SPHINGOLIPIDS AND PROSTATE CANCER: COMPLEX MOLECULES FOR A COMPLEX DISEASE

PEDRO FILIPE DA COSTA PINHEIRO
SÃO JOÃO DA MADEIRA, PORTUGAL

B.S. IN BIOLOGY, UNIVERSITY OF PORTO, 2011
M.SC. IN MOLECULAR ONCOLOGY, UNIVERSITY OF PORTO, 2013

A DISSERTATION PRESENTED TO THE GRADUATE FACULTY OF THE UNIVERSITY OF VIRGINIA IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PATHOLOGY

UNIVERSITY OF VIRGINIA
MAY 2021

Not like the brazen giant of Greek fame,
With conquering limbs astride from land to land;
Here at our sea-washed, sunset gates shall stand
A mighty woman with a torch, whose flame
Is the imprisoned lightning, and her name
Mother of Exiles. From her beacon-hand
Glows world-wide welcome; her mild eyes command
The air-bridged harbor that twin cities frame.

"Keep, ancient lands, your storied pomp!" cries she
With silent lips. "Give me your tired, your poor,
Your huddled masses yearning to breathe free,
The wretched refuse of your teeming shore.
Send these, the homeless, tempest-tost to me,
I lift my lamp beside the golden door!"

Emma Lazarus, 1883

DEDICATION

This entire Thesis is dedicated to my partner in life, Katherine Still. We started this journey as class and tablemates, became friends, started to date, moved in together, and became cat-parents together. And what a journey it has been! I've always wanted to come to the US to get my PhD and that was my dream, what I was not aware of was that 3500 miles way I would find the best person I've ever met! Someone that was raised in a different country, but with similar values, and that is what makes our relationship so special!

Throughout our years together, you've been my biggest advocate making it clear to people that I was better than what credit they would give me or when I wouldn't be taken seriously. Early on, you took a lot of interest in me and devoted part of your time in helping me navigate life in a different country. But more than that, I want you to know that you are truly an inspiration for everyone that surrounds you, even if most people don't tell you that. You are the most thoughtful, talented, hardworking, deep-thinker, smart, intelligent, person I've ever met. And to top all of that, your values make you a person that we should all strive to copy.

Early on I understood that I had never met someone like you, you truly embody the spirit of UVa to go "all in for excellence". Your work ethic on every single day is still something that I can't really fathom to this day, but one thing is certain: it has made me better, trying to mimic it. Your thoughtfulness and deep thinking is unmatched, and has made me more aware of my surroundings, my past, our future, and myself. Your honest assessments are delivered with kindness, even when not necessarily granted, and that has led me to try to become calmer when addressing my frustrations with others. I sincerely admire you, and I still can't believe one day you told me I would have nothing to write about you, because that's who you are: an humble human being, always wanting to be better, and that my Darling is your best quality. And that's what I do every day: I follow your lead by trying to be better.

We also have similar tastes in music, food, shows and all our routines won't be specific to graduate school because they will continue throughout our life together, but will definitely leave a mark on these 6 years. The Covid-19 pandemic has afflicted millions of people, and it affected us as well; it brought us home for 3 months where we would only see each other. Despite all the challenges the world faced, I sincerely can't be more thankful for that time with you. Our Theses were written together, how many people have that privilege? We adopted our beloved cat Virginia "Ginny" Mae and together have tried our best to give her the best home possible. We had the

chance throughout all of our daily walks to learn more about each other, to talk deeply about subjects that matter to us, and to together, keep growing; these are priceless things. You helped me deal with missing Home; now, being with you makes me feel at Home.

I thank you for all of your scientific support, all our discussions about presentations, techniques, results; for all of your patience and never giving up on me; for letting me be myself even if that entails hour-long conversations about what animals are thinking; and specially, for all of your love. I won't say that I wouldn't finish my degree without you, but I can truly say that I wouldn't be the person I am today, that I wouldn't be as thrilled about my future, and that is way more important in life.

For all of these reasons and more, Thank you so much I will be forever grateful to whoever put you on my path in life! This one is truly for you, my Love!

ACKNOWLEDGEMENTS

Usually when acknowledging and thanking the help one is given throughout a journey, people refer to the fact that it takes a village to achieve something. Well, I've always dreamt big and have a flair for the grandeur, thus, this Thesis required people in two countries, in two different continents separated by the second largest Ocean on planet Earth. For this reason, this section will be written in two different languages, Portuguese and English.

À Professora Carmen Jerónimo e ao Professor Rui Henrique: por me terem dado a minha primeira oportunidade, e por todas as oportunidades que me proporcionaram durante todos estes anos. Apesar do ambiente científico em Portugal não ser o melhor, não posso estar mais orgulhoso do laboratório que me deu as ferramentas essenciais para chegar a este ponto na minha carreira. E para isso muito contribuiu a vossa confiança nas minhas capacidades e todo o vosso trabalho como mentores em me ajudar a desenvolver as mesmas! Todo o meu tempo no laboratório resultou num currículo que me permitiu ser competitivo em concursos internacionais, e que me abriu portas que dificilmente julgava possíveis. A ambos, um muito obrigado por tudo o que fizeram por mim!

À Comissão Fulbright, Portugal, pela pessoa da Otília Reis, e em memória da Paula Lemos: agradeço a oportunidade que me proporcionaram de cumprir o sonho de estudar e obter o meu Doutoramento nos EUA. Um obrigado por todo o apoio que me deram e dão aos Portugueses que viajam para os EUA em busca de melhores oportunidades e mostrar ao Mundo a qualidade dos profissionais do nosso País. Por isso e muito mais, o meu sincero Obrigado!

To Joel Hockensmith: you were the first person I met from UVa and at Charlotesville. I will never forget the time I landed in Charlottesville, and you were waiting for me at the airport, as well as our trip to buy food and sheets for my first week at UVa. From my first interview to the moment I joined the program you made me feel like I belonged at UVa and for being the biggest advocate of me joining BIMS, I will be forever grateful.

To Janet Cross: I can truly say that I've never met anyone that advocates as much as you do for the graduate students. You set the example for leadership, compassion, attention, and I'm very thankful that our paths have crossed. You have given me a lot throughout my time at UVa, not only from all the thoughtful questions during committee meetings and presentations, but very special to me were all our conversations about career, life, and our interests throughout these years. Thank you

for always supporting and helping me becoming not only a better scientist, but also a better person, always aware of others and how we can positively impact their lives. Thank you, Janet!

To my other committee members Dan Gioeli, David Wotton, and Christopher Moskaluk: I'm so glad that all of you accepted my invitation to be part of my committee and journey in graduate school. You have made this Thesis possible by your constant enquiries and interest in the work, and also pushing me to a higher level not only in productivity but also specially in my critical thinking of the literature and my own results/direction. Thank you for helping me reaching a higher level as a scientist.

To Richard Tanson: you have had to deal with all of my questions about visa status, traveling as a foreigner student, and your no-nonsense answers makes things so clear and helpful all the time. I want to thank you for all the work you did for me and all the advice, it made my experience a better one, and it made me a more informed international student in the US!

To John Lukens: thank you for embracing myself and my personality, for all the jokes, scientific help, soccer talk, but most importantly thank you for caring so deeply about Kate and myself! You have been a great inspiration for us, your support and friendship means a lot to me! Thank you Notorious Lukens!

To the members of the Kester lab, present and past: thank you for a great lab environment throughout the years and making the lab a great place to be!

To Todd Fox: I have to start by admitting that when I joined the lab, I thought you were quite intimidating. Throughout the years we have got closer, and your knowledge and intelligence have made my project and Thesis better, and I learned a lot from you. I will always appreciate your interest in myself and my science and all the knowledge and advice you have passed along. I can also add our time at NASA and the Mardi Gras parade in Galveston as some of the best work-related memories throughout grad school. All our conversations about science, projects, basketball, and other subjects will be dearly missed given how much relief and enthusiasm (most times) they provided! I hope that we can continue to work together in the future and having those conversations even if at a distance!

To the Kester boys, Kelly Drews, Logan Patterson, and Jeremy Shaw: thank you for welcoming me to the lab with arms wide open. Your presence and companionship made my time in lab so much easier and better. To Kelly: I will always appreciate your scientific acumen and knowledge, all of your help with presentations and meetings, game nights, our baseball talk (Go Yankees!), and all of

our soccer games (Kelly next!). To Logan: our work trips to Cashiers and Galveston, plus the drive for Kelly's wedding will remain as some of my fondest memories from graduate school. You were a blast to have around, and I am just really sorry that I suck at video games except Fifa. I hope you can forgive me for that! To Jeremy: how many hours did we talk during our time together in lab? To answer that question would be a very interesting exercise. All of our trips together for conferences and weddings, the fact that I was with you for your first international trip welcoming you to my home country, all the soccer games, the debates about the dodgeball rankings, the list goes on and on and on. Your scientific input made my project better, and I will always remember those conversations with great joy! Plus, who has ever give a joint talk at a conference? Thank you to the three of you, and I hope that we can continue our friendship for many years to come!

To Jeff Johnson: the biggest advantage of going to conferences is the friends one can make, and you were one of them. The sphingolipid community is a small community making it easier to get to know people well. But not everyone is like you: open immediately to the newcomers and taking an interest in their work. We have had quite a blast during those conferences and your visit to Charlottesville. You have always shown interest in knowing my story, and I'm very grateful for your friendship and support!

To Myles Cabot: the true King of sphingolipids (sorry Mark!), you have been a great friend and advocate from the moment we met at my first sphingolipid conference. You truly take the younger students under your wing and make them feel welcome into the sphingolipid community. Throughout the years I had the opportunity to attend different conferences with you, which helped us bond and strengthen our friendship. I will never forget how lucky I was to go through Old Jerusalem with you getting a history lesson from you and talking about your time in the city learning from your own history. You have been one my biggest advocates always encouraging, helping me with applications to different opportunities during my time in grad school, and above all we have had so much fun throughout the years! Muchas gracias amigo!

To Mark Kester, my mentor: mentor/friend, I guess? I couldn't have made a better decision when I decided to ask you to do my second rotation in your lab after being wowed by your nanoparticle-light show! At the time I was looking for a mentor that would give me freedom to pursue my own ideas, and in you I found that. To have the opportunity to develop my project I became a better scientist, and it has prepared me for the tough road ahead in my scientific journey. I will always appreciate that you

were open to novel ideas and pursuits, and that you always encouraged me to apply for different opportunities that could help me boost my curriculum and improve my skillset. From early on I could sense the trust and respect you had for my opinion and experience, which meant a lot and inspired me to continue to get better by giving everything I had for the lab and everyone's projects. You also allowed me to take time off to visit my family back home, never questioning that time or putting into jeopardy knowing that I would get the job done. I also don't know of anyone else in grad school that had the opportunity to go to so many conferences in different parts of the globe, and our trip to Israel (great tennis match!) will be at the top of my work-related experiences. You also have taught me a lot about the business world with our patents together as well as always being interested in sharing your knowledge of that side of things that students are not as familiar with. More than just a scientific mentor, I've had the privilege to develop a close relationship with you since we have so many interests in common. All the sports talk, all of your teaching on horse racing, attending UVA games, Super Bowl parties, the Saab, those are moments and things that most people are not lucky enough to get during their time as a graduate school. However, the most important piece for me is that you allowed me to be myself in lab even if it came at a cost for your mental health by how much I talk in lab meetings and how passionate I can be during scientific discussions. For all of this, I'm really grateful I started you as a mentor and end this journey with you as a mentor/friend, and thankful that you bet on me!

To my soccer teammates across the years: thank you for all the memories on the soccer field throughout the years! All of those games helped me stay in touch with something I love and more importantly allowed me to yell, curse, and vent the frustrations of tough weeks in grad school! It was a pleasure to share the field with all of you.

To Cat Lammert: you were one of the first people that I built a relationship in Charlottesville, and our love for soccer brought us together. You were very instrumental in my adaptation to the US, and I will always appreciate you for that! All the rides to parties and soccer games, all the games played and watched, those are very fond memories that I will always cherish. We had a great time even when we got together to do homework and attend class, and you were the one introducing me to Kate and Mike making our group a great source of entertainment and joy during the stress of core course. We also brought Silas and Mavy to our lives definitely enriching it further! I will never forget the hospitality of your family when we visited Maine,

and how proud I was when you and Kate crossed the finish line of your PhDs as well as the half ironman race! Thank you for your friendship, fellow November 7th baby!

To Mike Raymond: fellow member of the HTC and my good friend, thank you so much for everything! Our time together in graduate school made everything much easier; from countless flow runs, science talk, but mainly all of our fun and distracting conversations. Those moments were so helpful to get out of the stress caused by failed experiments and bad results. Our coffee runs followed by our discussions about several different subjects are still the envy of a lot of people. I feel like sometimes people don't take us seriously because of how much fun we try to have during work and having other interests than just science, and that I believe helped us develop a close friendship. All the fun texts, memes, and GIFs, the separate realities and personas we have built for the pets that belong to our lives still thrill me and make me laugh all the time. Basically, to understand our friendship people just have to connect the dots; it makes you think! I'm sure that we will continue building on our friendship and I'm thrilled to see what the future lays ahead for you as a scientist: one of the most creative people I've seen at the bench! Thank you my friend!

To the Coulson, Morais, and Ferreira family: I've known you since I was born (it has been a while!) and we have been family throughout these years. All the tales I would hear from you about the distant land where you lived are the major reason why I've always wanted to study and live in the United States. When I moved, I knew that even at a distance I would have you to support me and help me with anything I needed while away from my country. To have that trust for sure made it easier for me to get on this adventure. So many memories we have built together and to many more, I'm so thankful for all your friendship, support, and love for my family and myself. Thank you guys!

To the Still family: since Kate and I started dating, you have been family to me. You embraced me as one of your own, taking the time to learn more about my family and myself, as well as my country and culture. I'm very grateful for the way you included me in the family from the start, and how supportive you have been of Kate and I. We have lived great moments together so far and many more will come, but I will be eternally thankful for the person that you raised, the most important person in my life, Kate. You did and continue to do a fantastic job as parents and I'm so very lucky to be able to call you my own family. During the course of this work you were also interesting and asked relevant questions about the project what is ahead in my career. I'm very thankful for your support!

Aos meus amigos Diogo e Juca: geopolíticos do IPO, diferentes idades, diferentes maneiras de ser, mas uma curiosidade, paixão por conhecimento sobretudo histórico e geográfico que nos vem mantendo unidos. Aprendi, e aprendo imenso com vocês, e o vosso intelecto é um incentivo para quem vos rodeia estar sempre investido em querer saber mais! Além disso, pela vossa amizade ao longo dos anos, um muito obrigado!

Aos meus amigos Daniel, Fábio, Marco, Nuno: por tantas conversas mesmo à distância; por terem sempre tirado do vosso tempo para saberem de mim; por tantas jogatanas de GoT mesmo que isso implique choro e cartas atiradas para cima da mesa; por tantas jantaradas; acima de tudo, por toda a vossa amizade e carinho para comigo e com a minha família, muito obrigado, esta também é vossa! Maiores de sempre!

Ao grande Diogo: cedo críamos uma amizade quando nos conhecemos e essa amizade tem-se mantido ao longo de todos estes anos. Tanta conversa, jantarada, jogos do nosso FCPorto, filmes, ao longo dos anos que mantém a nossa amizade forte e a crescer! Em relação ao FCPorto, as conversas e jogos com o teu primo João e o grande Nuno Caniço são inolvidáveis, tamanha é a discrepância como que vemos os jogos e analisamos, acaba sempre ou por dar piada ou por dar para o "torto". Um muito obrigado por tudo, acima de tudo por nestes últimos anos teres tomado a iniciativa de te aproximar dos meus pais e os ajudado sempre que precisavam.

Ao grande João: longe vão os tempos em que te pisava o braço a distrair nas aulas. Longe vai o dia em que te pedi uma caneta no primeiro dia do nosso 10º ano ou todas as minhas vitórias no PES, longe vai o dia em que começamos a nossa amizade. É um prazer enorme crescer e ver-te crescer e lembrar os nossos tempos de sonhar com o futuro (acho que nos temos portado à altura daquilo que ambicionávamos). Um obrigado meu grande amigo, e ainda que com todos os sonhos, só consigo prever coisas ainda maiores no nosso futuro!

À minha cunhada: já lá vão uns aninhos desde que entraste na minha vida, e confesso que no início foi um bocadinho difícil ter de partilhar a atenção do meu irmão (tolice, eu sei!). Mas com o tempo fomos tornando-nos amigos e melhorando no nosso passatempo favorito, gozá-lo! Tal como me viste crescer, também eu te vi a ti, desde jogadora de andebol e um tanto ou quanto rebelde, até à fantástica Mãe que és, com um grande orgulho na pessoa que és! Obrigado por estares sempre presente na minha vida, por te preocupares, e por te interessares na minha vida profissional e pessoal, mas acima de tudo por seres um modelo de constante aprendizagem e dedicação.

À Leonor: quando nasceste estava numa reunião para decidir a resposta aos revisores de um artigo, quando saí do País tinhas pouco mais de 1 ano; continuas a mesma pequena de sempre, aos olhos do Tio serás sempre assim. É difícil não acompanhar o teu crescimento diariamente e mais presencialmente, mas é um orgulho ver-te formar a tua personalidade e ver-te crescer mesmo que seja à distância. Que o meu caminho te ensine que não há limites ao que nós queremos atingir, quando desde pequeninos pomos isso na cabeça. Literalmente mudaste o mundo de todos os que te rodeiam quando nasceste, e continuar a ver-te crescer é uma sensação de alegria constante. A ti só te tenho a agradecer existires e seres essa fonte de energia que todos nós adoramos!

Ao meu Mano: fim da Primavera e início do Verão de 2008, o mundo estava prestes a entrar em colapso financeiro mas a minha mais importante decisão até esse momento avizinhava-se: qual o curso a tirar na Universidade? Lembro-me como se fosse hoje de num jantar de família anunciar que me ia candidatar ao curso de Biologia, e de ser imediatamente recebido com um "Vais trabalhar para o Pingo Doce?" de tua parte. Será caso, 13 anos depois, para dizer que não tem corrido mal essa decisão? Essa tua pergunta é apenas um exemplo da tua devoção e preocupação comigo e com o meu futuro. Muitas pessoas que se cruzam no meu percurso falam da minha maturidade acima da idade, e a pessoa mais responsável por isso foste tu. Desde cedo me puseste debaixo da tua asa e me mostraste o mundo pelo prisma de alguém quase uma década mais velho. O facto de me teres incluído em saídas com amigos, viagens, festas, idas ao cinema, etc permitiram-me ir aprendendo como agir no mundo de adultos que ainda não estava próximo de entrar. E também acho que eu fui sempre te permitindo ter uma conexão com os teus tempos de criança, tantos jogos de hóquei de sala, defesas do Marchegiani, jogos de Fifa e PES. Para mim sempre foi fácil crescer no sentido que tenho sempre um ponto de guia em ti, e isso são coisas que não se aprendem na escola nem se ganham com diplomas. A tua ética profissional, o teu altruísmo, a tua dedicação, são coisas que só consigo almejar mas sei que não consigo alcançar, porque tu estás no topo. Sem ti e sem o teu apoio esta Tese não existiria, nem eu seria como sou, eternamente grato pelo teu amor!

Aos meus pais: no dia 18 de Junho de 2015 despedi-me de vocês no aeroporto do Porto, e sabia que só vos voltaria a ver presencialmente pelo Natal. Muita gente chama a isto um sacrifício de quem parte; na minha opinião o verdadeiro sacrifício é dos que ficam. Quem parte, parte com o sonho de conquistar novos mundos, novos objetivos, conhecer novas culturas, explorar a sua própria identidade. Quem fica, fica com a saudade. E este foi apenas mais uma sacrifício que vocês fizeram durante toda

a minha vida para me proporcionar as melhores e mais importantes experiências, a melhor educação, e a certeza de que estão sempre comigo para o que eu precisar. Esta Tese e este Doutoramento são tão ou mais vossos do que meus. Foram vocês que desde cedo me incutiram a estudar, a ler, a ponderar, a questionar, mesmo que essas questões depois vos dessem dores de cabeça. E também foram os primeiros, sobretudo a Mãe, a me meter na cabeça a ideia de ser cientista, de tirar um Doutoramento e deste Doutoramento ser nos EUA (desculpa, não foi em Harvard, mas perto!). E se tudo isto não fosse suficiente, a educação que me deram não podia ter sido melhor e é ainda mais importante do que qualquer ano letivo; sempre me incutiram os valores de respeitar o próximo, da importância da família, de ouvir quem está a falar com atenção, de ter a noção do nosso lugar no mundo, de trabalhar arduamente sem nunca perder noção da importância dos que nos rodeiam, humanidade e humildade. Sem vocês, sem o vosso apoio, e sem todos os sacrifícios que fizeram, tenho a certeza que não estaria onde estou, e espero que este momento seja um momento de orgulho para vocês, e em que vocês possam tirar um momento e celebrar não o meu feito, mas sobretudo o vosso trabalho! Vocês são os meus heróis!

I also would like to thank NIH/NCI and the UVa Cancer Center for their financial support that made this work possible.

SUMMARY

Prostate cancer (PCa) is the second most incident cancer in men worldwide, and despite vast amounts of research it remains one of the major epidemiological burdens in oncology. The prostate gland is regulated by male androgens that activate the Androgen Receptor (AR), a transcription factor and a master regulator of several different signaling pathways, contributing towards normal development of the organ. In PCa, these hormones exert an important role in the onset and progression of the disease, and therefore have been the main therapeutic target for PCa patients. Anti-androgens are a common therapy in prostate cancer (PCa), targeting Androgen Receptor (AR) signaling or hormone production; however, these therapies fail due to selection of highly aggressive castration-resistant PCa cells (CRPCa). Therefore, novel and more efficacious therapeutics and better molecular understanding of this disease are extremely important to help PCa patients. In this Thesis, I explored the potential of ceramide-centric therapeutics in PCa and the mechanisms underlying the intersection between AR and sphingolipid metabolism. Ceramides are sphingolipids, a class of bioactive lipids that play structural and signaling roles in cells and have been shown to possess anti-tumor properties by triggering cell death of tumor cells. Because ceramides are hydrophobic, delivery is problematic. Enter, Ceramide nanoliposomes (CNL); these nanoparticles have now demonstrated great promise in multiple different tumor models, and more importantly in a completed multi-institutional Phase 1 NCI trial (NCT02834611). While ceramides have been speculated as highly selective apoptotic lipids, CNL is the only delivery modality to have completed Phase 1 testing, demonstrating enhanced pharmacokinetic (PK) parameters and minimal toxicities in patients.

Here, we demonstrate that elevating endogenous ceramide levels with administration of exogenous Ceramide Nanoliposomes (CNL) was efficacious in AR-negative prostate cancer cell lines with limited efficacy in AR-positive cells. This effect is mediated through reduced *de novo* sphingolipid synthesis in AR-positive cells. We show that anti-androgens elevate *de novo* generation of sphingolipids via *SPTSSB*, a rate-limiting mediator of sphingolipid generation. Moreover, pharmacological inhibition of AR increases the efficacy of CNL in AR-positive cells through *de novo* synthesis, while SPTSSB knockdown limited CNL's efficacy in AR-negative cells. Alluding to clinical relevance, SPTSSB is up-regulated in advanced PCa patients after anti-androgens treatment. We also show that AR stimulation leads to altered expression of several sphingolipid metabolic enzymes that can be explored in

future studies. Moreover, we determine that the anti-tumor effect of C6-ceramide in PCa cells is due to a specific chiral conformation of its lipid structure, suggesting that the bioactive lipid itself as well as its chiral-specific metabolism is crucial for the treatment efficacy.

These findings emphasize the relevance of AR regulation upon sphingolipid metabolism, support the potential of CNL as a PCa therapeutic, and open new avenues to be pursued concerning the importance of the intersection between sphingolipid metabolism, androgens, and AR in PCa.

TABLE OF CONTENTS

DEDICATION V

ACKNOWLEDGEMENTS VII

SUMMARY XV

TABLE OF CONTENTS XVII

LIST OF ABBREVIATIONS XIX

LIST OF FIGURES XXIII

CHAPTER 1: INTRODUCTION 27

1. THE PROSTATE 27

1.1 ANATOMY AND HISTOLOGY 27

1.2 DISORDERS AFFECTING THE PROSTATE GLAND 28

1.3 A MAJOR EPIDEMIOLOGICAL AND FINANCIAL BURDEN WORLDWIDE 29

1.4 DIAGNOSIS: A SET OF IMPERFECT, YET HELPFUL TOOLS 30

1.5 GLEASON SCORE: AN ARCHITECTURAL STAGING APPROACH 31

1.6 REVISED TUMOR STAGING BY TNM 32

1.7 TUMOR PROGRESSION: MOLECULAR PATHWAYS BEHIND THE CURTAIN 33

1.8 WHO, WHEN, AND HOW TO TREAT PROSTATE CANCER 34

1.9 MODELS FOR PROSTATE CANCER RESEARCH 36

1.10 PROSTATE CANCER BREAKS THE WARBURG RULE 38

2. ANDROGEN SIGNALING IN PROSTATE CANCER 41

2.1 STEROID HORMONES, ANDROGENS, AND COOPERSTOWN 41

2.2 ANDROGEN RECEPTOR: A DYNAMIC STRUCTURE 41

2.3 A POWERFUL REGULATOR 43

2.4 DISORDERS ASSOCIATED WITH THE ANDROGEN RECEPTOR 45

2.5 METABOLIC REGULATION: FEEDING THE UNCONTROLLABLE FIRE 46

2.6 BLOCKING THE RECEPTOR 48

2.7 THE SEEMINGLY UNAVOIDABLE RELAPSE 50

3. THE MOST ENIGMATIC LIPIDS: SPHINGOLIPIDS 55

3.1 UNCOVERING THE SPHINX 55

3.2 DIFFERENT ROUTES OF SPHINGOLIPID SUBWAY SYSTEM 56

3.2.1 The only entrance: *De novo* synthesis of sphingolipids (D) 56

Extra extra: SPTSSB, a small protein with great impact 59

3.2.2 Atypical route....60
3.2.3 Phosphorylated ceramides (P)....61
3.2.4 From ceramides to sphingomyelin and back (S)....62
3.2.5 Ceramides with sugar and beyond (G)....63
3.2.6 The salvage pathway and the Exit route (E)....66
3.3 CERAMIDES: A VERSATILE LIPID....69
3.4 CERAMIDE IN CANCER: THE ULTIMATE DEATH LINEUP....72
3.4.1 Conventional cancer therapies mediate sphingolipid metabolism....73
3.4.2 Ceramide: a major player in cell death....75
3.4.3 Ceramide: old lipid repurposed by cutting edge technology....77
4. COLLISION COURSE: ANDROGEN RECEPTOR AND SPHINGOLIPIDS IN PCA....79
4.1 WHAT IS KNOWN ABOUT THIS INTERSECTION?....79
4.2 ANDROGENS AND SPHINGOLIPIDS....82

CHAPTER 2: GOALS 85

1. RATIONALE....85
2. MAIN GOALS....85

CHAPTER 3: MATERIALS AND METHODS 87

CHAPTER 4: *ROLE OF SPTSSB-REGULATED DE NOVO SPHINGOLIPID SYNTHESIS IN PROSTATE CANCER DEPENDS ON ANDROGEN RECEPTOR SIGNALING* 97

