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# THE ROLE OF ENDOPLASMIC RETICULUM MEMBRANE CONTACT SITES IN HUMAN ENDOTHELIAL CELLS

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“That which can be asserted without evidence,
can be dismissed without evidence.”

— Hitchens’s razor, Christopher Hitchens

**ABSTRACT**

Age-related diseases such as neurodegeneration and cardiovascular disease are increasingly common. Growing evidence suggests that impaired lipid metabolism, often stemming from obesity and associated dyslipidemia, contributes significantly to these diseases. This dysfunction may involve disrupted intracellular lipid traffic as multitude of lipids are synthesized in the endoplasmic reticulum (ER) and mitochondria, from which they must be transported in a timely manner to ensure proper cellular function. Aberrations in cellular lipid traffic can result in activation of integrated stress pathways that have a plethora of downstream outcomes, such as inflammation or apoptosis. Lipids can be transported from cellular compartments by two main means, vesicular and non-vesicular traffic. Traffic by vesicles can take place between the ER and Golgi or through endosomal pathway from and to the cellular periphery. Non-vesicular transport often refers to the transport of lipids and other small molecules, between intracellular membrane compartments. In the non-vesicular pathways lipid transfer proteins traffic lipids between membrane compartments as individual molecules, or form membrane contacts sites (MCS) through interactions with other proteins. In this thesis, a multitude of methods, including transcriptomics and lipidomics, as well as functional assays were used to characterize changes in macromolecular pools of MCS protein-manipulated endothelial cells. The manipulations targeted oxysterol binding protein and oxysterol binding protein related protein 7 with small molecule inhibitors, as well as vesicle associated membrane protein associated proteins A and B with small hairpin RNA silencing. The results implicated that MCS proteins modulate the cellular cholesterol content by affecting on ER cholesterol homeostatic machinery and on lipid droplets, as well as trigger a ubiquitous initiation of integrated stress pathways and downstream upregulation of inflammatory process. Angiogenesis was shown to be inhibited *in vitro* consistent with the downregulation of cellular division associated genes. Lipidomics analysis implicated modulation in the viscosity of intracellular membranes. MCS manipulations were speculated to have an impact on membrane lipid composition, reducing membrane viscosity and accumulation of unfavorable lipids at membranes. Thus, the manipulations resulted in an increased integrated stress, downregulated cellular division and decreased angiogenesis. This thesis represents the first of its kind to study the macromolecule pools of endothelial cells with dysfunctional MCS to this extent. These data provide a basis for further research into how dysfunctional lipid transport affects endothelial cells and possibly disease such as atherosclerosis

**Table of Contents**

**ABBREVIATIONS**

| | |
|---|---|
| ABC | ATP binding cassette transporter protein |
| ATF6 | Activating transcription factor 6 |
| ATP | Adenosinetriphosphate |
| ApoA1 | Apolipoprotein A1 |
| Bcl-2 | B-cell lymphoma 2 |
| CDP-DAG | Cytidine diphosphate-DAG |
| CE | Cholesteryl ester |
| Cer | Ceramide |
| CoA | Co-enzyme A |
| COP | Coat protein complex |
| CYP | Cytochrome P450 enzyme |
| DAG | Diacylglycerol |
| DDIT3 | DNA Damage Inducible Transcript 3 |
| DKD | Double knock down |
| GDP-GTP | Guanosine diphosphate-guanosine triphosphate |
| EC | Endothelial cell |
| ECM | Extracellular matrix |
| eIF2α | Eukaryotic initiation factor 2 alfa |
| ER | Endoplasmic reticulum |
| ERAD | ER-associated degradation |
| ERGIC | Endoplasmic-reticulum-Golgi intermediate compartment |
| FA | Fatty acid |
| FasL | Fas ligand |
| FC | Free cholesterol |
| G3P | Glycerol-3-phosphate |
| GSEA | Gene set enrichment analysis |
| GPL | Glycerophospholipid |
| HDL | High density lipoprotein |
| HMG-CoA | β-hydroxy-β-methylglutaryl-CoA |
| HUVEC | Human umbilical cord vein endothelial cells |
| HSPA | Heat Shock Protein Family A |
| IRE1 | Inositol-requiring enzyme 1 |
| ISP | Integrated stress pathway |
| LPL | Lipoprotein lipase |
| LTP | Lipid transport protein |
| LXR | Liver X receptors |

| | |
|---|---|
| MAM | Mitochondria associated membrane |
| MCS | Membrane contact site |
| MOMP | Mitochondrial outer membrane permeabilization |
| mRNA | Messenger ribonucleic acid |
| ORP | Oxysterol binding protein related protein |
| ORP7 | Oxysterol binding protein related protein 7 |
| OSBP | Oxysterol binding protein |
| PA | Phosphatidic acid |
| PC | Phosphatidyl choline |
| PE | Phosphatidyl ethanolamine |
| PERK | Protein kinase RNA-like ER kinase |
| PI | Phosphatidyl inositol |
| PIPs | Phosphatidyl inositol phosphates |
| PI4P | Phosphatidyl inositol 4 phosphate |
| PM | Plasma membrane |
| PS | Phosphatidyl serine |
| ROS | Reactive oxygen species |
| S1/2P | site-1 and site-2 proteases |
| SCAP | SREBP cleavage-activating protein |
| shRNA | Short hairpin ribonucleic acid |
| SM | Sphingomyelin |
| SOAT1 | Sterol O-acyltransferase 1 |
| SREBP | Sterol regulatory element-binding proteins |
| STIM1 | Stromal Interaction Molecule 1 |
| UPR | Unfolded protein response |
| TC | Total cholesterol |
| TG | Triacylglycerol |
| TGN | *Trans*-Golgi network |
| TNF-α | Tumor necrosis factor-alpha |
| UPR | Unfolded protein response |
| VAPA | Vesicle associated membrane protein associated protein A |
| VAPB | Vesicle associated membrane protein associated protein B |
| VEGFB | Vascular endothelial growth factor B |
| XBP1 | X-Box Binding Protein 1 |

**LIST OF ORIGINAL PUBLICATIONS**

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Author's contributions to the publications:

I. Designed and performed most of the experiments apart from the lipidomic and transcriptomic high-throughput methods, collected and made all samples, analysed the data, and wrote first draft of the manuscript.

II. Designed and executed majority of the experiments excluding the lipidomic and transcriptomic high-throughput methods, gathered and produced all samples, analysed the data, and wrote first draft of the manuscript.

III. Designed and performed most of the experiments apart from the omics high-throughput methods, produced all samples, analysed the data, and wrote first draft of the manuscript as well as participated in funding acquisition.

# 1 INTRODUCTION

Out of the numerous intracellular organelles, the endoplasmic reticulum (ER) has the widest variety of functions, ranging from the synthesis of major macromolecules, such as proteins and lipids, to trafficking and storing them. The timely synthesis and trafficking of major macromolecules is vital for cells, therefore aberrations in ER function can lead to the induction of multiple types of cellular stress such as ER, Golgi, lipid and oxidative stress. ER stress can be triggered by the accumulation of unfolded proteins, toxic by-products, or lipids such as free cholesterol (FC). Acute ER stress can induce significant changes in cellular metabolism, by for example triggering unfolded protein response (UPR), or in the case of prolonged ER stress apoptosis or necrosis. Since the accumulation of ER products at the ER is deleterious it its vital to understand how macromolecules are trafficked into and from the ER. According to current knowledge there are multiple means, by which this trafficking occurs. Vesicular trafficking can occur via coat protein complex (COP) coated vesicles that bud of from the ER membrane and deposit their cargo at the Golgi apparatus by fusing to the Golgi membrane. Another means of intracellular trafficking is by transport membrane contact site (MCS) proteins that can traffic lipids and small molecules between two membranes without fusing together as in vesicle-mediated traffic. Other trafficking mechanisms include diffusion of small molecules through the cytosol or as bound to carrier proteins, as well as hemifusion of membrane leaflets. MCS have been shown to be pivotal for the exchange of lipids and signalling molecules between membrane compartments *in vitro*, but whether the exchange of lipids is limited to the bulk modulation of membrane lipid composition or if MCS have a more precise role in modifying the lipid environment of membrane raft domains remains unclear. What is certain is that MCS display a wide variety of distinct functions. Multiple protein families have been identified and shown to be specialized in the traffic of often a single lipid species between different membrane compartment pairs. The oxysterol binding protein related protein (ORP) family is a notable example of this, as most of the proteins in this family traffic cholesterol or phosphatidylserine (PS) in exchange for phosphoinositides (PIPs), between the ER and other members compartments. MCS dysfunction has been implicated as one key factor in the development of cardiovascular diseases, but research on MCS in endothelial cells (ECs) is very limited. This makes ECs MCS an opportune target for research as ECs can contribute to the development of atherosclerosis and various other cardiovascular complications. Therefore, this thesis focused on how the dysfunction of ER MCS affects ECs especially their lipid metabolism, and other functions such as inflammation and angiogenesis.

# 2 REVIEW OF LITERATURE

This literature review will discuss how MCS function, as well as their importance in cellular homeostasis. ER and its multiple roles in cells will be discussed, as well as how major lipid classes are synthesized, and how their homeostasis is regulated. Cellular stress responses are often the result of major

disturbances in cellular homeostasis, therefore this review also gives background on integrated stress pathways (ISP), as well as how they are initiated and ultimately resolved. Since this thesis focuses on ER MCS in ECs, some of their key functions are discussed.

### 2.1 ER functions

The ER is a versatile cell organelle, and it not only act as the hub for protein synthesis and folding, but also for lipid synthesis and storage, calcium homeostasis regulation, as well as detoxifies endogenous and exogenous compounds (Schwarz and Blower 2016). Even though most high school textbooks depict the ER as a static organelle stacked neatly next to the nucleus, it changes shape, mass, and area depending on a cell's needs (Woo, Williams, and Tsai 2023). The rough endoplasmic reticulum harbours ribosomes that are responsible for producing proteins from messenger ribonucleic acids (mRNA). It is the more static part of the ER whereas the smooth ER is a more dynamic organelle that can transform in response to both external and internal stimuli (Perkins and Allan 2021). Proteins are not the only macromolecules that are produced at the ER, and many lipids such as steroids, very-long-chain fatty acids, ceramides and glycerophospholipids are produced at the ER (Ventura and Isabel Hernández-Alvarez 2022). ER can also detoxify both intracellular and extracellular compounds, with ER localized cytochrome P450 family proteins (Crib et al. 2005). Another crucial task for the ER is to quality control protein synthesis, aberration of which can lead to formation of unfolded and misfolded proteins, which in turn causes ER stress (G. Chen et al. 2023).

### 2.2 ER stress

The literature on ER stress, and its causes and means to alleviate it, is extensive and multiple thorough reviews have been written on the topic over the years. ER stress occurs when the protein folding capacity of the ER is overwhelmed or disturbed that in turn leads to the accumulation of both misfolded and unfolded proteins in the ER lumen. Multiple environmental factors can lead to ER stress, these include nutrient deprivation, oxidative stress, calcium imbalance, viral infection, lipid disturbances, toxins, as well as protein overload during rapid cell growth (X. Chen et al. 2023).

Calcium balance at the ER is important for protein folding as many chaperones, such as Calnexin and Calreticulin, use calcium ions as cofactors to ensure correct protein folding (Leach and Williams 2013). Therefore, calcium imbalance can affect chaperone activity and subsequently protein folding capacity. ER calcium homeostasis is regulated by multiple factors, that include calcium channels (Lanner et al. 2010). Calcium is influxed to the ER from the cytosol by endoplasmic reticulum calcium adenosine triphosphate (ATP)-ase pumps that upkeep high ER calcium concentrations in an ATP dependent manner (Xu and Van Remmen 2021). Calreticulin, calnexin and other calcium binding chaperones

also maintain a constant storage of calcium at the ER. Therefore, ER calcium concentrations can be affected by dysfunction of either influx, efflux, or storage of calcium at the ER.

Cells produce reactive oxygen species (ROS) and nitrogen species as part of normal cellular metabolism, and at basal cellular conditions they are quickly stored or removed by anti-oxidative agents (Bardaweel et al. 2018). When the balance between generation and removal or storage of ROS is disturbed, excess oxidative ions and molecules can accumulate at the mitochondria or ER that in turn have multitude of detrimental effects (Burgos-Morón et al. 2019). In the context of ER stress, accumulation of ROS can oxidize amino acid residues (Juan et al. 2021) that leads to the misfolding or unfolding of proteins. In surplus, these un- or misfolded proteins lead to the upregulation of ER stress. Accumulation of ROS can have detrimental effects on other cellular function, and these include lipid oxidation that disrupts membrane integrity, DNA damage that causes mutation and genome instability, as well as damage to mitochondrial DNA that leads to dysfunction and further generation of ROS, resulting in a positive feedback loop (Juan et al. 2021).

Nutrient deprivation has a more indirect effect on ER stress, as lack of amino acids can affect protein synthesis. At first decreased protein synthesis capacity can be beneficial but it can also reduce the amount of chaperone proteins produced during longer starvation. Energy metabolism is also affected by lack of nutrients, as decreased amount of ATP can directly affect the protein synthesis and protein folding. Lack of ATP can reduce the potential to upkeep calcium homeostasis that can also be affected by the reduction in cellular influx of exogenous calcium. Redox balance may also be affected by nutrient deprivation as the lack of antioxidants can increase the concentration of reactive oxygen species (J. Zhu et al. 2023).

Viral infections are another source of ER stress, and viruses can appropriate the protein synthesis machinery and subsequently overwhelm the protein folding capacity of the ER (Walsh and Mohr 2011). Viral proteins can also disrupt ER calcium homeostasis, as well as induce inflammation and activation of immune response, that in turn induce ER stress through multiple pathways, such as the induction of tumor necrosis factor-alpha (TNF-α) and interleukin 1 as well as -6. These cytokines induce signalling cascades that can lead to the upregulation of UPR (Choi and Song 2019).

In response to ER stress, the morphology of the ER can undergo significant changes as part of UPR. Expansion of ER membrane is one of these events (Schuck et al. 2009; Yuanhao Guo et al. 2022), and cells use the expanding membrane to accommodate more mis- and unfolded proteins, but also to add more surface area for protein synthesis and folding. The expanding ER membrane needs a constant supply of membrane lipids. Therefore, expansion of the ER membrane also requires the upregulation of membrane lipid synthesis (Moncan et al. 2021). Expanding ER membrane is also used to generate ER stress granules that are aggregates of mis- and unfolded proteins as well as ER chaperones. These granules are deposited from the ER membrane to the cytosol to be further sequestered and degraded by the proteasome, which in turn assists in alleviating ER stress (Nicchitta 2024).

### 2.2.1 Unfolded protein response

Persistent ER stress can lead to apoptosis or contribute to the pathogenesis of various diseases, including neurodegenerative disorders, diabetes, and cancer (Bettigole and Glimcher 2015; Ghemrawi and Khair 2020; Yadav et al. 2014). Therefore, processes to reduce or eliminate ER stress as quickly as possible have evolved. One of these routes is UPR that is mediated by three main transmembrane proteins located in the ER membrane: inositol-requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6), and protein kinase RNA-like ER kinase (PERK). The UPR aims to restore ER homeostasis by increasing protein folding capacity, reducing protein synthesis, and eliminating irreversibly misfolded proteins through ER-associated degradation (ERAD) and promote autophagy. UPR can trigger apoptotic pathways to eliminate cells that are overwhelmed by stress, thereby maintaining tissue integrity and function.

One of the three main pathways that trigger ER stress is the IRE pathway that activates when heat shock protein family A (HSPA) Member 5 disassociates from the luminal domains of IRE1. Autophosphorylation of HSPA5 through oligomerisation triggers the alternative splicing of x-box binding protein 1 (XBP1) that in turn activates numerous proteins involved in ERAD, lipid metabolism and protein folding, thus helping to alleviate ER stress. Induction of IRE1 also activates a process called regulated IRE1-dependent decay, that degrades ER-localized mRNAs, further reducing protein synthesis and folding load at the ER. IRE1 also contributes to the crosstalk between multiple other signalling pathways in an indirect manner. An example is the activation of XBP1 that can regulate expression of genes related to immune response and inflammation (Siwecka et al. 2021).

ATF6 is another ER membrane localized protein, that is inactivated in non-stressful conditions. When ER stress accumulates ATF6 translocates to the Golgi where it is cleaved by site-1 and site-2 proteases (S1P, S2P), to form a truncated cytosolic fragment called ATF6f. This fragment functions as a transcription factor that can activate the transcription of genes, which have an ER stress response element in their promotors, chaperone proteins including calnexin, protein disulfide isomerases, ERAD-associated proteins, as well as lipid synthesis proteins, such as fatty acid synthase and stearoyl-CoA desaturase 1, both of which are important for the synthesis of fatty acids (Hillary and Fitzgerald 2018).

PERK pathway functions in a similar manner to the IRE1 pathway: it is activated by HSPA5 release and PERK is autophosphorylated. Activated PERK further phosphorylates the alpha subunit of eukaryotic initiation factor 2 alfa (eIF2α) that regulates protein synthesis initiation and reduce protein synthesis globally. PERK also phosphorylates eIF2B, decreasing its guanosine diphosphate-guanosine triphosphate (GDP-GTP) exchange activity leading to reduced protein synthesis. Downstream effects of eIF2α deactivation induce the expression of genes related to amino acid metabolism, redox homeostasis, anti-oxidative defence as well as chaperones and ERAD pathway proteins (Z. Liu et al. 2015).

ERAD is the quality control mechanism that ensures only properly folded and functional proteins are moved out of the ER. When mis- or unfolded proteins are detected at the ER lumen, they are moved to the cytosol to be degraded by the ubiquitin-proteasome system. Essentially the ERAD process has four distinct steps. The first is the recognition of aberrantly folded proteins, by chaperones and quality control proteins at the ER. The second phase is the retrotranslocation of the aforementioned proteins to the cytoplasm. In the third phase ubiquitin proteins tag aberrant proteins with ubiquitin that act as degradation signals for the proteasome. The fourth step is degradation where ubiquitinylated proteins are broken down to smaller peptides by the proteasome complex, thus returning peptides and amino acids back to protein synthesis (Gariballa and Ali 2020).

