Targeting Lipids to Regulate Endothelial Cell Senescence

Xingxing Han

A thesis submitted in fulfilment of the requirements for the degree of

Master of Philosophy (Medicine and Health)

Centenary Institute of Cancer Medicine and Cell Biology

Faculty of Medicine and Health

The University of Sydney

2023

Thesis statement of originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Xingxing Han

22th Jun 2023

Abstract

Endothelial cell senescence, marked by irreversible growth arrest, is a major factor in vascular aging and cardiovascular disease, such as atherosclerosis. One of the key drivers of senescence is oxidative stress, which leads to the activation of various signalling pathways involved in senescence progression, including p53/$p21^{WAF1/CIP1}$ and $p16^{INK4A}$/Rb pathways. Hydrogen peroxide (H_2O_2) is commonly used to achieve oxidative stress-induced premature senescence *in vitro*. Despite progress, the exact molecular mechanisms and regulatory factors governing this process are not fully understood.

Sphingolipids have drawn attention due to their potential role in controlling endothelial cell senescence. These diverse membrane-constituting and bioactive lipids regulate various cellular processes, including cell survival, proliferation, and inflammation. Disruption in sphingolipid metabolism and signalling have been implicated in the development of age-related diseases. Hence, understanding sphingolipid's role in endothelial cell senescence is crucial for unravelling cardiovascular disorders.

In this thesis, I aim to investigate the key sphingolipids that dictate premature senescence in endothelial cells induced by H_2O_2. To assess this, I employed several sphingolipid compounds and their metabolic enzyme inhibitors to alter the levels of certain sphingolipid species. I found increasing sphingosine levels by the addition of exogenous sphingosine or

inhibition of its catabolic enzyme sphingosine kinase 1 drove the establishment of endothelial cell senescence. Conversely, reducing sphingosine levels by inhibition of ceramide synthase or acid ceramidase inhibited H_2O_2-induced endothelial cell senescence.

This thesis identified sphingosine, but not ceramide or sphingosine 1-phosphate, as the key determinant of endothelial cell senescence, paving the way for future research into the molecular mechanisms and guiding the development of sphingosine-targeted senolytic therapeutics.

Acknowledgement

A research degree is challenging; however, it was a valuable experience in my life. I would like to express my gratitude to those who supported me during my candidature.

First of all, I would like to express my deepest gratitude to my supervisor Dr. Yanfei (Jacob) Qi, who led my way into the research field. My supervisor has provided me with excellent research mentoring. During my early days in the lab, I am immensely grateful to my supervisor for organizing training sessions for me, which played a crucial role in laying a strong foundation for my future projects. My supervisor also provides me opportunities to learn cutting-edge technologies, collaborate with other researchers, attend conferences, and engage in scientific discussions. These experiences have broadened my horizons, expanded my scientific network, and enhanced my overall research experience. I would also like to acknowledge his patience, encouragement, and continuous availability to discuss ideas, provide constructive suggestions, and offer guidance during challenging moments. His mentorship has not only nurtured my academic growth but also motivated me to strive for excellence. It is a true honour to have had him as my supervisor, and I extend my heartfelt gratitude for his exceptional guidance throughout this journey.

I would also like to express my gratitude to my co-supervisor, Prof. Jennifer Gamble. Her valuable suggestions and guidance have greatly contributed to the progress of my work and

have provided valuable directions for improvement. Additionally, thanks for her and her lab ensuring an uninterrupted supply of HUVEC cells and other experimental materials have been instrumental in enabling smooth and continuous progress in our research endeavours. Furthermore, I am also grateful for another co-supervisor Dr. Mary Kavurma. Her guidance, suggestions, and advice have significantly enhanced the quality of my work and provided valuable insights for my improvement.

I would thank other members of the team, Dr. Long Hoa Chung, Xin Liu, Darren Liu, Jerry Huang, and Celia Jiang, who have provided valuable assistance, advice, and support at various stages of my research journey.

I also like to thank the people at Centenary Institute, Charles Perkin Centre, and the University of Sydney. I deeply thank all the members from vascular biology group and Brain Lipidomics and Metabolism group for their friendship, encouragement, and technical expertise. Thanks for Dr. Paul Coleman and Dr. Ka Ka Ting for their valuable guidance and support on my projects. Thanks for Ms Dilini Gunatilake for ensuring the availability of essential research materials. Thanks for Dr. Collin Tran and Dr. Jonathan Teo for their shared knowledge and support. I would also like to acknowledge Sydney cytometry, Centenary animal house, technical support services team of Charles Perkins Centre Hub, who kindly trained me critical experimental techniques.

Last but not least, I would like to extend my heartfelt appreciation to my husband Yang Liu, my parents Yuchun Han and Yumei Wang, for their unwavering love, support, and encouragement throughout my academic journey. Their constant belief in my abilities and their sacrifices have been the motivation of my dream.

Table of Contents

List of Figures

List of Tables

Abbreviation

CerS1	Ceramide Synthase 1
CerS2	Ceramide Synthase 2
CerS3	Ceramide Synthase 3
CerS4	Ceramide Synthase 4
CerS5	Ceramide Synthase 5
CerS6	Ceramide Synthase 6
FA	Fatty Acyls
$^{\bullet}OH$	Hydroxyl Radicals
53BP1	P53-Binding Protein 1
8-OH-G	8-Hydroxyguanosine
ACER1	Alkaline Ceramidase 1
ACER2	Alkaline Ceramidase 2
ACER3	Alkaline Ceramidase 3
AD	Alzheimer's Disease

ASAH1	N-Acylsphingosine Amidohydrolase 1
ASAH2	N-Acylsphingosine Amidohydrolase 2
C1P	Ceramide-1-Phosphate
C1PP	C1P Phosphatase
CDKI	Cyclin-Dependent Kinase Inhibitor
Cer	Ceramide
CERK	Ceramide Kinase
CerSs	Ceramide Synthases
DAG	Diacylglycerol
DDR	Damage Response
Des	Desaturases
dhCer	Dihydroceramide
EC	Endothelial Cells
EC_{50}	Half-Maximal Effective Concentrations
EDHF	Endothelium-Derived Hyperpolarizing Factor

ER	Endoplasmic Reticulum
ET-1	Endothelin 1
FB1	Fumonisin B1
GL	Glycerolipids
GMZ	Gemcitabine
GP	Glycerophospholipids
GPCRs	G-Protein-Coupled Receptors
hAD-SCs	Human Adipose-Derived Stromal Cells
HDF	Human Diploid Fibroblasts
HFD	High-Fat Diet
hTERT	Human Telomerase Reverse Transcriptase
HUVEC	Human Umbilical Vein Endothelial Cells
IC_{50}	Half Maximal Inhibitory Concentration
ICAM1	Intercellular Adhesion Molecule 1
ILCNC	International Lipid Classification and Nomenclature Committee

KDSR	3-Keto-Dhphingosine Reductase
LDL	Low-Density Lipoprotein
MAPK	Mitogen-Activated Protein Kinase
MCP1	Monocyte Chemoattractant Protein 1
MiDAS	Mitochondrial Dysfunction-Associated Senescence
NcDase	Neutral Ceramidase
NF-κB	Nuclear factor kappa B
N-SMase	Neutral Sphingomyelinase
$^{\bullet}O_2^-$	Superoxide Radicals
OGD/R	Oxygen-Glucose Deprivation and Reoxygenation
OIS	Oncogene-Induced Senescence
oxLDL	Oxidized Low-Density Lipoprotein
PK	Polyketides
PKC	Inhibiting Protein Kinase C
PR	Phenol Lipids

p-RB	Phosphorylated Retinoblastoma Protein
PS	Premature Senescence
PS1	Presenilin 1
PUFA	Polyunsaturated Fatty Acid
RB	Retinoblastoma Protein
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
SA β-Gal	Senescence-Associated B-Galactosidase
SaL	Saccharolipids
SAPK	Stress-Activated Protein Kinase
SASP	Senescence-Associated Secretory Phenotype
SBB	Sudan-Black-B
SIPS	Stress Induced-Premature Senescence
siRNA	Small Interfering RNA
SL	Sphingolipids

SMS	Senescence-Messaging Secretome
SOD	Superoxide Dismutase
Sph	Sphingosine
SphK1	Sphingosine Kinase 1
SphK2	Sphingosine Kinase 2
SphKs	Sphingosine Kinases
SPL	S1p Lyase
SPPs	S1p Phosphatases
SPT	Serine Palmitoyltransferase
ST	Sterol Lipids
TLC	Tram-Lag1-Cln8
TNBS	Trinitrobenzene Sulfonic Acid
TNF	Tumour Necrosis Factor
TNFα	Tumor Necrosis Factor Alpha
TRAF2	Tumor Necrosis Factor Receptor-Associated Factor 2

VCAM1	Vascular Cell Adhesion Protein 1
VEGF	Vascular Endothelial Growth Factor
VSMCs	Vascular Smooth Muscle Cells
γ-H2AX	Phosphorylated Histone H2AX

Chapter 1. Literature Review

1.1 Endothelial Cell Senescence

1.1.1 Cellular senescence

Senescence occurs in a biological process when cells permanently cease dividing in reaction to diverse stress signals (1). The phenomenon of limited proliferation, referred to as 'cellular senescence', had been coined over half a century ago by Leonard Hayflick and Moorhead, who observed a significant decrease in the capacity of human fibroblasts to proliferate in culture (2). Subsequent experiments demonstrated that cell proliferation decreased with each cell passage until the cells lost their capacity to divide completely, despite the existence of nutrients and growth factors (2). Such a phenomenon led to the coining of the term 'cellular senescence', which has been linked to the ageing process (2). Hayflick's research has shown that conventional human cells in culture are constraint in their capacity to divide, referred to as replicative senescence, and stop the process of replication and division after 40 to 60 times of serial passaging, a phenomenon known as the Hayflick Limit (3, 4).

It is now widely accepted that replicative senescence, resulting from telomere shortening, is one form of cellular senescence. On top of that, multiple inducers exist, and they can all result in senescence, involving oncogene-induced senescence (OIS), DNA damage-

induced senescence, epigenetically triggered senescence, as well as mitochondrial dysfunction-associated senescence (MiDAS), in replication-competent cells. These forms of senescence are collectively referred to as premature senescence (5, 6).

1.1.2 Characteristics of endothelial cells senescence

The vascular endothelium is a monolayer of endothelial cells that lines the inner surface of blood vessels (7). It is critical in sustaining vascular homeostasis through regulating blood fluidity controlling vascular tone, modulating the production of pro-inflammatory molecules, facilitating pro-inflammatory immune responses, and promoting neovascularization (7). As a dynamic interface between the blood and surrounding tissues, the vascular endothelium is essential for the proper functioning of the cardiovascular system (7). However, endothelial cells are vulnerable to injury from circulating substances and pathogenic stressors, which can disrupt blood flow and barrier function, leading to tissue and organ dysfunction (8). Additionally, pro-inflammatory cytokines and chemokines produced by endothelial cells can send harmful signals in different types of cells found in blood vessels and in non-blood organs and tissues (8).

Endothelial cell senescence, depicted by permanent cell cycle arrest, is a protective mechanism when the endothelium has been damaged, preventing the unrestrained proliferation of damaged cells (9). Structure of the vascular system and the turnover rate

of endothelial cells are believed to contribute to the development of senescence in these cells (5, 10). Endothelial cells have been commonly regarded as one of the first cell types undergoing senescence as individuals age, and the highest number of senescent cells are seen in highly endothelial tissues (5).

When endothelial cells undergo senescence, they display several common features, which are summarized below (11):

1.1.2.1 Morphological changes

In comparison to cells undergoing proliferation, senescent cells frequently have an enlarged size as well as a flattened morphology (1, 11), as evidenced by previous studies (11). Senescent cells may also possess multiple nuclei and display significant vacuolization that the unfolded protein reaction causes endoplasmic reticulum stress. The state of the scaffolding protein caveolin 1 (12) and the Rho GTPases Rac1 and CDC42 are crucial for the occurrence of shape changes in senescent cells (13). In addition, senescent cells have been observed to transmit signals to other cells through the transfer of proteins across cytoplasmic bridges (14).

Senescent endothelial cells exhibit a distinctive morphological phenotype characterized by a larger, flatter shape and a reduced capacity to change their phenotype or realign as a reaction to laminar shear stress (15). Biochemically, senescent endothelial cells enhance

their adherence to basement membranes, which impedes their proper alignment with blood flow (15). This increased adhesion leads to resistance to detachment and enables cells to survive for prolonged periods (11, 15).

1.1.2.2 Cell cycle arrest

Senescent cells are depicted by a permanent cessation of proliferation (16). Unlike quiescence, which is a reversible growth arrest occurring in the G0 phase, G1 and G2 are the stages of the cell cycle when senescence often takes place (1, 16). In addition, senescent cells have lost their ability to respond to mitogenic or growth factor stimulation. This means that even when circumstances are optimal for cell development, they have no way of restarting the cell cycle (1). Cells that have reached their final differentiation stage, like senescent cells, are irreversibly prevented from re-entering the cell cycle (1, 5). Senescence is typically induced in response to cellular stress, while terminal differentiation results from a predetermined developmental program (1, 5).

Additionally, the activity of cell-cycle inhibitors induces the cessation of the cell cycle, which slow down the cell division process (17, 18). During this process, two major tumor suppressor pathways, p53/p21CIP1 and p16INK4a/Rb, are activated to regulate the exit from the cell cycle (16).

In the p53/p21CIP1 pathway, P53 is critical in the DNA Damage Response (DDR) pathways by regulating a transcriptional program (16). Upon receiving a DNA-damaging stimulus, P53 initiates the pathway by undergoing phosphorylation (p-p53) (16). This leads to the transient expression of cyclin-dependent kinase inhibitors (CDKIs), including p21CIP1, which accumulates due to the presence of p-p53 (16). Subsequently, p21CIP1 blocks the CDK2-cyclin E complex, allowing hypophosphorylated Rb to bind with E2F and leading to cell cycle arrest (Figure 1) (16). It is worth noting that the p53 protein undergoes various post-translational modifications, including ubiquitylation, phosphorylation, acetylation, sumoylation, methylation, and neddylation, which regulate its physiological function and activity and determine how it responds to different cellular signals (19). Acetylation of p53 enhances its protein stability, helps it attach to its specific boosters with lower affinity, promotes its interaction with other proteins, contributes to its antiviral activities, and is necessary for its DNA injury checkpoint reactions and oncogene activation (19). In the p16INK4a/Rb pathway, p16INK4A is transcribed from the CDKN2A gene, which is activated upon the INK4/ARF locus (20). p16INK4A is a cyclin-dependent kinase inhibitor (CDKI) that suppresses CDK4-CyclinD complex function, resulting in inhibition of Rb phosphorylation and preventing E2F from binding with Rb, thereby halting cell cycle progression (Figure 1) (21).

It is important to note that these two pathways do not function entirely independently; rather, crosstalk exists between certain regulatory factors (22). In fact, the p53 and Rb pathways regulate senescence by suppressing transcription factors (23). Specifically, the ultimate target of both pathways is the retinoblastoma protein (RB), existing within a hypophosphorylated form and binds to E2F (23). This binding inhibits E2F from targeting gene promoters, thereby inhibiting transcription and halting cell cycle progression (23).

The activation of p21 and p16 exhibits variability across cell types and stressors. However, in certain cell types, senescent cells expressing p21 and p16 converge in a similar manner, with injury initially causing a surge in p21 levels that subsequently decrease over a period, then an increase in p16 levels (24, 25). While the in vivo dynamics of p21 as well as p16 are unknown, animal model data indicate that stromal vascular fraction cells with elevated p21 levels accumulate in adipose tissue with obesity, which differs from cells with high p16 levels (26). Both the molecular response and cellular fate are contingent upon the type, extent, and duration of the injury (11). For instance, even minor cellular damage results in p53 stabilization, allowing for p21 transcription until the cells recover from the injury and re-enter the cell cycle (27, 28, 29). However, senescence, which is frequently accompanied by increased p16 expression, arises from delayed p21 initiation or more severe or prolonged damage (27, 28, 29).

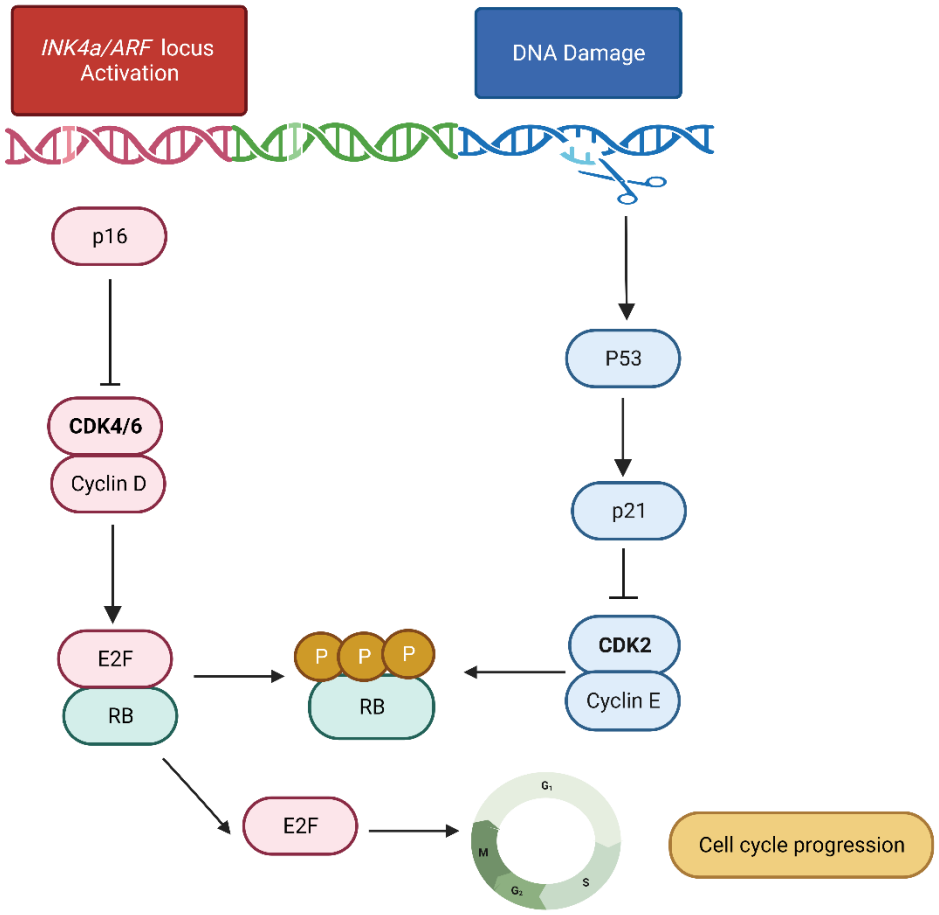

Figure 1.1 Schematic chart depicting the molecular pathway in the regulation of cell cycle arrest in senescence Cell cycle arrest can be regulated by activating either the $p16^{INK4a}$/Rb or p53/p21CIP1 pathways. Graph created by Biorender.

1.1.2.3 Senescence-Associated Secretory Phenotype (SASP)

Despite undergoing a state of permanent development stop characteristic of senescence, cells retain metabolic functions (30). A cell's ability to secrete a cocktail of chemicals that may impact the activities of neighboring non-senescent cells allows it to influence its immediate surroundings and interact with other cells (30, 31). Senescent cells exhibit significant changes in their secretome and a hypersecretory phenotype referred to as the Senescence Associated Secretory Phenotype (SASP) or Senescence-Messaging Secretome (SMS) (32), a fundamental hallmark of senescence (33, 34).

SASP comprises various types that are diverse and contingent on cell type and senescence processes. SASP is composed of several soluble signaling factors, such as proinflammatory cytokines, chemokines, angiogenic factors, growth modulators, proteases, matrix metalloproteinases, bioactive lipids, and extracellular matrix components, all constituting primary constituents of SASP (33, 35). Pathophysiological consequences of different types of senescent cells can only be mediated via SASP and is thus intricately connected to both their advantageous and harmful impacts (36, 37).

1.1.3 Causes of endothelial cell senescence

It is noteworthy that endothelial cells are situated within blood vessels, rendering them continuously exposed to oxygen, glucose, lipids, amino acid metabolites, immune cells,

reactive oxygen species (ROS), and inflammatory cytokines, all of which may contribute to senescence (38). The following sections delve into the different environmental factors that add to the process of senescence.

1.1.3.1 Physical forces

Due to their unique location, the haemodynamic environment, which refers to the physical forces and conditions exerted on endothelial cells by the flow of blood within blood vessels, are critical in endothelial cell function and can significantly impact vascular health (11). Shear stress and pulsatile pressure are two haemodynamic variables of blood flow which can accelerate EC senescence (39). Regions with dysfunctional blood flow experience high rates of endothelial cell turnover, placing these cells at an increased risk of being damaged by haemodynamic and replicative stress (39). Studies involving cell cultures and mouse arteries have yielded valuable insights into the significance of the haemodynamic environment in relation to endothelial cells. Specifically, these studies have indicated that when ECs have been exposed to disturbed flow patterns, they exhibit a higher rate of cellular turnover. This increased turnover ultimately leads to a state of replicative senescence in these cells (40, 41). In addition, findings derived from human tissue samples provide evidence that endothelial cells located near arterial bifurcations, which experience higher levels of haemodynamic stress, exhibit increased cell turnover and replicative

senescence compared to ECs from less stressed arteries, venous endothelial cells, and vascular smooth muscle cells (VSMCs) (42).

Another aspect related to location is that endothelial cells have a prolonged lifespan in regions with stable blood flow (39). However, cells with an extended lifespan are more susceptible to damage from exposure to harmful substances in the blood. Endothelial cells, regardless of their lifespan, may undergo senescence when exposed to certain stimuli (11).

1.1.3.2 Oxygen

Exposure of cells to a high partial pressure of oxygen has been widely recognized as a factor that accelerates the onset of cellular senescence (43, 44, 45). Notably, arterial endothelial cells are continuously exposed to a senescence-inducing stimulus, unlike other types of cells in the body. Arterial blood has a partial pressure of oxygen of approximately 100 mmHg, while venous blood has around 40 mmHg under normoxia, which is a normal oxygen condition (46, 47). In contrast, the definition of oxygen partial pressure in the spleen and skeletal muscle is 66 mmHg and 34 mmHg (46), respectively (46, 47). This unique characteristic of arterial ECs may explain the senescence onset observed in these cells.

1.1.3.3 Metabolites

In addition, the presence of amino acid metabolites and lipids may induce endothelial senescence both in vitro and in vivo (48, 49, 50). Specifically, the extended contact of HUVECS to homocysteine, a metabolite of the amino acid methionine, has been found to increase the expression of senescence markers, such as $p21^{cip1}$, $p16^{INK4A}$ and p53 (48). This may be because of demethylation of telomerase reverse transcriptase, which leads to telomere shortening, a characteristic feature of senescent cells (48). Moreover, exploring the relationship between lipids circulating in the bloodstream and the endothelium is crucial. Endothelial dysfunction, which triggers the development of atherosclerosis, involves impaired endothelial function, resulting in the accumulation of lipids beneath the endothelial barrier (51). Significantly, the senescence of endothelial cells may contribute to the accumulation of lipids by inducing a decreased expression of junctional proteins and impairing the barrier function of the endothelium (52, 53).

1.1.3.4 Chronic inflammation

Herein, another intricate interplay between inflammation and endothelial cell senescence can be observed. Chronic or excessive inflammation can trigger a cascade of molecular events that contribute to cellular senescence in endothelial cells. Studies have provided evidence supporting the notion that endothelial cell exposition to tumor necrosis factor alpha (TNFα) leads to early onset of replicative senescence (54). This exposure also results in increased expression of various molecules involved in inflammation, such as cytokines,

chemokines, as well as adhesion molecules including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM1) (54). Moreover, the premature senescence induced by TNFα and the resulting inflammatory environment can be alleviated by antioxidant administration or nuclear Factor κB (NF-κB inhibition (54). This suggests that targeting oxidative stress or the NF-κB signaling pathway may have potential therapeutic benefits in preventing or alleviating TNFα-induced endothelial cell senescence and associated inflammation.

The mechanisms underlying endothelial senescence involve the presence of inflammatory factors, which can play a role in DNA damage and DNA damage activation paths for responses (55). These processes are critical in driving the senescence of endothelial cells, triggering functional and phenotypic changes associated with senescence (11). In addition, senescent endothelial cells may be a reason for inflammatory cytokines, further promoting the inflammatory milieu. This might cause senescence in normally dividing cells (56).

1.1.4 Types of endothelial cell senescence

Despite possessing several unique characteristics, endothelial senescence shares many underlying processes with general senescence. Similar to senescence in other cells, endothelial senescence can be induced by multiple stressors, including intrinsic factors including telomere shortening, DNA damage, oxidative stress, oncogenic activation,

disturbed flow, and mitochondrial dysfunction, as well as various extrinsic factors including UV radiation and chemotherapeutic agents, etc (57).

Therefore, these stressors may induce a persistent DNA damage response (DDR) that produces cellular senescence by triggering a permanent halt in cell division (27, 58). It is worth noting that the aforementioned stimuli can induce endothelial senescence through both telomere-dependent and telomere-independent mechanisms.

1.1.4.1 Replicative senescence

The term "replicative senescence" was originally coined due to the belief that senescence reflected the limited ability for division seen by normal diploid cells during culture proliferation (3, 4). In large arteries, ECs are primarily renewed through mitotic cell division instead of being replaced by progenitor cells (59). Senescence, which is a consequence of repeated cell division, can be triggered by several factors, but telomere shortening stands out at the molecular level (60). Telomere loss, which occurs when cells divide rapidly through mitotic cell division, accelerates replicative senescence (42).

More specifically, the process of DNA synthesis could be categorised in two key stages:

a) During the first stage, DNA polymerases generate a new strand of complementary polynucleotides from the 5' to 3' end (60, 61).

b) the second stage, DNA synthesis is initiated by using an RNA primer that is subsequently eliminated. This process leads to the emergence of the 'end-replication problem', characterized by the sequences loss at the 5' end of linear molecules with each round of cell division (62, 63).

Telomeres play a vital role in this process, as they are made up of a repeating DNA sequence (TTAGGG) that caps the end of chromosomes (62, 63). Telomeres are crucial in preserving the integrity and stability of chromosomal ends, as they prevent the degradation and recombination of DNA ends (62, 63).

Telomeric DNA is typically lost incrementally with each instance of cell division (63). The traditional DNA polymerases can have blundered by failing to replicate the lagging strand, which accounts for this anomaly (61, 63). Approximately 100 base pairs are lost from telomeres with each successive round of division (64). The ribonucleoprotein telomerase can compensate for the loss of terminal sequences that occur during replication by synthesizing new telomeric repeats and appending them to the 3' end of chromosomal DNA (61). However, telomerase is missing or present at exceedingly low levels among the vast majority of primary human somatic cells (61). This results in the shortening of chromosomes with every cycle of cell division (61, 64). Besides, telomeric DNA is particularly susceptible to oxidative harm owing to its high concentration of GGG (65),

which can easily lead to single-strand breaks (65). As a result, cellular oxidative stress exhibits a significant effect on the degree of telomere erosion (65).

As a result, when telomeres reach a critical length threshold, cell division is inhibited by a DNA damage response (DDR) mechanism that safeguards the chromosomes from becoming impaired (64). Additionally, the cell cycle is permanently arrested (64), leading to a state of replicative senescence in these cells. In instances where telomeres become damaged and uncapped, a series of events is triggered that ultimately leads to cellular senescence through activating the p53/p21CIP1 tumour suppressor pathway and the DNA damage response (DDR) (66). Specifically, the DDR mechanism in relation to replicative senescence is reliant on telomeres and is associated with the uncapping of telomeres and a general shortening of telomeres (67).

Apart from telomere shortening, non-telomeric DNA damage can also induce senescence. The onset of cellular senescence induced by mitogenic stimuli occurs before the replicative limitations of cells are reached, rendering the replicative age of cells irrelevant. This occurrence is known as premature senescence and does not appear to be associated with telomere length. In vascular cells, early senescence triggered by the constitutive activation of Ras, a key signalling molecule implicated in atherogenic stimulation, is linked to increased levels of p53 and p16. This, in turn, exacerbates vascular inflammation both in vitro and in vivo (68). Extracellular signal-regulated kinase (ERK) may be crucial for Ras-

induced senescence, as inhibiting ERK can restore the cell growth stop caused by Ras activation (68). Conversely, activating ERK may trigger senescence in p53 and p16, leading to the aging process beginning prematurely (67).

Regarding endothelial senescence, several research laboratories have provided compelling evidence that endothelial telomeres undergo erosion with age, moreover, such a shortening is particularly announced within regions that are susceptible to atherosclerosis (42, 69, 70). Additionally, a study has shown that there is an accumulation of senescent endothelial cells in a rabbit carotid artery damage model (71). It is worth noting that a group of scientists from Austria conducted a study on replicative senescence in HUVECs in vitro, and they demonstrated that senescent endothelial cells exhibit an arrest in the G1 phase, which is another classic feature of senescence, along with an accumulation of 4N content suggestive of polyploidization (72).

1.1.4.2 Stress induced-premature senescence (SIPS)

In addition to telomere shortening, the induction of senescence can also be attributed to non-telomeric DNA damage (73, 74). It has been observed that the onset of cellular senescence induced by mitogenic stimuli is not dependent on the replicative age of cells, and occurs before the replicative limitations of cells are reached (73). Premature senescence describes this condition and is not believed to be associated with telomere

length (73, 74). Notably, the term "premature" underscores the rapidity with which the development progresses (73). Endothelial cells are constantly subjected to a variety of stresses, including activated oncogenes, inflammation, hemodynamic pressures, and toxic chemical exposure, with oxidative stress being particularly noteworthy, all of which trigger several signaling pathways that further induce cellular senescence (11).

The early onset of senescence in vascular cells correlates to elevated levels of p53 and p16, which are triggered by the constitutive activation of Ras, a key signaling molecule implicated in atherogenic stimulation that triggers vascular cell senescence, thus exacerbating vascular inflammation both in vitro and in vivo (68). Inhibition of extracellular signal-regulated kinase (ERK) activity rescues the cell growth arrest produced by Ras activation, demonstrating the importance of ERK activity for Ras-induced senescence (68). Conversely, p53 and p16 senescence may be accelerated by a stimulated version of ERK (67).

Senescence correlates to a persistent DNA damage response (DDR) that leads to irreversible DNA damage, which is a prominent feature of cellular senescence (58). This is notable through an elevated accumulation of γ-H2Ax (phosphorylated histone H2AX at Ser139) and p53-binding protein 1 (53BP1) in chromatin, and the initiation of a kinase cascade through serine/threonine-nonspecific kinases ATM and ATR, followed by

checkpoint serine/threonine kinases CHK1 and CHK2, the p53/p21CIP1 axis becomes activated (27, 75).

Oxidative stress, a significant trigger of SIPS, occurs while the creation of reactive oxygen species (ROS) overcomes the cell's capacity in removing them (76, 77). ROS comprises a class of highly reactive molecules, including superoxide anion, hydroxyl, and hydrogen peroxide (77). Notably, hydrogen peroxide is a widely utilized model for inducing oxidative stress-induced premature senescence in endothelial cells (78, 79, 80, 81). In this project, the hydrogen peroxide model was employed to induce senescence in HUVECs. Further details regarding this model will be discussed in the subsequent section.

Remarkably, endothelial cell senescence is a significant contributor to endothelial dysfunction, which impairs vascular function and leads to various vascular diseases, including atherosclerosis, hypertension, heart failure, and thrombosis (9, 82). Moreover, SIPS accelerates the aging process of endothelial cells (82), which further exacerbates vascular aging and leads to age-related changes such as reduced elasticity, increased stiffness, and impaired regenerative capacity of blood vessels (9, 82). Therefore, comprehending the fundamental processes of oxidative stress-induced senescence within endothelial cells is crucial for developing potential therapeutic strategies to mitigate age-related vascular diseases.

1.1.5 Pathophysiological roles of endothelial cell senescence

1.1.5.1 Angiogenesis and vascular repair

Angiogenesis, a process of creating new blood vessels, is a fundamental physiological activity (11). It is widely established that endothelial cells play a pivotal part in this procedure, which involves several factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor, and hypoxia-inducible factor 1α (11). However, endothelial cell senescence can impair the repairability of the endothelial lining due to persistent growth arrest (71). During endothelial senescence, protein modifications that affect cellular structure and cytoskeletal function, leading to changes in cell motility and impaired angiogenic potential due to compromised replicative ability (83, 84, 85, 86). Additionally, it has been established that the interference of telomeric repeat-binding factor 2 (TRF2) in senescent endothelial cells can also impair the ability to generate new vessels (87). Additionally, studies have shown that genetically deleting or inhibiting telomerase in mice and rats can disrupt angiogenesis, leading to impaired tissue repair ability (88, 89). At the mechanistic, reduced VEGF expression resulting from endothelial cell senescence may also contribute to diminished angiogenic ability (90). Moreover, EC senescence is linked to a variety of age-related diseases, involving decreased angiogenesis, such as atherogenesis (91). Atherosclerotic plaque development is greatly influenced by angiogenesis (92).

1.1.5.2 Vascular permeability

When healthy, the endothelium forms a mechanical barrier between the bloodstream and the arterial wall, controlling the flow of substances including tiny molecules, inflammatory cells, and nutrients (93). In response to oxidative and inflammatory stressors, vascular permeability is controlled by the spacing at EC junctions (93). If the EC is damaged, inflammatory cells and other tiny substances can enter and leave the blood artery unhindered (93). Notably, subjecting endothelial cells to prolonged culture until they reach a senescent state results in the disruption of key junctional proteins, including cadherin 5 (vascular endothelial cadherin) and tight junction protein ZO-1 (53). Additionally, senescent endothelial cells exhibit decreased levels of occludin and claudin 5, which are junction proteins (53). Moreover, tight connections in non-senescent cells have been shown to be faulty when co-cultured with senescent endothelial cells, according to one research (53). This phenomenon can be attributed to the senescence-associated secretory phenotype (SASP), considering that inflammation is generally accepted to enhance endothelial monolayer permeability (94). Additionally, one research suggests that aging ECs may contribute to a breakdown in the brain's protective blood-brain barrier (95). TNFα was found in much greater concentrations in the endothelial cells of aged mice compared to young animals, suggesting that inflammation contributes to a loss of tight junction complex formation and endothelial barrier function (95). Senescent endothelium cells contribute to

the progression of atherosclerotic disease by lowering the blood vessel's barrier function, making it easier for LDL cholesterol and immune cells to enter the bloodstream (53, 96).

1.1.5.3 Vascular vasodilatation

One of the critical functions of healthy endothelium is to regulate vascular tone, which plays a pivotal function in regulating blood pressure and circulation. This process is achieved through the release of several vasodilators and vasoconstrictors. Vasoconstrictor endothelin 1 (ET-1) and other vasodilator factors such as NO, prostacyclin, and Endothelium-Derived Hyperpolarizing Factor (EDHF) manage the delicate balance between vasoconstriction and vasodilation (97). However, one study has reported that increasing production of ET-1 in human endothelial cells is associated with cellular senescence (98), which explains the age-related damage in shear stress-induced vascular relaxation (99). Additionally, flow-mediated vasodilation, systolic volume, and NO production all decline with age (97). Additionally, several vascular disorders, including atherosclerosis, arterial stiffness, and hypertension, have been linked to ET-1 (100).

1.1.6 Endothelial cell senescence in atherogenesis

Reversible cell division stoppage, metabolic activity, and the generation of a senescence-associated secretory phenotype (SASP) are hallmarks of the irreversible process known as cell senescence (101). Tissue function, regeneration, and integrity can all be jeopardized

by an excess of senescent cells. The proinflammatory nature of the SASP makes it vulnerable to paracrine and systemic actions that promote persistent inflammation (102). Human atherosclerotic lesions include endothelial senescence and the SASP that is linked with it (103), while endothelial cells become more vulnerable to atherogenesis with age (104). Moreover, it is well-established that the production of inflammatory factors is the first step in the development of atherosclerosis (105). Important factors in the progression of atherosclerosis include proinflammatory cytokines produced by the SASP (106, 107, 108). More specifically, endothelial cell senescence aggravates endothelial dysfunction and inflammation, characterized by the activation of NF-κB and the expression of adhesion molecules, including ICAM-1 and monocyte chemoattractant protein-1 (MCP-1) (109, 110). At this point, monocytes are pulled from the circulation and deposited in the artery intima, where they differentiate into macrophage foam cells and aid in the formation of atherosclerotic lesions in their early stages (111). Because of their potential to be selectively eliminated or prevented by both naturally occurring and synthetic chemical substances, senescent cells and SASP are seen as promising therapeutic targets (112). A novel strategy for treating atherosclerosis can be found in the use of senolytics (drugs that eliminate senescent cells) or senomorphics (drugs that inhibit SASP) (113).

1.1.7 Detection of cellular senescence

It's worth noting that several of these features, alone or in combination, have been implemented to recognize senescent cells in vitro and in vivo. However, these markers are not specific to the senescent state and may show different results in different cell types and tissues (18, 114).

1.1.7.1 Senescence-associated β-galactosidase (SA β-gal) activity

A reliable method for identifying cellular senescence is the evaluation of Senescence Associated β-Galactosidase (SA-β-Gal) activity. SA-β-Gal, a lysosomal enzyme (115), facilitates the hydrolysis of β-galactosidase into monosaccharides, a process that is exclusive to senescent cells (116, 117). The enzymatic activity of SA-β-gal is detectable in numerous regular cells around pH 4.0-4.5, but it is dramatically increased in senescent cells because of their increased lysosomal mass (118). Within senescent cells, SA-β-gal can be detected through histochemical means at pH 6.0 (118, 119, 120). By catalyzing X-gal with β-galactosidase a blue deposition is generated, which is visible under bright field microscopy (119).

The augmented expression of SA-β-gal in senescent cells, which may be attributed to the increased lysosomal mass, is a hallmark of senescent cells (5, 119). This observation is in line with the formation of autophagic vacuoles in senescent cells, which harbor macromolecules and organelles that cannot be degraded (121). In addition to being linked

to replicative senescence and a loss of telomeric structural integrity, SA-β-gal is also present in cells that have undergone senescence as a result of other causes (118, 121). The pathological implications of the heightened lysosomal mass in the arterial wall remain unclear, and its etiology is yet to be determined (118, 119, 121).

The assessment of formalin-fixed tissues, such as human samples, using SA-β-gal staining has become a challenge, as most research on senescence relies on in vivo cultures or fresh samples. Despite its widespread use, the assay's reliability has been called into question due to its lack of specificity. Specifically, SA-β-gal activity can increase in Swiss 3T3 cells (fibroblast cells) incubated under low serum conditions (122) or in nontransformed fibroblast cultures with high density (123). Therefore, additional detection methods are necessary to be used in conjunction with SA-β-gal staining.

1.1.7.2 Cyclin-Dependent Kinase Inhibitors

Protein indicators of cellular senescence include the accumulation of p53 and the downregulation of phosphorylated Retinoblastoma protein (pRB), as well as the buildup of the CDK2 inhibitor p21WAF1/Clip1 (CDKN1A) and the CDK4/6 inhibitor p16INK4A (CDKN2A) (17, 23). These two primary pathways (p16Ink4a/RB and p/p21CIP1 axes) have separate functions in controlling cell cycle halt. Replicative senescence, senescence induced by the DNA damage response (DDR), senescence induced by reactive oxygen

species (ROS), and senescence induced by oncogenes (OIS) are all characterized by activation of the p53/p21CIP1 pathway (124, 125). In contrast, the p16Ink4a/RB pathway differs from the former, as it is not involved in DDR-induced senescence, although it is considered to be more strongly implicated in the maintenance of the senescent phenotype (124, 125). Therefore, p21, p16, P53, and pRb are frequently used as diagnostic markers for senescence due to their unique roles in senescent cells. Moreover, chronic DNA damage response (DDR) is linked to senescence induction, and its presence may directly promote p53 acetylation (126). Thus, acetyl-p53 can be regarded as a marker for the detection of senescence.

1.1.7.3 Other hallmarks of senescence

A novel biomarker, Sudan-Black-B (SBB) dye-specific lipofuscin staining, has been identified for the detection of replicative and stress-induced senescence in long-term formalin-fixed samples (127). Additionally, senescent cells exhibit characteristic morphological alterations, rendering LaminB1 a valuable biomarker for senescence (17). The nuclear lamina is mostly comprised of a protein called lamin B1, and its downregulation has been observed in ageing human and mouse cells (17). The significance of lamin B1 in preserving the nucleus' structural integrity is essential (21, 128). However, in senescent cells, this integrity is compromised, leading to the extrusion of cytoplasmic chromatin fragments outside of the nucleus (128). It is thought that the SASP is activated

by these fragments via the cGAS/STING pathway. Lamin B1 depletion can be identified using immunoassays, imaging, and immunoblotting techniques (21).