1. RATIONALE....97
2. RESULTS....98

CHAPTER 5: ANDROGEN RECEPTOR AND SPHINGOLIPID ENZYMES 113

1. RATIONALE....113
2. RESULTS....114

CHAPTER 6: CERAMIDE AND CHIRALITY – THE GOOD, THE BAD AND THE UGLY 121

1. RATIONALE....121
2. RESULTS....124

CHAPTER 7: DISCUSSION AND FUTURE PERSPECTIVES 133

1. DISCUSSION....133
2. LIMITATIONS OF THESE STUDIES AND CHALLENGES REMAINING....144
3. FUTURE PERSPECTIVES....146

REFERENCES 149

LIST OF ABBREVIATIONS

AAWS	Anti-androgen withdrawal syndrome
Abi	Abiraterone acetate
ACAC	Acetyl-coA carboxylase
ACER1-3	Alkaline ceramidase 1-3
ACLY	ATP-citrate lyase
AFMS	Anterior fibromuscular stroma
AJCC	American joint committee on cancer
AKT	AKT serine-threonine kinase
AMACR	Alpha-methylacyl-CoA racemase
ADT	Androgen-deprivation therapy
AR	Androgen receptor
ARE	Androgen responsive element
ASAH1	Acid ceramidase
ASAH2	Neutral ceramidase
B3GALT4	Beta-1,3-Galactosyltransferase 4
B4GALT6	Lactosylceramide synthase
BAK1	BCL2 antagonist killer 1
BAX	BCL2 associated X protein
BBB	Blood-brain barrier
BCL-XL	B-cell lymphoma extra-large
BPH	Benign prostatic hyperplasia
C1P	Ceramide-1-phosphate
CAS	Cellular apoptosis susceptibility
Cer	Ceramide
CERK	Ceramide kinase
CERS1-6	Ceramide synthases 1-6
ChIP	Chromatin Immunoprecipitation
ChIP-seq	Chromatin Immunoprecipitation sequencing
CNL	C6-ceramide nanoliposomes
CRPCa	Castration-resistant prostate cancer
CTD	C-terminal domain
CYP11A1	Cytochrome P450 Family 11 Subfamily A Member 1
CYP17A1	Cytochrome P450 Family 17 Subfamily A Member 1
DBD	DNA binding domain

DEGS1-2	Dihydroceramide Δ4-desaturase 1-2
dhCer	Dihydroceramides
DHEA	Dehydroepiandrosterone
dhSph	Dihydrosphingosine
DHT	Dihydrotestosterone
DOD	Department of Defense
DRE	Digital rectal examination
EBRT	External-beam radiation therapy
EMT	Epithelial to mesenchymal transition
ENPP7	Alkaline sphingomyelinase
EPR	Enhanced permeability retention
ER	Endoplasmic reticulum
ERG	ETS-related gene
ETS	Erythroblast transformation-specific
EZH2	Enhancer of zeste homolog 2
FASN	Fatty acid synthase
FDA	Federal Drug Administration
FOXA1	Forkhead box A1
$GABA_A$	G-aminobutyric acid receptor
GAL3ST1	Galactosylceramide sulfotransferase
GalCer	Galactosylceramide
Gb3	Glotriaosylceramide
GBA1-3	Glucosylceramidase 1-3
GEM	Genetically engineered mouse
GLA	α-galactosidase A
GlcCer	Glucosylceramide
GnRH	Gonadotropin-releasing hormones
GPCR	G protein-coupled receptors
GR	Glucocorticoid receptor
GS	Gleason score
GSL	Glycosphingolipids
HDAC	Histone deacetylase
HGPIN	High-grade prostatic intraepithelial neoplasia
HRPC	Hormone resistant prostate cancer
HSAN1	Hereditary sensory and autonomic neuropathy 1
IMRT	Intensity-modulated radiation therapy

ISUP	International society of urological pathology
KDSR	3-ketodihydrosphingosine reductase
LacCer	Lactosylceramide
LBD	Ligand binding domain
LPP	Lipid phosphate phosphatases
LSD1	Lysine-specific demethylases 1
MDR1	Multi-drug resistance 1
MDV3100	Enzalutamide
MLKL	Mixed lineage kinase domain-like protein
MTOR	Mechanistic target of rapamycin kinase
NCoR	Nuclear receptor corepressors
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEPC	Neuroendocrine prostate cancer
NIH	National Institutes of Health
NKX3.1	NK3 Homeobox 1
NR	Nuclear receptor
NTD	N-terminal domain
ORMDL1-3	ORMDL Sphingolipid Biosynthesis Regulator 1-3
PCa	Prostate cancer
PCRP	Prostate cancer research program
PDX	Patient-derived xenografts
PERMIT	Protein that mediates ER-mitochondria trafficking
PI3K	Phosphoinositide 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	Prostatic intraepithelial neoplasia
PK	Pharmacokinetic
PKA	Protein kinase A
PKC	Protein kinase C
PML	Promyelocytic leukemia protein
PP2A	Protein phosphatase 2A
PSMA	Prostate specific membrane antigen
PTEN	Phosphatase and tensin homolog
Rb1	Retinoblastoma 1
RIPK1-3	Receptor-interacting protein kinase 1-3
RP	Radical prostatectomy

S1P	Sphingosine-1-phosphate
S1PR1-5	Sphingosine-1-phosphate receptor 1-5
SCAP	SREBP cleavage activating protein
SGMS1-2	Sphingomyelin synthase 1-2
SGPL1	Sphingosine-1-phosphate lyase 1
SGPP1-2	Sphingosine-1-phosphate phosphatase 1-2
SM	Sphingomyelin
SMPD1-5	Sphingomyelin phosphodiesterase 1-5
SMRT	Silencing mediator of retinoid and thyroid receptors
SOX2	SRY-Box Transcription Factor 2
S1P	Sphingosine-1-Phosphate
Sph	Sphingosine
SPHK1-2	Sphingosine kinase 1-2
SPOP	Speckle Type BTB/POZ Protein
SPTLC1-3	Serine palmitoyltransferase long chain base subunit 1-3
SPTSSA-B	Serine Palmitoyltransferase Small Subunit A-B
SREBP	Sterol regulatory element-binding proteins
ST3GAL5	GM3 synthase
ST8SIAI	GD3 synthase
ST8SIA5	GT3 synthase
STAR	Steroidogenic Acute Regulatory Protein
TCA	Tricarboxylic acid cycle
TCGA	The Cancer Genome Atlas
TF	Transcription factor
TMPRSS2	Transmembrane protease serine 2
TNFA	Tumor necrosis factor alpha
TRAMP	Transgenic adenocarcinoma of the mouse prostate
TRUS	Transrectal ultrasound
UGCG	Glucosylceramide synthase
UGT8	Galactosylceramide synthase
VDAC2	Voltage dependent anion channel 2
KLK3	Kallikrein-3
WHO	World health organization

List of Figures

FIGURE 1 – ESTIMATED AGE-STANDARDIZED INCIDENCE RATE PER 100.000 IN 2020 IN MALES OF ALL AGES. **30**

FIGURE 2 – MODIFIED GLEASON SCORE SYSTEM FOR GRADING OF PCA BASED ON GLANDULAR HISTOLOGY OF THE TUMORS. **32**

FIGURE 3 – THE SPHINGO CITY METRO. **69**

FIGURE 4 – SPHINGOLIPID FAUCETS AND DRAINS. **72**

FIGURE 5 – CNL ARE EFFICACIOUS AGAINST HIGHLY AGGRESSIVE AR-NEGATIVE PCA CELLS. **99-100**

FIGURE 6 – EFFICACY OF CNL IS HIGHER IN HIGHLY AGGRESSIVE AR-NEGATIVE CELLS. **101**

FIGURE 7 – INTACT AR SIGNALING HINDERS CNL EFFICACY IN PCA CELLS. **103**

FIGURE 8 – INTACT AR SIGNALING HINDERS CNL EFFICACY IN PCA CELLS. **104**

FIGURE 9 – *DE NOVO* SYNTHESIS OF SPHINGOLIPIDS IS NEGATIVELY REGULATED BY AR. **107**

FIGURE 10 – CNL INDUCES *DE NOVO* SYNTHESIS OF SPHINGOLIPIDS IN AR-NEGATIVE CELLS. **109**

FIGURE 11 – SPTSSB EXPRESSION IS ELEVATED IN NEUROENDOCRINE PCA PATIENTS. **110**

FIGURE 12 – SPTSSB IS ESSENTIAL FOR CNL EFFICACY IN AR-NEGATIVE CELLS. **112**

FIGURE 13 – AR REGULATES THE EXPRESSION OF ENZYMES INVOLVED IN SPHINGOLIPID METABOLISM. **116**

FIGURE 14 – AR REGULATION OF SPHINGOLIPID ENZYMES' EXPRESSION. **119**

FIGURE 15 – REGULATION OF SPHINGOLIPID METABOLIC ENZYMES BY AR STIMULATION ACROSS TIME. **120**

FIGURE 16 – D-ERYTHRO-CERAMIDE IS THE MOST EFFECTIVE AGAINST PCA CELLS. **125**

FIGURE 17 – DIFFERENT CERAMIDE ANALOGS ARE METABOLIZED DIFFERENTIALLY BY LNCAP CELLS. **129**

FIGURE 18 – IMPAIRED METABOLISM OF SPHINGOLIPIDS LEADS TO REDUCED CELL VIABILITY. **131**

FIGURE 19 – REMOVING CNL FROM CELL CULTURE MEDIA HINDERS ITS EFFICACY. **132**

FIGURE 20 – SPTSSB IS AN ACHILLES' HEEL IN PCA. **143**

LIST OF TABLES

TABLE 1 - KEY RESOURCES USED IN THIS THESIS. **87**

TABLE 2 - LIST OF DOWNREGULATED SPHINGOLIPID ENZYME TRANSCRIPTS BY AR STIMULATION IN PCA CELLS LINES. **117**

TABLE 3 - LIST OF UPREGULATED SPHINGOLIPID ENZYME TRANSCRIPTS BY AR STIMULATION IN PCA CELL LINES. **118**

CHAPTER 1: INTRODUCTION

1. THE PROSTATE

1.1 ANATOMY AND HISTOLOGY

The prostate gland is a walnut-shaped small organ located in the pelvic region below the bladder and surrounding the urethra [3,4]. This gland is essential for the male reproductive system with its main role being secretion of seminal fluid that helps sperm to travel and survive [3,5]. In adults, the normal prostate reaches 25cm^3 and weighs about 20g [6]. The formation of prostate tissue during development depends on the signaling of male hormones, such as androgens that signal through the Androgen receptor (AR), which is one of the most important genes in the prostate in normal and cancer conditions [3].

After more than two centuries of controversies surrounding the establishment of the prostate anatomy, in 1968 John E. McNeal proposed a zonal structure [7,8]. McNeal worked on the zonal structure for 25 years reaching what is now commonplace: four nonhomogeneous and distinct zones- peripheral, central, transition and anterior fibromuscular stroma (AFMS) [6,9]. These zones are not only different anatomically but also histologically and biologically, which explains why they also differ when it comes to prostate disorders. The peripheral zone is the main area and comprises about 70% of the prostate, and most of the inflammation and prostate carcinomas (PCa) arise in this area. This zone is close to the rectum and palpable by digital rectal examination (DRE), a gold-standard screening test for PCa [3,6]. The central zone, which surrounds the ejaculatory ducts, makes about 25% of the prostate area and a small portion of PCa arise in this area, usually very aggressive and invasive tumors [10]. The transition zone surrounds the proximal urethra and is composed of two equal lobules that account for about 5% of the total prostate area. Between 10 and 20% of PCa cases arise in this zone, but age-related benign prostatic hyperplasia (BPH) is frequently limited to this area [3,6]. These three areas are responsible for the glandular function of the prostate; while AFMS is compose of muscle and fibrous tissue that support the gland. A thin layer of fibrous tissue involves these areas, the so-called prostate capsule [3,6]. Neuroendocrine cells are another component of the prostate gland [3,6,11].

1.2 DISORDERS AFFECTING THE PROSTATE GLAND

In an aging population, disorders that affect the prostate gland are very common as they are mostly associated with age, and these disorders can range from benign to malignant conditions [12].

The first broad study of prostate pathology dates back to 1900, and in 1925 Reischauer proposed that prostatic hyperplasia developed from mesenchymal proliferation and a reorganization of epithelial cells into a epithelial stromal proliferation [13]. Nowadays this condition is termed benign prostatic hyperplasia (BPH) and is projected to afflict about 62% of men 50 years old or older [12]. Interestingly, about 90% of 80-years old men have signs of BPH, which points to an age-related condition [14]. Despite being a benign condition, BPH can impact men's lives due to prostate enlargement [15]. Despite BPH and PCa frequently coexisting in patients there are no direct links in a progression from BPH to PCa.

Proliferative inflammatory atrophy (PIA) is characterized by chronic inflammation and high heterogeneity [3,16]. This type of lesion is thought to occur after inflammation and leads to regeneration and proliferation of epithelial cells in the prostate [16]. PIA shares several genetic and morphological abnormalities with high-grade prostatic intraepithelial neoplasia (HGPIN) suggesting it as a precursor for this condition [17,18]. Prostatic intraepithelial neoplasia (PIN) is a common pre-malignant condition afflicting the prostate with more direct evolutionary ties to PCa. PIN lesions can be distinguished between low-grade and high-grade PIN (HGPIN) based on the histological features [19]. HGPIN is considered to be the most likely precancerous region that leads to a significant number of cases of PCa [4,20,21]. High levels of proliferation and prominent nucleoli characterize HGPIN, however, basal cells which are absent in PCa, are present in this pre-malignant condition. This key difference is a helpful tool for distinction between these two disorders, but also shines a light on how HGPIN might be an intermediary between normal and prostatic cancer tissue [5,20,21]. When looking at the similarities between HGPIN and PCa it is important to mention that both occur in close proximity in about 70% of the prostatectomy specimens, and that both are multifocal and heterogeneous [20].

Prostate adenocarcinoma (PCa) is estimated to account for 95% of the total tumors that arise in the prostate, ranging from indolent to extremely aggressive entities [5,6,22]. This heterogeneity, also observed molecularly, combined with the lack of symptoms in initial stages of the disease, makes it imperative to find better therapeutic and diagnostic options for earlier stages, as well as determining which molecular pathways are behind this progression. Another important entity that

originates in the prostate are neuroendocrine tumors [11,23]. This represents a group of heterogeneous tumors including small cell carcinoma, PCa with focal differentiation, large cell neuroendocrine carcinomas, among others [24]. Neuroendocrine focal differentiation is described as occurring in 5-10% of localized tumors but is more prevalent (~25%) after hormonal treatment of PCa patients [11,23]. These cases termed neuroendocrine prostate cancer (NEPC) originate due to cell plasticity after inhibition of hormonal receptors by transdifferentiation of luminal PCa cells [11,25]. The common theme in all types of neuroendocrine tumors is the lack of effective therapeutics and being responsible for most of the mortality among PCa patients.

1.3 A MAJOR EPIDEMIOLOGICAL AND FINANCIAL BURDEN WORLDWIDE

Prostate cancer (PCa) was the second most incident tumor in men worldwide during 2018, accounting for 13.5% of cancer cases (Figure 1) [26,27]. During the same period PCa had the 5th highest cancer-related mortality rate in men worldwide at 6.7% [26]. Prostate cancer is also about three times more incident in regions of the world with higher economic development rates compared with lower development rates, while mortality rates don’t display the same pattern and are pretty much equal [26]. In the United States, the incidence rates for 2020 are estimated to be even higher and reaching about 21% of the new cancer cases in men, the leading tumor in that category [28]. Regarding mortality, PCa in 2020 will be responsible for 10.3% of the cancer-related deaths in men second only to lung cancer, a drop of 52% since 1993 [28].

Given the improvement of early diagnosis and treatments for PCa, patients tend to live longer but with side effects from the disease increasing the costs associated with PCa. PCa is now not only an epidemiological problem, but also a financial one for governments and families worldwide. Researchers have estimated that in 2020, PCa will have a total cost of $16.3 billion, which would represent a $4.5 billion increase since 2010 [29].

Taking into consideration the increasing incidence rate and increased costs this leads to an increased psychological burden for families and societies. Thus, it is crucial to find novel molecular mechanisms that lead to improvement of current therapeutics available or totally new therapeutic modalities for PCa.

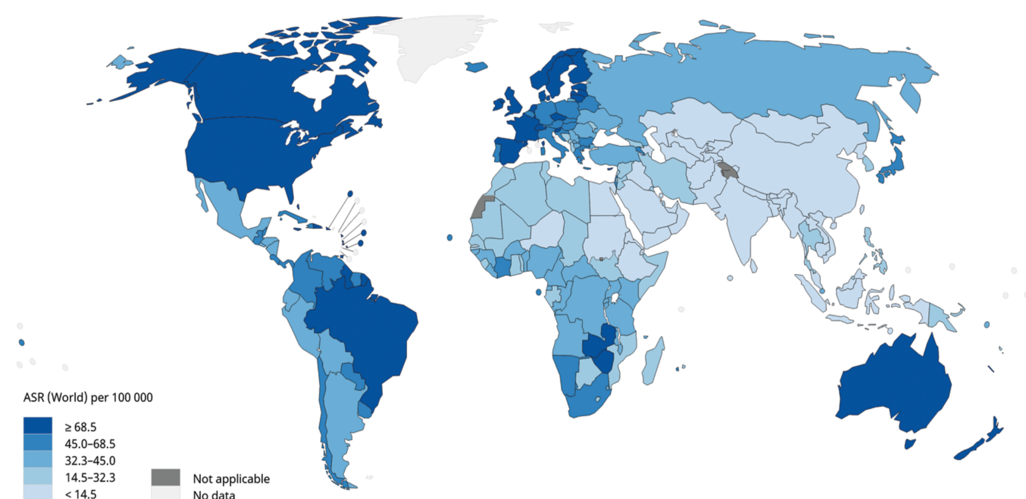


Figure 1 -Estimated age-standardized incidence rate per 100.000 in 2020 in males of all ages[26].

1.4 DIAGNOSIS: A SET OF IMPERFECT, YET HELPFUL TOOLS

As previously stated, most cases of PCa are asymptomatic until invasion occurs, therefore there have been decades of research into biomarkers and techniques for PCa diagnosis. There are several studies pointing to a lack of evidence regarding the benefits of widespread screening of PCa, however it is advised to offer early detection to well-informed men [30,31]. Currently, screening for PCa is recommended to start in men at age 50 and to be regularly performed until their seventies [32]. The gold standard for screening is a combination between digital rectal examination (DRE), and measurement of serum concentration of prostate specific antigen (PSA) [33]. DRE by itself is of limited value as it is highly dependent on physicians' experience, but it's an important adjuvant tool to PSA testing and is critical in determining clinical stage [34]. The US implemented serum PSA as a screening methodology in 1987 and saw an almost immediate rise in PCa incidence rates [26,28]. Incidence rates have since then dropped or stabilized.

PSA, or Kallikrein-3 (KLK3) is a glycoprotein produced and secreted by the prostate. The main problem with PSA testing is that PSA itself is organ-specific but not cancer-specific leading to fallible results. As an example, BPH, a common benign condition is known to lead to increased levels of PSA in men [34]. A traditional value for PSA of 4 ng/mL has been established as a cutoff to determine the need for a biopsy after abnormal or inconclusive DRE, with 70% of PCa patients having values higher than 4 ng/mL [34]. A cutoff such as the one for PSA can be deceiving and leads to under- and over-diagnosis, and currently a risk stratification approach has been

adopted. This strategy originates from a study that showed that 25% of patients with 3.1-4 ng/mL had high-grade tumors [35]. The overtreatment of indolent tumors can present a problem originated from fallible biomarkers, as only 10-20% of men are at risk of developing clinical PCa, while 60-70% are estimated to harbor the disease [34]. These issues with DRE and PSA have led to recommendations to not use them in every man, but let it be an individual choice [26]. Meanwhile, researchers are still trying to find new and more reliable biomarkers, especially for urine and circulating tumor cell detection [36,37].

After abnormal or inconclusive screening results, patients usually undergo imaging examination via ultrasound and/or transrectal ultra-sound (TRUS) biopsy. This technique allows physicians to evaluate prostate status (volume, inflammation) but also assist on obtaining material for histopathological evaluation [38].

The search for more reliable biomarkers that can limit the number of unnecessary biopsies and better predict aggressiveness and disease course is still essential for PCa.

1.5 GLEASON SCORE: AN ARCHITECTURAL STAGING APPROACH

In 1966, Donald Gleason proposed a grading system based on PCa architecture after biopsy that remains as one of the main tools for predicting patients' outcome [39-41]. This grading system has been coined as Gleason score (GS) and has evolved and changed through the years to incorporate new findings in the prostate cancer histology field. It is based in the architecture and differentiation of the tumors and does not include any cytological information [42]. Gleason proposed a 5-tier grading system that scores histologic patterns from 1-5 that reflects the differentiation level (Figure 2). In other words, the highest score represents less differentiated and therefore more aggressive tumors. Gleason and his colleagues earlier recognized the heterogeneity of most PCa and adapted this to encompass the two most common architectures observed, originating the GS that is the sum of both scores ranging from 2-10 [40,42]. Increased GS is correlated with tumor size, more advanced clinical staging, metastatic disease, and lower survival rates [42]. In 2014, the International Society of Urological Pathology (ISUP) established a new consensus on how to report GS to better represent the risk imposed by the disease [43]. One of the main issues that the 2014 consensus addresses is the difference in prognosis with GS 7, which will represent patients with 4+3 or 3+4 tumors. Thus, another 5-tier system was proposed as follows: GS ≤ 6 (Grade group 1); GS 3+4 (Grade group 2); GS 4+3 (Grade group 3); GS 8 (Grade group 4); GS 9-10 (Grade group 5) [23,43]. The authors

contend that this system, that has its foundation in the original work by Gleason, is better at predicting prognosis but also at reflecting PCa biology [43]. This system has also been adopted by the World health organization (WHO) [23].

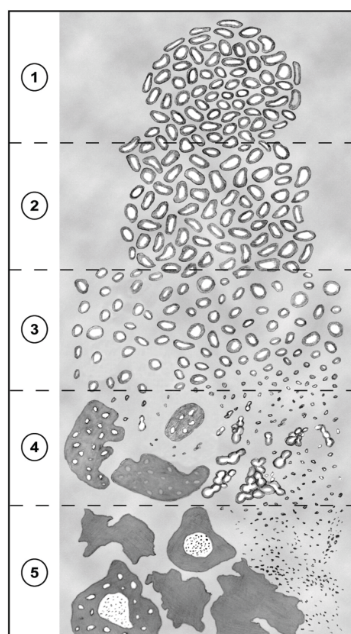


Figure 2 – Modified Gleason score system for grading of PCa based on glandular histology of the tumors (Adapted from reference 41).

1.6 REVISED TUMOR STAGING BY TNM

Tumor staging is of extreme importance, as it works as a guide by ascertaining the severity of the disease, estimating its prognosis, guiding treatment options, and helping clinicians all over the world to report their findings in the clinic [44]. Therefore, staging needs to be extremely accurate, and periodical revisions and adaptations are made to ensure this accuracy. The American Joint Committee on Cancer (AJCC) first published a cancer staging manual in 1977, and since then it has become the main option to stage solid tumors from all sites. In 2018, the 8th and latest update came into effect [44]. The AJCC staging system is termed TNM, and it consists in three different categories: T- extent of primary tumor, N- presence and extent of lymph node involvement, and M- presence and extent of distant metastasis. There are two main types of staging that take place in the clinic: clinical and pathological. Clinical staging occurs before intervention and includes DRE, serum PSA, imaging techniques, TRUS, and GS grade [44,45]. After the biopsy, criteria such as absence of basal cells by lack of p63 and cytokeratin 5/14 staining can confirm the diagnosis of PCa [5]. Elevated levels of alpha-methylacyl-CoA racemase (AMACR) are also common and represent a luminal phenotype characteristic of PCa [5]. Pathological

staging is usually performed after radical prostatectomy (RP) and involves macro and microscopic evaluation of the surgical specimen combined with regional lymph nodes [46]. The T category ranges from T1-T4 depending on the extent of the tumor in the prostate and evidence of local invasion, while N and M are binary, either absent (0) or present (1) [44]. Combining the clinical and pathological staging leads to assignment of patients to four different groups regarding their prognosis and guides clinicians towards the appropriate treatment options available [44].

1.7 TUMOR PROGRESSION: MOLECULAR PATHWAYS BEHIND THE CURTAIN

Cancer is a genetic disease where tumor suppressor genes become deactivated, and oncogenes activated leading to overgrowth of neoplastic cells; PCa is not an exception to this phenomenon [47]. Several pathways have been identified as cancer hallmarks [47]. With the advent of high-throughput techniques that allow analysis of the entire genome, transcriptome, and proteome it has become much easier to understand the underlying molecular alterations behind PCa progression. Currently, unlike other endocrine-related tumors such as breast and ovarian cancer, there are no established molecular subtypes that are applied in the clinic despite several efforts to achieve so.

As expected, some molecular events and players that play a role in PCa are similar to other tumor models. Genes involved in cell cycle regulation (p53, Rb), epigenetic machinery (*EZH2, HDACs)*, oncogene Myc, oxidative response (*GSTP*), signaling cascades regulated by mTOR and MAPK, and heat shock proteins among others, have all been reported as involved in PCa [5,48-50]. For the sake of brevity in this Thesis only the major frequent events that are specific to PCa will be detailed.

There are three genes that are crucial during the normal prostate development and maturation that are dysregulated in PCa: NK3 Homeobox 1 (*NKX3.1*), Forkhead box A1 (*FOXA1*), and *AR* [49]. *NKX3.1* is a transcriptional repressor located in a chromosome region (8p21) frequently displaying loss of heterozygosity in HGPIN and PCa [5,49,50]. Studies have shown that the loss of *NKX3.1* expression is partial and not sufficient to induce tumor formation in mouse models, but instead is related with development of PIN lesions, suggesting this event as an early occurrence in the progression from normal prostate to PCa [50]. *AR* is a nuclear hormone receptor that plays an important role as a transcription factor in normal prostate and PCa and will be discussed further in Section 2 of this Thesis (*Androgen signaling in Prostate cancer). FOXA1* is a transcription factor that combined with epigenetic mechanisms regulates AR expression as it has been shown to positively

and negatively regulate the signaling pathways activated by AR [51]. FOXA1 transcript has been shown to have elevated expression in PCa where levels are able to predict shorter time to death from PCa and have also been shown to be elevated in metastases of castration-resistant PCa (CRPCa) [52,53].

Another pathway that is frequently dysregulated in cancer in general is the PI3K/AKT/PTEN axis. Phosphatase and Tensin Homolog (*PTEN*) is a bona fide tumor suppressor gene that functions as a lipid phosphatase that is frequently lost in PCa patients, and thus has low expression in about 85% of patients, being a marker for poor prognosis [49,50]. Mouse models with knockout *PTEN* develop both PIN lesions as well as PCa [54]. The absence of *PTEN* is a crucial checkpoint on the oncogenic axis of PI3K/AKT that leads to more aggressive tumors.

A key molecular rearrangement occurring in PCa is the Transmembrane protease serine 2-erythroblast transformation-specific (*TMPRSS2-ETS*) fusion gene that is estimated to occur in a more than 50% of patients. *TMPRSS2* is an androgen-regulated gene and, in this gene fusion event, its 5' untranslated region is fused with a member of the ETS family [55]. This way the ETS family gene is under control from AR signaling activity maximizing its expression in prostate cells. The most common partner for fusion with *TMPRSS2* is ETS-related gene (*ERG*) and this fusion product is present in about 50% of the diagnosed PCa [56]. The overexpression of *ERG* in PCa has been associated with degradation of extracellular matrix and decreased cell adhesion, potentially contributing to a more aggressive phenotype [57,58]. Overexpression of *ERG* in mouse models has been shown to result in minimal and low-grade PIN lesions, but when this occurs in the absence of *PTEN* it leads to HGPIN and PCa [5]. This shows that temporal and spatial molecular alterations are important for the pathological progression in PCa.

It is important to note that, despite a vast amount of information on molecular alterations in PCa, molecular therapeutics remain centered in AR inhibition. Thus, novel and alternative pathways can still be explored for the benefit of PCa patients.

1.8 Who, when, and how to treat Prostate cancer

The complexity and heterogeneity of PCa makes it difficult for clinicians to determine a treatment plan that fits all patients. Several criteria need to be taken into consideration before a treatment decision is made, and those include: staging, grading, age, life expectancy and life quality post-treatment [18]. With the widespread use of PSA all over the world, more and more asymptomatic men have been subjected

to biopsies that disclosed early stage PCa [26,59]. The question that clinicians need to ask themselves (and their patient) frequently regarding PCa after its finding is to treat or not to treat? The key challenge becomes to determine who are the patients where treatment will change the course of the disease from those where treatment would be unnecessary [3].

A common strategy in PCa, given its association with patients' age, is active surveillance to avoid overtreatment. This is applied to very low-risk, locally confined PCa that are not treated but instead followed and, if there is increased risk of progression, then treatment becomes an option [60]. Active surveillance was developed when data pointed that men diagnosed with PCa live up to 20 years with a survival rate of 80-90% [60]. Changes in GS after new biopsy or a rise in PSA are the follow-up criteria for potential progression and lead to a new course of action [60]. A variant of active surveillance is watchful waiting which, instead of curative has palliative intent, and relies more on patients' symptoms than clinical tests [61].

