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AMERICAN UNIVERSITY OF BEIRUT

SMPDL3B MEDIATES RADIATION-INDUCED HUMAN GLOMERULAR ENDOTHELIAL CELL INJURY

by
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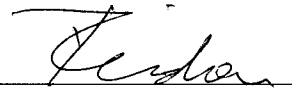at the American University of Beirut

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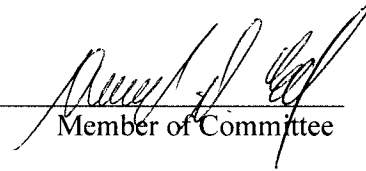## SMPDL3B MEDIATES RADIATION- INDUCED HUMAN GLOMERULAR ENDOTHELIAL CELL INJURY
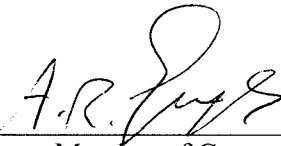
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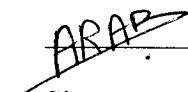# THESIS, DISSERTATION, PROJECT RELEASE FORM

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AN ABSTRACT OF THE THESIS OF

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Major: Physiology

Title: SMPDL3b Mediates Radiation-Induced Human Glomerular Endothelial Cell Injury

Background: Radiotherapy has been a milestone in the field of oncology, though, for many tumors, the cure comes at a price. Innocent tissue bystanders get their share of the radiation, the latter leading to cell death and compromising many bodily functions. One of the major concerns in treating abdominal and spine cancers is the collateral damage that the kidneys suffer. Renal failure can be a major cause of death in such patients even when cancer subsides. Our group has been long investigating the alterations in podocyte signaling pathways in radiation-induced renal damage as well as in other podocytopathies, with particular focus on lipidomics. A major player, sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b) has been identified in the chain of events leads to podocyte dysfunction upon exposure to radiation. Changes at the level of protein expression of this newly identified enzyme are linked to alterations in cellular sphingolipidomic profile and hence cellular function and survival. However, since podocytes are not the only determinants of the integrity of the glomerular filtration barrier (GFB) and kidney function, our project extends to explore other cell lines.

Aims: We aimed to establish a radiation dose-response curve of a relatively new cell line, referred to as conditionally immortalized human glomerular endothelial cells. We also aimed to reveal the effect of radiation on these cells by unraveling the signaling pathway that links proteomics, namely NADPH oxidases and oxidative stress, to the alterations in the sphingolipidomic profile and the subsequent effect of this on cell survival. Similar to our previous work on human podocytes, SMPDL3b will be at the center of our focus.

Materials and Methods: Human glomerular endothelial cells were be cultured as required. To establish the dose-response curve, a colony-forming unit assay will be used. The cells were irradiated at 4Gy and sent to mass spectrometry for analysis of the sphingolipid profile. The levels of expression of NOX1 and SMPDL3b post-radiation were studied as well as the change in oxidative stress during the course of 24 hours. The cells were treated with a GKT, a NOX inhibitor, with siRNA-specific to knockout SMPDL3b and cell survival was assessed. Finally, the levels of expression of SMPDL3b will be re-studied in the absence of NOX activity.

Conclusion: A dose of 8 Gy was enough to render all the plated endothelial cells unviable. Radiation increases NOX1 expression and causes significant oxidative stress. It also increased SMPDL3b levels and subsequently lead to a decrease in ceramide-1-phosphate and an increase in long-chain ceramide levels rendering the cells apoptotic. Cell survival could be restored by knocking out SMPDL3b. Finally, this entire chain of

events could be stopped by using GKT, a NOX inhibitor, which not only reduced oxidative stress but also diminished radiation-induced SMPDL3b induction.

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ABBERIVIATIONS

C1P ceramide-1-phosphate

CERK: ceramide kinase

CerS: ceramide synthase

DKD: diabetic kidney disease

FSGS: focal segmental glomerulosclerosis

CiGEnC: Conditionally immortalized glomerular endothelial cells

GFB: Glomerular filtration barrier

GFR: Glomerular filtration rate

NOX: NADPH oxidase

OS: Oxidative stress

ROS: Reactive oxygen species

RT: Radiation therapy

S1P: Sphingosine-1-phosphate

S1PR: Sphingosine-1-phosphate receptor

SM: Sphingomyelinase

SMase sphingomyelinase

SMPDL3b: Sphingomyelin-phosphodiesterase acid-like 3b

SphK: Sphingosine kinase

SPT: Serine-palmitoyl transferase

TBI: total body irradiation

CHAPTER I

INTRODUCTION

A. Introducing Sphingolipids

1. The sphingolipid Metabolic Pathway

Studies addressing bioactive lipids have extensively evolved in the past decade, revealing an unexpected role of the lipidome in cell biology which rivals that of the genome and proteome (Y. A. Hannun & Obeid, 2018). Bioactive lipids started gaining attention since the identification of the inositol phospholipids in the 1950s and continued to attract scientists in the field, with the identification of the signaling molecule diacylglycerol (DAG) in the 1980s (Y. A. Hannun & Obeid, 2008). Bioactive lipids, by definition, are lipid species that change levels acutely and/or chronically in response to stimuli, usually in time and/or dose-dependent manner. In addition to their structural roles in biological membranes and as energy storage molecules, lipids are now considered to serve as second messengers (Y. A. Hannun & Obeid, 2018). Rather than being mere spectators, it is currently well-established that lipids regulate the accommodation, fluidity and subdomain structures of such membranes, hence controlling countless aspects of cellular physiology. It suffices to say that the function and state of activation of many membrane-bound and associated proteins including receptors are highly influenced by the lipid composition of the membrane, the latter being dependent on a variety of intracellular and extracellular conditions.

First introduced to the literature in the late 19th century by Thudicum, after their isolation from rat brain extracts (Yamakawa, 1996), sphingolipids have gained increasing attention, and many modern biotechnological techniques have been

developed to gain an insight into their metabolism and mechanism of action. It is through these techniques that the structure of sphingosine has been elucidated. Sphingosine is the building block of sphingolipids that allows their distinction from all other lipid subtypes. Recent literature reveals that sphingolipids are a major class of bioactive lipids, serve key roles in cell signaling, and are important modulators of cellular interactions thus establishing a link between the sphingolipid metabolic pathways and a variety of physiological and pathophysiological conditions, called sphingolipidoses. Although the metabolic pathway of sphingolipids has been established and its compartmentalization has been laid out, with more than 40 enzymes involved in mammalian cells, researchers and clinicians still face numerous uncertainties in this field. To address these ambiguities, it is inevitable to define the biochemical and molecular interactions between sphingolipids and other cellular macromolecules like proteins. The implications of such interactions in safeguarding or disrupting cellular homeostasis are yet to be elucidated (Y. A. Hannun & Obeid, 2018). In addition, our schematic understanding of the various enzymes involved in the turn-over of these lipid species is frequently challenged by the identification of new players, one of which is the sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b) (Gorelik, Illes, Heinz, Superti-Furga, & Nagar, 2016).

Sphingolipid metabolism can be thought of as an interconnected network of biochemical reactions that starts from one point, diverges through synthesis and then converges again at breakdown into another common point. Thus, sphingolipids share the same catabolic and anabolic biochemical pathways. The most important bioactive sphingolipids that are known to be involved in cellular growth, senescence, apoptosis, migration, and differentiation among other cellular processes are sphingosine,

sphingosine-1-phosphate (S1P), ceramide and ceramide-1-phosphate (C1P) (Y. A. Hannun & Obeid, 2018). Ceramide and its phosphorylated form, C1P, are the most relevant signaling sphingolipids in the context of our work.

The synthetic process of sphingolipids starts in the endoplasmic reticulum from non-sphingolipid precursors to form 18-carbon amino-alcohol backbones. Modification of these backbones, namely sphingosine, dihydrosphingosine, and phytosphingosine by the addition of other organic groups is what adds layers to the diversity of the family of sphingolipids, which currently comprises more than a thousand naturally occurring molecules.

At baseline or upon the exposure of the cell to a stressor such as hypoxia, radiation, heat or chemical treatment, the sphingolipid metabolic pathway is triggered and ceramide, the simplest of all sphingolipids, is synthesized through one of three distinct pathways, one of which is *de novo* synthesis. The latter happens at the cytosolic surface of the intracellular endoplasmic reticulum through the condensation of L-serine and palmitoyl-CoA, catalyzed by serine-palmitoyl transferase (SPT) to produce 3-ketosphinganine. 3-ketosphinganine is further converted through a set of biochemical reactions involving the enzyme ceramide synthase (CerS) and a fatty acyl group into dihydroceramide and eventually ceramide. Ceramide can then be transported to the Golgi apparatus, either via vesicular or non-vesicular transport, where it is further processed depending on the metabolic needs of the cell to yield more complex sphingolipids. The Golgi apparatus is hence considered the site of synthesis of the complex sphingolipids such as glycosphingolipids and sphingomyelins. The newly synthesized lipids are then escorted to the plasma membrane (PM) through well-known mechanisms that are similar Golgi-PM transfer of proteins. CerS also catalyzes the

synthesis of ceramide from the backbone sphingosine through a second pathway, namely the salvage pathway. The latter serves to recycle the fatty acids in the cell. There are six isoforms of the enzyme ceramide synthase, all encoded by the same family of genes, but each of which produces a different ceramide subspecies unique in its fatty acid composition and degree of saturation. Finally, ceramide can be generated through the hydrolysis of sphingomyelins. This reaction is mediated by a family of enzymes called sphingomyelinases, producing phosphocholine as a by-product.

Once ceramide is generated, it can be dealt with by the cell in different ways, in addition to its use in the synthesis of complex sphingolipids. For example, a family of enzymes known as ceramidases breakdown ceramides into sphingosine. There are three known ceramidases, each of which functions at an optimum pH. Alternatively, ceramide can be phosphorylated through ceramide kinase (CERK) to produce ceramide-1-phosphate (C1P), another phosphorylated bioactive sphingolipid with an extensive array of functions. Figure 1 illustrates the sphingolipid metabolic pathway. Despite the intensive ongoing research on C1P, neither a C1P lyase nor C1P membrane-bound receptors have been identified yet.

Lipids are macromolecules that are not as readily eliminated by the cells owing to their hydrophobic nature. Hence, they tend to accumulate in the intracellular space if they are not metabolized. It is not surprising therefore that for every new complex sphingolipid that the cell synthesizes there is an opposing enzyme that can catabolize it into a simpler form that can be more readily metabolized and eliminated. Many lysosomal hydrolases cooperate in a coordinated fashion to breakdown complex sphingolipids. The absence of one enzyme dictates the accumulation of its substrate which often leads to a pathological condition. Indeed, defects in sphingolipid

catabolizing enzymes stand behind many of the identified lipid storage diseases such as Fabry's and Tay Sachs diseases.

Just like all complex sphingolipids start from simple precursors during anabolism, all end up with ceramide after catabolism. The only route out of the sphingolipid metabolic pathway is the deacetylation of ceramide into sphingosine by ceramidases. The latter can be lysed by S1P lyase in the ER into hexadecenal and phosphoethanolamine, thus marking the only known "exit" out of the sphingolipid metabolic cycle. The sphingolipid pathway has been extensively explored in a set of well-written reviews from which the discussed information was obtained (Abou Daher et al., 2017; Gault, Obeid, & Hannun, 2010; Y. A. Hannun & Obeid, 2008, 2011; Y. H. Zeidan & Hannun, 2007) and the mentioned fatty aldehyde products of sphingolipid clearance are sequentially metabolized by a set of newly identified enzymes (Wakashima, Abe, & Kihara, 2014).

It is important to note that several recent findings have increased the scope of our understanding of the sphingolipid metabolic pathways. Studies on hereditary sensory and autonomic neuropathy (HSAN1) have revealed that SPT has substrates other than serine such as alanine and glycine, albeit with less specificity, leading to the production of low levels of non-canonical by-products including deoxysphingoid and the 1-deoxymethylsphingoids (Murphy et al., 2013). Additionally, recently identified subunits of SPT have shown a remarkable specificity to fatty acids other than palmitate such as myristate and stearate. Hence, there has been increasing traction to replace the nomenclature of SPT by SAG/PMST to refer to its ability to use alanine, glycine, stearate, and myristate as substrates other than the major ones, serine and palmitate. Whether the generated non-canonical metabolites have physiological effects is still

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unknown to date, however, they have been implicated in neurotoxicity observed in HSAN1 patients (Bode et al., 2016; Harmon et al., 2013; Hornemann et al., 2009).

Besides, other studies have recently identified an unsuspected enzyme, named sphingomyelin synthase-related (SMSr). Instead of utilizing phosphocholine to generate sphingomyelin from ceramide, the latter utilizes phosphoethanolamine as a donor of ethanolamine phosphate and rather generates another sphingolipid called ceramide ethanolamine phosphate (Cabukusta et al., 2017).

Because of their involvement in many important cellular processes, there have been monumental efforts to translate the basic research conducted on sphingolipids into clinical applications. Sphingolipids are being increasingly implicated as key players in the pathogenesis of a wide range of human diseases including cancer, inflammation, insulin resistance and diabetic complications, and a variety of neurological and metabolic disorders. Unravelling the sphingolipidomic universe and developing small molecules that can interfere with sphingolipid metabolism have been milestones in understanding and treating inflammatory conditions, malignancies and autoimmune diseases (Y. H. Zeidan & Hannun, 2007). Indeed, the sphingosine analogue FTY 720, also known as Fingolimod, has been recently approved by the FDA under the tradename Gilenya® as the first oral treatment for adult and pediatric patients with relapsed multiple sclerosis (MS) in the United States (Strader, Pearce, & Oberlies, 2011).

2. Sphingolipids in Cell Signaling

It has become clear that sphingolipids are involved in major aspects of cell biology. Numerous lines of evidence implicate them in cellular growth, differentiation, adhesion and migration, cell cycle regulation, apoptosis, necrosis, autophagy, nutrient uptake and

metabolism, in addition to responses to stressful stimuli such as inflammation (Chaurasia & Summers, 2015; Galadari, Rahman, Pallichankandy, & Thayyullathil, 2015; Y. A. Hannun & Obeid, 2008; Spiegel & Milstien, 2011). Our comprehensive understanding of sphingolipids has been propelled by the use of advanced molecular technologies such as gene silencing, CRISPR-mediated knock-out of the enzymes involved in sphingolipid metabolism and through the use of model organisms (Y. A. Hannun & Obeid, 2018).

Our basic insight into the functions of sphingolipids comes from yeast studies in *Saccharomyces cerevisiae*. An increase in the temperature of the surrounding of yeast cells increases the flux into the *de novo* synthesis pathway and results in the generation of phosphorylated and non-phosphorylated sphingoid bases. Ceramides are the main response elements generated and they regulate nutrient permeability, arrest mRNA sequestration and translation and transiently halt the cell division. Ceramides have also been implicated in the iron transport, the acid sensitivity, and the regulation of cell cycle checkpoints in yeast cells. Furthermore, ceramides are involved in the responses of a yeast cell to DNA damage and to the accumulation of reactive oxygen species (ROS) (Chauhan, Visram, Cristobal-Sarramian, Sarkleti, & Kohlwein, 2015; Epstein & Riezman, 2013; Matmati et al., 2013; Montefusco, Matmati, & Hannun, 2014; Teixeira & Costa, 2016).

Recent data underscore the critical role of bioactive lipids in cellular adhesion, migration, and invasion. Sphingosine kinases 1 & 2 (Sk1 and Sk2), enzymes that generate sphingosine-1-phosphate, have been specifically implicated in the formation of filopodia in response to certain stimuli. This happens in an S1PR2-mediated mechanism that involves the phosphorylation of ezrin, a protein involved in cellular motility.

S1PR2 is G-protein coupled receptor (GPCR) and is a member of the S1P receptors family (S1PRs), a set of five cell surface receptors through which S1P exerts most of its known physiological effects on the cells (Adada et al., 2015; Bretscher, Edwards, & Fehon, 2002).

In addition, sphingolipids affect membrane dynamics. The literature supports the presence of specific domains in the cellular plasma membranes, including rafts, that are enriched with sphingolipids, with a remarkable abundancy of ceramides. Although the nature and importance of such domains have not been elucidated yet, there is evidence that they regulate membrane receptor functions and hence influence downstream signaling pathways that are involved in the response of the cells to endocrine, paracrine and autocrine stimuli. There is also evidence that supports the role of ceramides in contributing to and potentially enhancing the formation of such domains (Carreira, Ventura, Varela, & Silva, 2015).

A landmark study has suggested the involvement of sphingolipids in exocytosis. The study conducted by Trajkovic et al. (2008) supports the role of the plasma membrane neutral sphingomyelinase enzyme (nSMase) in the formation and secretion of exosomes (Trajkovic et al., 2008). Exosomes are a subtype of small secretory vesicles implicated in cell-cell communication and present possible therapeutic targets in many clinical settings on neural development and degeneration (Guo, Bellingham, & Hill, 2015; Kosaka et al., 2010). In accordance with these findings, another study conducted by Yuyama et al (2012) proved that inhibition of sphingomyelin formation, through the inhibition of SMS2, also promotes exosome secretion and may help neurons get rid of the toxic β-amyloid proteins, hence implicating sphingolipids in Alzheimer's disease (Yuyama, Sun, Mitsutake, & Igarashi, 2012). SMS2 also appears to play a role

in the recognition and interaction between the HIV viral particle and the host cell plasma membrane and the enveloping of the virus (Hayashi et al., 2014).

The role of sphingolipids in membrane traffic was further expanded by studies that implicated Sk1 and the generated S1P in the formation of recycling endosomes, in an S1PR-independent mechanism. Knockdown of *SK1* in such studies lead to defective endocytosis (Shen et al., 2014). It was also demonstrated that specific species of sphingomyelins, such as C_{18} sphingomyelins, interact with an integral component of the coat protein COPI and play a crucial role in vesicular trafficking (Contreras et al., 2012).

3. Ceramide and C1P in Cell Damage, Survival & Proliferation

Ceramide is generated from sphingoid bases by the action of a family of six ceramide synthases (CerSs 1-6), each of which has its fatty-acid specificity and hence generates a different endogenous ceramide; So rather than being a single lipid moiety, ceramides constitute a set of related molecules with distinct emerging functions (Y. A. Hannun & Obeid, 2018). In addition to the variety observed in sphingoid bases due to the incomplete specificity of SPT (Bode et al., 2016; Harmon et al., 2013; Hornemann et al., 2009), the variation in ceramides is further compounded by the variety of acyl groups that can serve as substrates for CerSs. While CerSs have been described to be mainly localized to the ER, current research hints at their presence in the mitochondria and nuclear membrane (Cingolani, Futerman, & Casas, 2016). The action of a family of elongases, hydroxylases, and desaturases that can either act on the sphingoid bases or the on fatty acyl groups further magnifies the complexity of ceramides by generating

different versions of the same molecule (Wakashima et al., 2014; Wegner, Schiffmann, Parnham, Geisslinger, & Grosch, 2016).

Unlike other sphingolipids, ceramides are neutral and hence do not require special transporters. They can reside in their site of generation and still be involved in many known functional changes by readily flipping across membrane leaflets (Y. A. Hannun & Obeid, 2008).

Ceramides are activated by a wide array of stressors such as genotoxic damage, heat shock, and oxidative stress. They are involved in anti-proliferative cellular processes including necrosis, apoptosis, and senescence, through activating or suppressing key effector molecules implicated in cellular division and survival (Galadari et al., 2015). For example, activation of CerSs 5 & 6 and the consequent accumulation of C_{16}-ceramide species have been implicated in the cytotoxic effects of ionizing-radiation (Mesicek et al., 2010). Indeed, UV-irradiation was shown to induce apoptosis in MCF-7 cells, a cell line of breast cancer, through the generation and accumulation of long-chain ceramide species including C_{16}-ceramides (Y. H. Zeidan, Wu, Jenkins, Obeid, & Hannun, 2008).

