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SPHINGOLIPID METABOLISM IN LUNG DISEASES

Ph.D thesis

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LIST OF ABBREVIATIONS

3-KDHR – 3-ketodihydrosphingosine reductase

AASM - American Academy of Sleep Medicine

ABC transporter – ATP-binding cassette transporter

AHI – apnea-hypopnea index

ALK – anaplastic lymphoma kinase

AP-1 – activator protein-1

$ApoM^{+}$- HDL - high-density lipoprotein bound apolipoprotein M

AUC – area under the curve

Bak – Bcl-2 homologous antagonist/killer

Bax – Bcl-2-associated-X protein

Bcl-2 – B-cell lymphoma protein 2

BcR – B-cell receptor

BMI – body mass index

BWF – bronchial washing fluid

C1P – ceramide-1-phosphate

CAPPs – ceramide-activated protein phosphatases

CDase – ceramidase

CERK – ceramide kinase

CerS – ceramide synthase

CERT – ceramide transfer protein

CGT – ceramide galactosyltransferase

COPD – chronic obstructive pulmonary disease

COX-2 – cyclooxygenase 2

CPAP – continuous positive airway pressure

CRP – C-reactive protein

DES – dihydroceramide desaturase

DLR− − negative diagnostic likelihood ratio

ECOG PS – Eastern Cooperative Oncology Group Performance Status Scale

EDTA – Ethylenediaminetetraacetic acid

EGFR – epidermal growth factor receptor

ELISA – enzyme-linked immunosorbent assay

ER – endoplasmic reticulum

ERK – extracellular signal-regulated kinase

ESS – Epworth Sleepiness Scale

GCS – glucosylceramide synthase

G-protein – guanin-nucleotide binding protein

HDAC 1 and 2 – histone deacetylase 1 and 2

HDL-C – high-density lipoprotein-cholesterol

HIF-1 – hypoxia inducible factor 1

HNSCC – head and neck squamous cell cancer

IAP – inhibitor of apoptosis protein

ICAM-1 – intercellular adhesion molecule–1

IH – intermittent hypoxia

IHC – immunohistochemistry

IL-1 – interleukin 1

JAK – Janus kinase

KRAS – Kirsten rat sarcoma viral oncogene

LCB – long chain base

LDL – low-density lipoprotein

LPP – lipid phosphate phosphatase

MAPK – mitogen-activated protein kinase

MEK – MAPK kinase

MinSatO2 – minimum oxygen saturation

MMP2 – matrix metalloproteinase 2

MOMP – mitochondrial outer membrane permeabilization

mTOR – mammalian target of rapamycin

NF-κB – nuclear factor kappa-B

NSCLC – non-small cell lung cancer

ODI – oxygen desaturation index

OS – overall survival

OSA – obstructive sleep apnea

PDGF – platelet derived growth factor

PDL1 – programmed cell death ligand 1

PKB/Akt – protein kinase B/Akt

PKCδ – protein kinase C delta

PKCζ – protein kinase C zeta

PP1 – protein phosphatase 1

PP2A – protein phosphatase 2A

PPARγ – peroxisome proliferator-activated receptor gamma

PSG – polysomnography

ROC – receiver operating characteristic analysis

ROS1 – c-ros oncogene 1

S1P – sphingosine-1-phosphate

S1PR1-5 – sphingosine-1-phosphate receptor 1-5

SCC – squamous cell carcinoma

SM – sphingomyelin

SMase – sphingomyelinase

SMS – sphingomyelin synthase

SNS – sympathetic nervous system

Sphk 1 and 2 – sphingosine kinase 1 and 2

SPNS2 – spinster homolog 2

SPP – S1P phosphatase

SPT – serine palmitoyltransferase

SPTi – sleep period time

STAT3 – signal transducer and activator of transcription 3

TAM – tumor associated macrophage

TLR – toll-like receptor

TME – tumor microenvironment

TNFα – tumor necrosis factor α

TPS – tumor proportion score

TRAF2 – TNF-receptor associated factor 2

TRAIL – TNF-related apoptosis inducing ligand

TST – total sleep time

TST90% – percentage of total sleep time spent with oxygen saturation below 90%

UV – ultraviolet

VEGF – vascular endothelial growth factor

vWF – von Willebrand factor

XIAP – X-linked inhibitor of apoptosis protein

1. INTRODUCTION

1.1. Sphingolipid metabolism

Sphingolipids are a vast class of lipid molecules bearing various functions in biological processes. They were discovered as important structural elements of eukaryotic cell membranes maintaining barrier function and fluidity. With the evolution of lipidomics in the last decades, they have been shown to exert diverse bioactive functions and play pivotal roles in cell signaling. Sphingolipid metabolism is a very delicate system that regulates cell fate, inflammation and stress mechanisms. The complexity of sphingolipid metabolism is a widely studied matter in molecular cell biology (1). The levels and interconversions of sphingolipids are regulated by an interconnected network of enzymes. Due to the hydrophobic nature of these molecules, they are mostly restricted to biological membranes. Therefore, their cellular and subcellular compartment-specific localization and transport is a major element of their signaling properties (2, 3).

1.1.1. Basic structure of sphingolipid molecules

Sphingolipids are a class of lipids that contain a long-chain amino alcohol base, referred to as a sphingoid backbone or long chain base (LCB). Sphingosine is the major sphingoid base in mammals. To this sphingoid base, an amide-bound long or very-long fatty acyl chain is added to form ceramide. In case of more complex sphingolipids such as sphingomyelin (SM), glycosphingolipids and galactosphingolipids, a polar head is attached to the C1-position. Structural heterogeneity arises from the variability of the headgroup and the hydrophobic sphingoid base and fatty acyl chain (1, 4).

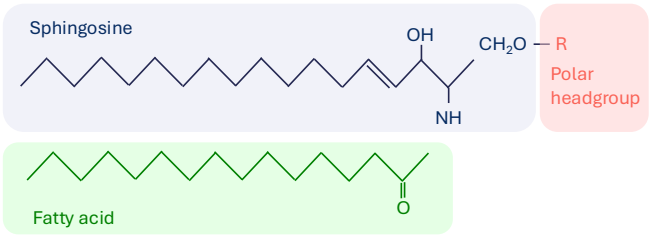

Figure 1 ***Basic structure of sphingolipids***. Sphingosine is the major base in mammals to which a fatty acid is linked by an amide bond. A variety of polar headgroups can be attached to the C1-position to form more complex sphingolipids. (Original figure prepared by the author for this thesis)

1.1.2. Formation and degradation of sphingolipid molecules

The generation, degradation, and interconversion of these molecules are effectuated by numerous well-regulated enzymes. *De novo* synthesis of the sphingoid base or LCB starts with the condensation of serine and palmitate by serine palmitoyltransferase (SPT) in the endoplasmic reticulum (ER), leading to the formation of dihydrosphingosine (4). This is the rate-limiting step of the *de novo* ceramide synthesis. Dihydrosphingosine is converted into dihydroceramide through amino acylation by ceramide synthases (CerS), which exist in 6 isoforms (CerS1-6) and add fatty acyl chains of different length to dihydrosphingosine (5). Dihydroceramide is then desaturated to form ceramide. Ceramide is considered to be the central hub of sphingolipid metabolism, as a precursor of all major sphingolipids. Ceramide is transported to the Golgi apparatus by ceramide transfer protein (CERT) (6) or vesicular transport (2). In the Golgi, with the addition of different headgroups, ceramide is transformed into complex sphingolipids such as SM and glycosphingolipids or can be phosphorylated by ceramide kinase (CERK) to generate ceramide-1-phosphate (C1P). Besides *de novo* synthesis, ceramide is also regenerated through the *salvage pathway*, the degradation of complex sphingolipids mostly in lysosomes. Some of the enzymes required for ceramide regeneration such as acid sphingomyelinase (SMase), neutral ceramidase (CDase), sphingosine kinase (Sphk), lipid phosphate phosphatases (LPPs) were also detected in the circulatory system suggesting that ceramide metabolism extends further outside of the cell (2, 7). An important metabolic pathway of ceramide is the formation of sphingosine-1-phosphate (S1P) through sphingosine. Sphingosine is generated from ceramide by CDases and then phosphorylated by Sphk type 1 or 2 to form S1P. S1P can be dephosphorylated into sphingosine and acylated again by CerS to form ceramide, or irreversibly degraded by S1P lyase. Both ceramide, sphingosine and S1P are bioactive molecules playing important roles in regulating cell fate. Their metabolic balance is often referred to as the 'sphingolipid rheostat' (2, 3, 8).

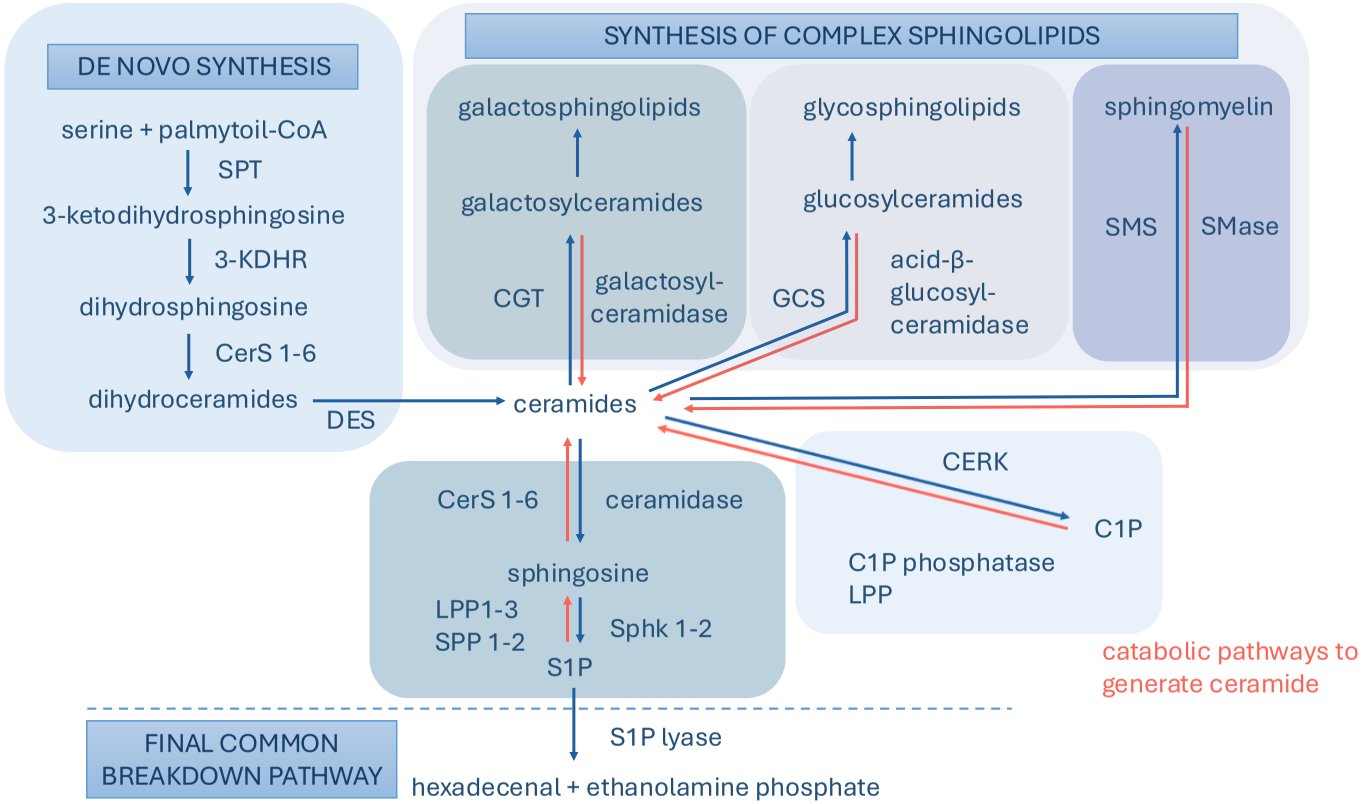

Figure 2 ***Schematic representation of sphingolipid metabolism.*** SPT – serine palmitoyltransferase, 3-KDHR – 3-ketodihydrosphingosine reductase, CerS – ceramide synthase, DES – dihydroceramide desaturase, CGT – ceramide galactosyltransferase, GCS – glucosylceramide synthase, SMS – sphingomyelin synthase, SMase – sphingomyelinase, CERK – ceramide kinase, C1P – ceramide-1-phosphate, LPP – lipid phosphate phosphatase, Sphk – sphingosine kinase, S1P – sphingosine-1-phosphate, SPP – S1P phosphatase (Original figure prepared by the author for this thesis)