### 2.2.2 Apoptosis, programmed self-destruction

Programmed cell death or apoptosis is a strictly regulated process of cellular destruction in contrast to necrosis that is a traumatic cell death resulting from acute cellular injury. There are two main signalling pathways that can trigger apoptosis, the intrinsic and extrinsic pathway, the latter of which is more dependent on external signals. Example of external signals are extracellular death ligands, such as fas ligand (FasL) or TNF-α, which bind to death receptors on the cell surface. The intrinsic pathway can be triggered by intracellular stress signals, such as DNA damage or oxidative stress. This pathway involves mitochondrial outer membrane permeabilization (MOMP), leading to the release of pro-apoptotic factors, such as Cytochrome C, from the mitochondria into the cytosol (Carneiro and El-Deiry 2020).

DNA Damage Inducible Transcript 3 (DDIT3) is one of the key transcription factors that induce ER stress related apoptosis. Induction of DDIT3 is regulated by PERK, ATF6 or ATF4, and DDIT3 activates the expression of the b-cell lymphoma 2 (Bcl-2) protein family that has both pro- and anti-apoptotic functions. DDIT3 can also activate death receptor ligands, and the components of the mitochondrial apoptotic pathway (H. Hu et al. 2019). During ER stress the balance between pro- and anti-apoptotic proteins is key for the initiation of MOMP, Cytochrome C release and eventual activation of, for example Caspase-9. Caspases are a group of protease enzymes that degrade cellular components in an orderly fashion (Svandova et al. 2023). Another activator of apoptosis are elevated cytosolic calcium levels that trigger the calcium-dependent apoptotic pathway (Sukumaran et al. 2021). Figure 1 summarizes the contributors and outcomes of both ER stress, ROS generation as well as other topics discussed in section 2.2 and its subheadings.

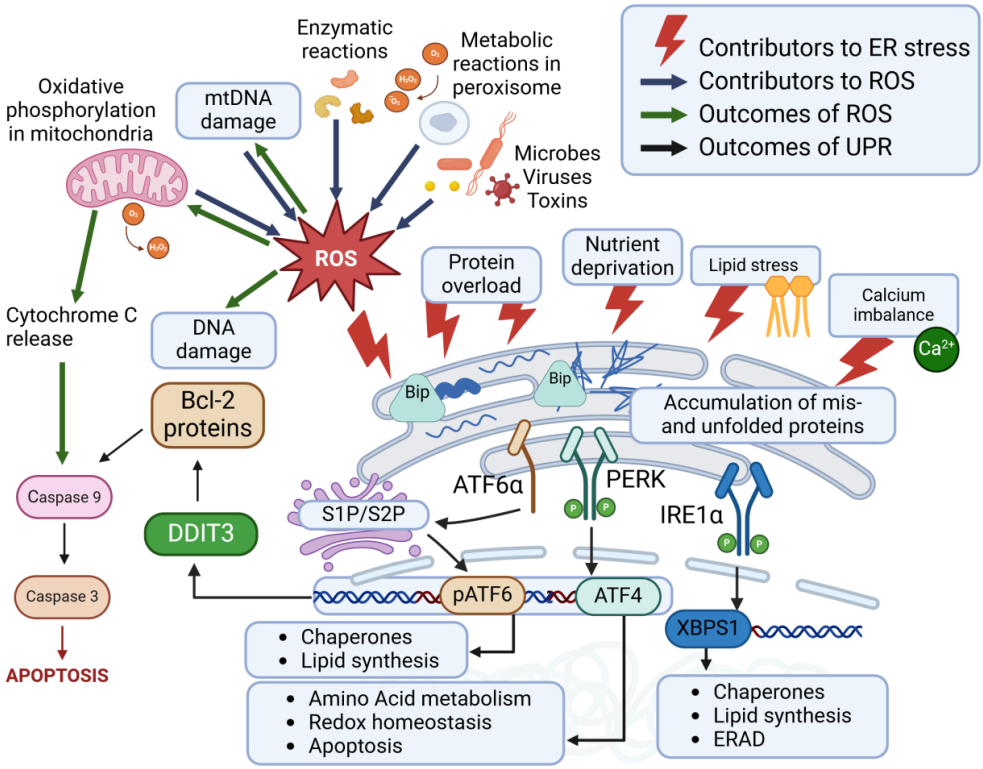


Figure 1 Image summarizing the contributors and outcomes of ER stress as well as reactive oxygen species. Legend distinguishing between contributors and outcomes has been added to the top right corner of the image. Abbreviations: UPR – Unfolded protein response, ROS – reactive oxygen species, ERAD – ER associated degradation. Created with BioRender.com.

### 2.3 ER mediated lipid synthesis and homeostasis.

The ER works in conjunction with the mitochondria to produce phospholipids at the mitochondria associated membranes (MAM), the function of which is not limited to simply facilitating the production of phospholipids but also has other functions, such as calcium uptake, lipid transport and autophagy initiation (X. Yang et al. 2023). The MAM is a closely apposed region of ER and mitochondria where exchange of lipids and calcium can be facilitated. The glycerophospholipids (GPL) phosphatidylcholine (PC), -ethanolamine (PE), -inositol (PI) and -serine (PS) are the main components of lipid bilayers in mammalian cells. Diacylglycerol (DAG) on the other hand is the main component of glycerophospholipids, and DAG is mostly generated from phosphatidic acid (PA) by enzymes at the plasma membrane (PM), ER and MAM (Hishikawa et al. 2014). This is the main route of newly synthesized glycerophospholipids but by no means the only one, as GPLs can also be generated from each other at the ER and mitochondria. The mitochondria need a constant supply of PS from the ER as they are unable to generate it and, vice versa, the mitochondria supply the ER with PE (Vance 2015). The ER

also synthesizes a multitude of other lipid classes, such as steroids, sphingolipids, PI and triacylglycerols (TG). The synthesis and homeostasis of the aforementioned lipid classes are discussed in the subsections below.

### 2.3.1 Regulation of cholesterol biosynthesis

Steroids are a broad lipid class that includes cholesterol and its biosynthetic intermediates as well as oxysterols and steroid hormones. Cholesterol is an essential lipid that has a multitude of functions within the cell, including regulation of membrane fluidity, formation of lipid rafts, regulation of membrane permeability, modulation of protein function and establishment of membrane curvature, such as formation of membrane invaginations and protrusions (Paukner, Lesná, and Poledne 2022). Generation of cholesterol at the ER is precisely balanced with its exit, as excess cholesterol at the ER can be toxic to cells and can result in ER stress (Bashiri et al. 2016).

Synthesis of cholesterol at the ER begins with glucose or fatty acid derived acetyl-CoA, which is converted into acetoacetyl-CoA, that is further converted into β-hydroxy-β-methylglutaryl-CoA (HMG-CoA) in a reaction catalysed by the enzyme thiolase. The next step in the pathway is the conversion of HMG-CoA into mevalonate facilitated by a rate limiting enzyme HMG-CoA reductase that also functions as the main regulatory factor in maintaining cholesterol homeostasis (Shi et al. 2022). HMG-CoA reductase is regulated by a feedback inhibition that is triggered by high intracellular cholesterol concentrations. Sterol regulatory element-binding proteins (SREBPs) and their associated proteins, such as SREBP cleavage-activating protein (SCAP) are responsible for this feedback inhibition. At low cholesterol conditions they actively promote the transcription of genes involved in cholesterol biosynthesis such as HMG-CoA reductase, whereas in high cholesterol conditions the aforementioned proteins are inhibited, thus reducing gene expression. In low cholesterol levels SCAP can bind to SERBPs, which SCAP ferries to the Golgi to be cleaved by proteolytic proteins site-1 and site-2 proteases (S1P and S2P). The cleaved active domain then acts as a transcription factor that initiates the transcription of cholesterol synthesis genes. Since cholesterol can competitively bind to SCAP, at high cholesterol concentrations cholesterol itself inhibits its own synthesis. Insulin induced gene can also prevent the upregulation of cholesterol synthesis by binding to SCAP and retaining the SCAP-SREBP complex in the ER (Shi et al. 2022).

*De novo* synthesis is only one of the many ways cells can acquire cholesterol, as cells can uptake lipoprotein particles from the blood stream, that are endocytosed through several receptors such as the low-density lipoprotein receptor (X. Zhang, Sessa, and Fernández-Hernando 2018). Cholesterol can also be taken from endogenous lipid droplets where it is stored as cholesteryl esters (CE). Cholesterol concentrations can subsequently be lowered by turning cholesterol back to CE and storing them in lipid droplets (LD)s (Olzmann and Carvalho 2019a). Another manner, by which cell modulate cholesterol concentrations is by efflux to extracellular lipoproteins through ATP binding cassette (ABC)

proteins, such as ABCA1 and ABCG1, that move intracellular cholesterol to Apolipoprotein A1 (ApoA1) and High-density lipoprotein (HDL), respectively (Hardy, Frisdal, and Le Goff 2017).

### 2.3.2 Cholesterol can be oxidized to form oxysterols

Cholesterol concentrations can also be modulated by turning cholesterol into oxysterol derivatives. Oxysterols are enzymatically or non-enzymatically oxygenated cholesterol products that are synthesized by different pathways depending on the cell type (Olkkonen, Béaslas, and Nissilä 2012). Oxysterols are derived from cholesterol by Cytochrome P450 enzymes, including CYP27A1 and CYP46A1 that produce 27-hydroxycholesterol (Pikuleva et al. 1998), and 24-hydroxycholesterol respectively (Lund et al. 2003). Lipoxygenases have also been shown to oxidize cholesterol, for example, ALOX15 can form 7α,15-dihydroxycholesterol and 7β,15-dihydroxycholesterol (Snodgrass et al. 2018). Cholesterol can also be oxidized by ROS during oxidative stress, and this form of oxysterol generation can produce, for example, 7-ketocholesterol and 25-hydroxycholesterol (Ghzaiel et al. 2023; Cao et al. 2020), which has been shown to inhibit the cholesterol transport of multiple ORPs as well as inhibit cholesterol synthesis by inhibition of HMG-CoA reductase (Suchanek et al. 2007).

The synthesis of oxysterols, particularly through enzymatic pathways involving CYPs and lipoxygenases, is regulated by multiple factors, including substrate availability, cofactor availability, transcriptional regulation of enzyme expression, and post-translational modifications (A. J. Brown, Sharpe, and Rogers 2021; Mutemberezi, Guillemot-Legris, and Muccioli 2016). Oxysterols can also undergo metabolism and conversion to other metabolites, contributing to their turnover and elimination. These metabolic pathways lead to the formation of bile acids, steroid hormones, and other biologically active molecules thus reducing the oxysterol levels (Mutemberezi, Guillemot-Legris, and Muccioli 2016). Oxysterols can also serve as ligands for various nuclear receptors, such as liver X receptors (LXRs) that are activated by intermediates in the cholesterol biosynthetic pathway, as well as other oxysterols (Janowski et al. 1999). These receptors play a central role in the regulation of cholesterol and lipid metabolism and the activation of LXRs induces the expression of genes involved in cholesterol efflux, particularly *ABCA1* and *ABCG1* (Bilotta et al. 2020). LXRs can have anti-inflammatory effects, particularly in macrophages. Activation of LXRs suppresses the expression of pro-inflammatory genes and cytokines, such as interleukin 6 (D. He et al. 2022). Oxysterols can be eliminated from cells through ABC transporters, which mediate the efflux of oxysterols (Tarling et al. 2010). As already mentioned, oxidative stress can lead to the accumulation of oxysterols through non-enzymatic oxidation of cholesterol. Therefore, maintenance of cellular redox balance plays a crucial role in both the prevention and formation of oxysterols.

### 2.3.3 Neutral lipids function as intracellular lipid storage

Neutral lipids consist of CEs, mono-, di- and triacylglycerols, and wax esters, which form the bulk of intracellularly stored lipids. These lipids function not only as stores of energy, but also protect the cells from toxic lipid species that could otherwise accumulate in cell organelles. Synthesis of neutral lipids uses Acetyl-CoA as a starting material, which is turned into fatty acid (FA) CoAs by Fatty acid synthase. These FA CoAs and glycerol-3-phosphate (G3P) are catalysed to form lysoPAs by ER resident Glycerol-3-phosphate acyltransferases. LysoPAs are subsequently converted into PA by lysophosphatidic acid acyltransferase. PAs are further transformed to DAGs, that takes place in the ER, and it is catalysed by the enzyme sterol O-acyltransferase 1 (SOAT1, formerly ACAT). Neutral lipids are incompatible with other membrane lipids and, thus, must be stored in lipid monolayer limited organelle called lipid droplet (LD). At low concentrations neutral lipids can also be stored in the ER bilayer, but once higher concentrations are reached, they demix from membrane GLPs to form a lipid lens that initiates lipid droplet genesis, i.e. formation of excess CEs and TGs are the initiators of lipid droplet genesis (Olzmann and Carvalho 2019b). LDs are dismantled in a process called lipophagy, that refers to the selective autophagy of LDs. Degradation of LDs can be achieved by at least two main routes; either by introducing lipases into the LDs, which then release FAs, or by the fusion of LDs with lysosomes, which also release FAs (S. Zhang et al. 2022). Thus, neutral lipid homeostasis is governed by the synthesis of neutral lipids and lipolysis as well as lipophagy.

### 2.3.4 Glycerophospholipids as membrane building blocks

PCs, PEs, PIs and PSs make up the bulk of all membrane GLPs in cells, while PIs and PAs also play crucial roles in cellular homeostasis either as a part of synthesis pathways or important lipid mediators of signalling. *De novo* synthesis of GLPs uses G3P and fatty acyl-CoA as starting material that are turned to lysoPA and further to PA. In subsequent reactions PA is further refined to either DAG or cytidine diphosphate-DAG (CDP-DAG), which are used to form either PE and PC or PIs, respectively. These lipid classes can be turned into their respective lyso species in the remodelling pathway where lysoacyltransferases cleave or transfer one fatty acid from or to another lipid. The *De novo* pathway also includes the reactions between PE, PS and PC, i.e. PE can be converted into PC or PS and PC to PS, and PS back to PE, whereas the CDP-DAG arm of the pathway, which includes PIs can further produce PI phosphates (PIPs), cardiolipins and phosphatidylglycerols (Vance 2015). As was the case for cholesterol, GPLs also efflux from the cells through ABC transporters to lipoprotein particles or by phospholipid transfer proteins (X. C. Jiang 2018). Another means of GPL efflux from the cells is by the exocytosis of vesicles and lipoprotein particles as the membranes of these organelles are made up of mostly GPLs (Dashti et al., n.d.; Ghadami and Dellinger 2023). Vesicle and lipoprotein endocytosis is also a means, by which cells can acquire exogenous GPLs.

### 2.3.5 Sphingolipid synthesis and homeostasis

Sphingolipids are complex lipids that play important structural and signalling roles in cell membranes, and synthesis of sphingolipids occurs primarily in the ER and Golgi apparatus (Quinville et al. 2021). The synthesis of sphingolipids begins with the condensation of palmitoyl-CoA and serine. This reaction is catalyzed by the enzyme serine palmitoyltransferase, resulting in the formation of 3-ketosphinganine. Next, 3-ketosphinganine is reduced by 3-ketosphinganine reductase to form dihydrosphingosine (also known as sphinganine). Dihydrosphingosine is then acylated by ceramide synthase, which adds a fatty acyl-CoA chain to the amino group of sphinganine, resulting in the formation of dihydroceramide (also known as sphinganine ceramide) (Quinville et al. 2021). Sphingomyelin is another major sphingolipid found in cell membranes, particularly in the plasma membrane and myelin sheaths of neurons. Sphingomyelin is synthesized by the transfer of a phosphocholine head group from phosphatidylcholine to ceramide. This reaction is catalysed by the enzyme sphingomyelin synthase, which is localized in the Golgi apparatus (Villani et al. 2008; K. Hu et al. 2024).

Sphingolipids, similar to other lipids, can be removed from cells through several processes. Cells can remove sphingolipids by packaging them into vesicles or lipoproteins that are then released from the cell through exocytosis (Ghadami and Dellinger 2023; Dashti et al., n.d.). For example, sphingolipids, including sphingomyelin, can associate with HDL particles in the bloodstream, and they may be exchanged between lipoproteins during lipid metabolism. Sphingolipids may also be removed from cells during membrane turnover processes, such as endocytosis and autophagy (Quinville et al. 2021). Lipid rafts are specialized microdomains in the plasma membrane enriched in cholesterol and sphingolipids (Grassi et al. 2020). Certain receptors or ligands associated with lipid rafts can be internalized into cells via lipid raft-mediated endocytosis. This process involves the invagination of lipid raft-containing membrane domains, leading to the formation of endocytic vesicles that are internalized into the cell thus internalizing both sphingolipids and cholesterol (El-Sayed and Harashima 2013). Figure 2 compiles simplified reactions and pathways of lipid homeostasis discussed in section 2.3.

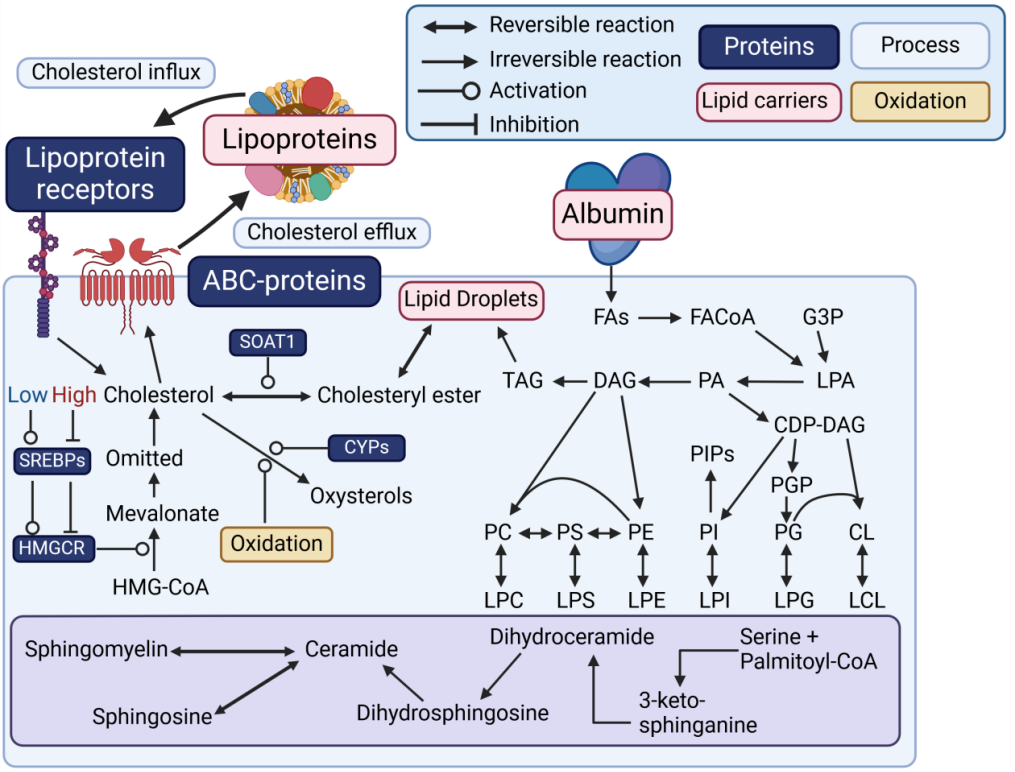


Figure 2 Image simplifying the reactions and pathways of lipid homeostasis discussed in section 2.3. A legend is shown at the top right corner of the image. Created with BioRender.com.