It is thought that ceramides exert their effects through the regulation of the cellular phosphoproteome via ceramide-activated serine/threonine protein phosphatases such as PP2A and PP1. Sit4, the PP2A analog in yeast cells, has been shown to play a remarkable role in the regulation of the cell cycle and mitochondrial functions (Teixeira & Costa, 2016). CAPPs bind to ceramides (Chalfant, Szulc, Roddy, Bielawska, & Hannun, 2004) and lead to the sequential regulation of other substrates such as the serine/threonine protein kinase B (AKT), protein kinase C and ezrin (Galadari et al.,

2015). There are different pools of ceramides in the cell, which tend to be organelle-specific. Compelling evidence implicates most of the known ceramide species in the induction of the apoptotic pathways, although there is a lack in the knowledge of the particular functions of specific ceramides species. Lysosomal ceramides, for example, activate cathepsin B and trigger the degradation of the X-linked inhibitor of apoptosis (Taniguchi et al., 2015). The mitochondrial ceramide pool has an independent role in the apoptotic pathway (Birbes et al., 2005); channeling ceramides into the mitochondrion was shown to be sufficient to induce apoptosis (Jain, Beutel, Ebell, Korneev, & Holthuis, 2017).

Ceramide-1-phosphate (C1P) was first described more than 2 decades ago in HL-60 human leukemia cells, where a group of researchers reported that sphingomyelin-derived ceramide can be phosphorylated by an intracellular enzyme functionally and physically different from diacylglycerol kinases (Kolesnick & Hemer, 1990). CERK is known to be different than but a close relative to sphingosine kinases (Sugiura et al., 2002), and its cloning has helped in unravelling many of the physiological functions of C1P. To date, CERK is the only known enzyme to produce C1P and the plasma concentrations of the latter can vary up to 20 µM (Boath et al., 2008), being mainly released from macrophages and damaged cells (Boath et al., 2008; C. Kim et al., 2013).

Numerous studies in the literature indicate that C1P is a major regulator of cell survival pathways. Gomez and co-workers have deciphered, through a series of experiments, the role of C1P in the survival of bone marrow-derived macrophages (BMDMs) under stress conditions, such as withdrawal of macrophage-colony stimulating factor (M-CSF). Although the withdrawal of M-CSF causes the death of macrophages, the addition of C1P was able to rescue these cells through the activation

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of the PI3K/AKT pathway and the downstream transcription factor NFκB, which leads to the production of anti-apoptotic proteins (Gómez-Muñoz et al., 2005). In earlier experiments as well, C1P has been shown to rescue these same cells through the inhibition of the acid-sphingomyelinase (ASMase) and the subsequent decrease in the production of pro-apoptotic ceramides (Gómez-Muñoz, Kong, Salh, & Steinbrecher, 2004).

In addition, studies that date back to 1995 and onwards have implicated the role of C1P in cellular proliferation in different cell types, starting with rat fibroblasts and moving on to different cancer cell lines such as A549 human lung adenocarcinoma, MCF-7 breast cancer, and mice leukemia cell lines (Gomez-Muñoz, 2018). The role of C1P in cellular proliferation was extensively studied by Gangoiti et al. in BMDMs. The mechanisms through which C1P stimulates cell proliferation involves the activation of a variety of signaling transduction including the upstream mitogen-activated kinase (MEK) and its downstream target, the extracellularly regulated kinases 1 & 2 (ERK1 & 2). AKT targets glycogen synthase kinase 3-beta (GSK 3β) and the downstream cyclin-D, c-Myc and c-Jun N-terminal kinases (JNKs) are also involved (Gangoiti et al., 2008). Subsequent studies by the same group demonstrated that the effect of C1P on BMDM proliferation is also mediated by the activation of the protein kinase C alpha (PKCα) and the phosphorylation and activation of the mTORC1 / P70S6K /RhoA/ ROCK pathway (Gangoiti, Granado, Arana, Ouro, & Gomez-Muñoz, 2010; Gangoiti et al., 2008). The production of a small number of reactive oxygen species (ROS) has been paradoxically implicated in the proliferative effects of C1P (Arana et al., 2012). In addition, a 2018 study conducted by Bernacchioni et al. revealed that the action of C1P on AKT and ERK1/2 is partially due to its activation of the lysophosphatidic acid

(LPA) signaling axis, including to the activation of PLA2, of major LPA G-protein coupled receptors (LPA$_1$ and LPA$_3$), and their downstream targets hence resulting in DNA synthesis and cellular regeneration (Chihwa Kim et al., 2013).

B. Sphingolipids in Health and Disease

Recent research implicates sphingolipids in a variety of disease states including inflammation, cancer, insulin resistance, diabetes and its complications, among many other conditions (Abou Daher et al., 2017; Y. A. Hannun & Obeid, 2018).

1. *Sphingolipids in Inflammation*

A strong case for the bioactive sphingolipid C1P has been established in inflammatory conditions. Inflammation is the body's natural response to foreign agents and allergens and is a mechanism by which the body tries to protect itself from invasion by potentially harmful organisms. On one hand, C1P was shown to mediate the translocation and activation of the cytosolic phospholipase A2 (cPLA2) in response to inflammatory stimuli such as the cytokine interleukin-1β (IL-1β) thus leading to the mobilization of arachidonic acid and the subsequent production of eicosanoids (Pettus, Bielawska, Spiegel, et al., 2003; Pettus, Bielawska, Subramanian, et al., 2003). On the other hand, C1P was shown to possess an anti-inflammatory function. In later experiments conducted by Hankins et al (2011), exogenous administration of C1P lead to a dose-dependent inhibition of LPS-mediated NFκB transcription and a consequent inhibition of LPS-mediated cytokine release by HEK293 cells and human peripheral mononuclear blood cells (Jody L Hankins, Todd E Fox, Brian M Barth, Kellee A Unrath, & Mark Kester, 2011).

S1P is another bioactive sphingolipid that has been extensively implicated in inflammation. It has been established that S1P acts in concert with C1P to regulate the mammalian inflammatory response. While C1P regulates the rate-limiting enzyme in the synthesis of eicosanoids, cPLA2 (Clark, Schievella, Nalefski, & Lin, 1995), S1P has been shown to regulate a later step through the enzyme cyclooxygenase-2 (COX2). COX2 utilizes the arachidonic acid that has been mobilized by cPLA2 to generate prostaglandins (Scott, Bryant, & Bidgood, 1999). The research on this pathway has extended recently to reveal that the sphingosine substrate used by SK to generate the S1P required for PGE2 production is generated by the hydrolysis of ceramide by acid ceramidase. This further implicates the intricate sphingolipid pathway in inflammatory reactions (Youssef H Zeidan et al., 2006).

Adaptive immunity depends on the trafficking of B and T lymphocytes between secondary lymphoid organs to keep antigens in check. This mechanism is highly dependent on the gradient of S1P acting through S1P1 receptors. Mice whose hematopoietic stem cells lack S1P1 receptors have revealed that S1PR1 and its substrate S1P are important not only in the recirculation and homing of lymphocytes but also in the development of T lymphocytes and the response of both B and T lymphocytes to chemotactic agents (Goetzl & Rosen, 2004; Matloubian et al., 2004).

S1P is highly involved in the cellular arm of the inflammatory reaction. In addition to what has been discussed earlier, neutrophil migration, extravasation, and priming rely on the generation of S1P (Ibrahim, Pang, & Melendez, 2004; MacKinnon et al., 2002). Also, mast cell degranulation, a critical step involved in allergic reactions, is dependent on the generation of C1P and S1P downstream of the interaction between IgE and its high-affinity receptor FcεR (Mitsutake et al., 2004; Prieschl, Csonga, Novotny, Kikuchi,

& Baumruker, 1999). Finally, the last example about the role of sphingolipids in immune reactions is the importance of C1P in macrophage survival and cytokine production (Gómez-Muñoz et al., 2004; J Turinsky, David M O'Sullivan, & Brian P Bayly, 1990).

2. *Sphingolipids in Cancer:*

An accumulating body of evidence suggests a critical role of ceramide and S1P-mediated signaling in tumorigenesis and chemotherapy response of breast, prostate and colon cancers. Tumorigenesis is the process through which the cell acquires numerous genetic aberrations that eventually lead to dysplastic morphology and unregulated growth (Furuya, Shimizu, & Kawamori, 2011).

The first study to point out a relationship between colon cancer and sphingolipids was conducted by Dudeja et al. in 1986. In this study, elevated levels of sphingomyelins were reported in the colonic mucosa of animals treated with 1,2 -dimethylhydrazine (DMH), an agent that is known to induce colon cancer (Dudeja, Dahiya, & Brasitus, 1986). In later studies, the oral administration of SM, ceramide analogs, or purified GSLs into different mice models of colon cancer revealed that these sphingolipids play a role in the malignancy and incidence of several types of colon cancer. Upon analysis of colon tumors, these effects were found to be achieved through mediating the expression of genes like E-cadherin, connexin 43, Beclin-2, and β-catenin (Lemonnier et al., 2003; Schmelz, Bushnev, Dillehay, Liotta, & Merrill Jr, 1997; Schmelz et al., 1999; Schmelz et al., 1996; Schmelz et al., 2001; Schmelz, Sullards, Dillehay, & Merrill Jr, 2000; Symolon, Schmelz, Dillehay, & Merrill Jr, 2004). Peroxisome proliferator-activated receptor γ (PPARγ) and post-transcriptional and translational modifications of

the gene expression profile of the cells involved are also thought to participate in the observed effect of the dietary-supplemented sphingolipids on the development and progression of induced colon carcinogenesis (Mazzei et al., 2011). Moreover, S1P and its synthetic enzymes and receptors (S1PR$_{1-5}$) have been implicated in colon cancer due to their mitogenic and angiogenic roles. This has been studied in detail with sphingosine kinase 1 (SphK1) due to the presence of SphK1 KO mice (Kawamori et al., 2009; Snider et al., 2009) and with sphingosine kinase 2 (SphK2) due to the presence of the SphK2-specific inhibitor ABC294649 (Kohno et al., 2006; Lynn W Maines et al., 2008), which has led to the new concept of using S1P antibodies as potential therapies (Allende & Proia, 2002; Hla, 2003; M.-J. Lee et al., 1999; Visentin et al., 2006).

Sphingolipids have also been implicated in drug resistance to breast cancer. The glucosylceramide content of tumors from chemotherapy-resistant breast cancer patients was significantly higher than the glucosylceramide content of breast tumors from chemotherapy-responsive ones. It has been exhibited that, in many cases of breast cancer, there is an overexpression and overactivation of glucosylceramide synthase, which catalyzes the conversion of the accumulated ceramide into glucosylceramide. In its turn, the accumulation of the glucosylceramide mediates the expression of proteins that are involved in multi-drug resistance such as P-glycoprotein and activates *cSrc* and β-catenin signaling (Antoon et al., 2010; Valerie Gouazé et al., 2004; Lavie, Cao, Bursten, Giuliano, & Cabot, 1996; Y.-y. Liu, Han, Giuliano, & CABOT, 2001; Y.-Y. Liu et al., 2004; Lucci et al., 1998). Indeed, the silencing of GCS with siRNA downregulates P-glycoprotein and re-sensitizes breast cancer cells to chemotherapeutic agents (Valérie Gouazé et al., 2005).

In addition, particular species of ceramide, such as the $C_{16:0}$ ceramide, have been associated with the malignancy and the metastatic potential of breast tumors (Antoon et al., 2009; Struckhoff et al., 2004). As a result, an accumulating body of evidence portrays ceramide (Antoon et al., 2010) as well as sphingosine kinase receptors (Goetzl, Dolezalova, Kong, & Zeng, 1999) as potential therapeutic targets in breast cancer, the latter being heavily involved in estrogen-induced tumorigenesis (Nava, Hobson, Murthy, Milstien, & Spiegel, 2002; Wang, Redmond, Watson, Condron, & Bouchier-Hayes, 1999).

Prostate cancer is one of the most lethal malignancies among males. The number of death from prostate cancer is on the rise mainly due to metastatic and/or recurrent malignancies that are chemo- and radioresistant; It is thought that sphingolipids play a critical role in the radiosensitivity of prostate cancer cells; particularly, the lack of ceramide production is implicated in the radio-resistance of the androgen-sensitive prostate cancer adenocarcinoma cell line LNCaP (Chmura et al., 1997). Since TNF-α, like ionizing radiation, triggers the hydrolysis of sphingomyelin (SM) into ceramide (Haimovitz-Friedman et al., 1994), treatment of LNCaP cells with TNF-α alongside irradiation enhanced the apoptosis in these cells, further implicating ceramide production in radio-sensitivity (Kimura, Bowen, Spiegel, & Gelmann, 1999). In addition to increasing the levels of ceramide, this treatment increased the level of sphingosine through activating the hydrolysis of S1P into sphingosine, leading to a remarkable drop in the intracellular levels of S1P. Indeed, treatment of LNCaP cells with exogenous sphingosine or inhibition of sphingosine kinases re-sensitized them to radiotherapy and elevated radiation-induced apoptosis (Nava et al., 2000).

In addition, overexpression of ceramidase, which converts ceramide into sphingosine is found in 60% of primary prostate cancer tissues and is associated with enhanced tumor proliferation and migration, bigger tumor volumes (Mahdy et al., 2009; Norris et al., 2006; Seelan et al., 2000), and resistance of tumors to conventional chemotherapeutic agents and radiotherapy (Mahdy et al., 2009; Saad et al., 2007).

In conclusion, sphingolipids, especially ceramides and S1Ps are major players in tumorigenesis and are target candidates for cancer treatment and prevention.

3. *Sphingolipids in Diabetes and Diabetic Complications:*

Diabetes is a metabolic disorder characterized by insulin resistance in muscle, liver, and adipose tissues. Initial observations that revealed the role of sphingolipids in the development of insulin resistance came from experiments that demonstrated an accumulation of ceramide in insulin-resistant tissues such as the liver and muscle tissues of Zucker obese rats (J. Turinsky, D. M. O'Sullivan, & B. P. Bayly, 1990). This was confirmed in later studies that reported elevation of ceramide levels in muscle biopsies obtained from obese insulin-resistant individuals (Adams et al., 2004). In addition, the adipose tissues of obese ob/ob mice showed increased mRNA levels of acid and neutral sphingomyelinases as well as of serine palmitoyl-transferase (SPT) (Samad, Hester, Yang, Hannun, & Bielawski, 2006).

Studies investigating the role of ceramide in promoting insulin resistance have discussed several ways through which ceramide can negatively affect insulin signaling through Akt and other less established targets in this signaling pathway. Ceramide can lead to the dephosphorylation and deactivation of Akt through PPA2, which can block Akt translocation to the plasma membrane by interacting with its pleckstrin-homology

domain and can also disrupt signaling through IRS1 and the PI3 kinase (Summers, 2006).

In addition, ceramide has been shown to lead to insulin resistance downstream of TNF-α and saturated fatty acids. Saturated fatty acids inhibit insulin signaling through activating the *de novo* synthesis of ceramide, which is why blockade of this pathway by fumonism B1 and myriocin, in C2C12 myotubes, restores proper insulin signaling even after treatment with palmitate (Summers, 2006). In addition to ceramides, the ganglioside GM3 and the enzymes involved in its synthesis such as GCS and GM3 synthase have also been implicated in insulin resistance downstream of TNF-α (Kabayama et al., 2005; Tagami et al., 2002; Yamashita et al., 2003).

Moreover, studies by Unger et al. have shown that the accumulation of ceramide by the *de novo* synthesis pathway is implicated in pancreatic β-cell damage and apoptosis (Kabayama et al., 2005); thus, proving that sphingolipids are involved in the initiating events of T2D such as peripheral insulin resistance and in β-cell failure and the resulting overt hyperglycemia (Ng, Wadham, & Sukocheva, 2017).

In addition to their role in insulin resistance and type 2 diabetes, sphingolipids have also been implicated in the development of type 1 diabetes. Recent evidence points at the importance of circulating sphingolipids as potential biomarkers for type 1 diabetes. Different animal models of type 1 diabetes such as streptozotocin-induced diabetic rats and Ins2Akita diabetic mice showed increased plasma levels of sphingosine-1-phosphate compared to the control group (J. L. Hankins, T. E. Fox, B. M. Barth, K. A. Unrath, & M. Kester, 2011). In fact, many previous studies have hinted at the role of the sphingosine kinase enzyme in improving hepatic insulin signaling in cases of obesity

and diabetes (Fox et al., 2011; S. Y. Lee et al., 2015; Osawa et al., 2011; Tao, Sifuentes, & Holland, 2014). Indeed, the mentioned animal models of type 1 diabetes showed decreased plasma and tissue levels of omega-9 esterified neuro- and cardioprotective sphingolipids such as omega-9 24:1 (nervonic acid) ceramide, sphingomyelin, and cerebrosides. It is worth mentioning that a high-fat diet exacerbated this decrease whereas treatment by insulin restored the normal levels of the mentioned sphingolipids (J. L. Hankins et al., 2011).

Based on the above evidence, one can deduce that pharmacological intervention or genetic ablations that manipulate sphingolipid levels by targeting metabolic enzymes might prove as potential therapeutic targets in ameliorating the lipotoxicity associated with obesity, type 2 diabetes and β-cell dysfunction (Holland & Summers, 2008).

In addition to their involvement in the development of different types of diabetes, research implicates the involvement of sphingolipids and their metabolism in the development and progression of diabetic macrovascular (Holland & Summers, 2008) and several microvascular complications including diabetic neuropathy (Abuhusain et al., 2013; Guan et al., 2011; Janes et al., 2014), retinopathy (L. W. Maines, French, Wolpert, Antonetti, & Smith, 2006; Xie et al., 2009) and nephropathy (Lan et al., 2011).

C. Sphingolipids in Renal Pathophysiology

It has become clear over the past decade that sphingolipids play a major role in kidney pathology. The accumulation of sphingolipids and their metabolites in renal tissue is characteristic of many genetic storage diseases that present with clinical nephropathy. Sphingolipids can also accumulate in functional kidney cells, however, eventually leading to kidney disease, even in the absence of a genetic predisposition.

The latter cases have been referred to as "acquired" sphingolipid storage diseases (Merscher & Fornoni, 2014).

Tay-Sachs and Sandhoff are two clinically indistinguishable lipid storage diseases characterized by genetic mutations in the subunits of hexosaminidase, an enzyme in the sphingolipid metabolic pathway, and the subsequent accumulation of its substrate, the ganglioside GM2. This leads to hepatic, nervous and renal dysfunction (Sandhoff, Andreae, & Jatzkewitz, 1968; Tatematsu et al., 1981).

Fabry's disease is characterized by a mutation in the gene encoding the enzyme alpha-galactosidase A (GLA), leading to the accumulation of globotriaosylceramide (Gb3) in many affected body tissues and fluids, mainly in the heart, kidneys and the central nervous system (Nance et al., 2006). In renal tissue, Gb3 accumulation occurs intracellularly in the endoplasmic reticulum, lysosomes and nuclei of kidney cells, leading to podocyte hypertrophy and acquisition of a characteristic foamy appearance, in addition to the formation of zebra-body inclusions resulting from the accumulated glycolipids, and mesangial matrix widening and foot processes effacement (Alroy, Sabnis, & Kopp, 2002; Askari et al., 2007). These microscopic observations have been directly associated with renal injury and proteinuria in patients with Fabry's disease (Najafian et al., 2011; Thurberg et al., 2002). Enzyme replacement therapy using recombinant human alpha-galactosidase A has proven to be efficient in halting disease progression and preventing renal insults that lead to kidney failure (Quinta et al., 2014; Thurberg et al., 2002).

Niemann-Pick is another genetic disorder of recessive autosomal inheritance pattern that is characterized by mutations in several genes, including the one coding for the

phosphodiesterase enzyme-1. These mutations lead to the accumulation of cholesterol and sphingomyelin in body tissues. Microscopic observations in SMPD1-KO mice reveal the presence of lipid-laden macrophages with a foamy appearance in the affected organs, such as the bone marrow, liver, and kidneys, a sign indicative of excessive inflammation (Briere et al., 1976; Kuemmel, Thiele, Schroeder, & Stoffel, 1997). Enzyme replacement therapy, also known as ERT, using a recombinant acid-sphingomyelinase enzyme proved to be effective in alleviating symptoms of Niemann-Pick disease, albeit at the level of animal models (Miranda et al., 2000).

Research on genetic disorders relating to sphingolipid metabolism and renal insults has initiated further investigations on the role of sphingolipids in non-genetic renal diseases.