1.1.3. Sphingolipid rheostat

The well-regulated balance of the ceramide-sphingosine-S1P metabolic pathway, the 'sphingolipid rheostat' is essential in determining cell fate (3, 9). These interconvertible bioactive sphingolipids bear opposing functions: while various ceramides are known to induce growth arrest, senescence and apoptosis (10), S1P promotes cell survival, proliferation, migration, angiogenesis and immune response (11). Their concentrations are strictly regulated and when perturbation occurs, it contributes to pathological processes and disease (12). With the development of sphingolipidomics, the theory of the rheostat was revealed to be more complex. Besides the intracellular conversion of these molecules, novel studies implicated the importance of extracellular autocrine and paracrine signaling as well (3): stimulation of transcription factor activator protein-1 (AP-1) by extracellular S1P was shown to increase the transcription of Sphk1, creating a positive feedback loop to sustain S1P-Sphk1 activity leading to progression of diabetic nephropathy in glomerular mesangial cells (13). Moreover, while ceramides are generally considered to promote apoptosis, in a recent study, ceramides of different chain length were shown to play opposing roles: $C\text{-}_{18}$ ceramide inhibited the growth of human head and neck squamous cell carcinoma (HNSCC) xenograft, while $C\text{-}_{16}$ ceramide inhibited ER-stress thus supporting cancer cell survival (14).

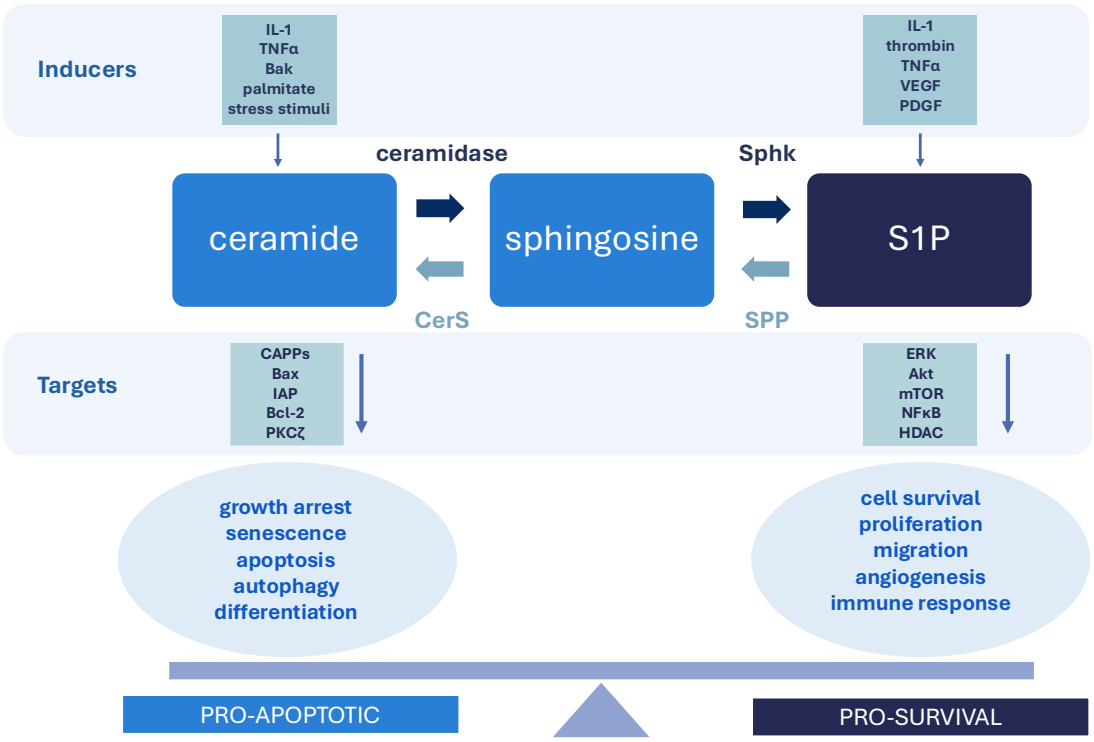

Figure 3 ***Schematic representation of the sphingolipid rheostat.*** The balance between ceramide and S1P synthesis regulates cell fate. IL-1 – interleukin-1, TNFα – tumor necrosis factor α, Bak – Bcl-2 homologous antagonist/killer, VEGF – vascular endothelial growth factor, PDGF- platelet derived growth factor, Sphk – sphingosine kinase, S1P – sphingosine-1-phosphate, CAPPs – ceramide-activated protein phosphatases, Bax – Bcl-2-associated-X protein, IAP – inhibitor of apoptosis protein, Bcl-2 – B-cell lymphoma protein 2, PKCζ – protein kinase C zeta, ERK – extracellular signal-regulated kinase, mTOR – mammalian target of rapamycin, NFκB – nuclear factor kappa B, HDAC – histone deacetylase (Original figure prepared by the author for this thesis)

1.1.3.1. S1P

S1P is an important bioactive lipid formed intracellularly from sphingosine by Sphk1 and Sphk2. The irreversible degradation of S1P by S1P lyase is the only exit from sphingolipid metabolism. The levels of S1P are strictly controlled by enzymes that regulate the formation of its substrate sphingosine, by Sphk-s and by S1P lyase.

S1P is produced intracellularly in different compartments, suggesting its diverse compartment-specific role (15). Sphk1 is found in the cytosol, associated to the plasma membrane, phagosomes and endosomal vesicles (16), while Sphk2 is located in mitochondria and in the nucleus, where it regulates gene transcription (17). The production of S1P is stimulated by growth factors like vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin-1 (IL-1) and thrombin (18).

S1P acts both intra- and extracellularly. After formation, S1P can follow three pathways: (a) recycled through phosphorylation to sphingosine, creating a pathway to regenerate ceramide, and subsequently more complex sphingolipids, (b) degraded irreversibly by S1P lyase, (c) transported extracellularly (16). Extracellular S1P effectuates autocrine, paracrine and endocrine actions through heterotrimeric guanine nucleotide–binding protein (G protein)-coupled receptors sphingosine-1-phosphate receptor 1-5 (S1PR1-5) (19). This signaling is called ‘inside-out signaling’ since S1P is generated intracellularly, then exported from the cell through specific transporters like ATP-binding cassette (ABCA1, ABCC1, ABCG2) and spinster homolog 2 (SPNS2) transporters to activate S1PRs (15, 20). The main source of S1P in circulation are red blood cells, platelets and endothelial cells (21). S1P can activate S1PRs on the same cell in an autocrine manner or is bound to chaperones like high-density lipoprotein bound apolipoprotein M ($ApoM^+HDL$) and albumin to exert paracrine and endocrine activities (22).

S1P spatial gradients, determined by export from the cell, extracellular metabolism and chaperone association, are crucial in specific biological activities (19, 23). Perturbations in S1P metabolism contribute to the development of pathologies like cancer, inflammatory diseases, autoimmune disorders, atherosclerosis, diabetes (11, 19).

S1PRs are known to activate pro-survival signaling routes such as the extracellular signal-regulated kinase (ERK) (24) and Akt pathways (25). S1PR3 activation was shown to suppress ceramide induced autophagy by activating the mammalian target of rapamycin (mTOR) pathway (26). Hypoxia induced S1P production by Sphk2 activated paracrine and autocrine effects of p42/44 mitogen-activated protein kinase (MAPK) resulting in chemoresistance in A549 lung carcinoma cells (27). S1P gradient created by SPNS2 was shown to regulate cardiac development in zebrafish (28), while in mice it promoted lymphocyte egress from lymphoid organs to the circulation (29). ApoM^{+}HDL bound S1P maintained vascular integrity through endothelial cell migration and formation of endothelial adherens junctions when binding to S1PR1, inducing its internalization and activation of MAPK and Akt signaling routes (22). In cultured human umbilical vein endothelial cells, binding of ApoM^{+}HDL-S1P resulted in the formation of anti-inflammatory S1PR1-β-arrestin 2 complex leading to suppressed nuclear factor kappa B (NF-κB) activation and inhibition of intercellular adhesion molecule 1 (ICAM-1) abundance by TNFα, resulting in decreased vascular inflammation (30).

S1PR independent intracellular activities of S1P are less known but are hypothesized to be as important as the receptor mediated effects. S1P generated by Sphk1 was shown to activate the TNFα associated NF-κB pathway via tumor-necrosis factor (TNF) receptor-associated factor 2 (TRAF2) independently of S1PRs (31). Sphk2 is predominantly located in the nucleus where it was shown to exert epigenetic gene regulation through inhibition of histone deacetylases 1 and 2 (HDAC1 and 2), resulting in increased histone acetylation and transcriptional activation of target genes such as p21 and c-fos (32).

1.1.3.2. Ceramide

Ceramide is the central element of sphingolipid metabolism. Generally, it consists of an LCB of 18 carbons and an amide-linked fatty acyl chain containing 14-26 carbons. It has been known that CerS 1-6 generate ceramides with different fatty acyl chains (5), and recent data have suggested that the LCBs also show variability with carbons ranging from 16 to 20 in number. Therefore, ceramide is more than one single molecule, but a family of related compounds. In general, ceramides are neutral sphingolipids, which by themselves are restricted to their site of formation. Although they have the ability to flip across membrane leaflets to exert specific functions, transport of ceramides in the cell is

only possible via assistance of CERT or vesicular trafficking (6, 8). The metabolism of ceramides is regulated by more than 20 enzymes in different subcellular compartments. These variations in the chemical structure and the distinct subcellular localization and trafficking of ceramide molecules warrant their diverse biological roles (2).

Besides being a metabolic precursor for other sphingolipid molecules, ceramides also exert bioactive functions. Ceramides are mostly known to mediate stress responses and anti-proliferative processes like cell senescence, growth arrest, apoptosis, autophagy, differentiation. Ceramide synthesis is induced by various stress stimuli such as heat shock, oxidative stress, anticancer drugs, inflammatory mediators like TNFα, IL-1, pro-apoptotic protein Bcl-2 homologous antagonist/killer (Bak) (33) and also by palmitate excess (2).

Many targets of ceramide mediated cell signaling have been identified, including ceramide-activated protein phosphatases (CAPPs) like protein phosphatases 1 (PP1) and 2A (PP2A) (34), protein kinase Cζ (PKCζ) (35), protein kinase B (PKB/Akt) (36), Bcl-2 protein family, mTOR (37), inhibitor of apoptosis protein (IAP) family (38) including survivin (39). Besides targeting intracellular molecules, ceramides also regulate signaling through the formation of ceramide rich platforms in plasma and mitochondrial membranes to cluster receptor molecules and facilitate signaling (40). PP1Cα induced by plasma membrane ceramide plays a role in cell migration and adhesion (41), while ceramide synthesized due to palmitate loading was shown to inactivate Akt via PP2A (42). In context of apoptosis, ceramide synthesis induced by pro-apoptotic Bak resulted in the inactivation of anti-apoptotic Bcl-2 proteins (43), while mitochondrial translocation of ceramide triggered Bcl-2-associated-X protein (Bax) dependent mitochondrial outer membrane permeabilization (MOMP) (44). Dysregulation of ceramide metabolism has been implicated in many pathological processes including diabetes (45), cardiovascular diseases (46), neurodegenerative disorders (47) and cancer (48).

