral interstitial fluid in parenchymal cells such as neurons. In contrast to vasogenic edema which causes a net increase in parenchymal fluids, cytotoxic edema does not alter the total cerebral fluid amount (Han et al., 2023). However, despite their differences, cytotoxic and vasogenic edema are interconnected as cytotoxic edema contributes to vasogenic edema and *vice versa*. The increased neuronal influx of sodium ions from the interstitial fluid during cytotoxic edema, result in parenchymal ionic imbalance causing sodium ions to flow from the blood to the cerebral interstitium. In turn, this chain

of events promotes water influx via aquaporin-4 channels resulting in vasogenic edema (Han et al., 2023; Krishnan et al., 2023). The development of cerebral edema is critical for the patient as it leads to increased intra-cranial pressure that may result in brain herniation and death (Han et al., 2023).

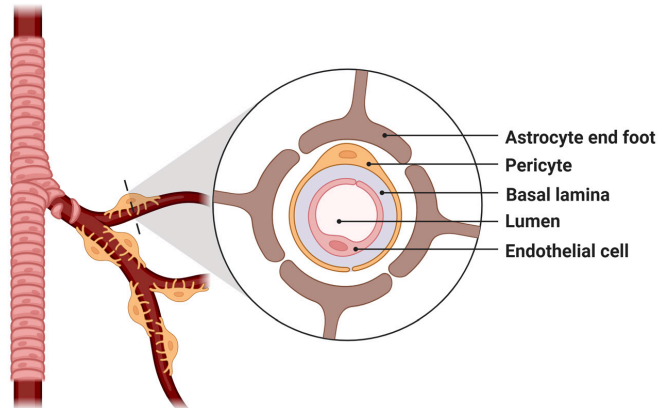


Figure 5: Illustration of the BBB. Surrounding the BECs is the basement membrane that anchor BECs, perivascular pericytes and astrocytic end feet together. These cells together restrict the passage of fluid and blood-borne substances into the brain parenchyma.

Malfunctioning of the BBB does not only contribute to cerebral edema but is also considered a major factor for the development and progression of hemorrhagic transformation (HT) (Bernardo-Castro et al., 2020). HT describe the cerebral bleedings or hemorrhagic infarctions after ischemic stroke (Hong et al., 2021; Jickling et al., 2014), believed to occur due to degradation of ECM components, the basal membrane and tight junction proteins therefore resulting in complete BBB breakdown (Bernardo-Castro et al., 2020). Indeed, BBB dysfunction is strongly associated with HT as reperfusion of capillaries with dysfunctional BBB can result in larger parenchymal hemorrhages (Bernardo-Castro et al., 2020; Hong et al., 2021; Jickling et al., 2014). Between 10 – 40% of all ischemic stroke patients develop HT and the resulting exposure of blood elements to the brain parenchyma contribute to increased infarct size, cerebral edema and ultimately mortality (Jickling et al., 2014). The mechanisms that mediate BBB permeability in ischemic stroke are therefore of great importance as they may ultimately increase the risk of larger neurological impairment as well as death.

1.3.4 Mechanisms of blood–brain barrier disruption

Ischemic stroke induces “opening” of the BBB where increased permeability present itself in distinct biphasic peaks. The initial opening, or first peaks of permeability, occur within the hyperacute and acute phase of stroke which take place during the first six and 72 hours respectively. The second peak is observed after 72 hours during what is known as the subacute phase (Bernardo-Castro et al., 2020). There are several factors that mediate BBB breakdown including cytotoxic edema, excitotoxicity, necrosis and inflammation. As discussed above, cytotoxic edema triggers an increased influx of ions from the blood. The balanced influx of ions, via ATP-independent ionic co-transporters located on BECs, is normally maintained by the continuous neuronal ionic efflux thereby counteracting BEC sodium uptake. However, the failure of neurons to maintain the ionic gradient increases BEC sodium uptake and secretion, during which a portion of the ions are retained in BECs. This retention causes BECs to swell thereby contributing to BBB breakdown (Abdullahi et al., 2018). Exacerbated neurotransmitter release also mediate BBB breakdown during the hyperacute phase. For example, abnormally high glutamate signaling increases calcium influx via glutamate receptors resulting in both calcium-mediated mitochondrial dysfunction as well as necrosis (Bernardo-Castro et al., 2020). In turn, mitochondrial dysfunction and necrosis-driven inflammation both promote the generation of ROS that can damage the cells of the BBB (Bernardo-Castro et al., 2020; Jickling et al., 2014). Ischemia-induced inflammation does not only contribute to ROS formation but also to the recruitment of immune cells via increased levels of cytokines and chemokines (Deb et al., 2010) such as IL-1 β , TNF- α and IL-8 (Clausen et al., 2020; Deb et al., 2010; Kostulas et al., 1999). The increased levels of inflammatory factors result in leukocyte adherence and infiltration as early as within 30 minutes of stroke onset in animals (Dereski et al., 1993), and as early as six hours in humans (Akopov et al., 1996). Leukocytes can promote breakdown of the BBB in multiple ways including occludin endocytosis and degradation (Murakami et al., 2009; Murakami et al., 2012) via activation of the protein kinase C β signaling pathway (Chou et al., 2004) as well as increased production of matrix metalloproteinases (MMPs) (Jickling et al., 2014).

1.3.4.1 Metalloproteinases

The MMPs degrade both tight junctions as well as the basal lamina and are therefore considered to be one of the main contributors to BBB breakdown (Bernardo-Castro et al., 2020). During increased BBB permeability, leukocytes are

believed to be an important source of MMP-9 as early expression has been linked to the infiltration of neutrophils in both humans (Rosell et al., 2008) and animals (Jickling et al., 2014). Other MMPs involved in early BBB breakdown include MMP-2 (Rosenberg et al., 1998) believed to be mostly derived from astrocytes (del Zoppo et al., 2012) and ECs (Reuter et al., 2013). Cerebral-derived MMP-9 and MMP-2 together with MMP-3, MMP-10, MMP-13 and MMP-14, are also important during later stages of BBB permeability for example during the acute and subacute phase (Jickling et al., 2014).

1.3.4.2 Vascular endothelial growth factor A

Another factor associated with BBB permeability after stroke is the pro-angiogenic factor VEGF-A, expressed by multiple parenchymal cells including cells of the BBB, inflammatory cells and neurons (Greenberg and Jin, 2013). Indeed, expression of both VEGF-A and its receptors VEGFR1 and VEGFR2 increases early during ischemia and is sustained over longer periods of time post ischemic onset (Greenberg and Jin, 2013). The contribution of VEGF-A to BBB breakdown and cerebral bleedings in ischemic stroke has been demonstrated in multiple studies. For example, intravenous administration of VEGF-A increases BBB leakage and cerebral bleedings in rats (Zhang et al., 2000), and inhibition of VEGFR2 reduces protein kinase C β -mediated phosphorylation of occludin thereby reducing BBB permeability in mice (Goncalves et al., 2022). Although the exact mechanisms remain unclear, it is suggested that VEGF-A induced breakdown of the BBB is mediated via the disassembly and degradation of tight junctions (Argaw et al., 2009) as well as via increased MMP-9 expression and activity (Lee et al., 2007; Valable et al., 2005).

1.3.4.3 Transcytosis and blood-brain barrier dysfunction

Besides the breakdown of junctional proteins, basal lamina and the subsequent increase in paracellular permeability, transcellular permeability has also been suggested to play an important role in early BBB disruption. In fact, increased transcellular permeability appears to precede the structural changes in tight junction proteins at the BBB. For example, using fluorescent microscopy, Krueger *et al.* detected normal presence of occludin and claudin-5 twenty-five hours post ischemic onset in an animal model of stroke despite increased extravasation of fluorescently conjugated albumin. Ultrastructural analysis using electron microscopy revealed intact tight junctions at the sites of albumin extravasation as well as increased vesicular formation in ECs. The same was true five hours post

middle cerebral artery occlusion (MCAO), and hence they attributed the early extravasation of albumin to transcellular rather than paracellular leakage (Krueger et al., 2013). Similar observations were made in a later study performed by Knowland et al. Utilizing two-photon imaging of *eGFP-claudin5* transgenic mice subjected to MCAO, Knowland et al. could only detect larger structural changes in tight junctions between 48–58 hours post ischemia onset. In contrast, an increased number of larger caveolae was detected as early as six hours post MCAO corresponding with an increase in transcellular transport of fluorescently conjugated albumin (Knowland et al., 2014). Interestingly, transcellular leakage appears not only to precede paracellular leakage but may in fact impact tight junction expression as demonstrated by Choi et al. Similar to the findings presented by Knowland et al., Choi et al. observed an increased Cav-1 expression as early as six hours post induction of focal cortical ischemia. Surprisingly, induction of stroke in *Cav-1^{-/-}* mice revealed an aggravated loss of tight junction proteins as compared to control mice presumably due to an increased activity of both MMP-2 and MMP-9 (Choi et al., 2016). Collectively, these observations suggest that transcellular leakage occur earlier than paracellular leakage during stroke (Knowland et al., 2014; Krueger et al., 2013) and that transcellular permeability may in fact impact paracellular permeability (Choi et al., 2016).

1.3.4.4 Tissue plasminogen activator and blood-brain barrier dysfunction

As previously discussed, therapeutic administration of rtPA unfortunately increases the risk of ICH and HT. The mechanisms of tissue plasminogen activator (tPA)-induced vascular permeability during stroke are thought to involve MMP-9 expression. The proposed importance of MMP-9 in tPA-mediated vascular leakage stem from experiments demonstrating reduced expression of MMP-9 in mice lacking tPA (Wang et al., 2003), increased MMP-9 activity in rats administered with rtPA post ischemic stroke (Kelly et al., 2006) and increased MMP-9 expression post rtPA treatment of *in vitro* cultured ECs (Wang et al., 2003). However, Yepes et al. demonstrated that one hour post MCAO, MMP-9 knockout mice do not display increased BBB integrity, while in contrast inhibition of tPA (using neuroserpin) or genetic deletion of tPA significantly reduced vascular permeability (Yepes et al., 2003). Furthermore, rtPA treatment increases vascular leakage 24 hours post MCAO in both wild-type and MMP-9 knockout mice (Copin et al., 2011). Collectively, these data suggest that at least up to 24 hours after ischemic onset, tPA increases vascular permeability independent of MMP-9. The early effect of tPA on BBB integrity has therefore been attributed to the activation

of other factors including platelet-derived growth factor C (PDGF-C) (Fredriksson et al., 2017). Indeed, the catalytic domain of tPA can cleave PDGF-C at the hinge region thus yielding active PDGF-C that can bind to and activate the PDGF receptor α (PDGFR α) resulting in downstream signaling events (Fredriksson et al., 2005). The role of PDGF-C as an important mediator for tPA-induced BBB permeability was first demonstrated by Su *et al.* in 2008. When administering activated PDGF-C into the cerebrospinal fluid of non-ischemic mice, they observed a tPA-independent increase in vascular leakage attributed to the activation of PDGFR α . The localization of PDGF-C was observed around arterioles while the PDGFR α appeared to be expressed by perivascular astrocytes, suggesting that the increased vascular leakage observed post administration of rtPA was mediated via perivascular PDGFR α positive astrocytes (Su et al., 2008). The model of a PDGF-C dependent tPA-induction of BBB leakage is further supported by other experiments demonstrating protein kinase C β -mediated phosphorylation of occludin upon PDGFR α activation resulting in occludin internalization and consequently vascular leakage (Goncalves et al., 2022). Collectively, these studies therefore demonstrate that PDGF-C can promote BBB permeability downstream of tPA.