Diabetic kidney disease (DKD) is the leading cause of renal failure in the United States; DKD is characterized by podocyte feet processes effacement and eventual podocyte loss (podocytopenia) and is associated with types 1 and 2 of diabetes (Meyer, Bennett, & Nelson, 1999; Pagtalunan et al., 1997; Steffes, Schmidt, McCrery, Basgen, & International Diabetic Nephropathy Study, 2001; Verzola et al., 2007; White et al., 2002). Recently, it has become clear that the levels of sphingolipids such as sphingosine (Gorska, Dobrzyn, & Baranowski, 2005), ceramide (Blachnio-Zabielska et al., 2012; Haus et al., 2009), glycosphingolipids (Kremer, Atzpodien, & Schnellbacher, 1975), and sphinganine (Blachnio-Zabielska et al., 2012; Gorska et al., 2005) are elevated in the plasma of diabetic patients. In addition, the renal diabetic complications have also been lately linked to the intracellular levels of sphingolipids in glomerular cells including the podocytes.

Since then, many studies have been conducted to elucidate the role of sphingolipids in diabetes-associated glomerular hypertrophy, cell proliferation, damage, and dysfunction. Rat models of STZ-induced type 1 diabetes were used and alterations in sphingolipid metabolism were observed as early as the 4th day after the induction of diabetes. Enzymes like neutral ceramidase and sphingosine kinase, responsible for conversion of ceramide into sphingosine and sphingosine-1-phosphate respectively, were increased in renal glomerular tissue and this was associated with the accumulation of the end-product S1P in the rat glomeruli (Geoffroy, Troncy, Wiernsperger, Lagarde, & El Bawab, 2005). In another study, an increase in the intracellular levels of glucosylceramide and GM3 was detected in the kidney cells of STZ injected rats 16 days after the induction of diabetes (Kwak et al., 2003). In addition, increased levels of production and activity of the serine-palmitoyl transferase (SPT) enzyme was detected in tubular epithelial and microvascular endothelial cells of diabetic rats. SPT is involved in the *de novo* synthesis of ceramide and subsequently, its overexpression lead to the accumulation of ceramide in the mentioned cells and the induction of the apoptotic pathway. Treatment with rapamycin, which targets the mTORC1 pathway, was able to reverse cell death and alleviate some of the symptoms of DKD such as proteinuria (G. Liu et al., 2011).

Puromycin aminonucleoside (PAN)-induced nephropathy is an animal model of human minimal change nephropathic disease. PAN injection in rats changes the sphingolipid profile of kidney cells in a dose and time-dependent manner; this is followed by proteinuria, hinting at a causative effect. The main sphingolipids involved are GD3 O-acetylated GD3 (Pawluczyk et al., 2014).

D. Radiation-induced Nephropathy

In a case report published in January 1967, Kaene et al. noted that the pathogenesis of radiation-induced renal injury is still not clear. Unfortunately, despite all the ongoing research, this statement still holds true today. Radiotherapy-induced renal disease was first described more than a century ago by Baerman and Linser and was later reported by many others, always presented with the same characteristic features. The emphasis on the presentations has been placed on vascular lesions. In their clinicopathologic study, Kaene and his colleagues reported their light and electron microscopic observations of renal biopsy specimens from two patients who showed up with renal insufficiency a year after receiving abdominal radiation for treatment of ovarian carcinoma. Their findings on light microscopy highlighted mild endothelial cell swelling and damage to the basement membrane. They further observed, using electron microscopy, sub-endothelial expansion with electron-lucent basement membrane-like material and loss of endothelial cell lining in some capillaries. Damage to the glomerular capillary loops was a consistent ultrastructural abnormality noted in both patients.

In another clinical study conducted in 1971 to demonstrate the long latency of radiotherapy-induced kidney injury, 67 patients without pre-existing hypertension were treated with approximately 20 Gy within 3 weeks, the treatment encompassing the left kidney. Almost 50% of these patients developed kidney toxicity between 8-19 years after radiotherapy. Of these, 5 patients developed fatal uremia and 2 developed malignant hypertension. Upon examining renal biopsies from these patients, atrophy of the left kidney was observed. The degenerative changes were most extensive in the medium and small arteries. This further implicates that the endothelial cells are major players in the sensitivity of the kidneys to radiotherapy.

The kidneys are organs with extremely vital functions. They maintain the body electrolyte/fluid balance and modulate blood pressure, filter waste metabolites into the urine, and produce erythropoietin to stimulate the production of red blood cells. Given their sensitivity to ionizing radiation, they remain to date the dose-limiting organs for using radiotherapy (RT) to treat gastrointestinal and gynecological malignancies and sarcomas and lymphomas of the upper abdomen in addition to total body irradiation (TBI). Although the incidence of RT-induced kidney injury remains underreported due to its long latency, it is divided based on the presentation into either clinical or subclinical with the latter being the acute injury that develops within three months after irradiation. However, the decrease in glomerular filtration rate (GFR) and increase in serum β_2-microglobulin are clinical signs that appear within the subacute period, defined as anywhere between 3 and 18 months after treatment. Chronic injury, usually presenting after 18 months from RT, is characterized by dyspnea, fatigue, nausea and vomiting, edema, malignant hypertension, elevated creatinine levels, and anemia among other detrimental symptoms. Chronic injury tremendously reduces patients' quality of life, requires dialysis and/or kidney transplantation, and often ends with coma and death. It is also noteworthy that RT-induced kidney injury decreases patients' reserves against future kidney insults hence rendering them more susceptible to renal failure.

All that said, radiation oncologists still face many technical problems during treatment in terms of defining the kidneys and calculating the overall dosage each kidney is receiving. Although the kidneys are easy to identify on computed tomography (CT) scan even without an intravenous (IV) contrast, the administration of the treatment using CT planning is still prone to many errors and more often than not the kidneys end up receiving a much higher share of radiation than originally planned or anticipated.

This is because even with modern techniques, such as intensity-modulated radiotherapy (IMRT), image-guided radiotherapy (IGRT) and four-dimensional CT scans, it is still inaccessible to accommodate for all the variables such as individual differences, the motion of the kidney during breathing, and the accompanied shifts in the location of the kidneys and that of the tumor. Finally, even in a hypothetical situation where all the aforementioned errors are either absent or accounted for, the magnitude of the inherent error introduced by including the "collecting system" of the kidney parenchyma in the calculation of the kidney volume can in no means be cleared.

According to the national cancer institute, around 1,735,350 patients are expected to be diagnosed with a malignant tumor in 2019 in the United States alone with an estimated mortality rate of 35%. On the top of the list comes a set of malignancies such as colon, kidney, and renal pelvis cancers and non-Hodgkin's lymphoma. Radiotherapy is a key element in the management of such tumors and is generally used in the treatment of almost half of the patients who are cured of cancer. RT offers a wide variety of advantages compared to or accompanied by other cancer management techniques. For example, some cancers can be treated with RT alone if detected in their early stages such as non-Hodgkin's lymphoma. Also, RT offers a reliable alternative to surgery in patients whose health or tumor conditions do not permit an invasive procedure for tumor resection especially those who suffer from chronic cardiovascular or respiratory diseases or those with tumors that are either unresectable or are otherwise present in close proximity to critical structures such as peripheral nerves or major blood vessels. In fact, RT is often used to improve local control over the tumor while keeping a lower morbidity rate. RT can also be used in combination with surgery in a variety of scenarios. Pre-operative RT is used to shrink the tumor and make it easier to operate on

whereas postoperative RT is used to mitigate the risk of cancer recurrence. In addition, RT can be used in a palliative manner to alleviate the pain of cancer patients with bone or brain metastases, spinal cord compression, or obstruction due to visceral metastases such as airway obstruction.

From what has been stated above, it is very clear that radiotherapy holds great and promising potential for fighting malignancies compared to the other available management techniques. However, there is no doubt that renal radiotoxicity can be fatal. There is a pressing need, now more than ever, to reveal the molecular and biochemical pathways involved in this toxicity to enable the forging of a radioprotective medication that can save the kidneys from the side-effects of the treatment and hence enable the delivery of higher doses of radiation to the tumor while maintaining integrity and function of the renal tissues.

E. Sphingomyelin Phosphodiesterase acid-like 3b (SMPDL3b)

The "lipid-centric" approach, discussed earlier in the introduction, dissects the lipid profile of the cell and allows the identification of the roles of the bioactive lipids. However, the current molecular era focuses mainly on an "enzyme-centric" approach. Scientists study the gene expression, protein and the enzymatic activity levels of the enzymes involved in signal transduction. This has allowed investigation of the mechanism of action and regulation of these proteins, appreciation of their cellular roles, and identification of molecules that can either augment or inhibit their activities, sometimes through modulating their expression levels. Examples of such enzymes are the NADPH oxidases (NOXs) and the newly identified sphingomyelin phosphodiesterase acid-like 3b (SMPDL3b).

However, we believe that through combining the classical "enzyme-centric" approach with the new and evolving "lipid-centric" one, we are capable of understanding the interaction between different cellular proteins and discovering where different sphingolipid species stand in the grand scheme of things (Yusuf A Hannun & Obeid, 2017).

Hence, in order to decipher the secrets of the cellular signaling pathways, it is crucial to connect what we already know about the familiar world of intracellular proteins to what is being discovered about the lipid universe; this can only be achieved by studying the interaction between the lipid-metabolizing enzymes and other cellular signaling proteins such as kinases and NADPH oxidases. This allows obtaining a comprehensive picture that would facilitate tuning into these cellular processes for therapeutic purposes.

1. *SMPDL3b in Renal Pathophysiology*

In the past decade, a group of scientists working from the Peggy and Harold Katz Family Drug Discovery Center at the University of Miami have extensively explored the role of SMPDL3b in glomerular diseases, with particular emphasis on podocytopathy. They have recently shown that the expression of SMPLD3b increases in the glomeruli of diabetic mice, in the glomeruli of patients with DKD and cultured human podocytes treated with sera from diabetic (db/db) animals. Increased levels of SMPDL3b were correlated with elevated RhoA kinase activity and cellular death. Concomitant with the observed increase in apoptosis, the increase in SMPDL3b has prevented cellular migration by blocking the interaction between $\alpha V\beta 3$ integrin and the soluble urokinase plasminogen activator receptor (suPAR); the plasma levels of the

latter are known to be higher in patients with and animal models of DKD compared to controls (Yoo et al., 2015). In addition, increased SMPDL3b levels were correlated with increased levels of sphingosine and S1P and decreased levels of ceramide in the renal tissues of db/db mice. The accumulation of the S1P and sphingosine intracellularly are tightly linked to the induction of apoptosis. This was also found to be the case in the glomerular mesangial and tubular cells of diabetic (db/db) mice (Brunskill & Potter, 2012; Ishizawa et al., 2014) and in the adipocytes of obese (ob/ob) mice (Kitiyakara, Eggers, & Kopp, 2004). It is worth noting that the observed lipid profile supports the sphingomyelinase role of SMPDL3b because it hints at a change in the profile of sphingomyelin metabolism and accumulation of simpler lipids (Merscher & Fornoni, 2014).

Focal segmental glomerulosclerosis (FSGS) is the most common cause of primary glomerular disease in adults, a leading cause of the nephrotic syndrome and a major cause of the end-stage renal disease (Kitiyakara et al., 2004). The research focus on FSGS has been mainly on the primary (idiopathic) form of the disease and recurrent FSGS after kidney transplantation, which happens in almost 40% of the patients (Baum, 2004; Hubsch et al., 2005; Senggutuvan et al., 1990). As in DKD, the research group from the University of Miami was able to reveal a critical role for SMPDL3b in FSGS. In a study conducted by Fornoni et al. (2011) on 41 patients with a high risk of recurrent FGSG, the number of SMPDL3b-positive podocytes post-reperfusion dropped in patients who later developed FSGS. The researchers thus hypothesized, based on the striking similarity between SMPDL3b and the acid-sphingomyelinase, that the accumulation of sphingomyelin might be a contributing factor to the pathogenesis of FSGS. Indeed, human podocytes treated with sera from patients with FSGS showed

decreased expression of SMPDL3b and decreased acid sphingomyelinase activity (ASMase). This was associated with cytoskeletal remodeling and apoptosis of podocytes and was linked to proteinuria and loss of proper kidney functioning. Overexpression of SMPDL3b or treatment with rituximab, a monoclonal antibody that binds to SMPDL3b in podocytes, showed to have a protective role against changes in renal podocyte morphology, such as loss of stress fibers, and against the compromise in kidney functioning. The mentioned evidence further supports the that sphingomyelin and the lipid-metabolizing enzyme, SMPDL3b, are heavily implicated in the pathogenesis of focal segmental glomerulosclerosis (Fornoni et al., 2011).

Along the same line, it was later demonstrated that the treatment of baboons with rituximab after pig-to-baboon xeno-kidney transplantation delayed the early development of proteinuria post-operation, albeit it was not effective in preventing its occurrence. Like in the study of Fornoni et al., this study revealed that treatment with rituximab was also effective against preventing podocyte injury and cell death through preventing the decrease in the levels of SMPDL3b (Tasaki et al., 2014) suggesting again a protective role of SMPDL3b and its associated lipid metabolites against kidney injury as is the case with FSGS.

It is hence clear that modulation of the levels of SMPDL3b mediates kidney injury in both diseases, FSGS and DKD although in different ways.

2. *SMPDL3b in Radiation-Induced Podocytopathy*

A recent study conducted by Ahmad et al. (2016) revealed that SMPDL3b mediates radiation-induced podocytopathy and renal dysfunction. After exposing cultured human podocytes to a radiation dosage of 8 Gy, the levels of several long-chain species of

ceramide increased, while the levels of sphingosine and sphingosine-1-phosphate, as well as the level of expression of SMPDL3b, dropped in a time-dependent manner. This was paralleled by changes in the morphology of the podocytes such as loss of filopodia and remodeling of the cortical actin due to the relocation of the actin-binding protein ezrin into the cytosol early after irradiation. Interestingly, podocytes that overexpress SMPDL3b were protected from radiation-induced cytoskeletal remodeling, which is thought to be a major player in compromising the glomerular function. Besides, treatment of the cultured podocytes with RTX partially prevented the radiation-associated loss of SMPDL3b. This was accompanied by remodeling of the cellular cytoskeleton. Experiments on animal models used in this study revealed the same results, emphasizing that SMPDL3b plays a radioprotective role in podocytes and its loss after irradiation contributes to podocytopathy (Ahmad et al., 2017).

F. NADPH Oxidase Enzymes (NOXs) and Oxidative Stress

Nicotinamide adenine dinucleotide phosphate-oxidases, also known as NADPH oxidases (NOXs), are a family of transmembrane enzymes that catalyze the reduction of oxygen by the addition of an electron to O_2, producing a superoxide anion (Haugen & Nath, 1999). Under normal physiological conditions, these enzymes are part of the intracellular polyol pathway; they reduce glucose into sorbitol to replenish the cellular supplies of glutathione (Bernobich et al., 2004).

Several subunits of NOXs have been identified over time. NOX2 was the first to be discovered (K. Bedard & K. H. Krause, 2007), and later on, an enzyme with striking homology to NOX2 was identified and referred to as NOX1 (A. Banfi et al., 2000; K. Bedard & K. H. Krause, 2007). Shortly afterwards, NOX 3 (Cheng, Cao, Xu, van Meir,

& Lambeth, 2001; Kikuchi, Hikage, Miyashita, & Fukumoto, 2000), NOX 4 (Geiszt, Kopp, Varnai, & Leto, 2000), and NOX 5 (B. Banfi et al., 2001) were respectively added to the family of NADPH oxidases. In addition, two large thyroid oxidases referred to as DUOX 1 and DUOX 2, have been classified by scientists in 1999 as part of the NOX family (De Deken et al., 2000).

The main isoforms of NOXs that can be found in the kidney cortices are NOXs 1, 2 and 4, with the latter being the most abundant (K. Bedard & K. H. Krause, 2007). In addition to their role in the production of ROS, NOXs are being recognized as key players in renal physiology and hence in maintaining the overall homeostatic status of the human body. First, NOXs are involved in the regulation of renal blood flow in multiple ways (Lopez, Salom, Arregui, Valero, & Fenoy, 2003). Also, NOXs alter the renal cellular fate and hence the fate of the entire renal tissue through activation of a wide array of signaling pathways (Rhyu et al., 2005) such as the ERK1/2 pathway (Gorin et al., 2004). Finally, NOX-dependent activation of several transcription factors such as NFκB is tightly involved in the regulation of renal gene expression and hence renal cellular physiology and function (Dorsam et al., 2000).

The main mechanism of action of NOXs is the transfer of electrons across biological membranes, hence producing reactive oxygen species (ROS). Low to moderate levels of ROS are essential for normal cellular functions. In fact, it was shown that ROS is involved in growth factor signaling, in mitogenic responses and in sensing the microenvironmental availability of oxygen (Geiszt et al., 2000). It is important to note that NOXs now thought to be the major source of ROS in the kidneys (K. Bedard & K.-H. Krause, 2007).

The overactivation of NOXs under pathological conditions, such as cellular stress, leads to the overproduction of ROS and overwhelms the inherent intracellular anti-oxidant machinery causing a state of oxidative stress (OS). OS results from a fundamental imbalance between the generated ROS and the ability of the body to rid itself from them, mainly due to the lack of the mechanisms of intracellular transport of ROS (Betteridge, 2000). In this case, ROS can inflict extensive biological damage by peroxidation of lipids, oxidation of proteins, and mutation cleavage of DNA (Niedowicz & Daleke, 2005). In addition, ROS induces a variety of transcription factors leading to irregular cellular proliferation and/or hypertrophy (Studer RK, 1997).

Several studies have revealed that OS leads to vascular dysfunction, which is a major contributing factor in kidney disease such as diabetic nephropathy (Vasavada & Agarwal, 2005). Also, cellular injury by OS targets critical kidney structures such as the glomerulus, interstitium, and renal vascularization. For example, arachidonic acid peroxidation due to the abundance of ROS can lead to renal vasoconstriction, subsequently causing a wide range of pathological symptoms (Montero A, 2000).

Finally, NOX enzymes have been extensively linked to radiotherapy-induced damage of healthy body tissues. The main mechanism of injury is thought to involve damage to stem cells responsible for repopulating the normal tissues as well as damage to the endothelial cells of the microvasculature supplying said tissue. The post-radiotherapy damage is also thought to be progressive and chronic due to accumulating free radicals and reactive oxygen species in the mentioned cells (Kim, Jenrow, & Brown, 2014).

Hence, when studying radiation-induced nephropathy, one cannot eliminate the role of NOXs. Exploring the state of expression and activation of the major NADPH oxidases and the effect of this on the structural and functional states, especially that of the endothelial cells in the tissue under investigation, is at the core of finding the root mechanisms of the radiotoxicity.

G. Aims and Hypothesis of This Study

The nephron is the basic functional unit of the kidney. It is made of two subunits: the glomerulus and the tubules. The glomerulus, forming the glomerular filtration barrier (GFB), is the first and most critical part of urine formation. Challenging the integrity of this barrier leads to end-stage renal disease (ESRD), which is characterized by proteinuria, hematuria and eventual kidney failure. The GFB is comprised of three functional layers. First, a layer of podocytes; These are specialized epithelial cells with voluminous cell bodies in the bowman's capsule and intermingled feet processes that extend into the mesangial matrix-forming tight pores. The second layer is a mesangial matrix; this is essentially a glycoprotein matrix on which the third layer rests. The latter is a layer of endothelial cells that form the capillaries which carry blood into the kidney glomerulus for filtration (Keane, 2000; Yoshikawa, Dube, & Wahby, 1994).

In their study, Ahmad et al. (2016) focused on radiation-induced nephropathy by examining the effect of X-Rays on human podocytes. To complement their work, we focus on our study on another key player in the GFB, the glomerular endothelial cells. These cells are as important functionally and structurally as the podocytes but are less studied due to the difficulty of isolating and handling endothelial cells in culture. It was

not until 2006 that Satchell was able to isolate and immortalize these cells, through *ex-vivo* culturing, by transfecting them with a temperature-sensitive gene by the SVLT40 virus, naming them conditionally immortalized human glomerular endothelial cells (CiGenc) (Singh et al., 2007).

In this study, we aim to establish an X-Ray irradiation dose survival curve of this newly isolated cell line, the CiGenc. We also aim to study the gene induction and protein expression and activity levels of the renal NADPH oxidases 1 & 4 in human glomerular endothelial cells after irradiation, and the effect of this on ROS production. In addition, we aim to study the gene induction and protein expression levels of our enzyme of interest, the sphingomyelin phosphodiesterase acid-like 3b in this same cell line and the effect of this on the lipid profile of the cell. Finally, we attempt to explore if there is any cellular pathway that links NOXs and the resulting oxidative stress to lipid metabolism through SMPDL3b and the effect of such pathway on the CiGenc survival when exposed to radiation.