### 2.4 ER mediated vesicle transport

Vesicle traffic between the ER and Golgi is mediated through COPI and COPII coated vesicles that ferry large amounts of cargo within a lipid bilayer bound vesicle. Furthermore, vesicles also mediate traffic from post-Golgi to the PM along membrane tubules (Gomez-Navarro and Miller 2016). This mode of transport is distinct from MCS transport in that the membranes fuse together there by releasing the cargo within the bilayer into the organelle lumen. This type of transport can move large amounts of cargo at a time and is not specialized to a specific molecule. The endosomal pathway is another route of transport mediated by distinct protein machineries, but COP coated vesicle transport remains the main route of transport between the ER and Golgi, and a discussion of the endosomal pathway remains out of the scope of this review. COPI and COPII coated vesicles play different roles in this transport as COPII is responsible for the anterograde transport of cargo from the ER to the endoplasmic-reticulum-Golgi intermediate compartment (ERGIC) whereas COPI moves cargo from the ERGIC to the ER in retrograde transport (Tang and Ginsburg 2023).

### 2.5 Intracellular traffic by membrane contact sites

The first descriptions of MCS were already made in the late 1950s and early 1960s (Copeland 1959; Porter 1957; Robertson 1960), and the field has evolved ever since. MCS traffic is distinct from vesicular traffic by the fact that the membranes in proximity, anywhere between 10 – 80 nm, do not fuse together (Scorrano et al. 2019). Current understanding on MCS has recognized at least four distinct functions they perform: signalling, regulation of organelle membrane dynamics, lipid transport and metabolic channelling (Prinz, Toulmay, and Balla 2019; Voeltz et al. 2024).

Non-vesicular lipid transport by lipid transport proteins (LTP) at MCS is one of the most well characterized functions of MCS. Lipid exchange at MCS has been postulated to have at least four different functions: Lipid mediated signalling, reorganization of membrane lipids, redundant lipid traffic apart from vesicular traffic and lipid channelling between compartments (Prinz, Toulmay, and Balla 2019; Prinz 2014). Especially lipid channelling between membranes has been under intense investigation as it can also mediate lipid signals from one membrane compartment to another. An example of lipid channelling is well exhibited by OSBP, which exchanges phosphatidyl inositol 4 phosphate (PI4P) for cholesterol against a concentration gradient at ER *trans*-Golgi (TGN) MCS (Mesmin et al. 2013). OSBP is tethered to the ER by Vesicle associated membrane protein associated protein A (VAPA), which binds OSBP's two phenylalanines in an acidic tract motif to the ER. On the other hand, the OSBP pleckstrin homology domain binds to TGN PI4P in a concentration depended manner. These tethers bring the ER and TGN to proximity, and exchange of ER cholesterol for TGN PI4P takes place, but the membranes do not fuse together even though they are in close contact. ER cholesterol is bound to the OSBP oxysterol binding pocket, and exchanged for PI4P at the TGN membrane, that is subsequently transported to the ER membrane. At the ER membrane PI4P is dephosphorylated to PI by an enzyme called SAC1, that maintains the concentration gradient between the ER and TGN. Once a sufficient drop in PI4P levels has been achieved the OSBP pleckstrin homology domain detethers from the TGN and the exchanges ends. This process is therefore autoregulated by TGN levels of PI4P and ER cholesterol (Antonny, Bigay, and Mesmin 2018; Mesmin et al. 2017; 2013).

MCS proteins can also act as tethers that bind two membranes into close contact. The aforementioned VAPA and other VAP protein family members, that include VAPB and the more recently characterized motile sperm domain containing 1 – 3 (Di Mattia et al. 2018; Cabukusta et al. 2020), are excellent examples of multifunctional tether proteins. Both VAPA and VAPB facilitate multitude of different MCS between the ER and other intracellular organelles (Murphy and Levine 2016; Cabukusta et al. 2020). The interactome of VAPA and VAPB (VAPome) thus far includes 350 interaction partners according to the IntAct database (Orchard et al. 2014; Murphy and Levine 2016; Cabukusta et al. 2020). A subset of this diversity of binding partners and membranes is shown in Figure 3, that exhibits ER membrane pairs as well as VAP MCS protein pairs. As is evident, the variety of MCS is substantial and although not shown, all organelle limiting membranes as well as other intracellular structures are included.

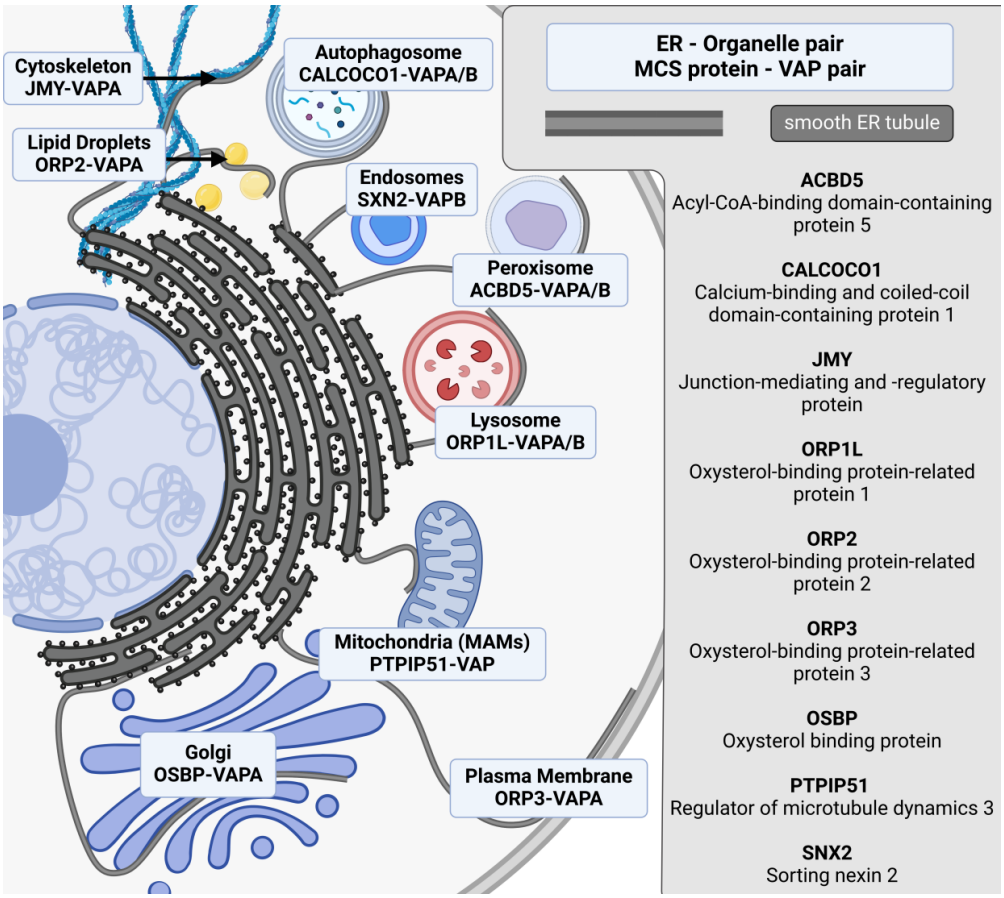


Figure 3 Illustration of the ER MCS diversity that VAPA and VAPB facilitate through the tethering of the ER and MCS proteins in other membrane compartments. Legend and abbreviations are shown on the rights side of the figure. ER organelle pair is displayed on top and protein paring below it. Modified from Taskinen et al. 2023 and created using BioRender.com.

In addition to lipid transport and metabolic channelling to specific membrane compartments, MCS are responsible for regulating calcium fluxes, such as store operated calcium entry. Stromal Interaction Molecule 1 (STIM1) is an ER membrane bound protein that oligomerises during low ER calcium conditions. STIM1 oligomerization increases its affinity with the PM localized calcium release-activated calcium modulator that allows the flow of extracellular calcium into the cell. In conjunction with ER calcium pumps and the close apposition of the ER and PM, this allows for replenishment of ER calcium levels (S. L. Zhang et al. 2005; Spassova et al. 2006; Liou et al. 2005). MCS proteins can also drive the release of ER calcium into mitochondria by for example through the tethering ER and mitochondria to close apposition by HSPA9 (formerly GRP75), that allows the inositol trisphosphate receptor released calcium to enter mitochondria through voltage-dependent anion channels (Csordás et al. 2010; Szabadkai et al. 2006; Rizzuto et al. 1993). Lipids can also traffic through metabolic channelling, and examples of channel proteins involved in these processes include the vps13-like or repeating b-groove

motif bridge-like family proteins, that have been speculated to facilitate the flow of lipids within a hydrophobic channel (Cai et al. 2022; Hanna, Guillén-Samander, and De Camilli 2023). Transport of lipids at channel MCS can be driven by the synthesis of a lipid at the MCS of the donor membrane (Kannan et al. 2017; Y. J. Kim, Guzman-Hernandez, and Balla 2011). MCS driven lipid exchange can also be increased by the translocation of MCS proteins to a MCS, which naturally rises the rate of traffic.

Cells rely on secondary messengers to exchange signals from the periphery to for example the nucleus. These signals can also be exchanged at MCS, that allows their quick translation. Many of these secondary messages are delivered by inositol 1,4,5 triphosphate that is a hydrolysis product of PI(4,5)P at the PM (Berridge and Irvine 1984). Another product of PIP hydrolysis is DAG, that along with inositol 1,4,5 triphosphate can initiate the release of intracellular calcium and activate protein kinases (Oh 2023). PI the backbone of PIPs is produced at the ER, but the kinases that produce PIPs are located at other membrane compartments (Balla 2013). This means that the ER-synthesized PI must be transported to appropriate membranes for phosphorylation, which can occur at MCS. Example of a signalling pathway that utilises PIPs as starting material for signalling is the PI3K pathway and its multiple downstream effects, have been extensively reviewed (Y. He et al. 2021; J. Yang et al. 2019). The phosphoinositide 3-kinase (PI3K) family consists of three subgroups that catalyse the reactions of different PIPs. The PI3K pathway begins with the production of PI(3,4,5)P that recruits 3-phosphoinositide-dependent kinase 1 to the PM, which subsequently phosphorylates and activates AKT resulting in the downstream effects of AKT activation, such as cell metabolism, growth, proliferation, and angiogenesis (Martins et al. 2021; Manning and Toker 2017). Thus, MCSs that transport PI to PM are vital for the replenishment of PI levels at the PM, that is used in the modulation of PIP content of the PM by PI-kinases, such as phosphatidylinositol 4 kinases and phosphatidylinositol 4 phosphate 5 kinases. Examples of MCS proteins that deliver PI from the ER to PM are NIR2 and C2CD2L (Chang and Liou 2015; Lees et al. 2017). PIPs can also be used to deliver cholesterol from the ER to the PM as is the case with OSBP-mediated delivery between the ER and TGN, and with ORP2, which delivers cholesterol to the PM (H. Wang et al. 2019; Koponen et al. 2020a).

MCS can generate subdomains within organelle membranes, thereby exerting control over membrane properties, organelle shapes, and functions. One of the functions of MCSs is to regulate organelle fission and fusion events. For instance, in mitochondria, MCSs facilitate continuous fission and fusion processes, crucial for maintaining optimal mitochondrial function. Key proteins involved in this regulation include dynamin-related protein 1 and inner membrane scission proteins. Before mitochondrial division, the ER wraps around mitochondria, facilitating actin-dependent mitochondrial constriction (Manor et al. 2015; Korobova, Ramabhadran, and Higgs 2013). Then adaptor proteins are recruited to the mitochondria that further constrict them and complete the fission (Smirnova et al. 1998; Arasaki et al. 2015; J. E. Lee et al. 2016; Osellame et al. 2016). Additionally, ER–mitochondria contacts have been associated with sites of mitochondrial fusion, although their direct role in fusion remains to be fully elucidated (Yuting Guo et al. 2018).

MCSs also impact organelle trafficking and positioning. MCSs can influence the transport and positioning of various organelles simultaneously. For instance, MCSs between the ER and endosomes or mitochondria are maintained during organelle trafficking along microtubules, allowing for coordinated movement (Friedman et al. 2010; Salogiannis, Egan, and Reck-Peterson 2016). Furthermore, MCSs can prevent vesicles from being transported by anchoring them to immobile organelles, such as the ER tethering endosomes (Jongsma et al. 2016). Moreover, MCSs contribute to autophagosome formation and endosomal cargo sorting (K. Suzuki et al. 2013; Graef et al. 2013; Okumura et al. 1990). Proteins like VAPA, VAPB, and VMP1 localize to these contacts and are essential for proper autophagosome biogenesis (Nascimbeni et al. 2017).

MCSs can establish functionally distinct subpopulations of organelles. For instance, specialized MCSs between the mitochondria and LDs create subpopulations with distinct lipid compositions and functions, such as increased ATP synthesis and reduced fatty acid oxidation. These examples underscore the significance of MCSs in cellular physiology and suggest a mechanism for segregating functional organelles from non-functional ones (Teixeira et al. 2018; Benador et al. 2018; Eisenberg-Bord et al. 2018). Proteins at MCSs can play crucial roles in determining organelle attachment and trafficking. For example, ORP1L serves as a cholesterol sensor linking endosomes to dynactin motor complexes or the ER, depending on cellular cholesterol levels (Rocha et al. 2009). Similarly, protrudin facilitates the transfer of kinesin 1 from the ER to late endosomes, aiding in endosome transport and neurite outgrowth (Raiborg et al. 2015).

MCSs are integral in coordinating cellular responses to various stresses, including lipid, mechanical, nutrient, oxidative, as well as ER stress. MCSs play crucial roles in alleviating lipid stress by facilitating lipid movement between organelles. For instance, the microtubule-binding nuclear protein is enriched at ER–LD contacts, preventing the toxic accumulation of lipid intermediates (Hariri et al. 2019). LDs can make prevalent contacts with the ER, mitochondria, and other organelles, aiding in lipid transport and storage. These contacts prevent FA stress during conditions like starvation-induced autophagy (Olzmann and Carvalho 2019b). MCSs are critical for responding to nutrient stress, particularly in selective autophagy pathways induced by nutritional changes (Böckler and Westermann 2014). Other MCSs, like ER–PM contacts, regulate TORC1 and TORC2 signaling pathways, coordinating stress responses and sterol homeostasis (Murley et al. 2017). During mechanical stress, cells can encounter forces like stretching, compression, or shear stress in blood vessels. MCSs contribute to mechanical stress responses by stabilizing cellular structures and membranes (Schapire et al. 2008; Pérez-Sancho et al. 2015). ER–mitochondria contacts play significant roles in responding to oxidative stress. ROS-mediated ER stress at these contacts can trigger apoptosis pathways, impacting cell survival (Yoboue, Sitia, and Simmen 2018Gilady et al. 2010; Raturi et al. 2016). ER–mitochondria contacts are also crucial in responding to ER stress by regulating calcium signalling and apoptosis. Additionally, ER–mitochondria contacts are implicated in coordinating lipid metabolism, essential for mitochondrial function during oxidative phosphorylation (Mourier et al. 2015; Subramanian et al. 2019).

### 2.5.1 VAMP associated proteins A and B

Most functional insights into a VAP-depleted phenotype stem from investigations into a rare form of dominantly inherited familial amyotrophic lateral sclerosis (ALS8), characterized by a VAPB P56S mutation (Aliaga et al. 2013; Nishimura et al. 2004). This mutation mimics a VAPB knockdown phenotype by mutant protein binding to the protein encoded by the wild-type allele, thereby impeding its ability to interact with its protein partners (H. Suzuki et al. 2009; Yamanaka et al. 2020). The depletion of VAPB and the effects of the P56S mutant have been associated with various cellular dysfunctions, extensively discussed in previous reviews. These changes include ER stress, elevated cholesterol, and TG levels, impaired UPR and autophagy, as well as neurodegeneration (Kors, Costello, and Schrader 2022; Borgese et al. 2021). Less is understood about the concurrent depletion of both VAPs, although most double knock down (DKD) studies demonstrate a significant impact on autophagy. For instance, Mao et al. observed an accumulation of PI4P at the Golgi following VAP DKD, leading to increased vesicles in endosomal, lysosomal, and autophagosome compartments (Mao et al. 2019). Similarly, individual knockdowns of either VAP resulted in increased autophagocytosis (D. Wu et al. 2018). Conversely, it has been reported that simultaneous depletion of both VAPs hindered autophagosome formation at the initiation stage (Zhao et al. 2018).

### 2.5.2 Oxysterol binding protein

The established lipid transport function OSBP was discussed in the previous section, but it does have other cellular functions (Lin et al. 2023). For example, OSBP overexpression increases cholesterol synthesis, inhibits CE formation, promotes TG synthesis and increases LDs in liver. Silencing of OSBP increases cholesterol efflux, lysosomal degradation of insulin granules and proinsulin. It also promotes cholesterol transport to lysosomes and increases autophagy in NPC1 deficient cells. Cholesterol traffic by OSBP can also facilitate viral RNA replication and has been shown to be a positive prognostic marker in cancers. The role of OSBP has not been well characterized in ECs and so far, only two studies have been done in an EC model. These studies have revealed that OSBP controls the activation of STAT3 and subsequent expression of profilin-1 and implicated OSBP in elevated CRP levels in rat ECs (Shmidt, Kazlauskas, and Romeo 2006; Romeo and Kazlauskas 2008).

### 2.5.3 Oxysterol binding protein like 7

ORP7 has received relatively little attention compared to its counterparts, notably OSBP, from which the family derives its name. The specific lipid transport and membrane pairs of ORP7 remain unclear, along with its other cellular roles. However, studies suggest its localization to the PM and ER, along with interaction with GABARAPL2 (Zhong et al. 2011; Lehto et al. 2004). Additionally, ORP7 has been implicated in the human macroautophagy pathway, interacting with unlipidated LC3B (Tu et al. 2022). Inhibition of ORP7 has been linked to increased ABCA1-dependent cholesterol efflux (Wright et al. 2021), and genetic studies associate certain variants with hypercholesterolemia (Abdul Murad et al.