1.1.3.3. Anti-ceramide antibody

Possible involvement of antibodies against sphingolipid molecules is under investigation in various diseases. Elevated anti-ceramide antibody levels were associated with increased plaque formation is mouse models with Alzheimer's disease (49) and have been

shown to be associated with nerve affection in patients with leprosy (50). 2A2, a monoclonal antibody against ceramide decreased mortality in gastrointestinal radiation syndrome in mice by preventing ceramide-induced endothelial apoptosis (51). In rodents, anti-ceramide antibody protected from diabetic retinopathy (52). Drug resistance in cancer therapy is a serious challenge, in mouse renal cell carcinoma xenograft models anti S1P monoclonal antibody sphingomab inhibited tumor growth both in treatment naïve and in sunitinib-resistant tumors (53). Antibodies against sphingolipids might prove to be promising therapeutic agents in various diseases in which altered sphingolipid metabolism is present. Moreover, since they don't impact intermediary sphingolipid metabolism, they might provide a better safety profile (52).

1.2. Sphingolipid metabolism in cancer

Sphingolipid metabolism is known to have major impact on regulating cancer cell death and survival (54). The theory of the sphingolipid rheostat, discussed above, illustrates the opposing roles of central molecules ceramide and S1P in regulating cell fate, cancer development and also cancer drug resistance (55).

Ceramide synthesis and accumulation mediates cancer-cell death through apoptosis, necroptosis, autophagy, ER stress, cell growth arrest (48). Apoptosis induced by ceramide can occur through the extrinsic pathway, via activation of transmembrane death receptors such as TNF receptor, TNF-related apoptosis inducing ligand (TRAIL) receptor, CD59 (Fas) surface receptor, or through the mitochondrial pathway, induced by cellular stress like heat shock, genotoxic damage, oxidative stress, anticancer drugs (37). During the mitochondrial pathway, ceramide induces Bax-dependent apoptosis via promoting MOMP and caspase activation (56). Long-chain ceramide-dependent proteasomal activation, inactivation of X-linked inhibitor of apoptosis protein (XIAP), induction of effector caspases and subsequential production of very long-chain ceramides have been observed during B-cell receptor (BcR)-triggered apoptosis (57). Ceramide has also been shown to inhibit the expression of matrix metalloproteinase 2 (MMP2), an enzyme that is involved in cancer invasion and metastasis (58). Autophagic cell death is another mechanism induced by ceramide involving the mTOR and Bcl-2 pathway (59). Due to its robust anti-tumor effect, ceramide metabolism is commonly altered in cancer cells limiting its cytotoxicity and cell death inducing activity (60).

Numerous downstream targets of ceramide have been identified which provide possible therapeutic targets in cancer treatment. Several studies have implicated the role of ceramide regarding cell response and sensitivity to chemotherapeutic agents and drug resistance (37). Stress stimuli like ultraviolet (UV) and ionizing radiation and the ligation of chemotherapeutic agents and death receptor ligands promote acid SMase activation via reactive oxygen species, nitrosative stress and protein kinase C-delta (PKCδ) activation. Ceramide is thought to be generated by acid SMase through the salvage pathway from sphingosine in lysosomes and cell membrane, resulting in cathepsin D activation in the lysosomes and possibly mediating receptor capping and microdomain formation in the cell membrane (2). *De novo* ceramide synthesis was induced by doxorubicin and camptothecin in follicular thyroid carcinoma cell line (61). Nanoliposomal ceramide had antitumor effect in pancreatic cancer when combined with gemcitabine or glucosylceramide synthase (62). In combination with doxorubicin, C6 ceramide induced chemo-sensitization in multiple cancer cell lines (63). Ceramide analogues and inducers alone or in combination with chemotherapy and immunotherapy are currently under investigation in cancer therapy. α-Galactosylceramide showed promising results in phase I-II trials activating invariant natural killer T-cells (64, 65). Ceramide nanoliposomes in combination with vinblastine showed therapeutic efficacy in AML in a phase I study (66), while pyridinium ceramide promoted mitochondrial apoptosis in pancreatic cell lines (67).

Chemotherapeutic drugs and radiation therapy have been shown to induce endogenous ceramide synthesis leading to cancer cell death. On the other hand, the shift towards the production of pro-survival sphingolipids such as S1P, results in resistance to these therapies (68, 69) and aggressive tumor behavior in various cancers (70).

Disruptions in sphingolipid metabolism lead to elevated levels of S1P in tumor tissues, thereby promoting cancer development. S1P produced intracellularly in cancer cells is exported to the tumor microenvironment (TME) where it interacts with cancer and non-cancer cells and other non-cellular components of the TME. Through complex signaling and regulating cell-cell communication, S1P enhances tumor cell growth and motility (68). Persistent activation of STAT3, induced by the SphK1/S1P/S1PR1 axis through the NF-κB-mediated proinflammatory pathway involving IL-6 and TNFα, has been shown to

link chronic intestinal inflammation to the development of colon cancer. (71). In ovarian cancer cells, S1P promoted chemotaxis and invasion (72). S1P has also been shown to exert pro-survival effects through regulating pro- and anti-apoptotic proteins. S1P inhibited mitochondrial translocation of Bax during apoptosis through the MAPK kinase (MEK)/ERK pathway (73). In human leukemia HL-60 cells, S1P acting on S1PR3 inhibited autophagy through activation of the mTOR pathway (26). Besides directly affecting cancer cells, S1P also modifies the behavior of TME components. S1P has been demonstrated to enhance angiogenesis and lymphangiogenesis (74), promote the recruitment of Treg cells in tumors via Janus kinase (JAK)/STAT signaling, and limit CD8+ T cell infiltration and activation (75) and enhance tumor associated macrophage (TAM) dependent metastatic spread through S1PR1 downstream signaling (76).

The S1P metabolic route is under investigation as a therapeutic target in various diseases. S1PR modulators are approved for multiple sclerosis and ulcerative colitis and are being tested in psoriasis and graft versus host disease (77). Agents targeting the S1P pathway are being developed including Sphk inhibitors, S1P analogs, S1P-specific neutralizing antibodies. Most preclinical data is available on S1P analog fingolimod or FTY720. Though this drug was implicated in potentiating sensitivity to chemotherapy and suppressing tumor progression, due to its impact on T-cell function its use in clinical setting is questionable (78). Sphk inhibitors were shown to enhance the efficacy of enzalutamide on prostate cancer cells (79). In a preclinical study, anti-S1P monoclonal antibody reduced tumor progression on multiple tumor cell lines (80).

The role of sphingolipid metabolism in cancer pathogenesis, and its potential clinical use in anticancer therapy and as a biomarker have been studied *in vitro* and *in vivo* in different cancer types (55, 81, 82). S1P and ceramide both play crucial roles in cancer and are promising targets for novel antineoplastic therapies. Precision medicine is an emerging approach in cancer therapy, tailoring medication to the patients' specific genetic, biochemical and lifestyle factors, shifting the focus from the tumor-histology centered approach (83). Lung cancer is still the leading cause of cancer death (84) despite the evolution of screening methods and advances in anti-cancer therapy, and the majority of lung cancer patients are diagnosed in advanced stages when therapy is limited. Therefore,

identification of novel diagnostic and prognostic biomarkers and understanding the metabolic and signaling pathways determining carcinogenesis and progression is crucial.

1.3. Sphingolipid metabolism in OSA

Obstructive sleep apnea (OSA) is a chronic breathing disorder characterized by the recurrent collapse of the upper airways leading to intermittent hypoxia (IH). The severity of OSA is determined based on the apnea-hypopnea index (AHI) which is the total number of apneas and hypopneas per hour (85). OSA is known to be associated with several comorbidities including cardiovascular disease, diabetes, neurocognitive disorders. IH is implicated to be a major causative factor in OSA-related comorbidities driving systemic and vascular inflammation with endothelial dysfunction, metabolic disturbances, oxidative stress and arousal related sympathetic nervous system (SNS) activation (86).

Sphingolipids have been described to regulate and participate in inflammatory responses in various ways. Proinflammatory cytokines such as TNFα and IL-1β - which are also increased in OSA-related inflammation (87) – promote the synthesis of sphingolipids (88). Obesity predisposes to OSA and is linked strongly with inflammation and metabolic dysfunction. Excessive activation of the SNS is known to be associated with lipolysis and insulin resistance resulting in hypoxia-inducible factor 1 (HIF-1) pathway stimulation (89). Moreover, inflammation induced by IH is suspected to involve the NF-κB and HIF-1 pathways (90). Sphingolipids play a role in obesity-induced pathologies as well. CERK was shown to be upregulated during adipocyte differentiation which is known to promote obesity-related chronic inflammation. The silencing of CERK genes led to the decrease of leptin secretion, peroxisome proliferator-activated receptor gamma (PPARγ) expression and lipid droplet formation (91). Circulating ceramide bounds to low-density lipoprotein (LDL) and increases insulin resistance and inflammation via toll like receptor (TLR) induced macrophage activation and amplification (45). Inhibition of ceramide synthesis has been shown to reduce insulin resistance associated with obesity, fatty acids and glucocorticoids in mice (92). Palmitate loading resulted in the accumulation of ceramides and consequently the inactivation of Akt, an important modulator in insulin signaling and metabolic control (93).

Sphingolipids are involved in endothelial dysfunction leading to atherosclerosis which is a common comorbidity in OSA. Production of S1P is increased by TNFα, thrombin and VEGF (18) that are also elevated in OSA (94, 95). S1P contributes to atherosclerosis via activating the endothelial nitric oxide synthase (96) and induces Chemokine (C-C motif) ligand 20, a potent lymphokine (97) resulting in the release of pro-coagulant and adhesive P-selectin and von Willebrand factor (vWF) from platelets (18). P-selectin and vWF have been shown to be increased in OSA (98, 99).

Sphingolipid metabolism has not yet been investigated extensively in OSA, but several overlapping metabolic pathways and biological processes have been identified, suggesting its possible involvement in OSA-related pathological processes. Recent studies estimate the prevalence of OSA to be as high as 50% in men and 25% in women (100). With daytime symptoms and associated comorbidities such as hypertension, stroke, cardiac arrhythmias, diabetes, depression, it proposes a major public health concern (101). Considering the high prevalence and the associated health risks and limitations of the current first-line treatment of continuous positive airway pressure (CPAP) ventilation (102), discovering the metabolic pathways involved in OSA in order to better understand the pathogenesis and identify possible novel diagnostic biomarkers and targets for treatment is essential.

2. OBJECTIVES

This doctoral thesis investigates the possible role of the sphingolipid rheostat in lung diseases, with a particular focus on its proinflammatory aspects. The main objectives are as follows:

1. To investigate the potential of S1P and anti-ceramide antibody as biomarkers in unresectable and advanced stage non-small cell lung cancer (NSCLC):
   - via assessing circulating levels of S1P in NSCLC patients compared to control group
   - via assessing circulating levels of anti-ceramide antibody in NSCLC patients compared to control group
   - via assessing anti-ceramide antibody levels in bronchial washing fluid (BWF) of NSCLC patients compared to control group
   - via correlating survival data with S1P and anti-ceramide antibody levels

2. To investigate the possible role of S1P and anti-ceramide antibody in OSA:
   - via assessing circulating levels of S1P in OSA patients compared to control group
   - via assessing circulating levels of anti-ceramide antibody in OSA patients compared to control group
   - via correlating clinical variables with anti-ceramide antibody levels

3. METHODS

3.1. Study design

We conducted two parallel case-control studies to evaluate sphingolipid metabolism in OSA and NSCLC. The primary endpoint in both studies was to compare levels of S1P and anti-ceramide antibody between the control and patient groups.