In this study, we hypothesize that radiation increase the gene expression, protein and activity levels of the NADPH oxidases 1 & 4 as well as the levels of reactive oxygen species in human glomerular endothelial cells in a time-dependent manner. We also hypothesize that concomitant with this, and downstream of NOX signaling, there will be folds change in the gene induction and expression levels of SMPDL3b and that this is associated with a change in the lipid profile of the cell, with particular emphasis on long-chain ceramide species. We believe that this scheme will diminish cell survival and that interfering in this pathway at the level of either NOXs or SMPDL3b or the level of the intracellular lipid profile, to reverse some of the observed changes, will curb the radiation-induced cellular damage and enhance cell survival of the CiGenc cells.
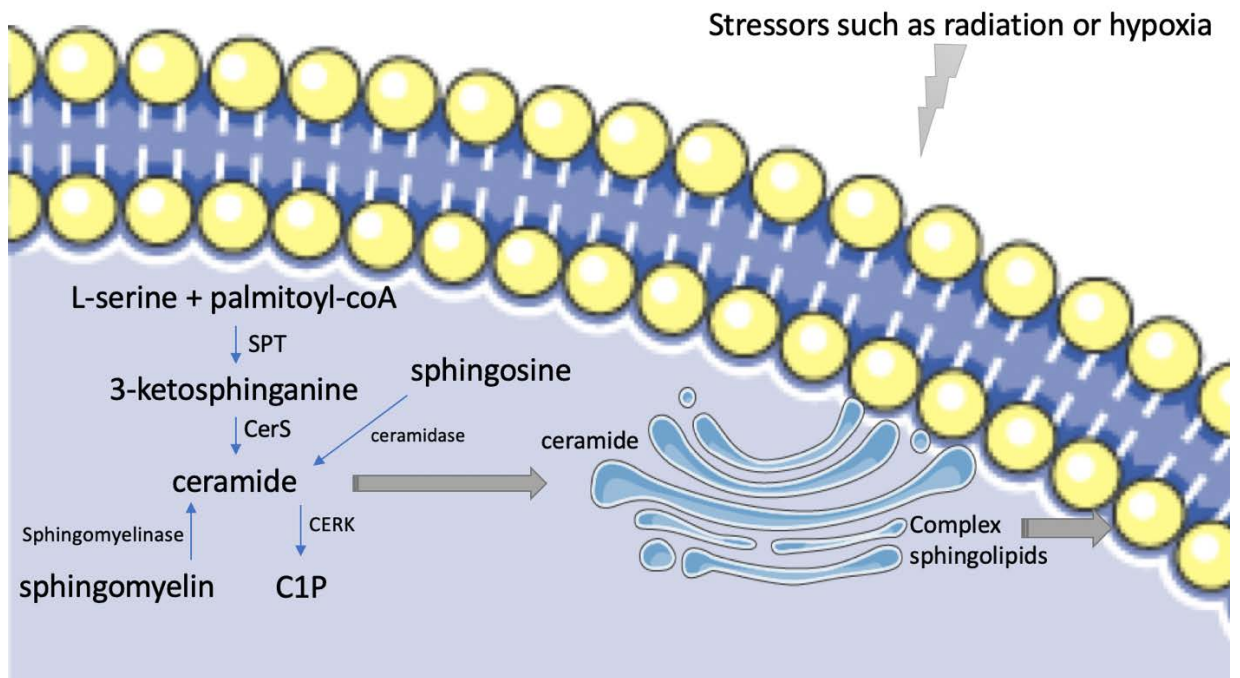
Figure 1. The sphingolipid metabolic pathway. Ceramide is a central molecule in this pathway. It can be synthesized in one of three ways, either *de novo* from L-serine and palmitoyl-coA by the action of SPT, or from sphingosine by the action of ceramidase, or through the degradation of sphingomyelin by the sphingomyelinase enzyme. Ceramide can be converted into more complex sphingolipids in the Golgi apparatus, phosphorylated to produce ceramide-1-phosphate (C1P) or degraded into sphingosine by the action of ceramidases.

Chapter II:

Materials and Methods

A. Materials

EBM 2 and EGM-2 were purchased from Lonza (Basel, Switzerland). PBS, penicillin-streptomycin, bovine serum albumin, RIPA lysis and extraction buffer, and MTT Cell Proliferation Assay kit were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was from Sigma-Aldrich (St. Louis, Missouri, USA). Anti-SMPDL3b was obtained from Genway (San Diego, CA, USA). DAPI (49,6-diamidino-2-phenylindole, dihydro- chloride) and DHE (dihydroethidium) were obtained from Thermo Fischer Scientific. Horseradish peroxidase (HRP)-conjugated anti-rabbit was form Promega (Madison, WI, USA). HRP-conjugated anti-mouse IgG, monoclonal anti-GAPDH antibody, protease, and phosphatase inhibitor cocktails were purchased from Calbiochem (San Diego, CA, USA). RNA extraction mini kit was from Qiagen (Valencia, CA, USA). NOX-1, NOX-4 and SMPDL3b forward and reverse primers were ordered from Basilky. Detergent-compatible protein assay kit, 4-20% SDS-PAGE gels, and 2X laemmli sample buffer were purchased from Bio-Rad (Hercules, CA, USA). Nitrocellulose membranes were purchased from Millipore (Billerica, MA, USA). Ceramide-1-phosphate was purchased from Avanti Polar Lipids (Alabaster, AL, USA). GKT137831was purchased from Cayman Chemical (Ann Arbor, Michigan, USA). FlexiTube SMPDL3b specific siRNA (5 nmol) (FlexiTube GeneSolution GS27293 for SMPDL3B) and Hi-Perfect transfection reagent were purchased from Qiagen (Hilden, Germany).

B. GEnc cell culture, irradiation and treatment

Human glomerular endothelial were cultured and differentiated in EBM-2 and EGM-2 with VEGF containing 2% FBS and 1% penicillin/streptomycin as previously described (Satchell et al., 2006). Briefly, conditionally immortalized endothelial cells were propagated at 33 degrees and then thermoshifted for differentiation for 5 days at 37 degrees. A single dose of irradiation was delivered from an RS2000 X-ray irradiator (225 kV) according to the manufacturer's specifications (Rad Source Technologies, Suwanee, GA, USA). The dose rate was adjusted to 195 cGy/min. For C1P treatment, cells were cultured in 96-well plates, and pretreated for 24 hours with 30 µM C1P. Prior to treatment, the C1P double-distilled water solution was sonicated at 4 degrees in to ensure proper dispersion. Cells where then irradiated at 4Gy treatment was stopped by removing the media and adding cold saline solution at the proper time points. For GKT treatment, cells were directly irradiated after treatment with 20 µM GKT dissolved in DMSO. An equal quantity of DMSO was added to the control samples.

C. Transfection with SMPDL3b-specific siRNA

For the purpose of transfection, GEnC were cultured in 6-well plates and transfected with SMPDL3b-specific siRNA and control siRNA as per the manufacturer's protocol. Briefly, 150 ng of siRNA were diluted in 100 µL of media, and 10 µL of the Hi-Perfect transfection reagent was added. The total amount of media per well was 2.4 mL, and the cells were incubated with the siRNA mixture for 24 hours after complete differentiation. Cells were then radiated, incubated again for 24 hours, and scraped for western blot.

D. Immunofluorescence with DHE and DAPI

For quantification of mean immunofluorescence (MIF), cells were stained with DHE for 30 minutes at 37° C, fixed and stained with DAPI then visualized using Zeiss confocal microscope (LSM710 Meta, Carl Zeiss, Inc., Thornwood, NY, USA). Data were analyzed using the LSM Image Browser Software.

E. Quantitative RT-PCR

Cells were washed with ice-cold PBS and RNA extraction was carried out according to the manufacturer's protocol of the RNA minieasy kit (Qiagen, Hilden, Germany). RNA was quantified by NanoDrop (Thermo Fischer Scientific) and converted to cDNA using a SuperScript III First-Strand Synthesis kit (Thermo Fischer Scientific). The cDNA was then diluted (1:25) and 2 µL were added per 25 µL of reaction. Using the Perfecta SYBR Green FastMix (Quantabio), the reaction was executed in real-time PCR system (Applied Biosystems, Foster City, CA, USA) as previously described (Fornoni et al., 2011). Real-time quantitative PCR was done for Nox1, Nox4 (results not shown) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following primer sequences were used (Table 1):

Primer	Sequence
H-GAPDH	F: 5'-gTCAgTggTggACCTgACCT-3'
	R: 5'-gTCAACggTACATCTggggA-3'
H-NOX1	F: 5'-CACAAgAAAAATCCTTgggTCAA-3'
	R: 5'-gACAgCAgATTgCgACACACA-3'

F. Protein Extraction and Western Blotting

Endothelial cells were homogenized in cold RIPA buffer supplemented with 20 µL protease and phosphatase inhibitor cocktail. Protein quantification was done using Lowry Reagent assay kit from Sigma-Aldrich. Samples were then prepared after quantification with 2X Laemmli sample buffer (Bio-Rad). An equal amount of proteins (25-30 µg) were then loaded into 12.5% SDS-PAGE gels (Bio-Rad) and transferred on nitrocellulose membrane overnight at 300 mA. The membranes were then blocked with 5% skimmed milk in Tris-saline solution for 1 hour at room temperature. The following primary antibodies were used, each according to the protocol suggested by the manufacturer: rabbit polyclonal anti-SMPDL3b (1:1000) (Genway Biotech, Inc., San Diego, CA, USA), mouse-monoclonal anti-GAPDH (1:1000)(Abcam), rabbit monoclonal Nox1 and Nox4 antibodies (1:250)(Abcam). The membranes were incubated with the primary antibodies overnight then washed 3 times for 10 minutes each in Tris-saline solution with 0.1% Tween 20. HRP conjugated secondary antibodies were used and the images were developed using enhanced chemiluminescence (Bio-Rad). Densitometry was performed using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

G. NADPH Oxidase Assay

The activity of the NADPH enzymes was assessed in cultured GEnC as previously described (Eid et al., 2010; Eid et al., 2009). Cultured endothelial cells were washed three times with ice-cold PBS and scraped from the plate. They were then centrifuged at 800g for 10 minutes at 4 degrees. Pellets were obtained and the supernatant was discarded. The pellet was suspended with a special lysis buffer (20 mMKH$_2$PO$_4$ [pH 7.0], 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml

aprotinin, and 0.5 μg/ml leupeptin). The homogenate was quantified using the Bio-Rad protein assay reagent. The assay was conducted on 50 μg of homogenates which were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin, and 100 μM NADPH. Light emission was measured after 30 seconds for 8 minutes in a luminometer. The first and last readings were discarded and a buffer blank was subtracted from each reading. Superoxide production was averaged and expressed as relative light units/min.mg of protein.

H. Liquid Chromatography-Mass Spectrometry Analysis

Cell pellets containing 10^6 cells per sample were subjected to liquid extraction. Liquid chromatography-mass spectrometry (LC-MS) analysis of sphingolipids was performed at the lipidomic core facility at the Medical University of South Carolina using electrospray ionization/tandem mass spectrometry on a mass spectrometer (Quantum; Thermo Fischer Scientific) as previously described (Bielawski, Szulc, Hannun, & Bielawska, 2006).

I. Statistical analysis

Results were expressed as the means ± SE. One-way ANOVA was used to compare groups and results were considered statistically significant if $P < 0.05$ (Graph Pad Prism software; La jolla, CA, USA).

Chapter III:

Results

A. Radiation decreases cell viability in a dose-dependent manner

In order to assess radiation-induced tissue damage on the glomerular endothelial cells, we conducted a clonogenic assay on different GEnC and established for the first time a survival response curve for the cell line under investigation. As anticipated, ionizing radiation induced a dose-dependent decrease in cell viability (Figure 2). The percentage of viable cells was assessed by the ability of each cell to form a detectable colony unit over a period of two weeks post-radiation. Cell viability dropped by 50% at 2 Gy compared to control. No colonies were detectable at 8 Gy.
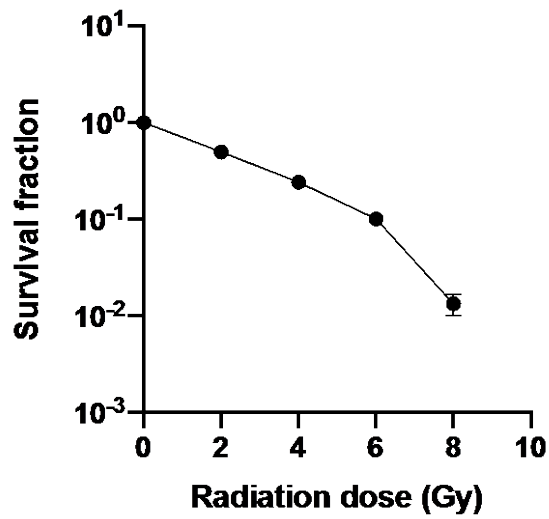
Figure 2. Logarithmic survival curve with increasing doses of radiation. Cultured endothelial cells were subjected to increasing doses of radiation, incubated at 37 degrees and the colony forming units (CFU) were counted under the microscope after staining with crystal violet. CFU were reported as percentage of control (non-irradiated cells). The results are representative of at least 5 independent experiments.

B. Radiation induces a change in the sphingolipidomic profile of GEnC

10^6 human glomerular endothelial cells were seeded and differentiated at the appropriate conditions. The control flasks received no radiation, whereas the experimental ones received a 4 Gy dosage of radiation. All flasks were incubated at 37°C for 24 hours. The cells were then lysed from sent for mass spectrometric analysis of their lipid content. Lipids were extracted at different time points of 30 minutes, 6 hours, 12 hours, 18 hours and 24 hours. Analysis of the results demonstrated a gradual time-dependent increase in the total ceramide levels, which peaked 24 hours post-radiation (Figure 3A). The main species of ceramide that contributed to this rise were the long-chain ceramides, specifically the ones with C16 and C24:1 acyl chains (Figure 3B). Concomitant with these changes, there was a parallel gradual and time-dependent drop in the levels of the ceramide-1-phosphate species, with the trough being at 24 hours post-radiation (Figure 3C).
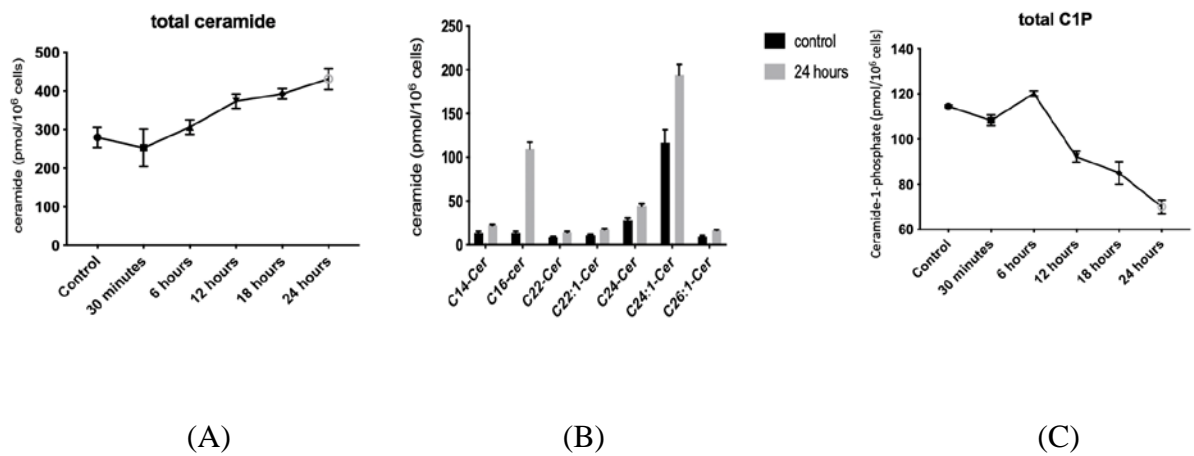
(A)   (B)   (C)

Figure 3. LC-MS analysis of radiation effects on sphingolipids. Cells were irradiated with 0 Gy (control) or 4 Gy, and pellets containing 10^6 cells were collected after 30 minutes, 6 hours, 12 hours, 18 hours and 24 hours. After extraction of lipids, the treatment groups were subjected to mass spectrometric analysis to determine the levels of total ceramide (A), the different ceramide sub-species (B), and the total level of C1P (C). Results represent the average of 3 independent experiments. . Results shown are the mean values of at least 4 independent experiments. *$P < 0.05$

C. Radiation increases the protein levels of SMPDL3b in GEnC

Due to the observed sphingolipidomic alterations post-radiation, we proceeded to investigate the changes in the levels of the lipid-modifying enzyme, SMPDL3b. In earlier experiments conducted by our group on human podocytes, radiation lead to a significant drop in the levels of SMPDL3b. Western blot on human endothelial cells showed results opposite to the ones which were obtained on podocytes. Irradiation of GEnC lead to a time-dependent, albeit delayed, increase in the protein levels of SMPDL3b, which peaked 24 hours post-radiation with a significant two-fold increase compared to the control (Figure 4), hence following the same pattern as that of ceramide.
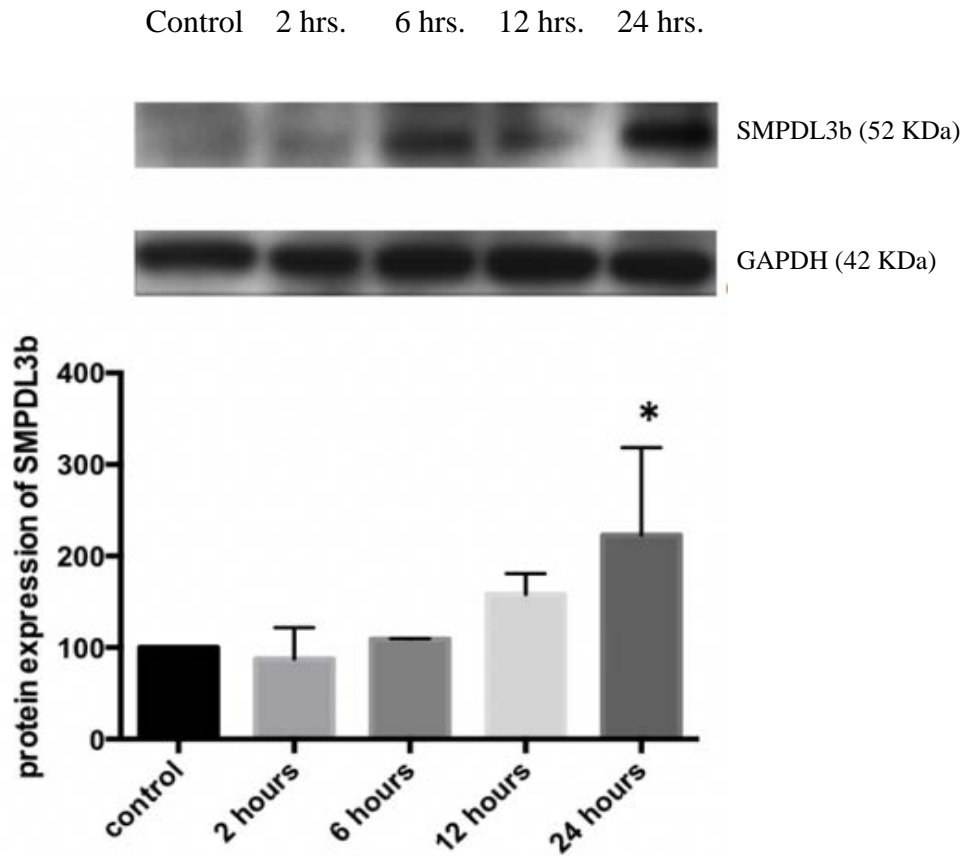
Figure 4. Changes in the level of protein expression of SMPDL3b post-radiation.