1.1.8 Strategies for Treating Senescence in the Vascular Endothelium

Considering the link between ageing and endothelial dysfunction, the prevention of endothelial senescence is a crucial objective. Senotherapeutics, a type of pharmacological therapy that specifically targets senescent cells (129), has garnered significant interest. The illness environment plays a critical role in determining whether cellular senescence is favoured, avoided, removed, regulated, or reverted (130). There are three primary types of pharmacological therapies that may be employed to prevent cellular senescence: senolytics, senomorphics, and senescent immunotherapy (129, 131, 132, 133). Senolytics weaken senescent cells' ability to avoid apoptosis, leading to cell death (131, 133). In contrast, Senomorphics interfere with senescence by inhibiting SASP rather than removing senescent cells, while senescent immunotherapy employs immune cells to eliminate senescent cells (131, 133).

In general, the use of senolytics to remove senescent cells is considered beneficial, but caution must be exercised as the removal of certain senescent cells can be harmful (11, 134, 135). Systemic administration can cause damage to other postmitotic cells that exhibit senescence markers and should not be eliminated (134, 135). Specifically, senescent

adipocytes, macrophages, or foam cells can be eliminated to address diseases such as atherosclerosis and metabolic disorders (135, 136, 137). Additionally, the removal of senescent glial cells can prevent cognitive dementia (138). However, the removal of senescence in beta cells can lead to diabetes (137, 139). Furthermore, the reduction of hypertrophy of cardiomyocytes and fibrosis has been shown to occur after the removal of senescent cells in a mouse model of heart failure, but it can also result in the formation of multiple nuclei in cardiomyocytes, which may contribute to hypertrophy and maladaptive remodelling in the long term (135, 140).

Therefore, the development and optimization of various techniques are necessary to enhance the selectivity with which senescent ECs are targeted (11, 129). The ideal method for targeting senescent ECs in age-related conditions and disorders remains a work in progress.

1.2 H_2O_2-induced premature senescence

1.2.1 The pathophysiology of oxidative stress

Free radicals, defined as atoms or molecules having at least one unpaired electron, are continually attacking the body (141). When an oxygen molecule loses an electron, it may "borrow" an electron from another molecule to regain stability (141). "Reactive oxygen species (ROS)" refers to a term used to describe a type of reactive molecule containing

oxygen, such as superoxide radicals (O2•−), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet ($^1O_2$) (141). In the biological context, ROS is produced as a byproduct of normal metabolic activity of oxygen, and helps tremendously in cell signaling and homeostasis (77). Normal cells maintain a low but steady level of ROS, as they are essential to cellular activities such as protein phosphorylation, transcription factor activation, apoptosis, etc. (77). Elevated production of reactive oxygen species (ROS) can cause harm to fundamental cellular components such as proteins, lipids, and DNA (142). Numerous studies suggest that oxidative stress contributes to the onset and/or progression of a variety of diseases, including metabolic disorders such as diabetes (143) and cardiovascular diseases such as atherosclerosis (76, 77). Evidence indicates that oxidative stress, rather than telomere shortening, is the primary factor triggering cellular senescence in endothelial cells isolated from chronic cigarette smokers with atherosclerosis (144). Moreover, genetic depletion of $p66^{shc}$ in mice can increase resistance to oxidative stress and decrease oxidative damage, resulting in an extended lifespan (145). These longer-lived mice exhibit greater resistance to the development of atherosclerosis when exposed to a high-fat diet (HFD) (145).

1.2.2 The Cell biology of oxidative stress

There are two primary sources of ROS generation: endogenous and exogenous. Endogenous ROS generation can occur during inflammation, infection, oxidative

phosphorylation, aging, and other processes (77, 141, 146). Exogenous free radicals can be formed due to exposure to environmental contaminants, radiation, tobacco smoke, certain drugs or chemicals, etc. (77, 141).

In the vascular endothelium, the majority of ROS, particularly hydrogen peroxide, are primarily produced by NADPH oxidase, xanthine oxidase, endothelial nitric oxide synthase, and mitochondrial respiration complexes (147, 148). During cellular respiration, NADPH oxidase is the primary generator of the radical superoxide anion (O2•)(77, 149, 150, 151, 152). Once generated, it contributes to the further generation of hydrogen peroxide (H_2O_2) through superoxide dismutase (SOD) (76, 148, 149, 150, 151). H_2O_2, a nonradical molecule, can react with O2•− to form the highly reactive ROS hydroxyl ion (OH•) through the Fenton or Haber–Weiss reaction (77, 149, 150, 151). These extremely reactive hydroxyl radicals interact particularly with the phospholipids found in proteins and cell membranes (77, 153), finally damaging cellular function. Whether generated endogenously or exogenously, these highly reactive ROS molecules can induce cell death and/or cellular senescence (77, 154).

A state of oxidative stress exists when there is a deficit in antioxidant defenses against free radicals, either because there are too many free radicals or because the body is unable to effectively dispose of them (149, 150, 155). Exposure to high levels of ROS typically leads to oxidative modification of cellular macromolecules, including lipids, proteins, as well as

DNA (146, 156). Low-density lipoproteins (LDLs) transport cholesterol throughout the body (157). LDL oxidation is a multi-step process that involves oxidative alterations to both the protein and lipids, which can lead to an increase in cholesterol levels (157). Based on evidence from the oxidative modification theory of atherosclerosis, oxidized low-density lipoprotein (oxLDL) is used as a biomarker of oxidative stress (158, 159). Polyunsaturated fatty acids (PUFAs) are another specific target of lipid peroxidation caused by hydroxyl and peroxyl radicals (160, 161). Additionally, both the membrane and structural integrity of the cell are vulnerable to attack from oxidant radicals (162, 163, 164). The oxidation of lipids can have far-reaching effects on fluidity, physiology, and membrane damage, as seen in various disorders (160, 161, 162). High levels of reactive oxygen species (ROS) can also alter the structure and function of proteins. Proteins are vulnerable to oxidative stress, which can cause structural modifications that may result in the loss or reduction of their enzymatic function (146, 164, 165).

Mitochondrial DNA, in particular, is highly susceptible to ROS damage because mitochondria are the primary source of intracellular oxidants (77, 166). Breaks in the double helix and an increase in nitrogen bases are two clear signs of oxidative stress-induced DNA damage (77, 166). The formation of 8-hydroxyguanosine (8-OH-G) is one of the most effects of oxidative stress on DNA (166), and it can be used as a biomarker to measure the extent of DNA damage caused by oxidative stress (77, 166).

In summary, ROS and oxidative stress can negatively impact tissue structure and function by inducing several cellular fates, including apoptosis, senescence, and autophagy (77). In this thesis, the focus is on oxidative stress-induced EC senescence.

1.2.3 H_2O_2-induced premature senescence

In point of fact, buildup of macromolecular oxidative damage, including lipids, DNA, and proteins, has been demonstrated to elicit cellular senescence in vascular endothelium (167). Although the precise mechanism of SIPS in endothelial cells remains elusive, accumulating data points to the role of reactive oxygen species (ROS) in DNA damage and the physiological mechanism that sets off the DNA damage response (DDR), ultimately leading to EC senescence (77, 168). This process entails the activation of $p16^{INK4a}$/pRB and p53/p21 pathways, which result in the upregulation of p53 and p16 (151, 169, 170).

Stress-induced premature senescence (SIPS), also referred to as premature senescence (PS), has been associated with oxidative stress caused by oxygen, hydrogen peroxide, and other stressors in both humans and mice (43, 45). Among these stressors, hydrogen peroxide (H_2O_2) has emerged as a potential inducer of senescence due to its ability to effectively mimic the oxidative environment in an aging population (171). Multiple cell lines have proven in vitro that H_2O_2 causes senescence, including fibroblasts (171) and ECs (74). However, the underlying mechanisms may vary widely and may even be cell-type specific.

In human diploid fibroblasts, sublethal concentrations of H_2O_2 induced premature senescence through persistent upregulation of p21 (169). Conversely, ECs undergo senescence in response to oxidative stress through an ATM/Akt/p53/p21-dependent signaling cascade that is activated by the DDR (172).

Activation of the DDR pathway by ROS can lead to a senescent growth arrest in vitro. This occurs when ATM or ATR kinases prevent cell cycle progression by stabilizing and activating p53, which then transcriptionally activates the cyclin-dependent kinase inhibitor (CKI) p21 (173, 174, 175). Persistent DNA damage, on the other hand, activates p16INK4a through dysfunction mediated by P38 Mitogen-activated protein kinase (MAPK) and ROS generation (176). The stress-activated protein kinases (SAPKs) family includes P38-MAPK (176, 177). As a result of experiencing a wide range of physiological stress, particularly oxidative stress, a set of kinases known as SAPKs are activated, inducing either apoptosis or senescence (176, 177). Following H_2O_2-induced senescence, p38MAPK is rapidly phosphorylated and activated as a response to the stress (73). It has been demonstrated that H_2O_2 induces p38(MAPK) phosphorylation in IMR-90 hTERT cells (178). To some extent, the increase in cells positive for SA-ß-Gal activity was mitigated by selective reduction of p38 (MAPK) activity (178). Additionally, inhibition of p38 (MAPK) activity ensuing in a low level of secretion of SASP factors in X-irradiation-induced senescence (179).

1.2.4 H_2O_2-induced endothelial cell senescence in age-associated diseases

As previously mentioned, increased levels of ROS resulting from oxidative stress can have detrimental effects on cellular and tissue physiology, as well as overall organismal health. EC senescence plays a crucial role in controlling age-related physiological and pathological characteristics, either directly or indirectly (168). Elevated levels of ROS have been linked to vascular aging (168), and recent findings have established a connection between vascular diseases and dysfunctional vascular endothelial cells (136). When senescent endothelial cells lose their ability to replicate, cell integrity is compromised, and angiogenesis ceases (53, 76, 180). Atherosclerotic plaques in the coronary arteries of humans and injured rabbit carotid arteries both include SA-β-Gal-positive cells (23, 71, 181), while endothelial and smooth muscle cells that are not growing show upregulation of p53 and p21 has been observed in human atherosclerotic lesions (182).

Furthermore, senescent cells are also thought to have a function in the development of neurological diseases including Parkinson's and Alzheimer's (167, 183). In numerous forms of neurodegeneration, senescent cells have been shown to accumulate. Elevated levels of p16, MMP, and IL-6 have been detected in brain tissue samples obtained from patients affected by neurodegenerative disorders, including Alzheimer's disease (AD) (138, 184, 185, 186).

1.3 The role of key sphingolipids in the regulation of senescence and other cellular processes

1.3.1 Sphingolipid metabolism

The International Lipid Classification and Nomenclature Committee (ILCNC) has defined eight categories for lipids (187), namely fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sterol lipids (ST), phenol lipids (PR), saccharolipids (SaL), polyketides (PK), and sphingolipids (SL) (187). Research on various model species, including humans, has revealed intriguing links between lipid levels and aging (188).

Sphingolipids, a crucial class of lipids, are involved in membrane construction and the regulation of nearly all cellular activities (189). They were first discovered by Thudichum 1884, who named them "sphingosins" after detecting them in brain tissue (190). Typically, sphingolipids contain sphingosine (Sph), which is a common sphingoid base (191). Additionally, they possess an aliphatic amino alcohol that typically consists of 18 carbons and serves as the nonpolar tail (192, 193). Ceramide (Cer) is synthesized when the sphingoid base forms an amide bond with a fatty acid (192, 193).

Over the course of the past thirty years of research, sphingolipids have been recognized as a crucial class of bioactive molecules that contribute significantly to the control of multiple cellular activities, including but not limited to cell cycle, senescence, proliferation, and

migration (194). Moreover, the potential implications of sphingolipids in the onset and progression of a diverse range of age-related pathologies, such as malignancies, metabolic and cardiovascular diseases, as well as neurological disorders, have been extensively studied (195).

This study endeavors to introduce the correlation between sphingolipids and EC senescence, as well as the associated pathologies. Novel and compelling findings of potential significance will be presented to furnish compelling evidence that substantiates the putative role of sphingolipids in the regulation of EC senescence.

1.3.1.1 Biosynthesis of sphingolipids

The metabolism of sphingolipids centers on the intricate processes of ceramide biosynthesis and catabolism, which can be initiated via either de novo synthesis or the salvage pathway (196). The de novo pathway happens at the endoplasmic reticulum (ER) membrane, and the production of a sphingoid base represents the initial step in the de novo biosynthesis of sphingolipids (197).

This process involves the catalysis of the condensation of serine and palmitoyl-CoA by Serine palmitoyltransferase (SPT), resulting in the formation of 3-ketodihydrosphingosine (198, 199, 200). There are three parts to SPT: SPTLC1, SPTLC2, and SPTLC3 (201, 202). On the cytoplasmic face of the ER, dihydrosphingosine is formed via the reduction of 3-

ketodihydrosphingosine, a process that is facilitated by the 3-keto-dhphingosine reductase (KDSR) (198, 203). Subsequently, the (dihydro) ceramide synthases (CerSs), also known as ceramide synthase, acetylate the dh-sphingosine to produce dihydroceramide (dhCer) (204). Finally, desaturases (Des) catalyze the desaturation of dhCer in the terminal stage of the de novo route, leading to ceramide production (205, 206).

1.3.1.2 Catabolism of Sphingolipids

Ceramide serves as a central hub in the intricate network of sphingolipid metabolism (196). It can be deacylated to sphingosine via the catalytic activity of ceramidases (207). Subsequently, S1P is produced when sphingosine is phosphorylated; sphingosine kinases 1 (SphK1) and 2 (SphK2) play key roles in this process (207, 208). Notably, SphK1 primarily functions to generate S1P at the plasma membrane (209, 210), while SphK2 predominantly carries out the same enzymatic activity in the nucleus, mitochondria, and ER (211, 212, 213, 214). The resulting product, S1P, can be irreversibly cleaved into phosphoethanolamine and hexadecenal by S1P lyase (SPL) (211). It is noteworthy that SPL is predominantly localized in the ER in both mice and humans (212, 213, 214), and is expressed in almost all tissues (215).

Nonetheless, it is important to highlight that within the salvage pathway, also known as the recycling pathway, the utilization of sphingosine, which is the byproduct of ceramide, can

lead to the formation of ceramide through reacetylation by CerS (216). It is noteworthy that CerS has the ability to directly convert Sph into Cer (217). Conversely, S1P, which is the product of sphingosine, can be converted back to Sph by S1P phosphatases (SPPs) (216).

1.3.1.3 Hydrolysis of complex sphingolipids

In mammals, sphingomyelin stands out as the most abundant sphingolipid (218, 219). The breakdown of sphingomyelin into ceramide is facilitated by sphingomyelinase (220). These enzymes can be separated into three categories, namely acid, neutral, and alkaline, according to their optimal pH (221, 222). Conversely, sphingomyelin can be synthesized from ceramide by sphingomyelin synthases (223). During this process, phosphatidylcholine is converted into diacylglycerol (DAG) (223). Currently, two types of sphingomyelin synthases have been identified, namely sphingomyelin synthase 1, which is located on the trans-Golgi cisterna, and sphingomyelin synthase 2, which is found in the trans-Golgi and plasma membrane (224, 225, 226).

On the other arm of the pathway, the Golgi apparatus is where increasingly sophisticated sphingolipids are synthesized (227, 228). Specifically, the glycosylation of ceramide leads to the production of glycoceramides (193). Conversely, glycoceramides can be hydrolyzed into ceramide by the acid β-Glucocerebrosidase (228). Additionally, it is worth noting that

phosphorylation of ceramide by ceramide kinase (CERK) generates ceramide-1-phosphate (C1P) (227, 229, 230). Conversely, C1P can be dephosphorylated to produce ceramide by C1P phosphatase (C1PP) (229, 230).

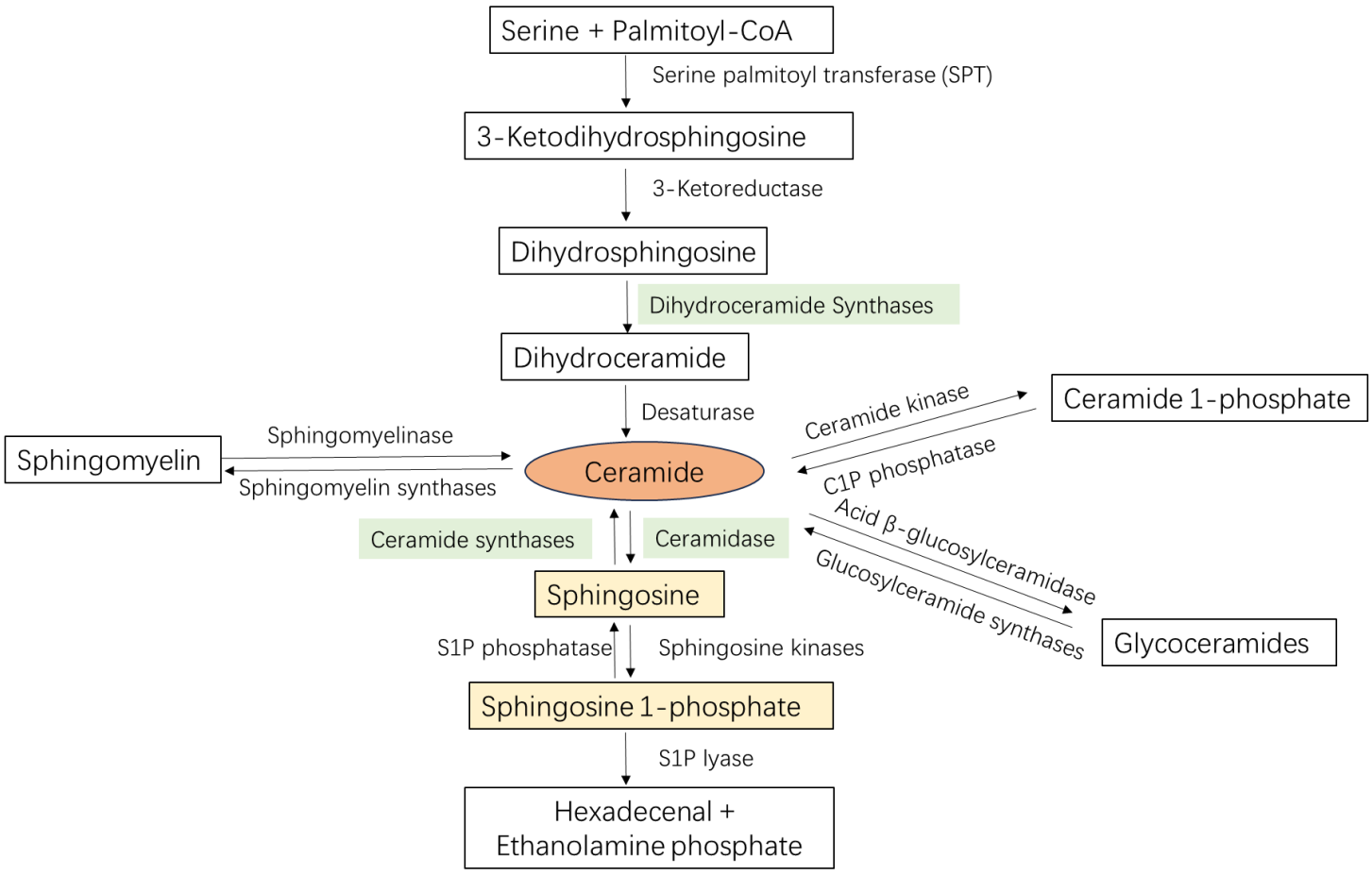

Figure 1.2 Sphingolipid metabolic network (231) Ceramide acts as a central hub in the intricate network of sphingolipid metabolism and can be synthesized through various enzymatic pathways involving multiple enzymes. Conversely, ceramide can undergo conversions to form complex sphingolipids, including sphingomyelin, glycoceramides, acylceramide, and ceramide 1-phosphate (231).

1.3.2 Key enzymes

1.3.2.1 Ceramide synthases and their related inhibitors

In addition to the aforementioned enzymes, there are several other types of enzymes that are of great importance. One such enzyme is ceramide synthase, which is a key focus of my entire project.

In mammals, there are six isoforms of ceramide synthase, namely CerS1-CerS6. Ceramides can vary in terms of their degree of saturation, α-hydroxylation and acyl chain length. As a result, each of the six ceramide synthases has a preference for different fatty acyl chain lengths to produce specific ceramides. Additionally, the expression of these isoforms varies across different tissues (232). Dihydroceramide (dhCer), which is a precursor of ceramide, is produced by CerS through the addition of fatty acyl-CoAs of varying chain lengths to the sphingosine backbone. Numerous studies have indicated that CerS primarily localizes to the endoplasmic reticulum (ER) (200, 233, 234, 235). However, recent research has shown that CerSs can also be found in mitochondria (236), mitochondria-associated membranes (237), the nucleus (238), and even perinuclear structures (239). There is still some debate regarding the region responsible for determining substrate specificity. One study has suggested that retaining CerS2 and CerS5 substrate specificity only requires a minimum region of 150 residues in the TLC (Tram-Lag1-CLN8) domain (240).

Interestingly, it has been discovered that CerS2 has the most abundant expression in mice compared to other ceramide synthase isoforms (241). However, another study has illustrated that CerS5 is the most abundant isoform in mice lung epithelia (242).

CerS1 is known to produce C18:0-ceramide, and it is highly expressed in the brain, skeletal muscle, and testis (243, 244, 245). Notably, CerS has been implicated in senescence, which is considered to be a type of stress response (232). A recent study has demonstrated that CerS1-derived C18:0 suppresses the promoter of telomerase reverse transcriptase (hTERT) in a human lung adenocarcinoma cell line. Suppressing hTERT, a functioning catalytic protein component of telomerase, causes cells to cease dividing and enter senescence because telomere production and recovery are impaired (232, 246). Additionally, CerS1 expression was found to be elevated in the brain tissue of Parkinson's disease patients (247).

CerS2 is predominantly expressed in the liver and kidney of murine models, where it catalyzes the synthesis of chain ceramides with extreme length, such as C22-C24 ceramides (243, 244, 245). In humans, CerS2 is also detected in the liver and kidney, as well as other organs, including the brain, heart, placenta, and lung (248, 249). A recent study utilizing Cers2-deficient mice has demonstrated that CerS2 participates in a diverse array of biological processes, including the preservation of myelin, maintenance of the histological structure of the cerebellum and kidney, and prevention of hepatocarcinoma (250).

Moreover, it has been suggested that the activity of CerS2 can be inhibited by S1P through the binding of S1P to an S1P receptor-like motif that is specifically present in CerS2 (241).

In general, CerS3 is responsible for synthesizing long chain ceramides, as it specifically utilizes very-long chain fatty acids, including C22-C26 fatty acyls. CerS3 is primarily expressed in the skin and testis, where it produces epidermis-specific ceramides and is highly expressed (243, 244, 245). Studies have shown that the expression of CerS3 increases during the differentiation of human keratinocytes, which involves the participation of PPARβ/δ (251). Notably, Cers3 mRNA is exclusively expressed in germ cells, with levels increasing by more than 700-fold during testicular maturation (252). This is supported by another study, which found that the expression of CerS3 was not detected in either the brain or the sciatic nerve using in situ hybridization (253).

With respect to CerS4, it exhibits a distinct preference for C18-C20 fatty acyl chains (244). Human tissue analysis has revealed the presence of CerS4 in the kidney (254) and breast (255), while Cers4 expression has been observed in various mouse tissues to varying degrees (239, 256). The role of CerS4 in Alzheimer's disease has been investigated, with findings indicating that increased expression of Cers2 and CerS4 is evident in the presenilin 1 (PS1) knock-in (PS1M146V) mouse model of Alzheimer's disease (257). Additionally, upregulation of CerS4 has been observed in the liver of HFD obesity/T2DM mouse models, suggesting a potential involvement of CerS4 in obesity and diabetes (258).

CerS5 is responsible for catalyzing the production of C16:0-ceramide in a specific manner (244). In humans, CerS5 has been detected in the lung (249), kidney (254), and breast tissue (255). In mice, Cers5 is expressed in the testis, kidney (256), liver (258), and macrophages (259), and is also present in brain tissue in rats (260). The expression of CerS5 mRNA increases in the developing brain after birth, suggesting a significant role of CerS5 in brain development (264). Apoptotic signals trigger the formation of ceramides, and CerS5 is a key player in this process (261, 262). Additionally, in response to upregulation of p53 expression, the synthesis of C16-ceramide, which is specifically produced by CerS5, increases in leukemia and colon cancer cells (263). Moreover, pharmacological inhibition of ceramide synthase using fumonisin B1 results in a significant reduction of ceramide during p53 upregulation, while other ceramide-generation pathways, such as the sphingomyelin pathway, are not involved in ceramide production in response to p53 upregulation (263).

Similar to CerS5, CerS6 exhibits a preference for C16-ceramide, while also favoring C14-ceramide (244). In humans, CerS6 has been detected in the kidney (254) and breast tissue (255), while in mice, it is present in multiple immune active and inflammatory cells, such as neutrophils (264). mRNA expression of CerS6 increases during postnatal brain development in rats, with CerS6 co-localizing to brain mitochondria and being elevated in

juvenile rat brain mitochondria (260). Additionally, CerS6 contributes greatly the migration of neutrophils and the inflammatory response in macrophages (265).

Fumonisin B1 (FB1), a structural analog of sphingosine, has been identified as an inhibitor of ceramide synthases. FB1 specifically targets ceramide synthase and interferes with its activity, leading to a disruption in the production of ceramide through both de novo and salvage pathways. This disturbance in sphingolipid metabolism can result in disease. By inhibiting ceramide synthase, free sphingoid bases, especially sphinganine, and sphingoid base 1-phosphates are both elevated by FB1, whereas decreasing dihydroceramide and complex sphingolipids, such as sphingomyelin (266, 267, 268).

Additionally, fumonisins are a group of mycotoxins that possess toxic and carcinogenic properties (268, 269). The disruption of sphingolipid metabolism caused by FB1 has been implicated in the development of FB1-induced toxicity (268, 269). In fact, FB1 is widely recognized as a potent carcinogen for the liver and kidneys in both rodents and humans. Accumulation of sphinganine and alterations in sphingolipid metabolism have been associated with the development of hepatotoxicity, immune system dysfunction, neurotoxicity, and other adverse health effects observed in animals and humans exposed to FB1 (270, 271, 272, 273).

In fact, numerous studies have provided support for the apoptotic-inducing and cell viability inhibiting effects of FB1. One study, for instance, has reported that treatment with 25 μM FB1 for 48 hr can promote apoptosis in sensitive porcine kidney epithelial (LLC-PK1) cells, whereas HEK-293 human embryonic kidney cells showed no such response. The study further revealed that HEK-293 cells exhibit resistance to FB1-induced apoptosis due to the fast S1P synthesis that promotes cell proliferation and provides survival (274). Another study, which employed 1.25 μM, 10 μM, and 20 μM FB1 to treat SNO cells, a type of osteoblastic cells, for 48 hr, performed the MTS assay and found that cell viability was unaffected by FB1 concentrations of 1.25 μM, but was significantly reduced by 10 μM and 20 μM FB1 treatment, suggesting an inhibition of cell proliferation by FB1 (275). In addition, another research team conducted a study on HL-7702 cells, a normal human liver cell line, by treating them with 0.1 μM, 1 μM, 10 μM, and 100 μM FB1 and a cell viability assay at 24h, 48h, 72h, and 96hr, respectively (276). The study initially found that FB1 had a significant inhibitory effect on the proliferation of HL-7702 cells. Nevertheless, with increasing duration of treatment, there was an observed increase in cell proliferation (276). Moreover, evidence suggests that FB1 influences the cell cycle of normal human liver cells via inducing variations in the expression levels of cyclin E and P21 (276).

On the other hand, the concentration of FB1 required to inhibit CerS can vary depending on specific experimental conditions, such as the CerS isoform, cell type, and assay

technique used. A study has shown that FB1 exhibits strong inhibitory activity against ceramide synthase in mouse brain microsomes. It has been observed that FB1 can inhibit more than 50% of ceramide synthase activity at a concentration as low as 75 nM in cultured cerebellar neurons (277). Interestingly, FB1 is a non-isoform-selective CerS inhibitor, with an IC50 of 0.22 μM ± 0.05 for human CerS1, 0.15 μM ± 0.08 for human CerS2, 0.92 μM ± 0.06 for human CerS4, and 0.29 M ± 0.05 for human CerS6 (278). Moreover, one study has pointed out that a concentration of 0.1 μM FB1 is capable of inhibiting half of the ceramide synthase activity in rat liver microsomes (279).

1.3.2.3 Ceramidases and their related inhibitors

Another type of enzyme that warrants attention is ceramidase. Currently, mammals have five identified ceramidase isoforms that can be categorized into three groups, namely acid, neutral, and alkaline, in light of their optimal pH, intracellular localization, and intracellular functions (231). In the acidic environment of lysosomes, acid ceramidase, which is encoded by the ASAH1 gene, undergoes proteolysis and begins deacylating ceramide (280, 281). A study has also revealed that ASAH1 performs a crucial function in proliferation and cancer development. Specifically, the expression of acid ceramidase is greater in proliferative melanoma cancer cells than in other types of cancer cells, despite the fact that it is expressed in normal human melanocytes (282). Additionally, overexpressing ASAH1 in L929 fibrosarcoma cells of mice has been shown to inhibit apoptosis caused by TNFα,

possibly by decreasing the level of ceramide (283). Conversely, blocking the pharmacological activity of acid ceramidase, which leads to an increase in ceramide levels, can promote apoptosis in L929 cells (287). Moreover, senescent cells have been reported to have abnormally high levels of ASAH1 (284). Evidence suggests that the protein levels of classic senescence markers, such as p53, p21, and p16, decrease in pre-senescent human fibroblasts that have silenced ASAH1 (288). More recently, a study has indicated that acid ceramidase contributes to Alzheimer's disease. It is evident that acid ceramidase activity and expression levels increase dramatically compared to other glycoproteins in Alzheimer's disease brain tissue (285).

On the other hand, ASAH2 is a gene that provides instructions for making an enzyme called neutral ceramidase, and this enzyme has been found to localize to the plasma membrane, where it plays a critical role in regulating sphingine and S1P signaling (219, 286). Neutral ceramidase has been found in significant concentrations in the intestinal epithelium, making it a focus of research on the breakdown of dietary sphingolipids (287). Moreover, DNA damage response has been shown to be linked to the control of neutral ceramidase (287). In a study, the treatment with the chemotherapeutic medication gemcitabine (GMZ) was discovered to cause cell growth inhibition during the G0/G1 phase and dephosphorylation of the retinoblastoma protein (Rb) by using a polyoma middle T transformed murine endothelial cell line, indicating cell cycle arrest (288). Mass

spectrometry testing showed that extremely long chain ceramides were elevated after GMZ therapy. Neutral ceramidase (NCDase) protein levels and activity were shown to be selectively decreased due to GMZ in the research. Further experiments using small interfering RNA (siRNA) targeting NCDase showed that its knockdown induced cell cycle arrest, increased total ceramide levels, and Rb dephosphorylation. These findings suggest that GMZ's mechanism of action involves downregulation of NCDase, leading to elevated ceramide levels, and subsequent cell cycle arrest (288). Moreover, another study has demonstrated that the accumulation of sphingosine in mitochondria occurs when traumatic brain injury occurs, presumably due to the activation of neutral ceramidase expressed in the brain (289). Furthermore, multiple stress triggers, such as NO, have been shown to lead to the downregulation of neutral ceramidase in mesangial cells (290, 291).

On the other hand, distinct cellular locations are occupied by three alkaline ceramidases to perform their functions (243). Alkaline ceramidase 1 (ACER1), residing in the endoplasmic reticulum (ER), can specifically hydrolyze C24:0 and C24:1 ceramide (292, 293). It is noteworthy that the human ACER1 gene exhibits significant similarity to the mouse Acer1 gene (294). ACER1 is predominantly located in the skin, specifically in the epidermal keratinocytes rather than the dermal fibroblast cells (295). Its presence is crucial for maintaining skin equilibrium and overall energy balance in the body (295). When ACER1 was overexpressed, it was shown to be localized to the endoplasmic reticulum

(294). In terms of its function, a study has revealed that the expression of human ACER1, as well as acid ceramidase, is elevated when human epidermal keratinocytes are stimulated by extracellular calcium (292). The increased ACER1 and acid ceramidase promote the breakdown of ceramides into sphingosine and S1P (292). The increased sphingosine and S1P cause the growth arrest and differentiation of keratinocytes (292). Thus, if ACER1 is inhibited or downregulated, keratinocyte development and differentiation may be disrupted (292).

In contrast to ACER1, ACER2 is located at the Golgi body and can deacylate monounsaturated C18, C20, and C24 ceramides (296, 297). Moreover, the placenta exhibits high levels of ACER2 expression. Interestingly, in response to DNA damage, ACER2 induces ROS production to mediate apoptosis. The researchers indicated that doxorubicin-induced DNA damage in tumor cells led to an increase in sphingosine levels by upregulating the expression of ACER2 (298). However, knocking down ACER2 significantly reduced the apoptotic and necrotic effects induced by doxorubicin (302). Furthermore, the introduction of additional ACER2 resulted in the fragmentation of the Golgi complex and halted the growth of HeLa cells (296). Conversely, low levels of ectopic ACER2 expression led to elevated levels of S1P without an increase in sphingosine levels (296). As a result, this condition facilitated cell proliferation in a serum-free medium (296). Therefore, ACER2 serves an important function in the production of S1P and S1P-related

cell proliferation and survival. However, sphingosine buildup, caused by overexpression of ACER2, can cause cell growth halt (296).

Concerning ACER3, it is located in both the ER and the Golgi body, where it aids in the breakdown of unsaturated long-chain ceramides and dh-ceramides (299). Additionally, ACER3 exhibits high expression levels in the placenta. Recently, a study demonstrated that downregulation of ACER3 resulted in increased expression of p21 and a further decrease in cell proliferation (300). Another study reported that depletion of ACER3 in human acute myeloid leukemia cells led to reduced cell growth and colony formation, increased apoptosis, and diminished AKT signaling (301).

Recent years have seen a massive uptick in study and investigation of ceramidases. Ceramidases hydrolyze ceramide to sphingosine in the catabolic pathway (302). By blocking the breakdown of ceramide, ceramide levels rise in the cells as a result of these inhibitors, whereas sphingosine and S1P levels fall (189, 302, 303). Altering the balance of the ceramide/sphingosine 1-phosphate axis by several ceramidase inhibitors can have significant implications for various cellular processes and disease pathways, such as cell proliferation, cell signaling, apoptosis, and inflammation (189).

Currently, by altering ceramide's structure and stereochemistry, several ceramidase inhibitors have been created. These modifications aim to enhance the potency and

specificity of the inhibitors, providing valuable tools for studying the role of ceramidases in various biological processes (302). ARN14974, a benzoxazolone carboxamide, is a compound that specifically inhibits acid ceramidase. Its inhibitory effects on acid ceramidase have been observed both in vitro and in vivo, with an IC_{50} of 79 nM (303). This inhibition has been shown to significantly decrease acid ceramidase activity in multiple organs (302, 303). The compound has been found to exhibit inhibitory effects on acid ceramidase activity in SW403 adenocarcinoma cells with an IC50 of 825 nM and RAW 264.7 murine macrophages with an IC50 of 400 nM at concentrations ranging from 0.1 to 20 mM (303). In vivo administration of ARN14974 at a dosage of 10 mg/kg intravenously has been shown to reduce acid ceramidase activity in various organs, including the brain, liver, heart, lungs, and kidneys. Additionally, this treatment has been found to elevate pulmonary ceramide levels in mice (303). It is worth noting that a separate study utilized a concentration of 20 μM ARN14974 to treat neonatal rat cardiomyocytes (nrCM) for 48 hours, resulting in the inhibition of acid ceramidase and subsequent accumulation of ceramide (304). Additionally, in a myocardial infarction mice model, this acid ceramidase inhibitor was administered intraperitoneally at a concentration of 10 mg/kg. Following injection, the compound exhibited a prolonged effect on enzyme activity reduction in mouse hearts, lasting for over seven hours (304).

(1S,2R-D-erythro-2-N-myristoylamino)-1-phenyl-1-propanol, also known as D-erythro-MAPP, is a potent inhibitor of alkaline ceramidase (305). The alkaline ceramidase from HL-60 human promyelocytic leukemia cells has been demonstrated to be selectively inhibited by this inhibitor, with an IC50 value of 1-5 μM, indicating its high potency in inhibiting the enzyme's activity. In addition, researchers have discovered that this inhibition causes a dose- and time-dependent slowing of cell growth along with a cell cycle arrest in the G0/G1 phase (305, 306). Conversely, a separate study has shown that D-erythro-MAPP also exhibits weak inhibition of acid ceramidase, with an IC_{50} value of 500 μM (307). This inhibitor has been found to induce a dose-dependent inhibition of acid ceramidase in both human melanoma cells and human HaCat keratinocytes. Interestingly, it does not exhibit significant inhibition towards alkaline ceramidases in either of the tested cells. As a result of this inhibition, an elevation of endogenous ceramide levels can be observed, after which apoptosis was induced and proliferation was suppressed in HaCaT keratinocytes (307).

C6-urea-ceramide (D-erythro-N-[2-(1,3-dihydroxy-4E-octadecene)]-N'-hexane-ure-sphingosine) has been identified as a potent inhibitor of neutral ceramidase, with an IC50 value of 366 nM in mouse embryonic fibroblasts (308). Another study has provided further evidence of the inhibitory effect of C6-urea-ceramide on neutral ceramidase. C6-urea-ceramide has been found to selectively enhance the total levels of ceramide in-type mouse

embryonic fibroblasts (MEFs) and HT-29 colon cancer cells. However, this increase in ceramide levels is not observed in MEFs that lack neutral ceramidase, indicating that C6 urea ceramide specifically targets and modulates the activity of this particular ceramidase isoform (309). C6-urea-ceramide inhibits HT-29 cell growth when present at doses between 5 and 10 μM, inducing apoptosis as well as autophagy specifically in these colon cancer cells. However, these effects are not observed in non-cancerous RIE-1 cells, suggesting a selective impact on cancer cells while sparing normal cells (309). Furthermore, in an HT-29 mouse xenograft model, administration of C6 urea ceramide at doses of 1.25, 2.5, and 5 mg/kg for five consecutive days has been found to result in a reduction in tumor growth. Additionally, certain ceramide species, such as C16, C18, C20, and C24, are upregulated in tumor tissue after treatment with C6 urea ceramide (309). It is worth noting that the inhibition of neutral ceramidase does not promote cellular senescence in HT29 cells when treated with C6 urea ceramide at 5 and 10 μM (309).

1.3.2.3 Sphingosine Kinases and their related inhibitors

Furthermore, in sphingolipid metabolism, there exist two distinct types of sphingosine kinase, namely Sphingosine kinase 1 (SphK1) and Sphingosine kinase 2 (SphK2) (310), which are accountable for the production of S1P. S1P, in fact, plays a crucial role as a survival factor for vascular endothelial cells, stimulates the expression of surface molecules on endothelial cells, and regulates vascular permeability (311). The synthesis of S1P at the

plasma membrane is primarily mediated by sphingosine kinase 1 (312, 313), whereas SphK2 exerts its function in the nucleus, mitochondria, as well as ER (314). Additionally, SphK1 is a major contributor to S1P synthesis in the circulatory system and malignant tissues (314, 315, 316), while SphK2 is the more prevalent isoform in the liver, kidney, heart, pancreatic islets, and brain (317). Sphingosine kinases have been linked to various cellular processes such as cell division, cell survival, migration, regulation of calcium homeostasis, development of the cardiovascular and nervous systems, inflammation, immunity, and the growth of cancer cells (318, 319). A study has indicated that the deficiency of S1P due to SphK1/ SphK2 double-knockout in mice can significantly disrupt neurogenesis, including neural tube closure, and angiogenesis, ultimately leading to embryonic death (320). It is worth noting that the SphK1−/− mice exhibited normal viability and fertility without any apparent abnormalities, despite a significant but incomplete reduction in total sphingosine kinase activity. These results indicate that SphK1 and SphK2 may serve similar purposes, and the deficiency in SphK1 activity can be compensated for by SphK2 (324). Additionally, the pro-inflammatory cytokine TNFα, which has a significant impact on the endothelium, triggers the activation of SK and the production of S1P in endothelial cells. Inhibition of sphingosine kinase leads to a decrease in TNFα-induced expression of adhesion molecules, including E-selectin and vascular adhesion molecule-1 (321).