1.3.5 Blood-brain barrier disruption as a potential therapeutic target

Dysfunction of the BBB is a major hallmark for the progression of ischemic stroke pathology and unfortunately limits the use of rtPA as discussed above. Preserving the integrity of the BBB is therefore of great interest in the context of reducing rtPA-mediated side effects and extending the therapeutic time window. Interestingly, administration of a neutralizing anti-PDGF-C antibody or inhibition of PDGFR α using imatinib, a small tyrosine kinase receptor inhibitor, significantly reduces vascular leakage after MCAO, and imatinib treatment reduces ICH in mice treated with rtPA five hours post onset of ischemia (Su et al., 2008). These together with other observations suggest that targeting the PDGF-C pathway as an adjuvant co-treatment to thrombolysis with rtPA may limit the risk of HT. Indeed, a phase II clinical trial evaluating the safety and efficacy of imatinib in patients treated with intravenous thrombolytics, determined that imatinib administration in stroke patients is safe and shows promising results in the improvement of neurological outcome (Wahlgren et al., 2017).

Another proposed therapeutic target in stroke is VEGF-A. In animal models, expression of both VEGF-A and its corresponding receptors increases early during MCAO and is sustained over a longer period of time (Greenberg and Jin,

2013). However, the exact role of VEGF-A in ischemic stroke remains unclear as VEGF-A has been reported to both convey neuroprotection and to exacerbate ICH and edema via increased BBB leakage (Greenberg and Jin, 2013). For example, Zhang *et al.* demonstrated that intravenous administration of recombinant VEGF-A one hour post focal cerebral embolic ischemia in rats increases BBB leakage and the risk of HT as well as the size of the ischemic lesion. In contrast, administration of VEGF-A 48 hours post stroke onset increased angiogenesis in the ischemic penumbra and improved neurological recovery (Zhang *et al.*, 2000). Likewise, VEGF-A removal via decoy receptor sequestering reduced edema formation and infarct size in a mouse model of cortical ischemia (van Bruggen *et al.*, 1999), while intraventricular infusion of anti-VEGF-A antibody increased infarct volume after ischemia in rats (Bao *et al.*, 1999). In addition, direct application of VEGF-A to the ischemic area via topical administration reduced infarct volume and edema formation after MCAO (Hayashi *et al.*, 1998). Collectively, these studies therefore demonstrate both a detrimental and a protective role of VEGF-A in ischemic stroke, likely due to an insufficient understanding of the role of this factor in ischemia-related pathology. VEGF-A therefore remains an unsuitable therapeutic target to prevent BBB leakage and improve stroke outcome.

VEGF-B has also been suggested to play a role in stroke pathology. Indeed, VEGF-B deficient mice subjected to permanent MCAO display increased infarct volume and neurological impairment (Sun *et al.*, 2004), while administration of VEGF-B in wild-type animals exposed to transient ischemia reduces early BBB leakage and neuronal damage via pericyte expression of VEGFR1 (Jean LeBlanc *et al.*, 2018). These studies suggest a neuroprotective effect of VEGF-B that may be beneficial when attempting to improve outcome after ischemic stroke. However, considering that earlier generations of the *Vegfb* knockout mice display congenital defects including smaller hearts, impaired coronary angiogenesis (Bellomo *et al.*, 2000) and impaired neurogenesis (Sun *et al.*, 2006), it is unclear if the observed increase in infarct volume is directly linked to the removal of VEGF-B or if these animals are in general more susceptible to stroke-related injury. Likewise, that VEGF-B signaling via VEGFR1 expressed on pericytes result in reduced vascular leakage is questionable since *in vivo* expression of VEGFR1 is mainly restricted to endothelial cells while expression in pericytes is either low or completely absent (He *et al.*, 2018). Hence, the role of VEGF-B in ischemic stroke remains unclear and more studies are needed to elucidate any potential beneficial or detrimental effects of VEGF-B manipulation in stroke-related pathology.

1.4 Diabetic kidney disease

Another major complication of T2D is the development of diabetic kidney disease (DKD), associated with severely reduced quality of life and increased risk of mortality. Roughly 50% of all T2D patients develop DKD which may further progress into end stage renal disease thus limiting the treatment options to dialysis or kidney transplant. Since DKD is associated with a severe reduction in life quality and increased risk of death, therapeutic management of T2D therefore often focus on preventing or halting DKD progression (Thomas et al., 2015). Strategies employed in the management of both T2D and DKD pathology include reducing hyperglycemia by increasing glucose-urine secretion via administration of sodium-glucose transport protein 2 inhibitors, or reducing hyperglycemia by promoting both insulin release and weight loss via administration of glucagon-like peptide-1 receptor agonists (Yamazaki et al., 2021). DKD is best described as the progressive failure of the kidney to properly filter the blood, manifesting as increased leakage of macromolecules, such as albumin, into the urine as well as a decline in the glomerular filtration rate. In normal renal physiology, the filtration of water, salts and low molecular weight proteins is achieved by the kidney structure known as the glomeruli (Daehn and Duffield, 2021). This structure is found inside what is referred to as the Bowman's capsule, and is composed of glomerular capillaries, basement membrane, podocytes and mesangial cells. The glomerular capillaries consist of fenestrated ECs covered in the carbohydrate-rich structure known as the glycocalyx. The glomerular basement membrane is found on the capillary abluminal side and serves as an anchor between the glomerular ECs and the podocyte foot processes. Logged in between glomerular capillaries are the mesangial cells that produce extracellular matrix proteins, provide structural support to the capillaries as well as protect them against increased glomerular pressure (Daehn and Duffield, 2021; Pollak et al., 2014). Outside the glomeruli is the Bowman's space, in which the primary glomerular filtrate enters, separated by parietal epithelial cells that form the outer layer of the Bowman's capsule (Pollak et al., 2014). The glycocalyx, fenestrated ECs, basement membrane and podocytes make up the glomerular filtration barrier, named so as it restricts the passage of molecules based on size and charge. The restriction of molecular passage into the primary urine is often attributed to the glycocalyx (that repels negatively charged molecules), the fenestrae of the glomerular ECs (that allow the passage of molecules below a certain size) and interconnected podocytes, which are tethered to the glomerular basement membrane via their foot processes. The foot processes of adjacent podocytes are connected via junctional proteins known as

slit diaphragms, that act as an additional barrier to macromolecules and provide mechanical support to counteract forces that arise during filtration (Daehn and Duffield, 2021). After entering Bowman's space, the primary glomerular filtrate drains into the renal structure known as the proximal tubule which is directly connected to the Bowman's capsule. In the tubule, fluids and molecules are re-absorbed from the primary urine by the tubular epithelial cells that therefore act as key components in maintaining systemic fluid homeostasis (Tian and Liang, 2021).

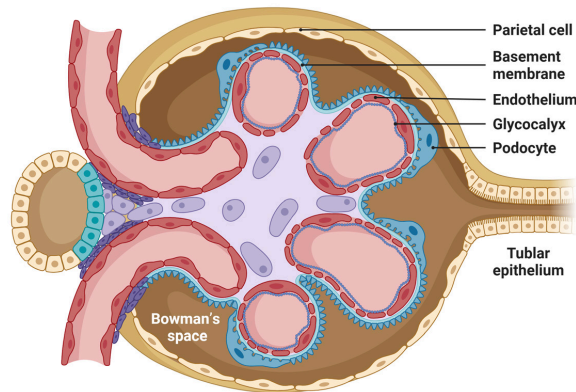


Figure 6: Illustration of the Bowman's capsule and the glomerulus. The glomerulus is composed of glycocalyx, capillary ECs, podocytes and mesangial cells, and give rise to the glomerular filtrate. The filtrate flows into Bowman's space and is subsequently drained into the tubule.

1.4.1 Pathological hallmarks of diabetic kidney disease

Due to its unique structure and function, it is not surprising that disease-mediated structural changes in the glomeruli are considered as hallmarks for DKD. These changes include glomerular basement membrane thickening, mesangial expansion and podocyte loss that together contribute to the breakdown of the filtration barrier. Injury to and loss of podocytes have been suggested to play a central role in disease development (Coward and Fornoni, 2015). For example, the loss of podocytes is associated with both increased albumin leakage as well as reduced glomerular filtration rate in T2D patients (Pagtalunan et al., 1997; Weil et al., 2012), and genetic removal or introduction of a loss of function mutation in podocin, a protein that take part in the assembly of the slit diaphragm, result in increased albuminuria and increased mesangial sclerosis in mice (Roselli et al., 2004; Tabatabaeifar et al., 2017). Another major hallmark in DKD is the

development of tubulointerstitial fibrosis often considered to be one of the strongest predictors for disease progression (Thomas et al., 2015) and is suggested to occur before glomerulopathy and microalbuminuria in early DKD (Golea-Secara et al., 2020).