Results were quantified by densitometry using Image J software, normalized to the

house-keeping gene GAPDH and expressed as percentage of control. Results shown are

the mean values of at least 4 independent experiments. *P<0.05

D. Radiation increases NADPH oxidase activity and subsequently superoxide anion generation

It is well-established in the literature that ionizing radiation leads to oxidative stress, which is partially responsible for mediating radiotoxicity in normal tissues (Riley, 1994; Robbins & Zhao, 2004). To that end, we investigated the production of reactive oxygen species (ROS) post radiation injury of GEnC. Cells were irradiated and stained with DHE and DAPI. The results showed a time-dependent increase in oxidative stress with significant ROS production at 2 and 24 hours after treatment. production at 2 and 24 hours after treatment (Fig. 5A& B).

NADPH oxidase enzymes (NOXs) are known to be the main source of intracellular ROS, including superoxide anions and hydrogen peroxide which mediate a variety of fundamental biochemical pathways (Lambeth, Krause, & Clark, 2008). To study the role of NOXs in radiation-induced oxidative stress in our cell line, we conducted the NADPH oxidase assay on differentiated endothelial cells after radiation exposure at different time points. Indeed, NADPH oxidase showed a time-dependent increase in activity post-radiation (Fig. 5C). This temporal pattern suggests that NOXs are a potential source of radiation-induced ROS production in human glomerular endothelial cells.
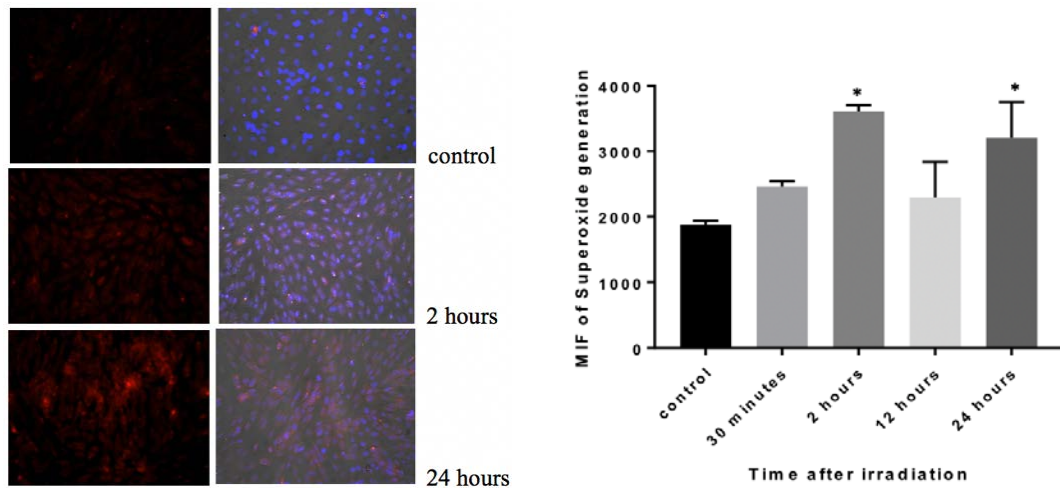
(A)

(B)
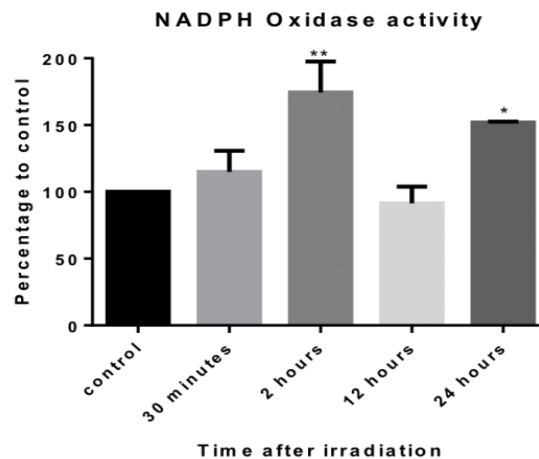
(C)

Figure 5. Radiation increases superoxide anion generation in glomerular endothelial cells by increasing NADPH oxidase activity. (A) Immunofluorescence staining with DHE and DAPI of endothelial cells radiation at 0 Gy (control) and at 4 Gy, 2 hours and 24 hours post-radiation. (B) Quantification of mean immunofluorescence at baseline (control) and at 30 minutes, 2 hours, 24 hours, and 24 hours post-radiation at 4 Gy. Cells were plated in T-25 flasks until 80% confluency, differentiated and then irradiated at 4 Gy. The pellets were collected at various time points. (C) NADPH oxidase activity

was assessed via a lucigenin-based assay. Photon emission was quantified as percentage of control (0 Gy) after subtracting blank. Results shown are the mean values of 3 independent experiments. *P<0.05

E. Radiation increases NOX1 gene expression and protein levels

A variety of the NADPH oxidase enzymes have been identified and discussed in the literature, ranging from Nox1 to Nox5. Despite their conversion at a functional level, the NOXs display an enormous variety in the protein composition and gene expression levels among different tissues in the body (K. Bedard & K. H. Krause, 2007). For our purpose, Nox1 and Nox4 are the two major isoforms expressed in human endothelial cells (Ago et al., 2005; Hu et al., 2005; Kobayashi, Nojima, Shibuya, & Maru, 2004). Therefore, we investigated the change in the expression levels of these two NOXs in response to radiation injury. Only the levels of Nox1 showed a significant time-dependent increase after radiation, peaking at 24 hours post-radiation (Fig. 6A). Our PCR results for Nox1 showed an incremental trend with a significant peak 2 hours post-radiation (Fig. 6B).
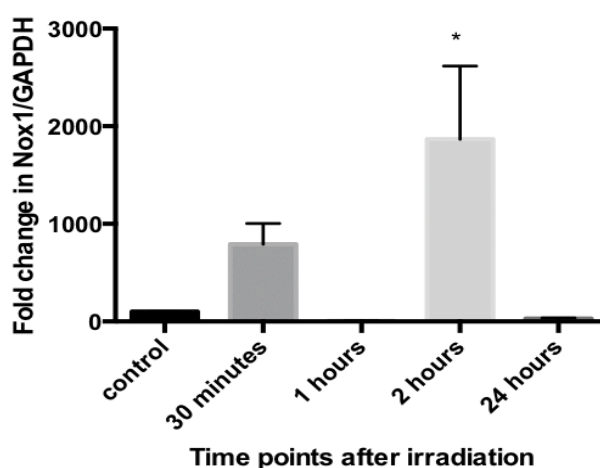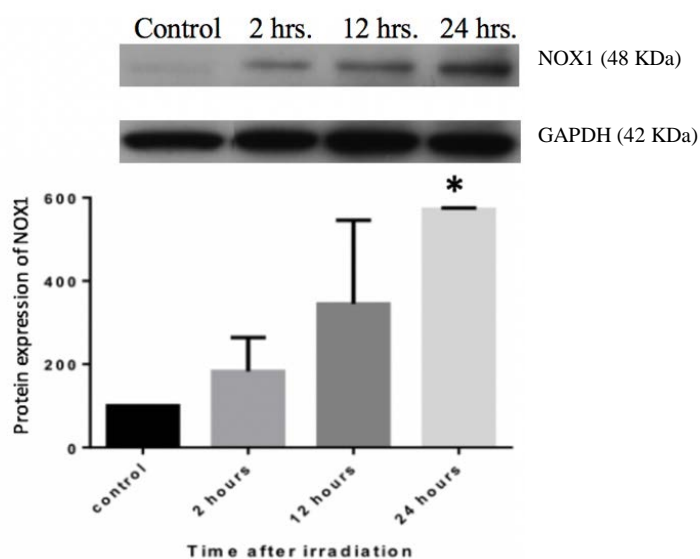
Figure 6. (A) NOX1 protein expression was assessed using western blot. Results were quantified by densitometry using Image J software, normalized to the house-keeping gene GAPDH and expressed as percentage of control. (B) NOX1 gene expression profile at 30 minutes, 1 hour, 2 hours and 24 hours post-irradiation. Results shown are the mean values of 3 independent experiments. *P<0.05

F. Treatment of GEnC with GKT, C1P or siRNA-induced KO of SMPDL3b improves cell survival post-radiation

We next wanted to investigate the role of the NADPH oxidase enzymes and the changes in the sphingolipidomic profile in endothelial cell survival. For this purpose, GEnC were irradiated and survival was assayed in presence or absence of GKT, a dual Nox1 and Nox4 inhibitor, or ceramide-1-phosphate (C1P) . In non-treated endothelial cells, survival dropped to around 35% after radiation. Treatment with GKT and C1P however were able to partially restore cell survival after radiation injury (Fig 7A). This suggests that oxidative stress by Nox1 and downregulation of C1P are key players in injury of GEnC upon irradiation. We further wanted to establish the role of SMPDL3b in radiation-induced tissue damage. Hence, we proceeded with silencing SMPDL3b expression in cultured GEnC using siRNA technology. We were able to achieve a 75% reduction in the protein expression levels of SMPDL3b (Fig. 7B), We then assessed the effect of SMPDL3b knockdown on radiation response using cleaved caspase-3 as a marker of cell survival. Indeed, knock down of SMPDL3b decreased caspase-3 cleavage and improved the survival of radiated glomerular endothelial cells (fig. 7C).
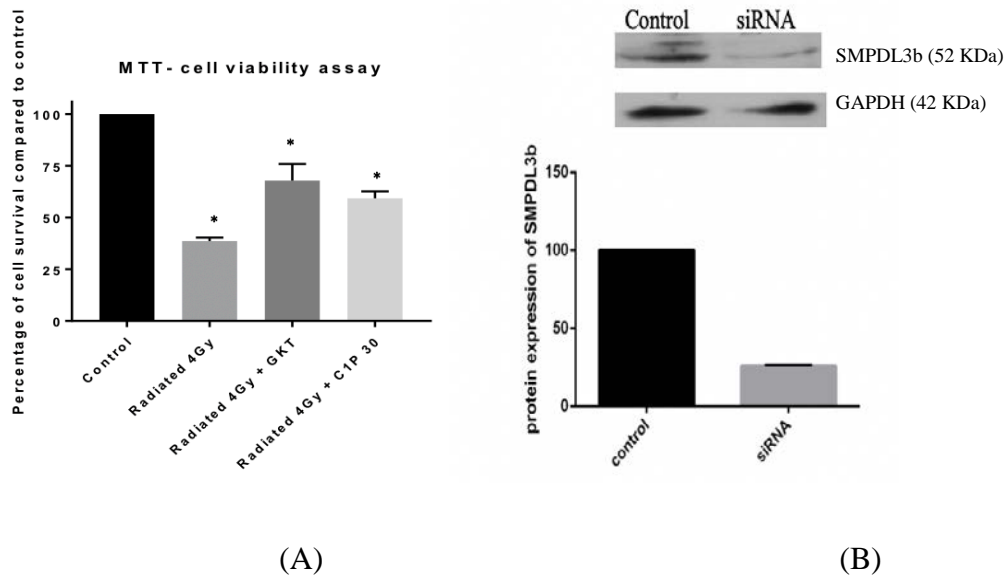
(A)

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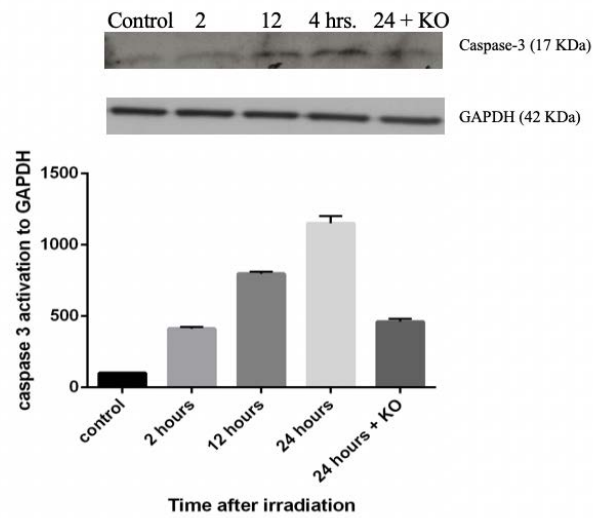
(C)

Figure 7. Endothelial cells were cultured in plated, differentiated, and treated either

with siRNA or with GKT or C1P before radiation at 4 Gy. (A) Cell survival was

assessed using MTT. Results were obtained by spectrophotometry. The readings were

subtracted from blank and expressed as percentage relative to control. (B) siRNA was

used to knockdown SMPDL3b and the protein expression levels of SMPDL3b were

analyzed after 24 hours of incubation. (C) SMPDL3b-knockout GEnC show improved

survival post-radiation as demonstrated by caspase-3 activation. Results are normalized to GAPDH and expressed as percentage to control. Results are the mean of at least three independent experiments each done in triplicates. *P<0.05

G. Treatment of GEnC with GKT decreases SMPDL3b overexpression post-radiation

Finally, we investigated the interplay between SMPDL3b and oxidative stress, as both are implicated in GEnC stress due to radiation. For that purpose, we studied the change in the levels of expression of SMPDL3b in radiated cells treated with GKT. Expression levels of SMPDL3b increased 24 hours post-radiation. However, pretreatment with GKT, restored levels of SMPDL3b post radiation almost back to normal levels (Fig. 8A). This suggests that ROS production by NADPH oxidase enzymes is upstream of SMDPDL3b.
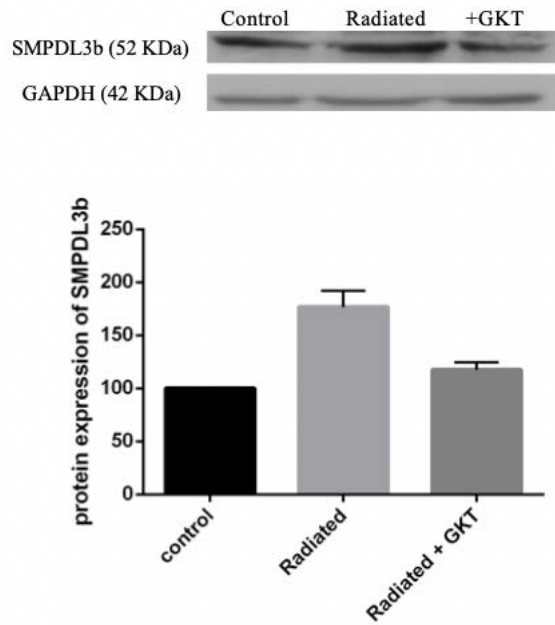
Figure 8. GKT treatment downregulates SMPDL3b protein expression post-radiation as shown by the western blot. Results are normalized to GAPDH and expressed as percentage to control. Results are the mean of at least three independent experiments each done in triplicates. *P<0.05

Chapter IV:

Discussion

Renal dysfunction is a detrimental consequence of cancer radiotherapy. It limits the ability of the treating physician to deliver optimal dosages of radiation which might ablate the tumor (Skinner, Kaplan, & Nathan, 2013). Besides, radiotherapy-induced acute kidney injury is associated with high morbidity and mortality. Such injury, even if reversible, might predispose for chronic damage in the form of chronic kidney disease (CKD) which compromises the quality of life of the patient (Goldstein & Devarajan, 2011). The Renal Insufficiency and Anticancer Medications (IRMA) study, a cross-sectional study conducted in 2007 involving 4,684 adults (mean age of 58) distributed among 15 centers in France found that 50-60% of the treated patients suffered from renal insufficiency at some point during and after their treatment regimen with an estimated mean glomerular filtration rate (eGFR) of <90 mL/min (Launay‐Vacher et al., 2007). Understanding of the pathogenesis of this outcome allows for the discovery and administration of renoprotective agents which would mitigate renal toxicity while improving the delivery and hence the efficiency of radiotherapy.

A group of researchers working in Miami have been studying nephropathy associated with diabetic kidney disease (DKD) and radiation-induced kidney damage through a "lipid-centric approach" and with a special focus on podocytopathy. Their work has revealed the role of SMPDL3b in glomerular disease. SMPDL3b levels were found to be elevated in the glomeruli of diabetic mice, in the glomeruli of patients with DKD and cultured human podocytes treated with sera from diabetic (db/db) animals. This increase in the levels of SMPDL3b was associated with the accumulation of S1P

and sphingosine, with the activation of signaling pathways known to trigger cell death and with the inhibition of tissue repair through impairing normal cellular migration (Yoo et al., 2015).

Another frequent cause of the glomerular disease is the idiopathic primary form of focal segmental glomerulosclerosis (FSGS) (Kitiyakara et al., 2004). As is the case with DKD, the Miami group was able to reveal that SMPDL3b is a key player in the pathogenesis of FSGS in patients receiving kidney transplantation. The study found that treatments which function to overexpress SMPDL3b played a protective role against podocyte apoptosis and the pathologic changes in podocyte morphology, such as cytoskeletal remodeling, which are highly associated with proteinuria and renal failure hence sparing kidney function in the setting of FSGS (Fornoni et al., 2011).

Given the role of SMPDL3b in glomerular diseases, our group went on to investigate whether alterations in SMPDL3b levels and the subsequent changes in the sphingolipidomic profile are involved in the radiation-induced glomerular injury, again with a particular emphasis on podocytopathy. In the study conducted by Ahmad et al. (2017), subjecting cultured human podocytes to 8 Gy of radiation lead to a drop in the levels of expression of SMPDL3b and a concomitant increase in the levels of the pro-apoptotic ceramide. These changes were associated with podocyte injury and cytoskeletal remodeling, both of which are thought to be important cytologic changes associated with renal insufficiency. Experiments on animal models in this same study further illustrated the radioprotective role of SMPDL3b and emphasized that radiation-induced decrease in SMPDL3b levels and the consequential changes in the lipid profile of the radiated podocytes are key players in radiation-induced nephropathy (Ahmad et al., 2017).

The scientific literature shows that one of the main mechanisms associated with radiation-induced injury of healthy tissues is the damage to the microvasculature supplying these tissues, particularly the endothelial cells lining the vessels. Since the glomerular endothelial cells are one of the major three layers of the glomerular filtration barrier, we hypothesized that injury to these cells in addition to podocytopathy might be a major player in radiotherapy-induced renal insufficiency (Vasavada & Agarwal, 2005).

The mechanism of injury to the microvasculature is thought to be due to the accumulation of reactive oxygen species (ROS) beyond the capacity of the cells to scavenge these toxins, ultimately resulting in a state of oxidative stress and cellular damage. The main cellular enzymes implicated in the generation of ROS are the family of NADPH oxidase enzymes (NOXs), two of which, namely NOXs 1 & 4, are of a major interest to us in the context of our research due to their high levels of expression in kidney glomeruli (K. Bedard & K.-H. Krause, 2007; Kim et al., 2014).

Integrating the literature with the latest work from our group, we came up with the current project. First, we embarked on investigating the role of the NOXs with the subsequent generation of ROS and the role of SMPDL3b with the subsequent changes in the lipid profile, on the response of cultured human glomerular endothelial cells (GEnC) to radiation. We then proceeded to investigate if there is an underlying connection between the proteomics and lipidomics of GEnC i.e. whether the changes at the level of SMPDL3b and the sphingolipidomic profile are dependent on the observed changes in NOXs and the generated ROS. Our experiments shed the light on a new pathway, which will further unfold during the discussion, and which poses a promising

potential in understanding and hence tackling the root of radiation-induced cell injury in GEnC.

Due to the difficulty of isolating the GEnC cell line and maintaining it in culture, there is very little research on it (Satchell et al., 2006). Hence, our starting point was to construct a dose-response curve to assess cell viability at different dosages of radiation. The observed results revealed, as expected, a remarkable decrease in cell clonality with increasing dosages of radiation. Survival dropped down to 50% at 2 Gy and continued to decrease gradually until hitting a bottom line of almost 0% at 8 Gy. Accordingly, we decided to conduct our experiments at a dose of 4 Gy, which would allow for enough cell viability to study the intracellular molecular pathways while magnifying the response to radiation so we can dissect the observations we are interested in.

As anticipated, radiating the cultured endothelial cells lead to a significant increase in the levels of gene expression and protein levels of NOX enzymes, particularly NOX1, and induced oxidative stress within the cells due to superoxide anion generation as illustrated by both spectrophotometric experiments, the immunofluorescence results using DHE and the NADPH oxidase enzyme assay using lucigenin. These results are in alignment with a huge body of literature which shows NOX activation and the accumulation of ROS as consequences of cell exposure to radiation, implicating this pathway in the observed decrease in cell viability (Ameziane-El-Hassani et al.; Cooper, Liu, & Hudson; Datta, Suman, Kallakury, & Fornace; Henri et al.; Mao, Nishiyama, Campbell-Beachler, et al.; Mao, Nishiyama, Pecaut, et al.; Martinez et al.; Yu, Sun, Gu, Wang, & Gao). This was evidenced through the GKT experiment whereby shutting NOX1 & 4 activities through the action of the dual inhibitor restored cell viability post-radiation.