PF543 is a compound recognized as a selective inhibitor of SphK1, with an IC50 of 2 nM (322). SphK1 is responsible for converting sphingosine into sphingosine-1-phosphate (S1) in cells(322). Notably, SphK1 and S1P play significant roles in controlling the process of angiogenesis (323). In vitro, DNA synthesis was shown to be induced by S1P in a dose-dependent manner, and chemotactic motility of HUVECs (323). By inhibiting SphK1, PF543 disrupts the production of S1P (322). PF-543 effectively inhibits SphK1 by competitively binding to sphingosine. It demonstrates a high selectivity for SphK1 over the SphK2 isoform, with more than a 100-fold difference in potency. The inhibition constant (Ki) of PF-543 against SphK1 is measured at 3.6 nM (322). PF-543 effectively suppresses the production of S1P in 1483 cells as well as human whole blood. The half-maximal effective concentrations (EC50s) for inhibiting S1P formation are measured at 8.4 nM in 1483 cells and 26.7 nM in human whole blood (326). It is interesting to note that although PF-543 can efficiently inhibit SphK1, reducing the levels of S1P to nearly undetectable levels, it is unable to cause a significant buildup of dihydroceramides (dhCers) and their metabolites in human gastric cancer cell lines (324). Additionally, cell proliferation is not inhibited and G0/G1 cell accumulation does not occur (324).

ABC294640, an inhibitor of SphK2, has been demonstrated to effectively reduce the generation of S1P in intact cells. The measured inhibition constant (Ki) for this compound is 9.8 μM (325). Notably, ABC294640 specifically inhibits the activity of SphK2 with an

IC_{50} value of 60 μM, while having no impact on the activity of SphK1 at concentrations up to 100 μM (325). In MDA-MB-231 cells, Evidence suggests that ABC294640 reduce the production of S1P in a dose-dependent manner (325). Additionally, ABC294640 has exhibited inhibitory effects on the proliferation of various cancer cell types, with IC50 values of 6 μM in HepG2 liver cells, 12.2 μM in A-498 kidney cells, 32.8 μM in PANC-1 pancreas cells, and 48.1 μM in HT-29 colon cells (325). Additionally, it has been found that ABC294640 can alleviate intestinal inflammation in both mouse and rat models of Crohn's disease induced by trinitrobenzene sulfonic acid (TNBS) (326). Furthermore, it has been observed that ABC294640 induces autophagic cell death rather than apoptosis in TIVE-LTC, a KSHV long-term-infected immortalized endothelial cell line (327). However, this effect was not observed in uninfected TIVE cells (327). Increased levels of the LC3B protein were shown to be connected to the triggering of autophagic cell death (327). Transcriptomic analysis of TIVE-LTC cells exposed to ABC294640 revealed significant changes in genes associated with cellular stress responses, cell cycle regulation and proliferation, and cellular metabolic processes. These alterations suggest that ABC294640 treatment affects various cellular pathways involved in stress adaptation, cell division, and metabolism within TIVE-LTC cells (327). Additionally, a study has shown that treatment with the Sphk2-specific inhibitor ABC294640 at a concentration of 80 μM for 8 hours effectively inhibits SphK2 in A549 lung cancer cells, leading to decreased stability of the human telomerase reverse transcriptase (hTERT) (328).

K145 is another specific inhibitor of SphK2, which has a Ki value of 6.4 μM (329). It exhibits IC50 values of 4.3 μM for SphK2, while the IC50 for SPHK1 inhibition is greater than 10 μM, indicating a lower potency towards SPHK1 compared to SPHK2 (329). In vitro, when used at a concentration of 10 μM, K145 effectively inhibits the activity of SphK2 and reduces the S1P levels in human leukemia U937 cells without a significant impact on the activity of Sphk1 (329). Additionally, K145 has been shown to exhibit anti-growth effects in U937 cells and induce apoptosis in these cells, which may be attributed to the block of downstream ERK and Akt signalling pathways (329). In another study, PF-543 (2 μM), a Sphk1-specific inhibitor, and K145 (10 μM), a Sphk2-specific inhibitor, were used to treat brain microvascular endothelial cells. The findings indicated that inhibition of the activity of SphK can lower the inflammatory response and mitigate endothelial barrier disruption following oxygen-glucose deprivation and reoxygenation (OGD/R) conditions (330). Moreover, treatment with K145 at a concentration of 6 μM for 48 hr has been found to efficiently inhibit the proliferation of the glioblastoma cell line, U-251 cells (331).

1.3.3 Cellular functions of ceramide, sphingosine, and S1P

Evidently, sphingolipid metabolism is essential for the regulation of virtually all biological and physiological activities. In general, ceramide and sphingosine are known to be associated with cell cycle arrest, senescence, apoptosis, and cell differentiation (332).

Conversely, S1P acts as a stimulant for cell survival, proliferation, migration, invasion, and angiogenesis (332).

1.3.3.1 Cellular functions of ceramide

Cell cycle arrest, cell proliferation, cell differentiation, and cell death are all regulated by ceramide. Very long-chain ceramides, in particular, have been linked to mitochondrial malfunction, which in turn triggers oxidative stress and cardiomyocyte mortality (333). In addition, a separate research team demonstrated that ceramide influences growth and differentiation in cultured DJM-1 cells, which belong to the squamous cell carcinoma cell line (334). Evidence suggests that a significant increase (up to 10-15-fold) in endogenous levels of ceramide caused 80% of Molt-4 leukemia cells to arrest at the G0/G1 phase (335).

It is worth noting that recent studies have identified ceramide as a new candidate for regulating senescence. According to recent research, endogenous levels of ceramide have significantly increased (4-fold) in senescent WI-38 human diploid fibroblasts (HDF) (336). Additionally, it was found that ceramide hindered DNA synthesis and mitogenesis in young HDF at doses of 10-15 μM, resulting in a senescent phenotype (336).

Furthermore, there is evidence to suggest that ceramide is associated with various vascular diseases, including hypertension, atherosclerosis, angiogenesis, and remodeling, making it a new focus of cardiovascular research (337). One study has demonstrated that ceramide

can block vasodilation and elevate blood pressure by inhibiting the release of NO in ECs (337).

1.3.3.2 Cellular functions of sphingosine

Sphingosine plays a crucial role in apoptosis. Evidence suggests that sphingosine levels increase in cancer cells induced by various factors, including environmental stress, chemotherapeutic treatment, and apoptotic stimuli (338, 339, 340, 341, 342). Additionally, the exogenous addition of sphingosine can induce apoptosis by inhibiting protein kinase C (PKC) (341, 343).

In addition, sphingosine also serves an important function in cell cycle regulation. Available data indicates that sphingosine levels increase in response to DNA damage by upregulating ACER2 (344). In a study, treatment with sphingosine in MOLT-4 cells, a type of hematopoietic cells, resulted in the dephosphorylation of Rb as early as 1 hour post-treatment. The complete transition to the dephosphorylated state of Rb was observed four hours after treatment. These effects were evident even at low concentrations of sphingosine, ranging from 100 to 500 nM. The maximum effects, however, were observed at concentrations of 1-2.5 μM. The sphingosine-induced dephosphorylation of Rb further led to the inhibition of cell cycle progression at the G0/G1 phase and a decrease in phosphorylated Rb (345). Additionally, increased sphingosine levels through upregulation

of Human Alkaline Ceramidase 1 and Acid Ceramidase affect the cell calcium-induced differentiation of Epidermal Keratinocytes (292).

1.3.3.3 Cellular functions of Sphingosine-1-phosphate (S1P)

S1P, a potent lipid mediator, is synthesized from sphingosine via sphingosine kinases (SphKs) and exerts a positive influence on several crucial processes, including cell proliferation and survival, which are in contrast to its precursors (sphingosine and ceramide) (346, 347). Thus, the balance between these sphingolipid metabolites, also known as theamide-S1P rheostat, plays a pivotal role in determining cell fate (348). Numerous studies have demonstrated that cancer cells, including those of hematopoietic origin, exhibit tolerance to chemotherapy and radiation treatment, in part due to an imbalance in the S1P/ceramide rheostat(347, 349, 350). Additionally, S1P has been shown to mitigate apoptosis induced by SphK1-I (an inhibitor of SphK1) in human leukemia cells(351).

Studies have demonstrated that red blood cells, platelets, and endothelial cells are the primary sites responsible for S1P synthesis (352). Moreover, S1P exported from erythrocytes and endothelial cells contributes to high levels of S1P in the bloodstream (352). Additionally, erythrocytes are the primary source of plasma S1P due to their ability to produce, store, and release S1P into the plasma efficiently. Research has shown that the majority (75%) of plasma S1P in adult mice is produced by erythrocytes, and nearly all

embryonic S1P is generated by erythrocytes (353). Furthermore, S1P is crucial for the formation of the embryonic vascular system because it controls the activity of receptors involved in this process (353, 354). Activated and aggregated platelets release S1P in response to endothelial cell damage, and this localized increase in S1P levels contributes to the healing of vascular injury (355, 356).

Following the production of intracellular S1P, it can be released into the extracellular environment through ATP-binding cassette transporters (ABC transporters) to exert its primary functions (354, 355, 357, 358, 359). However, several studies have also reported that S1P can interact with intracellular receptors, including histone deacetylase (360), prohibitin 2 (361), and human telomerase reverse transcriptase (hTERT) (328). Besides, S1P serves as a cofactor in TRAF2-mediated RIP1 ubiquitination, which further leads to the activation of NF-κB (209). TNF receptor-associated factor 2 (TRAF2) is a crucial player in NF-κB signaling induced by TNFα (209). Notably, the activation of NF-κB signaling is a hallmark of endothelial dysfunction and inflammation, which is caused by endothelial senescence (1). Additionally, another study demonstrated that SphK1 and its product S1P can protect HUVCSs from the apoptotic effect of TNFα (362). Moreover, it has been reported that a specific binding motif of TRAF2 with SphK1 causes the activation of NF-κB (210). Furthermore, the interaction between extracellular S1P and five G-protein-coupled receptors (GPCRs) can initiate several signaling pathways that are vital in

determining cell fate and behavior, including migration, adhesion, survival, and proliferation (363).

1.3.4 The potential links between sphingolipids and cellular senescence

Recently, numerous studies have identified that sphingolipids and their upstream and/or downstream metabolites are involved in the regulation of senescence. As a bioactive player, ceramide and S1P have opposite effects on the regulation of cell fate. Specifically, ceramide promotes cell death and negatively influences cell growth, whereas S1P promotes cell survival and proliferation and inhibits cell death (194). One study has shown that endogenous ceramide levels increase 2.4 times in HUVECs during the initiation of replicative senescence. Furthermore, they also demonstrated that long-term exogenous C6-ceramide treatment in low passage cells can induce senescence, which is characterized by the suppression of cell proliferation and DNA replication, as well as an increase in SA-β-gal expression (364). On the other hand, this research team also indicated that treatment with exogenous C6-ceramide can promote senescence in human fibroblasts(336). Similarly, in IMR-90, another human fibroblast line, ceramide levels were increased two times in senescent cells compared to young cells (365). Mechanistically, in human diploid fibroblasts, ceramide increases the level of P21, resulting in the inhibition of CDK2 (366). Additionally, ceramide results in Rb dephosphorylation (367), both of which have the potential to induce cell cycle arrest and senescence (366, 367).

Moreover, S1P, an additional bioactive molecule renowned for its pro-growth and pro-survival properties, has been identified as a potential contributor to anti-senescence (363). The production of S1P is facilitated by the phosphorylation of sphingosine by SphK1 and SphK2 (363). Previous research has shown that the reduced expression of SphK1/SphK2 leads to a decline in S1P levels, thereby promoting cellular senescence (368). Inhibition of SphK1 activity through pharmacological means or gene silencing impairs proliferation and induces senescence in human adipose-derived stromal cells (hAD-SCs) (368). However, treatment with exogenous S1P and the ceramide synthesis inhibitor fumonisin B1 can rescue SphK1 knockdown-induced cellular senescence (368). Recent research has also revealed that both human and mice fibroblasts contain S1P, and it binds to human telomerase reverse transcriptase (hTERT), thereby sustaining telomerase and promoting telomere durability and cell proliferation (328).

A recent study has demonstrated that chemical inhibitors targeting SphK1, SphK2, or dihydroceramide desaturase (Des1) can increase the levels of p53 and p21, two key markers of cellular senescence, in prostate cancer cells (369). Notably, siRNA-mediated knockdown of SphK1 or SphK2 fails to elevate p53 and p21 levels, while SphK1 silencing attenuates DNA synthesis in prostate cancer cells (369). Conversely, Des1 silencing results in an increase in p53 levels, and simultaneous knockdown of Des1 and SphK1 elevates p21 levels (369). Collectively, these results indicate that Des1 and SphK1 work together in a

substantial manner to induce senescence and control cell proliferation in prostate cancer cells through p53/p21-dependent and independent mechanisms (369).

Conversely, sphingosine's function in senescence has received little study. One study revealed that sphingosine induces MOLT-4 (human leukemic cell lines) cell cycle arrest in the G1 phase and dephosphorylation of pRb in a concentration- and time-dependent manner, ranging from 100nM to 500 nM (370). However, the underlying mechanisms remain unclear, and it is hypothesized that altered phosphatase activity leads to the dephosphorylation of Rb(370). Additionally, research has shown that sphingolipids, such as ceramide and sphingosine, accumulate in the liver and brain as individuals age (371, 372). Moreover, studies regarding the role of other sphingolipids in senescence have also been reported, including an eight-fold activation of neutral sphingomyelinase (N-SMase) in senescent fibroblasts (336).

In conclusion, the influence of specific sphingolipid metabolites and enzymes on cellular senescence has been established. However, the relationship between sphingolipids, particularly sphingosine, and EC senescence remains inadequately explored. Therefore, further research is necessary to fully elucidate the underlying mechanisms linking bioactive sphingolipids and EC senescence.

Chapter 2. Hypothesis and Aims

The role of particular sphingolipid metabolites and enzymes in the process of cellular senescence has been demonstrated. Given the these aspects, this thesis is structured to examine the regulatory role of sphingosine in H2O2-induced EC senescence, specifically its potential involvement in causing cell cycle arrest.

My project was consist of two parts:

Aim 1: To investigate the role of sphingosine on H_2O_2-induced EC senescence *in vitro*.

Aim 2: To identify the effect of sphingosine on cell cycle arrest in H_2O_2-induced EC senescence.

Chapter 3. Materials and Methods

3.1 Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords obtained from Royal Prince Alfred Hospital (ethics#2019/ETH06859). HUVECs were maintained in gelatin-coated (Sigma Aldrich, Cat#G9391) T25 flasks with Medium 199 (Sigma, Cat#M4530) supplemented with 20% foetal calf serum (Sigma, Cat#F9423), sodium bicarbonate (Invitrogen), 20 mM HEPES (Thermo Fisher, Cat#15630-080), 1 mM sodium pyruvate (Thermo Fisher, Cat#11360070), 1X MEM non-essential amino acids (Thermo Fisher, Cat#11140050), 0.1% Sodium Bicarbonate (Thermo Fisher, 25080-094), 22.5 μg/ml Endothelial Cell Growth Supplement (ECGS) (BD bioscience, Cat#354006) and 15 μg/ml heparin (Sigma, Cat#H3393) and 100 units/ml Penicillin/Streptomycin antibiotics (Thermo Fisher, Cat#15140122). Cells were cultured at 37 °C, 5% CO_2, and only passages 1-3 were used for experiments.

3.2 Cell treatment

Sphingolipids and their enzyme inhibitors (Table 3.1) were added to HUVECs at a confluence between 20-30% for 24 hr. When HUVEC confluence reached 30-50%, these compounds were withdrawn, followed by the addition of 0.3 mM hydrogen peroxide to induce senescence. Hydrogen peroxide was also withdrawn in 24 hr (Figure 3.1).

Table 3.1 The function and source of certain treatment

Name	Function	Source
ABC294640	Inhibitor of sphingosine kinase 2	Echelon Biosciences, Cat# B0025
ARN14974	Inhibitor of acid ceramidase	Cayman, Cat#17119
C2 Ceramide	Exogenous ceramides	Avanti Polar Lipids, Cat#860502P
C6 Ceramide	Exogenous ceramides	Avanti Polar Lipids, Cat#860506P
C6-urea-cer	Inhibitor of neutral ceramidase	Sigma-Aldrich, Cat# 857400P
D-erythro-MAPP	Inhibitor of alkaline ceramidase	Cayman, Cat#10165
Dimethyl Sulfoxide (DMSO)	Vehicle	Sigma-Aldrich, Cat#D2650
Fumonisin B1(FB1)	Inhibitor of ceramide synthases	Cayman, Cat#62580
K145	Inhibitor of sphingosine kinase 2	Cayman, Cat# 11691
PF-543	Inhibitor of sphingosine kinase 1	Cayman, Cat# 17034
Sphingosine	Exogenous sphingosine	Echelon Biosciences, Cat#S-1000
Sphingosine-1-phosphate (S1P)	Exogenous S1P	Avanti Polar Lipids, Cat#860492

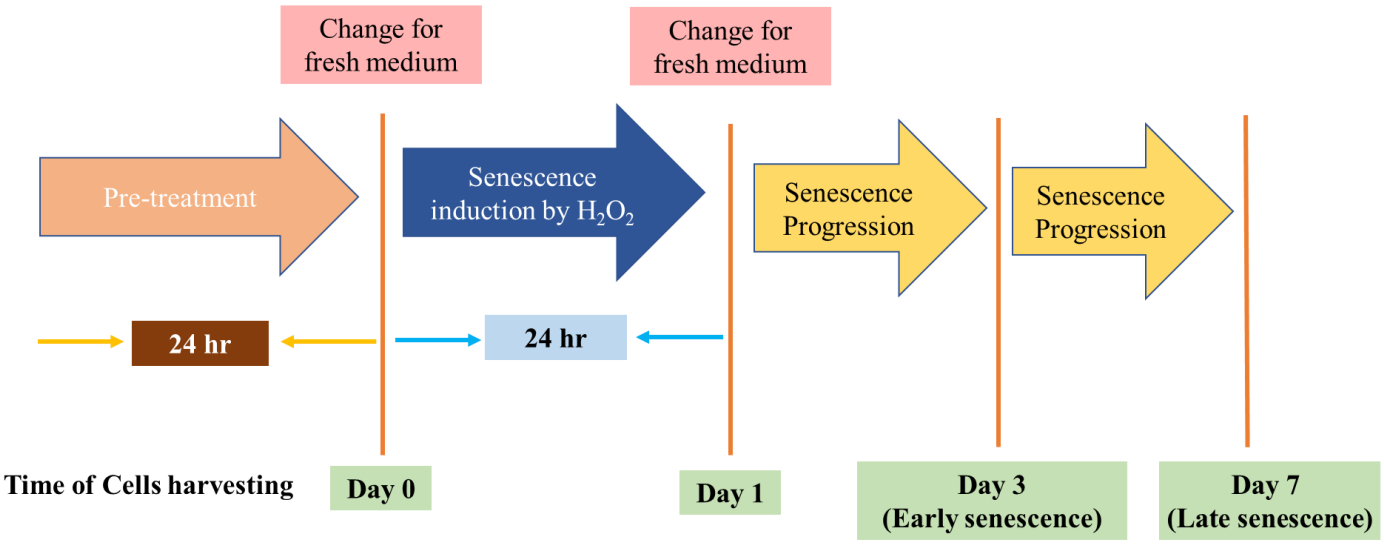

Figure 3.1 Timeline of treatment

3.3 Senescence associated β-galactosidase (SA-β-Gal) staining

SA-β-Gal staining was performed using a commercial kit (Cat#9860; Cell signalling Technology) following the manufacturer's protocol. After removal of the growth medium, HUVECs were washed once with 1X DPBS (Dulbecco's phosphate-buffered saline, Thermo Fisher, Cat#14190250). Then 1X Fixative solution was added. Cells were fixed for 15 min at room temperature. Next, cells were rinsed with 1X DPBS twice and stained with SA-β-Gal staining solution (PH 6.0, critical) for 24 hr at 37 °C in an incubator without CO_2. To avoid evaporation, I sealed the plates with parafilm. After staining, the SA-β-Gal staining solution was removed, and 70% glycerol was used for preservation. Cells positive for SA-β-Gal staining were imaged using a microscope at 200X magnification.

3.4 Cell viability assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, CAT#G1112), a colourimetric method, was employed to determine the cell viability. This assay detects the formazan dye resulting from the reduction of the MTS tetrazolium compound in metabolically active cells.

HUVECs were plated at 0.8 x 10^4 cells per well in a 96-well plate. After treatments, an MTS assay was performed by incubating cells in 100 µl fresh medium supplemented with 20 µl MTS working solution consisting of MTS (Promega, CAT#G1112) and phenazine

methosulfate (PMS, Sigma, P9625). MTS incubation lasted for 1 hr at 37 °C, and luminescence was examined at 490 nm on a TECAN Infinite M1000Pro plate reader.

3.5 Quantitative RT-PCR

After the desired treatment and washing with DPBS, 1 ml TRIzol (Invitrogen, CAT#15596026) was added to the T25 flask of HUVEC cultures and incubated for 8 min. Chloroform was added and mixed well by vigorous shaking. After settling at room temperature for 3 min, the two phases were separated. The top phase was carefully transferred into a new tube without touching the interphase. Then isopropanol was added to precipitate mRNA, followed by rinsing with 75% ethanol. After air-drying, the pellets were dissolved in RNase-free water (Sigma). Subsequently, the concentration and purity of mRNA were determined by a Nanodrop spectrophotometer (Thermo Fischer Scientific).

To remove any DNA contamination, DNase (NEB, CAT#M0303) was used as per the manufacturer's instructions. 10 µg RNA was added to a reaction mix, which contains 10 µl reaction buffer, 1 µl DNase I, and nuclease-free H_2O to a final volume of 100 µl. Next, the reaction was incubated at 37 °C for 10 min. Subsequently, the reaction was heat inactivated at 75 °C for 10 min, in the presence of 1 µl of 0.5 M EDTA.

RNA was reverse-transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher CAT#4368814). According to the manual, 1 µg mRNA

was added to a 20 µl reaction system consisting of 2 µl 10X RT buffer, 0.8 µl 25X dNTP, 2 µl 10X random Primers and 1 µl reverse transcriptase. The reverse transcription was carried out by Eppendorf Mastercycler Nexus Gradient under the following program: 25 °C 10 min, 37 °C 2 hr, 85 °C 5 min. Once finished, cDNA was diluted with an additional 40 µl of RNase-free water. qRT-PCR was carried out on a Roche Lightcycler 480 machine using SensiFAST™ SYBR Lo-ROX Kit (Bioline, London, UK, CAT #BIO-94005). A total 10 µl reaction system includes 0.2 µl forward primer (10 µM), 0.2 µl reverse primer (10 µM), 5 µl SensiFAST SYBR®Lo-ROX mix, 2 µl cDNA and 2.6 µl nuclease-free H_2O. cDNA was denatured at 95 ºC for 10 min, then amplified with 40 cycles of 95 ºC for 10 sec, 60 ºC for 15 sec, and 72 ºC for 20 sec. *β-actin* was used as the reference gene. Target gene expression was normalised to *β-actin* level. The primer sequences are listed below:

Human *ASAH1*

Forward sequence: GCACAAGTTATGAAGGAAGCCAAG

Reverse sequence: TCCAATGATTCCTTTCTGTCTCG (373).

Human *ASAH2*

1st time:

Forward sequence: TCAGAGAGCAAAGTCAAAAACAT

Reverse sequence: CCCTTCTGTTTTCCCCTGTGTA (designed by Primer-BLAST, National Center for Biotechnology Information, US)

2nd time:

Forward sequence: AGATGTGACTGTCTGGCTCAAT

Reverse sequence: CCCTTCTGTTTTCCCCTGTGTA (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *ACER1*

1st time:

Forward sequence: ATCCGCCTGGTCTTCATC

Reverse sequence: CTCCTTATTGCTGGTCTTCC (designed by Primer-BLAST, National Center for Biotechnology Information, US)

2nd time:

Forward sequence: CAGTTCATCCGCCTGGTCTT

Reverse sequence: TCCTTATTGCTGGTCTTCCTGT (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *ACER2*

Forward sequence: CCTGTTCTGCTGGATCAGTGAC

Reverse sequence: GTAGGCAAAGCATACACAGCCC (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *ACER3*

Forward sequence: CAATGTTCGGTGCAATTCAGAG

Reverse sequence: GGATCCCATTCCTACCACTGTG (374)

Human *CERS1*

Forward sequence: AGCAAAGCCGAGGAAGCA

Reverse sequence: TGGGTTCTCCCTTCCAAACA (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *CERS2*

Forward sequence: CCAGGTAGAGCGTTGGTT

Reverse sequence: CCAGGGTTTATCCACAATGAC (375)

Human *CERS3*

1st time:

Forward sequence: TCGCACAGATGGTGTCCTG

Reverse sequence: GCTTCCTGTTTCACCACTTGT (designed by Primer-BLAST, National Center for Biotechnology Information, US)

2nd time:

Forward sequence: GACATTTGGCTGGAGTCTGC

Reverse sequence: AAGGAAAAACAATGAGGCGGC (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *CERS4*

Forward sequence: TGGAGCTGGGGGACTGATTA

Reverse sequence: ACTGGACAGCATTCTCTGCT (designed by Primer-BLAST, National Center for Biotechnology Information, US)

Human *CERS5*

Forward sequence: CAAGTATCAGCGGCTCTGT

Reverse sequence: ATTATCTCCCAACTCTCAAAGA (375)

Human *CERS6*

Forward sequence: AAGCAACTGGACTGGGATGTT

Reverse sequence: AATCTGACTCCGTAGGTAAATACA (375)

Human *SPHK1*

Forward sequence: AGGCTGAAATCTCCTTCACGC

Reverse sequence: GTCTCCAGACATGACCACCAG (376)

Human *SPHK2*

Forward sequence: GCTGCTGCGCCTTTTCTTG

Reverse sequence: CCTGTAGCGGCCCATACTC (376)

Human *ACTB* (*β-actin*)

Forward sequence: CATGTACGTTGCTATCCAGGC

Reverse sequence: CTCCTTAATGTCACGCACGAT (376)

3.6 Western blotting

Proteins were extracted from HUVECs with or without treatments. Cells were harvested by a cell scraper into DPBS (Thermo Fisher). After removal of DPBS by centrifugation at 1000 rpm, lysis buffer containing 1% Triton X-100 was added to cell pellets. Then cells were sonicated by QSonica-Q800R2 for 10 min and centrifuged at 15000 rpm for 5 min at 4 °C. Protein concentrations were determined by Bicinchoninic Acid (BCA) assay. BCA working solution containing 200 µl bicinchoninic acid solution (Sigma B9643) and 4 µl copper (II) sulphate solution (Sigma C2284). Following a 15-min incubation at room temperature, the plate was read at 562 nm on a TECAN plate reader. After obtaining the protein concentration, 15 µg protein was mixed with NuPAGE LDS sample buffer (Thermo Fisher, NP0007) and NuPAGE Sample Reducing Agent (Thermo Fisher, NP0004) and loaded into Bolt 4-12% Bis-Tris Plus Gels in MOPS running buffer (Thermo Fisher NP0001) for electrophoresis. When electrophoresis was finished, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, IPVH00010) using 1X NuPage transfer buffer (Thermo Fisher, Cat#NP00061). Then PVDF was blocked with 5% skim milk for 45 min. Immunoblotting was performed with the following antibodies from Cell Signaling Technology: p21 Waf1/Cip1 (12D1) Rabbit mAb (Cat#2947S), Acetyl-p53 (Lys382) Antibody (Cat #2525), p53 (7F5) Rabbit mAb (Cat #25270, Phospho-Rb (Ser807/811) Antibody (Cat #9308T), Rb (4H1) Mouse mAb (Cat #9309T) and β-actin

Mouse mAb (Cat #3700). Immunodetection was carried out by chemiluminescence using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Cat#WBKLS0500) with a BIORAD ChemiDoc TOUCH imaging system. Quantification was carried out using Image J software.

3.7 Statistics

All data were presented as mean ± SD. One-way ANOVA was applied to compare multiple groups using GraphPad Prism version 9. P-values under 0.05 were considered statistically significant.

Chapter 4. Sphingosine is a *bona fide* promoting factor of H_2O_2-induced EC senescence

Vascular endothelial cell (EC) senescence and related vascular dysfunction contribute to the development of cardiovascular disease in ageing (377). The onset and progression of EC senescence and vascular disease in ageing have been linked to dysregulation of the cell cycle, induction of oxidative stress, vascular inflammation, altered calcium signalling and inhibition of angiogenesis (9). Recent research has shown that eliminating senescent cells may be a treatment option for ageing and a variety of age-related diseases (132, 378, 379). Nonetheless, therapeutic approaches to influencing cellular senescence remain in their infancy.

Sphingolipids are a class of essential lipids that involves in several significant cellular functions, particularly the cellular structure and cellular processes, such as cell cycle, senescence, proliferation, and migration (193). Hence, understanding how sphingolipids regulate endothelial cell senescence is particularly important for deciphering cardiovascular disorders, such as atherosclerosis (380). An increasing body of evidence has suggested that sphingolipids and their metabolism play an important role in the regulation of senescence (380). However, the majority of studies have focused on ceramide-induced senescence, and very little has been discovered about other sphingolipid-induced senescence.

In this chapter, I employed various sphingolipids and their metabolic enzyme inhibitors to investigate the major contribution of sphingolipids in the regulation of H_2O_2-induced premature senescence in HUVECs.

4.1 H_2O_2-induce premature senescence in HUVECs

To understand the dynamics of EC senescence process and to determine the optimal time point in the following tests of anti-senescence compounds, I treated HUVECs with 300 μM H_2O_2 for 4 hr, 8 hr, 24 hr, 3 days, and 7 days (Figure 4.1 A). Conventionally, suggested cell cycle regulators, such as p21, acetyl p53 and phosphorylated Rb, were examined as hallmarks of senescence. As shown in Figure 4.1 A and B, I observed a significant 2.6-fold increase in p21 protein levels as early as 4 hr after H_2O_2 treatment as compared with the untreated group (0 hr). It was further elevated to 3.1-fold at 8 hr and reached the peak of 3.5-fold at 24 hr. After that, it backed to 3-fold on day 3 and 3.2-fold on day 7.

In contrast, acetyl p53 levels exhibited a transient increase at both 4 hr and 8 hr by around 3.7 and 2.8 times, respectively. At the end of day 1 and day 3, acetyl p53 levels dropped to 59% and 58% of the untreated group, respectively. On day 7, the level was only 21% of the untreated group.

The expression level of p-Rb was decreased to 41% of the untreated control at 4 hr, and then it dropped to 30% at 8 hr. The lowest level was observed at 24 hr, which was only 10%

of the untreated. The level of p-Rb had some slight increase on day 3 and day 7, which were 13% and 28% of the untreated, respectively.

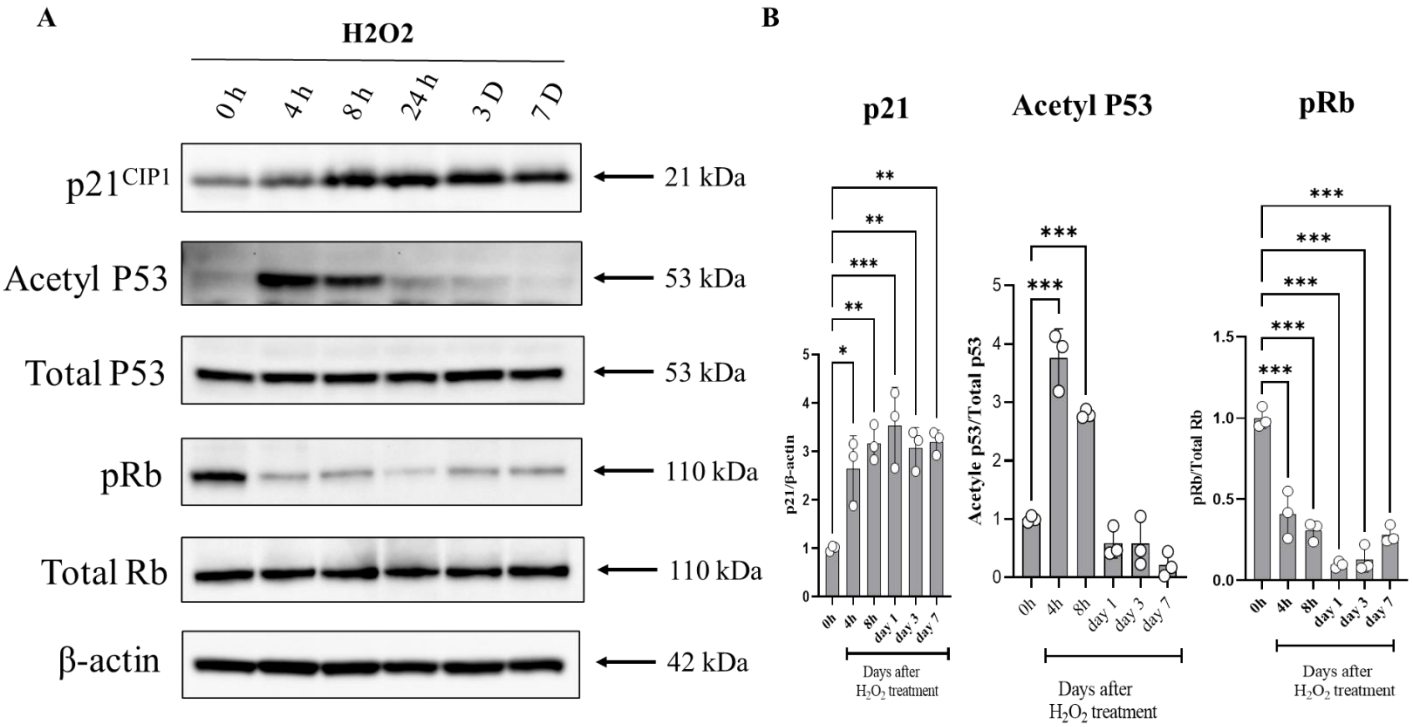

Figure 4.1 H_2O_2-induced premature senescence in HUVECs. (A) HUVECs were treated with 300 μM H_2O_2 for 24 hr and analysed using Western blotting at indicated time points. (B) Expression levels of the indicated proteins were quantified; n=3. Data are expressed as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; versus 0 hr.

4.2 mRNA levels of sphingolipid enzymes in normal HUVECs

A growing body of studies indicates that lipids, particularly sphingolipids, are critical players in the regulation of cellular senescence. To further understand the role of sphingolipids in this process, a panel of genes encoding sphingolipid enzymes were examined by qRT-PCR in normal HUVECs (Figure 4.2). I mainly targeted three subgroups of these enzymes, including ceramidases, ceramide synthases and sphingosine kinases, because of their significance in the regulation of the production of ceramide, sphingosine and S1P in the sphingolipid metabolic pathway.

Firstly, I examined the levels of all six ceramide synthases *CERS1-6*. Among them, the very long-chain ceramide (C22 and C24)-producing enzyme *CERS2* was most abundantly expressed, followed by C16 ceramide-producing enzymes *CERS5* and *CERS6* (Figure 4.2). However, the other three ceramide synthases exhibited an extremely low expression (Figure 4.2).

Secondly, I examined *ASAH1* (acid ceramidase), *ASAH2* (neutral ceramidase) and *ACER1-3* (alkaline ceramidases). To compare the abundance of these target genes, all of my data were normalised to *β-actin*. Among all ceramidases, *ASAH1* was the most abundant one, followed by *ACER3* (Figure 4.2). In contrast, *ASAH2* and *ACER1* were not detectable for up to 40 PCR cycles, which was confirmed using two sets of primers (Figure 4.2).

Last, I examined the expression levels of *SPHK1* and *SPHK2*. Clearly, *SPHK1* is the predominant sphingosine kinase isoform in normal HUVECs.

These results suggest which sphingolipid enzymes and their related sphingolipids are relatively more important to endothelial cell biology.

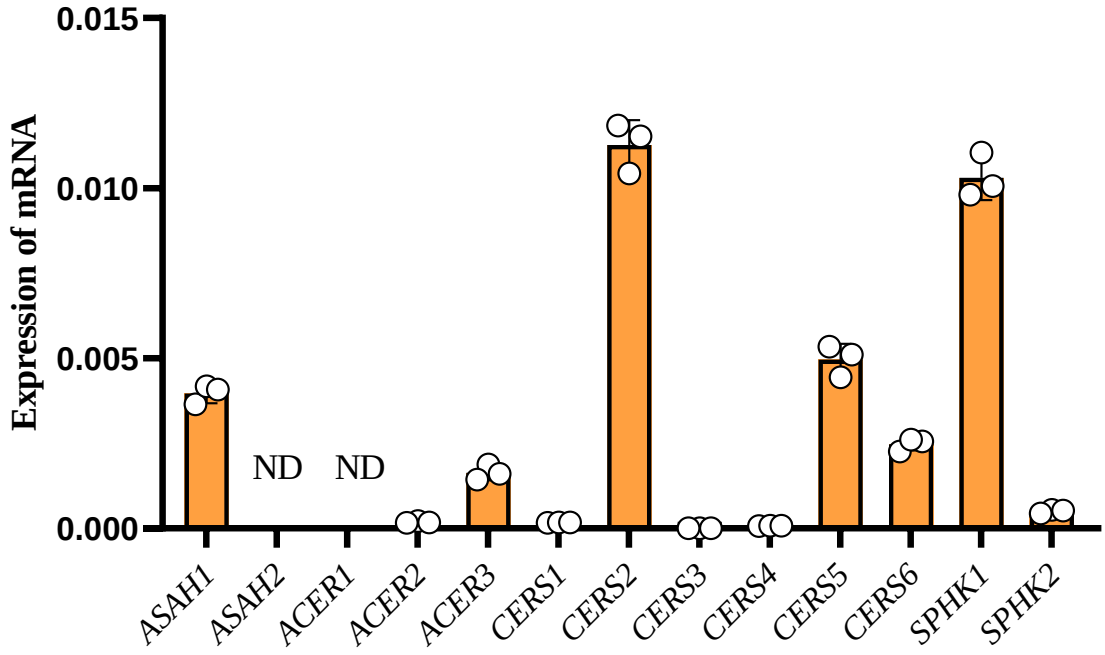

Figure 4.2 mRNA levels of sphingolipid enzymes in normal HUVECs. The mRNA expression level of sphingolipid enzymes in normal HUVECs was determined by qRT-PCR and then normalised to *β-actin*. Data are expressed as $2^{-\Delta CT}$, n=3. ND, not detectable

4.3 Ceramide Synthases are upregulated in H_2O_2-induced senescence

Having determined the abundance of sphingolipid enzymes of interest in HUVECs, I further examined whether they were altered in EC senescence at day 0 (normal, untreated cells), day 3 (early senescent cells) and day 7 (late senescent cells) after H_2O_2 treatment.