1.4.2 Diabetic kidney disease and dyslipidemia

The kidney is an organ with high metabolic activity that relies heavily on aerobic oxidation of glucose and FAs. This is partially due the high rate of ATP-dependent sodium potassium exchange in the tubular epithelial cells which facilitates the re-absorption of ions, amino acids, water and glucose from the primary urine (Tian and Liang, 2021). Considering the high metabolic rate of the kidney, T2D-associated insulin resistance, hyperglycemia and dyslipidemia as key factors for DKD development are especially interesting (Thomas et al., 2015). Although hyperglycemia has been proposed as a major risk factor (Thomas et al., 2015), the increased use of glucose lowering agents in treating diabetic patients has not reduced the incidence of DKD (de Boer et al., 2011) and the focus has therefore shifted from hyperglycemia to other disease mediators such as dyslipidemia. That hyperlipidemia and excessive renal lipid accumulation can mediate the development and progression of DKD was already proposed back in 1982 when the lipid nephrotoxicity hypothesis was introduced by John F Moorhead (Moorhead et al., 1982). Since then, the contribution of T2D-related insulin resistance, dyslipidemia and renal lipid accumulation to DKD development has gained increasing attention. In the healthy state, tubular epithelial cells tend to oxidize FAs while ECs, mesangial cells and podocytes mostly utilize glucose for energy production. However, glomerular cells increase their rate of FA uptake and oxidation during kidney disease (Mitrofanova et al., 2023). For example, diabetic animal models display increased gene expression of *Slc27a3* (FATP3) and *Slc27a4* (FATP4) (Falkevall et al., 2017) as well as increased expression of CD36 and FATP4 in the glomerulus (Hua et al., 2024), and FA exposure of *in vitro* cultured podocytes leads to increased CD36 expression further contributing to LD accumulation and ROS production (Hua et al., 2015; Hua et al., 2024). Patients suffering from DKD also present with increased lipid deposition in the kidney (Herman-Edelstein et al., 2014), presumably related to several factors including increased plasma lipid levels, increased renal FA uptake, increased renal lipogenesis, decreased renal FAO, increased renal cholesterol uptake and decreased cholesterol efflux (Schelling, 2022). Indeed, expression of CD36 (Hua et al., 2015) and *SLC27A3* (FATP3) (Cao et al., 2023) as well as expression of *de novo* FA synthesis genes

(Afshinnia et al., 2019) are increased in the kidney of human DKD patients. Furthermore, DKD patients display upregulation of VEGF-B in glomeruli (Falkevall et al., 2017; Woroniecka et al., 2011), tubules (Cao et al., 2023; Shen et al., 2021) and plasma (Wei et al., 2022). However, exactly which type of cells that express VEGF-B remains to be determined. In addition, a larger abundance of saturated LCFAs in plasma correlates with DKD progression (Afshinnia et al., 2018), and FABP1 and FABP2 have been suggested to act as biomarkers for DKD since higher plasma levels have been associated with reduced renal function in DKD patients (Tsai et al., 2020). The abnormalities in renal lipid metabolism present during disease may additionally be linked to systemic and renal insulin resistance. For example, glomerular insulin sensitivity is reduced in diabetic animal models (Tejada et al., 2008), and mice lacking the insulin receptor in podocytes develop DKD-associated pathologies (Welsh et al., 2010). In addition, podocyte overexpression of VEGF-B increases glomerular LD accumulation, glomerular insulin resistance and impairs renal function in high-fat diet (HFD)-fed mice (Falkevall et al., 2017). Furthermore, reduced insulin sensitivity in humans is associated with increased risk of albuminuria (Pilz et al., 2014) and DKD development in diabetic patients (Ahlqvist et al., 2018). The increase in renal FA uptake and reduced insulin sensitivity during DKD suggests a role for the endothelium in mediating disease progression and targeting endothelial FA transport may therefore ameliorate both DKD and general T2D pathology as will be discussed below.

1.5 Targeting the endothelium to ameliorate type 2 diabetes pathology

The endothelial barrier plays an important role in maintaining systemic fluid and metabolic homeostasis. It is therefore not surprising that pathological changes to the endothelium are suggested to underlie T2D development, especially when considering the complete dysregulation of systemic metabolism that occur during this disease. Indeed, pathological changes in the microvasculature often precedes the development of T2D, and both endothelial dysfunction and insulin resistance have been proposed to act as causative factors for disease development (Graupera and Claret, 2018; Hasan and Fischer, 2021). Furthermore, alterations in endothelial function and insulin sensitivity have been linked to an abnormal regulation of systemic metabolism, and the mechanisms of endothelial FA and glucose transport are therefore receiving increasingly more attention as potential novel drug targets that may ameliorate the pathophysiology of T2D. Indeed, modulating EC transport of FAs and glucose through genetic manipulation that remove the expression of transendothelial FA transport regulators, or by

enhancing signaling of factors that block the transport of FAs from the blood to tissues show promising results in animal studies. For example, stimulation of apelin signaling in HFD mice improves insulin sensitivity and increases glucose transcytosis thus consequently increasing glucose utilization (Hwangbo et al., 2017). In addition, angiopoietin-2 signaling shunts excess FAs into subcutaneous adipose tissue thus reducing ectopic lipid accumulation in mice further highlighting the important role of the adipose tissue in buffering a surplus in plasma lipids (Bae et al., 2020). Consistent with the aforementioned factors, mice with lower levels of 3-HIB display reduced accumulation of lipids in skeletal muscle as well as improved glucose tolerance (Jang et al., 2016). Targeting of VEGF-B is particularly promising for combating the development of T2D, as mice that lack VEGF-B appear healthy thus suggesting minimal adverse effects of VEGF-B removal (Hagberg et al., 2010). However, despite being well tolerated, a significant association between increased blood pressure and anti-VEGF-B treatment was demonstrated in a phase II clinical trial designed to assess the safety of anti-VEGF-B treatment in DKD (Cooper et al., 2024). Therefore, more studies are needed that specifically address the potential side effects of VEGF-B neutralization in humans. Nevertheless, animal models of T2D that either lack VEGF-B or are treated with VEGF-B neutralizing antibodies, display reduced ectopic lipid accumulation, restored insulin sensitivity, improved glucose tolerance and have reduced circulating blood lipids (Hagberg et al., 2012). Furthermore, systemic inhibition of VEGF-B reduces adipose tissue lipolysis and protects diabetic mice against the development of MAFLD (Falkevall et al., 2023), and ameliorates renal lipid accumulation correlating with improved glomerular function in diabetic mouse models (Falkevall et al., 2017). These together with other findings support the therapeutic potential of specific targeting of endothelial substrate transport modulators, and studies attempting to elucidate the cellular mechanisms responsible for their effects are ongoing.

2 Research aims

Rationale

Obesity and T2D are spreading at an alarming rate. T2D patients often present with metabolic dysfunctions manifesting as increased levels of circulating lipids, ectopic lipid accumulation in tissues and insulin resistance. The endothelium serves as a gate keeper for the transport of energy substrates from the blood to the parenchyma, and dysfunctions in the endothelium arise early during T2D disease development. Abnormalities in the endothelium can be coupled to alterations in energy substrate transport reflected by an increased FA and decreased glucose transport respectively. However, little is known regarding the mechanisms that underlie endothelial energy substrate selection and transport. Understanding these processes are therefore crucial when attempting to target endothelial FA transcytosis as a therapeutic strategy to combat T2D co-morbidities including ischemic stroke and diabetic kidney disease.

Overall aim

The overall aim of this thesis was to identify mechanisms that underlie endothelial FA and glucose uptake, and to evaluate the therapeutic potential of targeting VEGF-B in obesity and diabetes-related ischemic stroke and kidney disease.

Aim I

Identify both novel inducers of endothelial FA uptake and the cellular metabolic pathways that governs FA uptake in ECs during different physiological conditions.

Aim II

Determine the impact of VEGF-B on endothelial glucose uptake and elucidate the underlying mechanisms.

Aim III

Investigate effects of obesity and dyslipidemia on FA uptake and endothelial cell substrate partitioning, and their connection to BBB permeability and ICH complications arising from delayed thrombolysis treatment after ischemic stroke.

Aim IV

Evaluate the impact of systemic dyslipidemia and VEGF-B signaling in diabetic kidney disease.

3 Materials and methods

This section includes the principal methodologies that I developed during my doctoral studies, as well as a table describing the mouse strains. A detailed description of all the methodologies can be found in the research papers included in this thesis. All illustrations in this thesis were created with BioRender.com.

3.1 Glucose and fatty acid tracers

The 2-NBDG molecule is a deoxy-glucose derivative conjugated to the NBD fluorescent molecule. This tracer has an excitation/emission spectra of 465/540 nm, is resistant to phosphorylation by the glycolytic enzyme hexokinase and is therefore commonly used to analyze cellular glucose uptake. Since the 2-NBDG molecule is phosphorylation resistant it cannot be trapped intracellularly. 2-NBDG is therefore often combined with a glucose enantiomer (L-glucose) that cells are not able to internalize. The presence of L-glucose therefore maintains the extracellular concentration gradient during and post 2-NBDG uptake forcing the tracer to remain in the cellular cytosol. Likewise, since 2-NBDG can further escape during subsequent cell fixation and washing steps, all buffers used after tracer incubation contain D-glucose to maintain intracellular 2-NBDG levels. In contrast, the BODIPYTM 558/568-C₁₂ molecule consists of a 12-carbon long FA conjugated to a fluorescent BODIPY and is used to analyze cellular LCFA uptake.

3.2 Lipid and nuclear dyes

The BODIPYTM 493/503 is a fluorescent hydrophobic dye that stains hydrophobic compartments and is therefore commonly used to visualize and analyze cellular neutral lipids. Hoechst is a fluorescent non-intercalating dye with an excitation/emission spectra of 350/461 nm that binds preferably to DNA regions rich in adenine and thymine and is used for the visualization and analysis of cell nuclei.

3.3 Endothelial cell *in vitro* culture

Human umbilical vein endothelial cells (HUVECs) is a commonly used *in vitro* EC model system since HUVECs retain many typical characteristics displayed by ECs *in vivo*. For example, HUVECs express typical EC markers including CD31, adhesion molecules, selectins, and von Willebrand factor as well as display a functional LDL uptake. In brief, we expanded HUVECs in endothelial cell growth medium with antibiotics and passaged the cells when roughly 90% confluency had been

reached. Following passaging, the cells were seeded in tissue culture-treated 10 cm dishes, 6-well plates, 24-well plates, 96-well plates or permeable cell culture inserts with 0.4 μm pore size pre-coated with 0.1% gelatine.

3.4 Transendothelial electrical resistance

Transendothelial electrical resistance (TEER) measures electrical resistance across an endothelial monolayer using two electrodes. Cells are grown to confluency on a tissue culture-treated permeable membrane placed inside a well of a well-plate. One electrode is placed inside the well, while the other is placed directly above the endothelial monolayer. The electrical resistance is then measured over the surface area using two electrodes. For TEER measurements of ECs, HUVECs seeded in permeable cell culture inserts were supplemented with or without oleic acid, and TEER was measured using a Milli-cell-ERS electrode from Millipore pre-calibrated in reference medium.

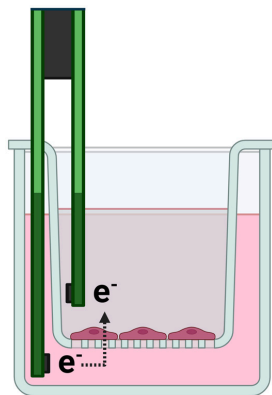


Figure 7: Illustration depicting TEER measurement. Electrical current flows between the electrodes. The electrical resistance produced when current passes through the EC monolayer can therefore be measured.

3.5 Neutral lipid content/lipolysis assay

To analyze alterations in cellular neutral lipid content in response to lipolysis, we designed a FA chase assay where HUVECs are pre-loaded with oleic acid prior to treatment with factors of interest. In brief, confluent HUVECs supplemented overnight with oleic acid-albumin conjugates, were deprived of growth factors in the absence or presence of pharmacological inhibitors. After growth factor deprivation, the cells were stimulated with VEGF-B₁₈₆ or treated with 3-HIB or lactate in the absence of oleic acid. The cells were then fixated in 4%

paraformaldehyde, washed with PBS and subjugated to short-term incubation with BODIPY™ 493/503 neutral lipid dye and hoechst. The cells were then directly imaged under a Zeiss Apotome microscope to evaluate neutral lipid content.