Radical prostatectomy (RP) is a surgical procedure that removes the prostate and has been used in localized PCa for over a century [59]. This approach has shown cancer-specific improvements in survival [60]. Cryotherapy, where freezing probes ablate tumors has also been used to treat localized PCa, especially with the help of ultra-sound technology to guide the probes [59,60]. In high-risk PCa, radiotherapy is usually an adjuvant treatment to RP with increased survival benefits [3,31]. Within radiotherapy there are several different modalities that have been used in PCa: external-beam radiation therapy (EBRT), intensity-modulated radiation therapy (IMRT), brachytherapy and proton beam therapy [31,59]. In low-risk PCa, some clinicians opt to perform radiation therapy in patients if they fit in the criteria necessary for it, and surgery would either be to risky or not predicted to show a significant health benefit compared with radiation [31].

Another major strategy for treatment of PCa, mainly for cases of advanced disease, is hormonal therapy or androgen-deprivation therapy (ADT). ADT was first reported back in the 1940s when Huggins and Hodges showed for the first time that after castration, PCa patients had relief of their symptoms and better prognosis [62]. ADT using either surgical or chemical castration (suppresses the secretion of androgens) or anti-androgens (blocks androgen action) is the core treatment option for advanced PCa and leads to symptom relief in 70-80% of patients [63]. Tumor volumes and PSA levels are rapidly decreased after implementation of ADT regimens [59]. Chemical castration is attained using luteinizing gonadotropin-releasing hormones by reducing testosterone levels [60]. The most common therapeutics to block

androgen signaling are AR antagonists, such as Enzalutamide (MDV3100), or inhibitors of androgen synthesis, such as Abiraterone acetate (Abi) [64].

However, relapse is inevitable and data shows that after 18-24 months virtually all patients relapse to either hormone resistant PCa (HRPC) or castration resistant PCa (CRPCa) [65]. In HRPC, patients no longer respond to any kind of hormonal therapy, while in CRPCa they are initially responsive to secondary hormonal ablations [31]. The molecular mechanisms of this relapse will be discussed in Section 2 of this Thesis (*Androgen signaling in Prostate cancer)*. This relapse leads to a stage of the disease where patients no longer have effective therapeutic options available besides palliative treatments. **Therefore, it is crucial to not only keep the focus on the resistance mechanisms to the standard of care chemotherapeutic approaches but also to find novel pathways that can be explored in synergistic combinations with the current status quo.**

1.9 Models for Prostate cancer research

As one of the most incident cancers worldwide, a lot of effort has been put into discovering new molecular mechanisms underlying PCa and therapeutics that can end up in the clinic benefiting patients. This effort is only possible because of the availability of *in vitro* and *in vivo* models that allow researchers to advance the knowledge of the disease. New and more advanced models are still needed to address issues such as disease progression and drug resistance.

Researchers have access to several PCa *in vitro* cell line models that are a quick and initial tool for researchers to test new compounds and important cellular pathways, but lack stromal components and microenvironment conditions that have been shown to play an important role in cancer [66]. There are four cell lines that represent the progression of PCa: LNCaP, C4-2, PC-3, and DU145 [67-69].

LNCaP cells were generated in 1977 from lymph nodes of a PCa patient [70]. This cell line is AR and PSA positive, PTEN negative, which is a common molecular signature of early PCa [69]. LNCaP cells have a mutated AR that increases its specificity to other androgens than just testosterone [68,69]. LNCaP has been used in xenografts studies for PCa, but its growth rate is slow, which led researchers to generate and establish sublines from it. One of the main sublines currently used is C4-2 cell line that was generated after passaging of parental LNCaP in a castrated mouse and then reinjecting into other castrated mice and harvested from bone metastasis [71]. C4-2 cells express AR but are androgen insensitive and have shown to have better engraftment and ability to metastasize in xenografts modeling of PCa [68,69]. Therefore,

C4-2 represents one step ahead of the PCa progression compared with LNCaP. In 1979, the PC-3 cell line was created from a bone metastasis of PCa patient [72]. This cell line represents AR negative tumors, as it lacks AR and PSA expression and is highly metastatic [68,69]. Moreover, PC-3 cells represent a neuroendocrine phenotype, that albeit rare are extremely aggressive in patients. PC-3 cells when xenografted in mice lead to osteolytic bone metastasis [68,69]. Another androgen insensitive cell line that is commonly used in PCa research is DU145, which was obtained from a brain metastasis [73]. These cells are more resembling of adenocarcinoma compared with PC-3 and still generate bone metastasis in xenograft models [68,69]. Several other cell lines have been generated and used in PCa research with different degrees of success and have been reviewed by Sobel & Sadar [74,75].

In addition to the disadvantages of using cell lines mentioned before, isolating cells and generating a novel cell line will lead to homogeneity which doesn't represent a disease like PCa that has high degrees of heterogeneity. A model that better represents this heterogeneity is patient-derived xenografts (PDX) [76]. PDXs have been shown to be able to resemble the progression of PCa towards drug resistance but lack the potential to metastasize in the first injection [69,76]. PDXs also have highly variable engraftment rates, a disadvantage that has hindered the widespread use of PDXs in PCa research [69,76]. One major disadvantage, similar to xenografts, is the fact that the mice used for these approaches are immunocompromised therefore lacking the role the immune system plays in patients. High-throughput alternatives to PDXs are organoids that are generated in a dish after cell collection from patients and allow for faster molecular and pharmacological studies [66,77].

With the advancement of genetic manipulation in the last 3 decades, genetically engineered mouse (GEM) models of PCa have been developed that circumvent some of the disadvantages that other methodologies have. These models account for tumor heterogeneity, stromal cells, immune response, and have been shown to represent the tumor progression seen in humans. One of the main caveats is the lack of PSA that doesn't allow this marker to be used in the study of targeted therapeutics to the prostate. GEM models can be grouped in four categories: (1) transgenic, (2) conditional knockout, (3) whole body knockout, and (4) gain of function models [78]. The most well studied transgenic mouse model in PCa research is the transgenic adenocarcinoma of the mouse prostate (TRAMP) where mice develop tumors within 18 weeks of age and are able to metastasize to lungs and lymph nodes, but not to the bone [69,78]. Castration has been shown to shrink tumors in TRAMP mice

in about 20% of the cases, while leading to aggressive tumors with neuroendocrine features in the rest of the animals [79]. A similar transgenic model named LADY has also been widely used [69]. More recent models are not based in the transgene approach but genetic knockouts that resemble PCa lesions. Models with deletion of PTEN and NKX3.1, either alone or combined, whole-body or prostate-specific have been developed with success [78]. Homozygous deletion of PTEN in mice prostate leads to development of hyperplasia, followed by PIN lesions within 6 weeks, and progression to PCa in 100% of the animals between 9-29 weeks [80]. Overexpression of the oncogene Myc with composite probasin promoter,ARR_2PB, yields a Hi-Myc mouse model that has been reported to progress from PIN to carcinoma at 13 weeks of age with invasiveness phenotype at 26 weeks [81]. Combination of PTEN and P53 in the prostate has been shown as a promising model to mimic human disease progression in all of its steps including the development of CRPCa and neuroendocrine phenotype [25].

Additional models have been used in PCa research in mouse models with different degrees of success and approximation to the human disease. Therefore, and with the constant technological advance, new models will in the near future help revealing novel mechanisms of onset and progression in PCa.

1.10 Prostate cancer breaks the Warburg rule

Cancer is a disease characterized by a chronic uncontrolled cell growth with the subsequent potential to invade and metastasize other tissues. This aberrant increase in cell proliferation means that cells need to ramp up their energy production to support the higher demand. In 1930 for the first time, Otto Warburg suggested that tumor cells not only have a higher metabolic rate, but also most importantly have a different metabolism than normal cells [82,83]. Otto Warburg found that cancer cells favor glycolysis and glucose consumption even in the presence of oxygen [82,83]. This finding led to the term “aerobic glycolysis” [47]. Meanwhile, non-neoplastic cells favor oxidative phosphorylation, and when in aerobic conditions use glucose to produce pyruvate and then carbon dioxide. Only in anaerobic conditions is glycolysis favored but the pyruvate produced by glucose consumption is not taken to the mitochondria at the same rate [47]. The switch that cancer cells undergo from regular aerobic conditions to aerobic glycolysis is counterintuitive and counterproductive as glycolysis produces less energy when compared to oxidative phosphorylation [47]. One of the mechanisms underlying the switch is up-regulation of glucose transporters that allow for increases in glucose uptake. In fact, clinicians have

used this as a diagnostic route by developing a radioactive scan (PET, positron emission tomography) based on radiolabeled glucose. As PET scans are based on glucose uptake, only cells that have high uptake of glucose will be radiolabeled and show a signal in this exam, allowing clinicians to discover or confirm the existence of tumor masses. Recently it has become evident that this metabolic switch is not static and that cells adapt to the conditions surrounding them, and tumors have been shown to have different cell populations that depend on different metabolic pathways, even showing symbiotic relationships between those pathways [84]. But studies have shown, that despite metabolic states being a constant flux in most cancer cells, there is one exception to the Warburg "rule", Prostate cancer.

The main function for the prostate gland is to produce prostatic fluid that composes semen. Thus, prostate metabolism is highly specialized in the production of citrate and subsequent release. In the 1940s, researchers found that normal prostate favors glycolysis even in aerobic conditions, a Warburg effect in normal cells [85]. Decades later, researchers found that the tricarboxylic acid (TCA) cycle is impaired in the prostate due to zinc accumulation that leads to enzymatic inhibition, not allowing for its oxidation but instead to its release [86]. Not surprisingly, hormonal regulation of metabolism in prostate has been widely studied [84,87,88]. Despite this difference in normal conditions compared with other tissues, PCa still undergoes a major metabolic switch compared with their normal counterparts. Just like metabolism is reversed in normal cells, the switch in PCa follows the reverse Warburg effect. Thus, PCa favors enhanced oxidative phosphorylation with high rates of lipogenesis and low rates of glycolysis both driven by hormones and AR [89-91]. PCa tumors have shown to be able to complete their TCA cycle, which happens by the down-regulation of zinc transporters: without zinc to block key enzymes in TCA, PCa cells are then able to oxidize citrate which then either helps TCA to come to an end or helps in lipogenesis [89]. This change in PCa metabolism helps to explain why PET scans are usually not helpful to detect the disease in patients. Nevertheless, a recent paper in a cohort of PCa patients with neuroendocrine disease showed that PET scans were able to detect 95% of nodal and visceral metastasis showing that metabolic changes also occur in the transition to independence from AR [92]. The presence of other cell types in the tumor microenvironment may represent an additional source of nutrients, and in PCa cancer associated fibroblasts have been shown to produce lactate which has been shown to be key for cells highly dependent on oxidative phosphorylation such as PCa cells [93-95]. This supports the idea that tumors include cell populations with different energetic needs or reliance on different metabolic

pathways, and that tumor cells might even hijack the nutrients secreted by those cells for their own proliferation.

Cancer cell metabolism is crucial to support disease progression and expansion to other areas of the body and constitutes an important avenue for treatments. It is thus important to reconcile that PCa might not benefit from the same metabolic treatments that most tumors would and that the reverse approaches might have to be applied. Moreover, AR, the major player in PCa and the focus of the next section of this Thesis regulates these dysfunctional metabolic pathways.

2. ANDROGEN SIGNALING IN PROSTATE CANCER

2.1 STEROID HORMONES, ANDROGENS, AND COOPERSTOWN

Hormones are signaling molecules produced by glands that form a communication system between organs and tissues to regulate physiological activities in the body. Glands that secrete hormones compose the endocrine system, and there are two types of glands: (1) secrete hormones into the bloodstream – endocrine; (2) secrete hormones into epithelial surfaces by the use of ducts – exocrine, such as prostate [96]. One of the three main classes of hormones is steroids that encompass cholesterol, steroid hormones (sex hormones and corticosteroids), and anabolic steroids (the synthetic anabolic steroids are responsible for increasing muscle synthesis and responsible for keeping many baseball players out of the Hall of Fame in Cooperstown) [96]. Steroid hormones, which include androgens, bind to nuclear receptors to initiate specific signaling pathways, a phenomenon that has been recognized for more than 50 years [97]. In fact, the discovery in the 1920s of sex hormones by Ruzicka and Butenandt led to their joint chemistry Nobel Prize. About 50 nuclear hormone receptors have been identified in humans, and the Androgen receptor is the natural receptor for androgens [98]. Androgens are synthesized in the adrenal glands and gonads using cholesterol as precursors [99]. Cholesterol is converted to pregnenolone by Cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1) that then gets hydroxylated by Cytochrome P450 Family 17 Subfamily A Member 1 (CYP17A1) to form 17OH-pregnenolone. CYP17A1 then through its lyase activity converts this molecule into dehydroepiandrosterone (DHEA). DHEA eventually becomes androstenedione and then testosterone, which is then secreted and taken up by prostate, liver, brain and skin. The prostate then transforms testosterone into dihydrotestosterone (DHT) by the activity of 5α-reductase (SRD5A1/2) [100]. Both testosterone and dihydrotestosterone are agonists of the AR, however their potency is not the same; DHT is a more potent agonist of AR [101]. The first reports on AR structure were published in the 1960s and since then, AR became the center of attention in the PCa field [102].

2.2 ANDROGEN RECEPTOR: A DYNAMIC STRUCTURE

Androgen receptor (AR) is a member of the nuclear receptors (NR) superfamily, a group of transcription factors (TF) that have a similar structure and function by regulating several key cellular pathways. This superfamily comprises four different classes: steroid receptors (Class I), RXR heterodimers (Class II),

homodimeric orphan receptors (Class III), and monomeric orphan receptors (Class IV) [103]. All NR contain a N-terminal domain (NTD), a DNA binding domain (DBD), a hinge region, ligand binding domain (LBD), and a C-terminal domain (CTD) [103]. Both DBD and LBD are the most conserved domains in the family, with LBD having specific changes for ligand selectivity and affinity [103,104]. The AR cDNA sequence was published for the first time in 1988 [105-108].

The NTD is the largest region in NRs' structure and the least conserved of all domains [98]. In AR, the NTD is disordered due to the high number of protein-protein interactions occurring in this area, conferring a high degree of plasticity to the protein [98,103]. The Activator Function-1 (AF-1) region is located in the NTD and it's the anchor for co-repressors and co-activators binding to AR and thus regulates transcription [104]. Moreover, the NTD is the target for the vast majority of post-translational modifications that include phosphorylation, acetylation, and SUMOylation [104]. Therefore, the NTD and its AF-1 region have a crucial role in the ability of AR to regulate transcription. Important evidence supporting this is shown by mutations in this area contributing to a weaker regulation of AR-related gene expression [109,110].

The DNA binding domain (DBD) is widely conserved and is the region responsible for the binding of AR to DNA. Three main zones constitute the DBD: two zinc fingers and a carboxyl-terminal extension [102]. The first zinc finger contains a P-box, a recognition helix that contributes to AR-DNA binding, and the second zinc finger contains a D-box, which allows AR dimerization [111,112]. The AR-DNA binding is not random; instead, it happens at the so-called androgen responsive elements (AREs). Chromatin immunoprecipitation (ChIP) experiments have shown and established that the canonical binding site for AR is 5'-AGAACAnnnTGTTCT [113-115]. The strength of this interaction depends on the amount of variability in the sequence, repeats of AREs and the co-repressors and/or activators assembled with AR at a given point [116].

The hinge region is the shortest in the structure of AR and it is a linker between the DNA and ligand binding domains. The hinge region contains a nuclear localization signal sequence that when exposed after ligand binding allows the translocation of the receptor from the cytoplasm to the nucleus [117,118]. The import of AR from the cytoplasm to the nucleus is accomplished with the help of importin-α [119]. Similar to the NTD, the hinge region is the target for several post-translational modifications [120].

The ligand binding domain (LBD) is the region where the interaction between testosterone and DHT occur, and its structure was first described two decades ago [121]. Upon binding of the ligand to a hydrophobic ligand-binding pocket, the receptor undergoes a conformational change that alters protein-interaction and localization of AR [122]. Akin to the NTD, the LBD contains an Activation Function (AF-2) region that facilitates interaction with co-regulators of AR [104]. The LBD also possesses a nuclear export signal that suppresses the nuclear localization signal present in the hinge region in the absence of ligand [116].

Despite the importance of natural ligands for AR function, in recent years several splice variants of AR have been described, mostly in the context of PCa [123]. Most of the variants described so far lack the LBD and are commonly constitutively active and able to bind to DNA and deploy an AR-signaling pathway even in the absence of ligand [123]. Combined with the importance of AF-1 for a strong regulation of transcription, this shows the plasticity of AR and its ability to regulate transcription without ligand.

Understanding how AR works and its structure has been of crucial importance to develop therapeutic strategies for PCa, and to make an educated attempt at predicting how the cells might develop resistance to these strategies. One of the main functions of AR is to act as a transcription factor and thus regulate expression of several genes that influence normal tissue development as well as cancer onset and progression.

2.3 A POWERFUL REGULATOR

Androgen receptor (AR) is the master regulator of prostate growth and function. AR in its normal length and without ligand binding forms a complex with heat-shock protein 90 (HSP90), and upon binding of the ligand changes its conformation and gets translocated to the nucleus where it can activate or repress gene transcription [124]. Given its role in the gland's growth and development, AR needs to regulate a wide array of cellular pathways. Around 20,000 full or half-site AREs have been described, and with the technological advances this number will only grow larger [124,125]. Most of these locations have been identified in gene enhancers and promoters, but recently intergenic regions have also been identified as a target for AR-regulation [125-128]. The most widely recognized AR targets are already used in clinic for diagnosis, prognosis or molecular stratification. In 1991, PSA (or KLK3) was first described as one of AR targets, and later in 1999 TMPRSS2, which is frequently translocated and fused in PCa, was also validated as an AR target [129,130]. Importantly,

a recent study has shown that AR has distinct programs depending on the cell cycle status of each cell [128]. When put into the context of cellular heterogeneity within prostate tumor masses, these cell cycle-related signaling programs might explain different dependence on various signaling pathways triggered by AR. The physiological regulation by AR is accomplished by two distinct mechanisms of action: (1) non-genomic, and (2) genomic.

Non-genomic regulation does not involve binding of AR to DNA, and instead occurs within seconds to minutes without translocation to the nucleus being necessary [131]. There are three basic criteria established to define non-genomic actions: (1) speed – it needs to occur between seconds to minutes, (2) membrane mediated – action that can be performed even if bound to repressor proteins, and (3) lack of transcription or translation machinery [132]. This mechanism of action is commonly associated with rapid induction of secondary messenger signal transduction cascades, such as free intracellular calcium, activation of protein kinase A and C (PKA-C), Mitogen-activated protein kinase (MAPK), and tyrosine kinase c-Src [133,134]. One important membrane structure that has been suggested to influence AR non-genomic function are caveolae [116,135]. Caveolae are structures in cell membranes rich in signaling proteins and membrane receptors, and have been shown to interact with AR in what could be a ligand-independent mechanism of activation of non-genomic AR [116]. It is possible that by activating kinases, AR is then phosphorylated itself promoting its activity without ligand in a feedback loop that would perpetuate AR's role in prostate cells. More studies addressing the non-genomic roles of AR are needed, but one thing is clear: these seem to be a highly important complement to the genomic role of the receptor.

As a nuclear receptor, and despite non-genomic actions, the current data suggests that AR acts mostly as a transcription factor. For this, AR needs to associate with the transcription machinery, co-activators and co-repressors [136]. A ratio between co-activators and co-repressors has been suggested as an important determinant of what kind of regulation by AR will be imposed in gene expression in a tissue- and temporal manner [137]. More than 169 co-regulators of AR have been described (reviewed in [136]). Co-activators of AR have been described as being part of different cellular pathways, which indicate a tissue-specific role in the activation of AR. These proteins act directly on the AR structure or recruit other partners to modify chromatin, NTD interactions with C-terminal for stabilization and enhanced activity, or recruitment of more co-activators [138]. Thus, it is not surprising that mouse models

with deletion of some of these co-activators have shown phenotypes similar to hormone deficiencies [139-141].

On the other hand, co-repressors act to negatively mediate expression of AR targets, however their *modus operandi* is similar to the co-activators: chromatin modification, inhibition of recruitment of co-activators, and blocking AR from translocating to the nucleus [138]. The most noteworthy are the mechanisms that still allow AR to bind to DNA, but the outcome is the repression of transcription, unlike what has been the primary study of AR signaling, its activator role. A putative molecular mechanism for this repression involves AR binding to a negative regulatory sequence and recruiting histone deacetylases (HDACs) that will lead to a more compact chromatin and impair transcription of that specific gene or loci [134]. The recruitment of HDACs happens after binding of nuclear co-repressors to AR: nuclear receptor corepressors (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT) [142]. Another mechanism of gene repression by AR comes from its co-expression with lysine-specific demethylases 1 (LSD1) that leads to demethylation of activating chromatin marks next to AREs enhancers [142]. On the contrary, AR has also been shown to associate with Enhancer of zeste homolog 2 (EZH2) that augments silencing of expression by increased methylation [142]. More studies are necessary to shed light on this important regulation, especially with evidence that AR can play both a tumor suppressor and oncogene role in PCa [143].

Taking into consideration the multitude of roles that AR can play, as well as the array of pathways and partners related with this nuclear receptor, dysregulation of either of these components can lead to altered phenotypes and end up in pathological conditions.

2.4 Disorders associated with the Androgen receptor

AR plays an important role in the signaling ability and is widely expressed in the body. Thus, changes in AR expression have been associated with different diseases, in the prostate and elsewhere. AR is located in the X chromosome; therefore, changes in its expression affect men differently than women, although these alterations can then be passed to the next generation. One of the most sociologically detrimental diseases resulting from AR deficiencies is male infertility, where a long (CAG)nCAA repeat diminishes the activity of AR and leads to defective spermatogenesis [102]. The same repeat has been associated with Kennedy's disease, as it results in degeneration of motor neurons in the lower body with subsequent muscle weakness [102,122]. Another condition related to AR is the androgen insensitivity

syndrome (AIS), where a mutation in the receptor leads to a lack of ability to respond to androgens [144]. AIS is a X-linked inherited disease first described in 1947 that results in impediment of males developing male sexual characteristics [102,122,144]. Development of cognitive impairment, bone disease, cardiovascular disease have all been linked with AR [116,122].

Despite all of these diseases being associated with AR, the one that has received most attention is PCa even though alterations in AR status is not sufficient to develop the disease [78]. Mouse models with overexpression of AR have been shown to develop PIN lesions [78]. One of the most interesting models concerns loss of AR in the stromal cells of the prostate in heterozygous PTEN mutants that led to repression of PIN formation [78]. The role of AR in disease onset and progression is undeniable, and several alterations have been described as mutations, overexpression, and gene amplification [102,116,122,145]. Given the importance of the androgens-AR signaling axis in PCa, several pharmacological approaches have been designed to block this important pathway (see section 2.6 of this Thesis), and evasion mechanism to these strategies will be explored in section 2.7 of this Thesis.

2.5 Metabolic regulation: Feeding the uncontrollable fire

As previously mentioned, metabolic changes are essential for cancer progression as cancer cells have a high demand for energy to sustain their proliferation. In PCa, development and progression are accompanied by higher rates of lipid synthesis and metabolism, mostly coordinated by AR [91,146,147]. In fact, studies have shown that AR is responsible for increased rates of beta-oxidation and lipogenesis and that both lead to higher proliferative rates [148,149]. One of the metabolic hallmarks of PCa is lipid accumulation, a phenomena happening in patients and models of study [84]. Enzymes responsible for fatty acid synthesis, such as fatty acid synthase (FASN), acetyl-coA carboxylase (ACAC), and acetyl-coA lyase have also been identified as overexpressed in PCa [91]. These newly generated fatty acids can then be used to generate membrane lipids [150] or used for energy production to sustain proliferation. Importantly, AR has been shown to regulate both FASN and ACAC in PCa [151]. Another metabolic branch regulated by AR is cholesterol biosynthesis. AR is able to regulate Sterol Regulatory Element-Binding Proteins (SREBP), a transcription factor responsible for cholesterol synthesis, which in itself is a precursor of steroid hormones clearing the way for a feedback loop where AR is able to regulate itself [91]. This is only accomplished by the ability that PCa cells have to esterify cholesterol and store them in lipid droplets where high levels of cholesterol

are no longer toxic to cells, and can't block the activation of SREBP [91]. AR is able to activate SREBP cleavage activating protein (SCAP) that then binds to SREBP, leading to its accumulation in the nucleus and thus activation of downstream targets involved in lipogenesis [84,152]. One other source of lipid accumulation is autophagy regulated by androgens and AR [153]. A recent study in mouse models of PCa showed that lipids promote PCa aggressiveness, as the deletion of PTEN and Promyelocytic leukemia protein (PML) led to activation of Mitogen-Activated Protein Kinase (MAPK) signaling and subsequent lipid production [154,155]. A minimally defined role for AR concerning lipid metabolism is the regulation of exogenous lipid uptake, which can generate a feedback loop: release of fatty acid from adipose cells or cells in tumor microenvironment, and uptake by the prostate cancer cells [147]. Uptake of exogenous lipids has been shown to be a biomarker for aggressiveness and worse prognosis [156,157]. However, more evidence is necessary to prove AR involvement in the enhanced uptake. Several therapeutic options have been tested to block lipogenesis with variable success [84,90,155].

Despite PCa being more lipogenic than most tumors, which rely on glycolysis, AR has been described as a regulator of glucose transporters, which can then feed into several key oncogenic pathways such as Phosphoinositide 3-kinases/AKT serine-threonine kinase/mammalian Target of Rapamycin kinase (PI3K/AKT/mTOR) [91]. The aforementioned relationship between AR and the epigenetics machinery is also an important regulator of metabolism, as the subsequent metabolites has direct impact in cells metabolism. Another important fuel source for cells, especially rapidly dividing cancer cells, is amino acid uptake and metabolism, as these are important for protein synthesis and energy production. AR has therefore also been linked to the up-regulation of amino acid uptake and metabolism in PCa, mainly glutamine, phenylalanine, tryptophan, tyrosine, lysine and arginine [158].

AR regulation of metabolism, and its ability to do so in the transition from latent to aggressive PCa is one of the reasons why so much emphasis has been put into developing effective therapeutics that target this receptor. However, the metabolic control and the ability to keep adding "logs to an uncontrollable fire" are the main reasons why tumors resist those same treatments. The continuous development of new methods and better equipment capable of more advanced metabolomics and lipidomics will keep adding to the understanding of the importance of AR's tight connection with metabolism.

2.6 Blocking the receptor

The role of androgens and the androgen receptor in the onset and progression of prostate lesions that culminate in prostate cancer is clear and long established. Thus, several inhibition strategies for the AR axis have been established with different degrees of success in the clinic. Independent of the specific strategies used, there are two main categories for this type of treatments: androgen synthesis inhibitors that block the synthesis of androgens and stop AR activity upstream of the receptor, and AR antagonists, which directly compete with androgens to block AR activity.

A standard treatment for patients with advanced PCa is to block luteinizing gonadotropin-releasing hormones (GnRH) leading to decreased production of testosterone [31,159]. After reducing the levels of serum testosterone, PCa shrinks but patients eventually relapse to the lethal phase of the disease [160]. However, this reduction only affects the testosterone produced in the testes, not impacting the adrenal gland and intratumor cells that are still able to produce DHEA via *de novo* steroid synthesis [159,161]. The DHEA can then be used by the prostate gland to generate testosterone and feed AR signaling [159]. Therefore this pathway is crucial to prevent resistance or to be used as a secondary treatment after initial resistance to GnRH inhibitors, and the Federal Drug Administration (FDA) has approved one inhibitor for this effect with others in advanced clinical trials [162]. The main enzyme involved in this pathway is CYP17A1, and its irreversible inhibitor Abiraterone acetate (Abi) is already approved by the FDA and in the clinic for PCa patients that relapsed following initial chemical castration [159,160]. Abi was developed after Abiraterone showed high toxicity in patients, with efforts resulting in 20 new compounds [161]. Abi was then shown to have even higher selectivity than Abiraterone, and moreover, as it inhibits the production of testosterone in the testes and adrenal gland, it is more effective than GnRH inhibition [161,163,164]. Abi has also been shown to be a partial antagonist of AR indicating that its benefits might come from targeting more than one pathway in the AR signaling pathways at any given time [165-167]. In clinical trials, Abi has shown an improvement in overall survival (~4 months) beyond standard of care for metastatic CRPCa but eventually patients relapse following development of resistance to the regimen [65,161]. Abi treatment induces the accumulation of steroid components upstream of CYP17A1 that have been shown to activate mutant AR forms, a potential evasion mechanism [65,160]. More enzymes involved in

steroidogenesis other than CYP17A1 are promising targets for PCa treatment and researchers have been working on it, albeit without any getting to the clinic [161].