I firstly examined ceramide synthase levels. The most abundant isoform *CERS2* was unaltered in EC senescence (Figur 4.3). Meanwhile, the *CERS5* and *CERS6* levels were only slightly changed by around 26% in particular stages of senescence progression (Figure 4.3). Interestingly, the minor isoforms *CERS3* and *CERS4* exhibited a more robust increase in EC senescence (Figure 4.3). However, considering their extremely low abundance, as shown in figure 4.2, this degree of gene upregulation seemed not sufficient to dramatically alter ceramide production and composition. Meanwhile, the *CERS1* level was marginally altered on day 3 of H_2O_2 treatment (Figure 4.3)

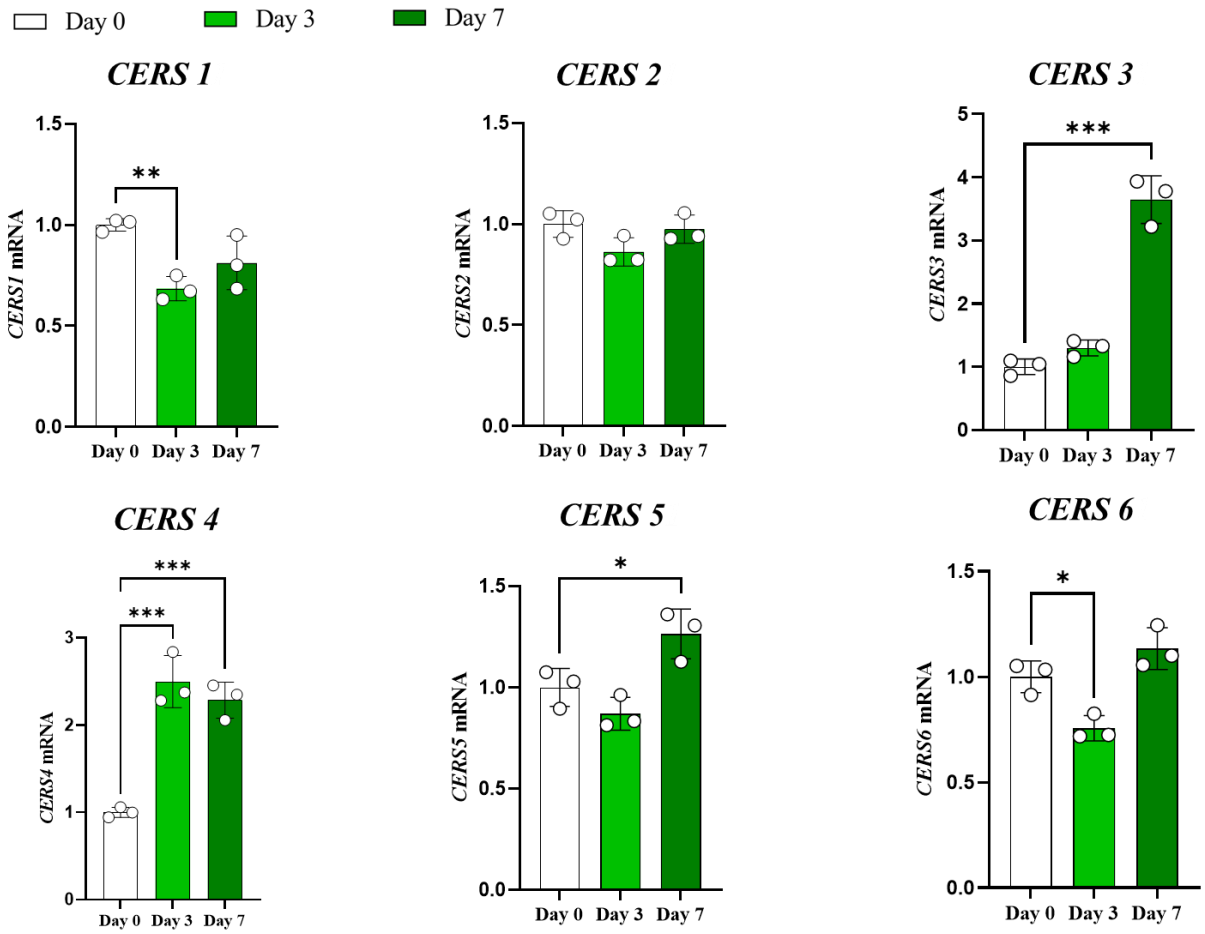

Figure 4.3 Ceramide Synthases are upregulated in H_2O_2-induced senescence. Senescence was induced by 300 μM H_2O_2 for 24 hr. The mRNA expression of *CERS1*, *CERS2*, *CERS4*, *CERS5* and *CERS6*, the gene encoding human ceramide synthase, was determined by qRT-PCR and then normalised to *β-actin*. Data are expressed as the fold-change compared with untreated control and shown as mean ± SD; n=3. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to day 0.

4.4 Acid ceramidase is upregulated in H_2O_2-induced EC senescence

I further examined the changes in ceramidases. Notably, the *ASAH1* mRNA levels increased temporally during senescence progression (Figure 4.4). Compared with day 0 control, it was elevated by 1.3-fold and 2-fold on day 3 and day 7, respectively (Figure 4.4). This trend was also observed in *ACER2* (Figure 4.4). However, as *ASAH1* mRNA was 21-fold more abundant than *ACER2*, my data indicates that *ASAH1* but not *ACER2*-mediated ceramide conversion to sphingosine might play a more important role in EC senescence. In contrast, the *ACER3* level was unchanged between time points (Figure 4.4). Same as shown in Figure 4.2, *ASAH2* and *ACER1* mRNA levels were not detectable.

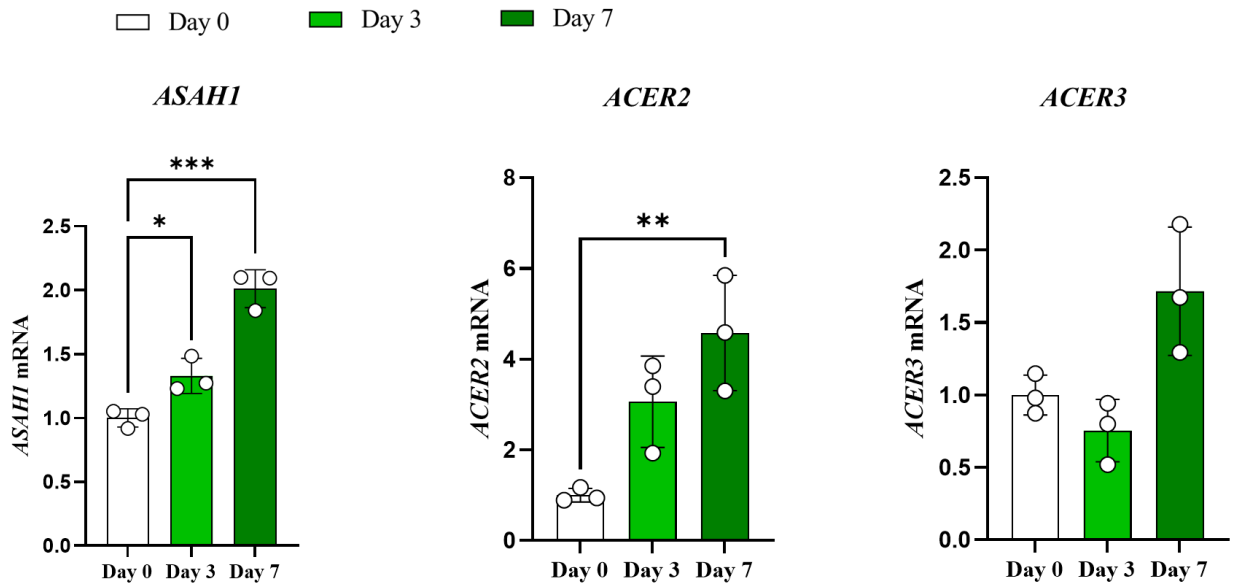

Figure 4.4 Acid ceramidase is upregulated in H_2O_2-induced EC senescence. Senescence was induced by 300 μM H_2O_2 for 24 hr. The mRNA expression of *ASAH1*, the gene encoding human acid ceramidase; *ACER2* and *ACER3*, the gene encoding human alkaline ceramidase, was determined by qRT-PCR and then normalised to *β-actin*. Data are expressed as the fold-change compared with untreated control and shown as mean ± SD; n=3. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, compared to day 0.

4.5 *SPHK1* and *SPHK2* levels were marginally altered in H_2O_2-induced EC senescence

Last, I examined whether *SPHK* levels were modulated in H_2O_2-induced senescence in HUVECs. The mRNA expression of *SPHK1*, the predominant sphingosine kinase isoform, was unaltered in EC senescence (Figure 4.5). Meanwhile, *SPHK2* level increased by 1.4-fold in early senescent HUVECs (Figure 4.5). These results imply that the rate of *SPHK*-mediated S1P production from sphingosine may not change in EC senescence.

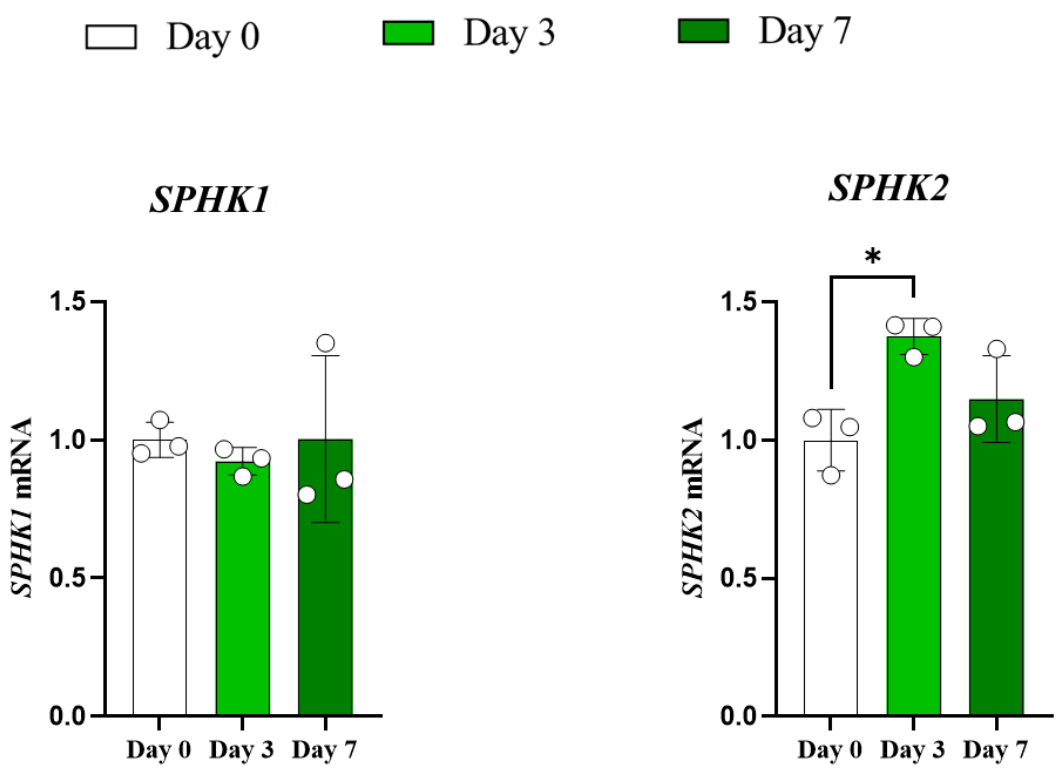

Figure 4.5 *SPHK1* and *SPHK2* levels were marginally altered in H_2O_2-induced EC senescence. Senescence was induced by 300 μM H_2O_2 for 24 hr. The mRNA expression of *SPHK1* and *SPHK2*, the gene encoding human Sphingosine Kinase, was determined by qRT-PCR and then normalised to *β-actin*. Data are expressed as the fold-change compared with untreated control and shown as mean ± SD; n=3. *$p < 0.05$; compared to day 0.

4.6 Inhibition of ceramide and sphingosine synthesis inhibits H_2O_2-induced EC senescence

Having examined changes in sphingolipid enzyme expression, I further interrogated whether inhibiting certain enzymes could promote or block senescence development. To this end, I pre-treated sphingolipid enzyme inhibitors 24 hr prior to H_2O_2 treatment (Figure 4.6) and examined the senescence development using SA-β-Gal staining.

H_2O_2 treatment alone induced 58% of senescence in HUVECs, defined by SA-β-Gal positivity (Figure 4.7). FB1 inhibits the de novo synthesis of ceramide, which also results in reductions in ceramide, sphingosine and S1P levels, while ARN14974 inhibit acid ceramidase-mediated ceramide conversion to sphingosine, leading to increased ceramide and decreased sphingosine and S1P (Figure 4.6). Interestingly, pre-treatment with FB1 significantly reduced the senescence to 24% in HUVECs (Figure 4.7), while pre-treatment with ARN14974 resulted in a similar reduction of senescence to 20%. These data together indicate that decreased sphingosine and S1P levels, rather than altered ceramide levels, contributed to EC senescence development. In addition, neutral ceramidase inhibitor C6-urea ceramide and alkaline ceramidase inhibitor D-erythro-MAPP failed to alter the percentage of HUVECs senescence which were around 50% and 53%, respectively. This aligned with my findings in Figure 4.2 and Figure 4.4 that acid ceramidase *ASAH1* was the

predominant ceramidase isoform in normal HUVECs and exhibited significant increase in HUVECs senescence.

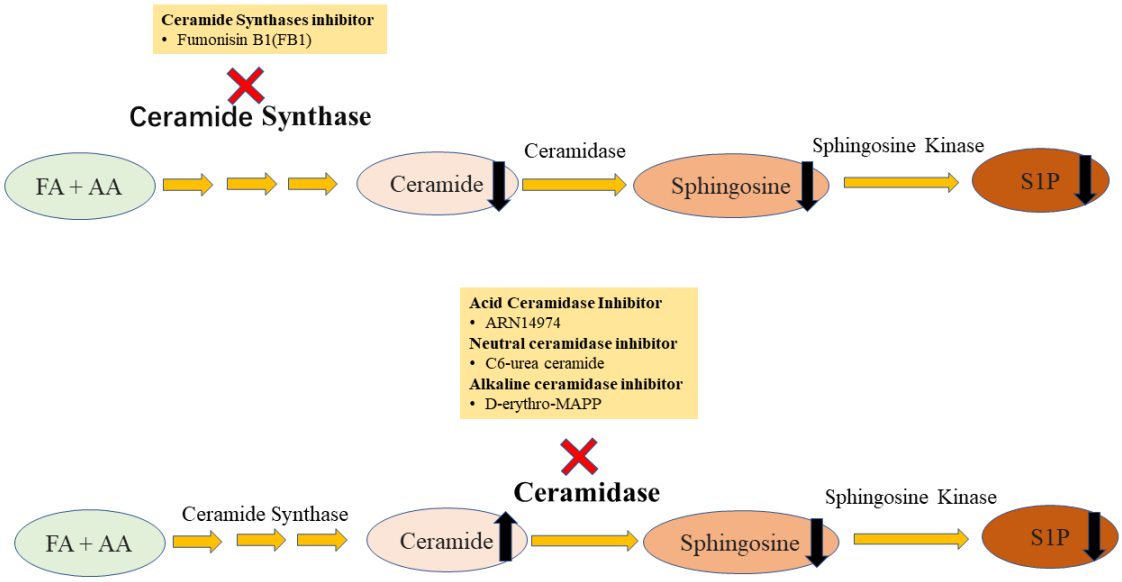

Figure 4.6 Schematic overview of the treatment design. Ceramide can be synthesised from amino acid and fatty acid by ceramide synthase through several processes. Then ceramide can be further catabolised to sphingosine by 3 types of ceramidases. Inhibitors of ceramide synthase and ceramidase were able to modify these enzyme activities and further alter the production of ceramide, sphingosine and S1P.

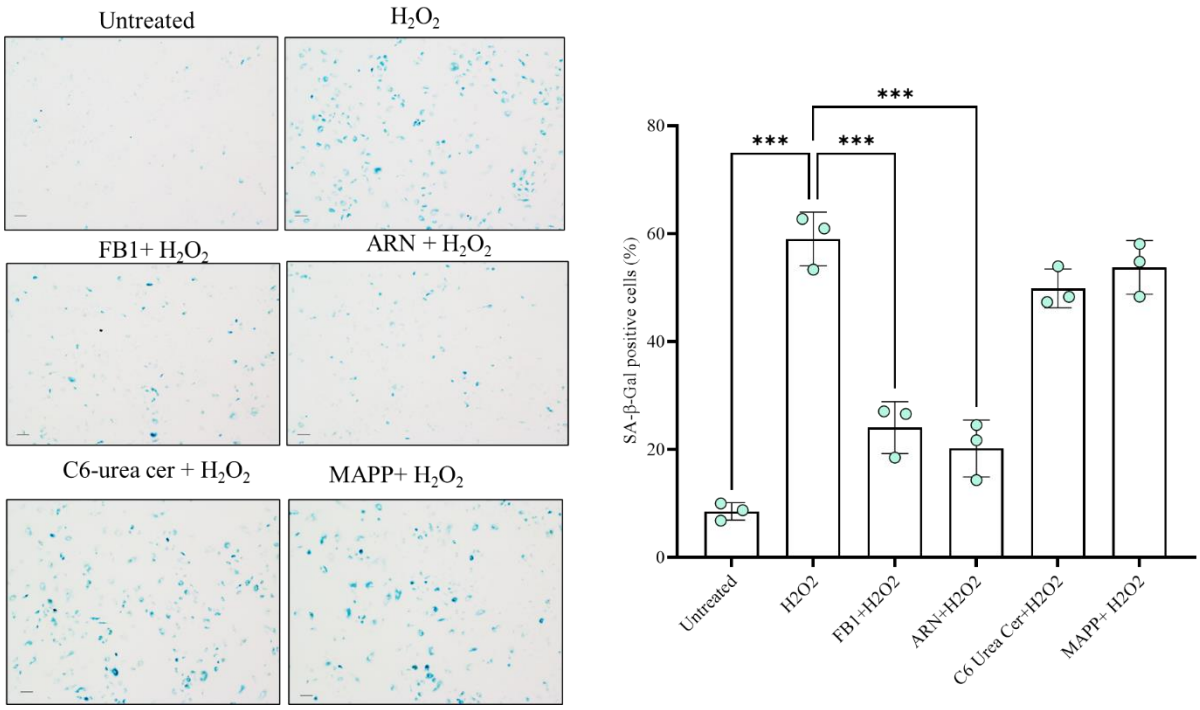

Figure 4.7 Inhibition of ceramide and sphingosine synthesis inhibits H_2O_2-induced EC senescence. (A) HUVECs were pre-treated with 10 μM FB1 for 24 hr, 10 μM ARN14974, 10 μM C6 urea ceramide and10 μM D-erythro-MAPP, then treated with 300 μM H_2O_2 for 24 hr. SA-β-Gal staining was performed at day 7. Senescent cells were stained with blue colour. Magnification: 400X; scale bar = 100 μm. (B) Data was expressed as mean ± SD; n=3. ***$p < 0.001$, compared to H_2O_2 group.

4.7 Sphingosine promotes H_2O_2-induced EC senescence

Next, I further confirmed that ceramide was not a regulator of EC senescence and dissected whether sphingosine or S1P played a leading role. Pre-treatment with C2 ceramide and C6 ceramide did not alter the percentage of senescence in HUVECs, which were 52% and 63% (Figure 4.8), confirming that ceramide was not attributable to EC senescence. In Figure 4.7, a significant inhibition of senescence has been observed with treatment of FB1 and ARN14974, which strongly suggest that decreased levels of sphingosine and S1P were associated with the senescence inhibition. Thus, I pre-treated HUVECs with sphingosine prior to H_2O_2. Sphingosine significantly increased senescence to 80% (Figure 4.8), which, together with FB1 and ARN14974 data, demonstrates that sphingosine promotes EC senescence. In contrast, the addition of exogenous S1P to cells profoundly reduced senescence to 20% (Figure 4.8). This indicates that S1P was an anti-senescence lipid. Meanwhile, although the reduced endogenous S1P was found in FB1 and ARN14974 pre-treated cells, it might not contribute to their anti-senescence effects.

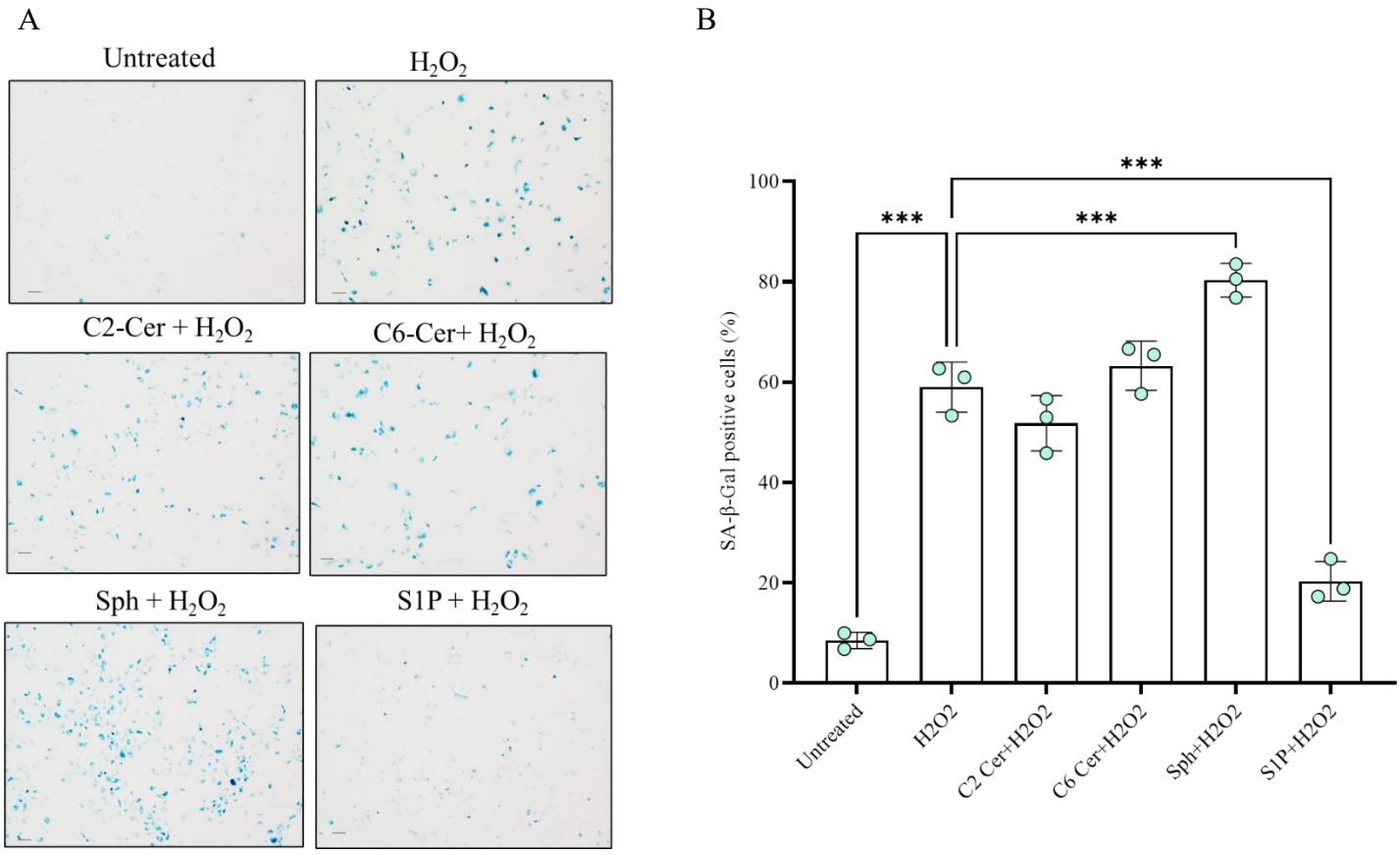

Figure 4.8 Sphingosine promotes H_2O_2-induced EC senescence. (A) HUVECs were pre-treated with 2 µM C2 Ceramide, 2 µM C6 Ceramide, 1 µM sphingosine and 1 µM S1P, then treated with 300 µM H_2O_2 for 24 hr. SA-β-gal staining was performed on day 7. Senescent cells were stained with blue colour. Magnification: 400X; scale bar = 100 µm. (B) Data was expressed as mean ± SD; n=3. ***$p < 0.001$, compared to H_2O_2 group.

4.8 Blocking sphingosine catabolism promotes EC senescence

I have identified that sphingosine was a driving factor for EC senescence via the inhibition of ceramide synthases and ceramidases as well as the addition of sphingosine. This led to testing whether blocking sphingosine conversion to S1P in the sphingolipid catabolic pathway would exhibit consistent phenotype. To this end, I pre-treated cells with SphK1 inhibitor PF-543 and SphK2 inhibitors ABC294640 and K145. Inhibition of SphKs caused an accumulation of endogenous sphingosine with a reduced S1P level (Figure 4.9). As shown in Figure 4.10, inhibition of SphK1 by PF-543 significantly increased senescence to 83%, comparable to the effects of sphingosine treatment (Figure 4.8). In contrast, inhibition of SphK2 by both compounds did not dramatically alter the senescence development (Figure 4.10). This is also consistent with that *SPHK1* was the predominant *SPHK* isoform in HUVECs (Figure 4.2).

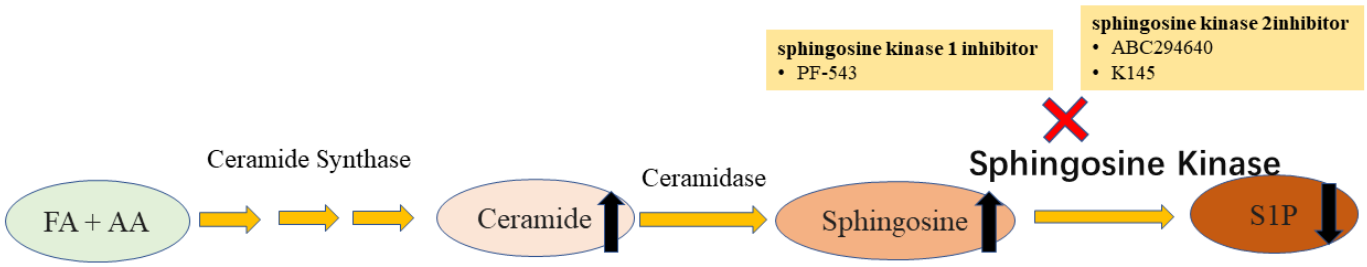

Figure 4.9 Schematic overview of the treatment design Sphingosine can be phosphorylated into S1P through SphK1 and SphK2. Inhibitors of SphK1 and SphK2 were able to modify these enzyme activities and further increase the sphingosine and decrease the S1P.

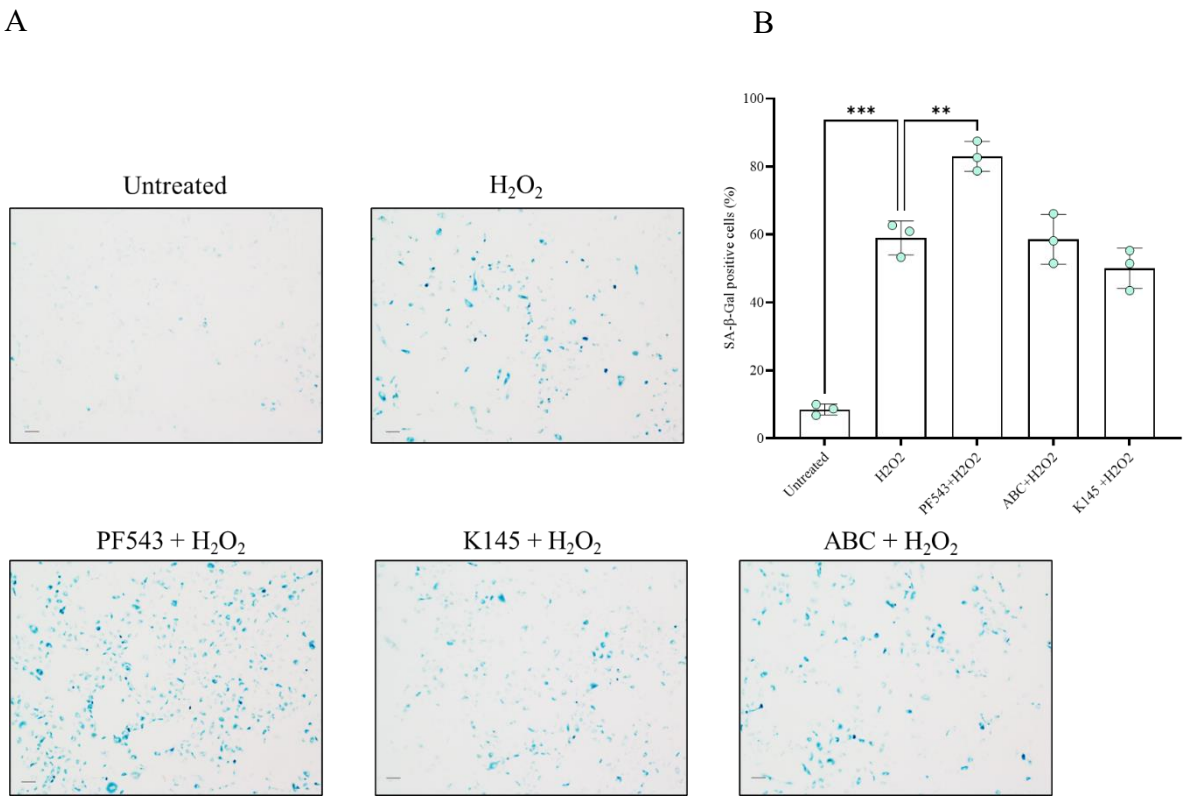

Figure 4.10 Blocking sphingosine catabolism promotes EC senescence (A) HUVECs were pre-treated with 2 μM PF-543, 10 μM K145, 10 μM ABC294640 for 24 hr, then treated with 300 μM H_2O_2 for 24 hr. SA-β-gal staining was performed on day 7. Senescent cells were stained with blue colour. Magnification: 400X; scale bar = 100 μm. (B) Data were expressed as mean ± SD; n=3. ***p < 0.01;* ****p < 0.001,* compared to H_2O_2 group.

4.9 PF-543 or sphingosine alone cannot induce EC senescence

From the experiments above, I have identified that the addition of exogenous sphingosine or accumulation of endogenous sphingosine (by PF-543 treatment) significantly promoted senescence development in H_2O_2-treated HUVECs. However, it is still unknown whether sphingosine itself had senescence-inducing effects or whether it determines cell susceptibility to H_2O_2-induced senescence. To address this, I treated HUVECs with sphingosine or PF-543 alone for 24 hr, without a follow-up H_2O_2 treatment, and then examined the senescence development at day 7. These two treatments alone did not induce EC senescence (Figure 4.11), suggesting that sphingosine sensitised HUVECs to H_2O_2-induced senescence.

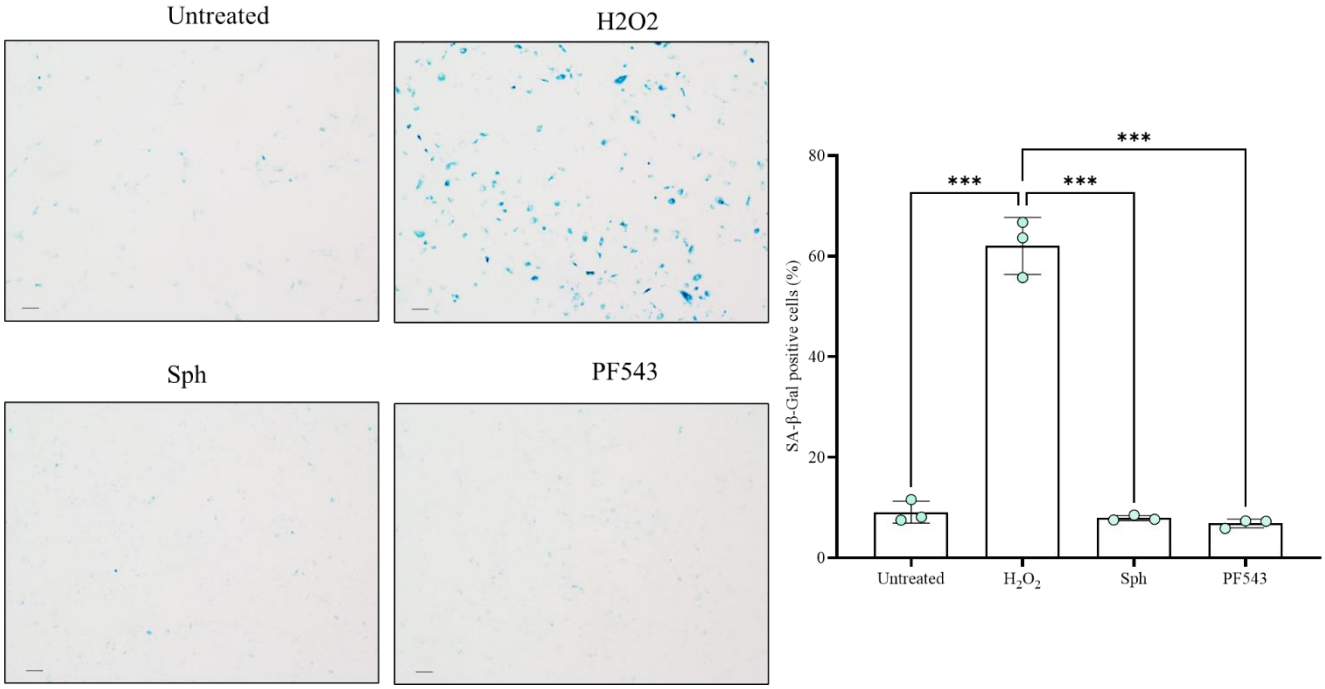

Figure 4.11 PF-543 or sphingosine alone cannot induce EC senescence (A) HUVECs were treated with 2 µM PF-543, 1 µM sphingosine and 300 µM H_2O_2 for 24 hr respectively. SA-β-Gal staining was performed on day 7. Senescent cells were stained with blue colour. Magnification: 400X; scale bar = 100 µm. (B) Data was expressed as mean ± SD; n=3. ***$p < 0.001$, compared to H_2O_2 group.

Chapter 5. Sphingosine promotes cell cycle arrest in H_2O_2-induced EC senescence

In the preceding chapter, I conducted a series of experiments to better understand how sphingolipids play a crucial role in controlling H_2O_2-induced senescence. After screening several sphingolipids and their metabolic enzyme inhibitors, I found that they may regulate H_2O_2-induced senescence positively or negatively. Based on the results of Chapter 3, sphingosine drives H_2O_2-induced EC senescence, whereas the inhibition of ceramide and sphingosine synthesis inhibits H_2O_2-induced EC senescence. Therefore, the subsequent efforts will focus on the role of sphingosine and related metabolic network in the regulation of EC senescence.

5.1 Sphingolipid regulation exhibits marginal impacts on cell viability in HUVECs

Sphingolipids, particularly ceramide, sphingosine and S1P, are implicated in the regulation of cell viability(189). Thus, initial treatment of HUVECs with these sphingolipids and their metabolic enzyme inhibitors might alter cell viability, contributing to the changes in cellular senescence.

To determine whether sphingolipid regulation affected the senescence outcomes through their cytotoxic or cytoprotective effects, I examined cell viability after the treatment of the related compounds in the absence or presence of H_2O_2. Please note, to simulate the condition in SA-β-Gal staining, I withdrew these treatments after 24 hr, leaving cells being maintained in treatment-free condition for up to 72 hr. Here I mainly focused on FB1, ARN14974 and sphingosine, as they caused the most significant changes in cellular senescence, as shown in Chapter 3.

Firstly, I treated HUVECs with sphingolipid-related compounds for 24 hr, 48 hr and 72 hr in the absence of H_2O_2. Among all treatments, only FB1 (ceramide synthase inhibitor) slightly reduced the percentage of viable cell number by 16.3% and 12.4% at 24 hr and 48 hr, respectively (Figure 5.1). When treated for 72 hr, C6-ceramide, FB1 and K145 (SphK2 inhibitor) marginally reduced cell viability by 11.8%, 15.3% and 10.1%, respectively (Figure 5.1). In contrast, treatment with sphingosine or ARN14974 did not affect cell

viability (Figure 5.1). These results indicate that sphingolipid regulation had minor impacts on cell viability in HUVECs. My findings also rule out the possibility that sphingosine promoted, whereas FB1 and ARN14974 inhibited, EC senescence via cell viability regulation.

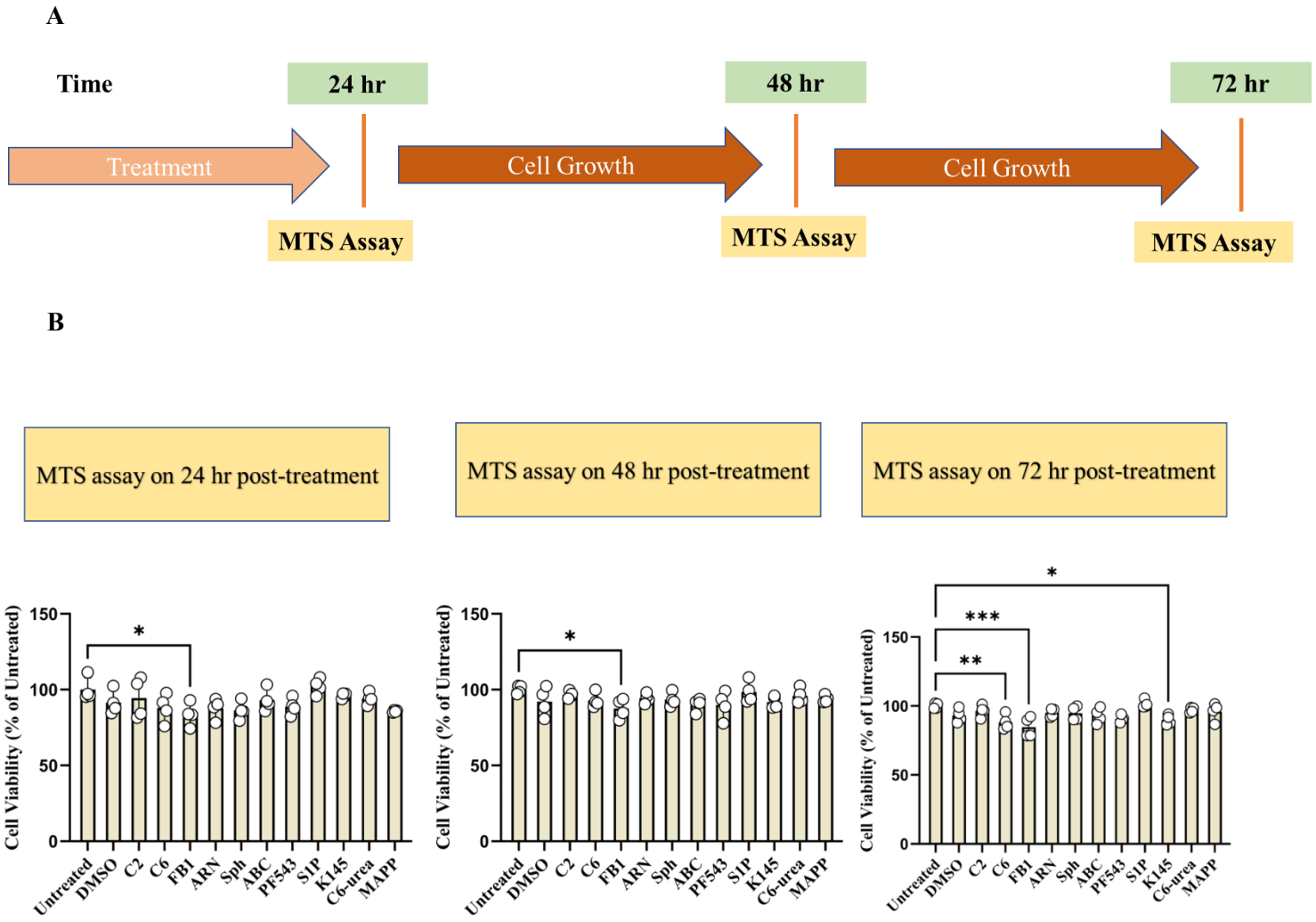

Figure 5.1 Sphingolipid regulation exhibits marginal impacts on cell viability in HUVECs. (A) Treatment timeline of MTS assay. HUVECs were treated with certain sphingolipid-related compounds for 24 hr, 0.1% DMSO, 2 μM C2 ceramide, 2 μM C6 ceramide, 10 μM FB1, 10 μM ARN14974, 1 μM sphingosine, 10 μM ABC294640, 2 μM PF-543, 10 μM K145, 10 μM C6-urea-cer, 10 μM D-erythro-MAPP. The percentage of cell viability was determined by MTS assay at the indicated time point. (B) Data are expressed as mean ± SD; n=4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; versus untreated.

5.2 Sphingolipid regulation exhibits marginal impacts on cell viability in H_2O_2-treated HUVECs

Next, I examined whether they affected cell viability under H_2O_2-induced oxidative stress. I chose the treatment schedule as that used in SA-β-Gal staining. In brief, I pre-treated HUVECs with sphingolipid-related compounds for 24 hr. After withdrawing these compounds, H_2O_2 was introduced for 24 hr. When H_2O_2 was withdrawn, the 24-hr cell viability was determined (Figure 5.2). Subsequently, cells were maintained in the absence of any treatments, until the 72-hr cell viability assay was carried out (Figure 5.2). At both cell viability assays, H_2O_2 induced 14.8% and 15.4% declines in cell viability, respectively (Figure 5.2). Pre-treatment with S1P protected cells from H_2O_2-induced cytotoxicity at 72 hr but not 24 hr (Figure 5.2). This data confirms the pro-cytoprotective roles of S1P. However, it also suggests this protection was not only effective when H_2O_2 was withdrawn. In contrast, despite their impacts on cellular senescence, FB1, ARN14974 and sphingosine did not affect cell viability in H_2O_2-treated HUVECs, uncoupling these two cellular events.