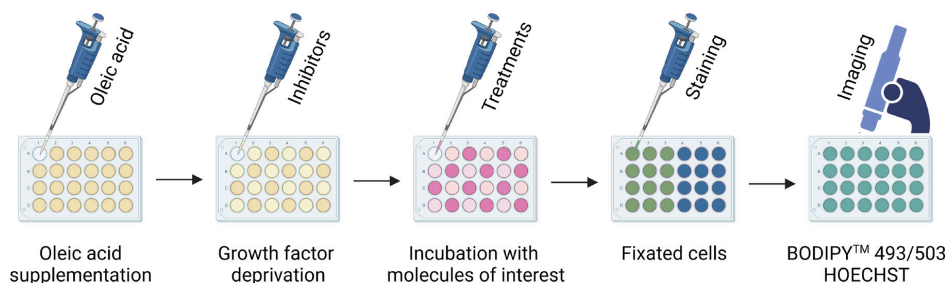


Figure 8: Schematic overview of the neutral lipid content assay. HUVECs are supplemented with oleic acid overnight prior to depriving the cells of growth factors in the presence of oleic acid and in the absence or presence of inhibitors. Treatments with either VEGF-B, 3-HIB or lactate are performed in the absence of oleic acid prior to cell fixation. Fixated cells are stained for lipids and nuclei using BODIPY™ 493/503 and hoechst prior to imaging.

3.6 Endothelial fatty acid uptake assay

To analyze endothelial FA uptake, we designed an assay based on short-term (6–7 min) incubation with BODIPY™ 558/568- C_{12} allowing us to visualize acute FA uptake. In brief, confluent HUVECs were deprived of growth factors for two hours in the absence or presence of various pharmacological inhibitors. The cells were then stimulated with VEGF- B_{186} or treated with lipolysis activators, oleic acid, lactate or 3-HIB to induce FA uptake in ECs. Following stimulations and treatments, the cells were washed in 0.1% FA-free bovine serum albumin and subjugated to short-term incubation with BODIPY™ 558/568- C_{12} -albumin conjugates. The cells were then fixated in 4% paraformaldehyde and nuclei were counter-stained with hoechst. The cells were then washed with PBS and directly imaged using a Zeiss Apotome microscope.

3.7 Endothelial glucose uptake assay

To analyze endothelial glucose uptake, we utilized the 2-NBDG molecule as a glucose analogue. In brief, confluent HUVECs were deprived of growth factors for two hours in the absence or presence of inhibitors. The cells were then stimulated with VEGF- B_{186} or VEGF- B_{167} protein and washed in Krebs ringer solution without glucose prior to subjugating the cells to incubation with 2-NBDG and L-glucose.

The cells were then fixated in 4% paraformaldehyde containing D-glucose and hoechst. The cells were washed with PBS containing D-glucose and directly imaged using a Zeiss Apotome microscope.

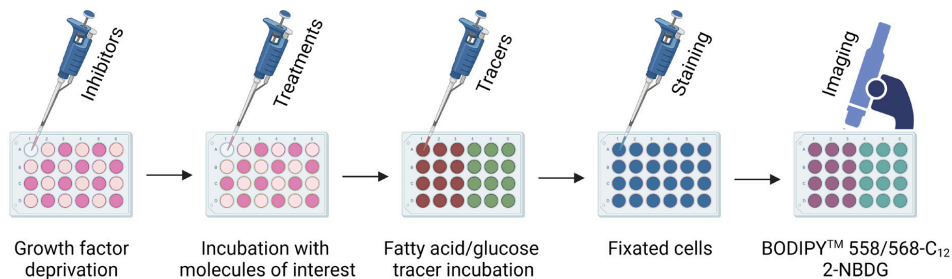


Figure 9: Schematic overview of the fatty acid or glucose uptake assay. Confluent HUVECs are deprived of growth factors in the absence or presence of inhibitors. After growth factor deprivation, the cells are stimulated with VEGF-B prior to incubating cells with either 2-NBDG or BODIPYTM 558/568-C₁₂. Following tracer incubation, the cells are fixated and stained with hoechst. Imaging is performed directly after nuclei counter-staining.

3.8 Live cell metabolic analysis

The Seahorse real-time metabolic analysis is based on measuring cellular flux of oxygen and hydrogen ions. This technology utilizes probes that fluoresce upon contact with either oxygen or hydrogen in the extracellular media adjacent to the cellular monolayer. Therefore, non-bicarbonate buffered media as well as oxygen degassing prior to analysis are required. Signal detection allows for measuring and comparing the total oxygen consumption and extracellular acidification rate as proxies for OXPHOS and anaerobic glycolysis respectively. There are several assays tailored according to analysis of interest. For example, to measure total ATP production, a series of mitochondrial electron transport chain complex inhibitors are injected at different times after initial measurements of both baseline oxygen consumption and extracellular acidification. The specific compounds used are oligomycin, rotenone and antimycin A which act as inhibitors of either ATP synthase, the electron transport chain complex I or the electron transport chain complex III respectively. The subsequent drop in oxygen consumption as well as the increase in extracellular hydrogen ions are then subtracted from the baseline measurements allowing one to analyze the amount of baseline OXPHOS and anaerobic glycolysis. Based on this the total ATP production can be calculated. Other assays include the mitochondrial stress test

designed to measure mitochondrial function and oxidation as well as maximum respiratory capacity.

To study if VEGF-B stimulation would impact ATP production in ECs, we performed the Seahorse XF real-time ATP rate assay. In brief, confluent HUVECs were stimulated with VEGF-B₁₈₆ for one hour in endothelial cell media followed by one hour stimulation in degassed HEPES-buffered Seahorse media. Seahorse metabolic analysis was initiated exactly two hours post VEGF-B stimulation using the Seahorse XFe96 analyzer. After baseline measurements had been performed, glucose was injected, and a series of new measurements were acquired prior to injection of first oligomycin and second rotenone combined with antimycin A and the nuclear staining dye hoechst. After analysis had been performed, the cells were fixated in 4% paraformaldehyde and cell nuclei were immediately imaged using a Zeiss apotome microscope. All values were normalized to nuclei count.

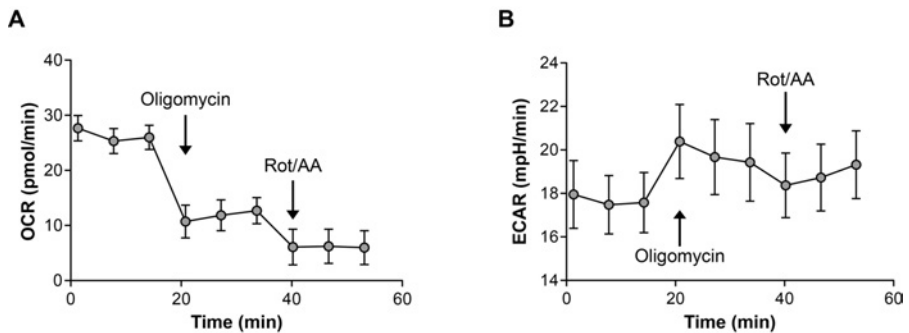


Figure 10: Graphs depicting injection strategy, oxygen consumption rate (A) and extracellular acidification rate (B) in HUVECs. Note the reduction in oxygen consumption rate (OCR) and the respective increase in extracellular acidification rate (ECAR) after injection of inhibitors of mitochondrial respiration.

3.9 Gene silencing

The siRNA gene silencing method is based on introducing siRNAs that are either custom designed or purchased commercially. These type of RNAs attach to their target mRNA molecules via complementary base pair binding leading to gene silencing by either directly interfering with protein translation or indirectly by initiating degradation of target mRNA. When performing siRNA gene silencing, a specially designed siRNA unable to bind any mRNA molecules produced in mammals is included as a negative control. The two most common methods used for introducing siRNAs into cells are lipofection or electroporation. Lipofection

refers to the utilization of a liposomal-based transfection reagent where cationic lipids enable the interaction between the RNA and the plasma membrane resulting in cellular endocytosis of the RNA molecule. In contrast, electroporation is based on exposing cells to electrical current resulting in reversible disruption of the cellular membrane as well as migration of negatively charged nucleic acids into the cell. In brief, lipofectamine and siRNA were mixed in special transfection medium (Opti-MEM™) and added directly to HUVECs grown to roughly 50% confluence. siRNA transfections were carried out for 24 hours and transfected cells were either used for endothelial FA uptake or glucose uptake experiments or for RNA isolation and gene expression analysis.

3.10 Statistical analysis

For statistical analysis of one variable between two groups, we used unpaired two-tailed *t*-test while for statistical analysis of one variable between three or more groups, one-way ANOVA with the appropriate post-hoc test was used. Two-way ANOVA was used for statistical comparison of two variables between three or more groups, while pairwise statistical analysis using two-way repeated measures ANOVA was used to analyze two variables between paired samples. Prior to all statistical analyses conformity to normality distribution of all datasets were determined. When applicable, appropriate non-parametric tests were used or conformity of data to log¹⁰ normality was determined prior to analysis of datasets with two variables. Values were considered significant when the acquired *p*-value was below the set threshold ($p < 0.05$). Detailed descriptions of statistical analysis are provided in the scientific papers of this thesis.

3.11 Animal models

Housing of animals and experimental procedures were in accordance with the guidelines stated by the European Community Council Directive and the Swedish National Board for Laboratory Animals. All animals were housed in groups at all times. Ethical permits were approved by the North Stockholm Animal Ethics Committee, and animal welfare guidelines as stated by the 3R principles (Russell and Burch, 1959) were followed at all times.

Model	Description
C57BL/6J mice	Wild-type mice
C57BL/6 <i>Vegfb</i> ^{-/-} mice	Harbors a constitutive deletion of <i>Vegfb</i> . Previously described (Aase et al., 2001).
BKS <i>Lepr</i> ^{db/db} (<i>db/db</i>) mice	Harbors a constitutive point mutation in the <i>Lepr</i> gene resulting in overfeeding. Used to model T2D pathology.
BKS.C57BL/6 <i>db/db Vegfb</i> ^{-/-} mice	Mixed background model previously described (Hagberg et al., 2012).
Adipoq ^{Cre+} / <i>Vegfb</i> ^{fl/+} mice	Transgenic model with adipocyte-specific under-expression of VEGF-B. Previously described (Falkevall et al., 2023). This model expresses the <i>cre</i> recombinase under the adipocyte-specific adiponectin promotor and harbors <i>cre</i> recognition sites (loxP) flanking exon 2–6 in one allele of the <i>Vegfb</i> gene.
Pod ^{Cre+} /VEGF-B ^{TG/+} mice	Transgenic model with specific VEGF-B overexpression in podocytes. Previously described (Falkevall et al., 2017). This model expresses the <i>cre</i> recombinase under the podocyte-specific podocin promotor and constitutively harbors, in one allele of the <i>Vegfb</i> gene, the rosa 26 <i>Vegfb</i> ₁₆₇ construct with loxP sites flanking the stop codon located downstream of the CAG promotor. CAG induced VEGF-B ₁₆₇ protein expression is therefore only initiated in podocytes.
HFD mice	Animals fed a HFD dietary regiment consisting of 60 kcal% fat, 20 kcal%, protein and 20 kcal% carbohydrates. Commonly used to model obesity.