In the NSCLC study, as a secondary endpoint, we analyzed survival data in relation to these biomarkers.

3.2. Study populations

3.2.1. NSCLC study

In this study, 34 control subjects for plasma sphingolipid measurements, 3 control subjects for bronchial washing fluid (BWF) sphingolipid measurements and 32 patients with stage III-IV NSCLC for plasma and BWF sphingolipid measurements were enrolled. Exclusion criteria were infections in the last 2 months and history of any malignancy in the control groups. Complete medical history was recorded, and venous blood samples were taken for sphingolipid and C-reactive protein (CRP) measurements from all groups. NSCLC patients were diagnosed at the Department of Pulmonology, Semmelweis University between October 7, 2021 and August 15, 2022. The diagnosis was based on histological and cytological evaluations conducted by experienced pathologists at the Department of Pathology, Semmelweis University. 30 patients had bronchoscopy due to endobronchial, peripheral lesions or enlarged mediastinal lymph nodes suspicious for malignancy, therefore either endobronchial biopsy, transbronchial biopsy or conventional transbronchial needle aspiration was performed, respectively. Bronchoscopy was not performed in 2 cases, when the diagnosis was based on cytological assessment of pleural effusion. During bronchoscopy, BWF samples were additionally collected for sphingolipid measurements. Independently of our study, as part of the routine diagnostic procedure, immunohistochemical (IHC) staining and comprehensive molecular genetic analysis were also performed at the Department of Pathology, Semmelweis University to support therapeutic decision making. The stage of NSCLC was defined based on the 8th Edition of the UICC TNM classification of Malignant Tumors (103). All patients had contrast enhanced chest and abdominal CT scans or PET-CT scan and brain imaging

(either contrast enhanced CT or MRI) for staging purposes. None of the patients were eligible for surgical resection.

BWF measurements were performed on samples from 20 subjects in the NSCLC group. Control subjects for BWF measurements (n=3) were limited in number due to the invasiveness of the procedure. These patients were referred to our Clinic because of incidental CT findings of pulmonary nodules and were diagnosed with non-malignant disease after thorough pulmonological examination.

All procedures involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study was approved by the local Ethics Committee (Semmelweis University, TUKEB 215/2021 and TUKEB 30/2014), and informed consent was obtained from all participating volunteers (104).

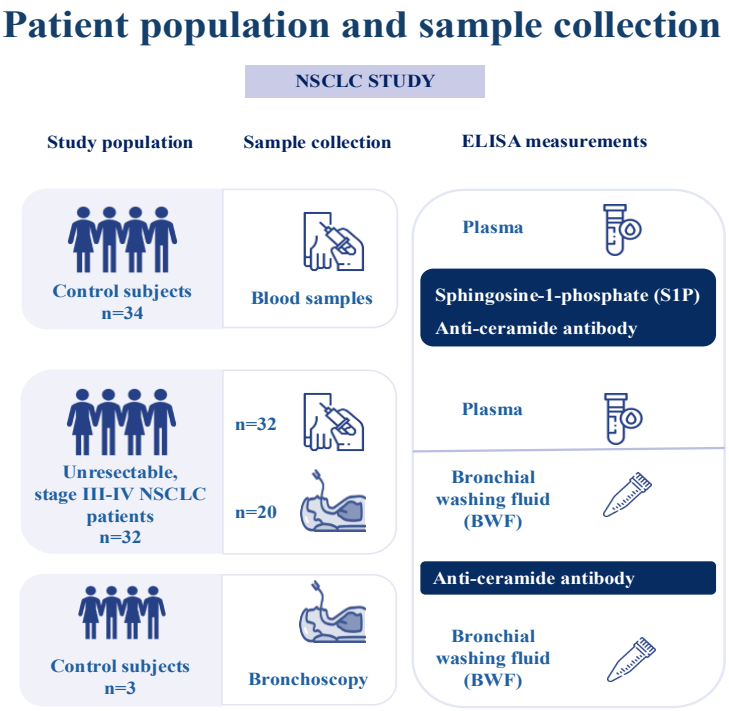

Figure 4 ***Patient population and sample collection in the NSCLC study.*** 34 control subjects for plasma, 3 control subjects for BWF and 32 patients with stage III-IV NSCLC for plasma and BWF sphingolipid measurements were enrolled. NSCLC – non-small cell lung cancer, BWF – bronchial washing fluid, ELISA – enzyme-linked immunosorbent assay (Original figure prepared by the author for this thesis)

3.2.2. OSA study

We recruited 68 subjects from the Sleep Unit of the Department of Pulmonology, Semmelweis University. The subjects were referred to our facility due to suspicion of OSA (i.e. snoring, witnessed apneas, daytime somnolence). None of the patients had previously been diagnosed with OSA, nor had they been treated with CPAP or mandibular advancement devices. Exclusion criteria included any uncontrolled chronic disease, history of any malignancy within 10 years, and infection within 2 months. Data for screen failures were not captured. All patients underwent a full night attended sleep study. On the night of the sleep study, medical history and the Epworth Sleepiness Scale (ESS) were recorded, and venous blood samples were taken for biomarker measurements.

Inpatient overnight polysomnography (n=41) and cardiorespiratory polygraphy (n=27) were performed using the Somnoscreen Plus Tele PSG and RC devices (Somnomedics GMBH Germany). Sleep stages, movements and cardiopulmonary events were scored manually according to the American Academy of Sleep Medicine (AASM) guideline (105). Total sleep time (TST), sleep period time (SPTi) and minimum oxygen saturation ($MinSatO_2$) were recorded. Apnea-hypopnea index (AHI), oxygen desaturation index (ODI) and the percentage of total sleep time spent with oxygen saturation below 90% (TST90%) were calculated. An AHI ≥5/hours was diagnostic for OSA (106).

The study was approved by the local Ethics Committee (Semmelweis University, TUKEB 30/2014 and RKEB 172/2018), and informed consent was obtained from all participating volunteers. All measurements were performed in accordance with the relevant guidelines and regulations (106).

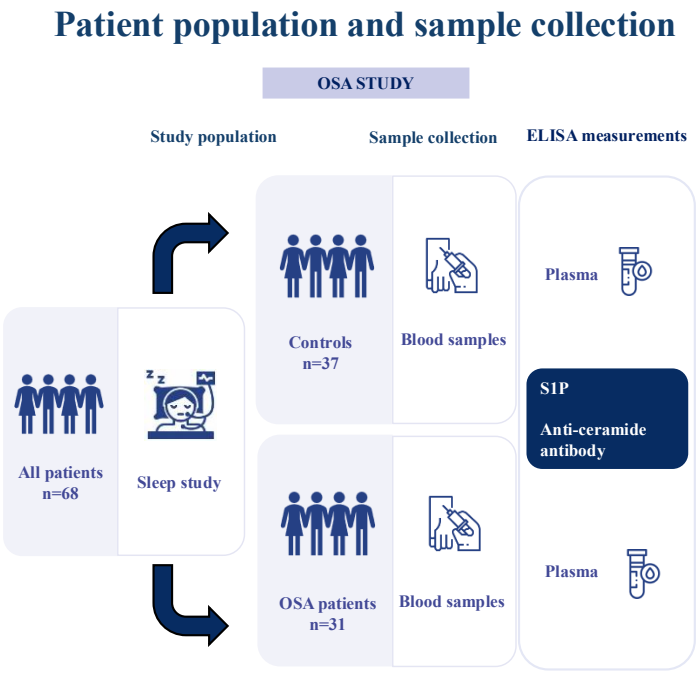

Figure 5 ***Patient population and sample collection in the OSA study.*** 68 patients underwent sleep study, which confirmed OSA in 31 cases. Plasma S1P and anti-ceramide antibody measurements were performed on samples from 31 OSA patients and 37 control subjects. (Original figure prepared by the author for this thesis)

3.3. S1P and anti-ceramide-antibody measurements

In both studies, venous blood samples were collected in EDTA tubes for plasma sphingolipid measurements. The samples were centrifuged at 1500 RPM for 10 minutes at 4 °C within 2 hours of collection. Plasma was separated into 250 µL aliquots which were stored at -80 °C until analysis. Samples were thawed just before the enzyme-linked immunosorbent assay (ELISA) measurements. In the NSCLC study, BWF was collected in tracheal suction sets and stored at -80°C. Commercially available ELISA kits were used for S1P and anti-ceramide antibody measurements (MyBioSource Inc., San Diego, CA, USA; Human S1P ELISA kit (MBS2516132) and Human Anti-Ceramide Antibody ELISA kit (MBS3804520)). The detection range was 3.13-200 ng/ml for the S1P kit and 12.5-400 ng/ml for the anti-ceramide antibody kit. Measurements were performed in duplicates according to the manufacturer's instructions and mean concentrations were used as inputs for analysis. The intra-assay variation coefficients were 8.5 % for the anti-ceramide antibody assay and 4.2 % for the S1P assay in the NSCLC study and 8.6 % for

the anti-ceramide antibody assay and 4.2 % for the S1P assay in the OSA study. Samples were used at a fourfold dilution for anti-ceramide antibody measurement and at a tenfold dilution for S1P measurements in the NSCLC study (104) and eightfold dilution in the OSA study (106).

3.4. Statistical analysis

The R statistical software program v. 4.1.3. (R Statistical Foundation, Vienna, Austria) and JASP 0.14.1 (University of Amsterdam, Amsterdam, The Netherlands) were used to perform statistical analysis. The sample size was estimated to detect differences of at least 70% of the standard deviation (0.70 effect size) in either anti-ceramide antibody or S1P levels between the two groups with a power of 0.80 and α error probability of 0.05 (107). P values of <.05 were considered significant. Data are expressed as median [range] or mean ± SD. Diagrams were plotted with the ggplot2 package for R. Shapiro-Wilk test showed normal distribution for anti-ceramide antibody and S1P levels in the NSCLC study, while in the OSA study the distribution was non-parametric. Therefore, the subsequent tests were different in the two studies.

In the NSCLC study, anti-ceramide antibody and S1P levels were compared between the two groups with linear regression. The values were adjusted for age, COPD and BMI. Categorical variables were compared with the chi-squared test. T-test was used to compare COPD patients with non-COPD patients in the NSCLC group regarding anti-ceramide antibody and S1P levels. Survival analysis was performed with the survival package of R which uses log-rank test (108). Kaplan Meier plots were drawn with the survminer package. We used receiver operating characteristic (ROC) analysis to calculate the cutoff value for anti-ceramide antibodies (rocit package of R). Using this cutoff, patients were classified as having high or low anti-ceramide antibody levels. In case of S1P, we used the median value as cutoff in determining high and low levels. We compared the survival of patients with and without KRAS mutation and PDL1 expression (104).

In the OSA group, data was not normally distributed. Mann-Whitney U-test and Chi-square test were used to compare clinical and demographic characteristics as well as biomarker levels between the OSA and control groups. We applied non-parametric analysis of covariance (ANCOVA) test adjusted for age, gender and BMI to investigate

the differences in biomarker levels between patients and controls. Plasma biomarker levels were correlated with clinical variables using Spearman's test and logistic regression analysis. P values <.05 were considered significant (106).

4. RESULTS

4.1. NSCLC study

4.1.1. Clinical characteristics of the study population

Data are summarized in Table 1. There was a significant difference regarding age, prevalence of smoking, chronic obstructive pulmonary disease (COPD), hypertension and levels of circulating CRP between the NSCLC and control groups (all $p<0.05$). The prevalence of COPD did not affect the levels of S1P or anti-ceramide antibody in the NSCLC group (104).