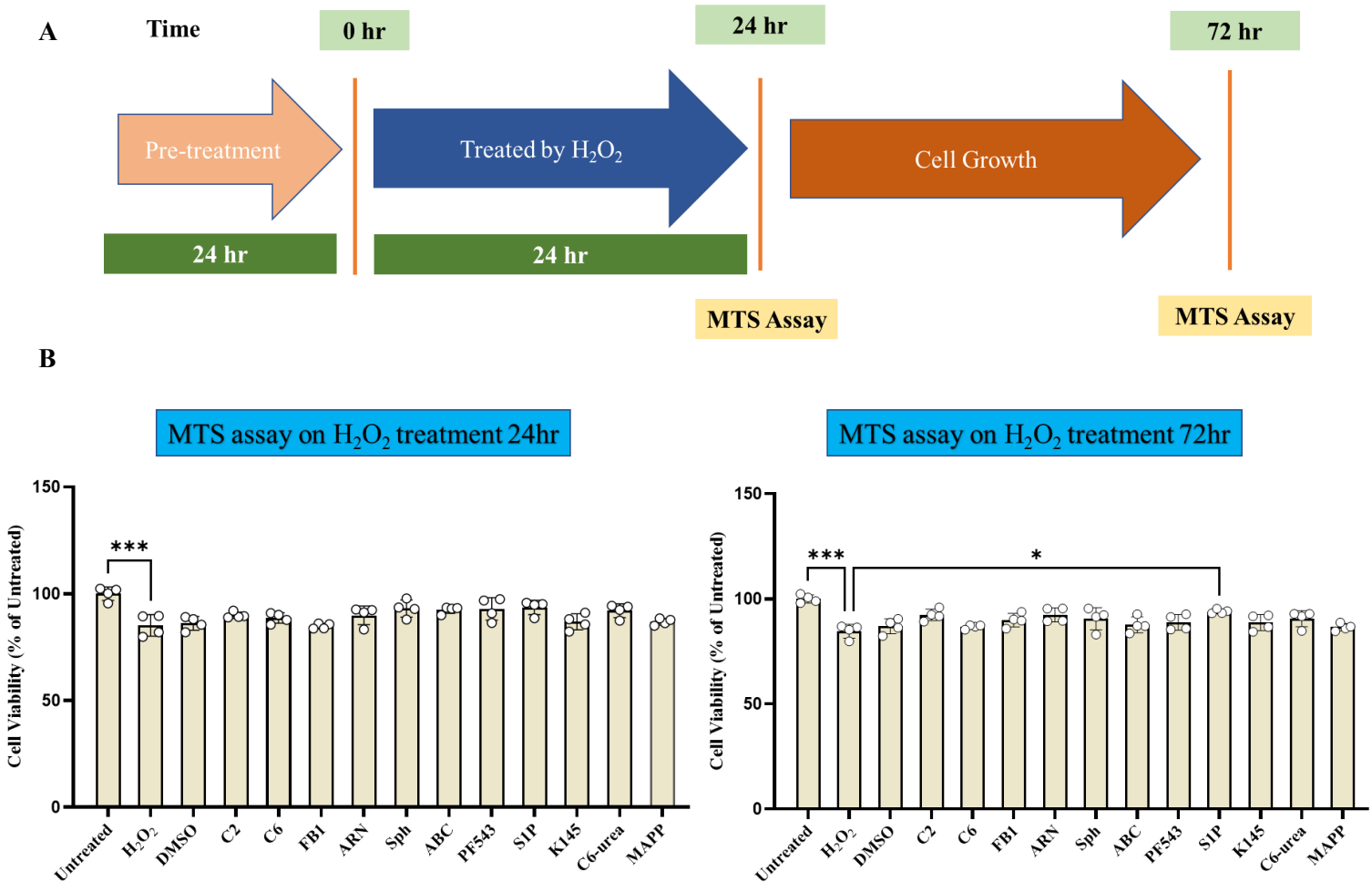

Figure 5.2 Sphingolipid regulation exhibits marginal impacts on cell viability in H_2O_2-treated HUVECs. (A) Treatment timeline of MTS assay. HUVECs were pre-treated with certain sphingolipid-related compounds for 24 hr, then treated with 300 μM H_2O_2 to induce senescence. The percentage of cell viability was determined by MTS assay at the indicated time point. (B) Data are expressed as mean ± SD; n=4. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; versus H_2O_2 group.

5.3 Effects of sphingosine on cell cycle regulators at the senescence initiation phase

Cellular senescence is a state of permanent cell cycle arrest(18). Any changes in cell cycle progression at the senescence initiation phase would alter the senescence outcome. I identified that sphingosine promoted H_2O_2-induced senescence in HUVECs, as demonstrated using FB1, ARN14974, sphingosine and PF-543 treatments (Figure 5.3). Thus, I first interrogated whether sphingosine pre-treatment caused cell cycle arrest, favouring H_2O_2-induced senescence initiation. To this end, I pre-treated HUVECs with sphingosine, followed by H_2O_2 treatment and examined the changes in senescence-associated cell cycle markers, including p21, acetyl-p53 and p-Rb. Acetyl-p53 levels were transiently elevated at 4 hr and 8 hr post-H_2O_2 treatment, but it returned to the baseline at 24 hr (Figure 5.3), indicating a transient induction of stress response that was associated with cell cycle arrest and senescence initiation (Figure 5.3). Meanwhile, H_2O_2 markedly upregulated p21 levels but reduced p-Rb levels as early as 4 hr, indicative of suppression of cell cycle progression (Figure 5.3). Pre-treatment with sphingosine for 24 hr did not change the baseline levels of p21, acetyl-p53 and p-Rb (as shown as 0 hr in Figure 5.3). This means that sphingosine did not induce cell cycle arrest prior to H_2O_2 treatment (Figure 5.3). When cells were treated with H_2O_2 for 24 hr, sphingosine pre-treatment caused an additional increase in p21 levels, which supports the notion that sphingosine pre-treatment potentiated HUVEC's susceptibility to H_2O_2-induced senescence. However, the overall

impact of sphingosine on the cell cycle at the senescence initiation phase was not pronounced, which could not explain its pro-senescence effects observed by SA-β-Gal staining at day 7.

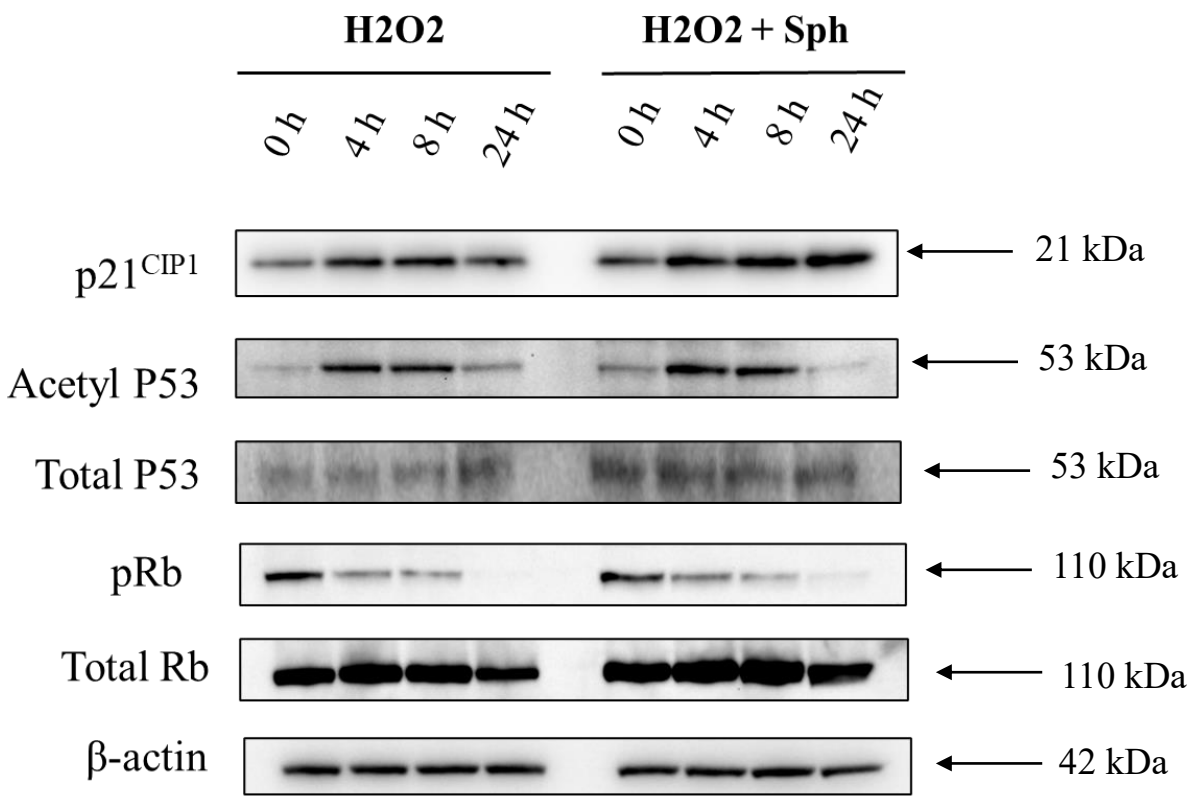

Figure 5.3 Effects of sphingosine on cell cycle regulators at senescence initiation phase.

HUVECs were pre-treated with 1μM sphingosine for 24 hr, prior to exposure to 300 μM H_2O_2 for 24 hr. Cell lysates were subjected to western blotting to analyse expression levels of the indicated proteins.

5.4 Effects of acid ceramidase inhibitor ARN14974 on cell cycle regulators at the senescence initiation phase

In Chapter 3, I found that expression of acid ceramidase *ASAH1* was increased in senescent HUVECs (Figure 4.4), and inhibition of ASAH1 by ARN14974 significantly suppressed EC senescence (Figure 4.7). Therefore, I interrogated whether ARN14974 protected HUVECs from H_2O_2-induced cell cycle arrest, contributing to its blockade of senescence development. Compared with H_2O_2 single treatment, ARN14974 pre-treatment caused a delayed upregulation of p21, reduced acetylation of p53 and a partial recovery of p-Rb level (Figure 5.4). Similar to sphingosine, the overall impacts of ARN14974 on the cell cycle were not dramatic. However, its effects on acetyl-p53 indicate an alleviated stress response to H_2O_2, which warrants further attention.

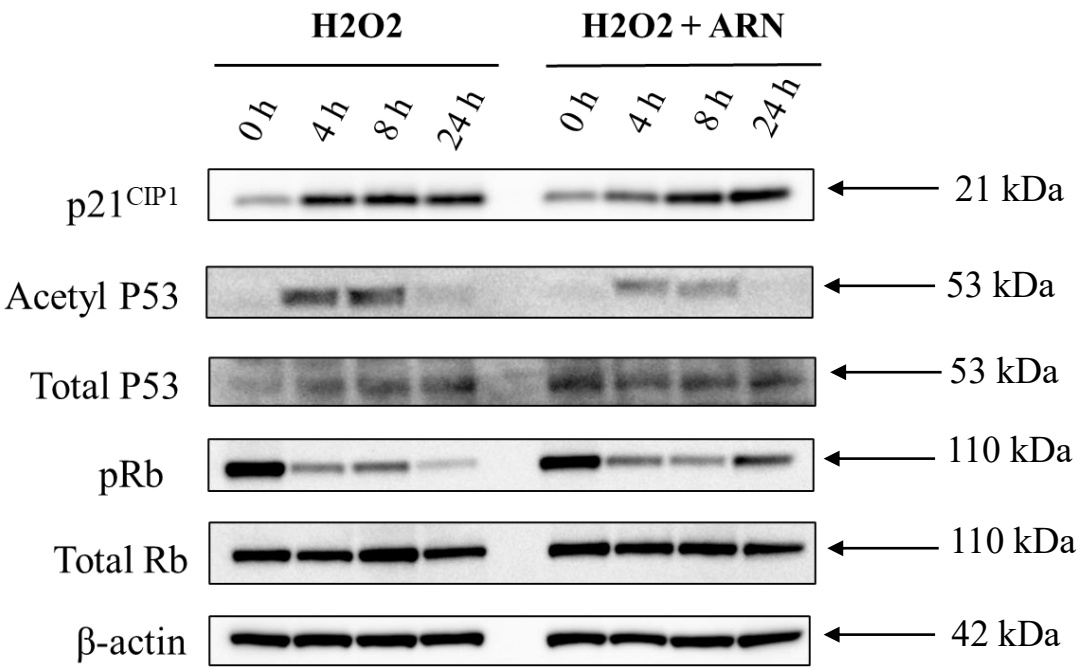

Figure 5.4 Effects of acid ceramidase inhibitor ARN14974 on cell cycle regulators at senescence initiation phase. HUVECs were pre-treated with 10 μM ARN14974 for 24 hr, prior to expose in 300 μM H_2O_2 for 24 hr. Cell lysates were subjected to western blotting to analyses expression levels of the indicated proteins.

5.5 Effects of ceramide synthase inhibitor FB1 on cell cycle regulators at the senescence initiation phase

In Chapter 3, I also found that inhibition of ceramide synthase by FB1 significantly suppressed EC senescence (Figure 4.7). Therefore, I also examined whether FB1 protected HUVECs from H_2O_2-induced cell cycle arrest, contributing to its blockade of senescence development. Compared with H_2O_2 single treatment, FB1 pre-treatment caused a dramatic reduction in p21 levels at all time points (Figure 5.5). This, together with the additional p21 upregulation after sphingosine treatment and delayed p21 elevation after ARN14974 treatment, suggests that sphingosine might slightly suppress cell cycle progression. Meanwhile, the effects of FB1 were much stronger than the other two treatments. Considering the major difference between these compounds is FB1, but not the other two can reduce cellular ceramide levels, this data suggests a critical role of ceramide in the regulation of the cell cycle. In addition, in line with ARN14974 treatment, FB1 also profoundly reduced acetyl-p53 levels (Figure 5.5). As both compounds inhibited sphingosine production, this result indicates that sphingosine but not ceramide was more important in mitigating stress in response to H_2O_2 treatment. In contrast, FB1 failed to reserve p-Rb levels in the presence of H_2O_2. In sum, FB1 exhibited protective effects against H_2O_2-induced cell cycle arrest via modulating the p53/p21 axis.

.

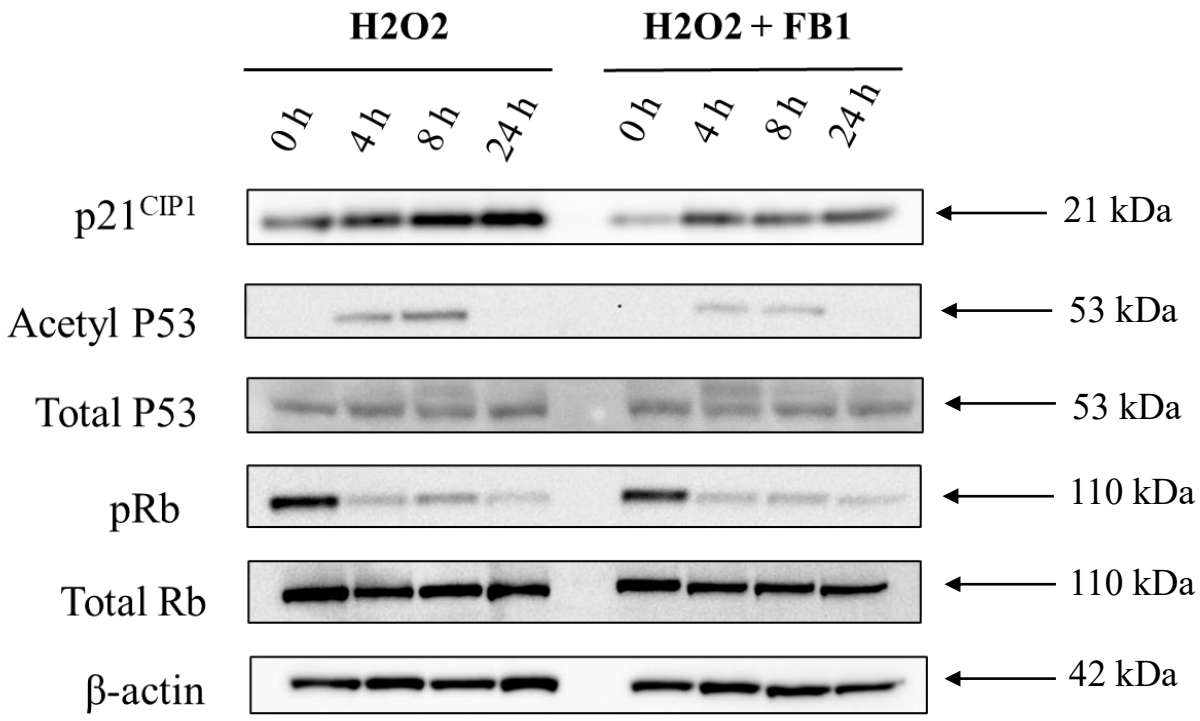

Figure 5.5 Effects of ceramide synthase inhibitor FB1 on cell cycle regulators at senescence initiation phase. HUVECs were pre-treated with 10 μM FB1 for 24 hr, prior to exposure to 300 μM H_2O_2 for 24 hr. Cell lysates were subjected to western blotting to analyse expression levels of the indicated proteins.

5.6 Sphingosine promotes cell cycle arrest associated with the establishment of senescence

Having demonstrated that sphingosine exhibited minor impacts on cell cycle regulators at the senescence initiation phase, I further examined whether it promoted cell cycle arrest at day 7 post-H_2O_2 treatment when cellular senescence was established. As acetyl-p53 only transiently elevated at 4 hr and 8 hr after the H_2O_2 challenge, I examined p21 and p-Rb levels in the following experiments on day 7. Consistent with Figure 4.3, sphingosine pre-treatment did not significantly alter p21 and p-Rb levels prior to H_2O_2 treatment (day 0) (Figure 5.6). In H_2O_2-treated HUVECs, p21 levels were upregulated by 2.7-fold, which was aligned with a pronounced senescence phenotype (Figure 5.6). In comparison, pre-treatment with sphingosine caused a 3.5-fold increase in p21 levels, which was significantly higher than H_2O_2 alone (Figure 5.6). In addition, at day 7, H_2O_2 reduced p-Rb levels to 32%, while sphingosine pre-treatment further lowered it to 11% of the untreated group (Figure 5.6). These data together indicate that sphingosine pre-treatment further impaired cell cycle progression at day 7 post-H_2O_2 treatment, confirming its impacts on cellular senescence.

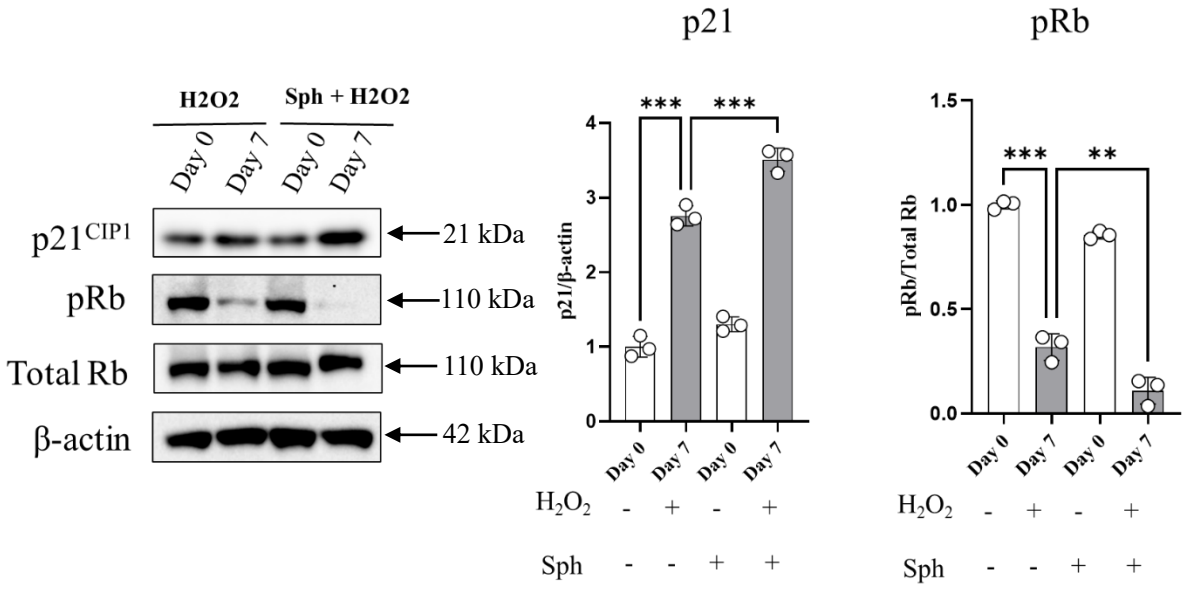

Figure 5.6 Sphingosine promotes cell cycle arrest associated with the establishment of senescence. (A) HUVECs were pre-treated with 1 μM sphingosine for 24 hr, prior to expose in 300 μM for H_2O_2 24 hr. Western blotting analyses of the indicated proteins at day 7. (B) Proteins expression levels of p21 and phosphorylated Rb were quantified; n=3. Data are expressed as mean ± SD. ***p < 0.01;* ****p < 0.001;* versus H_2O_2 day 7 group.

5.7 Acid ceramidase inhibitor ARN14974 promotes cell cycle progression and mitigates senescence

I examined whether ARN14974 improved cell cycle arrest at day 7 post-H_2O_2 treatment when cellular senescence was inhibited. Consistent with Figure 4.4, ARN14974 pre-treatment did not significantly alter p21 and p-Rb levels prior to H_2O_2 treatment (day 0) (Figure 5.7). In H_2O_2-treated HUVECs, p21 levels were upregulated by 2.6-fold, which was highly consistent with what I found in other experiments (Figure 5.6). In contrast, pre-treatment with ARN14974 only caused a 1.4-fold increase in p21 levels, which was significantly lower than H_2O_2 alone (Figure 5.7). In addition, at day 7, H_2O_2 reduced p-Rb levels to 28%, whereas ARN14974 pre-treatment restored it to 79% of the untreated group (Figure 5.7). These data together indicate that ARN14974 pre-treatment profoundly restored cell cycle progression at day 7 post- H_2O_2 treatment, which aligned with its impacts on cellular senescence inhibition.

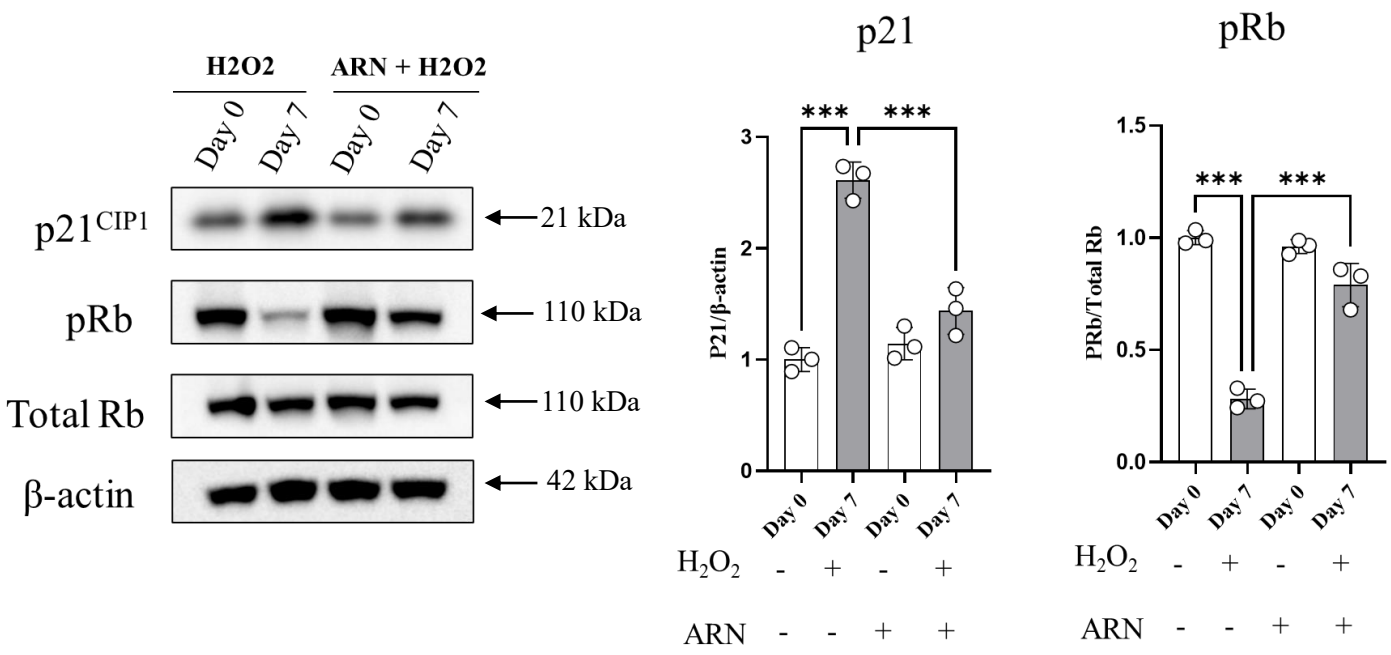

Figure 5.7 Acid ceramidase inhibitor ARN14974 promotes cell cycle progression and mitigates senescence. (A) HUVECs were pre-treated with 10 μM ARN14974 for 24 hr, prior to exposure to 300 μM of H_2O_2 for 24 hr. Western blotting analyses of the indicated proteins at day 7. (B) Proteins expression levels of p21 and phosphorylated Rb were quantified; n=3. Data are expressed as mean ± SD. ****p < 0.001;* versus H_2O_2 day 7 group.

5.8 Ceramide synthase inhibitor FB1 restores cell cycle progression associated with its blockade of senescence

Last, I examined whether FB1 improved cell cycle arrest at day 7 post-H_2O_2 treatment when cellular senescence was inhibited. In H_2O_2-treated HUVECs, p21 levels were upregulated by 2.8-fold. In contrast, pre-treatment with FB1 caused a 1.4-fold increase in p21 levels, which was significantly lower than H_2O_2 alone (Figure 5.8). In addition, at day 7, H_2O_2 reduced p-Rb levels to 26%, whereas FB1 pre-treatment restored it to 77% of the untreated group (Figure 5.8). These data together indicate that FB1 pre-treatment profoundly restored cell cycle progression at day 7 post-H_2O_2 treatment, which aligned with its impacts on cellular senescence inhibition.

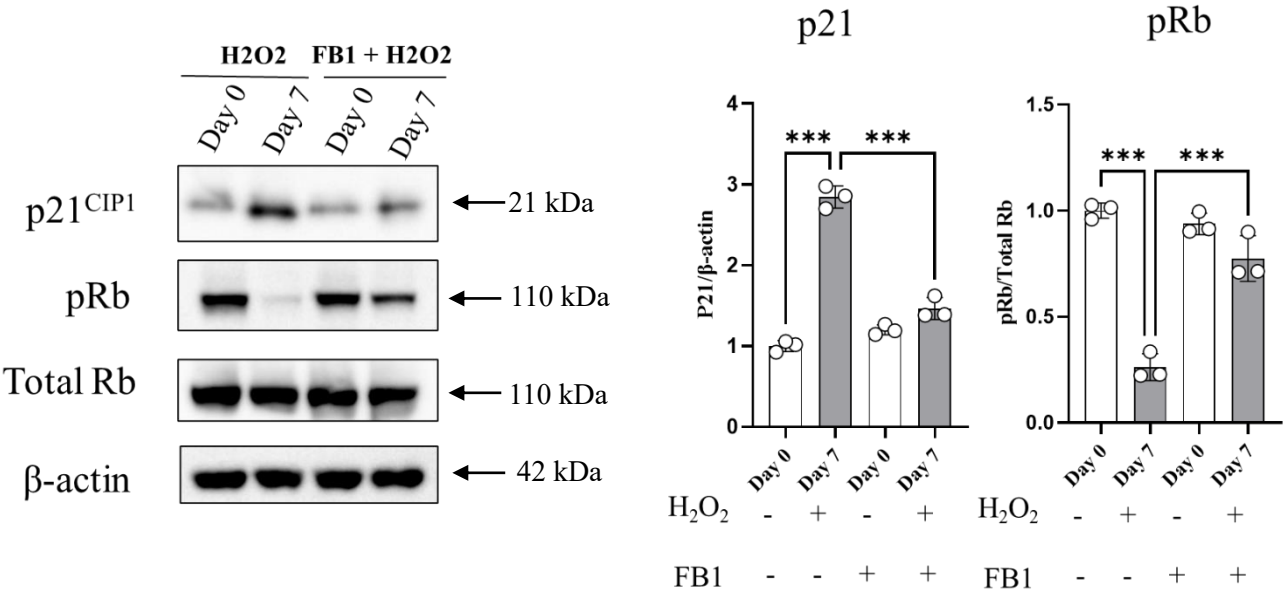

Figure 5.8 Ceramide synthase inhibitor FB1 restores cell cycle progression associated with its blockade of senescence. (A) HUVECs were pre-treated with 10 μM FB1 for 24 hr, prior to exposure to 300 μM H_2O_2 for 24 hr. Western blotting analyses of the indicated proteins at day 7. (B) Proteins expression levels of p21 and phosphorylated Rb were quantified; n=3. Data are expressed as mean ± SD. ****p < 0.001;* versus H_2O_2 day 7 group.

Chapter 5. Discussion

The endothelium is the interior lining of blood vessels, while ECs line the blood vessels interior. They are crucial for maintaining vascular homeostasis and regulating cardiovascular health (11). Within the context of normal vascular homeostasis, the functional endothelium exhibits the capacity to generate a diverse array of substances that govern vascular tone, cellular adhesion, thromboresistance, smooth muscle cell proliferation, and inflammation within the vessel wall, responding to both physical and chemical stimuli (381). The initial recognition of the endothelium's influence on vascular tone served as a catalyst for further study into its significance (381). Numerous vasoactive molecules are synthesized and secreted by the endothelium, thereby facilitating either vasodilation or vasoconstriction, while also responding to and modulating the effects of circulating vasoactive mediators such as bradykinin and thrombin (381). Additionally, endothelial cells play a critical role in angiogenesis, a fundamental process involved in blood vessel formation during embryonic development, growth, corpus luteum and endometrial production, regeneration, and wound healing (382). The process of endothelial ageing, however, can exert an impact on vascular function through various mechanisms, including diminished nitric oxide production, heightened oxidative stress, altered inflammatory responses, and compromised integrity of the endothelial barrier (11). It is worth noting that endothelial dysfunction represents a hallmark feature of numerous

cardiovascular conditions, such as atherosclerosis, hypertension, and vascular ageing (4). By disrupting multiple signaling pathways and impairing endothelial functions, senescent endothelial cells may actively participate in the initiation and progression of these pathological states (4). Therefore, comprehending the phenomenon of endothelial cell senescence assumes paramount importance in elucidating the underlying mechanisms that contribute to vascular dysfunction and its associated diseases (4).

HUVECs present a more faithful representation of human endothelial cell behavior in comparison to cell lines, rendering them a more dependable option for studying EC-related phenomena. Notably, the in vitro HUVEC model is frequently employed to scrutinize characteristics of the vascular endothelium and the cellular mechanisms underlying endothelial function (383). The accessibility of HUVECs is facilitated by their isolation from the umbilical cord (384). To explore the physiological and pathological ramifications of diverse stimuli, the HUVEC model can be employed either in isolation or in conjunction with other cell types, such as leukocytes and smooth muscle cells (385).

In contrast to replicative senescence, which is expedited by telomere attrition, premature senescence in HUVECs can be instigated by diverse stressors, including oxidative damage, oncogene activation, or UV irradiation (74, 386). It is worth noting that H_2O_2, a well-established approach for inducing oxidative stress and eliciting premature senescence-like characteristics in endothelial cells, has been extensively utilized in numerous studies (80,

387, 388). In a specific study, HUVECs were exposed to varying concentrations of H_2O_2 for a duration of 24 hours. The results demonstrated a dose-dependent escalation in the proportion of cells exhibiting positive staining for senescence-associated β-galactosidase (SA-β-gal) subsequent to H_2O_2 treatment (81). The percentage of positively stained cells reached approximately 40% for a concentration of 200 μM H_2O_2 and 50% for 400 μM H_2O_2 (81). Similarly, another study observed that a 2-hour treatment of H_2O_2 (200 μM) in HUVECs also led to an increase in the percentage of SA-β-gal staining positive cells, reaching approximately 50% at 3 days post-treatment (389). Furthermore, a separate study reported that the induction of premature senescence in HUVECs could be achieved by treating the cells with H_2O_2 at concentrations of 0, 25, 50, and 100 μM for a duration of 6 days, as evidenced by the escalating proportion of SA-β-gal staining positive cells (388). In another study, HUVECs were subjected to treatments of 10 μM and 100 μM H_2O_2 for a duration of 2 hours, followed by incubation in fresh HUVECs medium for either 24 or 48 hours. This experimental condition was found to induce senescence, as evidenced by the presence of positive SA-β-gal staining (390). Additionally, treatment with 75 μM H_2O_2 for 4 consecutive days, with daily medium changes, resulted in a 4.1-fold increase in the proportion of SA-β-Gal positive HUVECs compared to the control group (391). In a separate study, HUVECs were exposed to various concentrations of H_2O_2 (20, 40, 60, 80, and 100 μM) for a duration of 1 hour, followed by a 24-hour culture period. The objective was to evaluate the senescence-inducing potential of H_2O_2. The findings demonstrated that

HUVEC senescence could be effectively induced with a treatment of 60 μmol/L H_2O_2, as confirmed by SA-β-gal staining, which exhibited an approximate 50% increase in positive staining (80). Moreover, another study also reported that treating HUVECs with 60 μM H_2O_2 for 1 hour, followed by a 24-hour culture period, resulted in an approximately 60% increase in the proportion of SA-β-Gal positive cells (392).

Based on the available literature, the senescence model was established by subjecting HUVECs to a 24-hour treatment of 300 μM H_2O_2. Subsequently, SA-β-gal staining was conducted at 7 days post-treatment, revealing that the proportion of cells exhibiting positive SA-β-gal staining reached 58% following H_2O_2 treatment alone (Figure 4.7, Figure 4.8, Figure 4.10), consistent with findings reported in other studies. It is important to note that the experiments described in sections 4.6, 4.7, and 4.8 were conducted collectively. To facilitate comprehension of the results, these findings were divided into three distinct groups. Therefore, these three groups shared the same untreated control group and H_2O_2 treatment alone group.

Following the establishment of the senescence model, the objective was to investigate the temporal dynamics of EC senescence and identify the optimal time point. To achieve this, HUVECs were exposed to varying durations of 300 μM H_2O_2 treatment, ranging from 4 hours to 7 days (Figure 4.1 A). Notably, a previous study demonstrated that even at a low dose of 25 μM H_2O_2 treatment for just 1 hour, an upregulation of p21 levels and increased

SA-β-gal-positive cells were observed in HUVECs (393). Additionally, another research group treated HUVECs with 200 μM H_2O_2 for 2 hours and measured the p21 levels on day 3, revealing a significant increase (389). Consistent with these findings, the finding of this study revealed a 2.6-fold increase in p21 protein levels as early as 4 hours post H_2O_2 treatment, peaking at 3.5-fold after 24 hours, followed by a 3-fold rise on the third 3 and a 3.2-fold increase on the seventh day (Figure 4.1).

Recently, a report has indicated an association between the induction of senescence and the DNA damage response (DDR), suggesting that the presence of DDR may directly facilitate the acetylation of p53 (126). Additionally, the acetylation of p53 has been found to promote the activation of growth-suppressive genes and initiate cellular senescence (394). Notably, a study demonstrated that treating HUVECs with 100 μM H_2O_2 for six consecutive days resulted in an upregulation of acetylated p53 levels on day 6. In contrast, my findings revealed a transient increase in acetyl p53 levels at both 4 hours and 8 hours after a 24-hour treatment, followed by a decrease below the levels observed in the untreated group on day 1, day 3, and day 7 (Figure 4.1). Additionally, a previous study has demonstrated that the administration of 200 μM H_2O_2 for a duration of 2 hours leads to a notable reduction in the phosphorylated Rb level on day 3 (389). Consistent with these findings, my results indicate a significant decrease in p-Rb levels following H_2O_2 treatment. Specifically, the p-Rb level exhibited a decline as early as 4 hours, reaching 41% of the

untreated level, and subsequently reached its lowest point at 24 hours, corresponding to 10%. The expression level of p-Rb was further reduced to 13% on day 3 and 28% on day 7 (Figure 4.1). In conclusion, the results of my study reveal that the treatment of HUVECs with 300 μM H_2O_2 induces time-dependent alterations in cell cycle-related proteins. These findings provide valuable insights for determining the appropriate time points for conducting further tests on anti-senescence compounds.

To investigate the role of sphingolipids in EC senescence, an analysis was conducted on the expression of genes encoding sphingolipid enzymes, including ceramide synthases, ceramidase, and sphingosine kinase, in normal HUVECs using qRT-PCR (Figure 4.2). It is worth noting that the localization of these enzymes involved in sphingolipid metabolism may differ depending on their specific tissue distribution. A previous study appraised the expression of ceramide synthases in human lung microvascular endothelial cells and found that C24- and C16-ceramides were notably abundant in the lung, indicating the high expression of their respective synthetic enzymes, *CERS2* and *CERS5*. Conversely, the low expression levels of ceramide synthases *CERS1*, *CERS3*, and *CERS4* in HUVECs may be attributed to their predominant expression in specific tissues, e.g., the brain, skeletal muscle, skin, kidney, and breast (244). In this study, it was also observed that *CERS2*, responsible for the production of very long-chain ceramides (C22-C24 ceramides), exhibited high expression, while *CERS5* and *CERS6*, involved in the synthesis of C16 ceramides, showed

moderate expression (Figure 4.2). The remaining ceramide synthases displayed low expression levels.

According to previous studies, *ASAH1*, also known as acid ceramidase, demonstrates widespread expression across different tissues, with notably high expression levels observed in the heart and kidney. It is also expressed to a lesser extent in the placenta, lung, and skeletal muscle (395). In line with these findings, this study revealed that *ASAH1* was the primary ceramidase, exhibiting the highest abundance among all the examined ceramidases. Following *ASAH1*, *ACER3* displayed the next highest relative abundance (Figure 4.2).

In contrast, the detectability of *ASAH2* and *ACER1* remained elusive even after subjecting the PCR process to a maximum of 40 cycles, as evidenced by the utilization of two distinct primer sets (Figure 4.2). The expression pattern of *ASAH2*, a neutral ceramidase, exhibits prominence in the kidneys, liver, small intestines, and colon, while displaying relatively lower levels in the brain, lung, and heart (395). Conversely, *ACER1* demonstrates predominant expression within the epidermal keratinocytes of the skin (295), which may account for their absence in normal HUVECs. Additionally, during the present research of *SPHK1* and *SPHK2*, it was determined that *SPHK1* serves as the primary isoform of sphingosine kinase expressed in normal HUVECs (Figure 4.2). Notably, a study has reported the presence of SphK1 in rat brain endothelial cells (396). Moreover, research has

demonstrated that the overexpression of SphK-1 results in heightened release of SphK activity, thereby promoting angiogenesis and vascular maturation (397). Additionally, another study has provided evidence that the suppression of *SPHK1* expression significantly impedes the proliferation of HUVECs (398). These findings corroborate the observations made, indicating the presence of *SPHK1* in endothelial cells.

Previous studies have also demonstrated the involvement of sphingolipids, specifically ceramide, sphingosine, and S1P, in various cellular processes, including signal transduction, cell proliferation, cell death, cell migration, and inflammation (399). Conversely, cellular senescence is a multifaceted process characterized by sequential steps, wherein cells cease their division, followed by the recruitment of immune cells by tissues to eliminate senescent cells, thereby facilitating tissue regeneration (400, 401). Recent research has elucidated the significance of sphingolipids and their role in the regulation of cellular senescence (227). Therefore, a study was conducted to examine the impact of sphingolipid enzymes on H_2O_2 -induced senescence through the utilization of qRT-PCR and SA-β-Gal staining.

In a study, it was discovered that p53 specifically stimulates the synthesis of ceramide by activating CERS5 (263). Notably, the tumor suppressor p53 plays a crucial role in the regulation of the cell cycle and cellular senescence. CERS5 may be implicated in the cellular processes induced by p53. During the investigation of ceramide synthase in

senescent HUVECs on day 3 and day 7, no noticeable change in the expression level of CERS2, the most abundant isoform, was observed (Figure 4.3). However, relatively abundant isoforms, namely *CERS5* and *CERS6*, exhibited a slight change of approximately 26% at specific stages of senescence progression, suggesting their potential involvement in H_2O_2-induced senescence (Figure 4.3). Conversely, *CERS3* and *CERS4*, which are less abundant isoforms, demonstrated a more pronounced increase in EC senescence (Figure 4.3). Nevertheless, despite this upregulation, these isoforms were present in extremely low abundance, as depicted in Figure 3.2. Therefore, it was concluded that the extent of gene upregulation in *CERS3* and *CERS4* was unlikely to have a significant impact on ceramide production and composition.