4 Results

4.1 Aim I

Identify both novel inducers of endothelial FA uptake and the cellular metabolic pathways that governs FA uptake in ECs during different physiological conditions.

*In **Paper I** we demonstrate that activation of lipolysis can drive endothelial FA uptake by increasing the shunting of FAs into the mitochondria resulting in mitochondrial ATP generation. We further identify oleic acid and forskolin as novel inducers of FA uptake in ECs.*

When attempting to study the relationship between VEGF-B-induced endothelial FA uptake and LD accumulation, we discovered that VEGF-B activated lipolysis in ECs (Figure 1A and Supplementary Figure 1A) via increased phosphorylation of HSL (Figure 1B and Supplementary Figure 1C). Treatment with lipolysis inhibitors or siRNA knockdown of *PNPLA2* (ATGL) or *LIPE* (HSL) reduced FA uptake in ECs stimulated with VEGF-B (Figure 2A-C and Supplementary Figure 2A). Likewise, pharmacological activation of lipolysis induced endothelial FA uptake (Figure 2D and Supplementary Figure 2C-D) while lipolysis inhibition mitigated this effect (Supplementary Figure 3A-B). Intriguingly, oleic acid treatment induced endothelial FA uptake via activation of lipolysis (Figure 2G and Supplementary Figure 3G, K) while siRNA knockdown of lipolytic enzymes or treatment with lipolysis inhibitors significantly reduced FA uptake in oleic acid-supplemented ECs (Figure 2I-J and Supplementary Figure 3H). Inhibition of either mitochondrial FA shunting or mitochondrial ATP production reduced FA uptake in VEGF-B stimulated, oleic acid-supplemented or forskolin-treated ECs (Figure 2E-F, K-L and Supplementary Figure 3C-D). Likewise, inhibiting either the transport of ATP from the mitochondria to the cytosol or cytosolic FA-CoA conjugation prohibited the ability of VEGF-B, forskolin and oleic acid to induce endothelial FA uptake (Supplementary Figure 2G-H and Supplementary Figure 3E-F, I-J).

In addition, we show that conversion of pyruvate to lactate facilitates lactate-induced endothelial FA uptake, and that both lactate and 3-HIB relies on mitochondrial ATP when engaging ECs in FA uptake.

We included lactate and 3-HIB in our study to investigate if other inducers of endothelial FA uptake rely on different metabolic pathways to assert their effect. We discovered that pharmacological or negative feed-back inhibition of lactate dehydrogenase-mediated lactate to pyruvate conversion reduced lactate-

induced endothelial FA uptake (Figure 3B, E), and inhibition of either mitochondrial ATP generation, cytosolic ATP transport or FA-CoA conjugation reduced FA uptake in lactate or 3-HIB treated ECs (Figure 3C, G, H and Figure 4I-K).

Furthermore, we demonstrate that ECs rely on functional lipolysis and FA esterification when engaging in FA uptake, and that both lactate and 3-HIB can promote FA de novo synthesis in ECs.

Inhibition of lipolysis reduced lactate's and 3-HIB's ability to induce FA uptake in ECs independent of mitochondrial FA shunting (Figure 4A-B, F, G and Supplementary Figure 4A, C). Likewise, inhibiting FA incorporation into TAGs reduced lactate, VEGF-B, 3-HIB and forskolin-induced endothelial FA uptake (Figure 4C-D, H and Supplementary Figure 4B). Lactate or 3-HIB treatment increased neutral lipids in oleic acid treated ECs (Figure 5A, D and Supplementary Figure 5A, D) while inhibition of acetyl-CoA carboxylase restored neutral lipid levels in lactate and 3-HIB treated cells independent of FA uptake (Figure 5B, E and Supplementary Figure 5C, E).

Lastly, we demonstrated *in vivo* that higher plasma NEFA levels increased FA uptake and retention in the CNS (Figure 6B-C).

4.2 Aim II

Determine the impact of VEGF-B on endothelial glucose uptake and elucidate the underlying mechanisms.

In Paper II we demonstrate that VEGF-B reduces GLUT1-mediated endothelial glucose uptake without affecting the total ATP production. We could further show that VEGF-B signaling reduces cholesterol uptake and deposition in membranes, connecting endothelial cholesterol content to GLUT1-mediated glucose uptake.

Stimulating ECs with VEGF-B reduced glucose uptake and transcytosis without affecting total ATP production or expression of metabolic genes (Figure 1A-B, EV3A-D and EV4). In contrast, genetic deletion of VEGF-B or systemic inhibition of VEGF-B signaling using a neutralizing antibody increased cardiac glycogen storage and glucose uptake from the blood respectively (Figure 1C-E). siRNA knockdown of the signaling receptors for VEGF-B (*FLT1* (VEGFR1) and *NRP1* (Neuropilin-1)), or *SLC2A1* (GLUT1) prevented VEGF-B's ability to reduce glucose uptake in ECs (Figure 2D and EV2A), suggesting that VEGF-B signaling impairs GLUT1-facilitated glucose uptake in ECs.

However, VEGF-B signaling did not affect GLUT1 expression (Figure 3A-B) but instead shifted GLUT1 distribution in the plasma membrane, indicative of changes in the lipid membrane microenvironment. Using sucrose density gradient fractionation, we found that VEGF-B signaling shifted the distribution of GLUT1 from low-density to high-density membrane fractions, indicative of a decreased membrane cholesterol content (Figure 3C-D and Figure S2A-B). Correspondingly, we could show that VEGF-B signaling reduced cholesterol uptake and deposition in membranes, connecting endothelial cholesterol content to GLUT1-mediated glucose uptake.

To elucidate a potential connection between VEGF-B's ability to both induce FA uptake and impair glucose uptake in ECs respectively, we analyzed FA uptake in ECs after knockdown of VEGF-B receptors or GLUT1. siRNA knockdown of *Flt-1* (VEGFR1) and *Nrp1* (Neuropilin-1) impaired VEGF-B's ability to induce endothelial FA uptake as expected while *Slc2a1* (Glut1) knockdown had no effect (Figure EV2D-F). Pointing to the temporal and sequential order of events, VEGF-B reduced cholesterol uptake and content in ECs within ten minutes followed by a decrease in the uptake of glucose and an increase in the uptake of FAs after 30 minutes and two hours respectively (Figure 4A-F). Likewise, extracting cholesterol from the plasma membrane reduced endothelial glucose uptake independent of VEGF-B without affecting endothelial FA uptake (Figure 5A and EV5A-B). In contrast, glucose uptake was normalized to control levels in cholesterol-supplemented cells stimulated with VEGF-B (Figure 5B).

Furthermore, we demonstrate that VEGF-B reduces low-density lipoprotein receptor re-cycling to the surface therefore reducing cholesterol uptake.

Genetic deletion or siRNA-mediated knockdown of *LDLR1* removed VEGF-B's ability to modulate EC cholesterol content (Figure 6B, D), and blunted VEGF-B's effect on endothelial glucose uptake (Figure 6C). Consistently, VEGF-B stimulation reduced surface expression of the LDLR and reduced LDL surface binding and uptake (Figure 6F-I and Appendix figure S3). However, VEGF-B does not affect overall LDLR expression (Appendix figure S4A-B). Instead, ECs stimulated with VEGF-B display alterations in the intracellular distribution of LDLR consistent with reduced receptor recycling (Appendix figure S4C-E and S5).

Lastly, we show that VEGF-B regulates membrane cholesterol content in vivo.

Full or partial genetic deletion of *Vegfb* or *Flt1* (VEGFR1) respectively increased vessel-associated and total cardiac free (non-esterified) cholesterol content *in vivo* (Figure 7A–C) without affecting expression of genes associated with cholesterol homeostasis (Appendix figure S6B). Both *db/db* and HFD-fed mice display reduced cardiac free cholesterol content as compared to wild-type or chow-fed control animals, and VEGF-B removal, either via genetic deletion or anti-VEGF-B antibody treatment, significantly increased cardiac free cholesterol levels in diabetic animal models (Figure 7D–F).

4.3 Aim III

Investigate effects of obesity and dyslipidemia on FA uptake and endothelial cell substrate partitioning, and their connection to BBB permeability and ICH complications arising from delayed thrombolysis treatment after ischemic stroke.

In Paper III we show that LCFA exposure switches ECs from glucose to FA uptake and reduces EC barrier integrity.

Supplementation with LCFAs reduced glucose uptake and GLUT1 expression in ECs (Figure 1B–E), independent of VEGF-B (Figure 1O). In contrast, LCFA treatment increased endothelial FA uptake (Figure 1F, P), correlating with augmented mitochondrial capacity and LD formation (Figure 1G–J and Figure S1B). A corresponding switch in EC nutrient selection was also observed after VEGF-B stimulation (Figure 1O–P). Furthermore, FA treatment reduced TEER (Figure 1K) and promoted barrier weakening and increased transcytosis in ECs (Figure 1L–N).

In addition, we demonstrate that systemic inhibition of VEGF-B reduces plasma lipid levels and restores brain glucose uptake capacity in HFD mice.

VEGF-B is broadly expressed in the CNS (Figure S2A), while co-expression of VEGFR1 and neuropilin-1 is restricted to ECs (Figure S2B–C). HFD-feeding results in weight gain, development of hyperglycemia (Figure S3B–C) and reduced brain glucose uptake capacity independent of CNS expression of either GLUT1 or VEGF-B (Figure 2I and S3D, F). Interestingly, short-term systemic inhibition of VEGF-B reduced plasma NEFA and TAG levels in HFD mice (Figure 2B–C), correlating with reduced parenchymal LD accumulation and restored brain glucose uptake to levels comparable with chow-fed animals (Figure 2I–K).

Furthermore, we show that increased levels of circulating lipids induce brain ectopic LD accumulation in mice subjected to MCAO and that this does not

correlate with local VEGF-B signaling in the brain. We connect the accumulation of LDs with increased endothelial oxidative stress and permeability in the CNS.

HFD mice displayed increased infarct size and increased incidence and severity of ICH when subjected to MCAO (Figure 3B-C) and were more reperfusion resistant and more susceptible to thrombosis post stroke (Figure 3D, F). LD accumulation in the vascular wall increased in response to MCAO (Figure 3H), and HFD-fed mice subjected to ischemia display increased LD accumulation in both ischemic and non-ischemic brain tissue (Figure 3I-L) as well as within ECs (Figure 3K-L). Furthermore, endothelial LD accumulation in HFD mice correlated with increased EC mitochondrial swelling in the ischemic hemisphere (Figure 3M) and HFD feeding increased the overall vasogenic permeability in the brain when subjected to MCAO (Figure 3N-O and S3K-L). To elucidate a potential role of VEGF-B signaling in the CNS during MCAO we investigated changes in cerebral VEGF-B expression in HFD mice subjected to MCAO. VEGF-B expression only marginally increased in the penumbra region three and 24 hours post MCAO (Figure S3M), while global CNS expression of VEGF-B and VEGFR1 were not changed (Figure S3O-P).