Table 1 ***Clinical characteristics of controls and patients with NSCLC.*** Data are median [range] or numbers (%). NSCLC – non-small cell lung cancer, BMI – body mass index, COPD – chronic obstructive pulmonary disease, CRP – C-reactive protein (104)

	Control (n=34)	*NSCLC (n=32)*	*p-value*
Age, years	46 [20-74]	65.5 [45-92]	<0.001
Male	11 (32.35)	15 (46.88)	0.34
BMI, kg/m^2	24.14 [17.21-41.02]	24.35 [17.9-33.56]	0.46
Hypertension	9 (26.47)	25 (78.13)	<0.001
Diabetes	2 (5.88)	6 (18.75)	0.22
COPD	2 (5.88)	12 (37.50)	0.004
Asthma	2 (5.88)	1 (3.13)	1
Smoking (active/all)	1/3 (2.94/8.82)	16/24 (50.00/75.00)	both <0.001
CRP, mg/l	1.17 [0.22-5.59]	12.0 [0.4-271]	<0.001

4.1.2. Oncological characteristics in the NSCLC group

Oncological data are summarized in Table 2. Pathological diagnosis confirmed 23 cases (71.87%) of adenocarcinoma and 9 cases (28.12%) of squamous cell carcinoma (SCC). PDL1 IHC staining was positive (tumor proportion score (TPS) ≥1%) in 8 cases (88.89%) of SCC and 15 cases (65.22%) of adenocarcinoma. Molecular genetic analysis detected KRAS mutation in 14 cases (60.87%), ALK fusion in 2 cases (8.70%), EGFR mutation in 1 case (4.34%) and ROS1 translocation in 1 case (4.34%) of adenocarcinoma samples. In 2 cases, molecular genetic analysis is not available: one patient deceased shortly after histological sampling and one patient did not return to our facility, no further data is available on them in our database. At diagnosis, most of the patients presented with stage IV disease. None of the patients were eligible for surgical treatment (104).

Table 2 ***Summary of oncological data of NSCLC patients (n=32).*** Data are presented in numbers (%). NSCLC – non-small cell lung cancer, ECOG PS – Eastern Cooperative Oncology Group Performance Status Scale, SCC - squamous cell carcinoma, PDL1 – programmed cell death ligand 1, IHC – immunohistochemistry, TPS – tumor proportion score, ALK – anaplastic lymphoma kinase, KRAS – Kirsten rat sarcoma viral oncogene, EGFR – epidermal growth factor receptor, ROS1 – c-ros oncogene 1, NA – not available (104)

Stage (%)	
IIIA	4 (12.5)
IIIB	4 (12.5)
IV	24 (75)
ECOG PS (%)	
0-1	23 (71.88)
2	8 (25)
3-4	1 (3.12)
Histology (%)	
SCC	9 (28.13)
Adenocarcinoma	23 (71.87)
PDL1 IHC (%)	
SCC PDL1	
TPS <1%	1 (11.11)
TPS 1-50%	6 (66.67)
TPS >50%	2 (22.22)
Adenocarcinoma PD-L1	
TPS <1%	6 (26.09)
TPS 1-50%	11 (47.83)
TPS >50%	4 (17.39)
NA	2 (8.69)
Molecular oncological analysis – Adenocarcinoma (%)	
ALK fusion	2 (8.70)
KRAS mutation	14 (60.87)
p.G12C	12 (85.71)
p.G12V	2 (14.29)
EGFR mutation (exon 18, p.G719A)	1 (4.35)
ROS1 translocation	1 (4.35)
No targetable mutation detected	3 (13.04)
NA	2 (8.69)

4.1.3. Circulating S1P levels in NSCLC patients

Our ELISA measurements detected significantly higher plasma levels of S1P in the NSCLC group (3770.99 ± 762.29 ng/ml vs. 366.53 ± 249.38 ng/ml, patients with NSCLC vs. controls, respectively, $p<0.001$) (Figure 6). These levels were not affected by age, BMI or the prevalence of COPD ($p=0.13$, 0.27 and 0.12, respectively). No significant difference was observed between S1P levels regarding KRAS gene mutation status (3842.79 ng/ml [374.3-4190.4 ng/ml] vs. 3923.53 [3397.8-4349.3 ng/ml], KRAS mutant vs. KRAS wild type, respectively, $p=0.37$) or PDL1 status either (3691.76 ng/ml [3397.8-4281.4 ng/ml] vs. 3931.16 ng/ml [374.34-4349.33 ng/ml], PDL1 negative vs. PDL1 positive cases, respectively, $p=0.89$) (104).

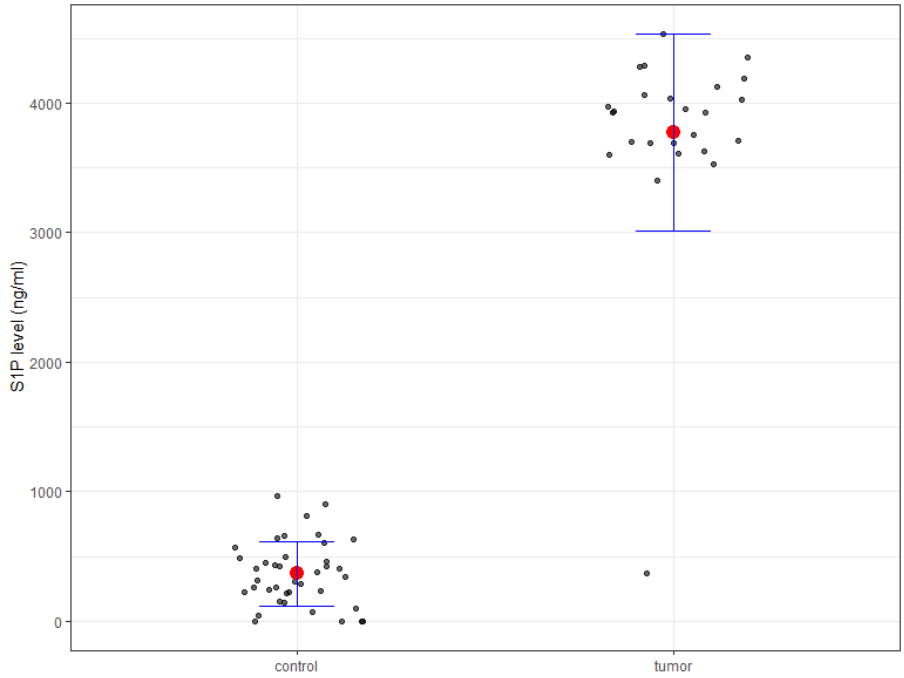

Figure 6 ***Plasma S1P levels.*** Circulating S1P levels were significantly higher in NSCLC patients than in controls (3770.99 ± 762.29 ng/ml vs. 366.53 ± 249.38 ng/ml, patients with NSCLC vs. controls, respectively, $p<0.001$). S1P – sphingosine-1-phosphate, NSCLC – non-small cell lung cancer (Original figure prepared by the author for this thesis)

4.1.4. Circulating anti-ceramide antibody levels in NSCLC patients

When comparing the NSCLC and the control groups, we found significantly elevated anti-ceramide antibody levels in the NSCLC group (278.70 ± 19.26 ng/ml vs. 178.60 ± 18 ng/ml, patients with NSCLC vs. controls, respectively, p=0.007) (Figure 7). Anti-ceramide antibody levels were not affected by age, BMI or the prevalence of COPD (p=0.11, 0.27 and 0.37, respectively). Also, there was no significant difference between anti-ceramide antibody levels in terms of KRAS gene mutation status (287.44 ng/ml [71.40-496.20 ng/ml] vs. 256.05 ng/ml [121.67-403.01 ng/ml], KRAS mutant vs. KRAS wild type, respectively, p=0.79) or PDL1 positive and negative NSCLC either (268.90 ng/ml [71.40-496.20 ng/ml] vs. 256.05 ng/ml [76.52-392.71 ng/ml], respectively, p=0.50) (104).

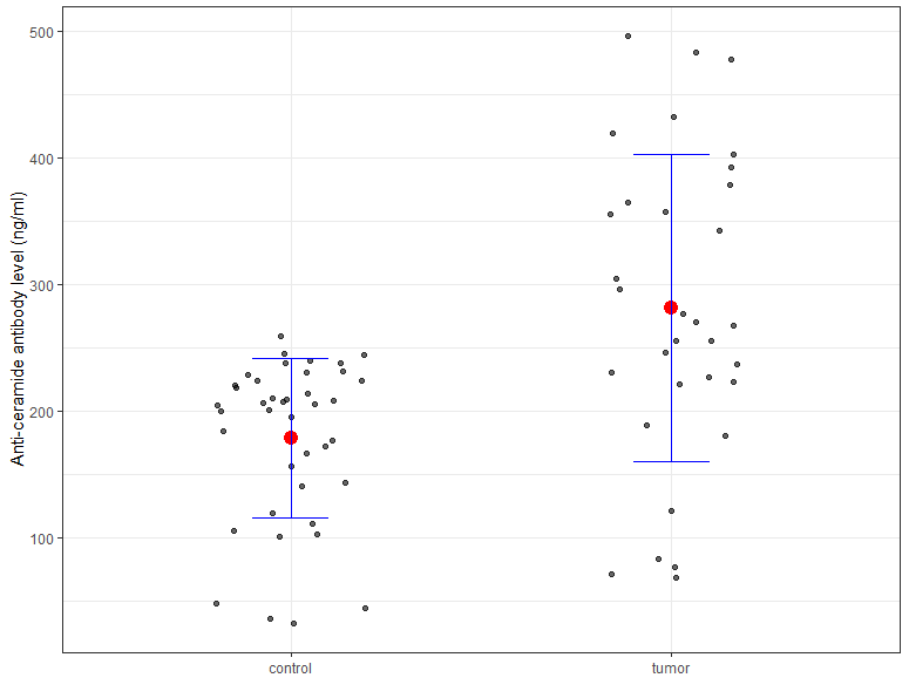

Figure 7 ***Plasma anti-ceramide antibody levels.*** Circulating anti-ceramide antibody levels in patients with NSCLC were significantly elevated compared to controls (278.70 ± 19.26 ng/ml vs. 178.60 ± 18 ng/ml, patients with NSCLC vs. controls, respectively, p=0.007). NSCLC – non-small cell lung cancer (Original figure prepared by the author for this thesis)

4.1.5. Anti-ceramide antibody levels in BWF

Regarding BWF samples, anti-ceramide antibody levels were significantly higher in the NSCLC group (155.29 ng/ml ± 27.58 ng/ml vs. 105.87 ng/ml ± 9.99 ng/ml, patients with NSCLC vs. controls, respectively, $p<0.001$). However, these results are limited due to the small number of cases (Figure 8) (104).

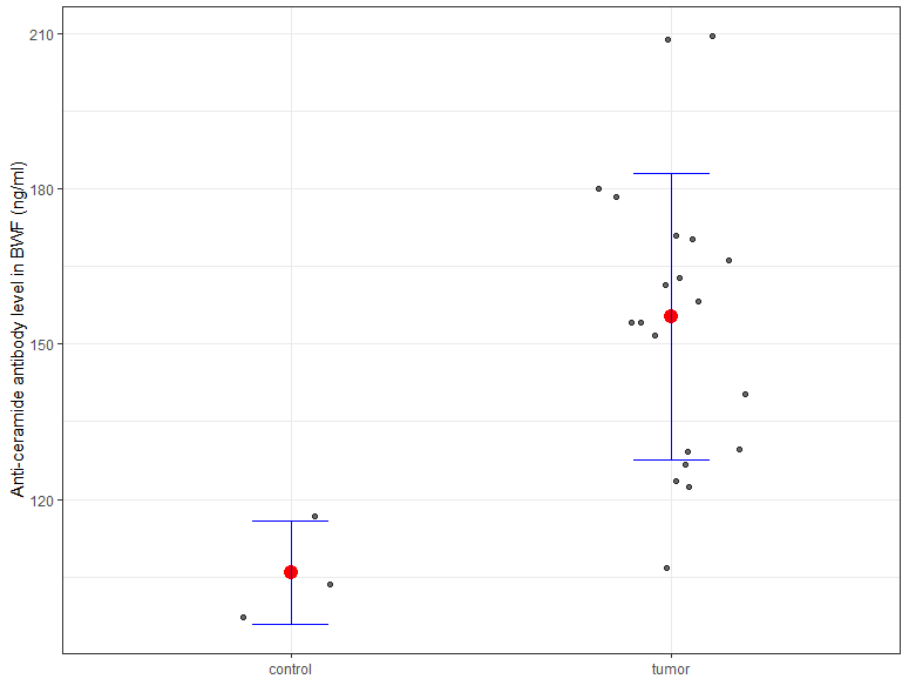

Figure 8 ***Anti-ceramide antibody levels in BWF samples.*** Anti-ceramide antibody levels in BWF samples were significantly higher in NSCLC patients compared to controls (155.29 ng/ml ± 27.58 ng/ml vs. 105.87 ng/ml ± 9.99 ng/ml, patients with NSCLC vs. controls, respectively, $p<0.001$); however, these results are limited due to small sample size. BWF – bronchial washing fluid, NSCLC – non-small cell lung cancer (Original figure prepared by the author for this thesis)

4.1.6. Survival analysis in the NSCLC group

We determined overall survival (OS) as a secondary endpoint in our study. The patients who were lost to follow up (n=6) were excluded from the survival analysis. Median OS was 13.36 months from the date of diagnosis (Figure 9).