However, the changes in the levels of SMPDL3b were rather surprising. Unlike in podocytes where SMPDL3b levels drop after exposure to radiation (Ahmad et al., 2017), gene expression and protein levels of SMPDL3b increased in a time-dependent manner post-radiation in GEnC. This opens up the possibility that SMPDL3b might play different roles in different cell lines. Despite its radioprotective effects in podocytes, SMPDL3b might be on the other hand involved in radiation damage of the glomerular endothelial cells. Indeed, using siRNA to knock down the expression of SMPDL3b in cultured GEnC restored cell viability suggesting a role for SMPDL3b in cellular injury downstream of radiotherapy in this particular cell line.

Another way to mitigate the levels of expression of SMPDL3b was to use GKT, a dual NOX inhibitor, therefore revealing for the first time that, in GEnC, SMPDL3b acts downstream NOX and the induced oxidative stress along the same cellular pathway eventually leading to cellular demise. The changes in SMPDL3b were accompanied by changes in the cellular lipidomic profile. In a nutshell, the levels of the anti-apoptotic C1P increased and that of the pro-apoptotic long-chain ceramides decreased tilting the balance towards cellular death. This is in alignment with a huge body of literature that supports the role of ceramide and C1P in cell death and cell survival, respectively (Yusuf A Hannun & Obeid, 2017). Finally, our work falls nicely in line with the most recent publication of Fornoni et al. (2019) which hints for the first time at the C1P lyase activity of the SMPDL3b enzyme (Mitrofanova et al., 2019). The decrease in C1P and the increase in ceramide, in endothelial cells post-radiation, following an increase in protein levels of SMPDL3b thereby hinting that SMPDL3b might be acting as a C1P lyase in our cell line, hydrolyzing it into ceramide. This establishes a chain of cellular events starting from NOX and reactive oxygen species, subsequent activation of

SMPDL3b and a change in sphingolipidomic profile, all of which serve to explain radiation-induced cell death.

This project paves the path to a new therapeutic potential where this SMPDL3b-centered pathway can be pharmacologically targeted to prevent radiation-induced renal damage (figure 9), which allows the delivery of an effective dosage of radiation without collateral damage to the kidneys. However, the study does not stand without its limitations. Given the paradoxical effect of SMPDL3b on the two major components of the glomerular filtration barrier, the communication between podocytes and endothelial cells need to be further explored under the effect of radiation, both through co-culture work and through in vivo animal models. The focus of our future work would be to explore the role of this pathway through a more holistic approach, revealing its position in the network of events that happens when the kidney tissue is irradiated, and trying to pinpoint the best stage at which medical intervention can yield favorable results.

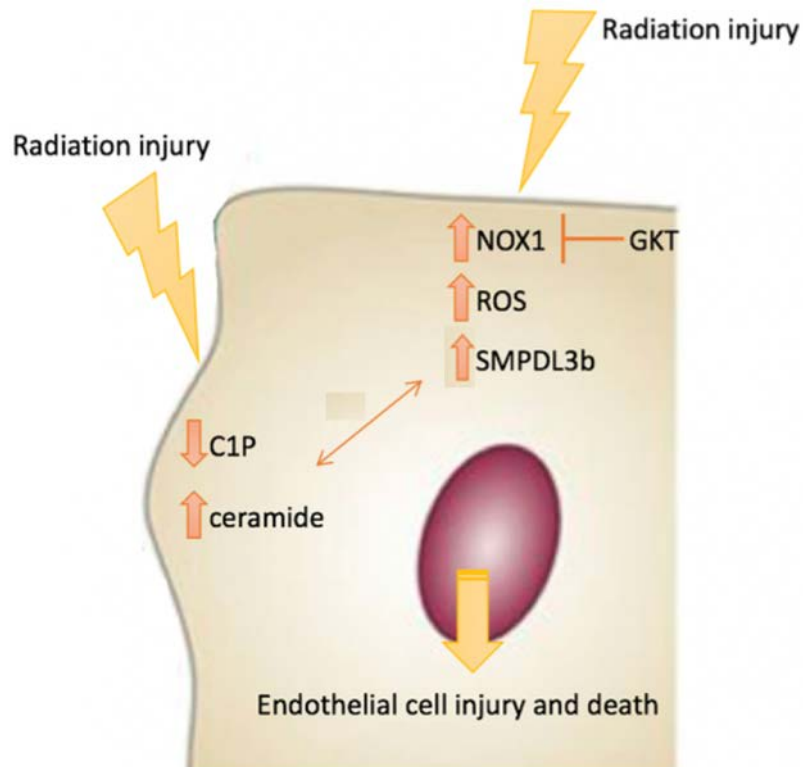**Figure 9.** Model of radiation-induced endothelial cell damage. The model depicts induction of NOX1 and NOX1-mediated reactive oxygen species (ROS) downstream radiation injury. The increase in SMPDL3b downstream of NOX1 is a key event in radiation injury of GEnC. This triggers changes in sphingolipid metabolism, including a drop in C1P and an increase in ceramide, which contributes to the damage phenotype.

REFERENCES:

Abou Daher, A., El Jalkh, T., Eid, A. A., Fornoni, A., Marples, B., & Zeidan, Y. H. (2017). Translational Aspects of Sphingolipid Metabolism in Renal Disorders. *Int J Mol Sci, 18*(12). doi:10.3390/ijms18122528

Abuhusain, H. J., Matin, A., Qiao, Q., Shen, H., Kain, N., Day, B. W., . . . Don, A. S. (2013). A metabolic shift favoring sphingosine 1-phosphate at the expense of ceramide controls glioblastoma angiogenesis. *J Biol Chem, 288*(52), 37355-37364. doi:10.1074/jbc.M113.494740

Adada, M. M., Canals, D., Jeong, N., Kelkar, A. D., Hernandez-Corbacho, M., Pulkoski-Gross, M. J., . . . Obeid, L. M. (2015). Intracellular sphingosine kinase 2-derived sphingosine-1-phosphate mediates epidermal growth factor-induced ezrin-radixin-moesin phosphorylation and cancer cell invasion. *FASEB J, 29*(11), 4654-4669. doi:10.1096/fj.15-274340

Adams, J. M., 2nd, Pratipanawatr, T., Berria, R., Wang, E., DeFronzo, R. A., Sullards, M. C., & Mandarino, L. J. (2004). Ceramide content is increased in skeletal muscle from obese insulin-resistant humans. *Diabetes, 53*(1), 25-31.

Ago, T., Kitazono, T., Kuroda, J., Kumai, Y., Kamouchi, M., Ooboshi, H., . . . Iida, M. (2005). NAD(P)H oxidases in rat basilar arterial endothelial cells. *Stroke, 36*(5), 1040-1046. doi:10.1161/01.STR.0000163111.05825.0b

Ahmad, A., Mitrofanova, A., Bielawski, J., Yang, Y., Marples, B., Fornoni, A., & Zeidan, Y. H. (2017). Sphingomyelinase-like phosphodiesterase 3b mediates radiation-induced damage of renal podocytes. *FASEB J, 31*(2), 771-780. doi:10.1096/fj.201600618R

Allende, M. L., & Proia, R. L. (2002). Sphingosine-1-phosphate receptors and the development of the vascular system. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids, 1582*(1), 222-227.

Alroy, J., Sabnis, S., & Kopp, J. B. (2002). Renal pathology in Fabry disease. *J Am Soc Nephrol, 13 Suppl 2*, S134-138.

Ameziane-El-Hassani, R., Talbot, M., de Souza Dos Santos, M. C., Al Ghuzlan, A., Hartl, D., Bidart, J. M., . . . Dupuy, C. NADPH oxidase DUOX1 promotes long-term persistence of oxidative stress after an exposure to irradiation. *Proceedings of the National Academy of Sciences of the United States of America, 112*(16), 5051-5056.

Antoon, J. W., Liu, J., Gestaut, M. M., Burow, M. E., Beckman, B. S., & Foroozesh, M. (2009). Design, synthesis, and biological activity of a family of novel ceramide analogues in chemoresistant breast cancer cells. *Journal of medicinal chemistry, 52*(18), 5748-5752.

Antoon, J. W., Liu, J., Ponnapakkam, A. P., Gestaut, M. M., Foroozesh, M., & Beckman, B. S. (2010). Novel d-erythro N-octanoyl sphingosine analogs as chemo-and endocrine-resistant breast cancer therapeutics. *Cancer chemotherapy and pharmacology, 65*(6), 1191-1195.

Arana, L., Gangoiti, P., Ouro, A., Rivera, I.-G., Ordoñez, M., Trueba, M., . . . Gomez-Muñoz, A. (2012). Generation of reactive oxygen species (ROS) is a key factor for stimulation of macrophage proliferation by ceramide 1-phosphate. *Experimental cell research, 318*(4), 350-360.

Askari, H., Kaneski, C. R., Semino-Mora, C., Desai, P., Ang, A., Kleiner, D. E., . . . Schiffmann, R. (2007). Cellular and tissue localization of globotriaosylceramide in Fabry disease. *Virchows Arch, 451*(4), 823-834. doi:10.1007/s00428-007-0468-6

Banfi, A., Muraglia, A., Dozin, B., Mastrogiacomo, M., Cancedda, R., & Quarto, R. (2000). Proliferation kinetics and differentiation potential of ex vivo expanded human bone marrow stromal cells: Implications for their use in cell therapy. *Exp Hematol, 28*(6), 707-715.

Banfi, B., Molnar, G., Maturana, A., Steger, K., Hegedus, B., Demaurex, N., & Krause, K. H. (2001). A Ca(2+)-activated NADPH oxidase in testis, spleen, and lymph nodes. *J Biol Chem, 276*(40), 37594-37601. doi:10.1074/jbc.M103034200

Baum, M. A. (2004). Outcomes after renal transplantation for FSGS in children. *Pediatr Transplant, 8*(4), 329-333. doi:10.1111/j.1399-3046.2004.00181.x

Bedard, K., & Krause, K.-H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiological reviews, 87*(1), 245-313.

Bedard, K., & Krause, K. H. (2007). The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev, 87*(1), 245-313. doi:10.1152/physrev.00044.2005

Bernobich, E., Cosenzi, A., Campa, C., Zennaro, C., Sasso, F., Paoletti, S., & Bellini, G. (2004). Antihypertensive treatment and renal damage: amlodipine exerts protective effect through the polyol pathway. *J Cardiovasc Pharmacol, 44*(3), 401-406.

Betteridge, D. J. (2000). What is oxidative stress? *Metabolism, 49*(2), 3-8.

Bielawski, J., Szulc, Z. M., Hannun, Y. A., & Bielawska, A. (2006). Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Methods, 39*(2), 82-91. doi:10.1016/j.ymeth.2006.05.004

Birbes, H., Luberto, C., Hsu, Y. T., El Bawab, S., Hannun, Y. A., & Obeid, L. M. (2005). A mitochondrial pool of sphingomyelin is involved in TNFalpha-induced Bax translocation to mitochondria. *Biochem J, 386*(Pt 3), 445-451. doi:10.1042/BJ20041627

Blachnio-Zabielska, A. U., Pulka, M., Baranowski, M., Nikolajuk, A., Zabielski, P., Gorska, M., & Gorski, J. (2012). Ceramide metabolism is affected by obesity and diabetes in human adipose tissue. *J Cell Physiol, 227*(2), 550-557. doi:10.1002/jcp.22745

Boath, A., Graf, C., Lidome, E., Ullrich, T., Nussbaumer, P., & Bornancin, F. (2008). Regulation and traffic of ceramide 1-phosphate produced by ceramide kinase: comparative analysis to glucosylceramide and sphingomyelin. *J Biol Chem, 283*(13), 8517-8526. doi:10.1074/jbc.M707107200

Bode, H., Bourquin, F., Suriyanarayanan, S., Wei, Y., Alecu, I., Othman, A., . . . Hornemann, T. (2016). HSAN1 mutations in serine palmitoyltransferase reveal a close structure-function-phenotype relationship. *Hum Mol Genet, 25*(5), 853-865. doi:10.1093/hmg/ddv611

Bretscher, A., Edwards, K., & Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol, 3*(8), 586-599. doi:10.1038/nrm882

Briere, J., Calman, F., Lageron, A., Hinglais, N., Emerit, J., & Bernard, J. (1976). [Adult Niemann-Pick disease: a 26 years follow-up. Report of a case with isolated visceral involvement, excess of tissue sphingomyelin, and deficient sphingomyelinase activity (author's transl)]. *Nouv Rev Fr Hematol Blood Cells, 16*(2), 185-202.

Brunskill, E. W., & Potter, S. S. (2012). Changes in the gene expression programs of renal mesangial cells during diabetic nephropathy. *BMC Nephrol, 13*, 70. doi:10.1186/1471-2369-13-70

Cabukusta, B., Kol, M., Kneller, L., Hilderink, A., Bickert, A., Mina, J. G., . . . Holthuis, J. C. (2017). ER residency of the ceramide phosphoethanolamine synthase SMSr relies on homotypic oligomerization mediated by its SAM domain. *Sci Rep, 7*, 41290. doi:10.1038/srep41290

Carreira, A. C., Ventura, A. E., Varela, A. R., & Silva, L. C. (2015). Tackling the biophysical properties of sphingolipids to decipher their biological roles. *Biol Chem, 396*(6-7), 597-609. doi:10.1515/hsz-2014-0283

Chalfant, C. E., Szulc, Z., Roddy, P., Bielawska, A., & Hannun, Y. A. (2004). The structural requirements for ceramide activation of serine-threonine protein phosphatases. *J Lipid Res, 45*(3), 496-506. doi:10.1194/jlr.M300347-JLR200

Chauhan, N., Visram, M., Cristobal-Sarramian, A., Sarkleti, F., & Kohlwein, S. D. (2015). Morphogenesis checkpoint kinase Swe1 is the executor of lipolysis-dependent cell-cycle progression. *Proc Natl Acad Sci U S A, 112*(10), E1077-1085. doi:10.1073/pnas.1423175112

Chaurasia, B., & Summers, S. A. (2015). Ceramides - Lipotoxic Inducers of Metabolic Disorders. *Trends Endocrinol Metab, 26*(10), 538-550. doi:10.1016/j.tem.2015.07.006

Cheng, G., Cao, Z., Xu, X., van Meir, E. G., & Lambeth, J. D. (2001). Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene, 269*(1-2), 131-140.

Chmura, S. J., Nodzenski, E., Beckett, M. A., Kufe, D. W., Quintans, J., & Weichselbaum, R. R. (1997). Loss of ceramide production confers resistance to radiation-induced apoptosis. *Cancer Res, 57*(7), 1270-1275.

Cingolani, F., Futerman, A. H., & Casas, J. (2016). Ceramide synthases in biomedical research. *Chem Phys Lipids, 197*, 25-32. doi:10.1016/j.chemphyslip.2015.07.026

Clark, J. D., Schievella, A. R., Nalefski, E. A., & Lin, L.-L. (1995). Cytosolic phospholipase A2. *Journal of lipid mediators and cell signalling, 12*(2-3), 83-117.

Contreras, F. X., Ernst, A. M., Haberkant, P., Bjorkholm, P., Lindahl, E., Gonen, B., . . . Brugger, B. (2012). Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. *Nature, 481*(7382), 525-529. doi:10.1038/nature10742

Cooper, K. L., Liu, K. J., & Hudson, L. G. Enhanced ROS production and redox signaling with combined arsenite and UVA exposure: contribution of NADPH oxidase. *Free Radical Biology & Medicine, 47*(4), 381-388.

Datta, K., Suman, S., Kallakury, B. V., & Fornace, A. J., Jr. Exposure to heavy ion radiation induces persistent oxidative stress in mouse intestine. *PLoS ONE [Electronic Resource], 7*(8), e42224.

De Deken, X., Wang, D., Many, M. C., Costagliola, S., Libert, F., Vassart, G., . . . Miot, F. (2000). Cloning of two human thyroid cDNAs encoding new members of the

NADPH oxidase family. *J Biol Chem, 275*(30), 23227-23233. doi:10.1074/jbc.M000916200

Dorsam, G., Taher, M. M., Valerie, K. C., Kuemmerle, N. B., Chan, J. C., & Franson, R. C. (2000). Diphenyleneiodium chloride blocks inflammatory cytokine-induced up-regulation of group IIA phospholipase A(2) in rat mesangial cells. *J Pharmacol Exp Ther, 292*(1), 271-279.

Dudeja, P. K., Dahiya, R., & Brasitus, T. A. (1986). The role of sphingomyelin synthetase and sphingomyelinase in 1, 2-dimethylhydrazine-induced lipid alterations of rat colonic plasma membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 863*(2), 309-312.

Eid, A. A., Ford, B. M., Block, K., Kasinath, B. S., Gorin, Y., Ghosh-Choudhury, G., . . . Abboud, H. E. (2010). AMP-activated protein kinase (AMPK) negatively regulates Nox4-dependent activation of p53 and epithelial cell apoptosis in diabetes. *J Biol Chem, 285*(48), 37503-37512. doi:10.1074/jbc.M110.136796

Eid, A. A., Gorin, Y., Fagg, B. M., Maalouf, R., Barnes, J. L., Block, K., & Abboud, H. E. (2009). Mechanisms of podocyte injury in diabetes: role of cytochrome P450 and NADPH oxidases. *Diabetes, 58*(5), 1201-1211. doi:10.2337/db08-1536

Epstein, S., & Riezman, H. (2013). Sphingolipid signaling in yeast: potential implications for understanding disease. *Front Biosci (Elite Ed), 5*, 97-108.

Fornoni, A., Sageshima, J., Wei, C., Merscher-Gomez, S., Aguillon-Prada, R., Jauregui, A. N., . . . Burke, G. W., 3rd. (2011). Rituximab targets podocytes in recurrent focal segmental glomerulosclerosis. *Sci Transl Med, 3*(85), 85ra46. doi:10.1126/scitranslmed.3002231

Fox, T. E., Bewley, M. C., Unrath, K. A., Pedersen, M. M., Anderson, R. E., Jung, D. Y., . . . Kester, M. (2011). Circulating sphingolipid biomarkers in models of type 1 diabetes. *J Lipid Res, 52*(3), 509-517. doi:10.1194/jlr.M010595

Furuya, H., Shimizu, Y., & Kawamori, T. (2011). Sphingolipids in cancer. *Cancer and Metastasis Reviews, 30*(3-4), 567-576.

Galadari, S., Rahman, A., Pallichankandy, S., & Thayyullathil, F. (2015). Tumor suppressive functions of ceramide: evidence and mechanisms. *Apoptosis, 20*(5), 689-711. doi:10.1007/s10495-015-1109-1

Gangoiti, P., Granado, M. H., Arana, L., Ouro, A., & Gomez-Muñoz, A. (2010). Activation of protein kinase C-α is essential for stimulation of cell proliferation by ceramide 1-phosphate. *FEBS letters, 584*(3), 517-524.

Gangoiti, P., Granado, M. H., Wang, S. W., Kong, J. Y., Steinbrecher, U. P., & Gómez-Muñoz, A. (2008). Ceramide 1-phosphate stimulates macrophage proliferation through activation of the PI3-kinase/PKB, JNK and ERK1/2 pathways. *Cellular signalling, 20*(4), 726-736.

Gault, C. R., Obeid, L. M., & Hannun, Y. A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv Exp Med Biol, 688*, 1-23.

Geiszt, M., Kopp, J. B., Varnai, P., & Leto, T. L. (2000). Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A, 97*(14), 8010-8014. doi:10.1073/pnas.130135897

Geoffroy, K., Troncy, L., Wiernsperger, N., Lagarde, M., & El Bawab, S. (2005). Glomerular proliferation during early stages of diabetic nephropathy is associated with local increase of sphingosine-1-phosphate levels. *FEBS Lett, 579*(5), 1249-1254. doi:10.1016/j.febslet.2004.12.094

Goetzl, E. J., Dolezalova, H., Kong, Y., & Zeng, L. (1999). Dual mechanisms for lysophospholipid induction of proliferation of human breast carcinoma cells. *Cancer research, 59*(18), 4732-4737.

Goetzl, E. J., & Rosen, H. (2004). Regulation of immunity by lysosphingolipids and their G protein–coupled receptors. *The Journal of clinical investigation, 114*(11), 1531-1537.