Systemic inhibition of VEGF-B prior to stroke onset decreases cerebrovascular LD accumulation resulting in improved BBB integrity thereby reducing stroke-associated pathology. Likewise, systemic VEGF-B neutralization post stroke improves the efficacy and safety of delayed thrombolytic reperfusion therapy in HFD mice subjected to MCAO.

Short-term pre-treatment with a neutralizing anti-VEGF-B antibody reduced circulating NEFA levels (Figure 2B) coinciding with reduced infarct volume and ICH in both chow and HFD-fed mice subjected to MCAO (Figure 4B-C). The reduction in circulating NEFAs correlated with improved penumbra perfusion (Figure 4E-F), reduced transcellular and paracellular leakage (Figure 4G-H and S4E-I) and reduced cerebrovascular LD accumulation (Figure 4I-J and S5D).

Administration of the anti-VEGF-B antibody in a therapeutic setting (one hour post-MCAO) neither reduced stroke-associated infarct nor ICH (Figure S6B-C). Consistent with a local rather than a systemic effect, a single injection of anti-VEGF-B antibody did not affect circulating lipid levels (Figure S6K-N) despite improving BBB integrity and reducing both vascular leakage and cerebrovascular LD accumulation (Figure S6E-J). In combination with five-hour delayed intravenous thrombolysis with rtPA, administration of anti-VEGF-B antibody one

hour post MCAO however reduced the infarct volume and ICH and improved the overall survival in HFD mice (Figure 5B-F). We discovered that delayed administration of rtPA increased the levels of circulating NEFAs post-MCAO, correlating with increased LD formation in the ischemic hemisphere, and that VEGF-B neutralization inhibited this effect (Figure 5H-J).

We further show that both stroke and intravenous thrombolysis with rtPA activates white adipose tissue lipolysis, and that adipose tissue-specific downregulation of VEGF-B is sufficient to improve stroke outcome and inhibit rtPA-induced adipose tissue lipolysis.

White adipose tissue lipolysis is activated in response to MCAO and is further aggravated by rtPA treatment, while systemic VEGF-B inhibition blunted both MCAO and rtPA-mediated adipose tissue lipolysis (Figure 6B-D and S7B-D). Furthermore, rtPA activated white adipose tissue lipolysis independent of MCAO resulting in increased release of NEFAs but not TAGs (Figure 6F-J and S7F-G), correlating with increased LD deposition in the brain (Figure 6K-L). Adipose tissue-specific haploinsufficiency of VEGF-B reduced infarct size and ICH in HFD-fed mice, (Figure 7B-C) and inhibited rtPA-mediated NEFA release in naïve chow-fed animals (Figure 7E). In addition, systemic removal of VEGF-B either via anti-VEGF-B antibody treatment four hours prior to rtPA administration or via genetic deletion reduced the levels of circulating NEFAs in naïve chow-fed mice treated with rtPA (Figure 7F, H).

Lastly, we demonstrate that rtPA increases plasma NEFA levels in human stroke patients presumably by activating white adipose tissue lipolysis.

Human stroke patients display increased plasma NEFA levels post rtPA treatment (Figure 7J) while, in contrast, time after stroke symptom onset did not affect plasma NEFA levels (Figure 7K) consistent with a specific effect of thrombolytic rtPA on white adipose tissue lipolysis.

4.4 Aim IV

Evaluate the impact of systemic dyslipidemia and VEGF-B signaling in diabetic kidney disease.

*In **Paper IV** we demonstrate that renal accumulation of LDs contributes to the progression of DKD in mice and that renal VEGF-B signaling is activated in diabetic animals. Furthermore, genetic deletion of Vegfb reduces LD accumulation in the glomeruli and improves renal function in diabetic animals.*

With age, *db/db* mice display increased LD accumulation within the glomeruli, lower abundance of podocytes (Figure 1A and S1A), increased albuminuria and hyperglycemia (Figure S1B), and increased *Vegfb* and *Slc27a4* (FATP4) expression (Figure 1B). Furthermore, VEGFR1 and neuropilin-1 are mainly co-expressed by ECs in the glomeruli in both *db/db* and control animals (Figure S5H-K), consistent with paracrine VEGF-B signaling regulating FA transport from the vasculature to the glomeruli. Indeed, genetic deletion of *Vegfb* reduced glomerular LD accumulation and FATP4 expression (Figure 1C-D), reduced albuminuria (Figure 1E), lowered the systemic blood pressure (Figure 1F), improved overall glomerular morphology (Figure 1G-H), reduced collagen deposition and reduced both podocyte injury (measured via synaptopodin expression) and vascular injury (measured via pecam expression or arteriolar thickness) in *db/db* (Figure 1I and S1I-K) as well as HFD mice (Figure 1J and S2A-G).

Administration of anti-VEGF-B treatment reduces glomerular LD accumulation and renal lipotoxicity, rescues the glomerular filtration barrier and improves renal function in both db/db and HFD mice.

Systemic inhibition of VEGF-B via anti-VEGF-B antibody administration reduced glomerular and podocyte LD accumulation (Figure 2A, J, S3A and S3B), prevented the development of macroalbuminuria (Figure 2B), lowered the systemic blood pressure and increased glomerular filtration rate when compared to isotype IgG control treated *db/db* mice (Figure 2C-D). In addition, inhibiting VEGF-B signaling in *db/db* mice reduced plasma levels of ketone bodies and TAGs but did not appear to mediate any major effect on plasma NEFA levels (Figure S3C), blood glucose levels or insulin sensitivity (Figure 2E and S3D-H) when compared to isotype IgG control. Furthermore, systemic anti-VEGF-B antibody treatment improved the overall glomerular morphology and reduced collagen deposition and podocyte and vascular injury (Figure 2F-I, S3J-R and S4A) as well as reduced the

renal levels of several lipid species when compared to isotype IgG control treated *db/db* mice (Figure 2K-O).

In HFD mice, systemic inhibition of VEGF-B reduced glomerular lipid accumulation and plasma NEFAs (Figure 3A and S4C-D), reduced albuminuria and blood pressure (Figure 3B-C and S4E) and improved glucose tolerance and insulin sensitivity (Figure S4F-G). Furthermore, systemic anti-VEGF-B antibody treatment reduced glomerular sclerosis (Figure 3E) and podocyte and vascular injury (Figure 3F and S4I) as well as reduced the deposition of collagen (Figure S4J) when compared to isotype IgG control treated HFD-fed animals. Furthermore, the effect of anti-VEGF-B treatment appeared to be dose dependent (Figure 3G and S4K-M).

We show that VEGF-B expression is higher in DKD patients and declines with disease progression, likely reflecting increased pathology in advanced DKD.

Genetic expression of *VEGFB* increases in the glomeruli of DKD patients but not in the tubule when compared to healthy control (Figure 5A-B). Furthermore, VEGF-B protein expression is higher in the glomeruli of patients with mild, moderate and severe DKD (Figure S5G) when compared to healthy controls. However, although higher compared to healthy individuals, expression levels of VEGF-B appear to decline during DKD progression (Figure 5C).

Finally, we demonstrate that increasing VEGF-B expression locally in the kidney aggravates renal damage thus contributing to DKD progression.

In response to HFD feeding, animals overexpressing *Vegfb* specifically in podocytes displayed increased albuminuria (Figure 6B), increased glomerular LD accumulation (Figure 6D and S7B), increased mesangial expansion and sclerosis (Figure 6E) and increased podocyte and vascular injury (Figure 6F-G) when compared to control animals. Increased local VEGF-B signaling in the glomeruli did not impact body weight (Figure S6G), glucose tolerance (Figure S6H), insulin sensitivity (Figure S6I) or plasma levels of NEFAs, TAGs or ketone bodies (Figure S7D).

5 Discussion

This thesis proposes that glucose and FA uptake by the endothelium is regulated by selective metabolic pathways in ECs, and that local and systemic regulation of endothelial energy substrate selection contributes to ischemic stroke and DKD pathology. The studies included in this thesis present evidence for the underlying mechanisms and evaluate the therapeutic potential of altering endothelial energy substrate transport via VEGF-B targeting when attempting to ameliorate disease pathology.

For example, in **Paper I** we demonstrate that activation of lipolysis can drive endothelial FA uptake, and that LD metabolism is crucial for the buffering of intracellular FAs. We suggest that VEGF-B activates lipolysis in ECs and that VEGF-B's ability to induce endothelial FA uptake is dependent on endothelial lipolysis. The observation that VEGF-B signaling can trigger HSL-mediated lipolysis in ECs is novel but evidence from other cell and tissue types support our findings. For example, studies have shown that overexpression of VEGF-B specifically in adipocytes results in increased HSL phosphorylation (Falkevall et al., 2023) and that VEGF-B stimulation of *in vitro* cultured myocytes increases the expression of both *Pnpla2* (ATGL) and *Lipe* (HSL) (Li et al., 2019a). However, since our data show that siRNA knockdown of *PNPLA2* (ATGL) also prohibits VEGF-B-induced endothelial FA uptake, it is unclear whether VEGF-B specifically acts on HSL or ATGL or both. Considering that HSL activity has been suggested to depend on ATGL during lipolysis (Bezaire et al., 2009), ATGL removal would therefore likely reduce lipolysis independently of VEGF-B and the exact role of VEGF-B in ATGL-mediated lipolysis therefore remains to be determined. Nevertheless, our data demonstrates that pharmacological activators of lipolysis can induce endothelial FA uptake illustrating that lipolysis can drive FA uptake in ECs. This is further highlighted by the identification of oleic acid as a novel inducer of endothelial FA uptake. Like VEGF-B stimulation, oleic acid treatment increases phosphorylation of HSL while pharmacological inhibition of lipolysis or siRNA knockdown of *PNPLA2* (ATGL) and *LIPE* (HSL) decreases oleic acid-mediated FA uptake in ECs. However, unlike VEGF-B, siRNA knockdown of the aforementioned lipolytic enzymes was not able to completely abolish oleic acid's ability to increase endothelial FA uptake when compared to non-treated cells. Since oleic acid has been demonstrated to increase the expression of both HSL and ATGL in ECs (Kuo et al., 2017), increased mRNA levels of *PNPLA2* and *LIPE* in response to oleic acid treatment may reduce the siRNA knockdown efficacy resulting in more ATGL and HSL expression as

compared to non-oleic acid treated cells. However, since no protein expression analysis was conducted post siRNA treatments this remains highly speculative. Nonetheless, that oleic acid activates lipolysis is supported by both our data demonstrating an oleic acid-mediated induction of HSL phosphorylation as well as by Kuo *et al.* demonstrating increased ATGL and HSL expression in response to oleic acid treatment (Kuo *et al.*, 2017).