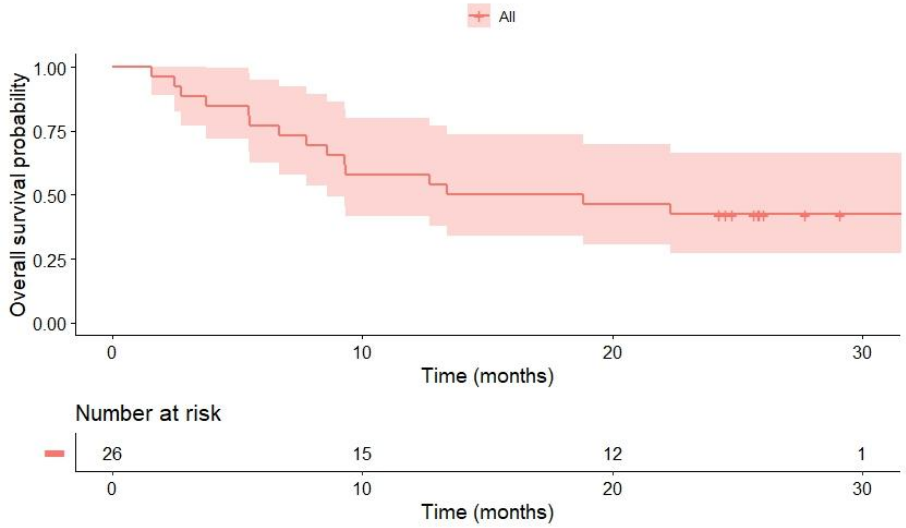

Figure 9 ***Kaplan-Meier curve for OS in the NSCLC group.*** Median OS was 13.36 months from the date of diagnosis. OS – overall survival, NSCLC – non-small cell lung cancer (Original figure prepared by the author for this thesis)

We observed a tendency for prolonged OS in patients who had high levels of anti-ceramide antibody (8.6 months [1.56-29.13 months] vs. 24.3 months [2.5-33.63 months], patients with low vs. high anti-ceramide antibody levels, respectively, p=0.098) (Figure 10). ROC analysis was used to calculate the cutoff value for anti-ceramide antibodies. The area under the curve (AUC) was 0.788 (95% CI: 0.668, 0.908). Two optimal cutoff values were determined using the negative diagnostic likelihood ratio (DLR−): at a cutoff of 184.5, sensitivity was 81.3% and specificity was 37.5%, with a DLR− of 0.5. At a cutoff of 270.3, specificity reached 100%, but at the cost of sensitivity dropping to 50%, maintaining the same DLR− of 0.5. Based on this analysis, we chose the 184.5 ng/ml value as cutoff, therefore patients who were true positives were considered to have high levels of anti-ceramide antibody, and false negatives were considered low level patients (104).

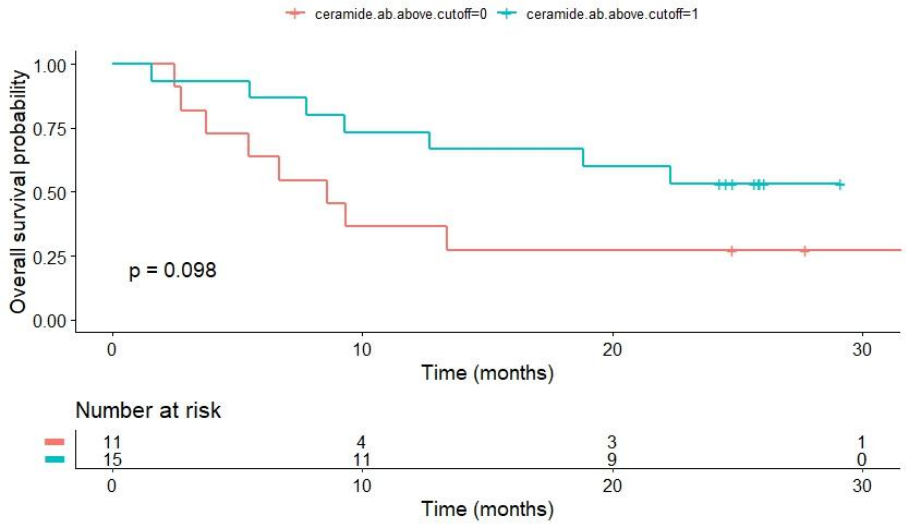

Figure 10 ***Kaplan-Meier curve shows OS of patients with NSCLC according to anti-ceramide antibody levels.*** Patients with high levels of anti-ceramide antibody had a tendency for prolonged OS (8.6 months [1.56–29.13 months] vs. 24.3 months [2.5–33.63 months], patients with low vs. high anti-ceramide antibody levels, respectively, p=0.098). Patients were classified as having high anti-ceramide antibody levels if they were higher than 184.5 ng/ml [68.81-496.21 ng/ml] based on ROC analysis. OS – overall survival, NSCLC – non-small cell lung cancer, ROC – receiver operating characteristic (104)

S1P levels did not affect OS (9.3 months [2.77-33.63 months] vs. 22.3 months [1.56-29.1 months] in the low S1P and high S1P groups, respectively, p=0.17). The median value of S1P concentrations (3926.52 ng/ml [374.34-4532.19 ng/ml]) was used as cutoff (Figure 11) (104).

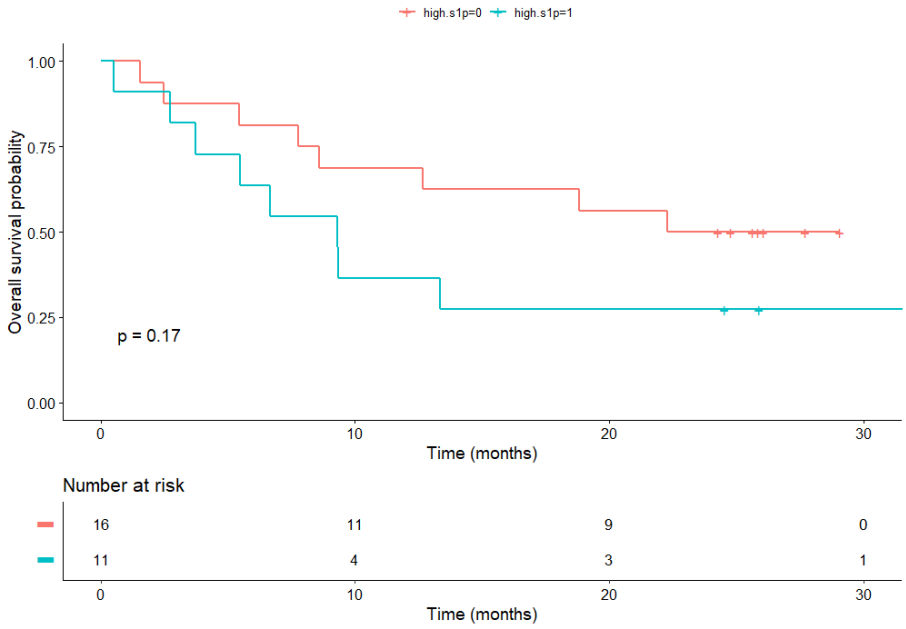

Figure 11 ***Kaplan Meier curve shows OS of patients with NSCLC according to S1P levels.*** We found no significant difference in OS (9.3 months [2.77-33.63 months] vs. 22.3 months [1.56-29.1 months] in the low S1P and high S1P groups, respectively, p=0.17). The median value of S1P concentrations 3926.52 ng/ml [374.34-4532.19 ng/ml] was used as cutoff. OS, overall survival; NSCLC, non-small cell lung cancer; S1P, sphingosine-1-phosphate (104)

4.2. OSA study

4.2.1. Demographic characteristics

Subject characteristics are summarized in Table 3. Patients diagnosed with OSA were older, had higher BMI, higher prevalence of hypertension, more elevated CRP, triglyceride levels, AHI and ODI and lower high-density lipoprotein-cholesterol (HDL-C) levels. There was no significant difference between the ESS scores and in the distribution of diabetes mellitus type 2 or cardiovascular disease (106).

Table 3 ***Basic characteristics of controls and patients with OSA.*** Data are median [range] or numbers (%). OSA – obstructive sleep apnea, BMI – body mass index, ESS – Epworth Sleepiness Scale, HDL-C – high-density lipoprotein-cholesterol, LDL-C – low-density lipoprotein-cholesterol, CRP – C-reactive protein, AHI – apnea-hypopnea index, ODI – oxygen desaturation index, TST – total sleep time, SPTi – sleep period time, MinSatO2 – minimum oxygen saturation, TST90% - total sleep time spent with oxygen saturation below 90% (106)

	Control (n=37)	***OSA (n=31)***	***p-value***
Age, years	47 [20-74]	60 [34 – 69]	< 0.001
Male	12 (32.43)	14 (45.16)	0.4
BMI, kg/m^2	24.34 [17.21-41.01]	31.22 [20.82 - 47.86]	< 0.001
Hypertension	15 (40.54)	21 (67.74)	0.04
Diabetes	2 (5.4)	6 (19.35)	0.16
Cardiovascular disease	2 (5.4)	5 (16.13)	0.15
ESS	6.21 ± 3.74	5.68 ± 3.17	0.57
Total cholesterol, mmol/l	5.34 ± 0.97	5.23 ± 1.19	0.79
HDL-C, mmol/l	1.87 ± 0.58	1.45 ± 0.81	<0.001
LDL-C, mmol/l	2.99 ± 0.94	3.07 ± 0.97	0.91
Triglycerides, mmol/l	1.1 ± 0.42	1.81 ± 0.84	<0.001
CRP, mg/l	1.12 [0.05-5.59]	3.95 [0.14-45.9]	< 0.001
AHI, 1/h	2.3 [0.0-4.8]	20.4 [6.8-106.7]	< 0.001
ODI, 1/h	0.8 [0.0-4.0]	19.4 [2.1-105.2]	< 0.001
TST, min	404.5 [278.0-486.5]	409.25 [101.5-504.0]	0.58
SPTi, min	426.5 [288.0-538.0]	435.5 [109.5-519.0]	0.87
MinSatO2, %	91 [87-95]	84 [52-94]	< 0.001
TST90%	0.0 [0.0-1.0]	3.6 [0.0-75.1]	< 0.001
Anti-ceramide antibody, ng/ml	209.55 [36.02-1725.29]	892.17 [4.02-1494.21]	< 0.001
S1P, ng/ml	290.35 [41.68-1760.0]	1760.0 [99.13-1760.0]	< 0.001

4.2.2. Anti-ceramide antibody levels in OSA patients

Anti-ceramide antibody levels were significantly elevated in OSA patients (892.17 ng/ml [4.02-1494.21 ng/ml] vs. 209.55 ng/ml [36.02-1725.29 ng/ml], patients with OSA vs. controls, respectively, ($p<0.001$) (Figure 12). The difference remained significant following adjustment for age, gender, and BMI ($p<0.001$) (106).