2023). In terms of gene expression, ORP7 shows correlations with other ORPs, with implications in cancer, eczema and amyloid β accumulation (B. H. Kim et al. 2023; Chou et al. 2021; Grosche et al. 2021).

### 2.6 Endothelial cells and lipid metabolism

ECs form and maintain the walls of both blood and lymphatic vessels and play a crucial role not only in regulating vascular permeability, but also participate in the regulation of blood flow, angiogenesis, tissue inflammation, and response to injury, such as blood coagulation (G. Li et al. 2024; Krüger-Genge et al. 2019). ECs also participate in the overall metabolic function of surrounding tissues by regulating the uptake of lipids, glucose, amino acids, and other metabolites. This barrier function is extremely important, as it is crucial that only select molecules can pass from the bloodstream to the adjacent tissues (Rodrigues and Granger 2015). Molecules pass through the endothelium by two routes, by transcellular or paracellular transport. Transcellular transport happens by, for example, active transport of lipids by vesicles as well as carrier, receptor, and transport proteins, through the EC cytoplasm (Fung, Fairn, and Lee 2018). Paracellular transport on the other hand occurs at the tight and adherent junctions between EC. This passive transport is driven by a concentration gradient between the vessel lumen and adjacent tissues, and only small molecules such as water, ions and glucose can pass through junctions into tissues (Yazdani et al. 2019;Claesson-Welsh, Dejana, and Mcdonald 2021).

ECs are not a single cell type but consist of diverse cell populations and this heterogeneity is driven by both genetic and epigenetic factors from their microenvironment (Minami, Muramatsu, and Kume 2019). Single cell RNA sequencing studies have implicated multiple EC types that show variability in their gene expression patterns based on organ specific microenvironments (F. Wang et al. 2022; Jambusaria et al. 2020). These studies have shown that ECs adapt to the needs of the organs they are adjacent to. These adaptations include a high energy phenotype at heart, skeletal muscle and adipose tissues, which efficiently transcellularly transport FAs from the lumen (Bagchi et al. 2022).

In capillary endothelium, EC lipid uptake primarily occurs as FAs, which are either albumin-bound or hydrolysed from lipoproteins by lipoprotein lipase (LPL). FA transport in ECs is regulated by paracrine factors including 3-hydroxyisobutyrate (3-HIB), Vascular endothelial growth factor B (VEGFB), apelin, and angiopoietin-2 (ANGPT2). 3-HIB, ANGPT2 and VEGFB, secreted by skeletal muscle, heart, white and brown adipose tissue enhance FA uptake in ECs, whereas Apelin inhibits it. Glycosylphosphatidylinositol-anchored high-density lipoprotein-binding protein 1 transports LPL, synthesized in parenchymal cells, from the subendothelial to the luminal side of ECs, where it breaks down lipoproteins. The entry of FAs into ECs is facilitated by cluster of differentiation 36 and fatty acid binding proteins. These proteins direct FAs to various cellular sites for oxidation, membrane synthesis, and signal transduction. ECs can also internalize lipoproteins directly via endocytosis, followed by hydrolysis in

lysosomes (B. Kim and Arany 2022). Once internalized, FAs are conjugated to CoA, using ATP produced by mitochondria, and are utilized for FA oxidation, TG synthesis and formation of LDs, or transported out of the cell. Acetyl-CoA carboxylase converts acetyl-CoA to malonyl-CoA, which is then elongated to FAs by fatty acid synthase and other elongases and desaturases. FAs are crucial for angiogenesis as inhibiting FA synthesis by blocking acetyl-CoA carboxylase or fatty acid synthase impairs angiogenesis (Bagchi et al. 2022).

Since MCS function as one major traffic route of intracellular molecules from and to the cell surface, it is important to understand how aberrations in their dysfunction can affect EC barrier function and transcellular transport. The literature on MCS in ECs is very limited, and focus on OSBP, ORP2 and protrudin (Koponen et al. 2020b; Arora et al. 2022a; Romeo and Kazlauskas 2008; Shmidt, Kazlauskas, and Romeo 2006). Similarly, a data base on MCS proteins (Pan et al. 2024) which as of summer 2024 has about 7000 annotated entries, includes only FUNDC1, SYTL2, and RAB27 that have been validated in ECs. Given the heterogenic nature of ECs and the lack of information on MSC in ECs, studies such as those included in this thesis are crucial for better understanding of ECs with dysfunctional MCSs.

### 2.6.1 Angiogenesis

Angiogenesis is the formation of new vasculature from pre-existing vessels. Angiogenesis is crucial for development and growth of new tissues, as they need oxygen and nutrients, as well as for wound healing as new vessels help repair damaged tissues. Angiogenesis can also be detrimental, as abnormal angiogenesis can help tumours grow, as well as make cardiovascular plaques “brittle” by burrowing into the plaque.

Induction of angiogenesis is dependent on the interplay of ECs and chemotactic stimuli from hypoxic tissues. These signals, VEGF being a major angiogenesis regulator, form a concentration gradient that activates the underlying EC layer. From these activated ECs few form tip cells, and this selection depends on their affinity of VEGF, as well as on their expression level of DLL4. Once selected, tip cells begin a signalling cascade that is driven through VEGF receptor 2, which represses its neighbouring cells from becoming tip cells. This lateral inhibition is mediated by the DLL4/NOTCH signalling pathway that transforms neighbouring cells into a proliferating stalk cell phenotype. The proliferating stalk cells form the bulk of the newly formed vessel lumen wall, which either pushes the vessel into the tissues, or is pulled by the tip cells, which guide the vessel towards the source of the growth gradient (Mühleder et al. 2020; Geudens and Gerhardt 2011; Eelen et al. 2020). Tip cells move the vessel into the tissue using actin filament filled PM protrusions called lamelli- and filopodia. These membrane protrusions probe the surrounding tissues for cues and respond by either polymerizing or depolymerizing the actin filaments, thus elongating or retracting them. Both protrusions attach to extracellular matrix (ECM) through focal adhesions that enable tip cells to adhere to ECM, facilitating their migration within tissues. Cell movement is driven by the contraction of intracellular actin

microfilaments known as stress fibres, which pull the cell toward focal adhesions. As cells are anchored to the ECM, tip cells must degrade it to invade tissues. This degradation is facilitated by matrix metalloproteinases produced by tip cells.

The formation of a functional blood vessel requires the presence of a lumen, and the mechanism of lumen formation during sprout elongation remains debated. Several theories have been proposed, including intracellular vacuole coalescence, intracellular vacuole exocytosis, and luminal repulsion models. Intracellular vacuole coalescence involves the fusion of intracellular vacuoles, forming a larger void within the cell that eventually merges with voids in neighbouring cells to create the lumen. Intercellular vacuole exocytosis describes the fusion of exocytotic vacuoles outside the cells, generating intercellular space that eventually forms the lumen between two cells. Luminal repulsion occurs when negatively charged CD34-sialomucins repel each other at VE-Cadherin sites between two cells, creating an intercellular void that fuses with neighbouring cells to form the lumen (Mühleder et al. 2020; Geudens and Gerhardt 2011; Eelen et al. 2020). Perivascular fibroblasts also contribute to angiogenesis by producing growth factors that promote the proliferation and migration of ECs, as well as recruiting pericytes and smooth muscle cells to stabilize the newly formed blood vessels. They also help to stabilize new blood vessels by producing and aligning ECM components that direct ECs to form proper vessel networks (L. S. Brown et al. 2019; Newman et al. 2011). ECs can also sense mechanical forces exerted by blood flow through mechanotransduction. For example, shear stress activates endothelial nitric oxide synthase and promotes the release of nitric oxide, which has vasodilatory effects and can stimulate angiogenesis (Flournoy, Ashkanani, and Chen 2022).

Angiogenesis also plays a significant role in cardiovascular diseases. Atherosclerotic plaque formation triggers angiogenesis due to hypoxia resulting from increased oxygen demand from inflammation, and reduced oxygen supply due to decreased blood flow caused by narrowing of the vessel lumen. These events can lead to the growth of malformed capillaries within an atherosclerotic plaque, causing intraplaque hemorrhage rendering the plaque unstable. Angiogenesis also plays a role in the development of heart failure and cardia ischemia, as both insufficient vessel growth and abnormal vessel regression can affect both. For example, during cardiomyocyte hypertrophy, insufficient angiogenesis can result in hypoxia and cardiac malperfusion. This mismatch between cardiomyocyte growth and angiogenesis, or simply put oxygen demand and supply, leads to a transition from adaptive to pathological cardiomyocyte hypertrophy and eventually to hearth failure or other cardiovascular diseases (Hemanthakumar and Kivelä 2020). The limited literature on MCS proteins in ECs have shown that they have a significant role in tube formation. Mainly FUNDC1, Protrudin and ORP2 knock out as well as knock down experiments have confirmed that reducing intracellular levels of these proteins reduces angiogenesis both *in vitro* and *in vivo* (C. Wang et al. 2021; Arora et al. 2022b; Koponen et al. 2020c).

### 2.6.2 Inflammatory response in endothelial cells

During early stages of inflammation ECs are activated by chemo- and cytokines secreted by nearby tissues and immune cells. Upon activation ECs upregulate the expression of adhesion molecules such as ICAM1 and VCAM1, integrins ITGA4 and ITGA5, as well as selectins such as P- and E-Selectin. Upregulation and presentation of these molecules at the cell surface help leukocytes adhere to the EC surface. Activated ECs also produce cytokines such as interleukins, TNF-α, and interferons, which amplify the inflammatory response, and activate nearby ECs in a positive feedback loop (Theofilis et al. 2021). Once circulating leukocytes, mostly neutrophils and monocytes have adhered, they migrate through the endothelium layer and infiltrate to the underlying tissue. This infiltration involves both leukocyte surface integrins and EC junction proteins that allow leukocytes to transverse through junctions. Leukocytes are attracted to migrate to the tissues by the same chemo- and cytokines that activated the ECs in the first place (Schimmel, Heemskerk, and van Buul 2017). ECs also facilitate the resolution of inflammation by modulating the endothelial barrier. They, for example, remove apoptotic cells and express receptors for anti-inflammatory molecules, thereby downregulating inflammatory gene expression, and promoting tissue repair. The barrier function also modulates the movement of immune cells from the tissue back to the vessel and controls excessive leakage of molecules and fluids from the inflamed tissues (Xue et al. 2023; Kadl and Leitinger 2005).

In conditions like, hypertension and hypercholesterolemia, where inflammation is heightened, MAMs and its MCSs may play a role in the initiation and progression of cardiovascular diseases by influencing inflammasome formation and elimination (H. Liu et al. 2020). MCS facilitate the assembly of the NLRP3-inflammasome, a protein complex crucial for the inflammatory response, by bringing together NLRP3 and apoptosis-associated speck-like protein. This assembly at MCS leads to the activation of caspase-1, which in turn promotes proinflammatory cytokines like interleukin-1β and -18. MAMs might also mediate downstream signals from pattern recognition receptors, which recognize molecules found in pathogens (Misawa, Takahama, and Saitoh 2017). Dysregulation of MCS can also lead to impaired organelle crosstalk, which has been associated with various neurodegenerative diseases such as hereditary spastic paraplegia and Parkinson's disease (Benarroch 2022). Therefore, MCS not only contribute to resolving inflammation, but dysfunctional MAM MCSs might also mediate inflammatory signals.

## 3 AIMS OF THE STUDY

I. To study the role of MCS in ECs.

II. Investigate effects of VAPA and VAPB knock down in ECs.

III. Elucidate the role of OSBP in EC function and lipid metabolism.

IV. Uncover the functions of ORP7 in ECs.

## 4 MATERIALS AND METHODS

A chart with a simplified experimental workflow used in each study included in this thesis is shown in Figure 4, in order to give the reader a better understanding of the experimental work done for this thesis.

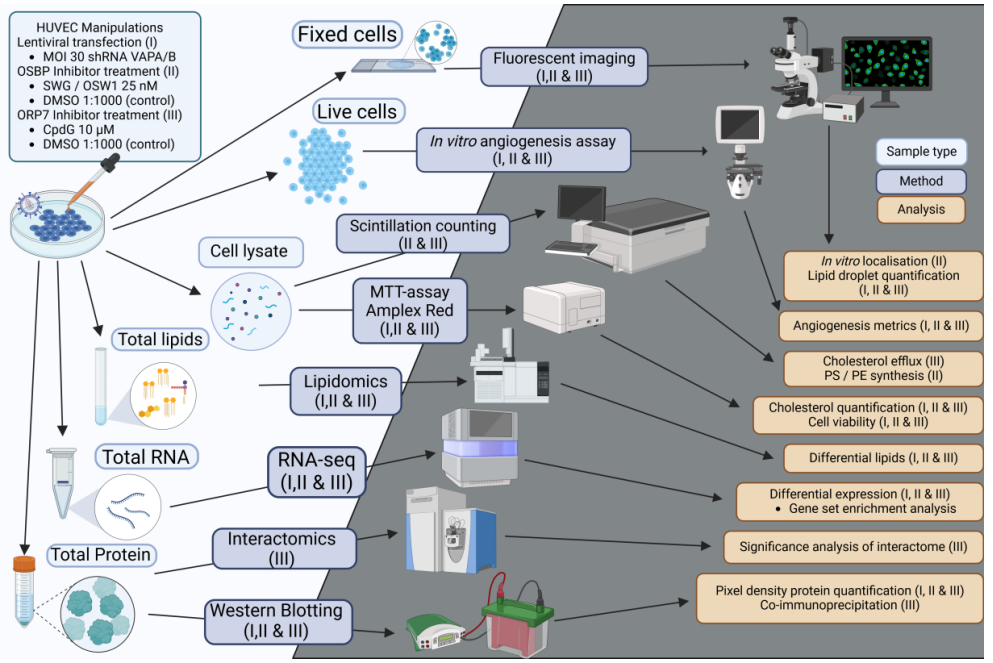


Figure 4 Simplified experimental workflow of samples and methods included in this thesis. Each sample type is depicted in pale blue boxes and each method used with the corresponding study in roman numerals are shown in dark blue boxes. Analysis types are depicted in beige boxes. Modified from Taskinen et al. 2023. Created with BioRender.com

*Cell culture (I, II & III)*

HUVECs were grown in EGCM2 media (PromoCell cat #: C-22111) without antibiotics, supplemented with endothelial growth mix 2 (PromoCell cat #: C-39216) unless otherwise specified. Cells were used mostly at passage 6.

*Transduction (I, II & III)*

HUVECs at passage 6 were transduced using lentiviral particles. Cells were incubated for 48 hours with an approximate MOI of 30 for each shRNA specific lentiviral particle, encoding VAPA shRNA (TRC0000380105, Sigma) and VAPB shRNA (TRCN0000152520 Sigma) or non-targeting shRNA

(SHC002, Sigma). After lentiviral transduction, cells were selected using media with puromycin (1:4000 of 10 mg/mL stock, Gibco), for 72 hours. Amaxa Transfections in were done as follows. Passage 6 HUVECs were transfected with mCherry-OSBP, mCherry-ORP4 and empty eGFP according to manufacturer's instructions (Lonza, Amaxa™ HUVEC Nucleofector™ Kit, cat #: VPB-1002, lot #: F-13812), using 1 ug of each plasmid and 1*10^6 cells per reaction. Each reaction was divided to two 6-well plate wells. HUVECs at passage 2 on 6-well plates were transduced with 200 µL of fresh frozen lentiviral particles (approx. MOI 15) that had either ORP7-mycBirA (oexORP7) or mycBirA (oexMYC) construct and incubated for 48h. After incubation cells were selected for 5 days using 1:1000 dilution of 10 mg /mL blasticidin, after which cells were left to recover for 48h. After recovery cells were expanded from the 6-well plates by splitting for two passages. Selected cells at passage 4 were then either frozen or further expanded to passage 6 and used for various experiments. All lentiviruses were produced by the Helsinki Biomedicum Virus Core supported by HiLIFE and the Faculty of Medicine, University of Helsinki, and Biocenter Finland.

*MTT assays (I, II & III)*

Metabolic activity was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega cat #: G358C), according to the manufacturer's instructions. Absorbance at 490 nm was measured using a PerkinElmer EnSpire Multimode Plate Reader. Percentage of metabolic activity was calculated by the following formula: $100 + (\frac{control-sample}{control} * 100)$, where control is the absorbance of cells treated with 1:1000 DMSO, transduced with non-targeting shRNA particles or oexMYC construct, and samples are cells treated with different concentrations of OSW1, SWG or CpdGORP7 as well as transduced with VAPA and VAPB lentiviral particles or oexORP7 construct.

*Western Blotting (I, II & III)*

Transduced or inhibitor treated cells were washed twice with ice cold PBS, then lysed to 200 µL lysis buffer (150 mM NaCl, 0.5 mM MgCl2, 0.5 % Triton X-100, 0.5% sodium deoxycholate (v/v) with 1 X EDTA free protease inhibitor cocktail (Merck cat #: 04693159001) for 20 min at +4°C. Lysates were collected to a fresh tube and spun for 15 min at 13 000 x G at +4°C, after which supernatant was moved to a fresh tube. Sample protein concentrations were measured with Thermo Fisher Pierce™ BCA Protein Assay Kit (cat #: 23225), and the samples were blotted with a BioRad Trans-Blot Turbo Transfer System. Membranes were blocked for 1 h at room temperature (RT) in 5 % milk TBST or for 5 min in BioRad EveryBlot solution and then probed with different antibodies overnight at +4°C. Membranes were washed thrice for 10 min with 1 X TBST at RT and then incubated with 1:2000 dilutions of HRP-conjugated secondary antibodies in Milk-TBST or EveryBlot for 1h at RT. Membranes were washed as before and detected using Thermo Fisher Pierce ECL Western blotting substrate (cat #: 32106, lot #: WJ335099) according to manufacturer's instructions.