Downstream of lipolysis, we show that mitochondrial FA shunting and mitochondrial ATP generation is crucial for VEGF-B and oleic acid to promote FA uptake in ECs. This is in line with the observations made by Ibrahim *et al.* demonstrating that local mitochondrial ATP is utilized by FATP4 to mediate FA-CoA conjugation therefore facilitating endothelial FA uptake (Ibrahim *et al.*, 2020). Indeed, prohibiting either mitochondrial ATP generation, mitochondrial ATP transport or FA-CoA conjugation reduces both VEGF-B and oleic acid-induced endothelial FA uptake. However, it is important to note that blocking mitochondrial ATP impacts basal endothelial FA uptake, and it is therefore not clear if VEGF-B downstream of VEGFR1 specifically mediates mitochondrial ATP generation or if inhibiting mitochondrial ATP production hinders endothelial FA uptake independent of VEGF-B. Indeed, in **Paper II** we demonstrate that VEGF-B does not significantly alter the total ATP amount and only a weak effect on oxygen consumption was observed. However, this experiment was carried out in conditions with low access to FAs and was designed to investigate VEGF-B's effect on the total ATP production in the context of endothelial glucose uptake. New experiments, looking specifically at mitochondrial respiratory capacity in response to VEGF-B stimulation in the absence or presence of FAs, are therefore needed to investigate any potential effects of VEGF-B on OXPHOS in ECs. Nevertheless, since hindering mitochondrial FA shunting via CPT1A inhibition prohibits VEGF-B mediated endothelial FA uptake, this strongly suggests that VEGF-B activation of lipolysis promotes FAO therefore increasing mitochondrial ATP generation. This is strengthened by the observation that VEGF-B and oleic acid both appear to converge on similar pathways to induce endothelial FA uptake. Our data that suggest that oleic acid-mediated activation of lipolysis increases OXPHOS via β -oxidation of FAs is further supported by Kuo *et al.* demonstrating reduced oxygen consumption specifically in CPT1A-inhibited ECs treated with oleic acid (Kuo *et al.*, 2017). Hence, our study together with others (Ibrahim *et al.*, 2020; Kuo *et al.*, 2017) support the idea that lipolysis activators increase endothelial FA uptake via increased FAO.

Since there are multiple ways of promoting OXPHOS independent of FAO, other factors reported to increase FA uptake in ECs may not require FA β -oxidation to do so. Indeed, neither 3-HIB nor lactate appear to rely on FAO to promote endothelial FA uptake, despite their dependency on mitochondrial ATP production. This suggests that both lactate and 3-HIB, at least initially, promote OXPHOS independent of FAs. Indeed, we attributed lactate's ability to induce mitochondrial ATP generation specifically to the conversion of lactate into pyruvate. Since it has been suggested that conversion of lactate to pyruvate via mitochondrial lactate dehydrogenase promotes NADH generation in the mitochondria (Young et al., 2020), we hypothesize that increased mitochondrial NADH availability drives lactate-induced endothelial FA uptake via NADH oxidation by the mitochondrial complex I (Sharma et al., 2009). Future experiments, using either mitochondrial complex I inhibitors or cells subjected to siRNA knockdown of *SLC25A51*, the gene coding for the protein responsible for NAD^+/NADH mitochondrial transport in mammalian cells (Luongo et al., 2020), should be carried out in order to determine the validity of our hypothesis.

Just as it is not fully understood exactly how lactate promotes mitochondrial ATP synthesis; it also remains unclear if and how 3-HIB can promote mitochondrial ATP generation. 3-HIB is a metabolite generated during catabolism of the amino acid valine, and can be further metabolized into propionyl-CoA that via subsequent reactions form methylmalonyl-CoA and finally succinyl-CoA which can enter the TCA cycle (Adeva-Andany et al., 2017; Jang et al., 2016). However, as demonstrated by Jang *et al.*, reducing the expression of 3-hydroxy-isobutyrate dehydrogenase, responsible for further metabolization of 3-HIB, does not affect endothelial FA uptake (Jang et al., 2016). Despite this, it is possible that accumulation of 3-HIB may force it into alternative metabolic pathways involved in amino acid metabolism. Hence, one should study the effect of 3-HIB in ECs post siRNA knockdown of mRNA transcripts encoding the propionyl-CoA carboxylase that promotes the conversion of propionyl-CoA to succinate (Wongkittichote et al., 2017). Furthermore, experiments analyzing mitochondrial respiratory capacity in response to either lactate or 3-HIB treatment should be carried out to determine if ECs increase OXPHOS in response to either of these two factors. However, since these experiments have not been conducted it remains unclear how lactate and 3-HIB promote mitochondrial ATP generation. Regardless of exactly how these factors promote OXPHOS, our data clearly indicates that neither lactate nor 3-HIB rely on FAO to do so. Despite this, pharmacological inhibition of lipolysis reduces

both lactate's and 3-HIB's ability to induce FA uptake in ECs demonstrating that LD metabolism is crucial for endothelial FA uptake. We hypothesized that inhibition of lipolysis would diminish the ability of LDs to incorporate newly formed TAGs, and that esterified FAs therefore would accumulate in the endoplasmic reticulum. In turn, this would likely result in a concentration-dependent negative feedback loop where TAG accumulation would further prohibit upstream FA esterification thus raising concentrations of FA-ester intermediaries including FA-CoA in the endoplasmic reticulum. Ultimately, the accumulation of FA-CoA conjugates would prohibit new FA-CoA conjugation resulting in reduced endothelial FA uptake. Supporting this idea is our data demonstrating that DGAT1 inhibition, prohibiting FA esterification into TAGs in the endoplasmic reticulum, reduces the ability of 3-HIB, lactate, VEGF-B and forskolin (pharmacological activator of lipolysis) to induce FA uptake in ECs. Collectively, these observations suggest that endothelial FA uptake is regulated via feedforward and negative feedback loops that rely on concentration gradients established by the amount of FA intermediaries in the endoplasmic reticulum and the number of TAGs in LDs. However, to truly investigate this one must conduct complex experiments attempting to measure the concentrations of FA-esters in the endoplasmic reticulum compartment as well as TAG concentration in LDs post lipolysis inhibition. Since no such experiments have been conducted, the idea of a feedforward and negative feedback mechanism controlling endothelial FA uptake is yet to be determined.

VEGF-B and oleic acid does not only alter FA uptake in ECs but additionally affects endothelial glucose uptake as demonstrated in **Paper II** and **Paper III**. Indeed, ECs reduce their uptake of glucose in response to VEGF-B stimulation, and systemic inhibition of VEGF-B increases cardiac glucose uptake in HFD mice. We attributed this effect to changes in GLUT1 plasma membrane distribution resulting from reduced cholesterol uptake via the LDLR. Although we hypothesize that the changes in cholesterol uptake are connected to receptor re-cycling, it is difficult to determine if VEGF-B increases the internalization of the LDLR or if prolonged VEGF-B signaling in fact promotes LDLR degradation. Regardless of the exact effect on the LDLR protein, VEGF-B signaling reduces LDLR surface expression as well as cellular cholesterol uptake and content, therefore establishing a clear link between VEGF-B signaling and EC cholesterol homeostasis. This link is further highlighted by our data demonstrating increased cardiac free cholesterol content

in both diabetic (*db/db* and HFD) and non-diabetic *Vegfb* knockout and anti-VEGF-B antibody treated mice.

Like with the LDLR, VEGF-B does not alter GLUT1 expression but rather change GLUT1 plasma membrane distribution. This distributional change was further linked to a reduction in plasma membrane cholesterol content. The GLUT1 protein has been shown to carry a cholesterol interaction motif (Malenda et al., 2012), and GLUT1-dependent glucose transport has been suggested to be mediated via a rocker-switch-like mechanism (Deng et al., 2014). We therefore hypothesized that the VEGF-B mediated reduction in plasma membrane cholesterol content would reduce GLUT1's ability to "flip-flop" glucose from the extracellular environment inwards to the cytosol. Ideally, to demonstrate the link between VEGF-B, cholesterol and GLUT1 function, one would design experiments where mutations are introduced in the cholesterol binding motifs of GLUT1 and stimulate ECs carrying mutated GLUT1 with VEGF-B or control. However, no such experiments were conducted and hence it is difficult to determine exactly how VEGF-B affects GLUT1-mediated glucose uptake and transport.

Importantly, VEGF-B is not the only factor that affects both glucose and FA uptake in ECs. As demonstrated in **Paper III**, oleic acid treatment reduces endothelial glucose uptake independently of glucose availability. Again, we attributed this effect to GLUT1 since oleic acid treatment reduced EC GLUT1 expression. The observation that two independent activators of lipolysis drive FA uptake while simultaneously reducing glucose uptake in ECs suggest that lipolysis and FAO might be an underlying mechanism for this effect. More importantly, it supports the idea that ECs undergo a metabolic switch in response to altered nutrient flux and that this switch appears to be determined by FAO. Supporting this are observations made by others as well as our own data. For example, Chaube *et al.* demonstrate that siRNA knockdown of *ANGPTL4* in ECs results in increased surface TAG lipolysis and CD36-mediated FA uptake with a simultaneous decrease in EC glucose uptake (Chaube et al., 2023). Furthermore, our own data demonstrate that although having a profound effect on endothelial glucose uptake, neither siRNA knockdown of *SLC2A1* (GLUT1) nor changes in the cellular cholesterol content alters VEGF-B induced FA uptake in ECs. In addition, oleic acid's ability to diminish glucose uptake in ECs is not affected by glucose availability and inhibiting LD metabolism blocks both 3-HIB and lactate-mediated FA uptake despite their lack of dependency on FAO. Hence, the decisive factor for

mediating the endothelial metabolic switch appears to be linked to FA metabolism.

The concept of a FA-mediated endothelial metabolic switch is corroborated by several of our *in vivo* findings exemplified by the following observations. Mice subjected to overnight fasting increase their uptake of FAs in the brain (**Paper I**) and HFD feeding induces LD accumulation in the brain vasculature while simultaneously reducing glucose uptake (**Paper III**). Furthermore, systemic VEGF-B inhibition reduces LD accumulation in the brain of HFD mice and normalizes brain glucose uptake when compared to chow-fed control. These experimental data presented in **Paper I and Paper III** are further supported by other studies demonstrating that short term HFD feeding reduces EC *Slc2a1* (GLUT1) mRNA expression in skeletal muscle tissue (Zhang et al., 2024) as well as reduces expression of BEC GLUT1 in the brain (Jais et al., 2016). Despite these observations, we did not see any changes in brain *Slc2a1* expression post long-term HFD feeding, likely reflecting that HFD-induced changes in GLUT1 expression occur acutely and appear to normalize between seven and 28 days (Jais et al., 2016; Zhang et al., 2024). Nevertheless, our combined findings presented in **Paper I, II and III** together with the observations of others suggest that exposing the endothelium to FAs results in altered energy substrate selection therefore reducing glucose uptake while promoting the uptake, utilization and transport of FAs.