Experimental data demonstrates a decline in the protein levels of p53, p21, and p16, which are well-established markers of cellular senescence, in pre-senescent human fibroblasts upon the silencing of *ASAH1* (284). Temporal analysis revealed an increase in *ASAH1* mRNA levels during senescence progression, consistent with previous studies. Similarly, the expression level of *ACER2* exhibited a similar trend (Figure 4.4). However, considering the 21-fold higher abundance of *ASAH1* mRNA compared to *ACER2* (Figure 4.2), these findings suggest that the conversion of ceramide to sphingosine mediated by ASAH1, rather than ACER2, may play a more significant role in EC senescence.

The SphK/S1P system was widely appraised in studies and shown to be critical in various cellular functions, such as cell motility, proliferation, cell growth, survival, and response to stress (402). Moreover, emerging evidence suggests that the SphK/S1P axis is critical in the development and functioning of the cardiovascular system (402, 403). Activation of the SphK/S1P- S1P receptor (S1PR) system has demonstrated protective effects on both the heart and the vasculature (402, 403, 404). In this study of mRNA expression, I observed that the predominant isoform of sphingosine kinase, *SPHK1*, remained unaltered in EC senescence (Figure 4.5). However, the less abundant isoform, *SPHK2*, exhibited a 1.4-fold increase in early senescent HUVECs (Figure 4.5). Considering their abundance in normal HUVECs (Figure 4.2), these findings suggest that the rate of *SPHK*-mediated S1P production from sphingosine may not undergo significant changes in EC senescence.

According to previous studies, FB1 has been identified as a non-isoform selective inhibitor of ceramide synthase, and its application has been observed in various cell lines, particularly endothelial cells (405, 406). By impeding the de novo synthesis of ceramide, this pharmacological inhibitor induces a decrease in the levels of ceramide, sphingosine, and S1P. Conversely, ARN14974 is a specific inhibitor that impedes the conversion of ceramide to sphingosine mediated by acid ceramidase, leading to an increase in ceramide levels and a decrease in sphingosine and S1P levels (303, 407). It has been documented that treatment with 10 μM ARN14974 for 24 hours can effectively inhibit acid ceramidase

in hepatocytes, resulting in a reduction in sphingosine production (407). Following the administration of FB1 and ARN14974, a notable reduction in H_2O_2-induced EC senescence was observed (Figure 4.7). Conversely, treatment with the neutral ceramidase inhibitor C6-urea ceramide and the alkaline ceramidase inhibitor D-erythro-MAPP did not result in any significant changes in the percentage of senescent HUVECs. These findings align with the results presented in Figure 4.2, which demonstrate that acid ceramidase *ASAH1* is the primary isoform in normal HUVECs, as well as the findings in Figure 4.4, which indicate a substantial increase in *ASAH1* levels in senescent HUVECs.

The differed consequence observed in the treatment of FB1 and ARN14974 lies solely in the ceramide level. Nevertheless, the findings indicate that the progression of endothelial cell senescence is primarily influenced by the diminished levels of sphingosine and S1P, rather than alterations in ceramide levels. It is worth noting that previous studies have demonstrated a significant increase in endogenous ceramide levels in senescent WI-38 human diploid fibroblasts (HDF) (336). Therefore, an additional experiment was conducted wherein HUVECs were subjected to exogenous administration of ceramide, sphingosine, and S1P to determine their respective roles in the regulation of EC senescence.

The experimental results revealed that the administration of C2 ceramide and C6 ceramide did not yield a substantial impact on the advancement of EC senescence (Figure 4.8), thereby supporting the notion that ceramide does not contribute to the progression of EC

senescence. Intriguingly, the treatment of sphingosine has been noted to result in a remarkable rise in senescent cells (Figure 4.8). This finding, in conjunction with the data presented in Figure 4.7, underscores the crucial role played by sphingosine in facilitating the progression of EC senescence. Additionally, previous reports have indicated that S1P possesses the ability to promote cell proliferation, survival, migration, and differentiation (399). In line with these findings, this study revealed a drop in the proportion of senescent cells post the administration of exogenous S1P (Figure 4.8). This observation is consistent with the existing literature on the subject. However, even though it has been demonstrated in numerous studies that FB1 and ARN14974 can reduce endogenous S1P levels (277, 303, 408), it remains uncertain whether this decline in S1P is directly associated with the efficacy of these agents in combating senescence. It is essential to conduct a comprehensive sphingolipidomic assessment in further studies to examine the changes in the levels of endogenous S1P and other bioactive sphingolipids, such as sphingosine and ceramide, upon the treatment with these inhibitors.

The conversion of sphingosine into S1P can be impeded through pharmacological inhibition of sphingosine kinase. Notably, the highly selective SphK1 inhibitor PF-543 has exhibited significant efficacy in reducing S1P production by inhibiting sphingosine kinase in human whole blood, with an EC50 of 26.7 nM (322). In the context of Human retinal endothelial cells, the SphK2 inhibitor ABC294640 has been employed at a concentration

of 10 μg/mL (409). Additionally, the SphK2 inhibitor K145 has demonstrated its ability to effectively inhibit SphK2 activity and reduce S1P levels in human leukemia U937 cells at a concentration of 10 μM (329). As for my study, upon pre-treating the cells with 2 μM PF-543, a notable increase in H_2O_2-induced senescence was observed (Figure 4.10), comparable to the impact observed with sphingosine treatment (Figure 4.8). In contrast, the inhibition of SphK2 by ABC294640 and K145 did not cause a significant alteration in the development of EC senescence (Figure 4.10). This finding further supports the notion that *SPHK1* is the primary isoform of sphingosine kinase in HUVECs (Figure 4.2). Additionally, a study has demonstrated that the overexpression of *SPHK1* in HUVECs promotes cell survival, highlighting the significance of SphK1 in endothelial cells (409).

Regarding the question as to whether sphingosine possesses direct senescence-inducing properties or if it modulates cellular susceptibility to H_2O_2-induced senescence, it is observed in this study that the independent administration of sphingosine or PF-543 did not elicit senescence in ECs, as evidenced by the observations presented in Figure 4.11. These findings imply that sphingosine augmented the vulnerability of HUVECs to senescence triggered by H_2O_2.

Previous research has proposed that sphingolipids, specifically ceramide, sphingosine, and S1P, exert an impact on cell viability (269, 398, 410). Notably, sphingosine has been identified as a potential intracellular mediator of apoptotic signals in mast cells (411).

Additionally, a separate study has reported the ability of sphingosine to induce programmed cell death in rhabdomyosarcoma cells (412). Moreover, the involvement of sphingolipid enzymes, including FB1 and K145, has also been suggested in relation to cell viability. Therefore, the initial administration of these sphingolipids and inhibitors to HUVECs may potentially modulate cell viability, thereby influencing the observed alterations in cellular senescence.

FB1, a mycotoxin with a low molecular weight, is synthesized by Fusarium fungi (413). Its high toxicity poses a threat to the well-being of both humans and animals (414). Numerous studies have demonstrated that FB1 exhibits a dose- and time-dependent inhibitory effect on cell viability in human and animal endothelial cells (405, 406, 415). Notably, a research revealed that even at a concentration of 10 μmol/L, FB1 treatment caused a marginal drop in cell viability in HUVECs after 24 hours, whereas a significant reduction in cell viability had been noted at both 24 and 48 hours when the concentration was 50 μM/L (415). Similarly, another study demonstrated that treatment with 50 μg/mL of FB1 inhibited cell viability in porcine hip artery endothelial cells (406). Furthermore, exposure to FB1 at various concentrations resulted in a marginal reduction in cell viability in swine umbilical vein endothelial cells after 24 hours at a concentration of 10 μg/mL (405). Subsequently, at concentrations of 20, 40, and 60 μg/mL, a significant drop of cell viability had been noted at both 48 and 72 hours (405). In contrast, previous studies have

reported that K145 exhibits growth-inhibitory effects and induces apoptosis in human leukemia cells (416). Additionally, another study revealed that K145 induces cell death in various human myeloma cell lines in a dose-dependent manner, with IC50 values between 3 and 7 μM at 24 hours (417).

In relation to the findings, a slight reduction in cell viability at 24 hours and 48 hours in the absence of H_2O_2 was observed solely with FB1 (Figure 5.1). C6-ceramide, FB1, and K145 exhibited a marginal decline up to 72 hours (Figure 5.1), which aligns with previous studies. Notably, sphingosine and ARN14974 did not significantly diminish cell viability (Figure 5.1). These findings suggest that the impact of sphingolipid regulation on cell viability in HUVECs is minimal. Furthermore, the results indicate that sphingosine does not promote senescence, while FB1 and ARN14974 do not impede senescence in ECs through the regulation of cell viability.

Subsequently, a study was conducted to assess the impact of these sphingolipid compounds on the cell viability of HUCECs under oxidative stress induced by H_2O_2. A study revealed that a 1-hour exposure to H_2O_2 led to a significant reduction in HUVECs viability at 24 hour, with increasing concentrations ranging from 40 to 100 μmol/L (80). Another study demonstrated that a 2-hour exposure to H_2O_2 at a concentration of 100 μM resulted in a substantial decrease in HUVECs cell viability at 24 hour, 48 hour, and 72 hour (418). Furthermore, when HUVECs were subjected to H_2O_2 at concentrations of 100, 300, or 500

μM, a dose-dependent decline in cell viability was observed after 6 and 12 hours of treatment (419). In line with these previous studies, the present findings indicated a significant decrease in cell viability at both 24 hour and 72 hour following single treatment with H_2O_2 (Figure 5.2). Upon comparing the H_2O_2 single treatment group with other groups, it was observed that pre-treating cells with S1P conferred protection against H_2O_2-induced cytotoxicity at 72 hours. However, this protective effect was not evident at 24 hours (Figure 5.2). These findings corroborate the pro-cytoprotective roles of S1P, as suggested by previous studies (346, 363, 403). Notably, the protective effect of S1P persisted even in the absence of H_2O_2. Conversely, FB1, ARN14974, and sphingosine exhibited no impact on cell viability in H_2O_2-treated HUVECs, despite their influence on cellular senescence. Thus, these results establish a dissociation between these two cellular processes (Figure 5.2).

Cellular senescence refers to a state characterized by the irreversible cessation of the cell cycle (18). It is well-established that cellular senescence can be triggered through two primary signaling pathways: the $p16^{INK4a}$/Rb pathway and the $p53/p21^{cip}$ pathway (16). Modulations in cell cycle progression during the initiation phase of senescence can profoundly influence the final outcome of senescence (18). Additionally, it has been reported that sphingosine possesses the ability to inhibit the activation of protein kinase C, a key regulator of various cellular processes, including cell proliferation (420). Moreover, treatment with sphingosine leads to the dephosphorylation of Rb in early-stage

hematopoietic cells, thereby further inhibiting cell cycle arrest in the G0/G1 phase (345). However, a separate study demonstrated that treatment with 40 μg/mL FB1 induces cell cycle arrest in the G0/G1 phase in swine umbilical vein endothelial cells (405). Therefore, the impact of pre-treatment with sphingosine, ARN14974, or FB1 on cell cycle-related proteins at the initiation stage of H_2O_2-induced senescence was investigated. Following pre-treatment with sphingosine, it was observed that HUVEC exhibited heightened sensitivity to H_2O_2-induced senescence, as evidenced by a subsequent increase in p21 levels at 24 hours post-treatment with H_2O_2 (Figure 5.3). However, the overall effects of sphingosine on the cell cycle during the initial phase of senescence were not substantial enough to account for the pro-senescence influence observed at day 7, as indicated by SA-β-Gal staining (Figure 5.8).

Furthermore, the data presented in this study indicates that pre-treatment with ARN14974 at the initiation of EC senescence did not exhibit a noticeable effect on the overall progression of the cell cycle (Figure 5.4). However, it is worth noting that a delayed upregulation of p21, reduced acetyl-p53 levels, and a partial recovery of p-Rb levels were observed (Figure 5.4). The observed impact on acetyl-p53 suggests a diminished stress response to H_2O_2, warranting further investigation (Figure 5.4).

Upon investigating the impact of FB1 on cell cycle-related proteins, a striking decrease in p21 levels was observed at 4 hours, 8 hours, and 24 hours (Figure 5.5). This substantial

reduction in p21 levels (Figure 5.5), coupled with the observed increase in p21 levels following pre-treatment with sphingosine (Figure 5.3) and the delayed elevation of p21 levels observed after pre-treatment with ARN14974 (Figure 5.4), suggests that sphingosine may have a marginal inhibitory effect on cell cycle progression. It has been reported in previous studies that ceramide has the ability to induce cell cycle arrest in various cell lines (335, 421, 422). The present findings demonstrate that the influence of FB1 on cell cycle regulators surpasses that of ARN14974 and sphingosine. These observations align with previous research in this field. Similar to the effect of ARN14974, a notable decrease in acetyl-p53 levels can also be observed following pre-treatment with FB1 (Figure 5.5). This suggests that FB1, like ARN14974, has the ability to suppress sphingosine levels. Interestingly, this indicates that sphingosine, rather than ceramide, may play a more significant role in responding to H_2O_2-induced stress. Additionally, it is worth noting that FB1 does not restore p-Rb levels in the presence of H_2O_2 (Figure 5.5). Collectively, these findings suggest that FB1 may protect cells from H_2O_2-induced cell cycle arrest by regulating the p53/p21 axis, which is consistent with previous studies in this area.

In addition, the impact of sphingosine, ARN14974, and FB1 pre-treatment on the cell cycle regulator was examined at day 7 following the establishment of cellular senescence induced by H_2O_2. Considering the transient elevation of acetyl-p53 levels only at 4 hours and 8 hours post-treatment of H_2O_2 as shown in timecouse (Figure 4.1), an assessment was

made regarding the changes in the levels of p21 and p-Rb on day 7. The data obtained from the study revealed a significant augmentation in p21 levels and a reduction in p-RB levels on day 7 when cells were pre-treated with sphingosine, as compared to cells treated with H_2O_2 alone (Figure 5.6). These findings confirm that the pre-treatment of sphingosine intensifies cell cycle arrest at day 7 following H_2O_2 treatment, thereby providing additional evidence of its involvement in cellular senescence. Furthermore, a noteworthy decrease in p21 levels was observed following the pre-treatment of ARN14794 on day 7 subsequent to H_2O_2 treatment (Figure 5.7). In contrast, the p-Rb level exhibited a remarkable restoration. These findings indicate that the pre-treatment with ARN14974 effectively counteracted the disruption of the cell cycle at day 7 after H_2O_2 treatment, thereby highlighting its potential in inhibiting cellular senescence. Similar to the results observed with the pre-treatment of ARN14974, the pre-treatment of FB1 also led to a significant decrease in p21 levels compared to H_2O_2 treatment alone (Figure 5.8). Conversely, it exhibited a substantial restoration of the p-Rb level (Figure 5.8). Consistent with its demonstrated ability to suppress cellular senescence, the findings of this study indicate that FB1 pre-treatment effectively revitalized cell cycle progression at day 7 post-H_2O_2 treatment.

FB1 is a potent pan-inhibitor of ceramide synthase, which reduces the production of ceramides. This leads to a critical question that whether the addition of exogenous C6-ceramide compromised the effects of FB1 in cells. In a noteworthy study, A549 cells were

subjected to the treatment with 20 μM C6-ceramide, in the presence or absence of 50 μM FB1. FB1 treatment effectively suppressed the generation of endogenous longer-chain ceramide levels induced by C6-ceramide (217). In a separate investigation, MCF-7 cells received treatment with 50 μM FB1 one hour before the addition of C6-Cer (25 μM). Exogenous introduction of C6-Cer led to heightened levels of endogenous longer-chain ceramides, which was also significantly negated by FB1 (423). Given these, a combined treatment of C6-ceramide and FB1 cannot address whether the anti-senescence actions of FB1 was attributed to the reduction in ceramide. In contrast, I demonstrated that addition of C6-ceramide promoted senescence, whereas ARN14974 treatment that increases ceramide levels mitigated senescence in HUVECs, indicating that ceramides were not key regulators of this process.

Chapter 6. Conclusions

In conclusion, the findings of this study highlight the critical role of sphingosine in H_2O_2-induced premature senescence. The discovery revealed that sphingosine drives H_2O_2-induced endothelial cell senescence. Additionally, impeding sphingosine catabolism by sphingosine kinase 1 inhibitor PF-543 can promote endothelial cell senescence. On the other hand, inhibition the synthesis of ceramide and sphingosine through the use of the ceramide synthase inhibitor FB1 and acid ceramidase inhibitor ARN14974 can effectively prevent H_2O_2-induced senescence in endothelial cells. This thesis identified sphingosine, but not ceramide or sphingosine 1-phosphate, as the key determinant of endothelial cell senescence, which shed light on further exploration into the molecular mechanism involved and offer potential targets for therapeutic interventions aimed at mitigating premature senescence-related disorders.

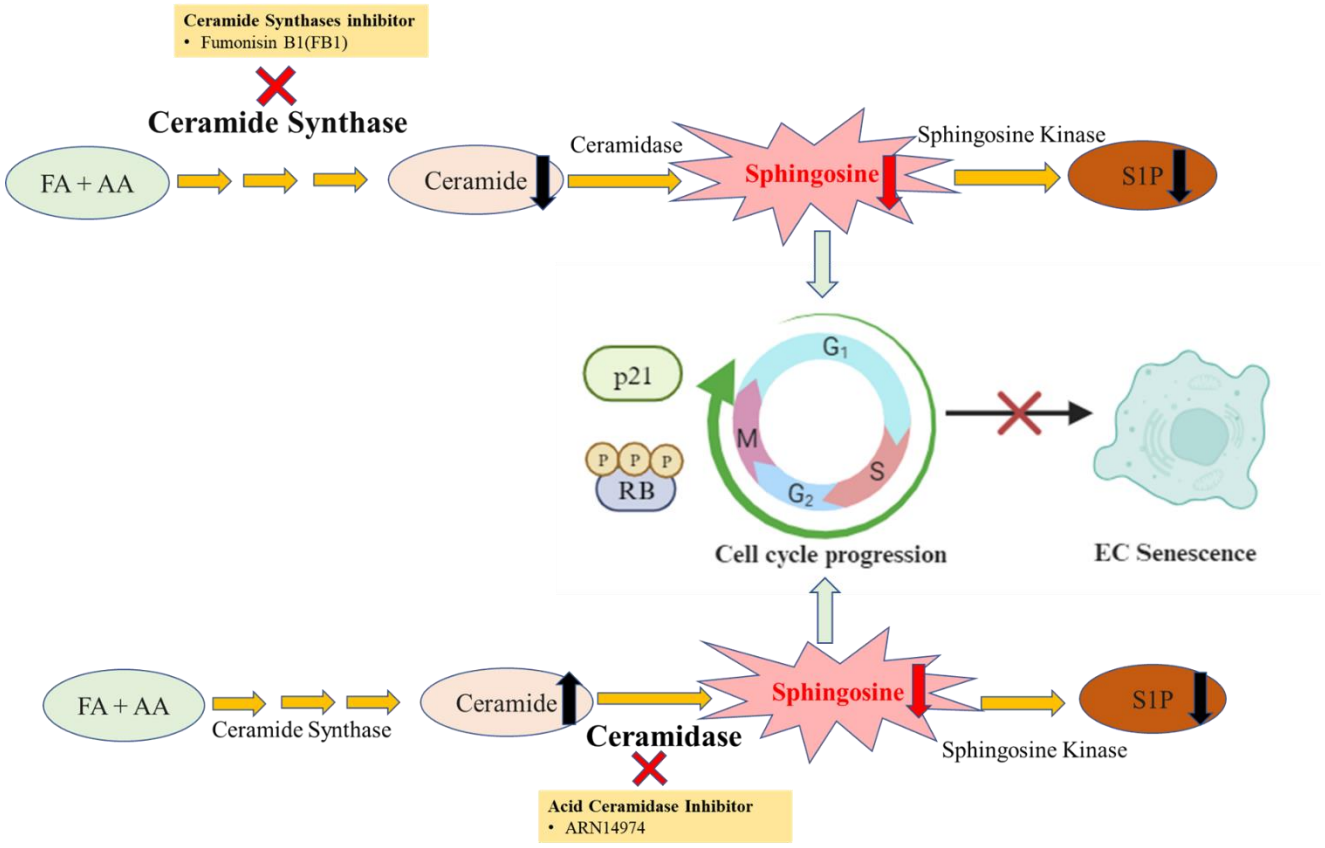

Figure 6.1 Summary of main findings. Sphingosine plays a critical role in H2O2-induced premature senescence. Blocking the production of ceramide and sphingosine using the ceramide synthase inhibitor FB1 and the acid ceramidase inhibitor ARN14974 has been shown to efficiently hinder premature senescence induced by H_2O_2 in endothelial cells.

Chapter 7. Potential limitations and future direction

In my thesis, I have provided evidence for the crucial significance of sphingosine in the progression of premature senescence induced by H_2O_2. Nevertheless, there are several unresolved matters that require further exploration in future investigations.

7.1 Profiling sphingolipidome in senescent ECs

My study has demonstrated a subset of sphingolipids, notably sphingosine, and their related enzymes as potential regulators of EC senescence. To further elucidate their roles, it is imperative to quantify the changes in these sphingolipids during the process of HUVEC senescence (days 0, 3 and 7) under the interventions of ARN114974, FB1 and PF-543. Analysing the association between sphingolipid changes and senescence status will provide deeper insights into sphingolipid regulation of EC senescence and evaluate the mechanism of actions of effective anti-EC senescence interventions.

7.2 Precise determination of EC senescence by ImageStreamX

A technical limitation of this study is inherent in the method used for quantitating senescence. The quantitation in this study primarily relied on SA-beta-Gal staining, which was subsequently validated through Western blotting of cell cycle arrest markers. However, this conventional approach to sequentially analysing senescence may inadvertently lead to

an overestimation of the number of senescent cells, due to the duplicate counting of SA-beta-Gal or p21 single positivity. In addition, this method did not take into account distinctive morphological changes observed in senescent cells, such as enlargement and flattening, which are unique features of these cells. To overcome this limitation, it is advisable to employ ImageStreamX imaging flow cytometry technology. This advanced technology allows for the simultaneous quantitation and imaging of multiple markers on single cells, including fluorescent SA-beta-Gal, p21, cell size and aspect ratio in single cells (424, 425). Meanwhile, ImageStreamX combines the advantages of high-throughput analysis capabilities of flow cytometry (424, 425). Therefore, it can offer a more accurate and comprehensive analysis of EC senescence and draw a more robust conclusion of sphingolipid-targeted anti-EC senescence approaches (424).

7.3 Investagation of mechanistic links between EC senescence and sphingolipid metabolism

My work has revealed that sphingosine drives H_2O_2-induced endothelial cell senescence, while ceramide synthase inhibitor FB1 and acid ceramidase inhibitor ARN14974 can effectively prevent H_2O_2-induced senescence in endothelial cells. However, the underlying mechanism behind these effects remains unknown. It has been reported that the lysosome plays a vital role in facilitating the impact of sphingolipids on the aging process (426). Therefore, my future plan is to determine whether sphingosine drives EC senescence

through impairing lysosomal integrity and functions. This can be identified through LysoTracker deep red and CellEvent™ Senescence Green Detection Kit to quantify size and number of lysosomes under confocal microscopy (427, 428). Furthermore, the conventional SA-β-Gal staining lacks the capability to provide accurate quantification of senescent cells (424). Hence, I plan to perform ImageStream X, a cutting-edge imaging flow cytometer, which combines the quantitative capabilities of flow cytometry with the analytical power of high-content image analysis (424, 429). Therefore, it possesses the ability to generate numerous high-resolution fluorescent and bright-field (BF) images of each individual cell directly within the flow (424). Additionally, this method offers a means to integrate multiple markers, such as SGP, p21, enabling a more dependable identification of senescent cells (425, 429).

7.4 Defining physiological role of acid ceramidase in EC senescence *in vivo*

Having demonstrated the *in vitro* anti-senescent effects, it is imperative to extend our examination to understand the *in vivo* impacts of AC ablation and the two pharmacological interventions, ARN and FB1. *In vivo* studies are particularly pivotal for understanding EC senescence due to the intricate regulatory interactions occurring between ECs and other cell types within the circulation and blood vessels (129). To this end, we have introduced p16-3MR mice obtained from Unity Biotechnology US (430). The p16-3MR (trimodality reporter) mouse is a sophisticated reporter model designed for identifying $p16^{+}$ senescent

cells with high luciferase activity, targeting their removal with ganciclovir, and isolating them using red fluorescent protein (431). p16 tdTom mice possess a reporter allele where the tandem-dimer Tomato (tdTomato) is integrated into the natural p16INK4a locus (432). Recently, researchers have investigated the dynamics and diversity of senescent cells *in vivo* by employing innovative p16 reporter mice and conducting single-cell transcriptome analysis (433). Therefore, utilising this mouse strain, we can precisely identify and quantify senescent cells *in vivo*. When coupled with endothelial specific marker CD31 staining (434), this approach enables the accurate quantification of senescent ECs following interventions *in vivo*.

However, it is imperative to acknowledge the potential adverse reactions associated with ARN and FB1. Injection of ARN14974 (10 mg kg^{-1}, i.p) in mice causes a substantial reduction in AC activity in multiple organs, including heart, lungs, brain, liver, and kidney (303). Another study has also found a reduced ASAH1 activity in mouse hearts over more than 7 hours after intraperitoneal injection of ARN14974 at a dose of 10 mg/kg (435). There are no available reports on its in vivo biosafety. Conversely, FB1 presents some immunotoxicity *in vivo* (435). The intragastric delivery of FB1 at 80 mg/kg to mice over a two-week period results in a decrease in spleen weight and induced a 12.9% apoptosis of thymocytes (436). Additionally, administering FB1 (100 mg/kg) to BALB/c mice for two weeks leads to elevated levels of interleukin-10 (IL-10) and interleukin-4 (IL-4) mRNA in

the spleen, along with reduced expression of interferon-γ (IFN-γ) and TNFα mRNA (436). Furthermore, multiple investigations have indicated that FB1 exhibits specific toxic effects on various organs, including the liver, lungs, kidneys, heart, and intestines, across different animal species (337). Additionally, it has been demonstrated that intraperitoneal injection of FB1 in C57BL6 mice at a dose of 8 mg/kg body weight reduced the oxidative defense mechanisms in the liver and kidneys, with no discernible impact on the lungs (437). Given these considerations, focusing on ARN14974 for further studies appears to be a more practical approach.

Besides the pharmacological testing of the ASAH1 inhibitor ARN14974, we also plan to ablate AC in mice. AC-knockout mouse models have been established (438, 439). *Asah1*$^{+/-}$ mice had regular lifespans but experienced a gradual lipid storage disorder that affected their skin, lungs, liver, and bones (439). These findings were associated with a two-fold or less increase in ceramide content in these tissues and a decrease in acid ceramidase activity (439). Notably, one study has reported that mice with a homozygous *Asah1* gene deletion (*Asah1*$^{-/-}$) experienced embryonic lethality before embryonic day (E) 8.5 (440).

As for another method, conditional *Asah1* knockout mice has been shown in one study. By utilising tamoxifen (TM) to induce the nuclear translocation of Cre recombinase in mice carrying a LoxP-flanked (floxed) Asah1 gene construct, researchers breed homozygous floxed offspring with Cre transgenic mice. This breeding strategy generated animals

referred to as conditional *Asah1* homozygous knockout mice (441). Upon knocking out the *Asah1* gene, the levels of acid ceramidase decreased in the ovaries, leading to a concurrent increase in ceramide content (441). Subsequently, this mouse model was employed to investigate the function of acid ceramidase in hepatic fibrosis and non-alcoholic fatty liver disease (NAFLD) (441, 442). Conditional *Asah1* knockout mice has also been established specifically in ECs (443). They were generated by crossbreeding floxed *Asah1*^{tmEx1} mice with Tie2-Cre transgenic mice (443). Crucially, this research indicated that lysosomal acid ceramidase plays a role in controlling the release of endothelial exosomes, subsequently influencing the secretion of NLRP3 inflammasome products, which are related to the innovative mechanism for dysfunction in vascular endothelial cells in diabetic conditions (443). However, Cdh5Cre is considered more specific to ECs relative to Tie2-Cre (444). In my MPhil study, I have generated a new mouse strain Cdh5Cre^{ERT2} carrying p16-3MR alleles. This can be further crossbred with floxed Asah1 mice to study the EC specific role of ASAH1 in senescence *in vivo*.

Hence, integrating *in vitro* and *in vivo* approaches can offer a more comprehensive understanding of the involvement of sphingolipids in endothelial cell senescence. This combined approach holds the potential to uncover novel therapeutic interventions for various vascular diseases.

Reference List

1. Herranz N, Gil J. Mechanisms and functions of cellular senescence. The Journal of Clinical Investigation. 2018;128(4):1238-46.
2. Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Experimental cell research. 1961;25(3):585-621.
3. Hayflick L. The limited in vitro lifetime of human diploid cell strains. Experimental Cell Research. 1965;37(3):614-36.
4. Shay JW, Wright WE. Hayflick, his limit, and cellular ageing. Nat Rev Mol Cell Biol. 2000;1(1):72-6.
5. Campisi J. Cellular senescence as a tumor-suppressor mechanism. Trends Cell Biol. 2001;11(11):S27-S31.
6. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. Int J Biochem Cell Biol. 2005;37(5):961-76.
7. Jia G, Aroor AR, DeMarco VG, Martinez-Lemus LA, Meininger GA, Sowers JR. Vascular stiffness in insulin resistance and obesity. Frontiers in physiology. 2015;6:231.
8. Rossman MJ, Kaplon RE, Hill SD, McNamara MN, Santos-Parker JR, Pierce GL, et al. Endothelial cell senescence with aging in healthy humans: prevention by habitual exercise and relation to vascular endothelial function. American journal of physiology-Heart and circulatory physiology. 2017;313(5):H890-H5.
9. Jia G, Aroor AR, Jia C, Sowers JR. Endothelial cell senescence in aging-related vascular dysfunction. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease. 2019;1865(7):1802-9.
10. Uryga AK, Bennett MR. Ageing induced vascular smooth muscle cell senescence in atherosclerosis. The Journal of Physiology. 2016;594(8):2115-24.
11. Bloom SI, Islam MT, Lesniewski LA, Donato AJ. Mechanisms and consequences of endothelial cell senescence. Nature Reviews Cardiology. 2023;20(1):38-51.
12. Cho KA, Ryu SJ, Oh YS, Park JH, Lee JW, Kim H-P, et al. Morphological adjustment of senescent cells by modulating caveolin-1 status. J Biol Chem. 2004;279(40):42270-8.
13. Denoyelle C, Abou-Rjaily G, Bezrookove V, Verhaegen M, Johnson TM, Fullen DR, et al. Anti-oncogenic role of the endoplasmic reticulum differentially activated by mutations in the MAPK pathway. Nat Cell Biol. 2006;8(10):1053-63.
14. Biran A, Perelmutter M, Gal H, Burton DGA, Ovadya Y, Vadai E, et al. Senescent cells communicate via intercellular protein transfer. Genes Dev. 2015;29(8):791-802.
15. Chala N, Moimas S, Giampietro C, Zhang X, Zambelli T, Exarchos V, et al. Mechanical Fingerprint of Senescence in Endothelial Cells. Nano Letters. 2021;21(12):4911-20.
16. McHugh D, Gil J. Senescence and aging: Causes, consequences, and therapeutic avenues. J Cell Biol. 2018;217(1):65-77.

17. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular Senescence: Defining a Path Forward. Cell. 2019;179(4):813-27.
18. Salama R, Sadaie M, Hoare M, Narita M. Cellular senescence and its effector programs. Genes Dev. 2014;28(2).
19. Reed SM, Quelle DE. p53 acetylation: regulation and consequences. Cancers. 2014;7(1):30-69.
20. Farooq U, Notani D. Transcriptional regulation of INK4/ARF locus by cis and trans mechanisms. Front Cell Dev Biol. 2022;10:948351.
21. González-Gualda E, Baker AG, Fruk L, Muñoz-Espín D. A guide to assessing cellular senescence in vitro and in vivo. The FEBS Journal. 2021;288(1):56-80.
22. Mohammadzadeh A, Mirza-Aghazadeh-Attari M, Hallaj S, Saei AA, Alivand MR, Valizadeh A, et al. Crosstalk between P53 and DNA damage response in ageing. DNA Repair. 2019;80:8-15.
23. Minamino T, Miyauchi H, Yoshida T, Tateno K, Kunieda T, Komuro I. Vascular cell senescence and vascular aging. Journal of molecular and cellular cardiology. 2004;36(2):175-83.
24. De Cecco M, Ito T, Petrashen AP, Elias AE, Skvir NJ, Criscione SW, et al. L1 drives IFN in senescent cells and promotes age-associated inflammation. Nature. 2019;566(7742):73-8.
25. Uryga AK, Grootaert MO, Garrido AM, Oc S, Foote K, Chappell J, et al. Telomere damage promotes vascular smooth muscle cell senescence and immune cell recruitment after vessel injury. Communications biology. 2021;4(1):1-16.
26. Wang L, Wang B, Gasek NS, Zhou Y, Cohn RL, Martin DE, et al. Targeting p21 highly expressing cells in adipose tissue alleviates insulin resistance in obesity. Cell Metab. 2022;34(1).
27. D'adda Di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. Nature Reviews Cancer. 2008;8(7):512-22.
28. Childs BG, Baker DJ, Kirkland JL, Campisi J, van Deursen JM. Senescence and apoptosis: dueling or complementary cell fates? EMBO reports. 2014;15(11):1139-53.
29. Hsu C-H, Altschuler SJ, Wu LF. Patterns of early p21 dynamics determine proliferation-senescence cell fate after chemotherapy. Cell. 2019;178(2):361-73. e12.
30. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153(6):1194-217.
31. Sun Y, Coppé J-P, Lam EW-F. Cellular senescence: the sought or the unwanted? Trends in Molecular Medicine. 2018;24(10):871-85.
32. Kuilman T, Peeper DS. Senescence-messaging secretome: SMS-ing cellular stress. Nature reviews cancer. 2009;9(2):81-94.
33. Coppé J-P, Kauser K, Campisi J, Beauséjour CM. Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. Journal of Biological Chemistry. 2006;281(40):29568-74.
34. Gorgoulis V, Adams PD, Alimonti A, Bennett DC, Bischof O, Bishop C, et al. Cellular senescence: defining a path forward. Cell. 2019;179(4):813-27.

35. Freund A, Orjalo AV, Desprez P-Y, Campisi J. Inflammatory networks during cellular senescence: causes and consequences. Trends in molecular medicine. 2010;16(5):238-46.
36. Rodier F, Campisi J. Four faces of cellular senescence. Journal of Cell Biology. 2011;192(4):547-56.
37. Kuilman T, Michaloglou C, Vredeveld LC, Douma S, van Doorn R, Desmet CJ, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. Cell. 2008;133(6):1019-31.
38. Erusalimsky JD. Vascular endothelial senescence: from mechanisms to pathophysiology. Journal of Applied Physiology. 2009;106(1):326-32.
39. Woywodt A, Bahlmann FH, De Groot K, Haller H, Haubitz M. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. Nephrology Dialysis Transplantation. 2002;17(10):1728-30.
40. Kotla S, Vu HT, Ko KA, Wang Y, Imanishi M, Heo K-S, et al. Endothelial senescence is induced by phosphorylation and nuclear export of telomeric repeat binding factor 2–interacting protein. JCI insight. 2019;4(9).
41. Warboys CM, de Luca A, Amini N, Luong L, Duckles H, Hsiao S, et al. Disturbed flow promotes endothelial senescence via a p53-dependent pathway. Arteriosclerosis, thrombosis, and vascular biology. 2014;34(5):985-95.
42. Chang E, Harley CB. Telomere length and replicative aging in human vascular tissues. Proceedings of the National Academy of Sciences. 1995;92(24):11190-4.
43. Chen Q, Fischer A, Reagan JD, Yan L-J, Ames BN. Oxidative DNA damage and senescence of human diploid fibroblast cells. Proceedings of the National Academy of Sciences. 1995;92(10):4337-41.
44. von Zglinicki T, Saretzki G, Döcke W, Lotze C. Mild hyperoxia shortens telomeres and inhibits proliferation of fibroblasts: a model for senescence? Experimental cell research. 1995;220(1):186-93.
45. Parrinello S, Samper E, Krtolica A, Goldstein J, Melov S, Campisi J. Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. Nat Cell Biol. 2003;5(8):741-7.
46. Richardson RS, Duteil S, Wary C, Wray DW, Hoff J, Carlier PG. Human skeletal muscle intracellular oxygenation: the impact of ambient oxygen availability. The Journal of physiology. 2006;571(2):415-24.
47. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. Cancer research. 1989;49(23):6449-65.
48. Zhang D, Sun X, Liu J, Xie X, Cui W, Zhu Y. Homocysteine accelerates senescence of endothelial cells via DNA hypomethylation of human telomerase reverse transcriptase. Arteriosclerosis, thrombosis, and vascular biology. 2015;35(1):71-8.
49. Oh S-T, Park H, Yoon HJ, Yang SY. Long-term treatment of native ldl induces senescence of cultured human endothelial cells. Oxidative medicine and cellular longevity. 2017;2017.

50. Shi Q, Hubbard GB, Kushwaha RS, Rainwater D, Thomas III CA, Leland MM, et al. Endothelial senescence after high-cholesterol, high-fat diet challenge in baboons. American Journal of Physiology-Heart and Circulatory Physiology. 2007;292(6):H2913-H20.
51. Gimbrone Jr MA, García-Cardeña G. Endothelial cell dysfunction and the pathobiology of atherosclerosis. Circulation research. 2016;118(4):620-36.
52. Yamazaki Y, Baker DJ, Tachibana M, Liu C-C, Van Deursen JM, Brott TG, et al. Vascular cell senescence contributes to blood–brain barrier breakdown. Stroke. 2016;47(4):1068-77.
53. Krouwer VJ, Hekking LH, Langelaar-Makkinje M, Regan-Klapisz E, Post JA. Endothelial cell senescence is associated with disrupted cell-cell junctions and increased monolayer permeability. Vascular cell. 2012;4(1):1-10.
54. Khan SY, Awad EM, Oszwald A, Mayr M, Yin X, Waltenberger B, et al. Premature senescence of endothelial cells upon chronic exposure to TNFα can be prevented by N-acetyl cysteine and plumericin. Scientific Reports. 2017;7(1):39501.
55. Kandhaya-Pillai R, Miro-Mur F, Alijotas-Reig J, Tchkonia T, Kirkland JL, Schwartz Jr S. TNFα-senescence initiates a STAT-dependent positive feedback loop, leading to a sustained interferon signature, DNA damage, and cytokine secretion. Aging (Albany NY). 2017;9(11):2411.
56. Barinda AJ, Ikeda K, Nugroho DB, Wardhana DA, Sasaki N, Honda S, et al. Endothelial progeria induces adipose tissue senescence and impairs insulin sensitivity through senescence associated secretory phenotype. Nature communications. 2020;11(1):481.
57. Hwang HJ, Kim N, Herman AB, Gorospe M, Lee J-S. Factors and Pathways Modulating Endothelial Cell Senescence in Vascular Aging. International Journal of Molecular Sciences. 2022;23(17):10135.
58. Fumagalli M, Rossiello F, Clerici M, Barozzi S, Cittaro D, Kaplunov JM, et al. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat Cell Biol. 2012;14(4):355-65.
59. McDonald AI, Shirali AS, Aragón R, Ma F, Hernandez G, Vaughn DA, et al. Endothelial regeneration of large vessels is a biphasic process driven by local cells with distinct proliferative capacities. Cell stem cell. 2018;23(2):210-25. e6.
60. Greider CW. Telomeres and senescence: the history, the experiment, the future. Current Biology. 1998;8(5):R178-R81.
61. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990;345(6274):458-60.
62. Chan SW, Chang J, Prescott J, Blackburn EH. Altering telomere structure allows telomerase to act in yeast lacking ATM kinases. Current Biology. 2001;11(16):1240-50.
63. Smogorzewska A, de Lange T. Regulation of telomerase by telomeric proteins. Annual review of biochemistry. 2004;73(1):177-208.
64. De Lange T, Shiue L, Myers RM, Cox DR, Naylor SL, Killery AM, et al. Structure and variability of human chromosome ends. Mol Cell Biol. 1990;10(2):518-27.