The other main group of inhibitors includes direct AR antagonists, and several have been discovered and approved throughout the years. AR is in the top 8% of druggable targets in regard to quantity of compounds that have been predicted to bind to it [168]. Within this group, the first generation included flutamide, nilutamide, and bicalutamide, which prevented binding of ligand and subsequent translocation of AR to the nucleus [161]. These three compounds had different degrees of affinity to AR with bicalutamide being the strongest and the only of these that hasn't been replaced by more potent inhibitors [160,161,169]. A key issue surrounding the first-generation AR inhibitors was their agonist/antagonist switch, where there was evidence for these drugs to potentiate AR action. In 2012, the FDA approved for metastatic PCa the first of the second generation of AR inhibitors, Enzalutamide (MDV3100) [169]. This new inhibitor resulted from a screen of about 200 nonsteroidal anti-androgens using LNCaP cells as the model of study [170,171]. This study showed that MDV3100 had higher affinity than bicalutamide (5-8 times) and was able to block ligand binding and slow down translocation of the receptor to the nucleus, binding of AR to DNA, and interactions of AR with co-activators [170]. Most importantly, MDV3100 was believed to not have agonist effect on AR, a key difference from the first-generation drugs. However, this has been disproved in the case of AR(F786L) mutations where MDV3100 has the ability to increase AR activity [170,172]. Moreover, MDV3100 does carry a novel side effect not seen with the first generation inhibitors, as this drug penetrates the blood-brain barrier (BBB), inhibits g-aminobutyric acid receptor ($GABA_A$) causing seizures in patients [161]. Taking this into consideration, researchers have tried to develop new compounds that act like MDV3100 without the neurological side effects or agonist switch. In 2018 and 2019, the FDA approved Apalutamide and Darolutamide respectively, for the treatment of metastatic PCa [161,172,173]. Despite both drugs displaying less brain penetrance, Apalutamide treatments are still subject to agonist switch; meanwhile, Darolutamide has no agonist switch properties, an improvement over every other AR inhibitor [161,172].

A lot of scientific effort and investment has been placed in finding efficacious treatments for PCa. Strikingly, the drug market value of PCa drugs is expected to reach $10B in 2026, with AR inhibitors accounting for half of that amount [168]. The biggest caveat remains despite all of the effort: disease progression. Regardless of the promise brought by the drugs above described, PCa remains elusive to treat as tumors find escape mechanisms to the targeted therapeutics. Antiandrogen

withdrawal syndrome (AAWS) has been described in patients treated with androgen synthesis inhibitors such as Abi, first and second-generation AR blockers [174]. This syndrome is defined as a drop in PSA serum levels after removing the patients from androgen deprivation medication, a parameter that is usually used to indicate that those same compounds are working [174]. Determining when to administer these drugs to patients has been a common quest for physicians, as a recent trial for Abi showed improvement in survival and disease progression in patients that were hormone naïve [175]. Moreover, to understand how AAWS arises, what mechanisms accompany such phenotype that ultimately results in disease progression, will guide physicians and help determine combination treatments potentially outside of the AR pathway that will help PCa patients long-term.

2.7 The seemingly unavoidable relapse

Cancer treatments in general have been shown to be effective, however this efficacy is usually short-term with recurrence always looming over physicians and patients. Prostate cancer due to its cellular and molecular heterogeneity it is difficult to determine and predict the best therapeutic options for patients. However, and taking into consideration the role androgens play in the development of prostate gland, blocking the production of these hormones by chemical castration or surgery (ADT) has been the main targeted therapeutic for PCa. Eventually patients relapse to the first stage of castration-resistance, hormone resistant PCa (HRPC), where these treatments are no longer effective. The main therapeutic option for HRPC is a combination of taxanes (e.g. docetaxel) and second-generation AR inhibitors (MDV3100, apalutamide, darolutamide) or Abiraterone acetate. Nonetheless, tumors adapt and relapse to the second stage, which is commonly termed castration-resistant PCa (CRPCa) that ends up being responsible for most deaths by PCa. One strategy with which physicians have been experimenting is to deploy combination treatments in early stages of the disease instead of incremental and sequential treatments [64]. Several models of resistance have been proposed for the ability of tumors to adapt to these treatments.

Mechanisms of resistance have been described for tumors and there are general mechanisms that also occur in PCa. Multi-drug resistance 1 (MDR1) is a membrane protein that pumps drugs out of cells (especially docetaxel) contributing to resistance [64]. Tumors exist in harsh conditions and one of the hallmarks is a leaky vasculature [47]. Leaky vasculature can impair the drug uptake by tumor cells due to defective transport [176]. However, the principle of leaky vasculature is behind the

promise and success of nanotechnologies that can still be transported and because of their size and deficient vasculature benefit from enhanced permeability retention (EPR) in tumors [177]. Another source of drug resistance related with vasculature is the hypoxic tumor environment, where overexpression of hypoxia-responsive genes upregulate pathways related to proliferation and survival, including phosphorylation of sphingosine to sphingosine-1-phosphate [178,179]. The production of interleukins such as IL-6, IL-23, IL-1β and IL-10 by stromal cells and infiltrating macrophages have also been associated with worse prognosis after treatment, with subsequent increased AR activity [180-183].

Recent studies have shown that after treatment of PCa, especially after targeting the hormonal axis, tumors elevate proliferation pathways bypassing the effect of treatments. In PCa the main evasion mechanism is still related to AR signaling as most metastatic CRPCa retain elevated levels of AR and downstream targets [5]. As previously described, mutations in AR lead to lack of efficacy of AR antagonists, and about 10-30% of PCa have gain of function mutations [5]. Several outcomes derive from these mutations including hypersensitivity to the natural agonists, protein stability, increased affinity for other steroids or growth factors, ligand-independent activity, and increased ability to recruit co-activators [5,64,159]. These phenomena suggest a pressure on cells to adjust their AR status in order to circumvent treatments.

Another common alteration regarding AR is splice variants that have been described as constitutively active even in the absence of circulating androgens [123]. The splice variant AR-V7, which lacks the LBD, was first described in 2008 and recent studies have linked this variant with resistance to Abi and MDV3100, and decreased survival rates [184-186]. Interestingly, ADT has been shown to drive splicing factors to AR pre-mRNA and increase levels of AR-V7 [187]. Despite AR-V7 being the most commonly found variant, others exist that lack the ability to translocate to the nucleus [123]. Thus, it is possible that these variants can have a predominant non-genomic role and trigger signaling cascades as described in section 2.3 of this Thesis.

Other transcription factors, including steroid hormone receptors such as glucocorticoid receptor (GR) can also compensate for AR signaling. Up-regulation of GR has been shown to be present in patients after treatment with MDV3100 and is associated with poor clinical response [188]. A molecular explanation for this is the overlap of about 50% between genes activated and repressed by AR and GR [64,188]. Moreover, oncogenic pathways such as the PI3K/AKT/mTOR cascade have also been described as elevated in PCa and can play a role in evasion of AR signaling inhibitors

[189]. Of importance, researchers have shown PI3K/AKT/mTOR can then positively regulate AR generating a feedback loop that in the absence of PTEN can be extremely detrimental for patients [64]. Given tumors instability, changing the ratio of co-activators/co-repressors plus epigenetic changes can also potentiate the resistance to AR inhibition and progression to both stages of CRPCa [5].

The main goal of GnRH inhibitors is to decrease the number of androgens produced in the body and to slow down AR activation and signaling. It has become apparent that PCa cells are able to circumvent this blockade by increasing the number of androgens produced from cholesterol, adrenal precursors or progesterone generating an intracrine and paracrine feedback loop of constant activation [145,190]. Despite lack of a definitive molecular mechanism for why tumors relapse to AR inhibition via intratumoral androgen synthesis, researchers have suggested that the higher affinity of testosterone and DHT to AR, compared with AR inhibitors, results in "out-competition" and AR activation [145]. Evidence supporting this theory comes from the up-regulation of key enzymes in the *de novo* steroidogenesis pathway, such as CYP17A1, suggesting why Abiraterone acetate is then efficacious in HRPC patients [145].

Lastly, lineage switching, and plasticity is of the utmost importance in the adaptation that occurs within tumors and that allows their function after treatment regimens. Part of this adaptation involves a transition from adenocarcinoma and AR-dependent phenotype towards a mixed phenotype between adenocarcinoma with neuroendocrine differentiation (NEPC) [2,191]. Recent efforts supported by advanced mouse models from the Abate-Shen lab have revealed that the progression to NEPC arises from transdifferentiation of luminal prostate adenocarcinoma cells after Abi treatment [25]. The authors of this study propose a model where there are neuroendocrine cells in the prostate, which have low proliferation rates (focal) and overt neuroendocrine phenotype that arise after Abi treatment and that is highly proliferative and lack AR expression [25]. Neuroendocrine differentiation is characterized by small cell morphology, the presence of chromogranin A, synaptophysin, and neural cell adhesion molecule [64,159]. Cell plasticity is then responsible for a lineage switching that renders androgen synthesis and AR inhibitors as useless for PCa treatment. Neuroendocrine phenotype occurs in about 25% of patients with metastatic CRPCa while infrequent in primary tumors [11]. Importantly, whole-exome sequencing studies comparing AR-dependent CRPCa and NEPC showed that these tumors are genomically similar, while the epigenetic profile is very different suggesting a role for epigenetic alteration in the progression to

neuroendocrine phenotype [64]. In fact, EZH2 overexpression has been shown in several models of neuroendocrine differentiation as well as patients [2,191,192]. Two of the main players in this progression seem to be overexpression of SRY-Box Transcription Factor 2 and 11 (SOX2/11) [25]. SOX2 regulates several key pathways that fit the model of progression and phenotype of neuroendocrine cells: stem cell-like properties, epithelial to mesenchymal transition (EMT), and neuroendocrine differentiation [193,194]. Inhibition of SOX2, SOX11 or EZH2 has been shown to reverse the lineage switching phenomena as well as the resistance to MDV3100 [25,195,196]. These data suggest that inhibitors of these proteins might be relevant to use as a combination treatment with already established treatments for PCa in order to stop the phenotype switch towards a more aggressive disease.

Despite all the efforts towards fine-tuning the treatments for PCa patients and avoid relapses, for now that has been unavoidable. A better understanding of pathways that are at the intersection of different activated or repressed mechanisms in tumor development or progression is crucial. More advanced techniques and models will continue to allow researchers to find novel therapeutic targets.

It is our understanding that sphingolipids are extremely well positioned to play an important role in all stages of PCa progression, as well as potential therapeutics. These lipids are important for membrane structure, as well as signaling cascades showing their relevance in cellular adaptability to the harsh conditions the tumor faces, and how they can be explored as therapeutic avenues. **The work in this Thesis is focused in understanding the intersection between sphingolipid biology, AR and Prostate cancer.**

3. The most enigmatic lipids: Sphingolipids

3.1 Uncovering the Sphinx

Lipids are biomolecules that are soluble in nonpolar solvents, and initially were thought to only play a role in cell energy and membrane structure. However, since the 1950s it became clear to investigators that some lipids were also involved in cell signaling [197]. These are so-called bioactive lipids, which respond to stimulations and that result in activation of downstream signaling cascades both intra- and extracellularly, being an integral component of signaling circuitry [198]. Given this role for lipids, it has become clear the lipidome of a cell is of extreme importance: the synthesis of metabolites and lipids represent the last steps after the DNA, to RNA, to protein progression, and therefore could be more indicative of phenotype.

"What is the creature that walks on four legs in the morning, two legs at noon, and three legs in the evening?" That was the riddle posed by the mythic creature, Sphinx. In both Egyptian and Greek mythology, the sphinx represented guardians of temples. According to the Greek mythology, Oedipus solved the riddle and defeated the Sphinx: the answer was man, who crawls in infancy, walks as an adult and needs a can in old age. In 1884, Johann Ludwig Wilhelm Thudicum published is acclaimed work "A treatise on the chemical constitution of the brain" and named one lipid that startled him after the Sphinx: sphingosine [198]. After describing the two first sphingolipids, sphingosine and sphingomyelin, Thudicum is widely considered the "Father of Sphingolipids". The term "sphingolipids" was coined in 1947 as it referred to lipids that contained a sphingoid base [199]. It took close to 100 years until investigators discovered the bioactive roles of sphingolipids, and just like in the Sphinx legend, when this riddle was solved Hannun and colleagues determined that adding these sphingolipids to cells led to death by suicide [200,201]. Sphingolipids are one of the main classes of bioactive lipids and at the membrane play a role in barrier function and membrane integrity [202].

All sphingolipids are built upon the same building block, sphingosine that can vary in its chain length [203]. However, free sphingosine is present at small amounts in mammalian cells as it is commonly N-acylated with a fatty acid CoA of varied carbon length to originate ceramides [203]. Complex sphingolipids such as sphingomyelin and glycosphingolipids (GSL) are formed by modifying and adding groups to ceramide, which is thus considered to be at the center of sphingolipid metabolism [204]. In 2008, Hannun and Obeid proposed a rule of thumb for the mass of sphingolipids usually present in human cells [197]. Briefly, per 10,000 molecules of sphingomyelin (SM) there

are 1,000 of ceramide (Cer), 30 of sphingosine (Sph) and 0.3 of sphingosine-1-phosphate (S1P) [197]. With the advancement of analytical methods to quantify sphingolipids, it has become evident that these proportions are merely indicative and not dogmatic. The sphingolipid mass is variable between tissues, stages of development as well as different insults cells have been exposed to. The different role played by the diverse sphingolipids in cell biology highlights that dynamic composition in cells.

The sphingolipid pathway is an intricate subway system, with different stops (lipids) being "end-points" or just mere intermediary stops in different metabolic routes (Figure 3). Interestingly, this subway system has only one entry and one exit known to researchers. Several key enzymes are involved in the following pathways: *de novo* synthesis, sphingomyelin hydrolysis, salvage pathway, ceramide glycosylation, ceramide phosphorylation and the exit pathway. The sphingolipid train needs to keep moving; when the train is stopped it originates sphingolipidoses, lipid storage disorders where certain lipids accumulate causing toxicity to cells (reviewed in [205]). Sphingolipids have been associated with so many cellular pathways that their involvement in cancer is natural [198]. The generation of different sphingolipids via different pathways, their role in cancer and potential therapeutic avenues to be pursued will be the subject of this section.

3.2 DIFFERENT ROUTES OF SPHINGOLIPID SUBWAY SYSTEM

3.2.1 THE ONLY ENTRANCE: *DE NOVO* SYNTHESIS OF SPHINGOLIPIDS (D)

The typical sphingolipid subway system starts in the endoplasmic reticulum (ER) with one simple reaction: condensation of L-serine and acyl-CoA (palmitoyl-CoA being the most abundant) to produce 3-ketosphinganine [206]. This synthesis process was described in two publications in back-to-back years [207-209]. However, just like in any other metabolic pathway there are exceptions to the rule; mammalian cells can use L-alanine or L-glycine instead of L-serine and therefore create atypical sphingolipids, or 1-deoxysphingolipids [210]. Serine palmitoyltransferases (SPTLC1-3) catalyze this rate-limiting step, in combination with serine palmitoyltransferase small subunits (SPTSSA-B). Originally, SPTLCs were thought to function as a single subunit, but further studies have shown that SPTLCs act as heterodimers to confer specificity to the sphingoid base generated [211]. Moreover, SPTSSA-B confers maximum activity to the heterodimers formed by SPTLCs, increasing its activity by 100-fold [211]. Despite initially being thought as not substrate specific, the different heterodimers possible to assemble between SPTLCs and SPTSSs have been shown to

impact which sphingoid bases are generated. Han *et al.* described the following heterodimers and corresponding specificity: SPTLC1-SPTLC2-SPTSSA lead to C18 sphingoid bases, the most common in mammalian cells, by utilizing C16-CoA as substrate; SPTLC1-SPTLC3-SPTSSA generate C16 and C18 sphingoid bases by consuming C14- and C16-CoA with preference for C14-CoA; SPTLC1-SPTLC2-SPTSSB complex has a strong preference for C18-CoA substrate generating C20 sphingoid bases; and SPTLC1-SPTLC3-SPTSSB has the ability to use a wide range of acyl-CoAs, without preference [211-213]. Therefore, the availability of different fatty acid-CoA and serine can impact the availability of substrates for the SPTLC-SPTSS complex to assemble. In fact, in parts of the brain, D-serine has been shown to be abundant and to competitively inhibit sphingolipid metabolism [214]. Another mechanism for inhibiting this pathway is the presence of exogenous sphingolipids that shuts down the SPTLC complex [215]. Hereditary sensory and autonomic neuropathy 1 (HSAN1) is characterized by loss of pain and temperature sensation caused by a mutation in SPTLC1 [216,217]. The SPTLC1 mutations in HSAN1 patients have shown to increase the specificity for L-alanine and L-glycine and subsequent generation of deoxysphingolipids that accumulate and cause neurotoxicity [218,219]. A mutation in the SPTSSB gene in mice has been reported as leading to accumulation of sphingolipids with C20 sphingoid bases inducing neurodegeneration [220]. The correlation between mutations and neurodegeneration is important and the Wattenberg laboratory recently published a temporal dynamic expression of SPTLCs and SPTSSs during myelination in rat oligodendrocytes that can explain why these are crucial molecules for normal brain function [221]. More studies are necessary to elucidate these mechanisms, however and given that over and underregulation of this pathway leads to pathological conditions, it seems important that organisms keep this entrance in check; enter ORMDL Sphingolipid Biosynthesis Regulator 1-3 (ORMDL1-3).

ORMDL proteins were found in 2002 and located to the cytosolic surface of ER, where they sense sphingolipid levels and interact with SPTLCs [222-226]. The Breslow study showed that depletion of ORMDLs leads to increase *de novo* synthesis of sphingolipids, providing evidence for ORMDLs negative regulation of SPTLCs complex [223]. Two important issues remain to be clarified in the ORMDL world: the role of the different isoforms regarding sphingolipid recognition, and how the lipid sensing occurs. Answering these two important questions will elucidate how cells respond to different sphingolipid fluctuation and stop the synthesis at a given time to prevent toxicity from occuring.

The next stop after the formation of 3-ketodihydrosphingosine is its rapid NADPH-dependent reduction to form dihydrosphingosine (dhSph) by the enzyme 3-ketodihydrosphingosine reductase (KDSR) [206,227,228]. KDSR reduces 3-ketodihydrosphingosine at its ketone group to a hydroxyl group and is present at the cytosolic leaflet of the ER [227,229]. Defects in KDSR have been associated to the onset of a skin disorder named progressive symmetric erythrokeratoderma, bovine muscular atrophy, and type II chronic lymphatic leucemia [228].

The progression in the subway leads dihydrosphingosine to dihydroceramides (dhCer) by N-acylation at the C2 amino group of dhSph and is executed by 6 different (dihydro)ceramide synthases (CERS1-6) [228,229]. CERS are specialized enzymes with preference for different fatty acid-CoA substrates, albeit this preference overlaps between some of the enzymes [202,228]. It is commonly accepted that CERS1 originates C18 (dihydro)ceramides; CERS2 creates C22-C26 (dihydro)ceramides; CERS3 is mostly expressed in testes and skin and leads to very long-chain ceramides (C28-32); CERS4 mostly responsible for generating C18-C20; CERS5 and CERS6 create C14 and C16 (dihydro)ceramides [202,204]. Different chain length and different saturation result in ceramides that play various roles in human cells, which will be discussed in Section 3.3 of this Thesis.

The first (dihydro)ceramide synthase was discovered in yeast and was found to be associated with longevity, as its deletion resulted in prolonged survival [230]. This enzyme in yeast was also responsible for the generation of ceramides [230]. About twenty years later, between 2002 and 2006, all human CERS were identified and characterized [231-234]. Despite most studies pointing to presence of CERS mostly at the ER in close connection with the upstream players in the *de novo* pathway, expression of CERS in other organelles, such as mitochondria has also been found [202,235]. It is also important to note that as it is the case for CERS3, other CERS are differently expressed in the human body, which enriches tissues with different ceramides according to the tissue need and function of those lipids [236]. The idea that sphingolipids are enigmatic, and therefore full of riddles, had its origins in the 1800s but to this day they can still provoke surprise in investigators. That is the case for the dimerization of CERS, where CERS have been found to modulate each other and being able to alter the sphingolipid composition of a particular tissue or cell through those same dimers [235,237]. Cellular sub-localization of CERS appears to be a dynamic process and not static and determined by a cell's necessity or external insults and still need much more clarification.

After dihydroceramides are generated, we reach the final stop of the *de novo* synthesis of sphingolipids pathway; a one-way ride where lipids formed might accumulate because of enzymatic deficiencies but can't go backwards at any point. Dihydroceramides are then desaturated by the dihydroceramide Δ4-desaturase (DEGS1-2). First DEGSs introduce a hydroxyl group at the C4-position of sphingoid backbone at the expense of oxygen, to generate the sphingosine backbone [229]. This step is followed by dehydration using NADPH or NADH, where a double bond is introduced between carbons C4-and C-5 to generate a ceramide (Cer) [229]. DEGSs are embedded in the cytosolic leaflet of the ER membrane, making it the primary place for *de novo* synthesis of sphingolipids, from L-serine and acyl-CoA to ceramides [229,238]. DEGS1 has been described as only being able to generate fully desaturated ceramides from dihydroceramides. On the contrary, DEGS2 can produce phytoceramides, which lack the double bond and are highly abundant in plants, yeast, and human intestines, skin and kidneys [239].

Ceramides are the culmination of 4 enzymatic processes and the end-lipid of *de novo* synthesis route. A ceramide cannot go back and become dihydrosphingosine or dihydroceramide, but it is at the front and center of the myriad of pathways that constitute the sphingolipid subway system. From this point on, there are multiple two-way routes that will be discussed in the following Subsections. It is important to recognize that more studies are necessary concerning the *de novo* synthesis pathway and its potential to render therapeutic benefits and molecular insights into the ceramide field.

EXTRA EXTRA: SPTSSB, A SMALL PROTEIN WITH GREAT IMPACT

A major player in the *de novo* synthesis of sphingolipids is SPTSSB, which consists of 76 amino acids and is a 9kDa protein. SPTSSB has three different aliases, C3ORF57, SSSPTB, and ADMP, and Singh and colleagues identified this gene in 2005 [240]. These authors were utilizing a mouse model that lacks testicular production of testosterone, *hpg* that had high transcript levels of SPTSSB in the prostate [240,241]. The expression of this gene was then down-regulated by adding exogenous testosterone[240]. Therefore, it indicates a potential connection between SPTSSB expression and the development of hormone-refractory prostate cancer. Nonetheless, the relevance of SPTSSB in this process is still unknown and two hypotheses might be suggested: it may represent a biomarker for this progression from hormone dependence to independence, or an active player by regulating signaling pathways. In fact, the authors of this study showed that SPTSSB was more expressed in the brain

of normal mice and that this expression was decreased in *hpg* mice [240]. This suggests an androgen regulation that is tissue-dependent and could be explained by the presence of co-factors that differently impact transcription.

In 2009, as previously mentioned, Han *et al.* showed that the presence of SPTSSB in the SPTLC complex is critical for maximum activity and synthesis of ceramide via the *de novo* pathway [211]. This elevated activity reaches 100-fold in comparison with normal levels of the enzyme [211]. The complex of SPTLC1-SPTLC2-SPTSSB preferentially generates C20 sphingoid bases, while SPTLC1-SPTLC3-SPTSSB uses a wide range of acyl-CoAs, without preference [211,212,242]. Coordinated regulation of SPTSSB and SPTSSA is also possible, as these two enzymes are reported as leading to different sphingoid bases in the *de novo* pathway [211]. This suggests that the assembly of the complex or differential expression can be established with a feedback loop with the availability of substrates in a given tissue.

Not a lot is known when it comes to human pathologies and SPTSSB, and in cancer there have been no studies published. In the neuroscience field, mutations in the SPTSSB gene in mice lead to neurodegeneration by accumulation of sphingolipids with C20 sphingoid bases [220]. The correlation between mutations and neurodegeneration is important and the Wattenberg laboratory recently published a temporal dynamic expression of SPTLCs and SPTSSs during myelination in rat oligodendrocytes that can explain why these are crucial molecules for normal brain function [221].

These data combined suggest a role for SPTSSB in the neuronal development and function, and a negative regulation by androgens in prostate. Taking into consideration that neuroendocrine differentiation occurs in resistance to anti-hormonal treatments, we speculate that SPTSSB might play a role in this resistance mechanism given its importance in cells of neuronal origin.

3.2.2 Atypical route

Despite most sphingolipids being generated by condensation of L-serine with a fatty acid-CoA, there is a class of atypical sphingolipids generated by L-alanine or L-glycine, called 1-deoxysphingolipids [243]. The use of L-alanine generates 1-deoxy-dihydrosphingosine, while L-alanine creates 1-deoxy-methyldihydrosphingosine [213,243]. A crucial difference between these classes of sphingolipids is that atypical sphingolipids lack the C1-hydroxyl group present in dihydrosphingosine [213,243]. The lack of C1-hydroxyl group stops addition of any complex groups (carbohydrates, phosphates) that are included in the more complex typical sphingolipids. Thus, these

sphingolipids cannot be degraded via the same routes as typical sphingolipids, and instead are metabolized by cytochrome P450 enzymes [244,245]. As described above, HSAN1 patients carry mutations in SPTLC1 that lead to a substrate preference to L-alanine and L-glycine and accumulation of 1-deoxysphingolipids, which have slower metabolism and display more toxicity [218,219]. Interestingly, studies with short-chain labeled 1-deoxyceramides showed a rapid localization to the mitochondria unlike labeled short-chain ceramides that localized to the Golgi and plasma membrane [245]. The authors did report that 1-deoxyceramide localized to the ER and Golgi at later time points, and reported changes in mitochondria fragmentation [245]. The ER signal at later time points was associated with morphological changes in the organelle and subsequent ER stress [245]. The stress sensitivity to 1-deoxysphingolipids seems to be dependent on the ability that certain tissues and cells have in processing these lipids into polyunsaturated and hydroxylated products [243]. Authors have reported that depletion of L-serine in cultured cells starts a shift towards higher usage of L-alanine and L-glycine, indicating a potential dead end for a cell's fate [246]. More studies are necessary to determine how the SPTLC complex might me more promiscuous than previously thought and able to use 3 amino acids. Given the toxicity of these atypical lipids, it is imperative to determine their role in regulating or being regulated by the typical sphingolipid metabolites as well as their impact in human disease.

3.2.3 PHOSPHORYLATED CERAMIDES (P)

In 2002, Sugiura *et al.* discovered the human gene that encodes the enzyme responsible for phosphorylating ceramides, ceramide kinase (CERK) [247]. This enzyme converts ceramides into ceramide-1-phosphate (C1P), a lipid that was discovered in 1989 in rats and in 1990 in humans by the Kolesnick laboratory [248,249]. Interestingly, CERK does not use sphingosine as a substrate and is specific to ceramides [247]. The phosphorylation reaction takes place in the trans-Golgi and is enhanced by the presence of both calcium and magnesium [229]. Upon phosphorylation, C1P can be transported to plasma membrane and other cell organelles by the action of a recently discovered transfer protein, ceramide-1-phosphate transfer protein [250]. C1P has been shown to be important for induction of eicosanoid synthesis by activating phospholipase A2 (PLA2α) [251]. C1P is an important lipid messenger that activates several key pathways such as phagocytosis, proliferation, vesicular trafficking, and calcium levels [252-255]. However, C1P is still an understudied sphingolipid and more context-dependent roles might be uncovered in the future, as sphingolipids and lipid messengers become a more prominent field. The route that takes ceramides to C1P is

not a one-way ticket, and in the early 1990s researchers identified what they thought were ceramide-specific phosphatase enzymes [256,257]. However, it is now known that these are lipid phosphate phosphatases (LPP) that despite having C1P as substrate, end up dephosphorylating other lipids [258-260].