Table 1. Antibodies used in this thesis.

| Protein | Dilution | Manufacturer | Catalog number | Lot number |
|---|---|---|---|---|
| ORP7 | 1:1000 | Sigma Aldrich | HPA036076 | NA |
| pFAK | 1:500 | Invitrogen | 44-626G | NA |
| FAK | 1:1000 | Invitrogen | AHO0502 | NA |
| pAKT1 | 1:1000 | Cell Signaling | 4051S | NA |
| AKT1 | 1:1000 | Invitrogen | AH01112 | YG373683 |
| ACTB | 1:5000 | Sigma Aldrich | A2066-2ml | 106m4770V |
| cMYC | 1:1000 | Santa Cruz Biotechnology | sc-40 | NA |
| OSBP | 1:1000 | Sigma Aldrich | HPA039277 | A106684 |
| ORP4 | 1:1000 | Sigma Aldrich | HPA021514 | R09314 |
| VAPA | 1:500 | Sigma Aldrich | HPA039277 | A106684 |
| VAPB | 1:500 | Atlas Antibodies | HPA013144 | NA |
| YIPF5 | 1:500 | Atlas Antibodies | HPA073622 | 000005592 |
| TGN46 | 1:1000 | Sigma Aldrich | T7576 | 126K4838 |

*Angiogenesis assay (I, II & III)*

Angiogenesis assay (Millipore cat #: ECM625, lot #: 3183813) was performed according to the manufacturer's instructions with 25 nM OSW1 and SWG OSBP inhibitors, 10 µM CpdG ORP7 inhibitor or VAPA/B silenced HUVECs. Cells were imaged using a bright-field set-up of EVOS M5000 (Invitrogen) microscope after 8 h on the matrix. Angiogenesis metrics were quantified from binary images ImageJ (Schindelin et al. 2012) Angiogenesis analyzer plugin (Carpentier et al. 2020).

*Measurement of cholesterol concentration (I, II & III)*

Amplex Red™ cholesterol assay (Invitrogen, cat#: A12216, lot #: 2422702) was performed according to manufacturer's instructions from 1:4 dilutions of cell lysates made as in section "*Western Blotting"* with (total cholesterol, TC) or without (free cholesterol, FC) cholesterol esterase treatment, and cholesterol concentrations were normalized to protein concentrations as measured in section Western blotting.

*Cholesterol efflux to ApoA1 and HDL (III)*

HUVECs treated with DMSO vehicle control, Sandoz 58-035 SOAT1 inhibitor T0901317 or CpdG ORP7 inhibitor (CpdG) were plated at 50,000 cells per 12-well plate well and left to adhere for 24 hours, after which cells were washed twice with 1 PBS and then 1 mL of media with 0.2 µCi/mL of $^{3}$H-cholesterol and 5 mg/mL T0901317 was added. After a 24 h incubation media was removed and cells were washed thrice with 1 X PBS, after which 2 mL of FBS free EGCM (Sigma Aldrich, cat #: 211F-500) media with 1 mg/mL of BSA was added. To the HUVEC media either 10 µM of T0901317, 10 µM CpdG or 1:1000 (v/v) DMSO was added and incubated for 24 h. After incubation, a 1 mL aliquot was

taken from each well and spun at 800 x g for 5 min, and the supernatant was collected to a scintillation tube. For ApoA1 mediated efflux 100 µL of FBS free media with 50 µg/mL ApoA1 was added and for HDL mediated efflux 111 µL of 450 µg/mL HDL isolated from human plasma and dialyzed to PBS was added and incubated for 24 h. Finally, media was collected and spun as before, and supernatant was collected to scintillation tubes. Cells were lysed to 300 µL of 1% SDS and collected to scintillation tubes. To each scintillation tube 3 mL of scintillation cocktail was added and tubes were mixed briefly before they were subject to liquid scintillation counting with a Wallac Winspectral 1414 counter.

*Measurement of triacylglycerol concentration (II)*

Triacylglycerols were measured using GPO-PAP assay kit (Cobas, Roche/Hitachi Tokyo, Japan) according to manufacturer's instructions; OD510 was measured using PerkinElmer EnSpire Multimode Plate Reader instrument.

*Measurement of PS synthesis (II)*

PS synthesis was measured as [$^3$H]-serine incorporation into PS in the following manner: Cells were grown for 18 h on 6-well plates in EGCM media with 25 nM of OSW1, SWG or 1:1000 of DMSO, after which media was removed from half of the wells, and the cells were washed twice with PBS. HBSS media with 1 X MEM amino acids (Gibco, cat #: 11130-051, lot #: 212135), 1 X MEM vitamins (Gibco), 1 µL/mL of [$^3$H]-serine (Perkin-Elmer, cat #: NET248250UC, lot #: 2662745), and 25 nM inhibitors or 1:1000 of DMSO was added (6 h time point). After 4 h of incubation the other half of wells with EGCM2 media (with inhibitors or DMSO) was removed, cells were washed, and above-mentioned media added and incubated for 2 h. After incubation, cells were washed and scraped to 2 % NaCl in $H_2O$. Lipids were extracted with a Bligh-Dyer protocol (BLIGH and DYER 1959), separated on Merck Silica TLC plates by using $CHCl_3$:methanol:acetic acid:formic acid:$H_2O$ (70:30:6:2:1) as running solution. The PS and PE spots were identified based on movement of appropriate lipid standards, scraped, and subjected to liquid scintillation counting. Data were normalized to total cell protein.

*mRNA quantification by quantitative real-time PCR (I, II & III)*

Quantitative real time PCR (qPCR) was performed on cDNA generated in section "cDNA synthesis" with the Roche Lightcycler™ 480 II instrument by using Sybr Green chemistry, Roche qPCR Master mix and primers specified in Table 2. RPLP was used as housekeeping control mRNA. Fold-changes in gene expression were calculated by employing the -DDC$_T$ method.

Table 2. Primer sequences, their targets included in this thesis.

| # | Sequence | Restriction site or gene | Direction | Use |
|---|---|---|---|---|
| 1 | TTTT-GAATTCTTATGGAACAAAAACTCATCTCAG | EcoR I | Forward | Cloning mycBirA to ORP7 in pEGFP-N1 |
| 2 | TTTTGAATTC-TACTTCTCTGCGCTTCTCAGG | EcoR I | Reverse | Cloning mycBirA to ORP7 in pEGFP-N1 |
| 3 | TTTTA-GATCTATGGACTTCCAAGAGAGGGA | Bgl II | Forward | Cloning oexmycbirA to pENTR2B |
| 4 | TTTT-GCGGCCGCGCCCAACTTTGTACAAGAAAGC | Not I | Reverse | Cloning oexmycbirA to pENTR2B |
| 5 | TTTT-GTCGACATGGACTTCCAAGA | Sal I | Forward | Cloning oexORP7-mycbirA to pENTR2B |
| 6 | TTTTGCGGCCGC-TACTTCTCTG | Not I | Reverse | Cloning oexORP7-mycbirA to pENTR2B |
| 7 | ACCCACCCTATGAACAACATGA | ABCA1 | Forward | qPCR |
| 8 | GAGTCGGGTAAC-GGAAACAGG | ABCA1 | Reverse | qPCR |
| 9 | CGGCC-TACTCCTCCACATACC | OSBPL7 | Forward | qPCR |
| 10 | ACTGTTCCCACAGGCACAATC | OSBPL7 | Reverse | qPCR |
| 5 | TGGTCATCCAG-CAGGTGTTCGA | RPLP | Forward | qPCR |
| 6 | ACAGACACTGG-CAACATTGCGG | RPLP | Reverse | qPCR |

*Immunofluorescence staining, microscopy, and lipid droplet quantification (I, II & III)*

HUVECs were seeded at 10 000 cells/coverslip and left to adhere for 24 h in 50 µL of EGCM2. Cells were fixed with 4% PFA in PBS, blocked in 1% BSA in PBS, and probed with antibodies against OSBP or TGN46. Lipid droplet staining was performed by incubating fixed cells for 1 hour at RT with 1:1000 of BODIPY493/503 stock (50 mM) and mounted with Mowiol:Dabco:DAPI solution. Imaging of the specimens was carried out on a Zeiss Observer.Z1 fluorescence microscope with a 63 X immersion oil objective. Lipid droplets were quantified as specified in (Adomshick, Pu, and Veiga-Lopez 2020).

*Co-immunoprecipitation (III)*

Oex cells or HUVECs were grown on 10 cm plates until 80% confluent, after which cells were washed twice with 1X cold PBS on ice. Cells were lysed to 1 mL of the lysis buffer described in section 2.3 that included both protease and phosphatase inhibitors and incubated for 30 min at + 4°C. Lysates were spun for 15 at 13,000 x g at +4 °C and supernatant was collected to a fresh tube. Magnetic beads were washed twice with 1 X TBST, after which 25 µL were added to 500 µL of cell lysates and mixed

on a roller for 1h at +4 °C. Either 5 µL of Mouse HRP antibody or 10 µL of AKT1 antibody (see section 2.3) were added to 25 µL of washed beads, and mixed on a roller for 2h at +4 °C. Beads were removed from each mix by incubating on a magnetic rack for 2 min at +4 °C, after which antibody coupled beads were added to the pre-cleared lysates and incubated o/n at +4 °C. Lysates were removed to a fresh tube by incubating on a magnetic rack and beads were washed thrice for 5 min with lysis buffer at +4 °C. To the washed beads 40 µL of 2 X Laemmli buffer (BioRad) was added and incubated for 10 min at +50 °C. Magnetic beads were removed as described before and to each elution 1 µL of 2-β-mercaptoethanol was added, and samples were boiled for 5 min at +100 °C. Samples were spun down for Western Blotting.

*Lipidomics (I, II & III)*

Lipids were extracted according to Folch *et al.* (Folch, Less and Sloane Stanley, 1957). Solvents were evaporated and the lipid extracts immediately dissolved in chloroform/methanol 1:2 (by volume) and just before mass spectrometry $NH_4OH$ was added (to give 2% solution) together with a standard mix containing representative standards for each lipid class analysed. The samples were infused into the electrospray source of a triple quadrupole (ESI-QQQ) mass spectrometer (Agilent 6410 TripleQuad, Agilent Technologies, Santa Clara, CA) and their lipids species were identified and quantified using lipid class-specific detection modes, as previously described (Ruhanen et al. 2017). Retrieved spectra were processed by Mass Hunter Workstation qualitative analysis software (Agilent Technologies, Inc.), and individual lipid species were quantified using the internal standards and LIMSA software (Haimi et al. 2006).

*Determination of 27-hydroxycholesterol. (I & II)*

27-hydroxycholesterol was quantified from cell pellets stored in 2% $NaCl_2$ by gas chromatography-mass spectrometry (GC-MS/MS) using stable isotope dilution analysis (Matysik, Klünemann, and Schmitz 2012). GC-MS/MS was performed on a triple quadrupole MS instrument TQ8050 equipped with a multifunctional autosampler AOC-6000, and an SH Rxi-5Sil MS column (30 m, 0.25 mm, 0.25 µm) (all Shimadzu Deutschland GmbH).

*Transcriptomics (I, II & III)*

RNA was extracted from cells with the RNAeasy Mini kit (Qiagen cat #: 74104) according to the manufacturer's instructions, with the following modifications: Samples were homogenized by passing lysate through a 200 µL pipette tip 10 times, on-column DNAse 1 digestion was performed according to manufacturer's instructions, and samples were eluted to 30 µL of RNAse free $H_2O$. RNA integrity, library preparation and RNA sequencing were performed according to the following instructions by GENEWIZ Germany GmbH (Leipzig, Germany). RNA samples were quantified using Qubit 4.0

Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with RNA Kit on Agilent 5300 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing libraries were prepared using the NEB Next Ultra RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. Sequencing libraries were validated using NGS Kit on the Agilent 5300 Fragment Analyzer (Agilent Technologies, Palo Alto, CA, USA), and quantified by using Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA). The sequencing libraries were multiplexed and loaded on the flow cell on the Illumina NovaSeq 6000 instrument according to manufacturer's instructions. The samples were sequenced using a 2x150 Pair-End (PE) configuration v1.5. Image analysis and base calling were conducted by the NovaSeq Control Software v1.7 on the NovaSeq instrument. Raw sequence data (.bcl files) generated from Illumina NovaSeq was converted into fastq files and de-multiplexed using Illumina bcl2fastq program version 2.20. One mismatch was allowed for index sequence identification.

*cDNA synthesis (I, II & III)*

cDNA was synthesized from RNA extracted as described in section "*Transcriptomics"* with the Vilo kit (Thermo Fisher, cat#: 11754050) by normalizing RNA concentrations to 500 ng or the highest possible amount by measuring RNA concentrations with the NanoDrop instrument (Thermo Fisher) and then following the manufacturer's instructions. The following PCR-program was used and produced cDNA was diluted 1:10 with molecular biology grade $H_2O$.

*Biotin proximity labeling interactomics (III)*

Interactomics was performed on HUVECs transfected with a pENTR2B-oexORP7-mycBirA or control pENTR2B-oexmycBirA vectors and cloning primers for them are shown in Table 2 (primers 1 – 6). Transfected cells on 6-well plate were selected for approximately 5 days with 10 µg/mL of blasticidin until all wild type HUVECs were dead, and then left to grow until confluent, after which cells were expanded to passage 6. Samples were created as described in (X. Liu et al. 2020). LC-MS/MS was performed by University of Helsinki, Proteomics Unit, Viikki (Helsinki, Finland) as described in (X. Liu et al. 2020).

*Bioinformatics and statistics (I, II & III)*

RNA-seq analysis was performed according to the following pipeline using the Chipster suite (Kallio et al. 2011): Pair ended reads were clipped with Triommatic (phred ≥ 30) (Bolger, Usadel, and Lohse 2014), then aligned to the human genome (GRCh38) with STAR (Dobin et al. 2012) and aligned reads

were counted using HTSeq (Pyl, Anders, and Huber 2014). Differential expression-analysis was performed using DESeq2 (Love, Huber, and Anders 2014) and log2 fold change shrinkage was estimated using apeglm (A. Zhu, Ibrahim, and Love 2019). Overrepresentation analysis (ORA) was performed using all genes that had an adjusted p-value of less than 0.05, gene set enrichment analysis (GSEA) was performed using the whole RNAseq data set and ranked according to log2 fold change and ties were resolved at random. Both ORA and GSEA were performed using the R package ClusterProfiler (Yu et al. 2012). Lipidomic data analysis was performed using the Bioconductor package LipidR (Mohamed A 2021) with the following caveats: Lipid concentrations were normalized to a normalization factor calculated by dividing each sample protein concentrations by the median of all sample protein concentrations. Concentrations were then multiplied by 1000 and log2 transformed for further analysis. All p-values in omics results were corrected using Benjamini-Hochberg correction. T-test and ANOVA comparisons between groups were made using R package ggpubr (stat_compare_means function). Volcano plots were made using the EnhancedVolcano R package, all pseudogenes, long non-coding RNAs and microRNAs as well as any ENSEMBL IDs that have no defined HGCN symbol were removed from the volcano plot analysis, due to the lack of database information on many of these RNAs and genes. Interactomics analysis was performed using the Crapome server for SAINT (Mellacheruvu et al., 2013).

# 5 RESULTS

## 5.1 Manipulations of ER MCS are well tolerated by HUVECs (II & III)

Inhibitors are a convenient way to study how the disruption of proteins at physiological levels affect cellular functions. In this thesis titration of inhibitors, as well as effects of shRNAs and lentiviral transfection were assessed by measuring the metabolic activity of manipulated HUVECs with MTT-assay. The DKD of VAPA and B (DKD VAPA/B) caused a very modest -20 % reduction in metabolic activity, even though a corresponding reduction of -50 % and - 67 % in protein levels of VAPA and B was seen (I, Figure 3). Measuring the metabolic activity of HUVECs at different concentrations of the inhibitors revealed that HUVECs tolerate high concentrations of OSW1 or SWG (II, Figure 1 B) and OSW1 reduces metabolic activity by 50% and SWG by 40% at 10 µM concentration. Inhibition of ORP7 with CpdG (CpdGORP7) demonstrated a similar pattern where a 50% reduction was seen only at 250 µM concentration (III, Figure 3 A). Our chosen concentration of 25 nM for OSW1 and SWG did not display a large reduction in metabolic activity as only 25% and 10% reductions were seen with OSW1 and SWG, respectively, whereas the 10 µM concentration chosen for CpdG did not show any alteration in metabolic activity.

*Inhibitors affect OSBP and ORP4 protein levels and localization (II & III)*

In study II the effects of inhibition of OSBP on its protein levels and intracellular localization were studied, Furthermore, off target effects of OSW1 and SWG on ORP4, a close homolog of OSBP, were investigated. Treatment of HUVECs with either OSW1 or SWG lead to the degradation of OSBP and ORP4 with both inhibitors resulting in an approximate 90% and 50 % reductions in both proteins, respectively. This coincided with an increase in transcript counts of both proteins (II, Figure 2). As OSBP has been demonstrated to disassociate from the TGN upon inhibition in other cell models (Péresse et al. 2020), the intracellular distribution of OSBP upon OSW1 and SWG inhibition was examined in HUVECs. OSW1 inhibition was shown to scatters OSBP and TGN46 from a perinuclear condensed localization much faster compared to SWG, OSW1 showed a clear peri-nuclear co-localisation of TGN46 and OSBP at 4h after inhibition, whereas OSBP demonstrated more scattered pattern. After a longer 24h timepoint inhibition OSW1 exhibited a marked lack of OSBP expression, corresponding to the aforementioned degradation, whereas SWG inhibition scattered the remaining OSBP and TGN46 to the cytosol (II, Figure 3 B and C). Since the inhibition of OSBP induced drastic reductions in its protein levels it was considered prudent to also investigate how the levels of ORP7 behave upon CpdG inhibition. No detectable differences were evident in the protein levels of ORP7 (III, Figure 15).

### 5.2 MCS dysfunction leads to altered regulation of diverse cellular functions (I, II & III)

The study designs in this thesis relied heavily on hypothesis free omics methods. Studies I and III used a similar workflow to each other, using effect size estimation with apeglm, but in study II a different log2 fold change shrinkage method was used, i.e. default DEseq2 method. As log2 fold change shrinkage can influence log2 fold change of genes with low transcript counts, a reanalysis of study II transcriptomics data using apeglm log2 fold change shrinkage estimation was warranted, to compare the GSEA results from each study in this thesis result section. To this end dot plots (Figures 5 and 6) compiling results from Wikipathways and Uniprot subcellular localisation GSEA results from each study were made, where each pathway was annotated to cellular function as described on top of each facet. It is important to note that comparing normalized enrichment scores (NES) is not necessarily a valid method. One can only remark whether or not the majority of genes in each gene set have been up- or downregulated, but not by how much or how many genes have been regulated using the differences in NES.