65. Von Zglinicki T. Oxidative stress shortens telomeres. Trends in biochemical sciences. 2002;27(7):339-44.
66. Roake CM, Artandi SE. Control of cellular aging, tissue function, and cancer by p53 downstream of telomeres. Cold Spring Harbor perspectives in medicine. 2017;7(5):a026088.
67. Lin AW, Barradas M, Stone JC, Van Aelst L, Serrano M, Lowe SW. Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. Genes Dev. 1998;12(19):3008-19.
68. Minamino T, Yoshida T, Tateno K, Miyauchi H, Zou Y, Toko H, et al. Ras induces vascular smooth muscle cell senescence and inflammation in human atherosclerosis. Circulation. 2003;108(18):2264-9.
69. Aviv H, Yusuf Khan M, Skurnick J, Okuda K, Kimura M, Gardner J, et al. Age dependent aneuploidy and telomere length of the human vascular endothelium. Atherosclerosis. 2001;159(2):281-7.
70. Okuda K, Khan MY, Skurnick J, Kimura M, Aviv H, Aviv A. Telomere attrition of the human abdominal aorta: relationships with age and atherosclerosis. Atherosclerosis. 2000;152(2):391-8.
71. Fenton M, Barker S, Kurz DJ, Erusalimsky JD. Cellular Senescence After Single and Repeated Balloon Catheter Denudations of Rabbit Carotid Arteries. Arteriosclerosis, Thrombosis, and Vascular Biology. 2001;21(2):220-6.
72. Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Jansen-Dürr P. Replicative senescence of human endothelial cells in vitro involves G1 arrest, polyploidization and senescence-associated apoptosis. Experimental gerontology. 2001;36(8):1327-47.
73. Debacq-Chainiaux F, Ben Ameur R, Bauwens E, Dumortier E, Toutfaire M, Toussaint O. Stress-Induced (Premature) Senescence. In: Rattan SIS, Hayflick L, editors. Cellular Ageing and Replicative Senescence. Cham: Springer International Publishing; 2016. p. 243-62.
74. Chen J, Goligorsky MS. Premature senescence of endothelial cells: Methusaleh's dilemma. American Journal of Physiology-Heart and Circulatory Physiology. 2006;290(5):H1729-H39.
75. D'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, et al. A DNA damage checkpoint response in telomere-initiated senescence. Nature. 2003;426(6963):194-8.
76. Sohal RS, Weindruch R. Oxidative stress, caloric restriction, and aging. Science. 1996;273(5271):59-63.
77. Pole A, Dimri M, Dimri GP. Oxidative stress, cellular senescence and ageing. AIMS molecular science. 2016;3(3).
78. Chen J-H, Ozanne SE, Hales CN. Methods of cellular senescence induction using oxidative stress. Biological aging: Methods and protocols. 2007:179-89.
79. Suo R, Zhao Z-Z, Tang Z-H, Ren Z, Liu X, Liu L-S, et al. Hydrogen sulfide prevents H2O2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation. Molecular medicine reports. 2013;7(6):1865-70.

80. Song Z, Liu Y, Hao B, Yu S, Zhang H, Liu D, et al. Ginsenoside Rb1 prevents H2O2-induced HUVEC senescence by stimulating sirtuin-1 pathway. PloS one. 2014;9(11):e112699.
81. Wu G, Li S, Qu G, Hua J, Zong J, Li X, et al. Genistein alleviates H2O2-induced senescence of human umbilical vein endothelial cells via regulating the TXNIP/NLRP3 axis. Pharmaceutical Biology. 2021;59(1):1386-99.
82. Katsuumi G, Shimizu I, Yoshida Y, Minamino T. Vascular senescence in cardiovascular and metabolic diseases. Frontiers in cardiovascular medicine. 2018;5:18.
83. Chang MW-F, Grillari J, Mayrhofer C, Fortschegger K, Allmaier G, Marzban G, et al. Comparison of early passage, senescent and hTERT immortalized endothelial cells. Experimental cell research. 2005;309(1):121-36.
84. Kamino H, Hiratsuka M, Toda T, Nishigaki R, Osaki M, Ito H, et al. Searching for genes involved in arteriosclerosis: proteomic analysis of cultured human umbilical vein endothelial cells undergoing replicative senescence. Cell structure and function. 2003;28(6):495-503.
85. Shelton DN, Chang E, Whittier PS, Choi D, Funk WD. Microarray analysis of replicative senescence. Current biology. 1999;9(17):939-45.
86. Vasile E, Tomita Y, Brown LF, Kocher O, Dvorak HF. Differential expression of thymosin β-10 by early passage and senescent vascular endothelium is modulated by VPF/VEGF: evidence for senescent endothelial cells in vivo at sites of atherosclerosis. The FASEB Journal. 2001;15(2):458-66.
87. El Maï M, Wagner K-D, Michiels J-F, Ambrosetti D, Borderie A, Destree S, et al. The telomeric protein TRF2 regulates angiogenesis by binding and activating the PDGFRβ promoter. Cell Reports. 2014;9(3):1047-60.
88. Franco S, Segura I, Riese HH, Blasco MaA. Decreased B16F10 melanoma growth and impaired vascularization in telomerase-deficient mice with critically short telomeres. Cancer Research. 2002;62(2):552-9.
89. Zaccagnini G, Gaetano C, Della Pietra L, Nanni S, Grasselli A, Mangoni A, et al. Telomerase mediates vascular endothelial growth factor-dependent responsiveness in a rat model of hind limb ischemia. Journal of Biological Chemistry. 2005;280(15):14790-8.
90. Chang H, Rha SY, Jeung H-C, Park KH, Kim TS, Kim Y-B, et al. Telomerase-and angiogenesis-related gene responses to irradiation in human umbilical vein endothelial cells. International journal of molecular medicine. 2013;31(5):1202-8.
91. Ungvari Z, Tarantini S, Kiss T, Wren JD, Giles CB, Griffin CT, et al. Endothelial dysfunction and angiogenesis impairment in the ageing vasculature. Nature Reviews Cardiology. 2018;15(9):555-65.
92. Camaré C, Pucelle M, Nègre-Salvayre A, Salvayre R. Angiogenesis in the atherosclerotic plaque. Redox biology. 2017;12:18-34.
93. Jia G, Habibi J, Aroor AR, Hill MA, DeMarco VG, Lee LE, et al. Enhanced endothelium epithelial sodium channel signaling prompts left ventricular diastolic dysfunction in obese female mice. Metabolism. 2018;78:69-79.

94. Claesson-Welsh L, Dejana E, McDonald DM. Permeability of the endothelial barrier: identifying and reconciling controversies. Trends in molecular medicine. 2021;27(4):314-31.
95. Elahy M, Jackaman C, Mamo JC, Lam V, Dhaliwal SS, Giles C, et al. Blood–brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. Immunity & Ageing. 2015;12(1):1-9.
96. Yanaka M, Honma T, Sato K, Shinohara N, Ito J, Tanaka Y, et al. Increased monocytic adhesion by senescence in human umbilical vein endothelial cells. Bioscience, biotechnology, and biochemistry. 2011;75(6):1098-103.
97. Lantz J, Renner J, Länne T, Karlsson M. Is aortic wall shear stress affected by aging? An image-based numerical study with two age groups. Medical Engineering & Physics. 2015;37(3):265-71.
98. Olmos G, Martínez-Miguel P, Alcalde-Estevez E, Medrano D, Sosa P, Rodríguez-Mañas L, et al. Hyperphosphatemia induces senescence in human endothelial cells by increasing endothelin-1 production. Aging Cell. 2017;16(6):1300-12.
99. Ishibazawa A, Nagaoka T, Takahashi T, Yamamoto K, Kamiya A, Ando J, et al. Effects of shear stress on the gene expressions of endothelial nitric oxide synthase, endothelin-1, and thrombomodulin in human retinal microvascular endothelial cells. Investigative ophthalmology & visual science. 2011;52(11):8496-504.
100. Jia G, Aroor AR, DeMarco VG, Martinez-Lemus L, Meininger GA, Sowers JR. Vascular stiffness in insulin resistance and obesity. Frontiers in physiology. 2015;6:231.
101. Munoz-Espin D, Canamero M, Maraver A, Gomez-Lopez G, Contreras J, Murillo-Cuesta S, et al. Programmed cell senescence during mammalian embryonic development. Cell. 2013;155(5):1104-18.
102. Krizhanovsky V, Yon M, Dickins RA, Hearn S, Simon J, Miething C, et al. Senescence of activated stellate cells limits liver fibrosis. Cell. 2008;134(4):657-67.
103. Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H, Komuro I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. Circulation. 2002;105(13):1541-4.
104. Tian XL, Li Y. Endothelial cell senescence and age-related vascular diseases. J Genet Genomics. 2014;41(9):485-95.
105. Wu C-M, Zheng L, Wang Q, Hu Y-W. The emerging role of cell senescence in atherosclerosis. Clinical Chemistry and Laboratory Medicine (CCLM). 2021;59(1):27-38.
106. Childs BG, Baker DJ, Wijshake T, Conover CA, Campisi J, Van Deursen JM. Senescent intimal foam cells are deleterious at all stages of atherosclerosis. Science. 2016;354(6311):472-7.
107. Libby P, Ridker PM, Hansson GK, Atherothrombosis LTNo. Inflammation in atherosclerosis: from pathophysiology to practice. Journal of the American college of cardiology. 2009;54(23):2129-38.

108. Stojanović SD, Fiedler J, Bauersachs J, Thum T, Sedding DG. Senescence-induced inflammation: an important player and key therapeutic target in atherosclerosis. European Heart Journal. 2020;41(31):2983-96.
109. Sun X, Fu Y, Gu M, Zhang L, Li D, Li H, et al. Activation of integrin alpha5 mediated by flow requires its translocation to membrane lipid rafts in vascular endothelial cells. Proc Natl Acad Sci U S A. 2016;113(3):769-74.
110. Jia G, Aroor AR, Jia C, Sowers JR. Endothelial cell senescence in aging-related vascular dysfunction. Biochim Biophys Acta Mol Basis Dis. 2019;1865(7):1802-9.
111. Gimbrone MA, Jr., Garcia-Cardena G. Endothelial Cell Dysfunction and the Pathobiology of Atherosclerosis. Circ Res. 2016;118(4):620-36.
112. Lagoumtzi SM, Chondrogianni N. Senolytics and senomorphics: Natural and synthetic therapeutics in the treatment of aging and chronic diseases. Free Radical Biology and Medicine. 2021;171:169-90.
113. Stojanovic SD, Fiedler J, Bauersachs J, Thum T, Sedding DG. Senescence-induced inflammation: an important player and key therapeutic target in atherosclerosis. Eur Heart J. 2020;41(31):2983-96.
114. Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes Dev. 2010;24(22):2463-79.
115. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, et al. Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell. 2006;5(2):187-95.
116. Hall BM, Balan V, Gleiberman AS, Strom E, Krasnov P, Virtuoso LP, et al. Aging of mice is associated with p16(Ink4a)- and β-galactosidase-positive macrophage accumulation that can be induced in young mice by senescent cells. Aging. 2016;8(7):1294-315.
117. Debacq-Chainiaux F, Erusalimsky JD, Campisi J, Toussaint O. Protocols to detect senescence-associated beta-galactosidase (SA-betagal) activity, a biomarker of senescent cells in culture and in vivo. Nature Protocols. 2009;4(12):1798-806.
118. Lee BY, Han JA, Im JS, Morrone A, Johung K, Goodwin EC, et al. Senescence‐associated β‐galactosidase is lysosomal β‐galactosidase. Aging cell. 2006;5(2):187-95.
119. Erusalimsky JD, Kurz DJ. Endothelial cell senescence. Handb Exp Pharmacol. 2006(176 Pt 2):213-48.
120. Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, et al. A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci U S A. 1995;92(20):9363-7.
121. Kurz DJ, Decary S, Hong Y, Erusalimsky JD. Senescence-associated (beta)-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. J Cell Sci. 2000;113 (Pt 20):3613-22.
122. Yegorov YE, Akimov SS, Hass R, Zelenin AV, Prudovsky IA. Endogenous β-Galactosidase Activity in Continuously Nonproliferating Cells. Experimental Cell Research. 1998;243(1):207-11.

123. Severino J, Allen RG, Balin S, Balin A, Cristofalo VJ. Is β-Galactosidase Staining a Marker of Senescence in Vitro and in Vivo? Experimental Cell Research. 2000;257(1):162-71.
124. Dulić V, Beney GE, Frebourg G, Drullinger LF, Stein GH. Uncoupling between phenotypic senescence and cell cycle arrest in aging p21-deficient fibroblasts. Mol Cell Biol. 2000;20(18):6741-54.
125. Beauséjour CM, Krtolica A, Galimi F, Narita M, Lowe SW, Yaswen P, et al. Reversal of human cellular senescence: roles of the p53 and p16 pathways. EMBO J. 2003;22(16):4212-22.
126. Vigneron A, Vousden KH. p53, ROS and senescence in the control of aging. Aging. 2010;2(8):471-4.
127. Georgakopoulou E, Tsimaratou K, Evangelou K, Fernandez M-P, Zoumpourlis V, Trougakos I, et al. Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues. Aging (Albany NY). 2013;5(1):37.
128. de Oliveira Mann CC, Kranzusch PJ. cGAS Conducts Micronuclei DNA Surveillance. Trends Cell Biol. 2017;27(10):697-8.
129. Sun X, Feinberg MW. Vascular endothelial senescence: pathobiological insights, emerging long noncoding RNA targets, challenges and therapeutic opportunities. Frontiers in Physiology. 2021;12:693067.
130. Davan-Wetton CS, Pessolano E, Perretti M, Montero-Melendez T. Senescence under appraisal: hopes and challenges revisited. Cellular and Molecular Life Sciences. 2021;78(7):3333-54.
131. Kim E-C, Kim J-R. Senotherapeutics: emerging strategy for healthy aging and age-related disease. BMB reports. 2019;52(1):47.
132. Von Kobbe C. Targeting senescent cells: approaches, opportunities, challenges. Aging (Albany NY). 2019;11(24):12844.
133. Song P, An J, Zou M-H. Immune clearance of senescent cells to combat ageing and chronic diseases. Cells. 2020;9(3):671.
134. Jurk D, Passos JF. Senolytic drugs: Beyond the promise and the hype. Mechanisms of Ageing and Development. 2022;202:111631-.
135. Owens WA, Walaszczyk A, Spyridopoulos I, Dookun E, Richardson GD. Senescence and senolytics in cardiovascular disease: Promise and potential pitfalls. Mechanisms of Ageing and Development. 2021;198:111540.
136. Sweeney M, Cook SA, Gil J. Therapeutic opportunities for senolysis in cardiovascular disease. The FEBS Journal. 2023;290(5):1235-55.
137. Blagosklonny MV. Paradoxes of senolytics. Aging (Albany NY). 2018;10(12):4289.
138. Bussian TJ, Aziz A, Meyer CF, Swenson BL, van Deursen JM, Baker DJ. Clearance of senescent glial cells prevents tau-dependent pathology and cognitive decline. Nature. 2018;562(7728):578-82.

139. Helman A, Klochendler A, Azazmeh N, Gabai Y, Horwitz E, Anzi S, et al. p16Ink4a-induced senescence of pancreatic beta cells enhances insulin secretion. Nature medicine. 2016;22(4):412-20.
140. Anderson R, Lagnado A, Maggiorani D, Walaszczyk A, Dookun E, Chapman J, et al. Length-independent telomere damage drives post-mitotic cardiomyocyte senescence. EMBO J. 2019;38(5):e100492.
141. Phaniendra A, Jestadi DB, Periyasamy L. Free radicals: properties, sources, targets, and their implication in various diseases. Indian journal of clinical biochemistry. 2015;30:11-26.
142. Halliwell B, Gutteridge JM. Free radicals in biology and medicine: Oxford university press, USA; 2015.
143. Rani V, Deep G, Singh RK, Palle K, Yadav UCS. Oxidative stress and metabolic disorders: Pathogenesis and therapeutic strategies. Life Sciences. 2016;148:183-93.
144. Farhat N, Thorin-Trescases N, Voghel G, Villeneuve L, Mamarbachi M, Perrault LP, et al. Stress-induced senescence predominates in endothelial cells isolated from atherosclerotic chronic smokers. Canadian Journal of Physiology and Pharmacology. 2008;86(11):761-9.
145. Napoli C, Martin-Padura I, De Nigris F, Giorgio M, Mansueto G, Somma P, et al. Deletion of the p66Shc longevity gene reduces systemic and tissue oxidative stress, vascular cell apoptosis, and early atherogenesis in mice fed a high-fat diet. Proceedings of the National Academy of Sciences. 2003;100(4):2112-6.
146. Salisbury D, Bronas U. Reactive oxygen and nitrogen species: impact on endothelial dysfunction. Nursing research. 2015;64(1):53-66.
147. Cai H. Hydrogen peroxide regulation of endothelial function: Origins, mechanisms, and consequences. Cardiovascular Research. 2005;68(1):26-36.
148. Bretón-Romero R, Lamas S. Hydrogen peroxide signaling in vascular endothelial cells. Redox Biology. 2014;2:529-34.
149. Genestra M. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. Cellular signalling. 2007;19(9):1807-19.
150. Dröge W. Free radicals in the physiological control of cell function. Physiological reviews. 2002.
151. Finkel T. Oxygen radicals and signaling. Current opinion in cell biology. 1998;10(2):248-53.
152. Manea A. NADPH oxidase-derived reactive oxygen species: involvement in vascular physiology and pathology. Cell and Tissue Research. 2010;342(3):325-39.
153. Bedard K, Krause K-H. The NOX Family of ROS-Generating NADPH Oxidases: Physiology and Pathophysiology. Physiological Reviews. 2007;87(1):245-313.
154. Rodríguez-Mañas L, El-Assar M, Vallejo S, López-Dóriga P, Solís J, Petidier R, et al. Endothelial dysfunction in aged humans is related with oxidative stress and vascular inflammation. Aging Cell. 2009;8(3):226-38.
155. Willcox JK, Ash SL, Catignani GL. Antioxidants and prevention of chronic disease. Critical reviews in food science and nutrition. 2004;44(4):275-95.

156. Beckman KB, Ames BN. The free radical theory of aging matures. Physiological reviews. 1998.
157. Trpkovic A, Resanovic I, Stanimirovic J, Radak D, Mousa SA, Cenic-Milosevic D, et al. Oxidized low-density lipoprotein as a biomarker of cardiovascular diseases. Critical reviews in clinical laboratory sciences. 2015;52(2):70-85.
158. Steinberg D. Modification of low-density lipoprotein that increases its atherogenicity. N Engl j Med. 1989;320:915-24.
159. Frijhoff J, Winyard PG, Zarkovic N, Davies SS, Stocker R, Cheng D, et al. Clinical relevance of biomarkers of oxidative stress. Antioxidants & redox signaling. 2015;23(14):1144-70.
160. Tao L. Oxidation of polyunsaturated fatty acids and its impact on food quality and human health. Adv Food Technol Nutr Sci. 2015;1:135-42.
161. Yin H, Xu L, Porter NA. Free radical lipid peroxidation: mechanisms and analysis. Chemical reviews. 2011;111(10):5944-72.
162. De La Haba C, Palacio JR, Martínez P, Morros A. Effect of oxidative stress on plasma membrane fluidity of THP-1 induced macrophages. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2013;1828(2):357-64.
163. Wu JQ, Kosten TR, Zhang XY. Free radicals, antioxidant defense systems, and schizophrenia. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2013;46:200-6.
164. Frei B. Reactive oxygen species and antioxidant vitamins: mechanisms of action. The American journal of medicine. 1994;97(3):S5-S13.
165. Halliwell B. Biochemistry of oxidative stress. Biochemical society transactions. 2007;35(5):1147-50.
166. Jacob KD, Hooten NN, Trzeciak AR, Evans MK. Markers of oxidant stress that are clinically relevant in aging and age-related disease. Mechanisms of ageing and development. 2013;134(3-4):139-57.
167. Christen Y. Oxidative stress and Alzheimer disease. The American journal of clinical nutrition. 2000;71(2):621S-9S.
168. Chandrasekaran A, Idelchik MdPS, Melendez JA. Redox control of senescence and age-related disease. Redox biology. 2017;11:91-102.
169. Chen QM, Bartholomew JC, Campisi J, Acosta M, Reagan JD, AMES BN. Molecular analysis of H2O2-induced senescent-like growth arrest in normal human fibroblasts: p53 and Rb control G1 arrest but not cell replication. Biochemical Journal. 1998;332(1):43-50.
170. Graeber TG, Peterson JF, Tsai M, Monica K, Fornace Jr AJ, Giaccia AJ. Hypoxia induces accumulation of p53 protein, but activation of a G1-phase checkpoint by low-oxygen conditions is independent of p53 status. Mol Cell Biol. 1994;14(9):6264-77.
171. Toussaint O, Medrano EE, von Zglinicki T. Cellular and molecular mechanisms of stress-induced premature senescence (SIPS) of human diploid fibroblasts and melanocytes. Experimental gerontology. 2000;35(8):927-45.

172. Zhan H, Suzuki T, Aizawa K, Miyagawa K, Nagai R. Ataxia Telangiectasia Mutated (ATM)-mediated DNA Damage Response in Oxidative Stress-induced Vascular Endothelial Cell Senescence [S]. Journal of Biological Chemistry. 2010;285(38):29662-70.
173. Macip S, Igarashi M, Berggren P, Yu J, Lee SW, Aaronson SA. Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol Cell Biol. 2003;23(23):8576-85.
174. Lee AC, Fenster BE, Ito H, Takeda K, Bae NS, Hirai T, et al. Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. Journal of Biological Chemistry. 1999;274(12):7936-40.
175. Al Bitar S, Gali-Muhtasib H. The role of the cyclin dependent kinase inhibitor p21cip1/waf1 in targeting cancer: Molecular mechanisms and novel therapeutics. Cancers. 2019;11(10):1475.
176. Iwasa H, Han J, Ishikawa F. Mitogen-activated protein kinase p38 defines the common senescence-signalling pathway. Genes to Cells. 2003;8(2):131-44.
177. Freund A, Patil CK, Campisi J. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J. 2011;30(8):1536-48.
178. Zdanov S, Debacq-Chainiaux F, Remacle J, Toussaint O. Identification of p38MAPK-dependent genes with changed transcript abundance in H2O2-induced premature senescence of IMR-90 hTERT human fibroblasts. FEBS Lett. 2006;580(27):6455-63.
179. Freund A, Patil CK, Campisi J. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J. 2011;30(8):1536-48.
180. Bahorun T, Soobrattee M, Luximon-Ramma V, Aruoma O. Free radicals and antioxidants in cardiovascular health and disease. Internet Journal of Medical Update. 2006;1(2):25-41.
181. Minamino T, Miyauchi H, Yoshida T, Ishida Y, Yoshida H, Komuro I. Endothelial cell senescence in human atherosclerosis: role of telomere in endothelial dysfunction. Circulation. 2002;105(13):1541-4.
182. Ihling C, Menzel G, Wellens E, Mönting JrS, Schaefer HE, Zeiher AM. Topographical association between the cyclin-dependent kinases inhibitor P21, p53 accumulation, and cellular proliferation in human atherosclerotic tissue. Arteriosclerosis, thrombosis, and vascular biology. 1997;17(10):2218-24.
183. Halliwell B. Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs & aging. 2001;18:685-716.
184. Golde TE, Miller VM. Proteinopathy-induced neuronal senescence: a hypothesis for brain failure in Alzheimer's and other neurodegenerative diseases. Alzheimers Res Ther. 2009;1(2):1-12.
185. Liu D, Hornsby PJ. Senescent human fibroblasts increase the early growth of xenograft tumors via matrix metalloproteinase secretion. Cancer research. 2007;67(7):3117-26.
186. Kritsilis M, V. Rizou S, Koutsoudaki PN, Evangelou K, Gorgoulis VG, Papadopoulos D. Ageing, cellular senescence and neurodegenerative disease. International journal of molecular sciences. 2018;19(10):2937.

187. Fahy E, Subramaniam S, Murphy RC, Nishijima M, Raetz CR, Shimizu T, et al. Update of the LIPID MAPS comprehensive classification system for lipids1. Journal of lipid research. 2009;50:S9-S14.
188. Bustos V, Partridge L. Good Ol'fat: links between lipid signaling and longevity. Trends in biochemical sciences. 2017;42(10):812-23.
189. Bartke N, Hannun YA. Bioactive sphingolipids: metabolism and function. Journal of lipid research. 2009;50:S91-S6.
190. Thudichum JLW. A Treatise on the Chemical Constitution of the Brain: Archon Books; 1962.
191. Carter HE, Haines WJ, Ledyard W, Norris WP. BIOCHEMISTRY OF THE SPHINGOLIPIDES: I. PREPARATION OF SPHINGOLIPIDES FROM BEEF BRAIN AND SPINAL CORD. Journal of Biological Chemistry. 1947;169(1):77-82.
192. Goñi FM, Alonso A. Biophysics of sphingolipids I. Membrane properties of sphingosine, ceramides and other simple sphingolipids. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2006;1758(12):1902-21.
193. Hannun YA, Obeid LM. Sphingolipids and their metabolism in physiology and disease. Nature Reviews Molecular Cell Biology. 2018;19(3):175-91.
194. Hannun YA, Obeid LM. Principles of bioactive lipid signalling: lessons from sphingolipids. Nature reviews Molecular cell biology. 2008;9(2):139-50.
195. Cutler RG, Mattson MP. Sphingomyelin and ceramide as regulators of development and lifespan. Mechanisms of ageing and development. 2001;122(9):895-908.
196. Lahiri S, Futerman AH. The metabolism and function of sphingolipids and glycosphingolipids. Cellular and Molecular Life Sciences. 2007;64(17):2270-84.
197. Merrill AH. De novo sphingolipid biosynthesis: a necessary, but dangerous, pathway. Journal of Biological Chemistry. 2002;277(29):25843-6.
198. Stoffel W. Studies on the biosynthesis and degradation of sphingosine bases. Chemistry and Physics of Lipids. 1970;5(1):139-58.
199. Merrill Jr AH. Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells. Biochimica et Biophysica Acta (BBA)-Lipids and Lipid Metabolism. 1983;754(3):284-91.
200. Mandon EC, Ehses I, Rother J, van Echten G, Sandhoff K. Subcellular localization and membrane topology of serine palmitoyltransferase, 3-dehydrosphinganine reductase, and sphinganine N-acyltransferase in mouse liver. Journal of Biological Chemistry. 1992;267(16):11144-8.
201. Hornemann T, Wei Y, von Eckardstein A. Is the mammalian serine palmitoyltransferase a high-molecular-mass complex? Biochemical Journal. 2007;405(1):157-64.
202. Hornemann T, Penno A, Rütti MF, Ernst D, Kivrak-Pfiffner F, Rohrer L, et al. The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. Journal of Biological Chemistry. 2009;284(39):26322-30.

203. Kihara A, Igarashi Y. FVT-1 is a mammalian 3-ketodihydrosphingosine reductase with an active site that faces the cytosolic side of the endoplasmic reticulum membrane. Journal of Biological Chemistry. 2004;279(47):49243-50.
204. Pewzner-Jung Y, Ben-Dor S, Futerman AH. When do Lasses (longevity assurance genes) become CerS (ceramide synthases)?: Insights into the regulation of ceramide synthesis. Journal of Biological Chemistry. 2006;281(35):25001-5.
205. Ternes P, Franke S, Zähringer U, Sperling P, Heinz E. Identification and characterization of a sphingolipid Δ4-desaturase family. Journal of Biological Chemistry. 2002;277(28):25512-8.
206. Beauchamp E, Goenaga D, Le Bloc'h J, Catheline D, Legrand P, Rioux V. Myristic acid increases the activity of dihydroceramide Δ4-desaturase 1 through its N-terminal myristoylation. Biochimie. 2007;89(12):1553-61.
207. Qi Y, Wang W, Song Z, Aji G, Liu XT, Xia P. Role of sphingosine kinase in Type 2 diabetes mellitus. Frontiers in Endocrinology. 2021;11:627076.
208. Adams DR, Pyne S, Pyne NJ. Structure-function analysis of lipid substrates and inhibitors of sphingosine kinases. Cellular Signalling. 2020;76:109806.
209. Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, et al. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. Nature. 2010;465(7301):1084-8.
210. Xia P, Wang L, Moretti PAB, Albanese N, Chai F, Pitson SM, et al. Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-alpha signaling. J Biol Chem. 2002;277(10):7996-8003.
211. Zamora-Pineda J, Kumar A, Suh JH, Zhang M, Saba JD. Dendritic cell sphingosine-1-phosphate lyase regulates thymic egress. Journal of Experimental Medicine. 2016;213(12):2773-91.
212. Van Veldhoven PP, Mannaerts GP. Subcellular localization and membrane topology of sphingosine-1-phosphate lyase in rat liver. Journal of Biological Chemistry. 1991;266(19):12502-7.
213. Ikeda M, Kihara A, Igarashi Y. Sphingosine-1-phosphate lyase SPL is an endoplasmic reticulum-resident, integral membrane protein with the pyridoxal 5′-phosphate binding domain exposed to the cytosol. Biochemical and biophysical research communications. 2004;325(1):338-43.
214. Reiss U, Oskouian B, Zhou J, Gupta V, Sooriyakumaran P, Kelly S, et al. Sphingosine-phosphate lyase enhances stress-induced ceramide generation and apoptosis. Journal of Biological Chemistry. 2004;279(2):1281-90.
215. Bektas M, Allende ML, Lee BG, Chen W, Amar MJ, Remaley AT, et al. Sphingosine 1-phosphate lyase deficiency disrupts lipid homeostasis in liver. Journal of Biological Chemistry. 2010;285(14):10880-9.
216. Kitatani K, Idkowiak-Baldys J, Hannun YA. The sphingolipid salvage pathway in ceramide metabolism and signaling. Cellular signalling. 2008;20(6):1010-8.

217. Ogretmen B, Pettus BJ, Rossi MJ, Wood R, Usta J, Szulc Z, et al. Biochemical mechanisms of the generation of endogenous long chain ceramide in response to exogenous short chain ceramide in the A549 human lung adenocarcinoma cell line. Role for endogenous ceramide in mediating the action of exogenous ceramide. J Biol Chem. 2002;277(15):12960-9.
218. Merrill Jr AH. Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. Chemical reviews. 2011;111(10):6387-422.
219. Hernández-Corbacho MJ, Salama MF, Canals D, Senkal CE, Obeid LM. Sphingolipids in mitochondria. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2017;1862(1):56-68.
220. D'Angelo G, Moorthi S, Luberto C. Role and function of sphingomyelin biosynthesis in the development of cancer. Advances in cancer research. 2018;140:61-96.
221. Milhas D, Clarke CJ, Hannun YA. Sphingomyelin metabolism at the plasma membrane: implications for bioactive sphingolipids. FEBS Lett. 2010;584(9):1887-94.
222. Beckmann N, Becker KA, Kadow S, Schumacher F, Kramer M, Kühn C, et al. Acid sphingomyelinase deficiency ameliorates Farber disease. International journal of molecular sciences. 2019;20(24):6253.
223. Adada M, Luberto C, Canals D. Inhibitors of the sphingomyelin cycle: Sphingomyelin synthases and sphingomyelinases. Chemistry and physics of lipids. 2016;197:45-59.
224. Huitema K, Van Den Dikkenberg J, Brouwers JF, Holthuis JC. Identification of a family of animal sphingomyelin synthases. EMBO J. 2004;23(1):33-44.
225. Halter D, Neumann S, van Dijk SM, Wolthoorn J, De Maziere AM, Vieira OV, et al. Pre-and post-Golgi translocation of glucosylceramide in glycosphingolipid synthesis. J Cell Biol. 2007;179(1):101-15.
226. Tafesse FG, Huitema K, Hermansson M, van der Poel S, Van Den Dikkenberg J, Uphoff A, et al. Both sphingomyelin synthases SMS1 and SMS2 are required for sphingomyelin homeostasis and growth in human HeLa cells. Journal of Biological Chemistry. 2007;282(24):17537-47.
227. Li S, Kim H-E. Implications of sphingolipids on aging and age-related diseases. Frontiers in Aging. 2022;2:87.
228. Parashuraman S, D'Angelo G. Visualizing sphingolipid biosynthesis in cells. Chemistry and Physics of Lipids. 2019;218:103-11.
229. Sugiura M, Kono K, Liu H, Shimizugawa T, Minekura H, Spiegel S, et al. Ceramide kinase, a novel lipid kinase: molecular cloning and functional characterization. Journal of Biological Chemistry. 2002;277(26):23294-300.
230. Lamour NF, Stahelin RV, Wijesinghe DS, Maceyka M, Wang E, Allegood JC, et al. Ceramide kinase uses ceramide provided by ceramide transport protein: localization to organelles of eicosanoid synthesis. Journal of Lipid Research. 2007;48(6):1293-304.
231. Chung LH, Liu D, Liu XT, Qi Y. Ceramide transfer protein (CERT): An overlooked molecular player in cancer. International journal of molecular sciences. 2021;22(24):13184.

232. Wegner M-S, Schiffmann S, Parnham MJ, Geisslinger G, Grösch S. The enigma of ceramide synthase regulation in mammalian cells. Progress in Lipid Research. 2016;63:93-119.
233. Jennemann R, Rabionet M, Gorgas K, Epstein S, Dalpke A, Rothermel U, et al. Loss of ceramide synthase 3 causes lethal skin barrier disruption. Human molecular genetics. 2012;21(3):586-608.
234. Venkataraman K, Riebeling C, Bodennec J, Riezman H, Allegood JC, Sullards MC, et al. Upstream of growth and differentiation factor 1 (uog1), a mammalian homolog of the yeast Longevity Assurance Gene 1 (LAG1), regulatesN-Stearoyl-sphinganine (C18-(Dihydro) ceramide) synthesis in a fumonisin B1-independent manner in mammalian cells. Journal of Biological Chemistry. 2002;277(38):35642-9.
235. Kageyama-Yahara N, Riezman H. Transmembrane topology of ceramide synthase in yeast. Biochemical journal. 2006;398(3):585-93.
236. Yu J, Novgorodov SA, Chudakova D, Zhu H, Bielawska A, Bielawski J, et al. JNK3 signaling pathway activates ceramide synthase leading to mitochondrial dysfunction. Journal of Biological Chemistry. 2007;282(35):25940-9.
237. Mesicek J, Lee H, Feldman T, Jiang X, Skobeleva A, Berdyshev EV, et al. Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. Cellular signalling. 2010;22(9):1300-7.
238. Gong M, You J, Cui X, Zheng J. Identification of nucleolar localization signal sequence of tumor metastasis suppressor gene-1. Zhonghua Bing li xue za zhi= Chinese Journal of Pathology. 2011;40(11):767-71.
239. Riebeling C, Allegood JC, Wang E, Merrill AH, Futerman AH. Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. Journal of Biological Chemistry. 2003;278(44):43452-9.
240. Tidhar R, Ben-Dor S, Wang E, Kelly S, Merrill AH, Futerman AH. Acyl chain specificity of ceramide synthases is determined within a region of 150 residues in the Tram-Lag-CLN8 (TLC) domain. Journal of Biological Chemistry. 2012;287(5):3197-206.
241. Laviad EL, Albee L, Pankova-Kholmyansky I, Epstein S, Park H, Merrill AH, et al. Characterization of ceramide synthase 2: tissue distribution, substrate specificity, and inhibition by sphingosine 1-phosphate. Journal of Biological Chemistry. 2008;283(9):5677-84.
242. Xu Z, Zhou J, McCoy DM, Mallampalli RK. LASS5 is the predominant ceramide synthase isoform involved in de novo sphingolipid synthesis in lung epithelia. Journal of lipid research. 2005;46(6):1229-38.
243. Ogretmen B. Sphingolipid metabolism in cancer signalling and therapy. Nature Reviews Cancer. 2018;18(1):33-50.
244. Levy M, Futerman AH. Mammalian ceramide synthases. IUBMB life. 2010;62(5):347-56.
245. Turpin-Nolan SM, Brüning JC. The role of ceramides in metabolic disorders: when size and localization matters. Nature Reviews Endocrinology. 2020;16(4):224-33.

246. Wooten-Blanks LG, Song P, Senkal CE, Ogretmen B. Mechanisms of ceramide-mediated repression of the human telomerase reverse transcriptase promoter via deacetylation of Sp3 by histone deacetylase 1. The FASEB Journal. 2007;21(12):3386-97.
247. Abbott SK, Li H, Munoz SS, Knoch B, Batterham M, Murphy KE, et al. Altered ceramide acyl chain length and ceramide synthase gene expression in Parkinson's disease. Movement Disorders. 2014;29(4):518-26.
248. Pan H, Qin W-X, Huo K-K, Wan D-F, Yu Y, Xu Z-G, et al. Cloning, mapping, and characterization of a human homologue of the yeast longevity assurance gene LAG1. Genomics. 2001;77(1-2):58-64.
249. Petrache I, Kamocki K, Poirier C, Pewzner-Jung Y, Laviad EL, Schweitzer KS, et al. Ceramide synthases expression and role of ceramide synthase-2 in the lung: insight from human lung cells and mouse models. PloS one. 2013;8(5):e62968.
250. Imgrund S, Hartmann D, Farwanah H, Eckhardt M, Sandhoff R, Degen J, et al. Adult ceramide synthase 2 (CERS2)-deficient mice exhibit myelin sheath defects, cerebellar degeneration, and hepatocarcinomas. Journal of Biological Chemistry. 2009;284(48):33549-60.
251. Mizutani Y, Sun H, Ohno Y, Sassa T, Wakashima T, Obara M, et al. Cooperative synthesis of ultra long-chain fatty acid and ceramide during keratinocyte differentiation. PLoS One. 2013;8(6):e67317.
252. Rabionet M, Van Der Spoel AC, Chuang C-C, von Tümpling-Radosta B, Litjens M, Bouwmeester D, et al. Male germ cells require polyenoic sphingolipids with complex glycosylation for completion of meiosis: a link to ceramide synthase-3. Journal of Biological Chemistry. 2008;283(19):13357-69.
253. Becker I, Wang-Eckhardt L, Yaghootfam A, Gieselmann V, Eckhardt M. Differential expression of (dihydro) ceramide synthases in mouse brain: oligodendrocyte-specific expression of CerS2/Lass2. Histochemistry and cell biology. 2008;129:233-41.
254. Marsching C, Rabionet M, Mathow D, Jennemann R, Kremser C, Porubsky S, et al. Renal sulfatides: sphingoid base-dependent localization and region-specific compensation of CerS2-dysfunction1 [S]. Journal of lipid research. 2014;55(11):2354-69.
255. Schiffmann S, Sandner J, Birod K, Wobst I, Angioni C, Ruckhäberle E, et al. Ceramide synthases and ceramide levels are increased in breast cancer tissue. Carcinogenesis. 2009;30(5):745-52.
256. Mizutani Y, Kihara A, Igarashi Y. Mammalian Lass6 and its related family members regulate synthesis of specific ceramides. Biochemical Journal. 2005;390(1):263-71.
257. Wang G, Silva J, Dasgupta S, Bieberich E. Long-chain ceramide is elevated in presenilin 1 (PS1M146V) mouse brain and induces apoptosis in PS1 astrocytes. Glia. 2008;56(4):449-56.
258. Lawton M, Tong M, Silbermann E, Longato L, Jiao P, Mark P, et al. Hepatic ceramide may mediate brain insulin resistance and neurodegeneration in type 2 diabetes and non-alcoholic steatohepatitis. Journal of Alzheimer's Disease. 2009;16(4):715-29.