Despite being the most understudied sphingolipid pathway, it is clear that C1P plays a role in balancing the levels of ceramide in cells and can signal through several critical pathways [261]. A homeostatic balance between levels of C1P and ceramide is therefore critical for a healthy cellular and tissue composition.

3.2.4 From ceramides to sphingomyelin and back (S)

Sphingomyelin (SM) is the most prevalent of sphingolipids and a vital component of cell membranes in humans. SM is mainly synthesized in the *trans* Golgi after ceramide is transported from the ER and a phosphocholine group is added to it [262]. This reaction is catalyzed by sphingomyelin synthases 1 or 2 (SGMS1-2) that transfer phosphocholine moiety from phosphatidylcholine to ceramide generating SM and diacylglycerol as a side product [263,264]. SGMSs have also been found to have the ability to add phosphoethanolamine to ceramide forming ceramide phosphoethanolamine instead of SM [265]. Interestingly, evidence points to a differential role for both SGMS enzymes: SGMS1 at the Golgi seems to be responsible for generating the majority of SM in the cells, while SGMS2 at the plasma membrane regulates the conversion of ceramide to SM upon local perturbations [265]. In 2004, researchers found a third protein, Sphingomyelin synthase related protein (SAMD8) that catalyzes the formation of ceramide phosphoethanolamine in the ER and has been described as a sensor for ceramide levels [266-268]. Given its prominent role in the plasma membrane, the synthesis of SM is tightly connected with cholesterol synthesis [269]. SM is also an important component of organelle membranes, and SM in the nucleus is thought to coordinate with cholesterol to act as an anchor for transcriptionally active chromatin and to protect double-strand RNA from degradation [270,271]. SM molecules in the plasma membrane of human cells are used as anchors by viruses, bacteria, and fungi through toxin interaction that usually forms pores that ultimately lead to infection or cell death [265]. Defects in the SGMS enzymes is detrimental for organisms given the prominent role played by SM in the membranes and the accumulation of ceramides in cells that can cause cellular death [272].

The synthesis of sphingomyelin does not render an endpoint in the sphingolipid metabolic pathway. In fact, sphingomyelin hydrolysis is a major source of ceramide

in cells. There are 5 sphingomyelinase phosphodiesterase (SMPD1-4 and ENPP7) that are categorized according to the optimal pH for them to carry out their function. Acid sphingomyelinase (SMPD1) is predominantly localized to the lysosome and metabolizes sphingomyelin present in endosomes and was the first sphingomyelinase to be discovered [229]. SMPD1 has also been found in the extracellular space where it can metabolize the sphingomyelin present in the outer leaflet of the plasma membrane [229]. Deficiencies in SMPD1 results in a genetic lysosomal storage disorder called Niemann-Pick type A and B disease due to accumulation of sphingomyelin in several organs and resulting neurological damage [273]. Alkaline sphingomyelinase (ENPP7) is the least understood enzyme of the group but it has been shown to be almost exclusive to the digestive system where it is suggested to play an anti-inflammatory role [274]. ENPP7 has been described as mutated in colorectal cancer patients leading to accumulation of sphingomyelin and reduced ceramide levels [275]. Neutral sphingomyelinases, SMPD2-4, have been identified, with SMPD5 as a pseudogene also playing a similar role in the mitochondria. Neutral sphingomyelinases have very specific locations among the group suggesting a role in hydrolyzing ceramide in different organelles. SMPD2 localizes primarily to the ER with small presence in the Golgi and nucleus; SMPD3 is mainly located in plasma membrane, Golgi and recycling compartments; SMPD4 is found predominantly in the ER and *trans* Golgi; and SMPD5 is located in the mitochondria [262,276]. Not only are neutral sphingomyelinases located in different compartments within the cells, but they are also expressed differentially depending on human tissue [262]. These enzymes have been shown to play important roles in the conversion of sphingomyelin to ceramides in response to chemotherapeutic agents in cancer, a subject that will be further addressed in Section 3.4.1.

A perfect balance between the levels of sphingomyelin and levels of ceramide is crucial for cell stability, membrane signaling, and overall health of tissues, as these are the two most predominant sphingolipids in cells. This balance is important and can be indicative of how effective cancer therapeutics might be.

3.2.5 Ceramides with sugar and beyond (G)

Ceramides are the basis for more complex lipids such as sphingomyelin, and glycosphingolipids: a ceramide with a carbohydrate attached to its polar head group by a glycosidic bound. Almost 400 species of glycosphingolipids have been described in mammals with different numbers of carbohydrate groups attached to a ceramide, as well as different carbohydrates [203]. The synthesis of glycosphingolipids starts with

the addition of a glucose or galactose group to the 1-hydroxyl position of ceramide by either glucosylceramide synthase (UGCG) or galactosylceramide synthase (UGT8) [277,278]. Both glucosylceramide (GlcCer) and galactosylceramide (GalCer) are the most abundant glycosphingolipids species and are usually called cerebrosides due to the presence of only one sugar residue attached to a ceramide [279].

UGT8 is a transmembrane protein present in the ER membrane and mostly expressed in brain tissue, kidneys, and testes [229]. Mouse models with UGT8 knockouts die prematurely most likely due to loss of nerve function, but when the expression of UGT8 is restricted to oligodendrocytes the phenotype is reversed suggesting an important role for GalCer in brain function [280]. Addition of sulfate groups to GalCer by Galactosylceramide sulfotransferase (GAL3ST1) in the Golgi produces acidic glycosphingolipids termed sulfatides, which are abundant in the kidney and brain tissue [203,279,281]. Sulfatides have been associated with several different pathologies, but the most established is the loss of sulfatides in the early stages of Alzheimer's disease that leads to accumulation of amyloid plaques in the brain by lack of clearance [282]. On the contrary, if a sialic acid is added by ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 5 (ST3GAL5) to GalCer that creates GM4 [283].

UGCG is a transmembrane protein located in the *cis*-Golgi that synthesizes GlcCer and is essential for mammalian development as knockout mouse models are embryonically lethal [284]. In fact, GlcCer is the first committed step in the glycosphingolipid pathway [285]. Adding exogenous GlcCer to the knockout mice rescues this phenotype [284]. GlcCer are essential components of skin as a precursor of skin ceramides, and plays important roles in different cellular pathways [203]. The enzymes glucosylceramidase 1-3 (GBA1-3) are responsible for converting GlcCer back to ceramide in the ER and keep a healthy pool of ceramide and GlcCer as well as more complex glycosphingolipids in cells [286]. In 1965, researchers identified the enzyme that catabolizes GlcCer into ceramide and associated the lack of this enzyme with Gaucher's disease, a lipid storage disorder [287]. This discovery led to a treatment for patients with the disease that consists of enzyme replacement therapy by administering GBA1 [287]. The accumulation of GlcCer and lack of conversion back to ceramide disrupts several pathways that lead to inflammation and cell stress [288,289]. UGCG has also been implicated in drug resistance in cancer where its higher expression after treatment correlated with increase in expression of Multidrug resistance 1 (MDR1) [290-293]. The increase in UGCG would therefore lead to an increase in the levels of GlcCer and subsequent higher order glycosphingolipids upon chemotherapy. This phenomenon is considered a mechanism for which cells evade

the accumulation of ceramide, again showing that a perfect balance in the sphingolipids subway routes is necessary for healthy cells and tissues.

A major intermediary in the synthesis of more complex glycosphingolipids is lactosylceramide (LacCer) generated by lactosylceramide synthase (B4GALT6) in the Golgi [294]. Upon formation of LacCer, sialic acid groups might be added to the sugar groups generating gangliosides, crucial components of the plasma membrane where they interact with transmembrane receptors and other lipids [295,296]. The initial steps of glycosylation occur in the *cis*-Golgi, while the subsequent steps take place in the *trans*-Golgi [294]. There are five groups of gangliosides (0, M, D, T, Q, P) depending on the number of sialic acid groups in the molecule (0-5), plus 4 series (0-, a-, b-, c-, d-) depending on the number of sialic groups in the galactose residue [294]. In the Golgi, GM3 synthase (ST3GAL5) adds a sialic acid group generating GM3 lipids, followed by GD3 synthase (ST8SIAI) and GT3 synthase (ST8SIA5) [294]. In order, the previous lipids belong to one of the 4 different series of gangliosides, and these are the precursors of each series. Several other enzymes are involved in this highly complex pathway, and have been found to be involved in different diseases (reviewed in [285,297]). Importantly, these membrane lipids are crucial for membrane structure, but also for proper signaling to occur in mammalian cells. One of the most interesting relationships between gangliosides and human disease is through their interaction with pathogens that bind to these lipids and use them as anchors for hijacking cellular machineries. These gangliosides are also altered in cancer and have been the focus of recent immunotherapeutic strategies developed to recognize and target cancer cells [298]. More technological developments will help determine the exact roles of each glycosphingolipids species as well as their importance in human diseases and their prevention.

The last glycosphingolipid are globosides, where instead of sialic acids, more carbohydrate groups are attached to LacCer. The globosides were initially found in the 1960s [299]. Glotriaosylceramide (Gb3) one of the most prevalent globosides, has been identified as accumulating in blood and organs of patients with Fabry disease [300]. This disease is caused by a deficiency in the enzyme that breaks down Gb3, α-galactosidase A (GLA) leading to toxicity by accumulation of Gb3 [300]. As it is the case for other lipid storage disorders, enzyme replacement therapy provides Fabry disease patients with functional GLA for normal process of Gb3 [300]. Globosides have been implicated in several diseases, but their role is still understudied, and more efforts are necessary to understand a class of lipids that plays a role as cell surface marker both for treatments as well as molecular understanding of diseases.

Once again, a traffic jam in the buildup or breakdown of highly complex glycosphingolipids originates and causes unhealthy tissues and cell scenarios. The clear role for these lipids to coordinate development and treatment response, makes it imperative that with technological advancement better methods are developed for quantification and analysis of their involvement in pathologies.

3.2.6 The salvage pathway and the Exit route (E)

All the different subway routes described above either generate ceramide or add more functional groups to the structure of ceramide generating more complex sphingolipids. In the late endosomes and lysosomes, ceramide can be degraded and generate sphingosine and free fatty acid [229]. This action takes place by the enzymatic activity of ceramidases that, just like sphingomyelinases, are organelle specific and depend on optimum pH [229]. There are three groups of ceramidases: acid (ASAH1), neutral (ASAH2), and alkaline (ACER1-3). ASAH1 is predominantly present in the lysosome and hydrolyzes ceramide species that are degraded from the plasma membrane. ASAH1 is the most important ceramidase for normal development, as a full body knockout in mice ends with embryonic lethality [301]. Moreover, in humans, absence of functional acid ceramidase leads to development of Farber disease [285]. In 1995 by the Sandhoff laboratory purified this enzyme from the lysosome [302]. ASAH2 is a ceramidase that works at neutral pH to catabolize ceramide species either at the plasma membrane, Golgi or mitochondria. When at the plasma membrane ASAH2, has been shown to be important for the generation of sphingosine-1-phosphate (S1P) [303]. Knockout mouse models of ASAH2 have shown that this enzyme is important in the intestine epithelia cells for dietary ceramide degradation [304]. The three alkaline ceramidases (ACER1-3) differ not only in tissue expression, but also in subcellular expression, and substrate specificity [198]. ACER1 is mostly restricted to the epidermis, while both ACER2-3 are more widely expressed in the human body [229]. ACER1 localizes to the ER, ACER2 is mostly present in the Golgi, while ACER3 is expressed both in the ER and Golgi [305-308]. ACER2 and ACER3 are responsible for hydrolyzing phytoceramides, unlike ACER1 [229]. A knockout mouse model of ACER3 resulted in enhanced colitis with accumulation of glycolipids [309]. Ceramidases play an important role in the generation of sphingosine from ceramide. These free sphingosine and fatty acid can then be used by ceramide synthases (CERS1-6) to re-generate ceramide species. In fact, 50% of the sphingosine levels in cells are estimated to be converted back to ceramide for homeostasis [310]. This back and forth is important in order to

degrade and generate ceramide species and more complex sphingolipids keeping a cellular balance within sphingolipid species.

In cells, sphingosine can be phosphorylated by the action of sphingosine kinases (SPHK1-2) to generate sphingosine-1-phosphate (S1P), a potent metabolite mostly present in the plasma. S1P was first described in the 1960s and it has a short half-life in the blood, about 15 minutes, where it plays an important role in vascular integrity as well as immune cell trafficking [209,310]. SPHK1 has been predominantly found in the cytosol and plasma membrane of human cells, while SPHK2 can be found both in the cytosol but also in the nuclear membrane [311]. The different cellular distribution of these kinases as well as their expression being different during mice development shows that these two enzymes potentially have context-specific roles [311]. In the plasma, erythrocytes and vascular endothelial cells are the main producers of S1P, while in the lymphatic system lymphatic endothelial cells contribute the most to the pool of lymph S1P [312,313]. In response to stimulations, SPHK1 has been shown to translocate from the cytosol to the plasma membrane in order to phosphorylate sphingosine there to S1P and leading to release of this molecule [229]. Despite not being found in the nucleus most of the time, SPHK1 has two nuclear export signals in its structure, and deletion of those lead to accumulation of SPHK1 protein in the nucleus. SPHK2 has wider substrate specificity than SPHK1, using not only sphingosine, but also phytosphingosine and dihydrosphingosine [229]. Knockout mouse models for both enzymes have been generated, with SPHK2 knockout mice not displaying any overt abnormalities, while mice with absence of SPHK1 were shown to have less intestinal inflammation in the context of induced colitis [314]. Importantly, given the role of S1P in the vasculature, the simultaneous knockout of both enzymes results in embryonic lethality due to improper vascular development [315]. Thus, it is likely that SPHK1 and SPHK2 might be able to compensate each other’s role when one of them is absent or has limited expression for a healthy cellular status.

S1P is a potent signaling metabolite with 5 G protein-coupled receptors (GPCR) known, Sphingosine-1-phosphate receptor 1-5 (S1PR1-5). These receptors show tissue-specificity eliciting a role of S1P in activating different signaling cascades depending on where in the body its receptors are present [316]. S1PRs have been shown to be extremely important for immune cell development and trafficking in the body, with a S1P gradient being important for cell migration and trafficking [310,317]. Nonetheless, S1P has also been shown to be able to signal through other mechanisms independent of S1PRs. One of the most interesting of these S1P actions is by binding to HDAC1 and 2 in the nucleus and inhibiting their action, impacting overall

epigenetic machinery [318]. Interestingly, these S1P targets seem to be dependent on the SPHK and location of where S1P is generated, adding another layer to the role of S1P as a potent signaling mediator [202]. More work is still necessary to disclose the specific roles of each receptor, and the degree of biological redundancy played by these GPCRs.

Playing the reverse role of SPHKs, there are two enzymes, Sphingosine-1-phosphate phosphatase 1-2 (SGPP1-2), that will remove the phosphate group from cytosolic S1P to generate sphingosine in the ER [319,320]. This activity of SGPPs is crucial to salvage sphingoid bases for the synthesis of ceramides and more complex sphingolipids such as sphingomyelin and glycolipids. SGPP overexpression, however, leads to accumulation of ceramide levels enhanced by adding exogenous S1P, showing that SGPPs are critical for the salvage pathway [321]. Expectedly, the levels of extracellular S1P were markedly decrease in the presence of higher expression of SGPP, indicating that this feedback might have crucial roles in limiting S1P availability and can contribute to S1P-dependent pathologies [322]. Both phosphatases have ubiquitous expression, suggesting a potential for compensations mechanisms giving the importance of maintaining homeostatic S1P levels, and so far there are no studies showing different roles for either enzyme.

Currently, the only exit route from the sphingolipid metabolic pathway is through Sphingosine-1-phosphate lyase (SGPL1) by hydrolyzing S1P in the ER generating phosphoethanolamine and fatty acid aldehyde that can then be reused by different metabolic pathways [323]. This reaction occurs exclusively in the ER of cells and SGPL1 is able to degrade any canonical sphingoid base found in humans [324]. SGPL1 is widely expressed in the body, with higher prevalence in thymus and intestines and lowest in brain and skeletal muscle [325]. Given the importance of SGPL1 in degrading sphingolipids when they might not be necessary for a cell, a knockout mouse model showed that at 8 weeks of age mice were not viable due to numerous defects [326]. This data emphasizes the necessity a cell has of keeping the sphingolipid metabolic subway going with no stops or accumulations. As introduced in these subsections, any unprompted stop leads to pathological phenotypes or lethality. From the degradation of S1P there is no turning back into the other sphingolipid routes.

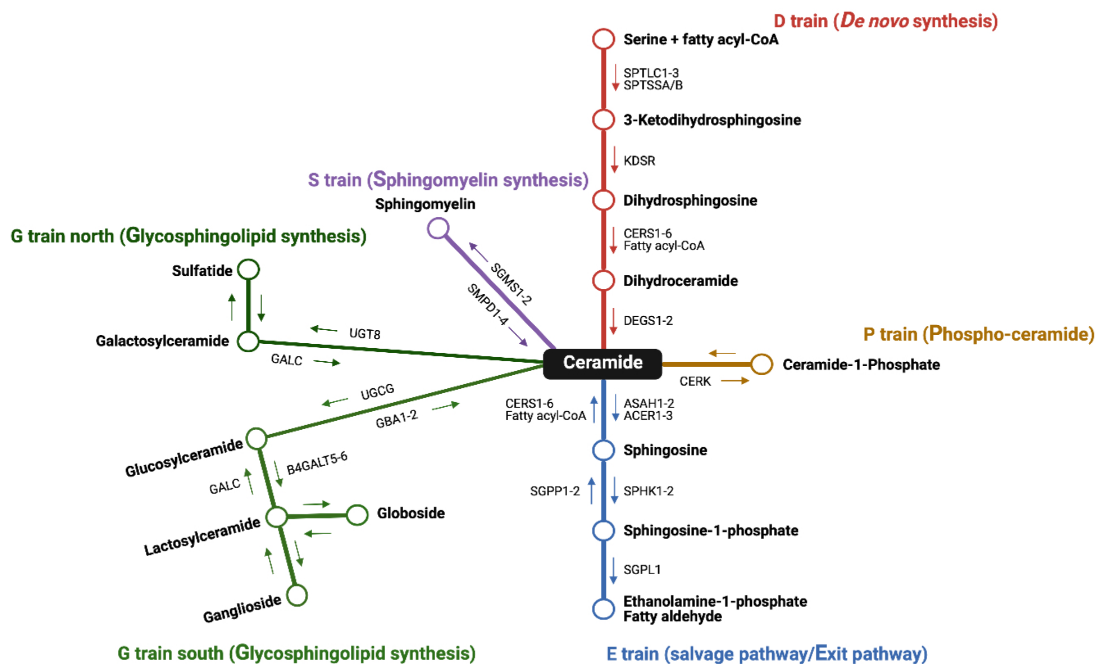


Figure 3 - The Sphingo City Metro: Representation of the pathways involved in the synthesis and metabolism of sphingolipids, as well as the respective enzymes responsible for each step. Any forced stops in the system lead to imbalanced cell homeostasis.

The main takeaway from sphingolipid metabolism is the intricate relationship between the lipid levels and the coordinated effort by enzymes in different organelles to achieve a healthy steady state. As briefly mentioned during this section, there are several sphingolipid-based diseases that have their origin in an accumulation of certain sphingolipids and the lack of others. Thus, it is not surprising that diseases like different cancers are characterized by sphingolipid imbalance and that researchers have attempted to address this with several therapeutics that target the sphingolipid metabolism pathways. The next section will focus on the role of ceramide in normal and cancerous cells and its use as a cancer therapeutic. The following reviews are more complete and provide insight into more lipids and mechanisms than the ones that are important for this Thesis [198,202,204,327].

3.3 Ceramides: a versatile lipid

Despite not having the most basic structure, ceramides are the center of the sphingolipid metabolic hub, from which more complex sphingolipids are built. As described above, ceramides are the product of 6 different enzymes: ceramide synthases (CERS1-6). These enzymes have different substrate specificities and generate ceramides with different chain length, in mammals mainly C14-C26.

Moreover, tissue distribution of these enzymes determines how much of each ceramide species a given tissue might have [236]. Ceramides are hydrophobic molecules and therefore their presence is vastly restricted to the plasma and organelle membranes where it can trigger signaling cascades and regulate membrane structure [328]. Ceramides consist of a fatty acid amide-linked to a sphingoid base, that can vary not only in length but also saturation and hydroxylation [329]. Each of these alterations in ceramides causes it to signal and regulate membranes differently [328]. Interestingly, Maula and colleagues have reported that when it comes to the chain length, differences cause minor changes in the bilayer properties of ceramide, indicating that these might be responsible for fine adjustments in lipid-lipid interaction and potentially signaling [328]. In fact, the levels of ceramide in the plasma membranes of resting cells is usually low, unlike the levels of sphingomyelin; however, upon certain stimuli and stress conditions rapid increases in ceramide levels are observed leading to activation of different signaling cascades in response [330].

Studies have also shown that ceramide generated by sphingomyelin hydrolysis in preformed membranes contributes to increased membrane permeability, vesicular aggregation and efflux of intravesicular components [331,332]. The fine-tuning caused by ceramide generation in the membranes has also been related to cell signaling by altering the dynamics of lipid-lipid, lipid-protein and protein-protein interactions. In fact, studies from Carrer *et al.* suggest that ceramide in the membrane is important for entrapping receptors and signaling molecules, favoring a stabilization of those receptors and facilitating their activation [225]. Regarding lipid-protein interaction, ceramide has been shown to associate with PKC, protein phosphatase A2 (PP2A), voltage dependent anion channel 2 (VDAC2) among others, thus regulating their activity [333-337]. In summary, the formation of ceramide-enriched domains contributes to the activation of signaling cascades by different mechanisms, showing the relevance not only of ceramide but also of the balance between sphingolipid species.

Accumulation of ceramides has long been associated with increased cell death; however, Stiban & Perera in 2015 showed that different ceramide species that vary in their hydrophobic tail have different abilities to promote cell death [338]. These authors reported that C16-ceramide has the ability to generate small and large pores in the mitochondria membrane initiating release of cytochrome C and activating apoptosis [338]. On the contrary, longer ceramide species such as C22- and C24-ceramides were also able to generate small pores, but were also reported to compete and destabilize pore formation by C16-ceramide, thus slowing down the initiation of

apoptosis [338]. These results are consistent with other studies showing that: B-cell lymphoma extra-large (BCL-XL) is able to inhibit formation of C16-ceramide-induced pores; at the beginning stages of cell death, C16-ceramide levels accumulate, while C24-ceramide is only increased in the latter stages of the process; and that overexpressing CERS4 and CERS6 that synthesize C16- C18-, C20-ceramides showed an anti-proliferative phenotype while CERS2 and C24-ceramide showed a pro-proliferative phenotype [339-342]. The advancement of high-resolution imaging and crystallography will keep advancing the field of biophysics, and more information will be gathered on how and why different ceramide species can trigger different pathways in cells.

A recent study from the Ogretmen laboratory added another layer of complexity to the ceramide field, while directing future studies to better understand not only the roles of ceramides, but also of the enzymes that synthesize them. The authors of this study showed that CERS1 is transported from the ER membrane to the mitochondria by a novel protein called protein that mediates ER-mitochondria trafficking (PERMIT) [343]. The CERS1 transported to the mitochondria is then able to synthesize mitochondria C18-ceramides that induce mitophagy-regulated cell death [343]. The spatiotemporal distribution of ceramide species was already known to be important, but this is one of the first studies showing that inter-organelle transport of ceramide synthases is crucial to determine role of ceramides and cellular responses upon stimulation or stress. However, more studies are needed with regards to other sphingolipid metabolic enzymes, as well as the localization of the lipids generated. Most studies are limited in their analysis of sphingolipid species mass as well as localization and time courses. Broad studies are thus necessary to address these issues and fully validate biological roles for both enzymes and lipids.

It is clear that different sphingolipids play opposite roles in the regulation of key cellular pathways, with the most striking example being ceramide (anti-proliferative) and S1P (pro-proliferative); this dichotomy originated the so-called ceramide-S1P rheostat [197]. How this balance is tipped might determine a cell's fate. However, with more emphasis in sphingolipid biology and the advent of CRISPR technology, the ceramide-S1P rheostat has become obsolete. As Marco Colombini has stated recently, there is a "tendency to seek the simplest interpretation (Ockham's razor) but with complex systems especially in Biology, the simplest interpretation is almost always wrong" [344]. With this in mind, in 2018 our laboratory proposed a novel model of interpretation for the different roles of sphingolipids [327]. Each cell has a pool of ceramide that can coordinate different cellular pathways and can be generated by

de novo synthesis or the conversion back from more complex sphingolipids such as C1P, sphingomyelin and glycosphingolipids ("faucets") (Figure 4). Given the prominent role of ceramide accumulation in contributing to cellular stress and death, cells then have different mechanisms to reduce the ceramide levels by conversion of ceramides to either less complex sphingolipids (sphingosine and S1P) or complex sphingolipids ("drains"). This balance between ceramide and all other sphingolipids is important to determine cell and tissue health. As previously mentioned, changing this homeostasis contributes to different pathologies; however, the understanding of how a cell must adapt to sphingolipid levels can also be used in favor of new therapeutics that explore this tipping point. **It is the goal of this Thesis to describe and understand how these two subjects have defined the importance of sphingolipids in cancer.**

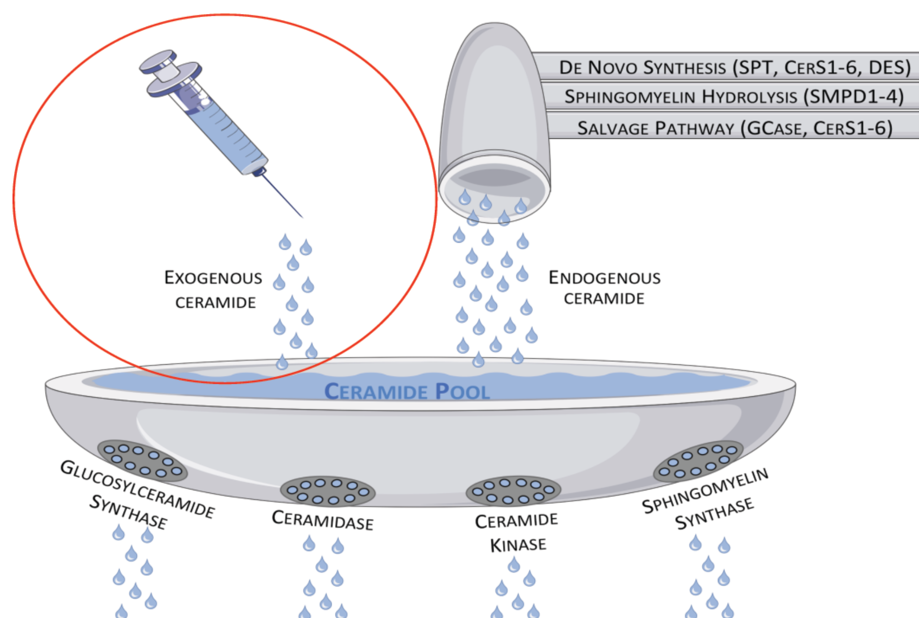


Figure 4 - Sphingolipid faucets and drains: Cells in homeostasis have a balance between synthesis of ceramide and the conversion to other sphingolipids to prevent excess of detrimental ceramides. Cancer cells have been described as overexpressing several pathways involved in the drainage of the ceramide pool. Ceramide NanoLiposomes are a mechanism to inundate this pool leading to accumulation of ceramide species and subsequent cell death in cancer cells (Adapted from reference 327).

3.4 Ceramide in cancer: the ultimate death lineup

It all started back in the early 1990s, when the late Lina Obeid demonstrated for the first time that ceramide led to programmed cell death in leukemia cells [345]. Since that publication, many others have connected ceramide to different types of cellular death in cancer. In Section 3.2 of this Thesis, the sphingolipid metabolic subway

concept was introduced, where any forced stoppage was linked to a human pathology. Cancer is no exception when it comes to alterations of the sphingolipid pathway. Ceramide accumulation has long been associated with increased cell death, and researchers have demonstrated that tumor cells have increased ability to metabolize ceramide in order to avoid its detrimental effects. In this Section, the subject of ceramides role in cellular death in cancer treatments and the ways tumors developed to evade those therapies will be explored.