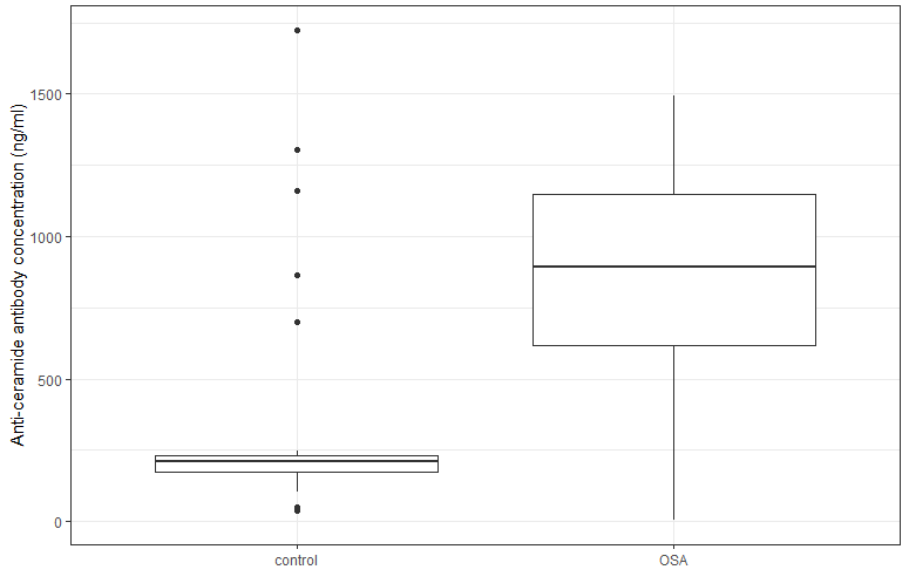

Figure 12 ***Plasma anti-ceramide antibody levels.*** Anti-ceramide antibody concentrations were significantly higher in OSA patients (892.17 ng/ml [4.02-1494.21 ng/ml] vs. 209.55 ng/ml [36.02-1725.29 ng/ml], patients with OSA vs. controls, respectively, $p<0.001$). OSA – obstructive sleep apnea (106)

4.2.3. Clinical variables and anti-ceramide antibody levels in OSA patients

In terms of clinical variables, we found significant correlation with anti-ceramide antibodies. BMI (ρ=0.25, p=0.04), CRP (ρ=0.36, p=0.005), AHI (ρ=0.43, p<0.001), ODI (ρ=0.43, p<0.001) and TST90% (ρ=0.35, p=0.004) showed positive correlation, while the lowest levels of oxygen saturation (MinSatO2) had a significant negative correlation (ρ=-0.37, p=0.001) (106).

Patients were divided into subgroups according to disease severity: mild (n=8), moderate (n=14) and severe (n=9) OSA. Anti-ceramide antibody levels were elevated in all OSA severity subgroups when compared to controls (p=0.02, p=0.02, p=0.008 for mild, moderate and severe subgroups, respectively) (Figure 13). However, when comparing the OSA subgroups, no correlation was detected based on markers of disease severity (106).

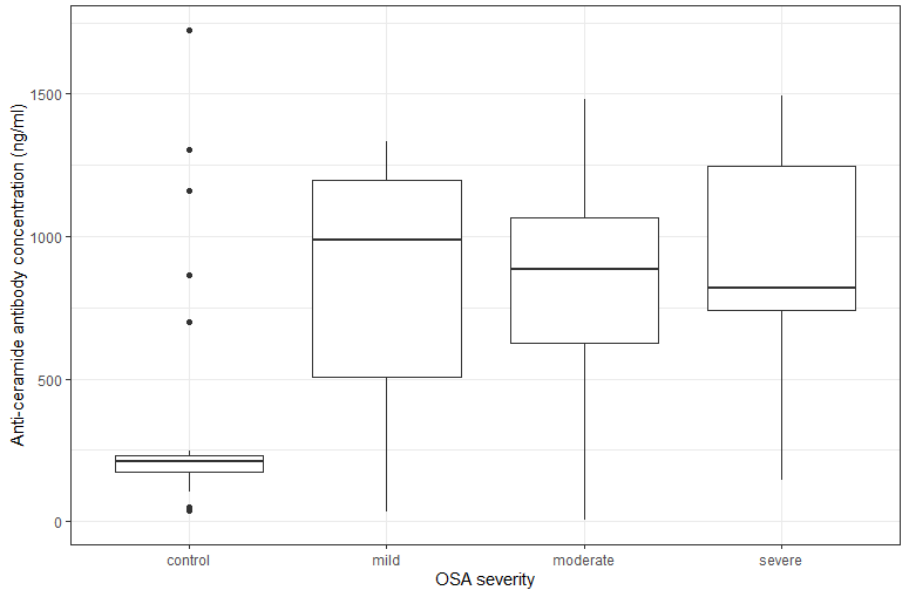

Figure 13 ***Anti-ceramide antibody levels according to OSA severity.*** Anti-ceramide antibody levels were elevated in all OSA severity subgroups when compared to controls (p=0.02, p=0.02, p=0.008 for mild, moderate and severe subgroups, respectively). OSA – obstructive sleep apnea (106)

4.2.4. S1P levels in OSA patients

Subjects in the OSA group had significantly higher S1P levels (1760.0 ng/ml [99.13-1760.0 ng/ml] vs. 290.35 ng/ml [41.68–1760.0 ng/ml] in patients with OSA vs. controls, respectively, p <0.001) (Figure 14). After adjustment for age, sex and BMI, the difference was still significant (p<0.001). Most S1P concentrations were above the higher detection limit of the ELISA kit, therefore these values were considered as the maximal mediator concentration. Due to this, further subgroup or correlation analysis was not feasible (106).

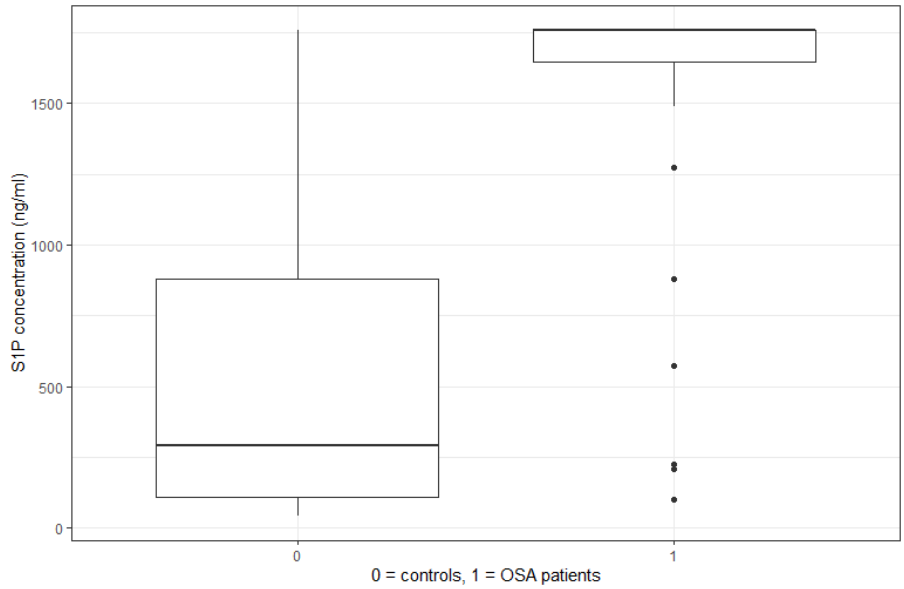

Figure 14 ***Plasma S1P levels.*** S1P levels were significantly higher in OSA patients (1760.0 ng/ml [99.13-1760.0 ng/ml] vs. 290.35 ng/ml [41.68-1760.0 ng/ml] in patients with OSA vs. controls, respectively, p <0.001). However, most values in the OSA group were above the upper limit of the ELISA assay. S1P – sphingosine-1-phosphate, OSA – obstructive sleep apnea, ELISA – enzyme-linked immunosorbent assay (106)

5. DISCUSSION

Besides prevention, early detection of diseases is the major factor for proper treatment. The studies that make the basis of this doctoral thesis focus on the possible involvement of sphingolipid molecules and their utility as biomarkers in two lung diseases where chronic inflammation plays a central role: advanced NSCLC and OSA. By measuring S1P and anti-ceramide antibody levels, we addressed the proinflammatory and pro-survival aspects of the sphingolipid rheostat.

The involvement of sphingolipid metabolism has been detected in many fundamental physiological processes, including the regulation of cell fate and inflammation. Consequently, its disturbance leads to the development of diseases. Chronic inflammation is known to be the basis of many pathological processes including OSA and NSCLC. Several common pathways have been identified in the pathology of these diseases and sphingolipid metabolism (11, 55).

5.1. The role of anti-ceramide antibodies in NSCLC and OSA

Our study is the first to demonstrate elevated circulating anti-ceramide antibody levels in both unresectable/advanced NSCLC and OSA. Although the underlying pathophysiology of these conditions differs – malignant transformation and progression in NSCLC, versus intermittent hypoxia, systemic inflammation, and metabolic dysregulation in OSA – our findings suggest a broader role of altered sphingolipid metabolism and immune recognition of ceramide in chronic inflammatory, hypoxia-related, and proliferative disease states.

In NSCLC, increased anti-ceramide antibody levels were detected in both plasma and BWF samples. The elevation in BWF indicates possible local production within the TME. Given that ceramides exert predominantly tumor-suppressive, pro-apoptotic, and anti-proliferative effects, we hypothesize that these antibodies may neutralize ceramide-mediated pro-apoptotic effects, thereby promoting tumor survival, similar to a previous study where anti-ceramide antibodies mitigated radiation-induced gastrointestinal apoptosis in murine models (51). Antibody levels were independent of KRAS mutation, PD-L1 status and COPD, suggesting a tumor-associated rather than a genotype- or comorbidity-driven mechanism. CRP, a non-specific marker of inflammation, was also elevated in NSCLC patients. Since active infection was an exclusion criterion at the time

of sample collection, CRP elevation was possibly the result of cancer-associated chronic inflammation (109).

The novelty of this observation is considerable, as to our knowledge anti-ceramide antibodies have not previously been reported in NSCLC. Analogies can be drawn from other studies investigating antibody–sphingolipid interactions in disease: for example, the presence of anti-ganglioside antibodies in Guillain–Barré syndrome (110) or in multifocal motor neuropathy (111), and anti-GD2 antibodies currently under investigation in cancer immunotherapy (112). Whether anti-ceramide antibodies in NSCLC contribute directly to immune evasion by disabling ceramide-mediated apoptosis, or are secondary byproducts of altered tumor lipid metabolism, remains to be determined. In situ localization studies, such as detection of anti-ceramide antibodies with IHC staining on tumor samples, will be necessary to confirm production sites and potential functional effects.

In OSA, anti-ceramide antibody elevation was observed even in mild disease. There was a significant correlation between antibody levels and CRP, BMI, and markers of overnight hypoxia. This pattern supports the link between chronic intermittent hypoxia–induced systemic inflammation (113), ceramide upregulation, and subsequent immune response (88). The correlation with BMI is consistent with previous findings of ceramides functioning as nutrient sensors and their accumulation during lipid overload (91, 114); however, the persistence of the anti-ceramide antibody elevation after adjustment for BMI indicates that obesity is not the sole driver.