Goldstein, S. L., & Devarajan, P. (2011). Acute kidney injury in childhood: should we be worried about progression to CKD? *Pediatric Nephrology, 26*(4), 509-522.

Gomez-Muñoz, A. (2018). The Role of Ceramide 1-Phosphate in Tumor Cell Survival and Dissemination. *Advances in Cancer Research.*

Gómez-Muñoz, A., Kong, J. Y., Parhar, K., Wang, S. W., Gangoiti, P., González, M., . . . Steinbrecher, U. P. (2005). Ceramide‐1‐ phosphate promotes cell survival through activation of the phosphatidylinositol 3‐kinase/protein kinase B pathway. *FEBS letters, 579*(17), 3744-3750.

Gómez-Muñoz, A., Kong, J. Y., Salh, B., & Steinbrecher, U. P. (2004). Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. *Journal of lipid research, 45*(1), 99-105.

Gorelik, A., Illes, K., Heinz, L. X., Superti-Furga, G., & Nagar, B. (2016). Crystal structure of mammalian acid sphingomyelinase. *Nat Commun, 7*, 12196. doi:10.1038/ncomms12196

Gorin, Y., Ricono, J. M., Wagner, B., Kim, N. H., Bhandari, B., Choudhury, G. G., & Abboud, H. E. (2004). Angiotensin II-induced ERK1/ERK2 activation and protein synthesis are redox-dependent in glomerular mesangial cells. *Biochem J, 381*(Pt 1), 231-239. doi:10.1042/bj20031614

Gorska, M., Dobrzyn, A., & Baranowski, M. (2005). Concentrations of sphingosine and sphinganine in plasma of patients with type 2 diabetes. *Med Sci Monit, 11*(1), CR35-38.

Gouazé, V., Jing, Y. Y., Bleicher, R. J., Han, T.-Y., Liu, Y.-Y., Wang, H., . . . Cabot, M. C. (2004). Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Molecular cancer therapeutics, 3*(5), 633-640.

Gouazé, V., Liu, Y.-Y., Prickett, C. S., Jing, Y. Y., Giuliano, A. E., & Cabot, M. C. (2005). Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs. *Cancer research, 65*(9), 3861-3867.

Guan, H., Song, L., Cai, J., Huang, Y., Wu, J., Yuan, J., . . . Li, M. (2011). Sphingosine kinase 1 regulates the Akt/FOXO3a/Bim pathway and contributes to apoptosis resistance in glioma cells. *PLoS One, 6*(5), e19946. doi:10.1371/journal.pone.0019946

Guo, B. B., Bellingham, S. A., & Hill, A. F. (2015). The neutral sphingomyelinase pathway regulates packaging of the prion protein into exosomes. *J Biol Chem, 290*(6), 3455-3467. doi:10.1074/jbc.M114.605253

Haimovitz-Friedman, A., Kan, C. C., Ehleiter, D., Persaud, R. S., McLoughlin, M., Fuks, Z., & Kolesnick, R. N. (1994). Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med, 180*(2), 525-535.

Hankins, J. L., Fox, T. E., Barth, B. M., Unrath, K. A., & Kester, M. (2011). Exogenous ceramide-1-phosphate reduces lipopolysaccharide (LPS)-mediated cytokine expression. *J Biol Chem, 286*(52), 44357-44366. doi:10.1074/jbc.M111.264010

Hankins, J. L., Fox, T. E., Barth, B. M., Unrath, K. A., & Kester, M. (2011). Exogenous ceramide-1-phosphate reduces LPS-mediated cytokine expression. *Journal of Biological Chemistry*, jbc. M111. 264010.

Hannun, Y. A., & Obeid, L. M. (2008). Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat Rev Mol Cell Biol, 9*(2), 139-150. doi:10.1038/nrm2329

Hannun, Y. A., & Obeid, L. M. (2011). Many ceramides. *J Biol Chem, 286*(32), 27855-27862. doi:10.1074/jbc.R111.254359

Hannun, Y. A., & Obeid, L. M. (2017). Sphingolipids and their metabolism in physiology and disease. *Nature reviews Molecular cell biology*.

Hannun, Y. A., & Obeid, L. M. (2018). Sphingolipids and their metabolism in physiology and disease. *Nat Rev Mol Cell Biol, 19*(3), 175-191. doi:10.1038/nrm.2017.107

Harmon, J. M., Bacikova, D., Gable, K., Gupta, S. D., Han, G., Sengupta, N., . . . Dunn, T. M. (2013). Topological and functional characterization of the ssSPTs, small activating subunits of serine palmitoyltransferase. *J Biol Chem, 288*(14), 10144-10153. doi:10.1074/jbc.M113.451526

Haugen, E., & Nath, K. A. (1999). The involvement of oxidative stress in the progression of renal injury. *Blood Purif, 17*(2-3), 58-65. doi:10.1159/000014377

Haus, J. M., Kashyap, S. R., Kasumov, T., Zhang, R., Kelly, K. R., Defronzo, R. A., & Kirwan, J. P. (2009). Plasma ceramides are elevated in obese subjects with type 2 diabetes and correlate with the severity of insulin resistance. *Diabetes, 58*(2), 337-343. doi:10.2337/db08-1228

Hayashi, Y., Nemoto-Sasaki, Y., Tanikawa, T., Oka, S., Tsuchiya, K., Zama, K., . . . Yamashita, A. (2014). Sphingomyelin synthase 2, but not sphingomyelin synthase 1, is involved in HIV-1 envelope-mediated membrane fusion. *J Biol Chem, 289*(44), 30842-30856. doi:10.1074/jbc.M114.574285

Henri, P., Beaumel, S., Guezennec, A., Poumes, C., Stoebner, P. E., Stasia, M. J., . . . Meunier, L. MC1R expression in HaCaT keratinocytes inhibits UVA-induced ROS production via NADPH oxidase- and cAMP-dependent mechanisms. *Journal of Cellular Physiology, 227*(6), 2578-2585.

Hla, T. (2003). Signaling and biological actions of sphingosine 1-phosphate. *Pharmacological research, 47*(5), 401-407.

Holland, W. L., & Summers, S. A. (2008). Sphingolipids, insulin resistance, and metabolic disease: new insights from in vivo manipulation of sphingolipid metabolism. *Endocr Rev, 29*(4), 381-402. doi:10.1210/er.2007-0025

Hornemann, T., Penno, A., Rutti, M. F., Ernst, D., Kivrak-Pfiffner, F., Rohrer, L., & von Eckardstein, A. (2009). The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. *J Biol Chem, 284*(39), 26322-26330. doi:10.1074/jbc.M109.023192

Hu, T., Ramachandrarao, S. P., Siva, S., Valancius, C., Zhu, Y., Mahadev, K., . . . Sharma, K. (2005). Reactive oxygen species production via NADPH oxidase mediates TGF-beta-induced cytoskeletal alterations in endothelial cells. *Am J Physiol Renal Physiol, 289*(4), F816-825. doi:10.1152/ajprenal.00024.2005

Hubsch, H., Montane, B., Abitbol, C., Chandar, J., Shariatmadar, S., Ciancio, G., . . . Zilleruelo, G. (2005). Recurrent focal glomerulosclerosis in pediatric renal

allografts: the Miami experience. *Pediatr Nephrol, 20*(2), 210-216. doi:10.1007/s00467-004-1706-7

Ibrahim, F. B. M., Pang, S. J., & Melendez, A. J. (2004). Anaphylatoxin Signaling in Human Neutrophils A KEY ROLE FOR SPHINGOSINE KINASE. *Journal of Biological Chemistry, 279*(43), 44802-44811.

Ishizawa, S., Takahashi-Fujigasaki, J., Kanazawa, Y., Matoba, K., Kawanami, D., Yokota, T., . . . Utsunomiya, K. (2014). Sphingosine-1-phosphate induces differentiation of cultured renal tubular epithelial cells under Rho kinase activation via the S1P2 receptor. *Clin Exp Nephrol, 18*(6), 844-852. doi:10.1007/s10157-014-0933-x

Jain, A., Beutel, O., Ebell, K., Korneev, S., & Holthuis, J. C. (2017). Diverting CERT-mediated ceramide transport to mitochondria triggers Bax-dependent apoptosis. *J Cell Sci, 130*(2), 360-371. doi:10.1242/jcs.194191

Janes, K., Little, J. W., Li, C., Bryant, L., Chen, C., Chen, Z., . . . Salvemini, D. (2014). The development and maintenance of paclitaxel-induced neuropathic pain require activation of the sphingosine 1-phosphate receptor subtype 1. *J Biol Chem, 289*(30), 21082-21097. doi:10.1074/jbc.M114.569574

Kabayama, K., Sato, T., Kitamura, F., Uemura, S., Kang, B. W., Igarashi, Y., & Inokuchi, J. (2005). TNFalpha-induced insulin resistance in adipocytes as a membrane microdomain disorder: involvement of ganglioside GM3. *Glycobiology, 15*(1), 21-29. doi:10.1093/glycob/cwh135

Kawamori, T., Kaneshiro, T., Okumura, M., Maalouf, S., Uflacker, A., Bielawski, J., . . . Obeid, L. M. (2009). Role for sphingosine kinase 1 in colon carcinogenesis. *The FASEB Journal, 23*(2), 405-414.

Keane, W. F. (2000). Proteinuria: its clinical importance and role in progressive renal disease. *Am J Kidney Dis, 35*(4 Suppl 1), S97-105.

Kikuchi, H., Hikage, M., Miyashita, H., & Fukumoto, M. (2000). NADPH oxidase subunit, gp91(phox) homologue, preferentially expressed in human colon epithelial cells. *Gene, 254*(1-2), 237-243.

Kim, C., Schneider, G., Abdel-Latif, A., Mierzejewska, K., Sunkara, M., Borkowska, S., . . . Ratajczak, M. Z. (2013). Ceramide-1-phosphate regulates migration of multipotent stromal cells and endothelial progenitor cells--implications for tissue regeneration. *Stem Cells, 31*(3), 500-510. doi:10.1002/stem.1291

Kim, C., Schneider, G., Abdel-Latif, A., Mierzejewska, K., Sunkara, M., Borkowska, S., . . . Ratajczak, M. Z. (2013). Ceramide-1-Phosphate Regulates Migration of Multipotent Stromal Cells and Endothelial Progenitor Cells—Implications for Tissue Regeneration. *Stem Cells, 31*(3), 500-510.

Kim, J. H., Jenrow, K. A., & Brown, S. L. (2014). Mechanisms of radiation-induced normal tissue toxicity and implications for future clinical trials. *Radiation oncology journal, 32*(3), 103.

Kimura, K., Bowen, C., Spiegel, S., & Gelmann, E. P. (1999). Tumor necrosis factor-alpha sensitizes prostate cancer cells to gamma-irradiation-induced apoptosis. *Cancer Res, 59*(7), 1606-1614.

Kitiyakara, C., Eggers, P., & Kopp, J. B. (2004). Twenty-one-year trend in ESRD due to focal segmental glomerulosclerosis in the United States. *Am J Kidney Dis, 44*(5), 815-825.

Kobayashi, S., Nojima, Y., Shibuya, M., & Maru, Y. (2004). Nox1 regulates apoptosis and potentially stimulates branching morphogenesis in sinusoidal endothelial cells. *Exp Cell Res, 300*(2), 455-462. doi:10.1016/j.yexcr.2004.07.023

Kohno, M., Momoi, M., Oo, M. L., Paik, J.-H., Lee, Y.-M., Venkataraman, K., . . . Sano, H. (2006). Intracellular role for sphingosine kinase 1 in intestinal adenoma cell proliferation. *Molecular and cellular biology, 26*(19), 7211-7223.

Kolesnick, R. N., & Hemer, M. R. (1990). Characterization of a ceramide kinase activity from human leukemia (HL-60) cells. Separation from diacylglycerol kinase activity. *J Biol Chem, 265*(31), 18803-18808.

Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F., Matsuki, Y., & Ochiya, T. (2010). Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J Biol Chem, 285*(23), 17442-17452. doi:10.1074/jbc.M110.107821

Kremer, G. J., Atzpodien, W., & Schnellbacher, E. (1975). Plasma glycosphingolipids in diabetics and normals. *Klin Wochenschr, 53*(13), 637-638.

Kuemmel, T. A., Thiele, J., Schroeder, R., & Stoffel, W. (1997). Pathology of visceral organs and bone marrow in an acid sphingomyelinase deficient knock-out mouse line, mimicking human Niemann-Pick disease type A. A light and electron microscopic study. *Pathol Res Pract, 193*(10), 663-671. doi:10.1016/S0344-0338(97)80025-8

Kwak, D. H., Rho, Y. I., Kwon, O. D., Ahan, S. H., Song, J. H., Choo, Y. K., . . . Jung, K. Y. (2003). Decreases of ganglioside GM3 in streptozotocin-induced diabetic glomeruli of rats. *Life Sci, 72*(17), 1997-2006.

Lambeth, J. D., Krause, K. H., & Clark, R. A. (2008). NOX enzymes as novel targets for drug development. *Semin Immunopathol, 30*(3), 339-363. doi:10.1007/s00281-008-0123-6

Lan, T., Liu, W., Xie, X., Xu, S., Huang, K., Peng, J., . . . Huang, H. (2011). Sphingosine kinase-1 pathway mediates high glucose-induced fibronectin expression in glomerular mesangial cells. *Mol Endocrinol, 25*(12), 2094-2105. doi:10.1210/me.2011-0095

Launay-Vacher, V., Oudard, S., Janus, N., Gligorov, J., Pourrat, X., Rixe, O., . . . Group, C. M. S. (2007). Prevalence of renal insufficiency in cancer patients and implications for anticancer drug management: the renal insufficiency and anticancer medications (IRMA) study. *Cancer, 110*(6), 1376-1384.

Lavie, Y., Cao, H.-t., Bursten, S. L., Giuliano, A. E., & Cabot, M. C. (1996). Accumulation of glucosylceramides in multidrug-resistant cancer cells. *Journal of Biological Chemistry, 271*(32), 19530-19536.

Lee, M.-J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., . . . Hla, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell, 99*(3), 301-312.

Lee, S. Y., Hong, I. K., Kim, B. R., Shim, S. M., Sung Lee, J., Lee, H. Y., . . . Park, T. S. (2015). Activation of sphingosine kinase 2 by endoplasmic reticulum stress ameliorates hepatic steatosis and insulin resistance in mice. *Hepatology, 62*(1), 135-146. doi:10.1002/hep.27804

Lemonnier, L. A., Dillehay, D. L., Vespremi, M. J., Abrams, J., Brody, E., & Schmelz, E. M. (2003). Sphingomyelin in the suppression of colon tumors: prevention versus intervention. *Archives of biochemistry and biophysics, 419*(2), 129-138.

Liu, G., Han, F., Yang, Y., Xie, Y., Jiang, H., Mao, Y., . . . Chen, J. (2011). Evaluation of sphingolipid metabolism in renal cortex of rats with streptozotocin-induced

diabetes and the effects of rapamycin. *Nephrol Dial Transplant, 26*(5), 1493-1502. doi:10.1093/ndt/gfq633

Liu, Y.-y., Han, T.-y., Giuliano, A. E., & CABOT, M. C. (2001). Ceramide glycosylation potentiates cellular multidrug resistance. *The FASEB Journal, 15*(3), 719-730.

Liu, Y.-Y., Han, T. Y., Yu, J. Y., Bitterman, A., Le, A., Giuliano, A. E., & Cabot, M. C. (2004). Oligonucleotides blocking glucosylceramide synthase expression selectively reverse drug resistance in cancer cells. *Journal of lipid research, 45*(5), 933-940.

Lopez, B., Salom, M. G., Arregui, B., Valero, F., & Fenoy, F. J. (2003). Role of superoxide in modulating the renal effects of angiotensin II. *Hypertension, 42*(6), 1150-1156. doi:10.1161/01.HYP.0000101968.09376.79

Lucci, A., Cho, W., Han, T., Giuliano, A., Morton, D., & Cabot, M. (1998). Glucosylceramide: a marker for multiple-drug resistant cancers. *Anticancer research, 18*(1B), 475-480.

MacKinnon, A. C., Buckley, A., Chilvers, E. R., Rossi, A. G., Haslett, C., & Sethi, T. (2002). Sphingosine kinase: a point of convergence in the action of diverse neutrophil priming agents. *The Journal of Immunology, 169*(11), 6394-6400.

Mahdy, A. E., Cheng, J. C., Li, J., Elojeimy, S., Meacham, W. D., Turner, L. S., . . . Liu, X. (2009). Acid ceramidase upregulation in prostate cancer cells confers resistance to radiation: AC inhibition, a potential radiosensitizer. *Mol Ther, 17*(3), 430-438. doi:10.1038/mt.2008.281

Maines, L. W., Fitzpatrick, L. R., French, K. J., Zhuang, Y., Xia, Z., Keller, S. N., . . . Smith, C. D. (2008). Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. *Digestive diseases and sciences, 53*(4), 997-1012.

Maines, L. W., French, K. J., Wolpert, E. B., Antonetti, D. A., & Smith, C. D. (2006). Pharmacologic manipulation of sphingosine kinase in retinal endothelial cells: implications for angiogenic ocular diseases. *Invest Ophthalmol Vis Sci, 47*(11), 5022-5031. doi:10.1167/iovs.05-1236

Mao, X. W., Nishiyama, N. C., Campbell-Beachler, M., Gifford, P., Haynes, K. E., Gridley, D. S., & Pecaut, M. J. Role of NADPH Oxidase as a Mediator of Oxidative Damage in Low-Dose Irradiated and Hindlimb-Unloaded Mice. *Radiation Research, 188*(4), 392-399.

Mao, X. W., Nishiyama, N. C., Pecaut, M. J., Campbell-Beachler, M., Gifford, P., Haynes, K. E., . . . Gridley, D. S. Simulated Microgravity and Low-Dose/Low-Dose-Rate Radiation Induces Oxidative Damage in the Mouse Brain. *Radiation Research, 185*(6), 647-657.

Martinez, R. M., Pinho-Ribeiro, F. A., Steffen, V. S., Caviglione, C. V., Fattori, V., Bussmann, A. J. C., . . . Casagrande, R. trans-Chalcone, a flavonoid precursor, inhibits UV-induced skin inflammation and oxidative stress in mice by targeting NADPH oxidase and cytokine production. *Photochemical & Photobiological Sciences, 16*(7), 1162-1173.

Matloubian, M., Lo, C. G., Cinamon, G., Lesneski, M. J., Xu, Y., Brinkmann, V., . . . Cyster, J. G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature, 427*(6972), 355.

Matmati, N., Metelli, A., Tripathi, K., Yan, S., Mohanty, B. K., & Hannun, Y. A. (2013). Identification of C18:1-phytoceramide as the candidate lipid mediator

for hydroxyurea resistance in yeast. *J Biol Chem, 288*(24), 17272-17284. doi:10.1074/jbc.M112.444802

Mazzei, J. C., Zhou, H., Brayfield, B. P., Hontecillas, R., Bassaganya-Riera, J., & Schmelz, E. M. (2011). Suppression of intestinal inflammation and inflammation-driven colon cancer in mice by dietary sphingomyelin: importance of peroxisome proliferator-activated receptor γ expression. *The Journal of nutritional biochemistry, 22*(12), 1160-1171.

Merscher, S., & Fornoni, A. (2014). Podocyte pathology and nephropathy - sphingolipids in glomerular diseases. *Front Endocrinol (Lausanne), 5*, 127. doi:10.3389/fendo.2014.00127

Mesicek, J., Lee, H., Feldman, T., Jiang, X., Skobeleva, A., Berdyshev, E. V., . . . Kolesnick, R. (2010). Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell Signal, 22*(9), 1300-1307. doi:10.1016/j.cellsig.2010.04.006

Meyer, T. W., Bennett, P. H., & Nelson, R. G. (1999). Podocyte number predicts long-term urinary albumin excretion in Pima Indians with Type II diabetes and microalbuminuria. *Diabetologia, 42*(11), 1341-1344. doi:10.1007/s001250051447

Miranda, S. R., He, X., Simonaro, C. M., Gatt, S., Dagan, A., Desnick, R. J., & Schuchman, E. H. (2000). Infusion of recombinant human acid sphingomyelinase into niemann-pick disease mice leads to visceral, but not neurological, correction of the pathophysiology. *FASEB J, 14*(13), 1988-1995. doi:10.1096/fj.00-0014com

Mitrofanova, A., Mallela, S., Ducasa, G., Yoo, T., Rosenfeld-Gur, E., Zelnik, I., . . . Sloan, A. (2019). SMPDL3b modulates insulin receptor signaling in diabetic kidney disease. *Nature Communications, 10*(1), 2692.