3.4.1 CONVENTIONAL CANCER THERAPIES MEDIATE SPHINGOLIPID METABOLISM

Ceramides, and all other sphingolipids, are not only important for their structural role in cell membranes but also because of their active role in regulating signaling cascades. Sphingolipids are important for all eukaryotes and the level of conservation among species is quite high, which has enabled researchers to identify the enzymes mentioned earlier in this Thesis, as well as their biological role. One of the earlier mechanisms found in yeast was that when under stress conditions, such as temperature, the organism adapts and increases its ceramide levels [346]. One of main general mechanisms by which chemotherapeutic agents work in cancer is through increased cell stress to an irreparable state. Given the evolutionary conservation of sphingolipid metabolism between species, it is thus not surprising that ceramide levels are also increased in humans after cell stress caused by those drugs [347]. Several chemotherapeutic classes have been found to increase ceramide generation via *de novo* synthesis [346,348-351]. Generation of ceramide after treatment via quick hydrolysis of sphingomyelin has also been reported to lead to cell death in cancer cells [352,353]. That is also the case for radiation treatments; in 1994 the Kolesnick laboratory found that the cell death induced by radiation was mediated by sphingolipid metabolism [354]. Specifically, authors found that after radiation there was a quick spike in sphingomyelin hydrolysis at the membrane resulting in increased ceramide levels and subsequent cell death [354]. In the following years the same laboratory showed that cells that lacked sphingomyelinase activity were insensitive to ceramide-induced cell death after radiation treatment, and that sensitivity was restored by re-expressing acid sphingomyelinase [355,356]. Activation of *de novo* synthesis of sphingolipids and of ceramide synthases have also been associated with increased response to radiation treatments in cancer cells [357-360]. However, the study by Mesicek and colleagues demonstrated that different ceramide species played opposite roles related with sensitivity to radiation treatment [359]. These authors showed that activation of CERS5

and CERS6 with corresponding increases in C16-ceramide led to enhanced response to radiation treatment, while CERS2 and one of its products C24-ceramide had the reverse effect [359]. As previously mentioned, more studies are important to determine the specific role of ceramide species, or if its role is dependent on cell status or localization of its increase [343,359].

Nonetheless, as mentioned before, tumor cells develop molecular strategies that allow them to adapt to treatment modalities and usually become more aggressive. In the sphingolipid realm, drains from the ceramide pool represent those strategies; up-regulation of ceramidases has been shown to confer resistance to radiation and chemotherapies, as well as lead to tumor progression in several models [361-366]. Another evasion mechanism is related to the association between overexpression of glucosylceramide synthase and MDR1 leading to resistance *in vitro* and *in vivo* to chemotherapeutics in different models [367-369]. One of the major mechanisms of drug resistance associated with ceramides follows the up-regulation of ceramidases and ends up with activation of sphingosine kinases and higher levels of S1P. S1P has generally been associated with poor prognosis by regulating cell survival, inflammation, and resistance to chemotherapy [198]. Sphingosine kinase 1 has been shown to be up-regulated in several different tumor models [370]. The up-regulation of SPHK1-2 then leads to resistance to chemotherapies by generating S1P in different tumor models [328,371,372]. On the other hand, SGPL the enzyme responsible for degrading S1P is down-regulated in tumor models contributing to increase S1P signaling [373]. It is therefore not surprising that several of these pathways have been the focus for the development of new therapeutic modalities that are in different phases in their pathway to the clinic. Albeit out of the scope for this Thesis, those strategies have been extensively reviewed in [327].

It is undeniable that ceramide plays an important role in cellular death of tumor cells and several strategies have been developed to deploy these “deadly” sphingolipid. Following the subway system model, forcing the train to fast progress from more complex sphingolipids to ceramide, or to add more ceramide mass *de novo* is a promising therapeutic strategy. Nevertheless, it is important to first understand how ceramides can lead to cell death, so the development of ceramide-based treatment strategies is based on informed decisions.

3.4.2 CERAMIDE: A MAJOR PLAYER IN CELL DEATH

Considering all the information and evidence gathered about ceramide and its role in activating cell death in cancer cells, it is important to understand through which mechanisms that occurs. The answer is not as simple as it seemed in the early 90s, and goes back to Marco Colombini's words that in science the simplest interpretation is almost always wrong [344]. Ceramide has been described as part of several different death pathways, and with more and more markers for different death pathways being discovered, it is possible that the involvement of ceramide in death mechanisms will keep growing.

The first study connecting ceramide with apoptosis in cancer (leukemia cells) was published in 1993 [345]. However, the lack of knowledge about other death pathways and especially the lack of reliable markers rendered these initial studies as limited. Nonetheless, later it became clear that radiation caused increased mitochondria membrane permeability with the involvement of ceramide channels in that organelle [340,374,375]. The activation of apoptosis was regulated by neutral sphingomyelinase inducing activity of pro-apoptotic proteins, such as BCL2 antagonist killer 1 (BAK1) and BCL2 associated X protein (BAX) [376]. Moreover, as previously mentioned, overexpressing CERS5 and CERS6 contributed to higher levels of C16-ceramide that were shown to sensitize cells to radiation [359]. However, to complicate matters, studies have also shown that accumulation of ceramide in the late endosome was able to block the activation of caspase 3, an effector protein in apoptosis [377]. This studies again point to different roles for sphingolipids depending on where in the cell they are located. Ceramide can also lead to cell death in the presence of pro-death signals such as tumor necrosis factor alpha (TNFα) [378].

Necroptosis is a programmed form of necrosis dependent on the sequential activation of receptor-interacting protein kinase 1-3 (RIPK1-3), and the pore-forming mixed lineage kinase domain-like protein (MLKL) without the participation of caspases [379]. Moreover, the interaction of lipids and MLKL is necessary for necroptosis [379]. CERS1 overexpression and its product C18-ceramide have been shown to induce necroptosis [380-383]. These studies showed that this effect correlated with PP2A activation that subsequently activated RIPK1 [380]. The full picture for ceramide activation of necroptosis is still yet to be painted and more studies are necessary for full understanding of this relationship.

Another cell mechanism that plays a dual role in cells is autophagy, which can be protective for cells and to keep a healthy status or detrimental and end up in cell death. Autophagy is a catabolic process that degrades and recycles damaged

organelles, proteins, and lipids via autophagosomes and lysosomes [384]. In cancer, accumulation of ceramide has been shown to induce autophagy resulting in death or survival depending on the context [385]. Sentelle and colleagues demonstrated that cell stress from chemotherapeutic agents led to accumulation of ceramides at the mitochondria [386]. This accumulation served as an "attractant" for autophagosomes to the mitochondria and induced autophagy of the mitochondria (mitophagy) [386,387]. The authors therefore proposed that CERS1-generated ceramide acts as a receptor on the mitochondrial membrane for autophagosomes to induce selective and lethal mitophagy in both head and neck cancer and leukemia cells [387]. Moreover, the Ogretmen laboratory recently showed that the synthesis of C18-ceramides in the mitochondria resulted in mitophagy in cancer cells [343]. For a more complete review on the role of ceramide and autophagy, the chapter published by Young *et al.* is recommended [384].

In 30 years, the fields of cell death and sphingolipids have come a long way, however more needs to be done when it comes to the intersection of both. The different roles ceramides play in different organelles and how this might influence cell death remain to be ascertained. Moreover, given this phenomenon, questions remain regarding ceramide's involvement in cell death in different models or upon different treatments: is it possible that multiple types of cell death come together at once? Can different ceramide species and sphingolipids metabolites regulate cell death differently? How is the regulation of cell death by ceramide occurring: lipid-lipid, lipid-protein, and/or biophysical interaction? Independent of the mechanism of cell death or how cell death is triggered, it is clear that ceramide plays a role in cancer cell death. Thus, in the last decades several efforts have been made to use ceramides as a treatment for cancer. Given that ceramide is a very hydrophobic lipid, especially with increased length of the fatty acid tail, its delivery in patients and even in *in vitro/in vivo* models is not easy. However, this has been bypassed by the use of exogenous short-chain ceramides that mimic the endogenous ceramides and still activate sphingolipid metabolism and results in similar phenotypes compared with endogenous ceramides.

3.4.3 CERAMIDE: OLD LIPID REPURPOSED BY CUTTING EDGE TECHNOLOGY

Ceramides are associated with cell death after conventional therapeutics; blocking sphingolipid metabolism in order to accumulate ceramides in cancer cells leads to cell death; endogenous ceramides are very hydrophobic and not soluble posing enormous problems for its use as drug; enter the short-chain exogenous ceramides. These exogenous ceramides are shorter and more soluble than the endogenous counterparts, and mainly three different lengths have been used: C2-, C8-, but mostly C6-ceramide. Short-chain ceramides are membrane-permeable and can translocate freely via the cytosol [388]. As previously mentioned, endogenous ceramides are usually bound to membranes in cells, and are rarely detected freely in the cytosol. This difference indicates that ceramide analogs affect the membrane properties of cells upon treatment in a different way than endogenous ceramides [389,390]. Nonetheless, the metabolism of these short-chain ceramides is very similar to the endogenous sphingolipids making them a good proxy [391]. Authors have shown that C6-ceramide is metabolized to endogenous sphingolipids by cells; nonetheless, this varies according to cell type, dose administered, and ability to metabolize sphingolipids [392]. The last point is important for future study designs; understanding how a cell/tissue will metabolize sphingolipid-based drugs is important to make informed clinical decisions.

In order to improve delivery and make these lipid solutions as water-soluble as possible, new formulations have been developed. The most important one for this Thesis are the C6-ceramide nanoliposomes (CNL) developed by the Kester laboratory, which have been shown to improve toxicity against cancer cells and allow for rapid distribution *in vivo* with increased intracellular delivery [393-395]. CNLs have a diameter of 85nm and are composed of 5 different lipids other than C6-ceramide which is the only pharmacologically active compound [396]. The nanoliposomal formulation is designed to accumulate within tumors due to their leaky vasculature and the enhanced permeability retention properties of the nanoparticle. Moreover, the PEGylated groups in the outside of the nanoliposomes protects it from engulfment by the immune system becoming a stealth agent in the body [391,393,394,397,398]. The delivery of the drug occurs via interlamellar bilayer movement of C6 ceramide and allows its delivery at the plasma membrane of cells [391,393-395,398]. *In vivo* studies have shown that CNL have a half-life of 11 to 35h, while non-liposomal C6-ceramide has a half-life of <15 minutes [396]. CNL have been shown in several different tumor models *in vitro* and *in vivo* to have anti-tumor properties [396,398]. *In* vitro, CNLs have been shown to inhibit phosphorylation of AKT and activate

apoptosis in a more potent fashion than non-liposomal C6-ceramide [393]. Moreover, studies have suggested that the ability of CNL to specifically induce death in cancer cells is related to the inhibition of glycolysis in those cells [399]. As previously mentioned, most cancer cells undergo a switch in their metabolism where they become more reliant in glycolysis than their normal counterparts, the Warburg effect. Thus, Ryland and colleagues suggest that it's the ability of CNL to block glycolysis that allows the specific effect against tumor cells [399]. It is important to note that not all tumors undergo that switch, with the main example being Prostate cancer. This phenomenon poses a risk for the benefit of CNLs in these tumors as a single agent. Nonetheless, these data indicate that cells have the ability to take up the payload and metabolize the drug. By inundating the ceramide pool with an exogenous source, the levels of endogenous ceramides will also be raised by re-acylation of the sphingoid base in C6-ceramide. Thus, the ceramide pool will flood, and the cells will undergo death. Given the specificity for tumor cells and the features that tumors have, such as leaky vasculature and fast growth rates, it elevates the potential of CNL as a cancer treatment.

More important than any preclinical studies, treatments with CNL have reached the clinic, and a Phase I clinical trial (NCT number NCT02834611) for solid tumors showed low overall toxicity even at concentrations three times above efficacious doses, and resulted in stable disease in 50% of the patients tested. This initial success is exciting and can be of extreme help for cancer patients worldwide. The lack of toxicity of these nanoliposomes, allows the combination with already existing therapeutics that might lack long-term efficacy [398,400]. **The work in this Thesis suggests a potential enhanced efficacy for a regimen treatment combining CNL with inhibition of AR for Prostate cancer patients.**

4. COLLISION COURSE: ANDROGEN RECEPTOR AND SPHINGOLIPIDS IN PCA

By now, the Reader should recognize the importance of Prostate cancer (PCa) as a burden worldwide, and a burden that is only increasing in its financial and sociological cost. It should also be clear how important metabolic switches are for cancer, and how PCa differs from other tumors. Moreover, the role that Androgen receptor (AR) plays in regulating several key pathways in PCa carcinogenesis should also be clear. The question that this Thesis tries to address is: how are sphingolipids related to AR in PCa? In this Section, potential connections between these two fields will be addressed.

4.1 WHAT IS KNOWN ABOUT THIS INTERSECTION?

The first study connecting alterations of sphingolipid metabolism in PCa was published in 1991, when researchers found that PCa samples had higher levels of sphingomyelin compared with BPH [401]. One year later, another study showed that, in *in vitro* models, the levels of sphingomyelin were elevated in cells with higher metastatic potential (DU145) compared with lower metastatic potential (LNCaP) [402]. The authors also found an increase in sphingomyelin synthase and a decrease of sphingomyelinase in the membranes of DU145 cells compared with LNCaP [402]. A similar study was published in 2004 where authors evaluated the mass of gangliosides and showed differences between AR-negative and AR-positive cells [403]. The authors reported increased levels of GM1b and GD1a in AR-negative cells when compared with AR-positive cells [403]. In 1996, researchers showed a synergistic effect between a PKC inhibitor and dihydrosphingosine in PCa cells [404]. The authors suggest that this synergism was determined by the presence of glucocorticoid receptor. Interestingly, the authors report an effect from dihydrosphingosine as a single agent in PC-3 cells but not in LNCaP [404]. A study that aimed at understanding the role of TNFα in sensitizing cells to radiation, showed that this occurred by elevating endogenous ceramide levels, which was shown by adding a C2-ceramide prior to radiation [405]. A similar result was later obtained with sphingosine increasing the effect of radiation in prostate cancer cells [406]. Another work in the late 1990s focused on the role of ceramides in the response to chemotherapeutics [407]. Camptothecin, a topoisomerase I inhibitor, was only effective in LNCaP cells that were able to increase ceramide levels in response to the treatment, unlike PC-3 cells [407]. However, 3 years later another study showed that cell death by camptothecin was independent of ceramide generation [408]. The use of short-chain C2-ceramide showed that both PC-3 and DU145 were equally sensitive to the treatment [409]. One of the main issues with

the sphingolipid publications especially when it relates to cell death is the lack of consistency. In 2001, Engedal *et al.* showed that C2-ceramide increased cell death in LNCaP cells, albeit at high doses, but the cells dying were not doing so uniquely through the apoptotic pathway [410]. This is somehow controversial as all studies before, and most of the literature after, claim that ceramide analogs kill cells by apoptosis. Thus, it is important to note this difference and point to an aspect of sphingolipid research that needs cleanup to avoid propagation of inconsistent conclusions regarding activated pathways.

Researchers drew a relationship between androgen levels and sphingolipid-mediated cell death, by growing LNCaP cells without hormones and showing an increase in C16-ceramide concomitant with cell death [411]. Moreover, recently Murdica *et al.* published a study showing increased levels of sphingomyelin and ceramide in LNCaP cells after Abiraterone acetate treatment and radiation [412]. In 2017, a study looking for lipid profiles and its connection with prognosis showed that higher levels C24:1-ceramide and C16-sphingomyelin were part of a three-lipid signature associated with worse prognosis [413].

The Knudsen laboratory published dihydroceramide desaturase (DEGS1) as a target regulated by the Androgen receptor, associating DEGS1 expression with increased migration and worse prognosis [128]. To date this is the only example of a validated molecular regulation by AR of a sphingolipid metabolic enzyme expression. Earlier on, the molecular focus regarding sphingolipid metabolic enzymes and PCa was placed on acid ceramidase (ASAH1). In the year 2000, this enzyme was shown to be up-regulated in PCa patients and cell lines; results that then were further validated by other laboratories [414,415]. Ectopic expression of ASAH1 on DU145 cells was reported as increasing tumor burden *in vivo* and associated with relapse from chemotherapeutic agents [416]. A follow-up study demonstrated that increased expression of ASAH1 increased the size of lysosome and led to autophagy that can be associated with treatment resistance, especially to sphingolipid-based drugs such as C6-ceramide [417]. Later studies showed an association between ASAH1 overexpression in patients and cells with the PI3K/AKT signaling axis and promotion of S1P production [370]. This connection between S1P and ASAH1 again shows the intricate relationship between sphingolipids and enzymes that can affect human pathologies. The enzymes responsible for generating S1P, SPHK1-2 have also been shown to be elevated in PCa, and higher levels are found in metastatic patients [418,419]. Thus, it is not surprising that several attempts have been made to target the sphingolipid pathways as PCa treatments, as reviewed in (Voelkel-Johnson, 2018). In fact, a recent

paper from the Voelkel-Johnson laboratory showed that ASAH1 inhibition blocked the growth and division of polyploid giant cancer cells in PCa, which are associated with therapy resistance and metastasis [366].

There are some converging points that although not studied in tandem, point to potential interactions and regulations between ceramide and AR. One of those interactions concerns importin-α; as previously mentioned, this protein is responsible for AR to be able to be translocated from the cytosol to the nucleus to display its genomic actions [119]. Ceramide has been shown to increase the expression of cellular apoptosis susceptibility (CAS), responsible for the export of importin-α from the nucleus [420]. Thus, we speculate that ceramide being able to regulate the localization of importin-α, indirectly can impact the localization of AR in the cell. Moreover, ceramide and S1P have been shown to interact and negatively regulate HDAC [318,421]. HDAC, as previously mentioned is one of the co-repressors associated with AR [142]. Therefore, it is possible that sphingolipid metabolism can regulate the association between HDAC and AR, impacting the signaling pathways regulated by both.

AR has both genomic and non-genomic actions; the non-genomic actions are associated with rapid induction of secondary messenger signal transduction cascades, such as free intracellular calcium, activation of protein kinase A and C (PKA-C), Mitogen-activated protein kinase (MAPK), and tyrosine kinase c-Src [133,134]. Caveolae have also been suggested as a regulator of these non-genomic actions of AR by promoting a ligand-independent activation of AR [116,135]. Ceramide has been reported as both an activator and repressor of caveolae formation [422,423]. Interestingly, ceramide has been shown to be involved in regulation of all these pathways, with no intersection with AR, but opening potential speculation for an inter-regulation between sphingolipids and AR activity. Ceramide has been shown to activate PKC; sphingolipids have been shown to regulate opening and closing of ion channels such as calcium flux; c-Src has been shown to regulate sphingolipid metabolism promoting tumor progression; and last but not the least, ceramide has been reported to activate MAPK cascades [336,424-426]. With all these effects in mind, as well as the biophysical properties of sphingolipids in general and ceramide in particular at the membrane level, we speculate that these pathways can be of interest for the intersection between the AR signaling axis and sphingolipid metabolism. More studies, especially those specific to PCa, are necessary to further elucidate if these potential interactions are relevant in the disease context.

It is important to note that most of the early publications concerning sphingolipids and prostate cancer lack the quantitative ability to identify specific sphingolipid species or conduct the broad identification of a full sphingolipid spectrum. Furthermore, the studies using short-chain ceramide analogs tended to utilize higher doses than what has been observed as efficient for ceramide nanoliposomes. Also, in recent years better understanding of the sphingolipid metabolic enzymes and the intricate pathways regulated by them has been acquired. Nonetheless, it is clear that sphingolipids play a role in PCa; a role that is still not very well understood, or at least broadly understood. With discovery of new enzyme and lipid species, painting a broader picture of sphingolipid metabolism in PCa is necessary. Importantly, given the prominent role of AR regulation for key pathways in PCa, more efforts should be put into place to understand what sphingolipid metabolic enzymes AR regulates and how to exploit this relationship for future therapeutics, or to better understand the lack of efficacy of the current AR inhibitors in PCa patients.

4.2 ANDROGENS AND SPHINGOLIPIDS

As mentioned above, normal prostate cells have a more glycolytic dependence than other tissues and undergo the reverse metabolic switch during tumor transformation in comparison with other tumors. It is not surprising then that AR regulates the basal metabolism and the metabolic switch that happens in the transformation of prostate cells [126,427]. One issue that has become clear is that AR inhibitors apply pressure on cancer cells and one of the major changes in plasticity implies metabolic changes [25,428]. Metabolic switches apply enormous stress in cells that need to adapt quickly and organelles involved in protein and lipid synthesis, such as ER, sense and respond to this stress [429,430]. In fact, AR has been reported as regulating this adaptation [431,432]. Metabolic switches are particularly important because metabolite pools then can impact transcription and translation, serving as feedback loops in cells. A critical example is that lipid turnover provides cells with acetyl groups by the action of ATP-citrate lyase (ACLY). ACLY generates a pool of acyl-CoA that can be further used for lipid synthesis [433]. ACLY has been shown to be an AR target and inhibiting this enzyme reduces histone acetylation and tumor growth in xenografts [434]. Previously, AR has been shown to regulate both FASN and ACACA in PCa [151]. Increasing the content of fatty acid availability might indicate an increase in sphingolipid metabolism, however this is purely speculation and needs further exploration.

AR can also regulate sphingolipid metabolism; but the opposite is also true, as bioactive lipids sphingolipids can regulate the synthesis of hormones. In 1996, Santana *et al.* showed that interleukin-1β induced hydrolysis of sphingomyelin and decrease in progesterone levels that was dependent on ceramide [435]. This result was independently validated three years later with the added layer of Steroidogenic Acute Regulatory Protein (STAR) suppression, a key enzyme in the start of steroid synthesis [436]. Importantly, in Leydig cells, ceramide was shown to repress STAR and subsequently decrease the levels of testosterone in those cells [437-439]. Short-chain ceramides have been shown to both suppress and enhance hormone secretion [440-442]. More clarification is then needed in the regulation of sphingolipids and their interaction with hormone release and hormonal receptors such as AR in prostate. Another example that indicates this potential relationship is the correlation between ASAH1 expression and estrogen receptor in breast cancer [443]. Ceramides have also been shown to be an important player in the glucocorticoid receptor regulation [444-446]. Importantly, the presence of sphingolipid metabolites in the nucleus has been shown to have an active role in transcription [318,447].

A better understanding of the interaction between sphingolipid metabolism and the Androgen receptor in a disease with a unique metabolism such as Prostate cancer is of extreme necessity and might provide clinicians with new treatment avenues. The role metabolism plays in cancer has already been recognized as a major hallmark and the technological advance of high-throughput analysis of lipids and metabolites is starting to identify new players in the carcinogenesis. **The work here presented aims at bridging the world of sphingolipid metabolism and AR signaling in PCa not only to uncover novel molecular pathways involved in the disease, but also to bring more efficient and long-lasting treatments to PCa patients.**

CHAPTER 2: GOALS

1. RATIONALE

Prostate cancer (PCa) is one of the most incident tumors worldwide, and despite huge amounts of investment in research the mortality rates aren't slowing down. Low specificity of the current biomarkers elevates the rates of over-diagnosis and treatment of indolent tumors. Additionally, several drugs have been developed for treatment of PCa with all resulting in relapse within 2 years. Thus, it is not surprising that the cost of care for PCa has been rising for several decades.

The role male hormones and especially the Androgen receptor (AR) play in normal prostate development and maturation as well as in PCa is clear and undisputed. With technological advancements it has become clear that AR is responsible for regulating virtually every cellular process in prostate cells, including lipid metabolism. One of the metabolic hallmarks of PCa is lipid accumulation and increased levels of enzymes involved in lipid synthesis. Sphingolipids have been associated as regulators and regulated by different cellular pathways with importance in human pathologies. In PCa, some examples of the importance of these bioactive lipids have been reported, with some accumulation of lipids after AR inhibition being of particular interest. Importantly, the use of synthetic ceramides has been of particular interest especially after a successful Phase I clinical trial for C6-ceramide NanoLiposomes (CNL) in solid tumors.

2. MAIN GOALS

- Determine the potential utility of CNL in PCa preclinical models as a single treatment or in combination with AR inhibitors
- Explore the regulation of sphingolipid metabolic enzymes by AR
- Uncover molecular Achilles heels within sphingolipid metabolism that can be used as therapeutic avenues for PCa

CHAPTER 3: MATERIALS AND METHODS

Table 1 - Key resources used in this Thesis.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
CellTiter 96 AQueous MTS Reagent Powder	Promega, Madison, WI	Cat #G1111
Phenazine methosulfate (PMS)	MilliporeSigma, Burlington, MA	Cat #P9625-1G
eBioscience™ Fixable Viability Dye eFluor™ 780	Thermo Fisher Scientific, Waltham, MA	Cat #65-0865-18
L-Serine-13C3	Toronto Research Chemicals, North York, ON, Canada	Cat #S270999
Glutamax	Thermo Fisher Scientific	Cat #35050061
D-erythro-C6-ceramide nanoliposomes	KeystoneNano, State College, PA	N/A
Ghost nanoliposomes	KeystoneNano, State College, PA	N/A
Abiraterone acetate	Selleckchem, Houston, TX	Cat #S2246
Enzalutamide (MDV3100)	Selleckchem	Cat #S1250
5α-Dihydrotestosterone (DHT)	MilliporeSigma	Cat #D-073-1ML
Lipofectamine 2000	Thermo Fisher Scientific	Cat #11668019
Opti-MEM™ I Reduced Serum Medium	Thermo Fisher Scientific	Cat #31985062
RPMI-1640	Thermo Fisher Scientific	Cat #11875-093
DMEM	Thermo Fisher Scientific	Cat #11965-092
DMEM/F12	Thermo Fisher Scientific	Cat #11330033
RPMI 1640 Medium w/o L-Glutamine, L-Serine, HEPES	US Biological,Salem, MA	Cat #R8999-15
Charcoal:Dextran Stripped Fetal Bovine Serum	Gemini Bio Products, West Sacramento, CA	Cat #100-119
Antibiotic Antimycotic (AA)	Thermo Fisher Scientific	Cat #15240062
Insulin-Transferrin-Selenium (ITS)	Corning, Corning, NY	Cat #25-800-CR
Fetal Bovine Serum	Gemini Bio Products	Cat #100-106

Keratinocyte-Serum-Free Media	Thermo Fisher Scientific	Cat #17005042
TRIzol	Thermo Fisher Scientific	Cat #15596018
TrypLE Select	Thermo Fisher Scientific	Cat #12563011
SYTOX™ Green Nucleic Acid Stain	Thermo Fisher Scientific	Cat #S7020
L-threo	Matreya, State College, PA	Cat #1828
D-threo	Matreya	Cat #1809
L-erythro	Matreya	Cat #1848
D16:1 Sphingosine	Avanti Polar Lipids, Alabaster, AL	Cat #860669
D18:1 Sphingosine	Avanti Polar Lipids	Cat #860490
D20:1 Sphingosine	Avanti Polar Lipids	Cat #860660
D18:0 Dihydrosphingosine	Avanti Polar Lipids	Cat #860498
D20:0 Dihydrosphingosine	Avanti Polar Lipids	Cat #860674
D18:1 1-deoxysphingosine	Cayman Chemical, Ann Arbor, MI	Cat #24515
D18:1 1-deoxydihydrosphingosine	Cayman Chemical	Cat #13511
D18:1 C6 1-deoxyceramide	Cayman Chemical	Cat #25493
Critical Commercial Assays		
iScript cDNA Synthesis Kit	Bio-Rad, Hercules, CA	Cat #1708891
iTaq Universal Probes Supermix	Bio-Rad	Cat #1725134
Human Cytochrome c Quantikine ELISA Kit	R&D Systems, Minneapolis, MN	Cat #DCTC0
CyQUANT™ NF Cell Proliferation Assay	Thermo Fisher Scientific	Cat #C35006
LDH-Cytotoxicity Assay Kit	Abcam, Cambridge, UK	Cat #ab65393
DC Protein assay	Bio-Rad	Cat #5000112
Deposited Data		
RNA-seq data Paschal lab	Jividen, 2018 [448]	GSE120660
LNCaP Timecourse	Takayama K.	GSE70150
Experimental Models: Cell Lines		
PC-3	Gioeli Lab at the University of Virginia	
LNCaP	Gioeli Lab at the University of Virginia	
DU145	Gioeli Lab at the University of Virginia	
VCaP	Gioeli Lab at the University of Virginia	
C4-2	Gioeli Lab at the University of Virginia	

22Rv1	Gioeli Lab at the University of Virginia	
PC-3/AR+	Paschal Lab at the University of Virginia	448
RWPE-1	Paschal Lab at the University of Virginia	
PPC-1	Voelkel-Johnson Lab at Medical University of South Carolina	
Oligonucleotides		
Silencer Select Negative Control No. 1 siRNA	Thermo Fisher Scientific	Cat #4390843
Silencer Select siRNA SPTSSB	Thermo Fisher Scientific	siRNA ID: s46587
PrimePCR FAM probe SPTSSA	Bio-Rad	qHsaCIP0029969
PrimePCR FAM probe SPTSSB	Bio-Rad	qHsaCEP0055358
PrimePCR FAM probe PSMB6	Bio-Rad	qHsaCEP0052321
PrimePCR FAM probe TBP	Bio-Rad	qHsaCIP0036255
PrimePCR FAM probe SPTLC1	Bio-Rad	qHsaCIP0039076
PrimePCR FAM probe SPTLC2	Bio-Rad	qHsaCIP0026692
PrimePCR FAM probe SPTLC3	Bio-Rad	qHsaCEP0054152
PrimePCR FAM probe KLK3	Bio-Rad	qHsaCEP0024737
PrimePCR FAM probe FKBP5	Bio-Rad	qHsaCIP0027271
PrimePCR FAM probe AR	Bio-Rad	qHsaCIP0026366
PrimePCR FAM probe ORMDL1	Bio-Rad	qHsaCEP0051492
PrimePCR FAM probe ORMDL2	Bio-Rad	qHsaCIP0027972
PrimePCR FAM probe ORMDL3	Bio-Rad	qHsaCEP0054129
Software and Algorithms		
GraphPad v5.0 for Mac	GraphPad Software Inc., La Jolla, CA, USA	https://www.graphpad.com/scientific-software/prism/
MassLynx Mass Spectrometry Software v4.1	Waters, Milford, MA	https://www.waters.com/waters/en_US/MassLynx-Mass-Spectrometry-Software

TargetLynx™ Application Manager v4.1	Waters	https://www.waters.com/waters/en_US/TargetLynx-/nav.htm?cid=513791&locale=en_PT
R studio	R Foundation	https://www.r-project.org/
Cytation 3 Gen5 2.09	BioTek, Winooski, VT	https://www.biotek.com/products/software-robotics-software/gen5-microplate-reader-and-imager-software/
CFX Manager 3.1	Bio-Rad, Hercules, CA	https://www.bio-rad.com/en-pt/sku/1845000-cfx-manager-software?ID=1845000
Attune NxT Flow Cytometer Software	Thermo Fisher Scientific	https://www.thermofisher.com/us/en/home/life-science/cell-analysis/flow-cytometry/flow-cytometers/attune-acoustic-focusing-flow-cytometer/attune-cytometer-software.html
Axio Imager.Z1	Zeiss, Oberkochen, Germany	https://www.zeiss.com/microscopy/int/products/imaging-systems/axio-scan-z1.html
FlowJo v.10	BD Biosciences, San Jose, CA	https://www.flowjo.com/

Cell culture

LNCaP and C4–2 cells were grown in DMEM:F12 with 5% Fetal bovine serum, 1% Insulin-Transferrin-Selenium and 1% Antibiotic-Antimicotic (AA). CWR22Rv1 (22Rv1), VCaP, PC-3 were grown in DMEM with 5% Heat-Inactivated serum and 1% AA. DU145 were grown in RPMI-1640 with 10% Heat-Inactivated serum. PC-3/AR+ were grown in RPMI-1640 with 5% Fetal bovine serum and 1% AA.