259. Halasiddappa LM, Koefeler H, Futerman AH, Hermetter A. Oxidized phospholipids induce ceramide accumulation in RAW 264.7 macrophages: role of ceramide synthases. PLoS One. 2013;8(7):e70002.
260. Novgorodov SA, Chudakova DA, Wheeler BW, Bielawski J, Kindy MS, Obeid LM, et al. Developmentally regulated ceramide synthase 6 increases mitochondrial Ca2+ loading capacity and promotes apoptosis. Journal of Biological Chemistry. 2011;286(6):4644-58.
261. Mullen TD, Jenkins RW, Clarke CJ, Bielawski J, Hannun YA, Obeid LM. Ceramide synthase-dependent ceramide generation and programmed cell death: involvement of salvage pathway in regulating postmitochondrial events. Journal of Biological Chemistry. 2011;286(18):15929-42.
262. Haimovitz-Friedman A. Radiation-induced signal transduction and stress response. Radiation research. 1998;150(5s):S102-S8.
263. Panjarian S, Kozhaya L, Arayssi S, Yehia M, Bielawski J, Bielawska A, et al. De novo N-palmitoylsphingosine synthesis is the major biochemical mechanism of ceramide accumulation following p53 up-regulation. Prostaglandins & other lipid mediators. 2008;86(1-4):41-8.
264. Barthelmes J, de Bazo AM, Pewzner-Jung Y, Schmitz K, Mayer CA, Foerch C, et al. Lack of ceramide synthase 2 suppresses the development of experimental autoimmune encephalomyelitis by impairing the migratory capacity of neutrophils. Brain, behavior, and immunity. 2015;46:280-92.
265. Schiffmann S, Ferreiros N, Birod K, Eberle M, Schreiber Y, Pfeilschifter W, et al. Ceramide synthase 6 plays a critical role in the development of experimental autoimmune encephalomyelitis. The Journal of Immunology. 2012;188(11):5723-33.
266. Wu W-I, McDonough VM, Nickels JT, Ko J, Fischl AS, Vales TR, et al. Regulation of Lipid Biosynthesis in Saccharomyces cerevisiae by Fumonisin B1∗. Journal of Biological Chemistry. 1995;270(22):13171-8.
267. Yoo H-S, Norred WP, Showker J, Riley RT. Elevated sphingoid bases and complex sphingolipid depletion as contributing factors in fumonisin-induced cytotoxicity. Toxicology and applied pharmacology. 1996;138(2):211-8.
268. Merrill Jr AH, Sullards MC, Wang E, Voss KA, Riley RT. Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. Environmental health perspectives. 2001;109(suppl 2):283-9.
269. Merrill Jr AH, Sandhoff K. Sphingolipids: Metabolism and cell signaling. New comprehensive biochemistry. 36: Elsevier; 2002. p. 373-407.
270. Chen J, Wei Z, Wang Y, Long M, Wu W, Kuca K. Fumonisin B1: Mechanisms of toxicity and biological detoxification progress in animals. Food and chemical toxicology. 2021;149:111977.
271. Riley RT, Enongene E, Voss KA, Norred WP, Meredith FI, Sharma RP, et al. Sphingolipid perturbations as mechanisms for fumonisin carcinogenesis. Environmental health perspectives. 2001;109(suppl 2):301-8.
272. Delongchamp R, Young J. Tissue sphinganine as a biomarker of fumonisin-induced apoptosis. Food Additives & Contaminants. 2001;18(3):255-61.

273. Riley RT, Voss KA. Differential sensitivity of rat kidney and liver to fumonisin toxicity: organ-specific differences in toxin accumulation and sphingoid base metabolism. Toxicological sciences. 2006;92(1):335-45.
274. Sharma N, He Q, Sharma RP. Sphingosine kinase activity confers resistance to apoptosis by fumonisin B1 in human embryonic kidney (HEK-293) cells. Chemico-biological interactions. 2004;151(1):33-42.
275. Khan R, Phulukdaree A, Chuturgoon A. Concentration-dependent effect of fumonisin B1 on apoptosis in oesophageal cancer cells. Human & experimental toxicology. 2018;37(7):762-71.
276. Wang S-K, Liu S, Yang L-G, Shi R-F, Sun G-J. Effect of fumonisin B1 on the cell cycle of normal human liver cells. Molecular medicine reports. 2013;7(6):1970-6.
277. Merrill Jr AH, Van Echten G, Wang E, Sandhoff K. Fumonisin B1 inhibits sphingosine (sphinganine) N-acyltransferase and de novo sphingolipid biosynthesis in cultured neurons in situ. Journal of Biological Chemistry. 1993;268(36):27299-306.
278. Turner N, Lim XY, Toop HD, Osborne B, Brandon AE, Taylor EN, et al. A selective inhibitor of ceramide synthase 1 reveals a novel role in fat metabolism. Nature communications. 2018;9(1):3165.
279. Wang E, Norred W, Bacon C, Riley R, Merrill Jr AH. Inhibition of sphingolipid biosynthesis by fumonisins. Implications for diseases associated with Fusarium moniliforme. Journal of Biological Chemistry. 1991;266(22):14486-90.
280. Gatt S. Enzymic hydrolysis and synthesis of ceramides. Journal of Biological Chemistry. 1963;238(9):PC3131-PC3.
281. Bernardo K, Hurwitz R, Zenk T, Desnick RJ, Ferlinz K, Schuchman EH, et al. Purification, characterization, and biosynthesis of human acid ceramidase. Journal of Biological Chemistry. 1995;270(19):11098-102.
282. Realini N, Palese F, Pizzirani D, Pontis S, Basit A, Bach A, et al. Acid ceramidase in melanoma: expression, localization, and effects of pharmacological inhibition. Journal of Biological Chemistry. 2016;291(5):2422-34.
283. Strelow A, Bernardo K, Adam-Klages S, Linke T, Sandhoff K, Krönke M, et al. Overexpression of acid ceramidase protects from tumor necrosis factor–induced cell death. The Journal of experimental medicine. 2000;192(5):601-12.
284. Munk R, Anerillas C, Rossi M, Tsitsipatis D, Martindale JL, Herman AB, et al. Acid ceramidase promotes senescent cell survival. Aging (albany NY). 2021;13(12):15750.
285. Huang Y, Tanimukai H, Liu F, Iqbal K, Grundke‐Iqbal I, Gong CX. Elevation of the level and activity of acid ceramidase in Alzheimer's disease brain. European journal of neuroscience. 2004;20(12):3489-97.
286. Ito M, Okino N, Tani M. New insight into the structure, reaction mechanism, and biological functions of neutral ceramidase. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2014;1841(5):682-91.

287. Carroll B, Donaldson JC, Obeid L. Sphingolipids in the DNA damage response. Advances in biological regulation. 2015;58:38-52.
288. Wu BX, Zeidan YH, Hannun YA. Downregulation of neutral ceramidase by gemcitabine: Implications for cell cycle regulation. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2009;1791(8):730-9.
289. Novgorodov SA, Riley CL, Yu J, Borg KT, Hannun YA, Proia RL, et al. Essential roles of neutral ceramidase and sphingosine in mitochondrial dysfunction due to traumatic brain injury. Journal of Biological Chemistry. 2014;289(19):13142-54.
290. Franzen R, Fabbro D, Aschrafi A, Pfeilschifter J, Huwiler A. Nitric oxide induces degradation of the neutral ceramidase in rat renal mesangial cells and is counterregulated by protein kinase C. Journal of Biological Chemistry. 2002;277(48):46184-90.
291. Franzen R, Pautz A, Bräutigam L, Geisslinger G, Pfeilschifter J, Huwiler A. Interleukin-1β induces chronic activation and de novo synthesis of neutral ceramidase in renal mesangial cells. Journal of Biological Chemistry. 2001;276(38):35382-9.
292. Sun W, Xu R, Hu W, Jin J, Crellin HA, Bielawski J, et al. Upregulation of the human alkaline ceramidase 1 and acid ceramidase mediates calcium-induced differentiation of epidermal keratinocytes. Journal of Investigative Dermatology. 2008;128(2):389-97.
293. Xu R, Sun W, Jin J, Obeid LM, Mao C. Role of alkaline ceramidases in the generation of sphingosine and its phosphate in erythrocytes. The FASEB Journal. 2010;24(7):2507.
294. Coant N, Sakamoto W, Mao C, Hannun YA. Ceramidases, roles in sphingolipid metabolism and in health and disease. Advances in biological regulation. 2017;63:122-31.
295. Liakath-Ali K, Vancollie VE, Lelliott CJ, Speak AO, Lafont D, Protheroe HJ, et al. Alkaline ceramidase 1 is essential for mammalian skin homeostasis and regulating whole-body energy expenditure. The Journal of pathology. 2016;239(3):374-83.
296. Xu R, Jin J, Hu W, Sun W, Bielawski J, Szulc Z, et al. Golgi alkaline ceramidase regulates cell proliferation and survival by controlling levels of sphingosine and S1P. The FASEB Journal. 2006;20(11):1813-25.
297. Sun W, Hu W, Xu R, Jin J, Szulc ZM, Zhang G, et al. Alkaline ceramidase 2 regulates β1 integrin maturation and cell adhesion. The FASEB Journal. 2009;23(2):656.
298. Xu R, Wang K, Mileva I, Hannun YA, Obeid LM, Mao C. Alkaline ceramidase 2 and its bioactive product sphingosine are novel regulators of the DNA damage response. Oncotarget. 2016;7(14):18440.
299. Hu W, Xu R, Sun W, Szulc ZM, Bielawski J, Obeid LM, et al. Alkaline ceramidase 3 (ACER3) hydrolyzes unsaturated long-chain ceramides, and its down-regulation inhibits both cell proliferation and apoptosis. Journal of Biological Chemistry. 2010;285(11):7964-76.
300. Hu W, Xu R, Sun W, Szulc ZM, Bielawski J, Obeid LM, et al. Alkaline ceramidase 3 (ACER3) hydrolyzes unsaturated long-chain ceramides, and its down-regulation inhibits both cell proliferation and apoptosis. J Biol Chem. 2010;285(11):7964-76.

301. Chen C, Yin Y, Li C, Chen J, Xie J, Lu Z, et al. ACER3 supports development of acute myeloid leukemia. Biochemical and Biophysical Research Communications. 2016;478(1):33-8.
302. Saied EM, Arenz C. Inhibitors of Ceramidases. Chemistry and Physics of Lipids. 2016;197:60-8.
303. Pizzirani D, Bach A, Realini N, Armirotti A, Mengatto L, Bauer I, et al. Benzoxazolone carboxamides: potent and systemically active inhibitors of intracellular acid ceramidase. Angewandte Chemie. 2015;127(2):495-9.
304. Song J-H, Kim G-T, Park K-H, Park W-J, Park T-S. Bioactive sphingolipids as major regulators of coronary artery disease. Biomolecules & Therapeutics. 2021;29(4):373.
305. Bielawska A, Greenberg MS, Perry D, Jayadev S, Shayman JA, McKay C, et al. (1S,2R)-D-erythro-2-(N-myristoylamino)-1-phenyl-1-propanol as an inhibitor of ceramidase. J Biol Chem. 1996;271(21):12646-54.
306. Bielawska A, Linardic C, Hannun Y. Ceramide-mediated biology. Determination of structural and stereospecific requirements through the use of N-acyl-phenylaminoalcohol analogs. Journal of Biological Chemistry. 1992;267(26):18493-7.
307. Raisova M, Goltz G, Bektas M, Bielawska A, Riebeling C, Hossini AM, et al. Bcl-2 overexpression prevents apoptosis induced by ceramidase inhibitors in malignant melanoma and HaCaT keratinocytes. FEBS Lett. 2002;516(1-3):47-52.
308. Bielsa N, Casasampere M, Aseeri M, Casas J, Delgado A, Abad JL, et al. Discovery of deoxyceramide analogs as highly selective ACER3 inhibitors in live cells. European journal of medicinal chemistry. 2021;216:113296.
309. García-Barros M, Coant N, Kawamori T, Wada M, Snider AJ, Truman J-P, et al. Role of neutral ceramidase in colon cancer. The FASEB Journal. 2016;30(12):4159.
310. Maceyka M, Sankala H, Hait NC, Le Stunff H, Liu H, Toman R, et al. SphK1 and SphK2, sphingosine kinase isoenzymes with opposing functions in sphingolipid metabolism. Journal of Biological Chemistry. 2005;280(44):37118-29.
311. Hla T. Physiological and pathological actions of sphingosine 1-phosphate. Semin Cell Dev Biol. 2004;15(5):513-20.
312. Alvarez SE, Harikumar KB, Hait NC, Allegood J, Strub GM, Kim EY, et al. Sphingosine-1-phosphate is a missing cofactor for the E3 ubiquitin ligase TRAF2. Nature. 2010;465(7301):1084-8.
313. Xia P, Wang L, Moretti PA, Albanese N, Chai F, Pitson SM, et al. Sphingosine kinase interacts with TRAF2 and dissects tumor necrosis factor-α signaling. Journal of Biological Chemistry. 2002;277(10):7996-8003.
314. Chen J, Wang W, Qi Y, Kaczorowski D, McCaughan GW, Gamble JR, et al. Deletion of sphingosine kinase 1 ameliorates hepatic steatosis in diet-induced obese mice: role of PPARγ. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2016;1861(2):138-47.

315. Allende ML, Sasaki T, Kawai H, Olivera A, Mi Y, van Echten-Deckert G, et al. Mice deficient in sphingosine kinase 1 are rendered lymphopenic by FTY720. Journal of Biological Chemistry. 2004;279(50):52487-92.
316. Zheng X, Li W, Ren L, Liu J, Pang X, Chen X, et al. The sphingosine kinase-1/sphingosine-1-phosphate axis in cancer: Potential target for anticancer therapy. Pharmacology & Therapeutics. 2019;195:85-99.
317. Alemany R, van Koppen CJ, Danneberg K, Ter Braak M, Meyer zu Heringdorf D. Regulation and functional roles of sphingosine kinases. Naunyn-Schmiedeberg's archives of pharmacology. 2007;374:413-28.
318. Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2006;1758(12):2016-26.
319. Taha TA, Hannun YA, Obeid LM. Sphingosine kinase: biochemical and cellular regulation and role in disease. J Biochem Mol Biol. 2006;39(2):113-31.
320. Mizugishi K, Yamashita T, Olivera A, Miller GF, Spiegel S, Proia RL. Essential Role for Sphingosine Kinases in Neural and Vascular Development. Mol Cell Biol. 2005;25(24):11113-21.
321. Xia P, Gamble JR, Rye KA, Wang L, Hii CS, Cockerill P, et al. Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. Proc Natl Acad Sci U S A. 1998;95(24):14196-201.
322. Schnute ME, McReynolds MD, Kasten T, Yates M, Jerome G, Rains JW, et al. Modulation of cellular S1P levels with a novel, potent and specific inhibitor of sphingosine kinase-1. Biochemical Journal. 2012;444(1):79-88.
323. Lee O-H, Kim Y-M, Lee YM, Moon E-J, Lee D-J, Kim J-H, et al. Sphingosine 1-phosphate induces angiogenesis: its angiogenic action and signaling mechanism in human umbilical vein endothelial cells. Biochemical and biophysical research communications. 1999;264(3):743-50.
324. Cingolani F, Casasampere M, Sanllehí P, Casas J, Bujons J, Fabrias G. Inhibition of dihydroceramide desaturase activity by the sphingosine kinase inhibitor SKI II [S]. Journal of lipid research. 2014;55(8):1711-20.
325. French KJ, Zhuang Y, Maines LW, Gao P, Wang W, Beljanski V, et al. Pharmacology and antitumor activity of ABC294640, a selective inhibitor of sphingosine kinase-2. Journal of Pharmacology and Experimental Therapeutics. 2010;333(1):129-39.
326. Maines LW, Fitzpatrick LR, Green CL, Zhuang Y, Smith CD. Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease. Inflammopharmacology. 2010;18:73-85.
327. Dai L, Bai A, Smith CD, Rodriguez PC, Yu F, Qin Z. ABC294640, A Novel Sphingosine Kinase 2 Inhibitor, Induces Oncogenic Virus–Infected Cell Autophagic Death and Represses Tumor GrowthABC294640 and Autophagic Death. Molecular cancer therapeutics. 2017;16(12):2724-34.
328. Panneer Selvam S, De Palma RM, Oaks JJ, Oleinik N, Peterson YK, Stahelin RV, et al. Binding of the sphingolipid S1P to hTERT stabilizes telomerase at the nuclear periphery by allosterically mimicking protein phosphorylation. Science signaling. 2015;8(381):ra58-ra.

329. Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, et al. Biological characterization of 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2, 4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. PloS one. 2013;8(2):e56471.
330. Wang J, Zhong W, Cheng Q, Xiao C, Xu J, Su Z, et al. Histone methyltransferase Smyd2 contributes to blood-brain barrier breakdown in stroke. Clinical and Translational Medicine. 2022;12(3):e761.
331. Neubauer HA, Tea MN, Zebol JR, Gliddon BL, Stefanidis C, Moretti PA, et al. Cytoplasmic dynein regulates the subcellular localization of sphingosine kinase 2 to elicit tumor-suppressive functions in glioblastoma. Oncogene. 2019;38(8):1151-65.
332. Gault CR, Obeid LM, Hannun YA. An overview of sphingolipid metabolism: from synthesis to breakdown. Sphingolipids as signaling and regulatory molecules. 2010:1-23.
333. Law BA, Liao X, Moore KS, Southard A, Roddy P, Ji R, et al. Lipotoxic very-long-chain ceramides cause mitochondrial dysfunction, oxidative stress, and cell death in cardiomyocytes. The FASEB Journal. 2018;32(3):1403.
334. Wakita H, Tokura Y, Yagi H, Nishimura K, Furukawa F, Takigawa M. Keratinocyte differentiation is induced by cell-permeant ceramides and its proliferation is promoted by sphingosine. Archives of dermatological research. 1994;286:350-4.
335. Jayadev S, Liu B, Bielawska AE, Lee JY, Nazaire F, Pushkareva MY, et al. Role for Ceramide in Cell Cycle Arrest (*). Journal of Biological Chemistry. 1995;270(5):2047-52.
336. Venable ME, Lee JY, Smyth MJ, Bielawska A, Obeid LM. Role of Ceramide in Cellular Senescence (*). Journal of Biological Chemistry. 1995;270(51):30701-8.
337. Zhang F, Jiang H, Ai D. Research Progress of the Role of Ceramides in Vascular Pathophysiology. Prog Pharm Sci. 2020;44:924-31.
338. Nava VE, Cuvillier O, Edsall LC, Kimura K, Milstien S, Gelmann EP, et al. Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. Cancer Research. 2000;60(16):4468-74.
339. Pchejetski D, Golzio M, Bonhoure E, Calvet C, Doumerc N, Garcia V, et al. Sphingosine kinase-1 as a chemotherapy sensor in prostate adenocarcinoma cell and mouse models. Cancer research. 2005;65(24):11667-75.
340. Ahn EH, Schroeder JJ. Induction of apoptosis by sphingosine, sphinganine, and C2-ceramide in human colon cancer cells, but not by C2-dihydroceramide. Anticancer research. 2010;30(7):2881-4.
341. Woodcock J. Sphingosine and ceramide signalling in apoptosis. IUBMB life. 2006;58(8):462-6.
342. Suzuki E, Handa K, Toledo MS, Hakomori S. Sphingosine-dependent apoptosis: a unified concept based on multiple mechanisms operating in concert. Proceedings of the National Academy of Sciences. 2004;101(41):14788-93.

343. Smith ER, Merrill Jr AH, Obeid LM, Hannun YA. [28] - Effects of Sphingosine and Other Sphingolipids on Protein Kinase C. In: Merrill AH, Hannun YA, editors. Methods in Enzymology. 312: Academic Press; 2000. p. 361-73.
344. Xu R, Wang K, Mileva I, Hannun Y, Obeid L, Mao C. Alkaline ceramidase 2 and its bioactive product sphingosine are novel regulators of the DNA damage response. Oncotarget 7: 18440–18457. 2016.
345. Chao R, Khan W, Hannun YA. Retinoblastoma protein dephosphorylation induced by D-erythro-sphingosine. J Biol Chem. 1992;267(33):23459-62.
346. Spiegel S, Milstien S. Sphingosine-1-phosphate: an enigmatic signalling lipid. Nature reviews Molecular cell biology. 2003;4(5):397-407.
347. Ogretmen B, Hannun YA. Biologically active sphingolipids in cancer pathogenesis and treatment. Nature Reviews Cancer. 2004;4(8):604-16.
348. Cuvillier O, Pirianov G, Kleuser B, Vanek PG, Coso OA, Gutkind JS, et al. Suppression of ceramide-mediated programmed cell death by sphingosine-1-phosphate. Nature. 1996;381(6585):800-3.
349. Hait NC, Oskeritzian CA, Paugh SW, Milstien S, Spiegel S. Sphingosine kinases, sphingosine 1-phosphate, apoptosis and diseases. Biochimica et Biophysica Acta (BBA)-Biomembranes. 2006;1758(12):2016-26.
350. Milstien S, Spiegel S. Targeting sphingosine-1-phosphate: a novel avenue for cancer therapeutics. Cancer cell. 2006;9(3):148-50.
351. Paugh SW, Paugh BS, Rahmani M, Kapitonov D, Almenara JA, Kordula T, et al. A selective sphingosine kinase 1 inhibitor integrates multiple molecular therapeutic targets in human leukemia. Blood, The Journal of the American Society of Hematology. 2008;112(4):1382-91.
352. Olivera A, Allende ML, Proia RL. Shaping the landscape: metabolic regulation of S1P gradients. Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2013;1831(1):193-202.
353. Xiong Y, Yang P, Proia RL, Hla T. Erythrocyte-derived sphingosine 1-phosphate is essential for vascular development. The Journal of clinical investigation. 2014;124(11):4823-8.
354. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. Science. 2007;316(5822):295-8.
355. Venkataraman K, Lee Y-M, Michaud J, Thangada S, Ai Y, Bonkovsky HL, et al. Vascular endothelium as a contributor of plasma sphingosine 1-phosphate. Circulation research. 2008;102(6):669-76.
356. Camerer E, Regard JB, Cornelissen I, Srinivasan Y, Duong DN, Palmer D, et al. Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. The Journal of clinical investigation. 2009;119(7):1871-9.
357. Hisano Y, Kobayashi N, Yamaguchi A, Nishi T. Mouse SPNS2 functions as a sphingosine-1-phosphate transporter in vascular endothelial cells. PloS one. 2012;7(6):e38941.

358. Yatomi Y, Ozaki Y, Ohmori T, Igarashi Y. Sphingosine 1-phosphate: synthesis and release. Prostaglandins & other lipid mediators. 2001;64(1-4):107-22.
359. Jones P, George A. The ABC transporter structure and mechanism: perspectives on recent research. Cellular and Molecular Life Sciences CMLS. 2004;61:682-99.
360. Hait NC, Allegood J, Maceyka M, Strub GM, Harikumar KB, Singh SK, et al. Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. Science. 2009;325(5945):1254-7.
361. Strub GM, Paillard M, Liang J, Gomez L, Allegood JC, Hait NC, et al. Sphingosine-1-phosphate produced by sphingosine kinase 2 in mitochondria interacts with prohibitin 2 to regulate complex IV assembly and respiration. The FASEB Journal. 2011;25(2):600.
362. Xia P, Wang L, Gamble JR, Vadas MA. Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. J Biol Chem. 1999;274(48):34499-505.
363. Proia RL, Hla T. Emerging biology of sphingosine-1-phosphate: its role in pathogenesis and therapy. The Journal of clinical investigation. 2015;125(4):1379-87.
364. Venable ME, Yin X. Ceramide induces endothelial cell senescence. Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease. 2009;27(8):547-51.
365. Meacci E, Vasta V, Neri S, Farnararo M, Bruni P. Activation of phospholipase D in human fibroblasts by ceramide and sphingosine: evaluation of their modulatory role in bradykinin stimulation of phospholipase D. Biochemical and biophysical research communications. 1996;225(2):392-9.
366. Lee J, Bielawska A, Obeid L. Regulation of cyclin-dependent kinase 2 activity by ceramide. Experimental cell research. 2000;261(2):303-11.
367. LEE JY, LEONHARDT LG, OBEID LM. Cell-cycle-dependent changes in ceramide levels preceding retinoblastoma protein dephosphorylation in G2/M. Biochemical Journal. 1998;334(2):457-61.
368. Kim MK, Lee W, Yoon G-H, Chang E-J, Choi S-C, Kim SW. Links between accelerated replicative cellular senescence and down-regulation of SPHK1 transcription. BMB reports. 2019;52(3):220.
369. McNaughton M, Pitman M, Pitson SM, Pyne NJ, Pyne S. Proteasomal degradation of sphingosine kinase 1 and inhibition of dihydroceramide desaturase by the sphingosine kinase inhibitors, SKi or ABC294640, induces growth arrest in androgen-independent LNCaP-AI prostate cancer cells. Oncotarget. 2016;7(13):16663.
370. Chao R, Khan W, Hannun YA. Retinoblastoma protein dephosphorylation induced by D-erythro-sphingosine. Journal of Biological Chemistry. 1992;267(33):23459-62.
371. Lightle SA, Oakley JI, Nikolova-Karakashian MN. Activation of sphingolipid turnover and chronic generation of ceramide and sphingosine in liver during aging. Mechanisms of ageing and development. 2000;120(1-3):111-25.
372. Kavok NS, Krasilnikova OA, Babenko NA. Increase in diacylglycerol production by liver and liver cell nuclei at old age. Experimental gerontology. 2003;38(4):441-7.

373. Lucki N, Sewer MB. The cAMP-responsive element binding protein (CREB) regulates the expression of acid ceramidase (ASAH1) in H295R human adrenocortical cells. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2009;1791(8):706-13.
374. Mao Z, Sun W, Xu R, Novgorodov S, Szulc ZM, Bielawski J, et al. Alkaline ceramidase 2 (ACER2) and its product dihydrosphingosine mediate the cytotoxicity of N-(4-hydroxyphenyl)retinamide in tumor cells. J Biol Chem. 2010;285(38):29078-90.
375. Ketteler J, Wittka A, Leonetti D, Roy VV, Estephan H, Maier P, et al. Caveolin-1 regulates the ASMase/ceramide-mediated radiation response of endothelial cells in the context of tumor–stroma interactions. Cell Death & Disease. 2020;11(4):228.
376. Tran C, Heng B, Teo JD, Humphrey SJ, Qi Y, Couttas TA, et al. Sphingosine 1-phosphate but not Fingolimod protects neurons against excitotoxic cell death by inducing neurotrophic gene expression in astrocytes. Journal of Neurochemistry. 2020;153(2):e14917.
377. Tian X-L, Li Y. Endothelial Cell Senescence and Age-Related Vascular Diseases. Journal of Genetics and Genomics. 2014;41(9):485-95.
378. Baker DJ, Wijshake T, Tchkonia T, LeBrasseur NK, Childs BG, Van De Sluis B, et al. Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. Nature. 2011;479(7372):232-6.
379. Baker DJ, Childs BG, Durik M, Wijers ME, Sieben CJ, Zhong J, et al. Naturally occurring p16Ink4a-positive cells shorten healthy lifespan. Nature. 2016;530(7589):184-9.
380. Trayssac M, Hannun YA, Obeid LM. Role of sphingolipids in senescence: implication in aging and age-related diseases. The Journal of clinical investigation. 2018;128(7):2702-12.
381. Deanfield JE, Halcox JP, Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation. 2007;115(10):1285-95.
382. Munoz-Chapuli R, Quesada A, Angel Medina M. Angiogenesis and signal transduction in endothelial cells. Cellular and Molecular Life Sciences CMLS. 2004;61:2224-43.
383. Han Y, Kim SY. Endothelial senescence in vascular diseases: current understanding and future opportunities in senotherapeutics. Experimental & Molecular Medicine. 2023:1-12.
384. Baudin B, Bruneel A, Bosselut N, Vaubourdolle M. A protocol for isolation and culture of human umbilical vein endothelial cells. Nature protocols. 2007;2(3):481-5.
385. Maciag T, Hoover G, Stemerman M, Weinstein R. Serial propagation of human endothelial cells in vitro. J Cell Biol. 1981;91(2):420-6.
386. Chowdhary S. The effects of oxidative stress on inducing senescence in human fibroblasts. Journal of the South Carolina Academy of Science. 2019;16(2):2.
387. Li R-L, Lu Z-Y, Huang J-J, Qi J, Hu A, Su Z-X, et al. SRT1720, a SIRT1 specific activator, protected H2O2-induced senescent endothelium. American Journal of Translational Research. 2016;8(7):2876.
388. Lin Y-j, Zhen Y-z, Wei J, Liu B, Yu Z-y, Hu G. Effects of Rhein lysinate on H2O2-induced cellular senescence of human umbilical vascular endothelial cells. Acta Pharmacologica Sinica. 2011;32(10):1246-52.

389. Lim S-H, Li BS, Zhu RZ, Choi B-M. 9-Hydroxy-6, 7-dimethoxydalbergiquinol suppresses hydrogen peroxide-induced senescence in human dermal fibroblasts through induction of sirtuin-1 expression. Asian Pacific Journal of Tropical Biomedicine. 2021;11(2):89.
390. Coleman PR, Hahn CN, Grimshaw M, Lu Y, Li X, Brautigan PJ, et al. Stress-induced premature senescence mediated by a novel gene, SENEX, results in an anti-inflammatory phenotype in endothelial cells. Blood, The Journal of the American Society of Hematology. 2010;116(19):4016-24.
391. Liu R, Liu H, Ha Y, Tilton RG, Zhang W. Oxidative stress induces endothelial cell senescence via downregulation of Sirt6. BioMed research international. 2014;2014.
392. Zheng Z, Wang M, Cheng C, Liu D, Wu L, Zhu J, et al. Ginsenoside Rb1 reduces H2O2‐induced HUVEC dysfunction by stimulating the sirtuin‐1/AMP‐activated protein kinase pathway. Molecular Medicine Reports. 2020;22(1):247-56.
393. Suo R, Zhao Z-Z, Tang Z-H, Ren Z, Liu X, Liu L-S, et al. Hydrogen sulfide prevents H_2O_2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation. Molecular Medicine Reports. 2013;7(6):1865-70.
394. Furukawa A, Tada-Oikawa S, Kawanishi S, Oikawa S. H2O2 accelerates cellular senescence by accumulation of acetylated p53 via decrease in the function of SIRT1 by NAD+ depletion. Cellular Physiology and Biochemistry. 2006;20(1-4):045-54.
395. Li C-M, Park J-H, He X, Levy B, Chen F, Arai K, et al. The human acid ceramidase gene (ASAH): structure, chromosomal location, mutation analysis, and expression. Genomics. 1999;62(2):223-31.
396. Pilorget A, Demeule M, Barakat S, Marvaldi J, Luis J, Béliveau R. Modulation of P‐glycoprotein function by sphingosine kinase‐1 in brain endothelial cells. Journal of neurochemistry. 2007;100(5):1203-10.
397. Ancellin N, Colmont C, Su J, Li Q, Mittereder N, Chae S-S, et al. Extracellular export of sphingosine kinase-1 enzyme: sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation. Journal of Biological Chemistry. 2002;277(8):6667-75.
398. Lai Y, Tian Y, You X, Du J, Huang J. Effects of sphingolipid metabolism disorders on endothelial cells. Lipids in Health and Disease. 2022;21(1):1-12.
399. Quinville BM, Deschenes NM, Ryckman AE, Walia JS. A comprehensive review: sphingolipid metabolism and implications of disruption in sphingolipid homeostasis. International journal of molecular sciences. 2021;22(11):5793.
400. Jeyapalan JC, Sedivy JM. Cellular senescence and organismal aging. Mechanisms of ageing and development. 2008;129(7-8):467-74.
401. Childs BG, Durik M, Baker DJ, Van Deursen JM. Cellular senescence in aging and age-related disease: from mechanisms to therapy. Nature medicine. 2015;21(12):1424-35.
402. Cannavo A, Liccardo D, Komici K, Corbi G, De Lucia C, Femminella GD, et al. Sphingosine kinases and sphingosine 1-phosphate receptors: signaling and actions in the cardiovascular system. Frontiers in pharmacology. 2017;8:556.

403. Peters SL, Alewijnse AE. Sphingosine-1-phosphate signaling in the cardiovascular system. Current opinion in pharmacology. 2007;7(2):186-92.
404. Pelletier D, Hafler DA. Fingolimod for multiple sclerosis. New England Journal of Medicine. 2012;366(4):339-47.
405. Li Q, Yuan Q, Wang T, Zhan Y, Yang L, Fan Y, et al. Fumonisin B1 inhibits cell proliferation and decreases barrier function of swine umbilical vein endothelial cells. Toxins. 2021;13(12):863.
406. Yuan Q, Jiang Y, Fan Y, Ma Y, Lei H, Su J. Fumonisin B1 induces oxidative stress and breaks barrier functions in pig iliac endothelium cells. Toxins. 2019;11(7):387.
407. Aji G, Huang Y, Ng ML, Wang W, Lan T, Li M, et al. Regulation of hepatic insulin signaling and glucose homeostasis by sphingosine kinase 2. Proceedings of the National Academy of Sciences. 2020;117(39):24434-42.
408. Lütjohann D, Meichsner S, Pettersson H. Lipids in Alzheimer's disease and their potential for therapy. Clinical Lipidology. 2012;7(1):65-78.
409. Maines LW, Fitzpatrick LR, French KJ, Zhuang Y, Xia Z, Keller SN, et al. Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. Digestive diseases and sciences. 2008;53:997-1012.
410. Zhang H, Desai NN, Olivera A, Seki T, Brooker G, Spiegel S. Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. J Cell Biol. 1991;114(1):155-67.
411. Itakura A, Tanaka A, Aioi A, Tonogaito H, Matsuda H. Ceramide and sphingosine rapidly induce apoptosis of murine mast cells supported by interleukin-3 and stem cell factor. Experimental hematology. 2002;30(3):272-8.
412. Phillips DC, Martin S, Doyle BT, Houghton JA. Sphingosine-induced apoptosis in rhabdomyosarcoma cell lines is dependent on pre-mitochondrial Bax activation and post-mitochondrial caspases. Cancer research. 2007;67(2):756-64.
413. Yu S, Jia B, Liu N, Yu D, Wu A. Evaluation of the Individual and Combined Toxicity of Fumonisin Mycotoxins in Human Gastric Epithelial Cells. International Journal of Molecular Sciences. 2020;21(16).
414. Arumugam T, Ghazi T, Chuturgoon A. Fumonisin B1 Epigenetically Regulates PTEN Expression and Modulates DNA Damage Checkpoint Regulation in HepG2 Liver Cells. Toxins. 2020;12(10).
415. Zhao X, Wang Y, Liu J-L, Zhang J-H, Zhang S-C, Ouyang Y, et al. Fumonisin B1 affects the biophysical properties, migration and cytoskeletal structure of human umbilical vein endothelial cells. Cell Biochemistry and Biophysics. 2020;78:375-82.
416. Liu K, Guo TL, Hait NC, Allegood J, Parikh HI, Xu W, et al. Biological characterization of 3-(2-amino-ethyl)-5-[3-(4-butoxyl-phenyl)-propylidene]-thiazolidine-2,4-dione (K145) as a selective sphingosine kinase-2 inhibitor and anticancer agent. PloS One. 2013;8(2):e56471.
417. Wallington-Beddoe CT, Bennett MK, Vandyke K, Davies L, Zebol JR, Moretti PA, et al. Sphingosine kinase 2 inhibition synergises with bortezomib to target myeloma by enhancing endoplasmic reticulum stress. Oncotarget. 2017;8(27):43602.

418. Zheng S, Zheng H, Huang A, Mai L, Huang X, Hu Y, et al. Piwi-interacting RNAs play a role in vitamin C-mediated effects on endothelial aging. International Journal of Medical Sciences. 2020;17(7):946.
419. Li LC, Wang ZW, Hu XP, Wu ZY, Hu ZP, Ruan YL. MDG‐1 inhibits H2O2‐induced apoptosis and inflammation in human umbilical vein endothelial cells. Molecular Medicine Reports. 2017;16(3):3673-9.
420. Hannun YA, Bell RM. Regulation of protein kinase C by sphingosine and lysosphingolipids. Clin Chim Acta. 1989;185(3):333-45.
421. Wang J, Lv X-W, Shi J-P, Hu X-S. Mechanisms involved in ceramide-induced cell cycle arrest in human hepatocarcinoma cells. World journal of gastroenterology: WJG. 2007;13(7):1129.
422. Dadsena S, Hassan DG, Holthuis JC. Unraveling the molecular principles by which ceramides commit cells to death. Cell Stress. 2019;3(8):280.
423. Hernández-Corbacho MJ, Canals D, Adada MM, Liu M, Senkal CE, Yi JK, et al. Tumor necrosis factor-α (TNFα)-induced ceramide generation via ceramide synthases regulates loss of focal adhesion kinase (FAK) and programmed cell death. Journal of Biological Chemistry. 2015;290(42):25356-73.
424. Biran A, Zada L, Abou Karam P, Vadai E, Roitman L, Ovadya Y, et al. Quantitative identification of senescent cells in aging and disease. Aging cell. 2017;16(4):661-71.
425. Gal H, Porat Z, Krizhanovsky V. A Multiparametric Assay to Evaluate Senescent Cells. Methods Mol Biol. 2019;1896:107-17.
426. Tang H, Huang X, Pang S. Regulation of the lysosome by sphingolipids: Potential role in aging. Journal of Biological Chemistry. 2022;298(7).
427. Clarke ST, Kelley A, Jackson T, Low Q, Calderon V, Huang T, et al., editors. Evaluation of cellular senescence through fluorescence characterization. Cancer Research; 2019: AMER ASSOC CANCER RESEARCH 615 CHESTNUT ST, 17TH FLOOR, PHILADELPHIA, PA ….
428. Ivanov A, Pawlikowski J, Manoharan I, van Tuyn J, Nelson DM, Rai TS, et al. Lysosome-mediated processing of chromatin in senescence. Journal of Cell Biology. 2013;202(1):129-43.
429. González‐Gualda E, Baker AG, Fruk L, Muñoz‐Espín D. A guide to assessing cellular senescence in vitro and in vivo. The FEBS journal. 2021;288(1):56-80.
430. Kaur G, Sundar IK, Rahman I. p16-3MR: A Novel Model to Study Cellular Senescence in Cigarette Smoke-Induced Lung Injuries. International Journal of Molecular Sciences. 2021;22(9).
431. Demaria M, Ohtani N, Youssef SA, Rodier F, Toussaint W, Mitchell JR, et al. An essential role for senescent cells in optimal wound healing through secretion of PDGF-AA. Developmental cell. 2014;31(6):722-33.
432. Liu J-Y, Souroullas GP, Diekman BO, Krishnamurthy J, Hall BM, Sorrentino JA, et al. Cells exhibiting strong p16 INK4a promoter activation in vivo display features of senescence. Proceedings of the National Academy of Sciences. 2019;116(7):2603-11.

433. Omori S, Wang T-W, Johmura Y, Kanai T, Nakano Y, Kido T, et al. Generation of a p16 reporter mouse and its use to characterize and target p16high cells in vivo. Cell Metab. 2020;32(5):814-28. e6.
434. Demaria M, O'Leary MN, Chang J, Shao L, Liu S, Alimirah F, et al. Cellular Senescence Promotes Adverse Effects of Chemotherapy and Cancer Relapse. Cancer Discov. 2017;7(2):165-76.
435. Hadas Y, Vincek AS, Youssef E, Żak MM, Chepurko E, Sultana N, et al. Altering sphingolipid metabolism attenuates cell death and inflammatory response after myocardial infarction. Circulation. 2020;141(11):916-30.
436. Abbès S, Ben Salah-Abbes J, Jebali R, Younes RB, Oueslati R. Interaction of aflatoxin B1 and fumonisin B1 in mice causes immunotoxicity and oxidative stress: Possible protective role using lactic acid bacteria. Journal of Immunotoxicology. 2016;13(1):46-54.
437. Dassi M, Souto NS, Braga AC, Freitas ML, Vasconcelos C, Oliveira MS, et al. Effects of repeated fumonisin B1 exposure on markers of oxidative stress in liver, kidneys, and lungs of C57BL/6 mice. Journal of Environmental Science and Health, Part B. 2018;53(12):840-5.
438. Kleynerman A, Rybova J, Faber ML, McKillop WM, Levade T, Medin JA. Acid Ceramidase Deficiency: Bridging Gaps between Clinical Presentation, Mouse Models, and Future Therapeutic Interventions. Biomolecules. 2023;13(2):274.
439. Li C-M, Park J-H, Simonaro CM, He X, Gordon RE, Friedman A-H, et al. Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. Genomics. 2002;79(2):218-24.
440. Li C-M, Park J-H, Simonaro CM, He X, Gordon RE, Friedman A-H, et al. Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. Genomics. 2002;79(2):218-24.
441. Eliyahu E, Shtraizent N, Shalgi R, Schuchman EH. Construction of conditional acid ceramidase knockout mice and in vivo effects on oocyte development and fertility. Cellular Physiology and Biochemistry. 2012;30(3):735-48.
442. Yu A, Cable C, Sharma S, Shihan MH, Mattis AN, Mileva I, et al. Targeting acid ceramidase ameliorates fibrosis in mouse models of non-alcoholic steatohepatitis. Frontiers in Medicine. 2022;9:881848.
443. Yuan X, Bhat OM, Lohner H, Zhang Y, Li P-L. Endothelial acid ceramidase in exosome-mediated release of NLRP3 inflammasome products during hyperglycemia: Evidence from endothelium-specific deletion of Asah1 gene. Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids. 2019;1864(12):158532.
444. Payne S, De Val S, Neal A. Endothelial-Specific Cre Mouse Models. Arteriosclerosis, Thrombosis, and Vascular Biology. 2018;38(11):2550-61.