Mitsutake, S., Kim, T.-J., Inagaki, Y., Kato, M., Yamashita, T., & Igarashi, Y. (2004). Ceramide kinase is a mediator of calcium-dependent degranulation in mast cells. *Journal of Biological Chemistry, 279*(17), 17570-17577.

Montefusco, D. J., Matmati, N., & Hannun, Y. A. (2014). The yeast sphingolipid signaling landscape. *Chem Phys Lipids, 177*, 26-40. doi:10.1016/j.chemphyslip.2013.10.006

Montero A, M. K., Khan RZ, et al (2000). F(2)-isoprostanes mediate high glucose-induced TGF-beta synthesis and glomerular proteinuria in experimental type I diabetes. *Kidney Int*(58), 1963-1972.

Murphy, S. M., Ernst, D., Wei, Y., Laura, M., Liu, Y. T., Polke, J., . . . Reilly, M. M. (2013). Hereditary sensory and autonomic neuropathy type 1 (HSANI) caused by a novel mutation in SPTLC2. *Neurology, 80*(23), 2106-2111. doi:10.1212/WNL.0b013e318295d789

Najafian, B., Svarstad, E., Bostad, L., Gubler, M. C., Tondel, C., Whitley, C., & Mauer, M. (2011). Progressive podocyte injury and globotriaosylceramide (GL-3) accumulation in young patients with Fabry disease. *Kidney Int, 79*(6), 663-670. doi:10.1038/ki.2010.484

Nance, C. S., Klein, C. J., Banikazemi, M., Dikman, S. H., Phelps, R. G., McArthur, J. C., . . . Desnick, R. J. (2006). Later-onset Fabry disease: an adult variant presenting with the cramp-fasciculation syndrome. *Arch Neurol, 63*(3), 453-457. doi:10.1001/archneur.63.3.453

Nava, V. E., Cuvillier, O., Edsall, L. C., Kimura, K., Milstien, S., Gelmann, E. P., & Spiegel, S. (2000). Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer Res, 60*(16), 4468-4474.

Nava, V. E., Hobson, J. P., Murthy, S., Milstien, S., & Spiegel, S. (2002). Sphingosine kinase type 1 promotes estrogen-dependent tumorigenesis of breast cancer MCF-7 cells. *Exp Cell Res, 281*(1), 115-127.

Ng, M. L., Wadham, C., & Sukocheva, O. A. (2017). The role of sphingolipid signalling in diabetesassociated pathologies (Review). *Int J Mol Med, 39*(2), 243-252. doi:10.3892/ijmm.2017.2855

Niedowicz, D. M., & Daleke, D. L. (2005). The role of oxidative stress in diabetic complications. *Cell Biochem Biophys, 43*(2), 289-330. doi:10.1385/cbb:43:2:289

Norris, J. S., Bielawska, A., Day, T., El-Zawahri, A., ElOjeimy, S., Hannun, Y., . . . Liu, X. (2006). Combined therapeutic use of AdGFPFasL and small molecule inhibitors of ceramide metabolism in prostate and head and neck cancers: a status report. *Cancer Gene Ther, 13*(12), 1045-1051. doi:10.1038/sj.cgt.7700965

Osawa, Y., Seki, E., Kodama, Y., Suetsugu, A., Miura, K., Adachi, M., . . . Seishima, M. (2011). Acid sphingomyelinase regulates glucose and lipid metabolism in hepatocytes through AKT activation and AMP-activated protein kinase suppression. *FASEB J, 25*(4), 1133-1144. doi:10.1096/fj.10-168351

Pagtalunan, M. E., Miller, P. L., Jumping-Eagle, S., Nelson, R. G., Myers, B. D., Rennke, H. G., . . . Meyer, T. W. (1997). Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest, 99*(2), 342-348. doi:10.1172/JCI119163

Pawluczyk, I. Z., Ghaderi Najafabadi, M., Patel, S., Desai, P., Vashi, D., Saleem, M. A., & Topham, P. S. (2014). Sialic acid attenuates puromycin aminonucleoside-induced desialylation and oxidative stress in human podocytes. *Exp Cell Res, 320*(2), 258-268. doi:10.1016/j.yexcr.2013.10.017

Pettus, B. J., Bielawska, A., Spiegel, S., Roddy, P., Hannun, Y. A., & Chalfant, C. E. (2003). Ceramide kinase mediates cytokine and calcium ionophore-induced arachidonic acid release. *Journal of Biological Chemistry*.

Pettus, B. J., Bielawska, A., Subramanian, P., Wijesinghe, D. S., Maceyka, M., Leslie, C. C., . . . Hannun, Y. A. (2003). Ceramide-1-phosphate is a direct activator of cytosolic phospholipase A2. *Journal of Biological Chemistry*.

Prieschl, E. E., Csonga, R., Novotny, V., Kikuchi, G. E., & Baumruker, T. (1999). The Balance between Sphingosine and Sphingosine-1-Phosphate Is Decisive for Mast Cell Activation after Fc∈ Receptor I Triggering. *Journal of Experimental Medicine, 190*(1), 1-8.

Quinta, R., Rodrigues, D., Assuncao, M., Macedo, M. F., Azevedo, O., Cunha, D., . . . Sa Miranda, M. C. (2014). Reduced glucosylceramide in the mouse model of Fabry disease: correction by successful enzyme replacement therapy. *Gene, 536*(1), 97-104. doi:10.1016/j.gene.2013.11.073

Rhyu, D. Y., Yang, Y., Ha, H., Lee, G. T., Song, J. S., Uh, S. T., & Lee, H. B. (2005). Role of reactive oxygen species in TGF-beta1-induced mitogen-activated protein kinase activation and epithelial-mesenchymal transition in renal tubular epithelial cells. *J Am Soc Nephrol, 16*(3), 667-675. doi:10.1681/ASN.2004050425

Riley, P. A. (1994). Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int J Radiat Biol, 65*(1), 27-33.

Robbins, M. E., & Zhao, W. (2004). Chronic oxidative stress and radiation-induced late normal tissue injury: a review. *Int J Radiat Biol, 80*(4), 251-259. doi:10.1080/09553000410001692726

Saad, A. F., Meacham, W. D., Bai, A., Anelli, V., Elojeimy, S., Mahdy, A. E., . . . Liu, X. (2007). The functional effects of acid ceramidase overexpression in prostate cancer progression and resistance to chemotherapy. *Cancer Biol Ther, 6*(9), 1455-1460.

Samad, F., Hester, K. D., Yang, G., Hannun, Y. A., & Bielawski, J. (2006). Altered adipose and plasma sphingolipid metabolism in obesity: a potential mechanism for cardiovascular and metabolic risk. *Diabetes, 55*(9), 2579-2587. doi:10.2337/db06-0330

Sandhoff, K., Andreae, U., & Jatzkewitz, H. (1968). Deficient hexozaminidase activity in an exceptional case of Tay-Sachs disease with additional storage of kidney globoside in visceral organs. *Life Sci, 7*(6), 283-288.

Satchell, S., Tasman, C., Singh, A., Ni, L., Geelen, J., Von Ruhland, C., . . . Mathieson, P. (2006). Conditionally immortalized human glomerular endothelial cells expressing fenestrations in response to VEGF. *Kidney international, 69*(9), 1633-1640.

Schmelz, E. M., Bushnev, A. S., Dillehay, D. L., Liotta, D. C., & Merrill Jr, A. H. (1997). Suppression of aberrant colonic crypt foci by synthetic sphingomyelins with saturated or unsaturated sphingoid base backbones.

Schmelz, E. M., Bushnev, A. S., Dillehay, D. L., Sullards, M. C., Liotta, D. C., & Merrill, A. H. (1999). Ceramide-β-D-glucuronide: synthesis, digestion, and suppression of early markers of colon carcinogenesis. *Cancer research, 59*(22), 5768-5772.

Schmelz, E. M., Dillehay, D. L., Webb, S. K., Reiter, A., Adams, J., & Merrill, A. H. (1996). Sphingomyelin consumption suppresses aberrant colonic crypt foci and increases the proportion of adenomas versus adenocarcinomas in CF1 mice treated with 1, 2-dimethylhydrazine: implications for dietary sphingolipids and colon carcinogenesis. *Cancer research, 56*(21), 4936-4941.

Schmelz, E. M., Roberts, P. C., Kustin, E. M., Lemonnier, L. A., Sullards, M. C., Dillehay, D. L., & Merrill, A. H. (2001). Modulation of intracellular β-catenin localization and intestinal tumorigenesis in vivo and in vitro by sphingolipids. *Cancer research, 61*(18), 6723-6729.

Schmelz, E. M., Sullards, M. C., Dillehay, D. L., & Merrill Jr, A. H. (2000). Colonic cell proliferation and aberrant crypt foci formation are inhibited by dairy glycosphingolipids in 1, 2-dimethylhydrazine-treated CF1 mice. *The Journal of nutrition, 130*(3), 522-527.

Scott, K. F., Bryant, K. J., & Bidgood, M. J. (1999). Functional coupling and differential regulation of the Phospholipase A2‐cyclooxygenase pathways in inflammation. *Journal of leukocyte biology, 66*(4), 535-541.

Seelan, R. S., Qian, C., Yokomizo, A., Bostwick, D. G., Smith, D. I., & Liu, W. (2000). Human acid ceramidase is overexpressed but not mutated in prostate cancer. *Genes Chromosomes Cancer, 29*(2), 137-146.

Senggutuvan, P., Cameron, J. S., Hartley, R. B., Rigden, S., Chantler, C., Haycock, G., . . . Koffman, G. (1990). Recurrence of focal segmental glomerulosclerosis in transplanted kidneys: analysis of incidence and risk factors in 59 allografts. *Pediatr Nephrol, 4*(1), 21-28.

Shen, H., Giordano, F., Wu, Y., Chan, J., Zhu, C., Milosevic, I., . . . De Camilli, P. (2014). Coupling between endocytosis and sphingosine kinase 1 recruitment. *Nat Cell Biol, 16*(7), 652-662. doi:10.1038/ncb2987

Singh, A., Satchell, S. C., Neal, C. R., McKenzie, E. A., Tooke, J. E., & Mathieson, P. W. (2007). Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. *J Am Soc Nephrol, 18*(11), 2885-2893. doi:10.1681/ASN.2007010119

Skinner, R., Kaplan, R., & Nathan, P. C. (2013). Renal and pulmonary late effects of cancer therapy. *Semin Oncol, 40*(6), 757-773. doi:10.1053/j.seminoncol.2013.09.009

Snider, A. J., Kawamori, T., Bradshaw, S. G., Orr, K. A., Gilkeson, G. S., Hannun, Y. A., & Obeid, L. M. (2009). A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. *The FASEB Journal, 23*(1), 143-152.

Spiegel, S., & Milstien, S. (2011). The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol, 11*(6), 403-415. doi:10.1038/nri2974

Steffes, M. W., Schmidt, D., McCrery, R., Basgen, J. M., & International Diabetic Nephropathy Study, G. (2001). Glomerular cell number in normal subjects and in type 1 diabetic patients. *Kidney Int, 59*(6), 2104-2113. doi:10.1046/j.1523-1755.2001.00725.x

Strader, C. R., Pearce, C. J., & Oberlies, N. H. (2011). Fingolimod (FTY720): a recently approved multiple sclerosis drug based on a fungal secondary metabolite. *J Nat Prod, 74*(4), 900-907. doi:10.1021/np2000528

Struckhoff, A. P., Bittman, R., Burow, M. E., Clejan, S., Elliott, S., Hammond, T., . . . Beckman, B. S. (2004). Novel ceramide analogs as potential chemotherapeutic agents in breast cancer. *Journal of Pharmacology and Experimental Therapeutics, 309*(2), 523-532.

Studer RK, C. P., DeRubertis FR. (1997). Antioxidant inhibition of protein kinase C-signaled increases in transforming growth factor-beta in mesangial cells. *Metabolism*(46), 918-925.

Sugiura, M., Kono, K., Liu, H., Shimizugawa, T., Minekura, H., Spiegel, S., & Kohama, T. (2002). Ceramide kinase, a novel lipid kinase. Molecular cloning and functional characterization. *J Biol Chem, 277*(26), 23294-23300. doi:10.1074/jbc.M201535200

Summers, S. A. (2006). Ceramides in insulin resistance and lipotoxicity. *Prog Lipid Res, 45*(1), 42-72. doi:10.1016/j.plipres.2005.11.002

Symolon, H., Schmelz, E. M., Dillehay, D. L., & Merrill Jr, A. H. (2004). Dietary soy sphingolipids suppress tumorigenesis and gene expression in 1, 2-dimethylhydrazine-treated CF1 mice and Apc Min/+ mice. *The Journal of nutrition, 134*(5), 1157-1161.

Tagami, S., Inokuchi Ji, J., Kabayama, K., Yoshimura, H., Kitamura, F., Uemura, S., . . . Igarashi, Y. (2002). Ganglioside GM3 participates in the pathological conditions of insulin resistance. *J Biol Chem, 277*(5), 3085-3092. doi:10.1074/jbc.M103705200

Taniguchi, M., Ogiso, H., Takeuchi, T., Kitatani, K., Umehara, H., & Okazaki, T. (2015). Lysosomal ceramide generated by acid sphingomyelinase triggers cytosolic cathepsin B-mediated degradation of X-linked inhibitor of apoptosis protein in natural killer/T lymphoma cell apoptosis. *Cell Death Dis, 6*, e1717. doi:10.1038/cddis.2015.82

Tao, C., Sifuentes, A., & Holland, W. L. (2014). Regulation of glucose and lipid homeostasis by adiponectin: effects on hepatocytes, pancreatic beta cells and adipocytes. *Best Pract Res Clin Endocrinol Metab, 28*(1), 43-58. doi:10.1016/j.beem.2013.11.003

Tasaki, M., Shimizu, A., Hanekamp, I., Torabi, R., Villani, V., & Yamada, K. (2014). Rituximab treatment prevents the early development of proteinuria following pig-to-baboon xeno-kidney transplantation. *J Am Soc Nephrol, 25*(4), 737-744. doi:10.1681/ASN.2013040363

Tatematsu, M., Imaida, K., Ito, N., Togari, H., Suzuki, Y., & Ogiu, T. (1981). Sandhoff disease. *Acta Pathol Jpn, 31*(3), 503-512.

Teixeira, V., & Costa, V. (2016). Unraveling the role of the Target of Rapamycin signaling in sphingolipid metabolism. *Prog Lipid Res, 61*, 109-133. doi:10.1016/j.plipres.2015.11.001

Thurberg, B. L., Rennke, H., Colvin, R. B., Dikman, S., Gordon, R. E., Collins, A. B., . . . O'Callaghan, M. (2002). Globotriaosylceramide accumulation in the Fabry kidney is cleared from multiple cell types after enzyme replacement therapy. *Kidney Int, 62*(6), 1933-1946. doi:10.1046/j.1523-1755.2002.00675.x

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., . . . Simons, M. (2008). Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science, 319*(5867), 1244-1247. doi:10.1126/science.1153124

Turinsky, J., O'Sullivan, D. M., & Bayly, B. P. (1990). 1,2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. *J Biol Chem, 265*(28), 16880-16885.

Turinsky, J., O'Sullivan, D. M., & Bayly, B. P. (1990). 1, 2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. *Journal of Biological Chemistry, 265*(28), 16880-16885.

Vasavada, N., & Agarwal, R. (2005). Role of oxidative stress in diabetic nephropathy. *Advances in chronic kidney disease, 12*(2), 146-154.

Verzola, D., Gandolfo, M. T., Ferrario, F., Rastaldi, M. P., Villaggio, B., Gianiorio, F., . . . Garibotto, G. (2007). Apoptosis in the kidneys of patients with type II diabetic nephropathy. *Kidney Int, 72*(10), 1262-1272. doi:10.1038/sj.ki.5002531

Visentin, B., Vekich, J. A., Sibbald, B. J., Cavalli, A. L., Moreno, K. M., Matteo, R. G., . . . Hall, H. S. (2006). Validation of an anti-sphingosine-1-phosphate antibody as a potential therapeutic in reducing growth, invasion, and angiogenesis in multiple tumor lineages. *Cancer cell, 9*(3), 225-238.

Wakashima, T., Abe, K., & Kihara, A. (2014). Dual functions of the trans-2-enoyl-CoA reductase TER in the sphingosine 1-phosphate metabolic pathway and in fatty acid elongation. *J Biol Chem, 289*(36), 24736-24748. doi:10.1074/jbc.M114.571869

Wang, J. H., Redmond, H. P., Watson, R. W., Condron, C., & Bouchier-Hayes, D. (1999). Involvement of tyrosine protein kinase in IFN-gamma-induced human endothelial cell apoptosis. *Shock, 11*(5), 311-318.

Wegner, M. S., Schiffmann, S., Parnham, M. J., Geisslinger, G., & Grosch, S. (2016). The enigma of ceramide synthase regulation in mammalian cells. *Prog Lipid Res, 63*, 93-119. doi:10.1016/j.plipres.2016.03.006

White, K. E., Bilous, R. W., Marshall, S. M., El Nahas, M., Remuzzi, G., Piras, G., . . . Viberti, G. (2002). Podocyte number in normotensive type 1 diabetic patients with albuminuria. *Diabetes, 51*(10), 3083-3089.

Xie, B., Shen, J., Dong, A., Rashid, A., Stoller, G., & Campochiaro, P. A. (2009). Blockade of sphingosine-1-phosphate reduces macrophage influx and retinal and choroidal neovascularization. *J Cell Physiol, 218*(1), 192-198. doi:10.1002/jcp.21588

Yamakawa, T. (1996). A reflection on the early history of glycosphingolipids. *Glycoconj J, 13*(2), 123-126.

Yamashita, T., Hashiramoto, A., Haluzik, M., Mizukami, H., Beck, S., Norton, A., . . . Proia, R. L. (2003). Enhanced insulin sensitivity in mice lacking ganglioside GM3. *Proc Natl Acad Sci U S A, 100*(6), 3445-3449. doi:10.1073/pnas.0635898100

Yoo, T. H., Pedigo, C. E., Guzman, J., Correa-Medina, M., Wei, C., Villarreal, R., . . . Merscher, S. (2015). Sphingomyelinase-like phosphodiesterase 3b expression levels determine podocyte injury phenotypes in glomerular disease. *J Am Soc Nephrol, 26*(1), 133-147. doi:10.1681/ASN.2013111213

Yoshikawa, T. T., Dube, W. F., & Wahby, V. (1994). Geriatric training in the Department of Veterans Affairs. *Am J Med, 97*(4A), 26S-28S.

Yu, H., Sun, S. Q., Gu, X. B., Wang, W., & Gao, X. S. Atorvastatin prolongs the lifespan of radiation-induced reactive oxygen species in PC-3 prostate cancer cells to enhance the cell killing effect. *Oncology Reports, 37*(4), 2049-2056.

Yuyama, K., Sun, H., Mitsutake, S., & Igarashi, Y. (2012). Sphingolipid-modulated exosome secretion promotes clearance of amyloid-beta by microglia. *J Biol Chem, 287*(14), 10977-10989. doi:10.1074/jbc.M111.324616

Zeidan, Y. H., & Hannun, Y. A. (2007). Translational aspects of sphingolipid metabolism. *Trends Mol Med, 13*(8), 327-336. doi:10.1016/j.molmed.2007.06.002

Zeidan, Y. H., Pettus, B. J., Elojeimy, S., Taha, T., Obeid, L. M., Kawamori, T., . . . Hannun, Y. A. (2006). Acid ceramidase but not acid sphingomyelinase is required for tumor necrosis factor-α-induced PGE2 production. *Journal of Biological Chemistry, 281*(34), 24695-24703.

Zeidan, Y. H., Wu, B. X., Jenkins, R. W., Obeid, L. M., & Hannun, Y. A. (2008). A novel role for protein kinase Cdelta-mediated phosphorylation of acid sphingomyelinase in UV light-induced mitochondrial injury. *FASEB J, 22*(1), 183-193. doi:10.1096/fj.07-8967com