Serum starvation in PC-3/AR+ was accomplished by switching from complete media to RPMI-1640 with 5% of Charcoal stripped FBS and 1% AA. RWPE-1 cells were grown in Keratinocyte-Serum-Free Media. Cells were maintained at 37°C and 5% CO^2 in a humidified chamber.

Cell viability assay

Cells were plated for 24 hours in 96-well plates before treatments. Cell viability was assessed by MTS assay according to manufacturer's instructions. Plates were incubated for 2 hours at 37ºC and absorbance (490nm) was measured after 2 hours using a Cytation 3. Data was normalized to vehicle controls as described in figure legends.

Cell death flow cytometry assay

Cells were plated for 24 hours in 6-well plates and then treated. Cells were ressuspended in Fixable Viability Dye 780 according to the manufacturers' instructions. The Attune Nxt Flow Cytometer was used to collect the data. Forward and side scatter measurements were used to gate for single cells and excluding cell debris. Control cells were incubated at 70ºC for 10 minutes for dead-cell gating. Single-stain compensation controls for viable and dead cells were collected Data were analyzed using FlowJo v.10 software.

Cell death imaging assay

Cells were plated for 24 hours followed by treatment with CNL and ghost for 22 hours. SYTOX™ Green Nucleic Acid Stain was then added to the cells according to manufacturer's instructions. Cells were then imaged using 20x magnification on an Axio Imager.Z1 for a total of 2 hours.

Quantitative reverse transcription PCR

Total RNA was obtained using TRIzol® reagent following the manufacturer's recommendations. RNA concentration and purity of samples were determined on a Cytation 3 plate reader. For the iScript cDNA Synthesis Kit 1μg of RNA was used. PrimePCR FAM probes used in this study are described in the Star methods section. Gene expression was quantified using iTaq Universal Probes Supermix according to manufacturer's instructions, and measured using CFX Connect Real-Time PCR Connection System. Data was analyzed using the comparative Ct method [449] with the average of PSMB6 and TBP as housekeeping control.

Cytochrome C ELISA

Cells were seeded in 6-well plates and 24h later treated. Cells were collected and lysed following the Human Cytochrome c Quantikine ELISA Kit manufacturer's instructions. After lysis and centrifugation, pellets were further incubated with 0.5% Triton-X100 for 10 minutes at 4ºC followed by centrifugation to obtain the mitochondria fraction. Protein levels from this fraction were then measured using the DC Protein assay and 1µg of protein was used. Cytochrome C levels were then assessed using the Human Cytochrome c Quantikine ELISA Kit with absorbance was measured at 450nm with wavelength correction at 540nm using a Cytation 3 plate reader.

Cell proliferation

Cells were seeded in 96-well plates and 24 hours later treated. Cell proliferation was then measured using CyQUANT™ NF Cell Proliferation Assay according to manufacturer's instructions. Fluorescence excitation was measured at 485nm and emission at 530nm using a Cytation 3 plate reader.

Cytotoxicity LDH assay

Cells were seeded in 96-well plates and 24 hours later treated. Release of lactate dehydrogenase (LDH) was measured using the LDH-Cytotoxicity Assay Kit according to manufacturer's instructions. Briefly, media was harvested from wells and incubated with LDH Reaction mix. Cell culture media was used as background control and lysed cells respective to each treatment as High control to account for cell number difference. The absorbance was measured at 450 nm on a Cytation 3 plate reader.

Mass spectrometry protocol

Lipid extraction and analysis was done using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). Cells were plated in 6-well plates and treated 24h after seeding. Lipids extraction was performed as previously reported (Tan, 2016). All data reported are based on monoisotopic mass and are represented as pmol/mg of protein extracted.

Isotope labeled serine tracing

Serine tracing was performed using isotope labeled L-Serine-13C3 (Toronto Research Chemicals, North York, ON, Canada). Cells were plated in complete media as described above in this section. After allowing cells to adhere for 24 hours, media was replaced with RPMI-1640 w/o L-Glutamine, L-Serine, HEPES with supplemented Glutamax and L-Serine-13C3 at the same concentration as complete RPMI-1640. Fetal bovine serum (10%) and AA (1%) were also added to this media. Cells were kept at 37°C and 5% CO_2 for 2 hours in this isotope labeled serine media, before being switched to their normal growth media; cells were then treated. Serine tracing was then done following the same mass spectrometry protocol described above.

C6-ceramide Nanoliposome

D-erythro-C6-ceramide nanoliposomes were obtained from KeystoneNano and manufactured according to previous literature (Kester, 2015). The vehicle control nanoliposomes (ghost) was formulated with the same lipid composition except for D-erythro-C6-ceramide. In this study, ghost control was used at a final concentration of 20µM in all experiments.

RNA-sequencing

To assess the gene expression of sphingolipid-related genes in a set of prostate cancer cell lines, we used the pre-processed data from GSE120660. In brief, reads were aligned to the hg38 genome using STAR, counts were determined using HTSeq, and differential expression was determined using the DESeq2 R package [448]. To measure changes in lipid-related gene over time, we downloaded the GSE70150 gene expression dataset, which included a LNCaP time course. Reads were aligned to the genome (hg38) using standard TopHat2 methods, counts were generated using HTSeq, and differential expression was determined using the DESeq2 R package. Z-score normalized gene expression changes for just sphingolipid-related genes were graphed using R with hierarchical clustering based on the Euclidian distance. We manually curated the sphingolipid-related genes with help from the Futerman laboratory at the Weizmann Institute, Israel.

Knockdown of SPTSSB

Cells were plated and incubated at 37ºC and 5% CO_2 for 16 hours. After 16 hours media was changed to DMEM without FBS and AA for 2 hours. Then, Lipofectamine 2000 was incubated with Opti-Mem according to manufacturer's instructions and added to cells. Silencer Select Negative Control No. 1 siRNA and Silencer Select SPTSSB siRNA-SPTSSB were used for cell transfection at a final concentration of 40nM. For drug treatments, cells were treated 24h after transfection for a total of 72 hours with mRNA levels evaluated every 24 hours according to the protocol above described for knockdown validation.

Prostate cancer patients' gene expression

Data available through the Cancer Genome Atlas (TCGA) (https://www.cancer.gov/tcga) for Prostate cancer (PRAD) dataset was accessed using the TCGA2STAT package using R software. Neuroendocrine datasets was accessed using cBioPortal for differences in expression of SPTSSB in patients [1,2,450]. Z-score cutoff used was ± 1.5.

Statistical analysis

Comparisons between two groups were performed using non-parametric Mann–Whitney U-test. A two-tailed t-test was used to assess differences for cytochrome C ELISA and mass spectrometry experiment following knockdown of SPTSSB. P values were considered statistically significant if lower than 0.05. All experiments were repeated with a minimum of three independent experiments. Mean and standard error of the mean (SEM) is shown in all figures unless stated. Markers *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively. Statistical analysis was performed using GraphPad Prism 5.0 software for Mac (GraphPad Software Inc., La Jolla, CA, USA).

Diagram design

Diagrams designed for this synthesis were generated using BioRender.com.

The following publication was used as a basis for the Chapters in this Thesis.

Pedro Costa-Pinheiro, Abigail Heher, Michael H. Raymond, Kasey Jividen, Jeremy JP Shaw, Bryce M. Paschal, Susan J. Walker, Todd E. Fox, Mark Kester. (2020) “Role of SPTSSB-Regulated *De Novo* Sphingolipid Synthesis in Prostate Cancer Depends on Androgen Receptor Signaling”. iScience 23.12 (2020): 101855

CHAPTER 4: *ROLE OF SPTSSB-REGULATED DE NOVO SPHINGOLIPID SYNTHESIS IN PROSTATE CANCER DEPENDS ON ANDROGEN RECEPTOR SIGNALING*

1. RATIONALE

Prostate cancer (PCa) is the most incident cancer in the United States male population, responsible for 20% of newly diagnosed cases [28]. Moreover, PCa is estimated to account for 10% of cancer-related deaths in the male population and has the second highest mortality rate [28]. Effective treatment of PCa remains elusive due to disease heterogeneity and complex disease progression [5]. Androgens are necessary for normal development and functional maintenance of healthy prostatic tissue, but also contribute to PCa onset and progression [451]. Androgens are natural ligands of Androgen Receptor (AR), a transcription factor that regulates several key cellular pathways such as proliferation and lipid metabolism [146]. Pharmacological inhibition of androgen synthesis or the receptor are currently the main therapeutic approaches for advanced PCa patients [63]. Despite symptom relief, virtually all patients develop recurrence within 24 months by progression to castration-resistant prostate cancer (CRPCa) [65,452]. Mechanisms of resistance to AR-signaling inhibitors in CRPCa patients include restored AR activity, hypersensitivity to androgens, and ligand-independent AR activation [18,453]. An alternative mechanism of resistance involves cell transdifferentiation to AR-negative neuroendocrine phenotype (CRPCa-NE) after androgen deprivation therapy (ADT) [18,25,453]. The emergence of this phenotype has been associated with worse prognosis and has features of both small-cell carcinoma and neuroendocrine differentiation [454]. Most PCa-related deaths occur due to CRPCa, which lacks any efficacious treatments [63]. Therefore, it is imperative to understand the mechanisms of resistance and develop new therapeutic modalities for CRPCa patients.

Cytotoxic agents, including AR-signaling inhibitors, and radiation therapy, have been shown to lead to an accumulation of sphingolipids, including pro-death ceramides [202,412,455,456]. Sphingolipids are bioactive lipids that have not only a structural role in organelle and plasma membranes, but also regulate signaling pathways [198,310]. Dysregulation of sphingolipid metabolic enzymes is associated with cancer progression [128,416,419,457,458]. Moreover, ceramide accumulation in tumors is associated with cancer cell death, which emphasizes the potential role of ceramide as a cancer treatment [198,202,310,327,388,459]. *De novo* synthesis of ceramide and other

sphingolipids is achieved by condensation of L-serine with an acyl-CoA substrate [226]. In mammals, several enzymes are involved in this process: three serine palmitoyltransferase long-chain (SPTLC1-3) subunits, two serine palmitoyltransferase small subunits (SPTSSA-B), and three ORMDL sphingolipid biosynthesis regulator (ORMDL1-3) [211]. The formation of a complex between 2 SPTLCs and SPTSSA or SPTSSB leads to the generation of dihydrosphingosine, a precursor sphingolipid that can result in accumulation of ceramide. Dihydrosphingosine is a bioactive lipid that influences cell signaling and has been associated with neurodegeneration (Zhao, 2015). These enzymes maintain tight control over the generation of ceramides, as sphingolipid accumulation can lead to several disruptions in cells and to various pathologies [202,460,461]. Not only do sphingolipids have a role in predicting patient outcomes in PCa, the sphingolipid profile also undergoes remodeling in response to conventional PCa treatments [412,413]. However, the underlying mechanisms and consequences of altered sphingolipid metabolism in PCa remain unclear.

Our laboratory has developed a non-toxic and biologically stable nanoliposome formulation that includes C6-ceramide (CNL), as a potential cancer therapeutic. CNL demonstrates selectivity for tumor cells in preclinical models and also has exhibited minimal toxicity in an ongoing Phase I clinical trial (NCT number NCT02834611) [391,396]. In the present work, we have discovered that CNL efficacy is determined by AR signaling in PCa. Through these findings, we determined that CNL treatment is more effective in highly aggressive AR-negative disease models. This efficacy occurs through elevation of SPTSSB-dependent *de novo* synthesis of ceramide, an understudied pathway in PCa cancer.

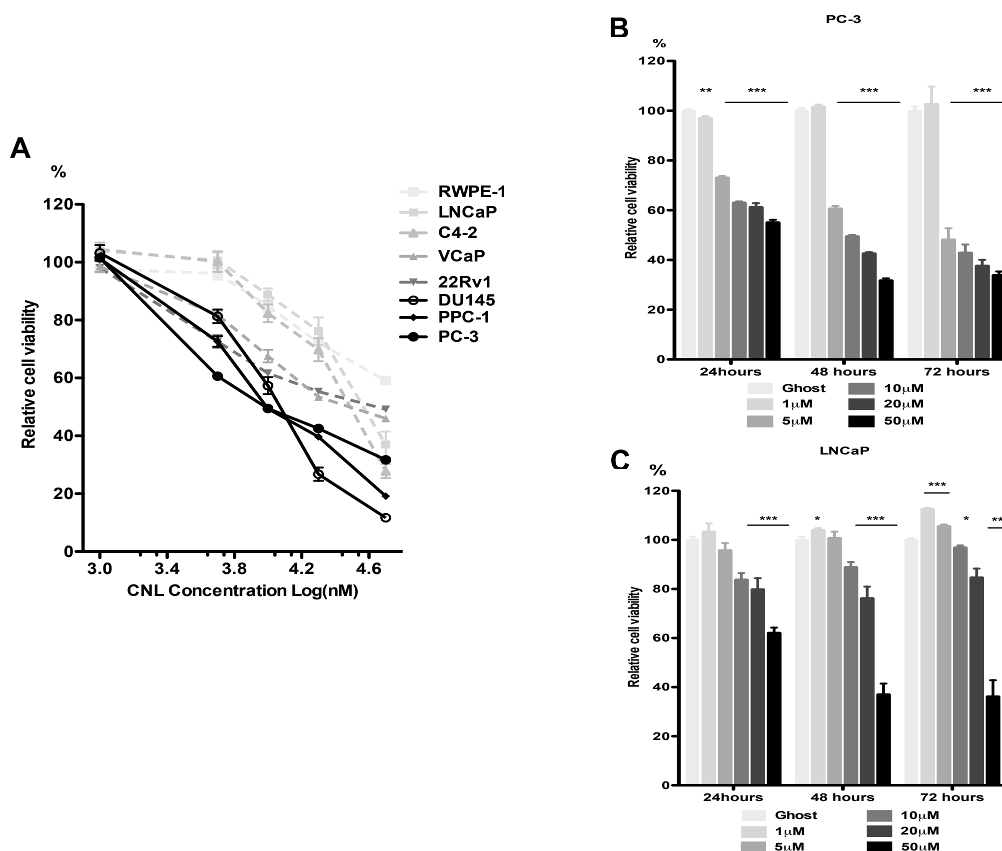
2. RESULTS

CNL is more efficacious against AR-negative than AR-positive PCa cells

To determine the efficacy of C6-ceramide nanoliposomes (CNL) in PCa, 7 PCa cell lines and a normal prostate epithelial cell line (RWPE-1) were utilized. These cells were treated with various concentrations of CNL for 72 hours and viability was determined relative to treatment with ghost nanoliposomes, the vehicle control that contains no bioactive C6-ceramide (Figure 5A, Figure 6A-F). Notably, cells that don’t express androgen receptor (AR), representative of most aggressive tumors, were the most sensitive to CNL (Figure 5B). Given the observed dichotomy in the response to CNL depending on AR status, we selected two of the most widely-studied representative cell lines of PCa: PC-3 (AR-negative) and LNCaP (AR-positive) for

further studies. We treated PC-3 and LNCaP cells with various concentrations of CNL across three different timepoints and observed that the CNL treatment was more efficacious in PC-3 cells in a time- and concentration-dependent manner (Figure 5B-C). These data demonstrate that CNL is most effective in the most aggressive form of CRPCa represented by lack of AR.

To further confirm our viability results, we measured the effect of CNL on different phenotypic readouts in the same cell lines. CNL increased cell death in AR-negative PC-3 cells compared with the control group, while cell death was not altered in AR-positive LNCaP cells (Figure 5D). The increase in cell death in PC-3 cells was observed to be concentration-dependent at 24 hours. CNL treatment in AR-negative cells also reduced mitochondrial cytochrome C levels, while these were unchanged in AR-positive cells (Figure 5E). Additionally, CNL induced greater release of lactate dehydrogenase (LDH) in AR-negative cells than in AR-positive cells showing differential toxicity in these cells (Figure 5F). Similarly, CNL reduced proliferation in PC-3 cells when compared with LNCaP cells (Figure 5G). Taken together, these results suggest the importance of AR status in affecting the efficacy of CNL in PCa cells, and the potential of CNL for treatment of the most aggressive neuroendocrine advanced PCa.



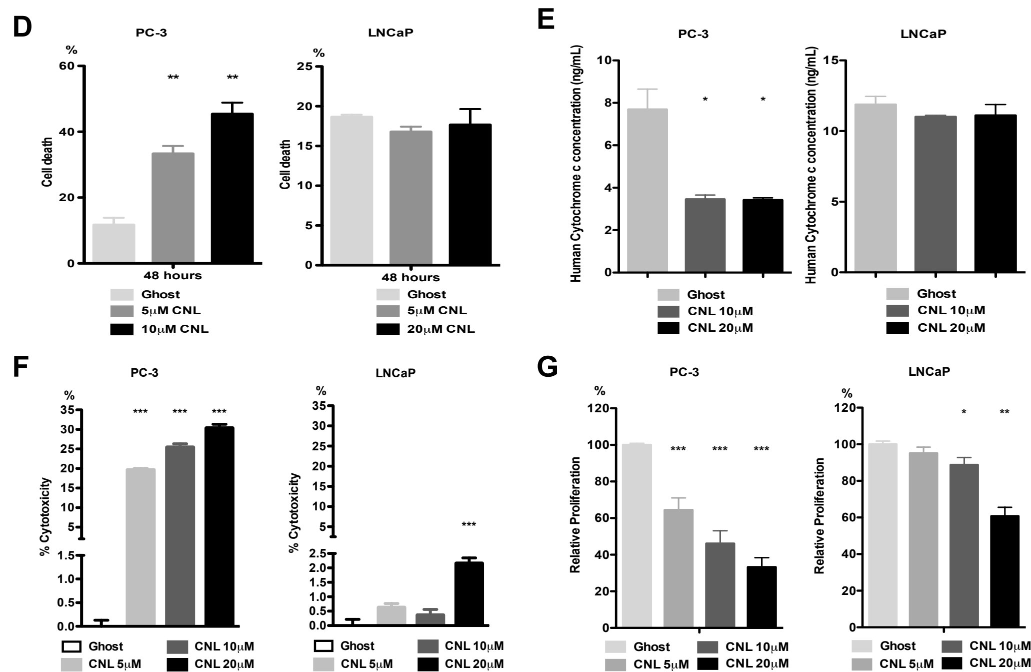


Figure 5 - CNL are efficacious against highly aggressive AR-negative PCa cells.

(A) Viability of 7 PCa cell lines and 1 normal prostate epithelial cell line (RWPE-1) after 48h of CNL treatment (1, 5, 10, 20, 50μM) compared to ghost control (20μM). Mean ± SEM from three independent experiments is represented. Grey- AR-positive cell lines; Black- AR-negative cell lines.

(B-C) Effect of time and concentration of CNL on the viability of (B) AR-negative cell line (PC-3) and (C) AR-positive cell line (LNCaP) compared to ghost control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

(D) Cell death measured after 48h of ghost and CNL treatment in PC-3 and LNCaP. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.

(E) Mitochondria cytochrome C in PC-3 and LNCaP cells treated with CNL and ghost control for 48h. Mean ± SEM from three independent experiments is represented. *P* values obtained using a two-tailed *t* test: * represent significance of p-values < 0.05.

(F) Cytotoxicity of CNL treatments in PC-3 and LNCaP after 48h compared to ghost control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.

(G) Cell proliferation measured in PC-3 and LNCaP cells treated with CNL and ghost control for 48h. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

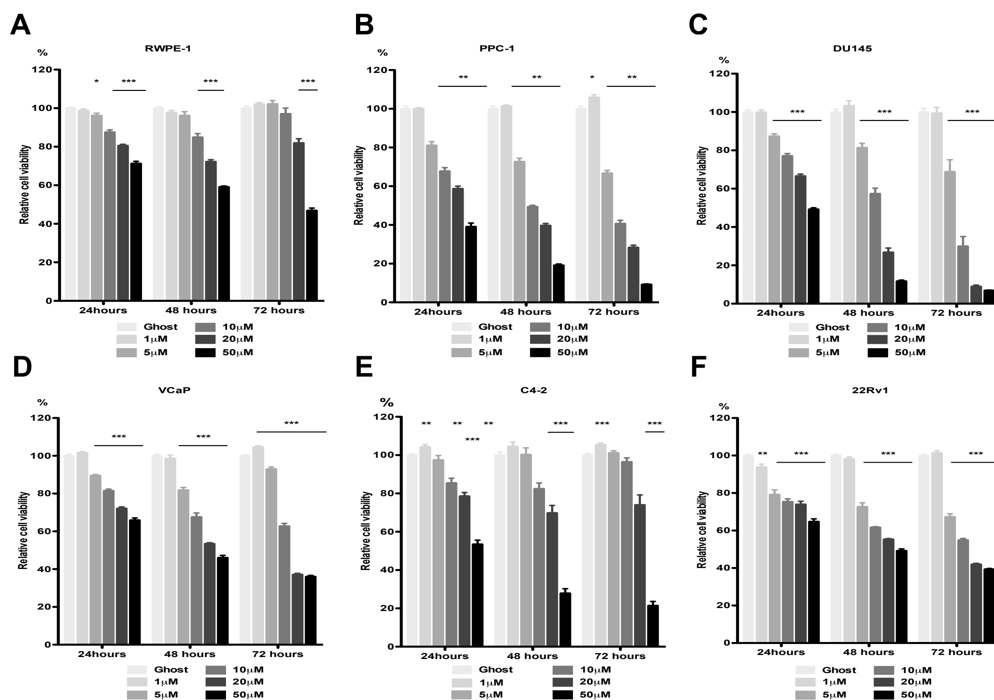


Figure 6 - Efficacy of CNL is higher in highly aggressive AR-negative cells.

(A-F) Time and concentration effect of CNL on the viability of AR-positive normal prostate cell line (A) RWPE-1; AR-negative PCa cell lines (B) PPC-1, (C) DU145; AR-positive PCa cell lines (D) 22Rv1, (E) VCaP, (F) C4-2, compared to ghost control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

CNL efficacy is enhanced in the presence of anti-androgens

Given the reduced efficacy of CNL in AR-positive cells, we sought to determine if inhibiting AR signaling would enhance the effectiveness of CNL as to mimic the effect observed in AR-negative cell lines. We combined CNL and Abiraterone acetate (Abi), an FDA-approved drug that suppresses production of androgens and is a partial AR antagonist [165]. We showed that combining Abi with CNL had an enhanced effect on reducing LNCaP cell viability (Figure 7A), and increased cell death (Figure 7B). The same phenomenon was observed when measuring cell proliferation, as combining both drugs significantly reduced cell proliferation (Figure 7C). The inhibitory effect of Abi on AR signaling was confirmed, as demonstrated by significant reductions of two downstream targets of AR, PSA (*KLK3*) and *FKPB5* (Figure 8A). We next tested C4-2 cells that are representative of AR-indifferent tumors, which don't rely on androgens for growth but are still positive for AR signaling. We observed the same response pattern as in LNCaP cells (Figure 7A), where the combination of Abi and CNL led to enhanced reduction in cell viability

of C4-2 cells (Figure 7D). Finally, we expanded our work to another FDA-approved drug for PCa: Enzalutamide (MDV3100), an AR antagonist. Validating the Abi results, the effect of MDV3100 and CNL was augmented by the combination of both drugs (Figure 8B-D). These data show that combining AR inhibitors with CNL facilitated cell death in AR-positive PCa cells.