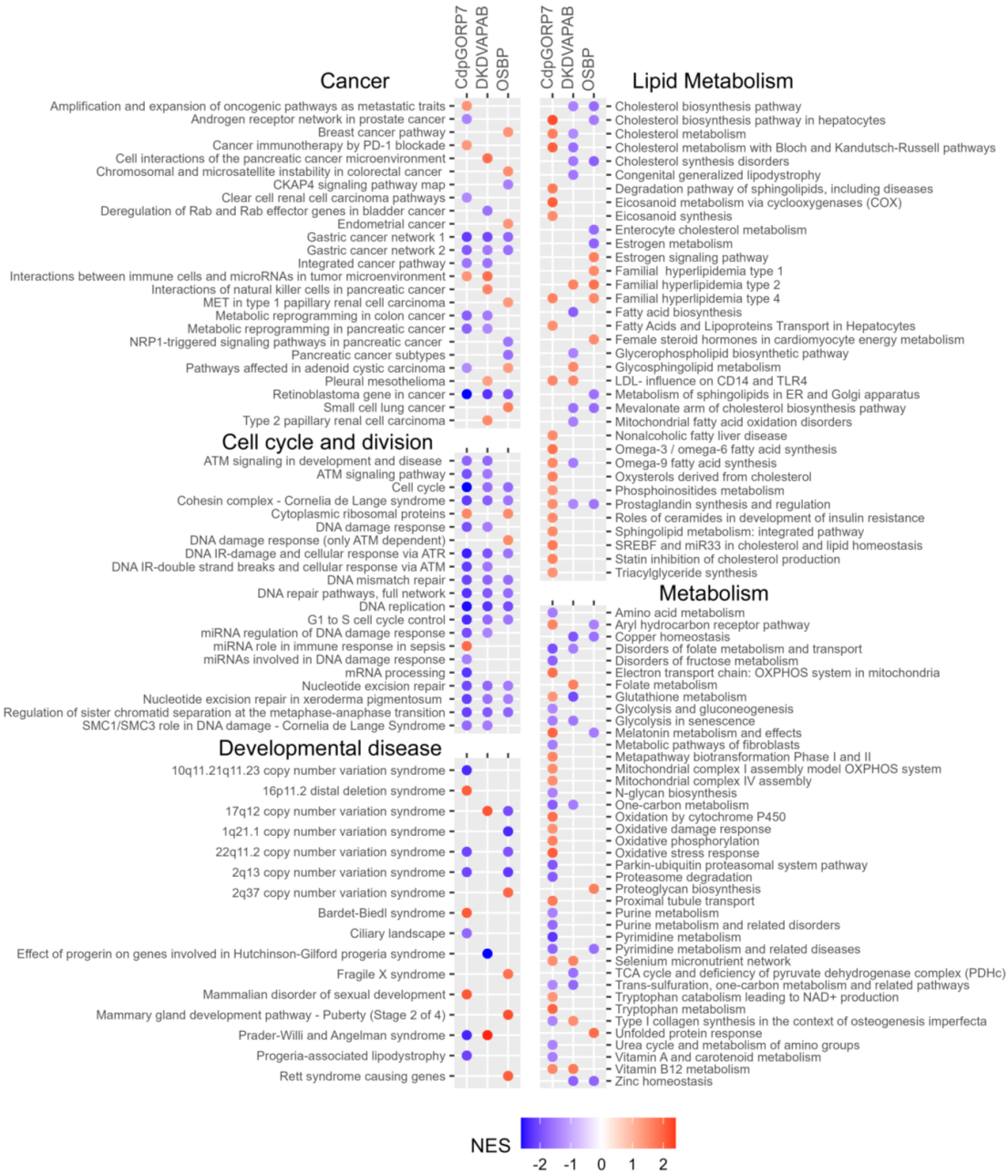


Figure 5 A dot plot compiling Wikipathways gene enrichment analysis (GSEA) results from each study. Each facet shows pathways annotated to a broader cellular function, such as cancer or lipid metabolism. X-axis exhibits each study and color of each dot the normalized enrichment score (NES) in that study. The Y-axis shows a pathway or a gene set. CpdGORP7 corresponds to a 24 h inhibition with CpdG, DKD VAPA/B the double knock down of VAPA and B and OSBP with 24 h inhibition with SWG

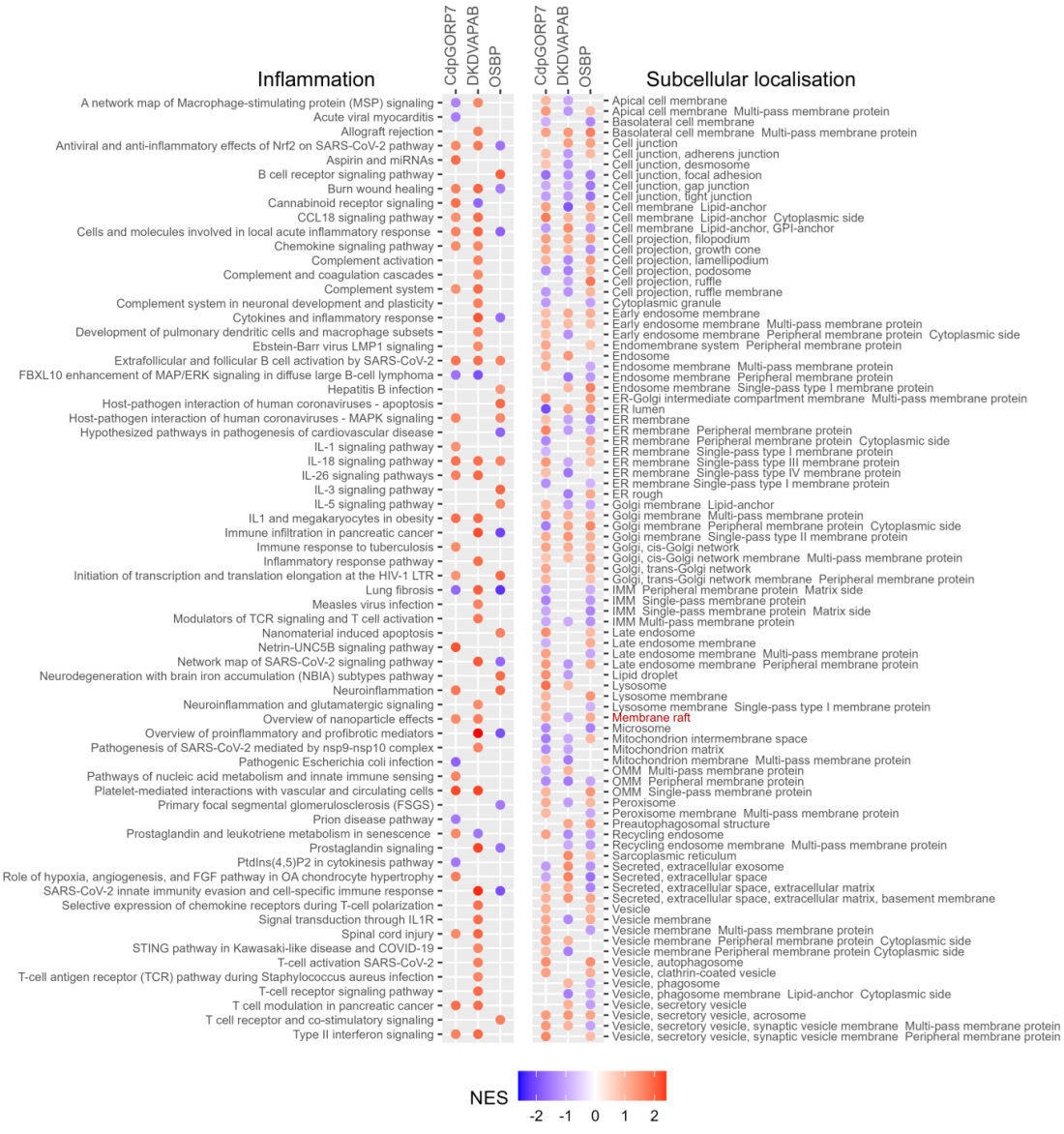


Figure 6 A dot plot compiling Wikipathways and Uniprot subcellular localization gene enrichment analysis (GSEA) results from each study. The left facet shows Wikipathways annotated to broader cellular functions of inflammation and the right facet a subset of all localization GSEA results. X-axis exhibits each study and color of each dot the normalized enrichment score (NES) in that study. The Y-axis shows a pathway or a gene set. Membrane raft gene set has been highlighted for convenience. CpdGORP7 corresponds to a 24 h inhibition with CpdG, DKD VAPAB the double knock down of VAPA and B and OSBP with 24 h inhibition with SWG

### 5.2.1 Selected commonly regulated cellular functions in ER MCS dysfunction

*Dysfunctional ER MCS led to downregulation in cell cycle and division genes (I, II & III)*

Downregulated gene sets in ORP7 inhibited (CpdGORP7) VAPA and B double knock down (DKD VAPA/B) and OSPB inhibited with SWG (OSBP), were related to cell division, cell cycle, DNA replication and nucleotide repair. This downregulation was not the result of the manipulation method used in the thesis, since the effect of was evident with both with inhibitor treatment and silencing used in studies I-III. Pathways included in the aforementioned functions are for example: *DNA repair pathways*, *Cell Cycle*, *DNA damage response*, that seem to be initiated through the *ATM/ATR pathways*, also shown to have negative NES.

*Inflammatory response is a common effect of ER MCS disturbance (I, II & III)*

A common effect of ER MCS dysfunction was activation of inflammatory signalling. These pathways included for example*, IL-(1,18,26) signalling pathways,* and *Neuroinflammation*. OSBP inhibited cells showed more variety in the regulation of inflammation related pathways as some sets had a negative NES, whereas ORP7 inhibition or DKD VAPA/B data sets had a positive NES such as, *Overview of proinflammatory and profibrotic mediators*. OSBP inhibition also exhibited independent positive NES in *IL-3 and -5 pathways*, and *B cell receptor signalling* pathway. ORP7 and DKD VAPA/B data sets displayed more consistent patterns of regulation as most inflammation related sets had a positive NES in at least one of the studies. Example gene sets include *CCL18 signalling pathway, Cytokines and inflammatory response, Cells and molecules involved in acute inflammatory response*.

*Genes localized to junctions, Golgi, inner mitochondrial membrane (IMM) and endosomes show common regulatory patterns (I, II & III)*

In Figure 6 the left facet displays different subcellular localisation gene sets according to the Uniport database. Commonly negative NES can be seen in location gene sets related to *cell junctions, Endosomes, Golgi, IMM, and lysosomes*. For example, Cell junctions such as, *Focal adhesion*, G*ap* and T*ight junctions* show negative NES in all studies, whereas *cis-Golgi network* related sets indicate positive NES.

### 5.2.2 Differential regulated cellular functions in ER MCS dysfunction

*Cholesterol metabolism shows differential regulation between studies (I, II & III)*

As displayed in Figure 5, DKD VAPA/B and OSBP inhibited cells presented a consistent pattern of negative NES in cholesterol metabolism related gene sets, whereas inhibition of ORP7 displayed

positive NES in cholesterol metabolism related sets. This pattern was evident in all gene sets that are related to cholesterol synthesis, and included sets, such as *Cholesterol biosynthesis pathway, Mevalonate arm of cholesterol biosynthesis pathway* and *Cholesterol synthesis disorders*.

*Inhibition of ORP7 leads to an increase in lipid metabolism (I, II & III)*

In Figure 5 different gene sets and their NES in each experiment related to lipid metabolism are presented. Remarkably, all gene sets annotated to this cellular function category had a positive NES in ORP7 inhibition and common sets between studies converged on *Familial hyperlipidemia* sets, where all studies indicated positive NES in at least one of the types. Notably, DKD VAPA/B and OSBP inhibition demonstrated decreases in most of the other sets annotated. A shared gene set between DKD VAPA/B and OSBP inhibition was *Prostaglandin synthesis and regulation*, whereas DKD VAP/B presented negative NES in *Fatty acid biosynthesis, Mitochondrial fatty acid oxidation disorders*, and *Omega-9 fatty acid synthesis*. OSBP inhibition on the other hand had a negative NES in *Metabolism of sphingolipids in ER and Golgi apparatus* as well as *Estrogen metabolism*.

*Diverse locations and membrane contact sites show differential regulation (I, II & III)*

Figure 6 left facet displays a variety of subcellular proteins that are differentially regulated between the studies. For example, *cell projection, ER, membrane raft, OMM (outer mitochondrial membrane)* and *mitochondria, peroxisomes, secreted* and *vesicle* related sets demonstrate differential regulation across studies. Further analysis was done on a novel database on MCS proteins, which was recently released (Pan et al. 2024), and these GSEA results have been compiled to Table 3.

Table 3. A table showing gene enrichment analysis results using membrane contact site protein database, where significant results have been marked with an asterisk and each column shows the normalized enrichment score in each study. CpdGORP7 corresponds to a 24 h inhibition with CpdG, DKD VAPA/B the double knock down of VAPA and B and OSBP with 24 h inhibition with SWG

| Description | ORP7 CpdG (III) | DKD VAPA/B (I) | OSBP SWG (II) |
|---|---|---|---|
| ER-Golgi | -0,8 | -1,2 | 1,4 |
| ER-Endosome | 0,9 | -1,2 | NA |
| ER-Lysosome | 1,1 | -1,8 * | NA |
| ER-Lipid droplet | 1,0 | -1,8 * | NA |
| ER-Peroxisome | -1,4 | -0,9 | NA |
| ER-PM | -1,2 | -1,2 | NA |

Although only a few MCS sets showed statistical significance, in DKD VAPA/B cells the ER-Lipid droplet and ER-Lysosome sets were statistically significant with the largest absolute NES as well. Other notably large NES can be seen in ORP7 and OSBP inhibited cells, in the ER-Peroxisome and

in ER-Golgi sets, respectively. Only the *ER-Golgi* set had values for all studies, it had a positive NES in OSBP inhibited cells and negative in other studies. MCS localized to small membrane organelles demonstrated a positive NES in ORP7 inhibited and a negative NES in DKD VAPA/B cells apart from the *ER-peroxisome* and *ER-PM contacts*, that had negative NES in both.

*Inhibition of ORP7 increases the expression of prostanoid synthesis genes (III)*

Prostanoids are lipid hormones that work as para- and autocrine signals for inflammation and are produced from membrane phospholipids. Hydrolysis of these phospholipids produces a lyso-species and a free fatty acid in the form of arachidonic acid (AA), that is in turn used as a substrate to produce prostanoids. Changes in ORP7 inhibited cells clearly upregulated the synthesis of prostanoids as the mRNA levels of key enzymes such as CYP1A1, SLOC2A1, and PTGS1/2 (Chan et al. 2002; Lu et al. 1996; Mesaros, Lee, and Blair 2010) increased significantly. This is also true at the gene set levels, as Wikipathways gene sets such as: *Eicosanoid metabolism via cyclooxygenases (COX)* and *Omega-3 / omega-6 fatty acid synthesis* had a positive NES (Figure 6, III). Omega-3 and -6 fatty acids are also used as precursors for eicosanoids such as EHA and DHA, that also mediate inflammation in a similar manner to prostaglandins produced by PTGS1/2, or its resolution (Wall et al. 2010).

*Inhibition of ORP7 showed activation of oxidative stress (III)*

Oxidative stress can have both beneficial and detrimental effects and thus, activation of oxidative stress can have multiple outcomes. In the transcriptomics data pathways such as *Oxidative damage response, Oxidative phosphorylation, Oxidative stress response* and pathways related to mitochondrial functions including *Mitochondrial complex I assembly model OXPHOS system*, or *Mitochondrial complex IV assembly*, demonstrated positive NES in ORP7 inhibited cells, whereas other treatments showed no enrichment.

*DKD of VAPA/B upregulates endosomal and vesicular genes (I)*

Vesicles and endosome play distinct roles in intracellular transport. COPI and II coated vesicles have been shown to mostly traffic between ER and the Golgi, whereas the endosomal compartment is mostly specialized in traffic between Golgi and the cellular periphery. Transcriptomics data on the DKD VAPA/B HUVECs indicated that the mRNA levels of numerous COPI and -II associated proteins had increased. The same data also demonstrated significant increases in genes, whose products localize to the ER, Golgi and Endosomal compartments such as lysosomes, late endosomes and preautophagosomes (I, Figure 10). Although mRNA levels can be an indicator of how protein synthesis will be regulated, they do not necessarily correlate with current levels of the protein they code, as the turnover of proteins can range from a few minutes to the lifetime of the cell. We investigated the protein levels of certain COPI and -II components shown to be upregulated based on transcriptomics (I, Table 2).

Although most of the COPI and -II components displayed no increases in their protein levels based on Western blotting (I, Figure S1), a decrease in YIPF5, a protein which recycles between ER and the Golgi, was clearly shown (I, Figure 12)

*OSBP inhibition leads to an upregulation in UPR (II)*

Stability of the ER is vital for cellular homeostasis, as it produces major macromolecules, such as lipids, as well as proteins for other cell compartments. UPR is one of the known mechanisms, by which cells deal with aberrations in ER functions and try to prevent damage to the cells from accumulating substrates or misfolded proteins. RNA sequencing data on the SWG inhibited HUVECs showed a marked overrepresentation in gene sets that are related to ER stress (II, Figure 10). Almost half of the top 30 overrepresented KEGG pathways could be linked to ER stress. This is also evident in Figure 5, where the Unfolded protein response had the largest positive NES of the Wikipathways gene sets shown in the metabolism function.

### 5.3 Cholesterol concentration is modulated from cholesteryl esters (I, II & III)

Cholesterol is a common lipid found in all intracellular membranes, but its concentration varies between organelles. Plasma membrane and the endosomal compartments harbour most of the intracellular FC, whereas the ER where FC is synthesized has only about 3 – 5 % of total cellular FC (van Meer, Voelker, and Feigenson 2008). Accumulation of FC at the ER has been shown to be toxic to cells and it can lead to a plethora of adverse outcomes, such as ER stress (Bashiri et al. 2016; Feng et al. 2003). The total intracellular cholesterol pool also includes stored cholesterol in lipid droplets in the form of CEs, that make up the core of these droplets along with TGs. Our studies uncovered that TC levels, i.e. the sum of FC and CEs, in HUVECs are kept at a constant when only one ER MCS is disturbed (II, Figure 7 & III, Figure 9), but when multiple ER and other MCS were disrupted simultaneously, HUVECs accumulate cholesterol in the form of FC (I, Figure 6). In either case HUVECs compensated for the change in TC or FC concentrations by modulating stored CEs. DKD VAPA/B led to a marked increase in intracellular levels of both TC and FC, that coincided with a decrease in CE levels but did not affect lipid droplet counts, suggesting no change in total TGs (I, Figures 6,7,8). OSBP inhibition led to a decrease in FC, but total cholesterol levels were kept constant by increasing CEs stored in lipid droplets as is evident from the concomitant increase in TGs and lipid droplets (II, Figures 5,7,9). Inhibition of ORP7 led to a decrease in CEs and a decrease in lipid droplet mean area but lipid droplet counts remained constant, which could be related to the increase in saturated and monounsaturated TGs (III, Figures 11 & 13). Interestingly, increases in 27-hydroxy cholesterol levels were seen in DKD VAPA/B and OSBP inhibited HUVECs (I, Figure 6. II, Figure 7), suggesting that ER cholesterol might be converted into oxysterols.