Mechanistically, antibodies may act as a counter-regulatory response to ceramide-induced apoptosis in endothelial and other tissues, possibly altering membrane raft signaling properties. This is supported by evidence that IH-induced ceramide synthesis is linked to Bax-dependent mitochondrial apoptosis (115) and that survivin, an inhibitor of apoptosis, is reduced in OSA (39). As in NSCLC, the functional direction – protective versus pathogenic – of these antibodies remains uncertain. They could theoretically mitigate tissue injury by neutralizing ceramide's pro-apoptotic signals or, conversely, interfere with beneficial apoptotic clearance of damaged cells.

Taken together, the elevation of anti-ceramide antibodies in both NSCLC and OSA points to a shared upstream trigger – persistent inflammation and cellular stress leading to

ceramide accumulation – followed by an immune response. While in NSCLC this may facilitate tumor progression, in OSA it may serve as a maladaptive or compensatory response to chronic vascular and metabolic stress. These antibodies may thus represent a unifying biomarker of chronic disease states with altered sphingolipid metabolism.

5.2. The role of S1P in NSCLC and OSA

S1P levels were significantly elevated in both NSCLC and OSA, highlighting its potential as a sensitive, non-invasive biomarker in distinct but inflammation-linked conditions. Our data suggest that increased sphingolipid turnover is a shared feature of both diseases.

In NSCLC, S1P elevation may reflect tumor-induced reprogramming of sphingolipid metabolism, shifting from pro-apoptotic ceramides towards pro-survival S1P. S1P levels were independent of KRAS mutation, PD-L1 status, and COPD, suggesting its potential as a general biomarker of tumor metabolic activity rather than a marker tied to specific genetic subtypes or inflammatory lung conditions. To our knowledge, our study is the first to quantify S1P levels specifically in unresectable and advanced NSCLC, adding to previous literature in other cancers, including hepatocellular (116), ovarian (117), lung (118, 119) and breast carcinoma (20, 120), where S1P elevation has been documented and, in some cases, associated with early disease detection or progression.

In OSA, S1P elevation is likely driven by mechanisms distinct from those in cancer. Red blood cells, platelets, and endothelial cells are major sources of circulating S1P (21), and both platelet activation and endothelial dysfunction are intensified in OSA (87, 121). Intermittent hypoxia and recurrent arousals lead to sympathetic activation, oxidative stress, and systemic inflammation (86, 113), all of which promote platelet activation and S1P release (21). S1P's role in OSA may be multifaceted: it can mediate immune cell recruitment (122), sustain chronic inflammation (71), regulate vascular tone, permeability (123), and steroidogenesis (124, 125) —pathways relevant to OSA-related hypertension, insulin resistance and cardiovascular disease (126, 127). S1P was shown to activate cyclooxygenase-2 (COX-2) via IL-6 and TNF-α (128), which was also implicated in the association between OSA and cancer (129). S1P also plays a dual role in atherosclerosis. Pro-atherogenic actions of S1P have been shown to mediate through S1PR3 via recruitment of monocytes and macrophages to atherosclerotic lesions and inhibition of smooth muscle cell proliferation and migration (130). At the same time, when bound to

ApoM+HDL, S1P can activate S1PR1 on endothelial cells, supporting vascular integrity (22) and potentially counteracting microvascular injury in OSA (131). Given its dual pro- and anti-inflammatory actions depending on receptor subtype and lipoprotein carrier, S1P may contribute both to vascular injury and to protective endothelial responses in OSA.

The parallel increase of S1P in NSCLC and OSA supports the hypothesis that sustained systemic inflammation and cellular stress drive sphingolipid pathway activation regardless of the primary disease trigger. However, the functional consequences of S1P elevation may differ: in NSCLC, fostering tumor progression; in OSA, contributing to both harmful vascular remodeling and possible compensatory vascular protection.

5.3. Integrated perspective and implications

The consistent elevation of both anti-ceramide antibodies and S1P in NSCLC and OSA indicates that dysregulated sphingolipid metabolism is a converging pathway in diseases with otherwise distinct primary etiologies but shared features of chronic inflammation, hypoxia, and systemic stress. While the specific drivers and functional outcomes differ – tumor progression in NSCLC versus cardiovascular and metabolic complications in OSA – the biochemical signature overlaps.

From a translational standpoint:

- Anti-ceramide antibody may serve as a novel biomarker of ceramide-targeted immune responses, with possible implications for therapeutic strategies involving ceramide analogues or inducers, both in oncology and in metabolic/inflammatory disease.

- S1P may function as both a diagnostic biomarker and a therapeutic target. Given that S1P pathway modulators (e.g., S1PR modulators, Sphk inhibitors) are already in clinical use for other conditions, repurposing them could be explored in NSCLC or OSA if mechanistic studies support a causal role.

Future research should prioritize longitudinal and interventional designs:

- In NSCLC, serial measurement of these biomarkers during therapy could clarify their prognostic and predictive value.

- In OSA, assessing biomarker dynamics before and after CPAP therapy or weight loss could determine reversibility and causal contribution to comorbidities.

- Mechanistic studies should address whether these molecules act primarily as pathogenic drivers, protective modulators, or bystanders of disease processes.

5.4. Limitations

Our studies have some notable limitations, mainly due to the limited number of subjects. Although the sample sizes in both studies were determined based on power calculations to detect differences in anti-ceramide antibody and S1P levels between groups, the overall number of subjects remains low. This limitation is particularly relevant for subgroup analyses and correlation assessments, rendering the survival data in the NSCLC study preliminary and exploratory. Therefore, further studies are needed with bigger sample sizes to confirm our results.

Regarding the NSCLC study, the differences between the control and NSCLC groups in age, smoking status and COPD might lead to bias. Furthermore, the study design is not suitable for determining the prognostic value of possible biomarkers S1P and anti-ceramide antibody, therefore further investigation is needed ie. in early stages of NSCLC, after completion of first line therapy.

In the OSA study, the study design limits the ability to establish a causal relationship between OSA and sphingolipid alterations. Another concern is the accumulation of ceramides in adipose tissue in obesity, which might cause a bias in the interpretation of our results. Although, elevated anti-ceramide antibody levels in OSA were independent of BMI, comparison should be performed in lean subjects with BMI<25 kg/m^2 as well. Finally, while both PSG and cardiorespiratory polygraphy were used for diagnostic testing, the latter may underestimate disease severity by failing to detect arousal-related hypopneas and potentially overestimating total sleep time (132), which could affect correlations with disease severity.

6. CONCLUSIONS

Our results show that sphingolipid metabolism is altered in lung diseases. We specifically investigated molecules S1P and anti-ceramide antibody which act on the pro-survival and proinflammatory part. In recent decades, advances in lipid biology have revealed this vast class of lipid molecules that serve not only as structural components of cellular membranes but also as bioactive mediators involved in key signaling pathways regulating essential cellular processes, including cell fate. The pathogenesis of both OSA and NSCLC is closely linked to chronic inflammation. Perturbations in sphingolipid metabolism have been implicated in sustaining a chronic inflammatory state. Furthermore, the regulation of cell survival and apoptosis through the balance of the sphingolipid rheostat has emerged as a critical factor in cancer development.

Our NSCLC study focused on patients with unresectable and advanced stage disease, in whom we detected both plasma S1P and anti-ceramide antibody levels to be significantly elevated. Measurements were performed prior to the initiation of therapy and our results are consistent with previous findings that pro-survival processes are upregulated in cancer. Additionally, anti-ceramide antibody levels were elevated in bronchial samples suggesting possible local production within the TME. Notably, higher anti-ceramide antibody levels showed a tendency for prolonged OS, which is a preliminary observation that warrants further investigation in larger cohorts.

Despite advances in cancer screening methods, lung cancer is still predominantly diagnosed at an advanced stage. Therefore, novel diagnostic approaches and sensitive biomarkers are needed for implementation in routine clinical practice. The findings of our study emphasize the potential of sphingolipids as possible sensitive and non-invasive biomarkers in NSCLC. Certainly, further studies are needed with a statistically more powerful cohort to confirm our results, and interventional studies may also be justified to assess the prognostic value of these markers. Besides the potential of sphingolipids as biomarkers, they are also investigated as targets in cancer therapy. Understanding the complex mechanisms by which sphingolipids mediate cancer cell fate might improve anticancer therapy.

OSA is chronic, highly prevalent sleep-related disorder that poses a significant public health concern due to the associated comorbidities. IH induced systemic inflammation is the major contributor to the pathology behind OSA. The metabolic pathways driving the inflammation that contributes to microvascular damage are not yet fully understood. While there are known overlaps in OSA and sphingolipid metabolism, the potential involvement of these molecules in OSA has not yet been investigated. We found that both S1P and anti-ceramide antibody levels were significantly elevated in patients with OSA. These findings align with the known diverse proinflammatory and immunomodulatory effects of sphingolipid metabolism. Furthermore, anti-ceramide antibody levels correlated with clinical variables, including a positive correlation with BMI. Ceramides have been shown to accumulate in white adipose tissue due to increased substrate uptake and alter cellular metabolism. However, the relevance of this finding is controversial, since the increase in anti-ceramide antibody levels remained significant after adjustment for BMI. Additional studies are needed to clarify the impact of obesity on altered sphingolipid metabolism in OSA. We also detected positive correlation of anti-ceramide antibody levels with AHI. Although anti-ceramide antibody levels were significantly increased even in mild OSA, there was no difference between OSA severity groups. Our findings support the possible involvement of sphingolipid metabolism in OSA and justify the need for further studies to reveal the exact role of sphingolipids in its pathogenesis.

In conclusion, the biomarker-based synthesis of our findings underscores the importance of sphingolipid metabolism as a cross-cutting biological axis linking cancer, sleep-disordered breathing, and potentially other chronic inflammatory conditions. This opens new possible perspectives for biomarker-based diagnostics and therapeutics that target lipid signaling pathways.

7. SUMMARY

Sphingolipid metabolism is a complex system regulating important cellular processes including cell fate and inflammation. The interconnected network of sphingolipids, the many enzymes that regulate their interconversion and the compartment-specific roles all contribute to the heterogeneity of their biological actions. Besides mediating indispensable biological processes, the perturbation of sphingolipid metabolism contributes to several diseases including cancer, inflammation, neurodegenerative disorders and metabolic disorders such as diabetes and atherosclerosis.

This thesis explores the possible role of sphingolipid metabolism – in particular the involvement of the sphingolipid rheostat – in two highly prevalent lung diseases, OSA and NSCLC, with a focus on the proinflammatory and pro-survival aspects. Significant elevation of both S1P and anti-ceramide antibody levels were observed in our studies.

In the NSCLC cohort, which included patients with unresectable, advanced-stage disease, increased pre-treatment S1P levels were consistent with previous studies implicating the role of S1P in promoting tumor cell survival and progression. As reliable biomarkers are needed in lung cancer diagnosis, S1P might be a promising non-invasive diagnostic biomarker based on our results. Anti-ceramide antibody levels were also elevated in NSCLC patients, both in circulation and in bronchial samples, suggesting that anti-ceramide antibody might be produced by the tumor and its microenvironment. We hypothesize that anti-ceramide antibodies might have a neutralizing effect against pro-apoptotic signaling of ceramides. Furthermore, higher anti-ceramide antibody levels showed a tendency for prolonged OS. Our results make a basis for following investigations exploring the diagnostic and prognostic value of sphingolipids in NSCLC.

In OSA, where chronic inflammation and microvascular damage are central to disease pathology, our findings similarly demonstrated elevated S1P and anti-ceramide antibody levels, even in patients with mild disease. Anti-ceramide antibodies positively correlated with BMI, suggesting an interaction with obesity-related metabolic alterations. However, the significance of this correlation remains uncertain, as elevated antibody levels persisted after adjusting for BMI. Based on our results, altered sphingolipid metabolism might possibly mediate pathological processes in OSA.

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9.2. Publications not related to the subject of the thesis

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9.3. Presentations/abstracts related to the subject of the thesis

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