More important is the role that the EC metabolic switch may play in metabolic disease pathology including obesity-related ischemic stroke and DKD. As demonstrated in **Paper III**, HFD mice present with increased circulating lipids, increased cerebrovascular LD accumulation and reduced brain glucose uptake capacity. In addition to these factors, HFD mice display alterations relevant for ischemic stroke pathology such as increased resistance to reperfusion and increased thrombogenicity. Indeed, HFD feeding increases infarct volume as well as ICH incidence post MCAO, attributed to reduced BBB integrity in the infarct region. The increased vasogenic edema could further be linked to LD accumulation since oleic acid treatment of ECs reduces TEER. Considering that we observed increased white adipose tissue lipolysis after MCAO, it is likely that increased endothelial FA exposure, resulting from a lipolysis-mediated increase in plasma NEFA levels, induces a switch in energy substrate selection at the BBB thus manifesting as increased cerebral FA uptake and reduced brain glucose uptake capacity. In turn, the increased FA uptake would promote LD accumulation and BBB "leakiness" thus increasing the infarct size as well as the incidence of ICH.

Supporting the idea of a reduction in the integrity of the endothelial barrier upon FA exposure, is the reduced integrity of the glomerular filtration barrier in HFD-fed mice demonstrated in **Paper IV**. HFD-fed mice display increased levels of plasma NEFAs resulting in increased LD accumulation in the glomeruli, similar to our observations in the brain. Like in stroke, the increase in LD accumulation was associated with increased disease pathology mediated by a reduction in vascular barrier integrity and dysfunctions in the glomerular filtration barrier coinciding with reduced renal function.

That endothelial substrate selection plays a role in ischemic stroke is further illustrated by the amelioration of stroke pathology observed upon systemic inhibition of VEGF-B. In **Paper III**, we connect this to systemic effects as post-treatment with anti-VEGF-B antibody did not impact infarct size nor ICH incidence. However, that local VEGF-B signaling in the brain may impact stroke outcome cannot be excluded since long-term effects were not evaluated. Considering that systemic VEGF-B inhibition after MCAO reduces LD accumulation and vasogenic edema in the brain, it is indeed possible that long-term improvements post stroke may manifest. Nevertheless, whether the effect is local or systemic or both, it is clear from our data that inhibition of VEGF-B prior to stroke or in combination with rtPA post stroke improves stroke outcome. In addition, systemic inhibition of VEGF-B may also ameliorate DKD as demonstrated in **Paper IV**. Indeed, genetic deletion of *Vegfb* or anti-VEGF-B antibody treatment improved renal function by reducing glomerular LD accumulation and lipotoxicity-associated damage to the glomerular filtration barrier in *db/db* animals. Since we observed no effect on plasma NEFA levels this again raises the question if these effects can be attributed to systemic inhibition or local inhibition of VEGF-B signaling. However, it is important to note that the *db/db* animal model presents with extreme metabolic disturbances making it difficult to draw any conclusions. Although systemic inhibition of VEGF-B only showed a modest effect on circulating lipids in *db/db* mice, HFD animals displayed reduced plasma NEFA levels in response to anti-VEGF-B antibody treatment. Combined with our data demonstrating that overexpression of VEGF-B in the podocytes of the kidney can drive DKD in transgenic mice, these observations collectively support a role for both local and systemic VEGF-B signaling in disease pathology. Despite this, it is difficult to determine the exact cellular source of VEGF-B in the kidney since mice lacking VEGF-B expression in the podocytes were not included questioning the physiological relevance. Nonetheless, the development of DKD in transgenic

animals promotes the general idea that driving endothelial FA transport locally via VEGF-B overexpression can induce organotypic damage regardless of cellular source.

Considering that it is impossible to determine exactly who will develop ischemic stroke prior to the actual event, the therapeutic relevance of pre-stroke therapeutics is questionable. In the clinic, patients are typically administered with rtPA (alteplase) as early as possible post stroke onset as delayed administration of thrombolytics is associated with poor stroke outcome. Our findings that rtPA-mediated activation of white adipose tissue lipolysis promote increased plasma NEFA levels in mice together with the observation that stroke patients present with increased plasma NEFA levels post administration of thrombolytics may therefore in part explain the negative effects associated with thrombolytics in general. Interestingly, anti-VEGF-B antibody treatment prohibits rtPA-mediated white adipose tissue lipolysis in mice further ameliorating stroke pathology associated with delayed thrombolytic administration. As we propose in **Paper III**, targeting VEGF-B as adjuvant therapy to thrombolysis may therefore be a viable strategy to reduce rtPA-mediated complications.

The contribution of rtPA and VEGF-B signaling in the adipose tissue to stroke pathology is intriguing as it strengthens the link between plasma NEFA levels and metabolic disease pathology. However, exactly how rtPA promotes white adipose tissue lipolysis remains unclear. The same is true for VEGF-B as adipocytes do not appear to express VEGFR1 and hence which cell is involved in regulating VEGF-B activation of white adipose tissue lipolysis is not fully understood. In a recently published study, Wang *et al.* propose that VEGF-B signaling in adipose tissue resident macrophages reduces local clearance of norepinephrine therefore increasing β -adrenergic induced lipolysis in adipocytes (Wang *et al.*, 2025). These findings corroborate previous findings demonstrating that transgenic animals overexpressing VEGF-B in the adipose tissue increases the release of NEFAs into the blood resulting in progression of MAFLD (Falkevall *et al.*, 2023). Despite these findings, the potential role of VEGF-B mediated endothelial FA transport in increasing the release of NEFAs from the adipose tissue to the blood has not been studied. Considering the mechanisms proposed to regulate endothelial FA uptake and transport presented in **Paper I**, it is possible that increased adipocyte lipolysis raises the concentration gradient of NEFAs in the adipocytes contra the blood therefore establishing a directional FA transport gradient from the adipocytes into the bloodstream. If true, then prohibiting endothelial lipolysis via VEGF-B inhibition

would therefore reduce endothelial FA uptake from the adipocytes therefore reducing the transport of FAs from the adipocytes over the endothelium into the blood. In turn, this would lower plasma NEFA levels and consequently the exposure of NEFAs to the endothelium resulting in reduced endothelial FA uptake in tissues. Reducing endothelial FA uptake in tissues would further promote endothelial glucose uptake and ameliorate ectopic lipid accumulation therefore restoring systemic insulin sensitivity. Nevertheless, these studies together with our own observations demonstrate that the capacity of the adipose tissue to buffer lipids seems to be crucial for maintaining systemic metabolic homeostasis. As our data suggest, increased white adipose tissue lipolysis results in increased FA transport and reduced glucose transport across the endothelial bed and may therefore partly explain the link between obesity, T2D and T2D-associated co-morbidities including DKD and ischemic stroke. It is therefore crucial for future therapeutic development that aim to ameliorate metabolic-associated disease pathology to account for the role of VEGF-B and the mechanisms that govern endothelial energy substrate selection, both on the local as well as systemic level.

6 Conclusions

In this thesis a novel mechanism regulating endothelial FA and glucose uptake is proposed. We demonstrate that endothelial lipolysis can drive FA uptake in ECs and further identify oleic acid as a novel inducer of endothelial FA uptake. We show that VEGF-B and oleic acid activates endothelial lipolysis and promotes FAO to drive endothelial FA uptake and that this reduces endothelial glucose uptake capacity. Likewise increased levels of circulating blood lipids increase FA uptake and reduces glucose uptake *in vivo*.

Experimental evidence provided further demonstrates that both MCAO and thrombolytic administration activates adipose tissue lipolysis in mice therefore promoting accumulation of FAs in the brain vasculature. We further show that accumulation of FAs in the cerebral vasculature is associated with reduced vascular integrity and increased vasogenic edema resulting in larger infarct size and increased incidence of ICH. In contrast, targeting VEGF-B reduces adipose tissue lipolysis and therefore improves stroke outcome post MCAO.

Intriguingly, we could demonstrate that administration of thrombolytics is associated with increased plasma NEFA levels in ischemic stroke patients, and that a more safe and efficacious thrombolytic treatment could be carried out in animals administrated with rtPA together with a neutralizing anti-VEGF-B antibody post MCAO. These data therefore propose that targeting VEGF-B as adjuvant therapy to thrombolytics may serve as a viable strategy to reduce rtPA-mediated complications.

Finally, we could associate increased LD accumulation in the glomeruli of the kidney with damage to the glomerular filtration barrier in mice. Evaluation of the effect of VEGF-B targeting in DKD animal models showed that systemic VEGF-B inhibition reduces glomerular LD accumulation, improves glomerular vascular and podocyte function and preserves the integrity of the filtration barrier coinciding with improved renal function. In summary, this thesis provides experimental evidence that demonstrates the importance of endothelial energy substrate selection and transport in the development of obesity and T2D associated ischemic stroke and kidney disease. We therefore suggest targeting endothelial FA uptake as a novel strategy to ameliorate metabolic disease pathology.

7 Points of perspective

The knowledge provided in this thesis concerns the mechanisms of endothelial energy substrate selection and identifies endothelial lipolysis as a facilitator for FA uptake in ECs. We have therefore mostly focused on targeting activators of lipolysis to promote metabolic homeostasis. Future studies should focus on identifying physiological repressors of endothelial lipolysis. Such repressors may be used to target the endothelium in order to prohibit endothelial FA uptake and transport and instead promote glucose uptake and transport by ECs. They would further be indispensable in the attempt to elucidate the connection between FA and glucose uptake in ECs which remain poorly understood.

The experimental evidence presented in this thesis points toward the endothelium as a disease mediator for both DKD and ischemic stroke. It is interesting to note the striking similarities between these two disorders especially when considering that both DKD and ischemic stroke involve vascular leakage and molecular extravasation, albeit with different consequences. Since targeting VEGF-B ameliorates pathology in animals modeling these diseases, it is therefore likely that endothelial FA uptake and transport is the common denominator. The connection between FA uptake and vascular dysfunction requires further study to elucidate how dyslipidemia may impact the endothelium.

The link between adipose tissue lipolysis and cerebrovascular FA uptake and LD accumulation has many future implications and requires further study. Despite that FA uptake in the human brain has been demonstrated (Karmi et al., 2010), the role of cerebrovascular FA uptake from the periphery in CNS pathology has until now largely been overlooked. That ischemic stroke is associated with FA uptake in the brain is therefore novel and suggests a relevance for brain FA uptake in other CNS-related disorders such as dementia. Especially when considering that obesity increases the risk for developing dementia (de Bruijn et al., 2015), and that the major risk allele for developing sporadic Alzheimer's disease is related to FA metabolism (Jansen et al., 2015). Of great interest would be to elucidate the role of VEGF-B in dementia and if reducing adipose tissue lipolysis by targeting VEGF-B can halt the progression of Alzheimer's disease. Our understanding of endothelial cell energy substrate selection may therefore ultimately lead to novel therapeutic strategies to combat obesity and T2D-related dementia in addition to stroke, DKD and other T2D co-morbidities.

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