### 5.3.1 ER MCS disturbance differentially regulates cholesterol synthesis (I, II & III)

Cholesterol synthesis takes place at the ER by a chain of multiple reactions and enzymes. The rate limiting enzyme in cholesterol synthesis is HMGCR, but other vital enzymes, such DHCR24 are also important in this synthesis pathway. The timely synthesis and exit of cholesterol from the ER are vital to avoid the toxic effects of cholesterol, which can lead to ER stress and other detrimental effects. Data in this thesis revealed that cholesterol biosynthesis was affected by the manipulation of ER MCS, especially OSBP and VAPA/B, whose manipulations displayed marked decreases in almost all steroid biosynthesis genes, as well as the enzyme SOAT1 that esterifies cholesterol to CEs (Qian et al. 2020). Commonly downregulated genes between all transcriptomic datasets included *EBP* and *DHCR24*, which act at the last steps in the synthesis of cholesterol. EBP converts Delta8-sterols to their corresponding Delta7-isomers that are subsequently transformed into cholesterol by SC5D, DHCR7 and finally by DHCR24 (Shi et al. 2022). SOAT1 showed downregulation in all but ORP7 inhibited cells, where a slight increase was present. Log2 fold change and adjusted p-values for the aforementioned genes, as well as those mentioned in the next subsection, have been added to Table 4.

Table 4. Table of metrics for genes related to cholesterol synthesis and efflux in all three studies. Increases compared to control are highlighted in red, reductions in blue.

| SYMBOL | VAPAB log2FC (I) | VAPAB padj | OSBP Log2FC (II) | OSBP padj | ORP7 Log2FC (III) | ORP7 padj |
|---|---|---|---|---|---|---|
| ABCA1 | 1,31 | 4,11E-34 | -0,46 | 1,80E-01 | 0,16 | 5,13E-03 |
| ABCG1 | -0,23 | 5,21E-04 | 2,55 | 2,05E-37 | 1,17 | 8,64E-59 |
| DHCR24 | -1,12 | 6,39E-62 | -1,34 | 6,97E-08 | -0,18 | 1,11E-05 |
| EBP | -0,34 | 2,24E-06 | -1,03 | 1,47E-07 | 0,11 | 2,87E-01 |
| HMGCR | -0,03 | 6,94E-01 | -0,37 | 2,48E-03 | -0,03 | 6,64E-01 |
| SC5D | 0,04 | 6,23E-01 | -0,79 | 3,78E-03 | 0,14 | 2,58E-02 |
| SOAT1 | -0,48 | 3,25E-13 | -0,54 | 3,88E-03 | 0,06 | 4,15E-01 |

The results discussed so far and some of those to come, have been compiled to Figure 7 shown below.

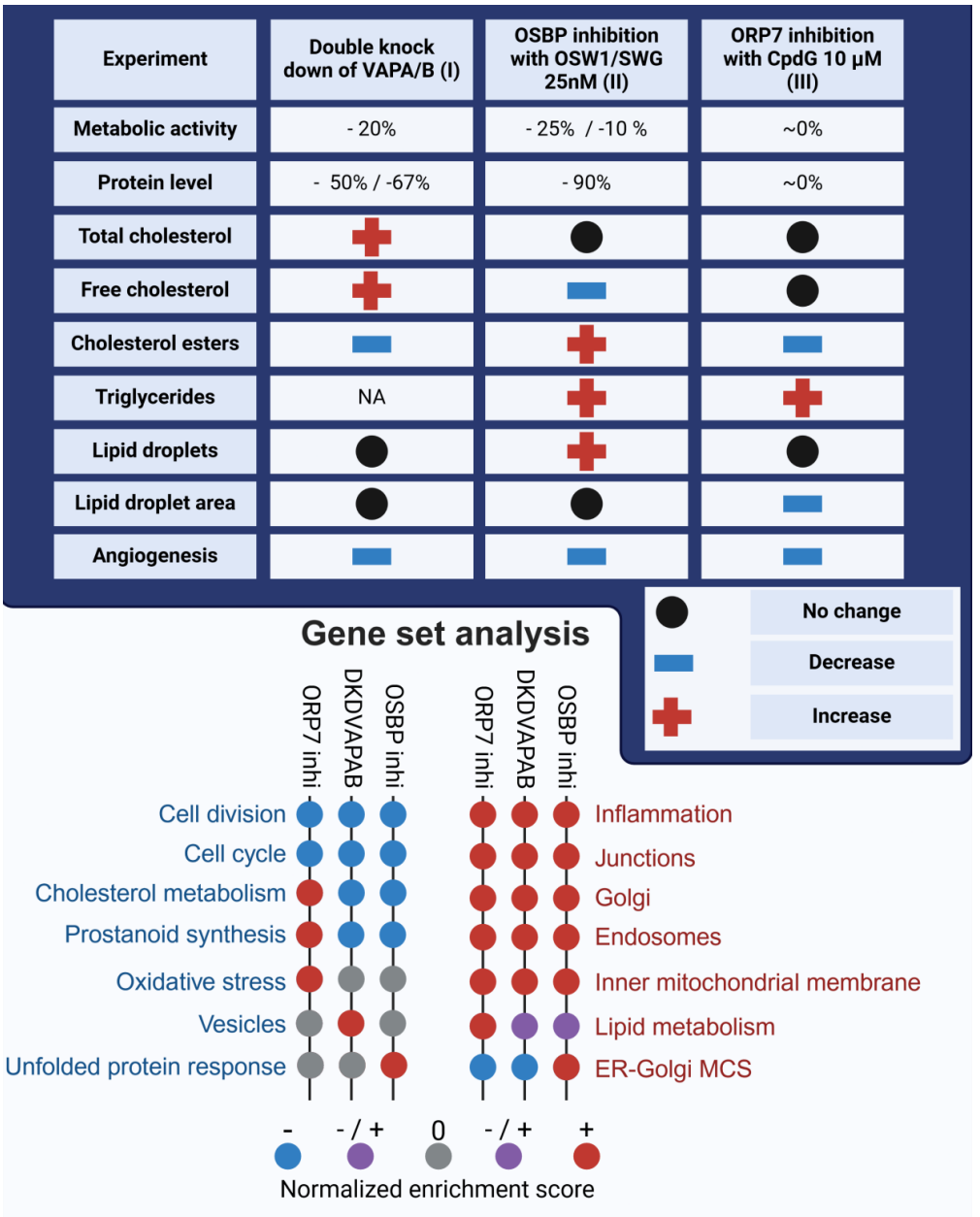


Figure 7 Image compiling the results from sections *5.1. Manipulations of ER MCS are well tolerated by HUVECS*, *5.2. MCS dysfunction leads to altered regulation of diverse cellular functions* to *5.3. Cholesterol concentration is modulated from cholesteryl esters* and *5.7. Angiogenesis is reduced by the manipulations of ER MCS*.. The table above shows the metric for each metric on the left most column of the table, whereas the legend for the table has been added to its right-hand bottom corner. Below the table is a simplified plot similar to Figures 5 and 6, the legend for it has been added below it. Blue symbolizing a negative normalized enrichment score (NES), purple, a mixed NES between gene sets, gray no NES available and red a positive NES.

### 5.4 ORP7 disruption leads to a decrease in cholesterol efflux (III)

Reverse transport is one way for cells to remove excess cholesterol. The cholesterol transporting ABC proteins move cholesterol to different types of lipoproteins. ABCA1-mediated cholesterol efflux moves cholesterol to ApoA1 whereas ABCG1 facilitates the movement of cholesterol to HDL particles. Of these two, ABCG1 is more highly expressed in ECs and is suspected to be the main route of cholesterol efflux (D. Li et al. 2010; Kennedy et al. 2005; Münch et al. 2014a). Since the inhibition of ORP7 was shown to increase ABCA1 dependent cholesterol efflux in THP1 cells (Wright et al. 2021), study III set out to confirm this result in ECs by investigating how ORP7 inhibition affects ABCA1 and -G1 dependent cholesterol efflux. A known LXR agonist T091317 (Dai et al. 2008) was used to induce ABCA1 mediated cholesterol efflux as a positive control for ApoA1 acceptor experiments. Study III uncovered that inhibition of ORP7 does not change ABCA1 mediated cholesterol efflux to ApoA1 but decreases ABCG1 dependent cholesterol efflux to HDL (III, Figure 10). This decrease also coincides with an increase in ABCG1 mRNA levels, whereas ABCA1 showed a more muted increase (III, Table 3). ABC proteins showed differential expression in other manipulations as well. In DKD VAPA/B cells the mRNA levels of ABCA1 had increased and ABCG1 decreased, whereas the OSBP inhibited cells showed increases in ABCG1 and a slight decrease in ABCA1. These results indicate that cholesterol efflux is affected by the manipulation of ER MCS.

### 5.5 Contact sites affect lipid homeostasis in a differential manner (I, II & III)

Lipid traffic is a major function of multiple ER MCS, and how their dysfunction affects intracellular lipid pools has been left with little attention by researchers. Membrane lipid homeostasis is vital for cells, and any disturbance can lead to complex outcomes. Especially in ECs the lipid composition of the plasma membrane is not only vital for angiogenesis but also for endothelial barrier functions. Therefore, we investigated with lipidomics how the manipulation of ER MCS affects the cellular lipid pools. Disrupting multiple ER MCS through VAPA/B DKD did not show significant changes in many lipid classes expect for the aforementioned decrease in CEs and an increase in PEs (I, Figure 8), whereas inhibition of OSBP led to a decrease in almost all lipid classes except for CEs and Cers (II, Figure 3). The inhibition of ORP7 led to an increase in Cers, LPCs and TGs as well as a decrease in CEs (III, Figure 11). At individual lipid species level, the DKD VAPA/B led to a decrease in very long chain highly unsaturated and saturated lipid species, where as an increase in relatively shorter chain unsaturated species were evident (I, Figure 9). Since the inhibition of OSBP showed such drastic reductions in all lipid levels, a clear patter did not emerge except in CEs where an increase in saturated, monounsaturated, and di-unsaturated species was seen and a decrease in longer tri- or more unsaturated species was observed, alongside with the reduction of all lipid species in other lipid classes (II) as also shown in Figure 8. Inhibition of ORP7 displayed increases in saturated and monounsaturated TGs and almost all LPCs and Cers. Decreases were shown in PCs 34:3 and 32:3 and shorter, highly unsaturated TGs such as 48:3 and 50:4. The rest of the changes in individual lipids were not

statistically significant (III, Figure 12). All the results from each study have been assembled to Figure 8, and notably there was a nomenclature change in SMs between studies I and III, which is reflected in Figure 8. For example, SM 16:0 corresponds to 34:1 species in the new Lipid Maps nomenclature. Cers were also represented in a differential manner between studies I and III, as in study III the total chain length was calculated for each species, i.e. study III Cer 32:1 corresponds to study I & II Cer d16:1/16:0, and this change was also considered when Figure 8 was compiled.

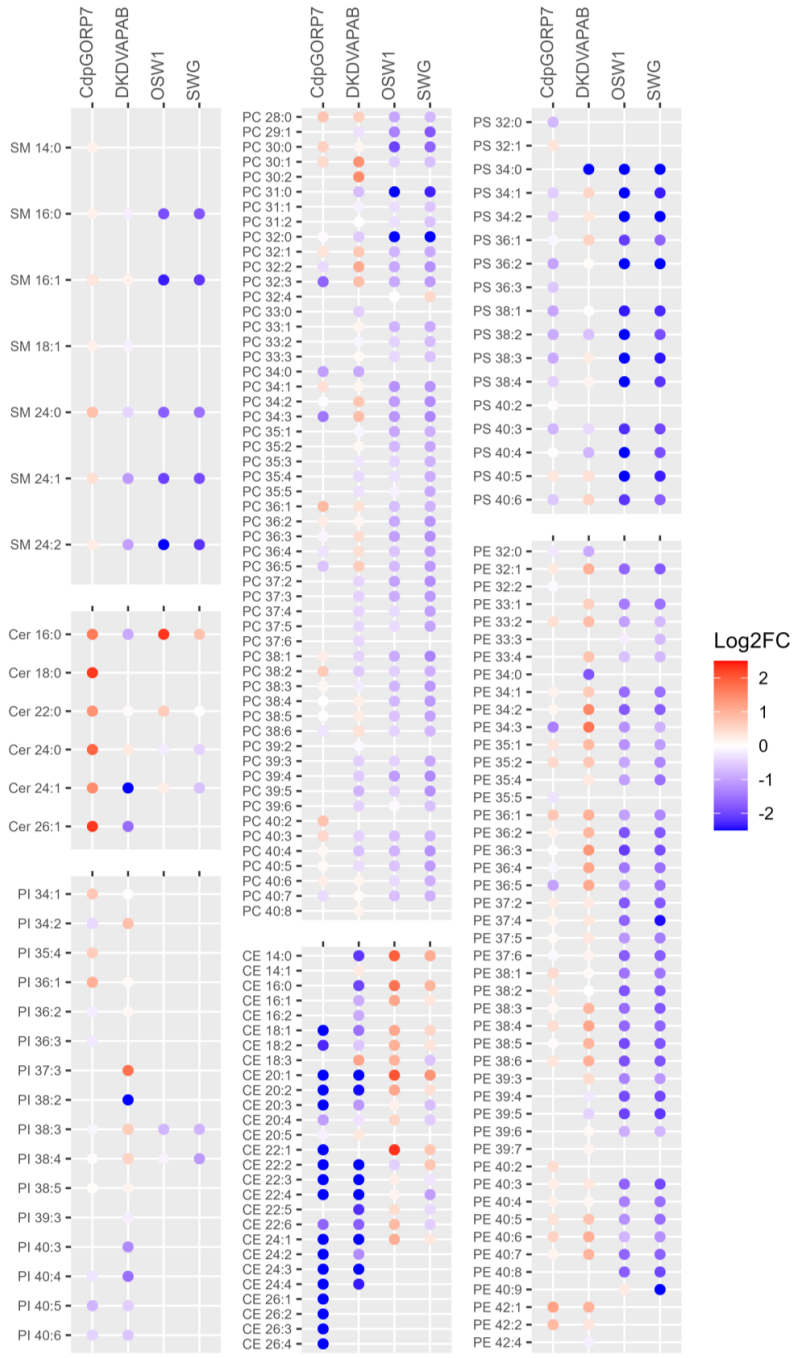


Figure 8 Dot plot exhibiting log2 fold changes of lipid species in each study. The Y-axis represents a lipid species, and each facet is a different lipid class. X-axis shows the log2 fold change of each lipid species as a colored dot in each experiment. A red colored dot represents an increase, and blue a decrease. Of note, Log2 fold change was capped at an absolute change of 2.5, to keep smaller changes still easily visible, which means that absolute fold changes larger than 2.5 were set to plus or minus 2.5.

From Figure 8 we can see that SMs have decreased in all manipulations except for the ORP7 inhibited cells, where almost all species show an increase. A similar pattern is also seen in Cers where ORP7 inhibition led to an increase in all species, whereas OSBP inhibition showed increases and DKD VAPA/B cells a decrease in 16:0 species. The relative lipid compositions of different lipid classes from each study are shown in Table 5.

Table 5. Relative lipid compositions of the manipulated HUVECs in each study and sample type, in percentages. Increases compared to control are highlighted in red, reductions in blue.

| Lipid class | SWG | OSW1 | DMSO | DKD VAPA/B | shNT | CpdGORP7 | DMSO |
|---|---|---|---|---|---|---|---|
| **PC** | 47,76 | 44,41 | 49,40 | 53,03 | 53,38 | 68,82 | 63,66 |
| **PE** | 16,50 | 14,98 | 23,11 | 28,63 | 18,68 | 15,92 | 14,51 |
| **PS** | 3,81 | 2,21 | 6,87 | 6,75 | 6,40 | 4,23 | 6,31 |
| **SM** | 6,28 | 4,85 | 10,98 | 9,66 | 16,02 | 9,96 | 9,20 |
| **CE** | 21,38 | 28,63 | 7,37 | 1,49 | 4,80 | 0,46 | 5,94 |
| **Cer** | 4,27 | 4,90 | 2,25 | 0,46 | 0,71 | 0,60 | 0,38 |

### 5.6 Inhibition of OSBP leads to a decrease in PS but not PE synthesis (II)

The lipidomics results for OSBP inhibition demonstrated decreases in all major glycerophospholipid classes, such as PC, PE, and PS, where the largest decrease was seen in PS corresponding to a mean log2 fold change of about 2.2. As glycerophospholipids are an essential part of all membranes, we suspected that a corresponding increase in PS or PE synthesis would be warranted to restore homeostasis. Our results indicated that PE synthesis did not change in either OSW1 or SWG treated cells, but a decrease in PS synthesis was evident contrary to our initial hypothesis of increased PS synthesis (Figure 8. II).

### 5.7 Angiogenesis is reduced by the manipulations of ER MCS (I, II & III)

Knowledge on how the manipulation of ER MCS affect angiogenesis is sparse, as very few studies have examined the role of MCS in ECs and their role angiogenesis. An angiogenesis inducing matrix was used to examine how the manipulations of ER MCS affect angiogenesis *in vitro*. This functional study was performed using all manipulations i.e. both VAPA/B silenced (I) and OSBP/ORP7 inhibitor treated (II & III) HUVECs. OSBP inhibition and DKD VAPA/B drastically reduced angiogenesis, as junction counts reduced to two digit counts with these manipulations. ORP7 inhibition depicted a smaller effect on angiogenesis as mean junction counts dropped by half compared to the DMSO control, and similar effect was uncovered on other angiogenic metrics such as branching and segment length. Our data also indicated that manipulation of multiple ER MCS (I) reduces angiogenesis more

than individual MCS as VAPA/B silenced and OSW1 treated cells (II) displayed a larger decrease compared to the more specific inhibitor of OSBP (SWG, II) and ORP7 (CpdG, III), that had similar reductions in junction counts.

### 5.8 ORP7 interaction with extracellular matrix components is mediated by AKT1 (III)

Since the interactome of ORP7 along with its other functions are poorly understood, proximity biotin labeling interactomics was performed with an oexORP7-mycBirA construct to uncover the ORP7 interactome in HUVECs. Our results revealed that ORP7 interacts with extracellular matrix components such as Collagens, Fibronectin, Perlecan and Multimerin as well as AKT1 (III, Table 4). We suspected that this interaction with the ECM components is not a direct interaction with ORP7, but is instead facilitated by AKT1, as ORP7 has not been shown to be secreted outside the cell where the ECM is located. We confirmed the AKT1 interaction with a conventional Co-immunoprecipitation assay, showing that AKT1 binds with ORP7 in both wild type and oexORP7-mycBirA expressing cells and showed no binding with a negative control (III, Figure 14) thus implicating that ORP7 might also have a role in regulating the cytoskeletal components.

## 6 DISCUSSION

This thesis used high dimensional omics data to examine how the manipulation of ER MCS affects the macromolecule pools and cellular functions of HUVECs. The studies demonstrated that each manipulation was well tolerated by HUVECs and there were no major reductions in metabolic activity, even though DKD VAPA/B and OSBP inhibition reduced their respective protein levels significantly. The transcriptomics data revealed that commonly regulated gene sets clustered to functions, such as inflammation, cell cycle and division. More diverse regulation was evident in lipid metabolism, including sterol and oxysterol synthesis. Other differences included the upregulation of endosomal and vesicular compartment genes in DKD VAPA/B HUVECs, upregulation of UPR in OSBP inhibited cells as well as lipid synthesis and oxidative stress in ORP7 inhibited cells.

### 6.1 Endothelial cells modulate cholesterol concentrations from lipid droplets

Cholesterol concentrations exhibited differential modulation between manipulation as increases in TC levels were seen in DKD VAPA/B HUVECs, but TC remained constant when inhibitors were used. FC concentrations were modulated from stored CEs in lipid droplets, the counts and mean areas of which changed in conjunction to TGs and CEs. Differential regulation of the ABC-proteins, shown to be vital for cholesterol efflux from the PM (Münch et al. 2014b; 2014a; Kennedy et al. 2005; Wright et al. 2021), suggested that manipulations used in this thesis result in differential efflux routes.

In DKD VAPA/B cells an increase in FC and a decrease in CEs were shown. This would suggest that cholesterol is trapped at the ER since cholesterol biosynthesis was downregulated. Cells also apparently modulated FC levels from CEs, hence the increased ER FC was not available for cells. Decrease in ABCA1-mediated cholesterol efflux may also have been taken place, as ABCA1 mRNA level was increased in DKD VAPA/B cells. Therefore, DKD VAPA/B cells not only show decreased cholesterol transport from the ER to other membranes but also a decreased CE transport from the ER to lipid droplets. The aforementioned increases cholesterol levels are in line with previously published reports, but our indirect measurements of lipid droplet amounts, and mean area in DKD VAPA/B cells (I) suggested no change in TGs, which conflicts with previous studies demonstrating that the VAPB P56S mutation increase TGs levels (Marques et al. 2006).

Data from study III would suggest that FC could be accumulating in membrane domains where it is inaccessible for other cellular functions. This is evident by the reduced ABCG1 cholesterol efflux as a possible increase in PM FC levels would rather suggest an increase in efflux (Juhl and Wüstner 2022). Other data pointing towards this conclusion is the increase in LPC, ceramides and FC that make up a significant portion of lipids in lipid rafts and raft like domains of MAM, and therefore could be accommodating an increase in FC-rich MAM raft-like domains (Grassi et al. 2020; Van Der Luit et al. 2002). The reduction of lipid droplet mean area and CEs in ORP7 inhibited cells, could be an indicator for a decrease in ER FC transport and thus the cells could be scavenging cholesterol to other membranes from lipid droplet CE stores. The unchanged ABCA1 efflux as well as TC levels had not change significantly, which are contrary results to the literature showing that ORP7 inhibition increases ABCA1 mediated efflux (Wright et al. 2021) and would suggest that ORP7 might not have a direct role in hypercholesterolemia (Abdul Murad et al. 2023).

Data suggesting an ER accumulation and reduced transport of FC in OSBP inhibited cells included the increase in CEs and lipid droplets. These data could imply that OSBP inhibited cells are removing excess FC from the ER to lipid droplets instead of TGN, which was proven to be scattered to the cytosol. Transcriptomics data suggested a reduction in ABCG1 efflux in OSBP inhibited cells as *ABGC1* was shown to be highly upregulated similar to study III. Increases in LDs and CEs as well as the decrease in cholesterol biosynthesis and increase in ER-stress related genes are in-line with data shown in previous studies (Oh-hashi et al. 2023; Mesmin et al. 2017).

### 6.2 Lipid homeostasis regulates membrane viscosity

Overall lipid homeostasis was also disrupted due to the manipulations used, and largest changes were seen in OSBP-inhibited cells where the concentrations of all lipids, apart from CEs and Cer were reduced. DKD VAPA/B cells demonstrated less changes in lipid classes but at lipid species level, increases in very-long-chain and saturated species were observed, whereas the intermediates between them decreased. ORP7 inhibited cells exhibited increases in saturated and mono-unsaturated

species whereas a decrease was detected in highly unsaturated species and intermediates between them showed very small or no changes at all.

As is evident in Figure 8, not all lipid species were present in each lipidomic experiment included in this thesis. There could be multiple reasons, of which variations in extraction procedure could be a possible explanatory factor. Even though the starting material for each experiment was the same, technical variation in extraction is possible, which could have led to the reduction in concentration of some lipid species, and therefore they could have been under the detection limit of the lipidomics platform. The lipid composition of growth media is obviously another variable that can affect the concentration of lipids in the final samples and, thus, which lipids can be detected.

OSBP-inhibited cells showed such drastic reduction in all lipid species that it is difficult to ascertain what types of modulations were taking place. What is very likely is that OSBP inhibited cells were reducing their membranes across all membrane compartments. One cause of these reductions might be the disassembly of the TGN, which is common in stress (Machamer 2015; J. Chen and Chen 2018). The relative lipid compositions in the inhibitor-treated and transfected cells are shown in Table 5, and interestingly, a larger decrease in PC, PE and PS was seen in the OSW1 treated HUVECs. OSW1 treated HUVECs also showed a larger degree of TGN scattering, which would suggest that the increased reductions in PC, PE and PS could indeed be due to a higher degree of TGN disassembly. The lipid class wide drop in PC, PE and PS is likely also due to increased ER FC levels, as reduction of these lipid classes would potentially make the ER membrane more hospitable for FC, similarly to an increase in ceramides (Garofalo et al. 2016). Similar reductions in PC, PE and PS can also be seen in ER stress induced yeasts, but it is questionable how well these results translate to mammals (Reinhard et al. 2020). Both OSW1 and SWG treated cells indicated a reduced PE to PC ratio (Table 5), which is puzzling as increased PE to PC ratios have been associated with decrease in membrane viscosity (Dawaliby et al. 2016). However, it cannot be ruled out whether ER membrane lipid ratios have decreased due to ER stress or TGN disassembly, as ER and Golgi have been shown to have somewhat similar lipid composition (Vance, 2015, Table 2). DKD VAPA/B HUVECs seem to be modulating membrane fluidity by removing saturated or longer more highly unsaturated species and replacing them with intermediates between these species. This could also represent a response to increased FC levels at the ER, especially the decrease in longer more unsaturated PCs species and the increase in almost all PE species. This would suggest an increased PE to PC ratio that might be used to offset the viscosity gain from excess FC similar to OSBP inhibited cells.

Lipidome changes in ORP7 inhibited HUVECs also suggest that membranes are being turned into a less fluid form, i.e. more saturated and monounsaturated species are being added to membranes whereas highly unsaturated species are being removed from them. Moreover, especially the increases in Cers would suggest that membrane composition has turned to a more rigid form. Where these lipids are being added or removed from remains a mystery, but some speculations can be made. Lipid rafts and raft-like domains harbour considerable amounts of both FC and Cers, which would offer an

explanation to the increase in Cers across all species (Fernandes et al. 2024; Garofalo et al. 2016). In study III an increase in all lysoPC species was demonstrated, which would also hint to an accumulation or rather the expansion of lipid rafts and raft-like domains, as lysoPCs can make the membrane environment more stable for increased Cers and FC (Van Der Luit et al. 2002). Raft accumulation of FC would make it inaccessible to other cellular functions and therefore lead to the scavenging of FC from lipid droplet CE stores.

### 6.3 Common regulatory patterns hint at an anti-proliferative mechanism

The results in section 5.2.1 exhibited clustering of downregulated genes in functions that are involved in cell cycle and division. This is likely due to induction of intracellular stress responses as, for example, ER-stress is known to cause G2/M cell cycle arrest, which would cause a decrease in cell cycle and cell division related genes (D. Lee et al. 2019a; 2019b). Activation of oxidative stress and subsequent release of cytochrome C is quite possible in ORP7 inhibited cell. Omics data from study III demonstrated a clear pattern of upregulation in oxidative stress pathways as well as an increase in C-18 ceramide, which has been shown be a crucial initiator of apoptosis and is needed for mitophagy (Sentelle et al. 2012; W. Jiang and Ogretmen 2013).

In OSBP inhibited cells the downregulation in cell division related sets could be the result of increased ER stress, which was also evident from the omics data from study II. Inhibition of OSBP likely induced ER stress by accumulation of cholesterol to the ER, since we saw the dispersion of TGN to the cytosol, which might reduce cholesterol uptake by the Golgi through OSBP mediated transport. The possible markers of excess ER cholesterol in study II were postulated to be the downregulation of cholesterol synthesis pathways and genes, as well as the increase in lipid droplet stored CEs. ORA results in study II also exhibited overrepresentation in ER stress related gene sets such as those related to neurodegeneration. Figure 3 also displayed an increase in UPR related genes in the OSBP inhibited HUVECs.

Study I demonstrated increases in ER/Golgi localized genes, which would suggest that DKD VAPA/B cells might also be suffering from ER stress. ER localized gene such as those coding for chaperone and proteasomal genes could be used to alleviate excess mis- and unfolded proteins, and therefore an increase in ER localized genes are visible. Lipidomics in study I also displayed increases in shorter and less saturated lipid species, which would suggest that DKD VAPA/B HUVECs are compensating for the increase in ER cholesterol by producing ER membranes that are less fluid and therefore more compatible with excess cholesterol.

### 6.4 Inflammation is a ubiquitous response to dysfunctional ER MCS

Manipulations of ER MCS showed clustering of upregulated genes in multiple different inflammatory functions, of which Interleukin signalling pathways showed the most consistent regulatory pattern. Considering that MCS are involved lipid trafficking, it is likely that the upregulation in inflammatory genes is the result of increase in inflammatory lipids, and the unfavourable lipid composition of membrane compartments. This is quite evident from the individual lipid species data, for example in ORP7 inhibition a considerable increase was seen in TGs, Cers and LPCs that can have inflammatory outcomes (Meade et al. 2023; Field, Gordillo, and Scherer 2020; Takaeko et al. 2021; Kajikawa et al. 2016; Law et al. 2019). Accumulation of ER cholesterol can also be another trigger for inflammation (Bashiri et al. 2016). Since the manipulated cells have likely increased ER or oxidative stress, it is difficult to ascertain whether or not the manipulation of ER MCS independently results in inflammation in ECs, i.e. if OSBP, VAPA, VAPB or ORP7 are directly involved in the inflammatory process. Most likely the increase in inflammation is the result of second order effects that are downstream from ER or oxidative stress pathways.

### 6.5 Angiogenesis requires functional ER MCS in ECs

Angiogenesis was reduced across all manipulations, of which OSBP inhibition showed the largest effect, while DKD VAPA/B and inhibition of ORP7 presented similar reductions in angiogenic metrics. This ubiquitous reduction in angiogenesis can be the result of multiple issues stemming from dysfunctional ER contact sites. The most likely explanation is the drastic reduction in proliferative potential of HUVECs as is evident from the reduction in cell cycle and division genes. Intracellular stress, be it ER, oxidative or lipid stress, can affect angiogenesis by reducing the potential for protein synthesis inducing more rigid PM membrane reducing expansion potential or mitochondrial dysfunction (T. Wu et al. 2023; Zechariah et al. 2013; J. Zhang et al. 2017; De La Haba et al. 2013). MCS involvement in shuttling ER synthesized lipid to the PM is also another likely explanation. This could be especially the case for DKD VAPA/B cells, since VAPA and VAPB form such a wide range of MCS. The defect of ER-PM MCS would reduce the traffic of cholesterol and GPLs to the PM, thus reducing the capacity for membrane expansion during the formation of lamelli- and filopodia protrusions. Another means by which DKD VAPA/B cells could have reduced lipid traffic from the ER to PM is a reduction of COPI and -II mediated transport, the compensatory upregulation in vesicle and COP related genes. Reduced vesicle budding from the ER to the Golgi could reduce the number of GPLs that are further added to the secretory and endosomal pathways and, thus, reduce the amount of GPLs that are trafficked to the PM. In a similar manner, the dysfunctional Golgi, i.e., scattering of TGN to the cytosol upon OSBP inhibition could lead to reduced GPL transport to the PM. Why ORP7 inhibition would reduce angiogenesis is somewhat puzzling. The inhibition of ORP7 could lead to the accumulation of FC at raft-like domains, which would make it inaccessible for other cellular functions.

The interaction between AKT1 and ORP7 was a novel finding but by no means unique in the ORP family, as also ORP2 and ORP9 have been demonstrated to bind to AKT1 (Lessmann et al. 2006; Ngo, Huber, and Ridgway 2006; Kentala, Koponen, Vihinen, et al. 2018). ORP2 in conjunction with AKT1 has been shown to regulate the actin cytoskeleton (Kentala, Koponen, Kivelä, et al. 2018). Whether or not ORP7 interacts with the ECM through AKT1 remains to be elucidated, but ORP7 could have similar effects to actin cytoskeleton as ORP7. Thus AKT1-ORP7 interaction could impact angiogenesis through stress fiber dynamics or through reduced focal adhesions binding to ECM. Although not measured in any of the studies included in this thesis, there is also a possibility that ER MCS dysfunction could influence EC permeability, as the subcellular localisation gene sets in Figure 6 showed a ubiquitous decrease in both gap and tight junction genes.

### 6.6 Limitations of single time point measurements in dynamic systems

Cells are by nature dynamic systems, where changes in, for example the transcriptome, result in changes in the proteome in a delayed manner. Transcript-level changes often occur at shorter time scales than protein level changes, ranging from a few minutes to the extreme of a few hours (C. Y. A. Chen, Ezzeddine, and Shyu 2008). Whereas changes at protein levels can take much longer as the halving time of proteins can range from the low of mere minutes to the mean of about 40 minutes all the way to the lifetime of a cell (Fornasiero and Savas 2023). Thus, transcriptomic data can reflect changes that are about to occur and not just those that are currently ensuing. This fact represents a problem of single time point measurements, as it can miss the dynamic mechanisms that are occurring as a response to any stimuli. Therefore, when interpreting the results of single time point transcriptomics results one must consider the possibility that the system might be still changing or has reached an equilibrium point where cells oscillate between two or more states. Since ER, lipid and oxidative stress are often intermediate step between recovery or apoptosis and necrosis, they can become catch of all terms that may be used as an explanation for all cellular dysfunctions. For example, one might conclude from the result shown that inhibition of OSBP causes ER stress, but the mechanism of ER stress initiation is left unanswered. Given that all manipulations used in this thesis employed relatively long incubation periods, namely at least 24 hours, the manipulated cells might have reached a point where intracellular stressors are the dominant feature at the time of mRNA extraction. In other words, cells might have reached an equilibrium where, for example, OSBP inhibition causes chronic ER stress and thus changes in ER stress related genes are the dominant result in transcriptomic data. Therefore, the direct changes caused by OSBP inhibition could be “drowned out” by the dominant changes due to ER stress. This problem is further exacerbated by GSEA, which gives more emphasis to pathways that have genes with large log2 fold changes, i.e. bigger NES values, and can obfuscate pathways with genes that have smaller log2 fold changes, in other words smaller NES values. Large log2 fold changes are of course very meaningful in biological sense, but so too are smaller log2 fold changes, as for example, small increases in the protein levels of enzymes can have large downstream effects.

In a similar manner small changes in MCS lipid transport proteins can have large downstream effects in the amount of lipid exchanged.

## 7 CONCLUSIONS

This thesis represents a first of its kind to characterize ER MCS in ECs to use transcriptomics and lipidomics in VAPA/B, OSBP or ORP7 manipulated HUVECs to this extent. This thesis also describes the first interactome on ORP7. These studies demonstrated that MCS could be vital for the transport of ER synthesised cholesterol and reductions in MCS proteins seem to induce ER and oxidative stress in HUVECs. ER MCS also have an integral role in modulating the levels of FC from lipid droplet stored CEs, and reduction in ER MCS could also hamper CE production from ER FC. MCS dysfunction was also linked to an increase in inflammatory signalling in ECs that was speculated to be a result of either cellular stress responses or an unfavourable lipid membrane composition due to defects in FC and GLP transport. Angiogenesis was also exhibited to be affected by the dysfunction of ER MCS as all studies demonstrated marked decreases in angiogenic processes that were hypothesized to be the result of ER and other stressors. These stressors reduced the proliferative capacity of HUVECs, increased inflammation and likely resulted in a disturbance of PM lipids for proper membrane expansion.

Even though this thesis extensively exhibited the changes in the macromolecule pools of HUVECs with multiple or single ER MCS dysfunction, numerous questions are still left unanswered. For example, how does the dysfunction of concurrent ER MCS and OSBP- or ORP7-facilitated MCS affect the cholesterol distribution in membrane compartments? Or which lipids ORP7 traffics, although cholesterol seems to be a likely candidate, and between which membranes? It remains also unknown whether these results translate to other EC types, given their heterogeneity and adaptations to specific microenvironments. The works included in this thesis serve as the foundation for further studies of MCS in ECs and hopefully the extensive omics data collected can be used make further hypotheses, help design robust and comprehensive experiments in this cell type. The results presented in this thesis give indications to the involvement of MCS dysfunction in cardiovascular diseases, metabolic disorders, and integrated stress related diseases such as neurodegeneration.

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