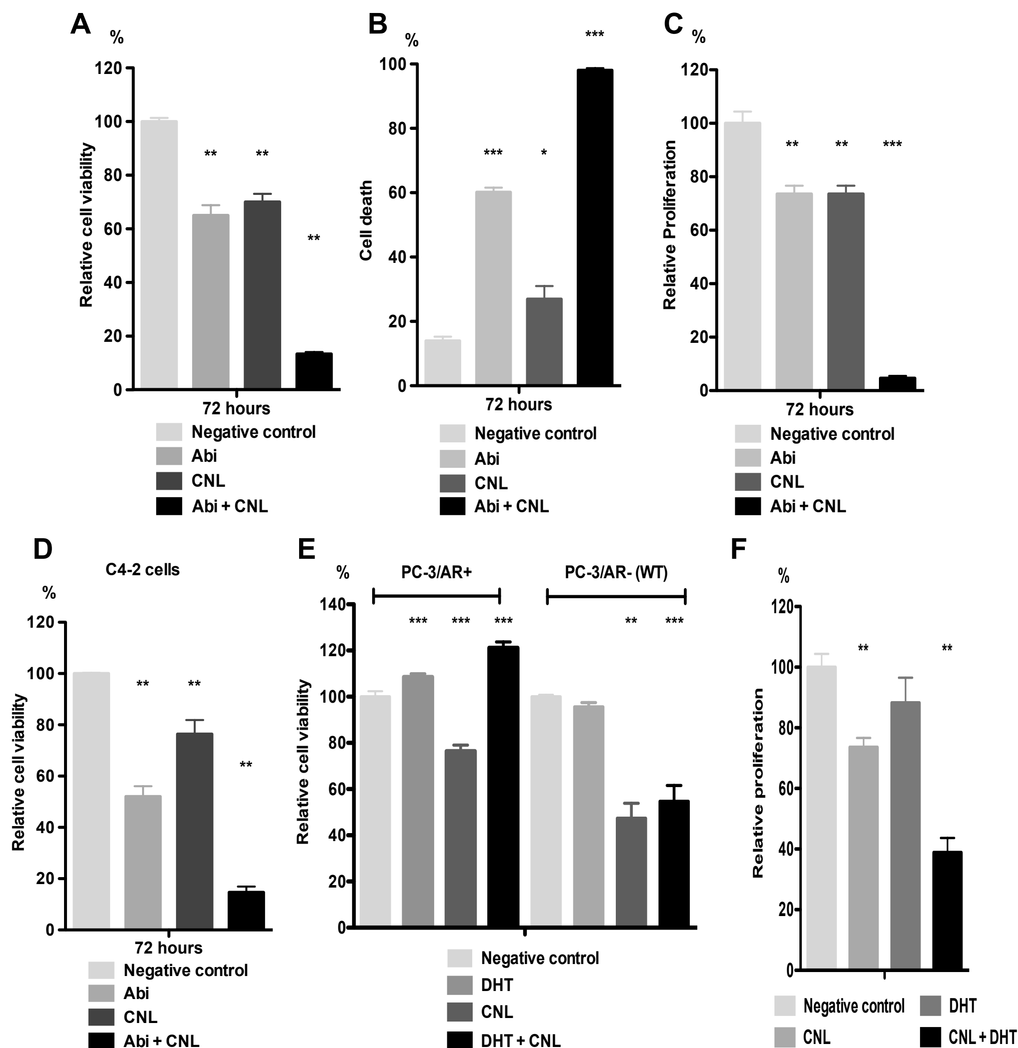


Figure 7 - Intact AR signaling hinders CNL efficacy in PCa cells.

(A) Viability of LNCaP cells measured after 72h of treatment with Abi (20μM), CNL (20μM), or Combo (20μM+20μM) compared to Negative control (EtOH+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.

(B) Cell death of LNCaP cells after 72h of treatment with Negative control (EtOH+ghost), Abi (20μM), CNL (20μM), or Combo (20μM+20μM). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: * and *** represent significance of p-values < 0.05 and 0.001 respectively.

(C) Proliferation of LNCaP cells treated with Abi (20μM), CNL (20μM), or Combo (20μM+20μM) for 72h and compared to Negative control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** and *** represent significance of p-values < 0.01 and 0.001 respectively.

(D) Viability of C4-2 cells measured after 72h of treatment with Abi (20μM), CNL (20μM), or Combo (20μM+20μM) compared to Negative control (EtOH+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.

(E) Viability of PC-3/AR+ and PC-3/WT cells after 24h of DHT (1nM) pretreatment followed by 48h of CNL (10μM) compared to Negative control (EtOH+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** and *** represent significance of p-values < 0.01 and 0.001 respectively.

(F) Proliferation of LNCaP cells treated with Dihydrotestosterone (100nM) for 24h followed by CNL (20μM) or ghost for 48h and compared to Negative controls. Mean ± SEM from three independent experiments is represented. P values obtained using non-parametric Mann–Whitney U-test: ** represent significance of p-values < 0.01.

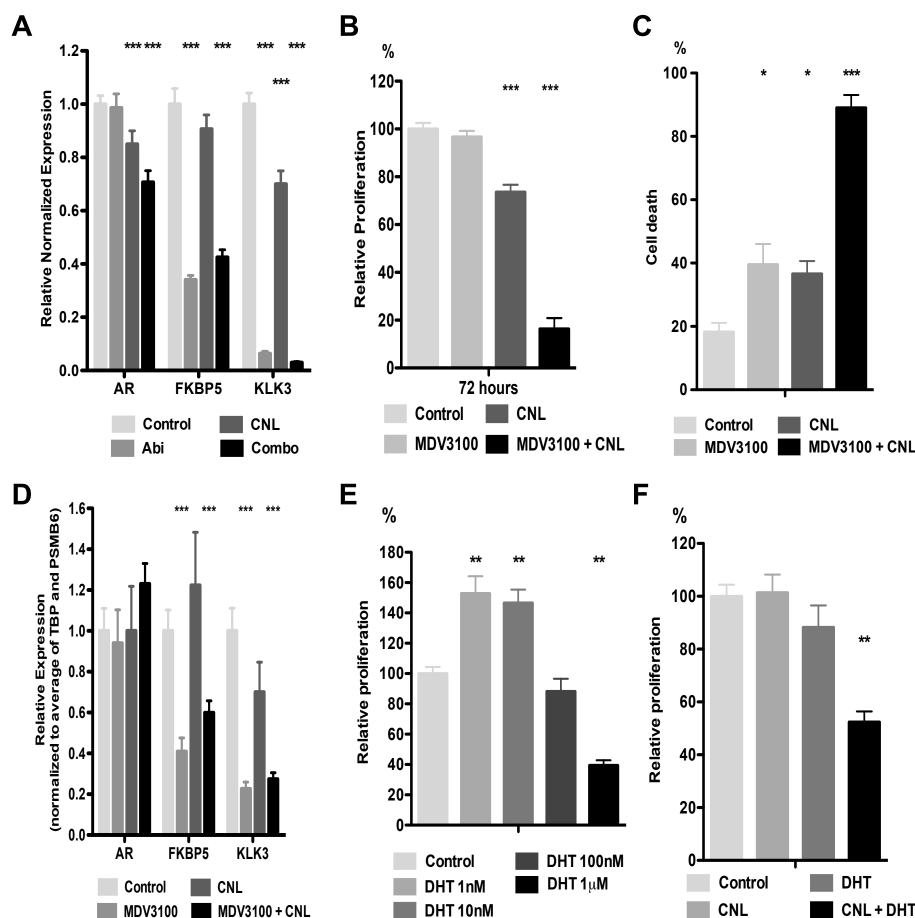


Figure 8 - Intact AR signaling hinders CNL efficacy in PCa cells.
(A) Gene expression of AR signaling targets in LNCaP cells treated with Abi (10μM), CNL (10μM), or Combo (10μM+10μM) for 48h compared to Negative control (EtOH+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.
(B) Proliferation of LNCaP cells after 72h of treatment with MDV3100 (30μM), CNL (20μM), or Combo (30μM+20μM) compared to Negative control (DMSO+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.
(C) Cell death of LNCaP cells after 72h of treatment with Negative control (DMSO+ghost), Abi (20μM), CNL (20μM), or Combo (30μM+20μM) compared to. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: * and *** represent significance of p-values < 0.05 and 0.001 respectively.
(D) Gene expression of AR signaling in LNCaP cells treated with MDV3100 (10μM), CNL (10μM), or Combo (10μM+10μM) for 48h compared to Negative control (DMSO+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.
(E) Proliferation of LNCaP cells treated with Dihydrotestosterone for 72h and compared to Negative control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.
(F) Proliferation of LNCaP cells treated with Dihydrotestosterone (100nM) for 24h followed by CNL (10μM) or ghost for 48h and compared to Negative controls. Mean ± SEM from three independent experiments is represented. P values obtained using non-parametric Mann–Whitney U-test: ** represent significance of p-values < 0.01.

AR signaling impairs the response to CNL treatment

To further test the importance of AR signaling in the response to CNL, we used PC-3 cells genetically engineered to express AR (PC-3/AR+) to determine if re-introduction of AR would decrease CNL efficacy. Under androgen-deficient conditions, we found no difference between PC-3/WT and PC-3/AR+ in the response to CNL. However, when we tested the effect of CNL on PC-3/AR+ cells in the presence of dihydrotestosterone (DHT) to stimulate androgen signaling, we found that PC-3/AR+ exhibited diminished efficacy towards CNL treatment (Figure 7E). These data suggest that activation of AR signaling is sufficient to impair CNL treatment in PCa cells.

In PCa cell lines, AR signaling is rapidly induced by androgen stimulation. However, due to a biphasic growth phenomenon, high doses of androgens can prevent AR-induced cell growth [462-464]. We confirmed these findings by showing that low concentrations of DHT (1nM and 10nM) significantly increased cell proliferation relative to vehicle control, in contrast to high dose of DHT (100nM and 1µM) where proliferation remained either unchanged or significantly decreased (Figure 8E). To further investigate the hypothesis that AR signaling facilitates resistance to CNL, we abrogated AR signaling with high androgen dosages followed by CNL treatment in AR-positive cells. The combination of CNL and blocking AR-driven cell growth with high dose DHT resulted in significantly lower rates of cellular proliferation (Figure 7F, Figure 8F). These data further support the idea that AR signaling impairs the efficacy of CNL in PCa cells.

Inhibition of AR signaling with abiraterone acetate increases de novo synthesis of sphingolipids

Given the enhanced effect of combining Abi and CNL in AR-positive cells, we sought to determine the impact of combining both drugs on sphingolipid metabolism. Mammalian cells have the ability to convert exogenous ceramides to naturally- occurring sphingolipids, adding importance to studies of sphingolipid metabolism after CNL treatment. C6-ceramide levels were measured to determine equivalent uptake between different treatment groups as well as metabolic conversion to endogenous ceramides after treatment. Interestingly, we observed that Abi led to more sustained levels of C6-ceramide compared to CNL treatment alone (Figure 9A). We also found the total mass of endogenous ceramides was significantly increased in the presence of both drugs (Figure 9B) relative to the negative control.

Moreover, we report for the first time a significant increase in dihydrosphingosine in AR-positive cells caused by Abi (Figure 9C), which was then augmented in the presence of CNL with Abi (Figure 9C). Importantly, dihydrosphingosine is a precursor of endogenous ceramides and is mostly generated by activation of *de novo* synthesis of sphingolipids. The accumulation of endogenous and exogenous ceramides, as well as dihydrosphingosine, in the presence of Abi and CNL is likely associated with enhanced cell death in LNCaP cells. These findings also suggested that AR signaling might negatively regulate *de novo* synthesis of sphingolipids.

To validate and trace augmented *de novo* sphingolipid synthesis, in the presence of anti-androgen, Abi, we implemented a novel mass spectrometry strategy. This technique requires the addition of isotope-labeled serine, a necessary amino acid for sphingolipid synthesis, to the cell culture growth media prior to anti-androgen treatment, thus enabling us to determine newly synthesized sphingolipid molecules. As expected, we observed a significant increase in dihydrosphingosine after Abi treatment in LNCaP cells and this increase in dihydrosphingosine comes from newly synthesized lipids via the *de novo* synthesis pathway, as measured by the serine-labeled mass (Figure 9D). These data support our hypothesis that blocking AR signaling leads to increase *de novo* synthesis of sphingolipids.

Anti-androgen abiraterone acetate increases the expression of enzyme regulators of de novo sphingolipid synthesis

Given that AR can regulate transcription of thousands of gene transcripts in different cell pathways, we measured the mRNA expression of 8 enzymes involved in the initiation of *de novo* synthesis of sphingolipids after Abi treatment, with or without CNL, in AR-positive LNCaP cells. We observed that after 48 hours, 6 of the 8 enzymes (*ORMDL1, ORMDL3, SPTLC1, SPTLC2, SPTLC3*, and *SPTSSB*) tested had elevated expression levels after combination treatment, while two (ORMDL2 and SPTSSA) did not (Figure 9E-G). Notably, Abi treatment increased the expression of SPTSSB by nearly 2-fold, which was then significantly augmented by CNL to nearly 3.5-fold, despite a lack of increase following treatment with CNL alone (Figure 9E). These data show that in AR-positive cells, the combination of Abi and CNL elevates expression of enzymes involved in the *de novo* synthesis of sphingolipids, consistent with the increased mass of dihydrosphingosine (Figure 9C).

To confirm regulation of SPTSSB by AR-signaling, we analyzed an RNA sequencing dataset of three different PCa cell lines stimulated for 24 hours with

R1881, an AR agonist. The expression of *SPTSSB* was not detected in LNCaP in this experiment. However, in two other AR-positive cell lines (PC-3/AR+, VCaP) *SPTSSB* expression was significantly decreased after AR stimulation (Figure 9H). These data also support AR signaling negatively regulating *de novo* sphingolipid synthesis through SPTSSB.

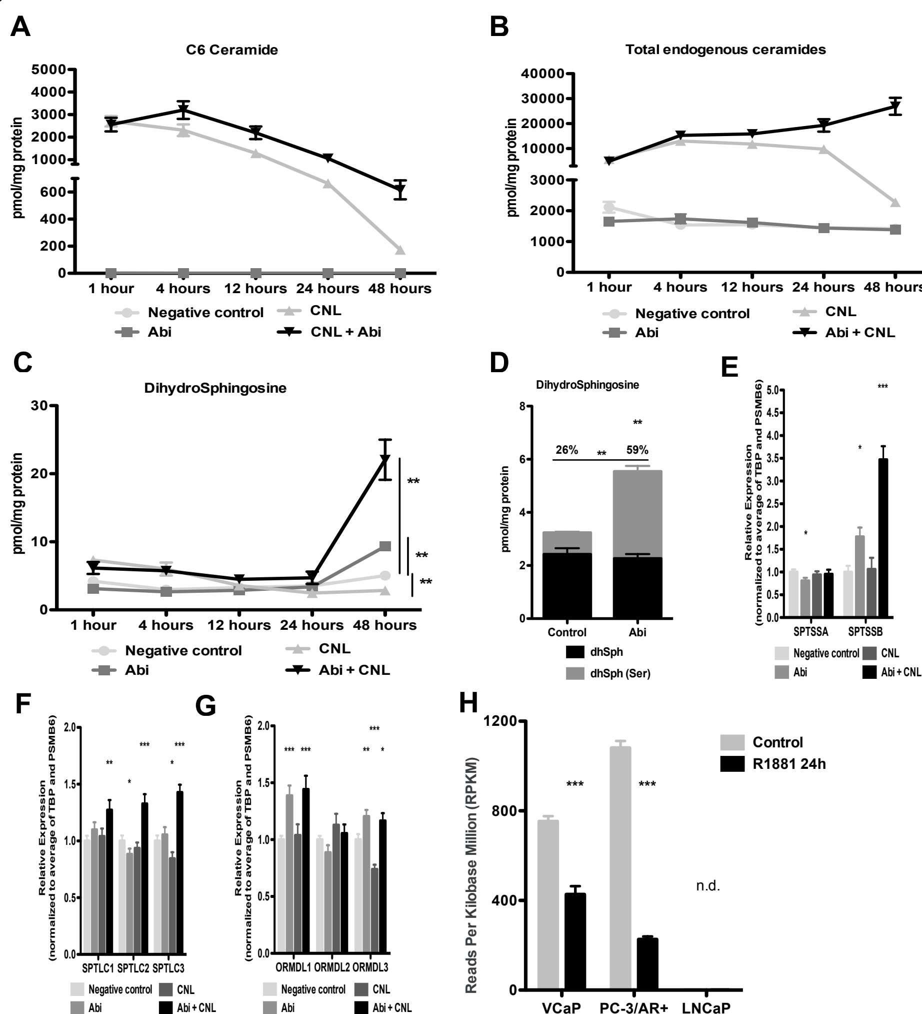


Figure 9 - *De novo* synthesis of sphingolipids is negatively regulated by AR. (A-C) Mass of (A) C6-ceramide, (B) Total endogenous ceramides, (C) Dihydrosphingosine in LNCaP cells treated with Negative control (EtOH+ghost), Abi (10µM), CNL (10µM), or Combo (10µM+10µM). Mean ± SEM (n=5) is represented.
(D) Mass of dihydrosphingosine d18:1 in LNCaP cells pulsed with L-Serine-13C3 for 2h followed by treatment with Negative control (EtOH) or Abi (10µM) for 48h. Mean ± SEM (n=5) is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.
(E-G) Gene expression of (E) *SPTSSA* and *SPTSSB*; (F) *SPTLC1, SPTLC2,* and *SPTLC3*; (G) *ORMDL1, ORMDL2,* and *ORMDL3* in LNCaP cells treated with Abi (10µM), CNL (10µM), or Combo (10µM+10µM) for 48h compared to Negative control (EtOH+ghost). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.
(H) *SPTSSB* expression from RNA-seq in VCaP, PC-3/AR+, and LNCaP after AR stimulation with R1881 (1nM) for 24h. Mean ± SEM from three independent experiments is represented. *P* values obtained using two-tailed *t* test: *** represent significance of p-values < 0.001 respectively.

CNL increases de novo synthesis of sphingolipids and accumulation of ceramides in AR-negative cells

To validate the importance of *de novo* sphingolipid synthesis driven by anti-androgens in in AR-positive cells, we sought to recapitulate these findings in AR-negative cells (PC-3) treated with CNL. After an initial increase in C6-ceramide following CNL addition, C6-ceramide levels decreased over time. However, after 48h of CNL treatment, C6-ceramide levels remained considerably high (Figure 10A). More importantly, the total endogenous ceramide mass, which excludes exogenous C6-ceramide, increased in CNL-treated PC-3 cells compared to control cells (Figure 10B). Concomitant with this increase in total ceramide levels, we observed a significant increase in the *de novo* precursor dihydrosphingosine that peaked at 48h (Figure 10C). This similar regulation of *de novo* synthesis of sphingolipids in AR-negative and AR-positive cells treated with Abi (Figure 9), support the notion that AR signaling can regulate CNL metabolism and ultimately determines CNL treatment response.

We then evaluated the impact of CNL treatment on the expression of de novo metabolic enzymes in PC-3 cells. We observed an overall increase in expression of most of these enzymes, which corroborates the increase in dihydrosphingosine mass observed (Figure 10D). Of particular interest, expression of *SPTSSB* was elevated in PC-3 cells after CNL treatment similarly to the combination between Abi and CNL in AR-positive cells (Figure 10E). In LNCaP, CNL treatment alone for 24 hours did not alter the expression of these proteins except for a modest increase of *SPTLC2* expression (Figure 10E). Overall, the findings in Figures 9 and 10 support the hypothesis that CNL treatment increases *de novo* synthesis of sphingolipids only in the absence of AR signaling.

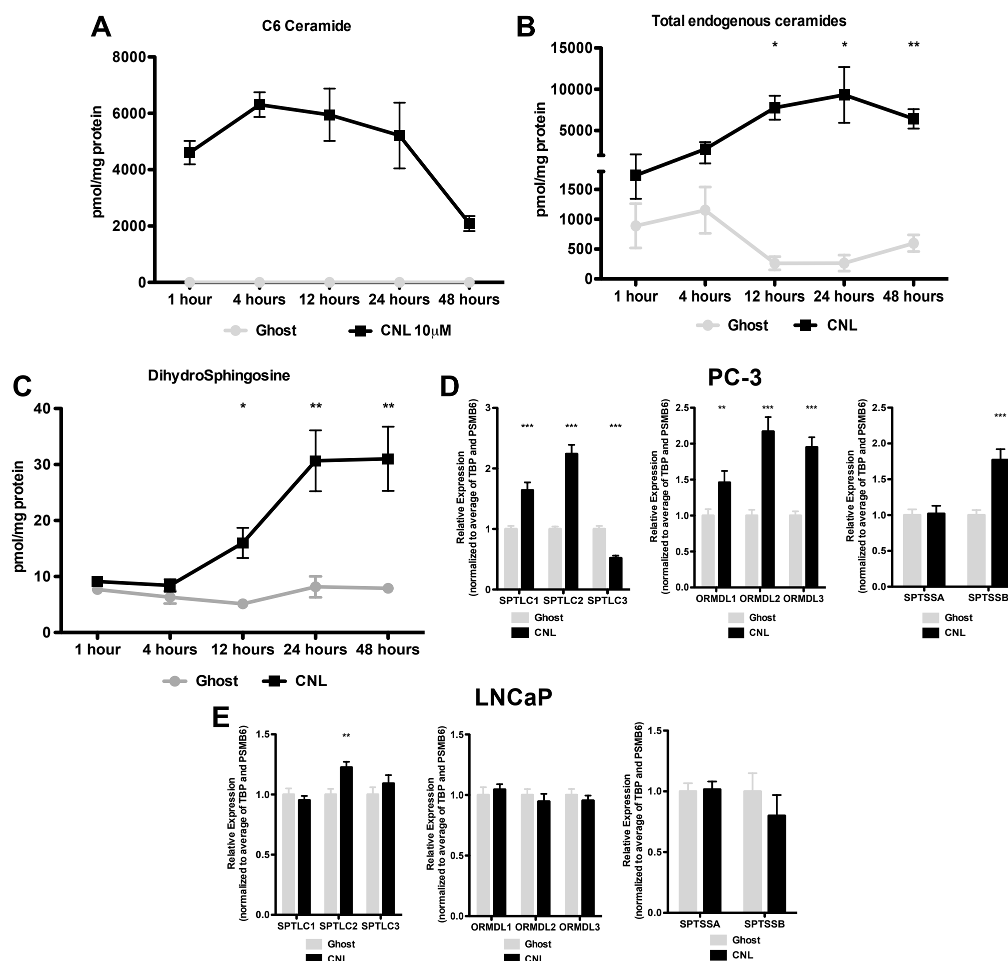


Figure 10 - CNL induces *de novo* synthesis of sphingolipids in AR-negative cells. (A-C) Mass of (A) C6-ceramide, (B) Total endogenous ceramides, (C) Dihydrosphingosine in PC-3 cells treated with ghost or CNL (10μM). Mean ± SEM (n=5) is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, and ** represent significance of p-values < 0.05, 0.01 respectively.
(D-E) Gene expression of SPTLC1-3, ORMDL1-3, SPTSSA, and SPTSSB in (D) PC-3 and (E) LNCaP cells after 24h of CNL (10μM) treatment compared to ghost control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

SPTSSB expression is up-regulated in advanced PCa patients

To determine the clinical relevance of our findings regarding SPTSSB, we evaluated its expression across available TCGA PCa patient datasets. Using a paired analysis of tumor versus normal tissue, we determined that the expression of SPTSSB was significantly down-regulated in tumors (Figure 11A) [450]. The comparison of all tumor samples versus the group of available normal samples also showed a reduction of *SPTSSB* expression in PCa (Figure 11B). Importantly, the expression analysis showed higher expression in samples with higher Gleason score, which represent more aggressive tumors (Figure 11C). Interestingly, in the TCGA original dataset,

patients' AR score was negatively correlated with SPTSSB expression (Figure 11D, Spearman correlation: -0.24; p<0.001) [465].

Surveying *SPTSSB* expression in advanced cases of PCa also provides evidence that SPTSSB plays an important role in PCa. In a neuroendocrine CRPCa dataset, we calculated that 28% of the patients had *SPTSSB* gene amplifications or increased mRNA levels (Figure 11E) [2]. A study from Fred Hutchinson Cancer Research Center that measured mRNA levels of genes from patients with disseminated PCa showed *SPTSSB* expression alterations, primarily increases, in 33% of patients that had received Androgen deprivation therapy (ADT) (17 of 63 cases) (Figure 11E) [1]. These results argue that elevated SPTSSB expression plays a significant role in aggressive, metastatic cases that have been treated with ADT.

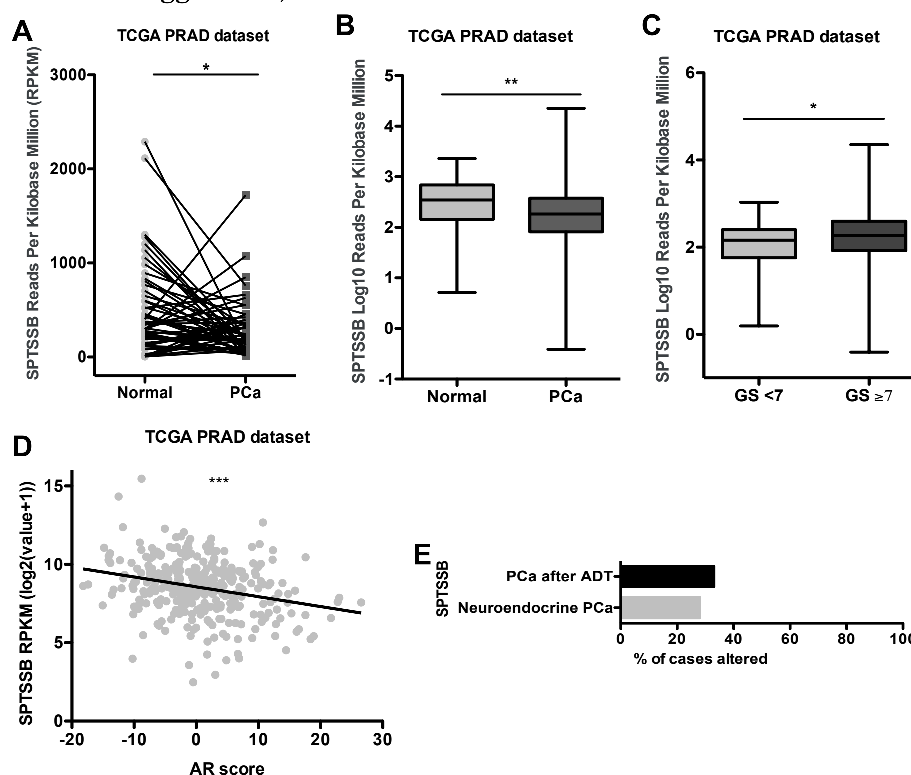


Figure 11 - SPTSSB expression is elevated in neuroendocrine PCa patients.

(A) SPTSSB expression from matched normal and tumor samples available in PRAD TCGA dataset (n=52). *P* values obtained using non-parametric Mann–Whitney *U*-test: * represent significance of p-values < 0.05.

(B) Log10 expression of SPTSSB in PRAD TCGA dataset (RNA-seq) in normal samples (n=52) and PCa patients (n=497). *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.

(C) Expression of SPTSSB in PRAD TCGA dataset segregated by Gleason score <7 (n=45) and ≥7 (n=452). *P* values obtained using non-parametric Mann–Whitney *U*-test: * represent significance of p-values < 0.05.

(D) Correlation between expression of SPTSSB and AR score in PRAD TCGA dataset (n=332). *P* values obtained using Spearman correlation: *** represent significance of p-values < 0.001.

(E) SPTSSB is altered in 33% of patients after ADT (27% with up-regulation or amplification) (n=63) [1], and amplified or up-regulated in 28% of neuroendocrine PCa samples (n=81) [2].

SPTSSB is necessary for regulating de novo synthesis of ceramide, which determines CNL efficacy in AR-negative cells

Given the prominent role that *de novo* synthesis plays in the efficacy of CNL in AR-negative cells and the observed elevation of *SPTSSB* in the aggressive ADT-treated PCa tumors, we examined the effect of CNL treatment after knock down of *SPTSSB* in AR-negative PC-3 cells. PC-3 cells transfected with siRNA targeting *SPTSSB* showed a reduction in *SPTSSB* expression (Figure 12A). Interestingly, *SPTSSB* knockdown in PC-3 cells diminished cell viability without increasing cellular death (Figure 12B-C). The efficacy of CNL was significantly hindered after *SPTSSB* knockdown as the effect on cell death and viability was reduced in cells treated with siRNA-*SPTSSB* compared to the control siRNA-scramble (Figure 12B-C). We confirmed that knockdown of *SPTSSB* was not compensated for by an overall increase in the expression of other enzymes involved in *de novo* synthesis of sphingolipids (Figure 12D). These data show that SPTSSB is necessary for maximum efficacy of CNL in AR-negative cells.

We next sought to determine the importance of SPTSSB in sphingolipid generation. After 24h of SPTSSB knock down, PC-3 cells were challenged with CNL for 24h and sphingolipid masses were measured. We observed that knocking down SPTSSB for 48 hours did not change sphingolipid mass in PC-3 cells (Figure 12E-G). However, upon CNL treatment, the knockdown of SPTSSB reduced the levels of C6-ceramide (Figure 12E) and total endogenous ceramides (Figure 12F). Notably, the mass of dihydrosphingosine, indicative of de novo synthesis of sphingolipids, was reduced in the siRNA-SPTSSB cells treated with CNL compared with scramble RNA (Figure 12G). The lower levels of ceramide accumulated in the siRNA-SPTSSB cells after CNL treatment correlates with reduced efficacy of CNL. This shows, for the first time, that SPTSSB dependent de novo synthesis of sphingolipids is critical for maximum CNL efficacy in AR-negative PCa cells.

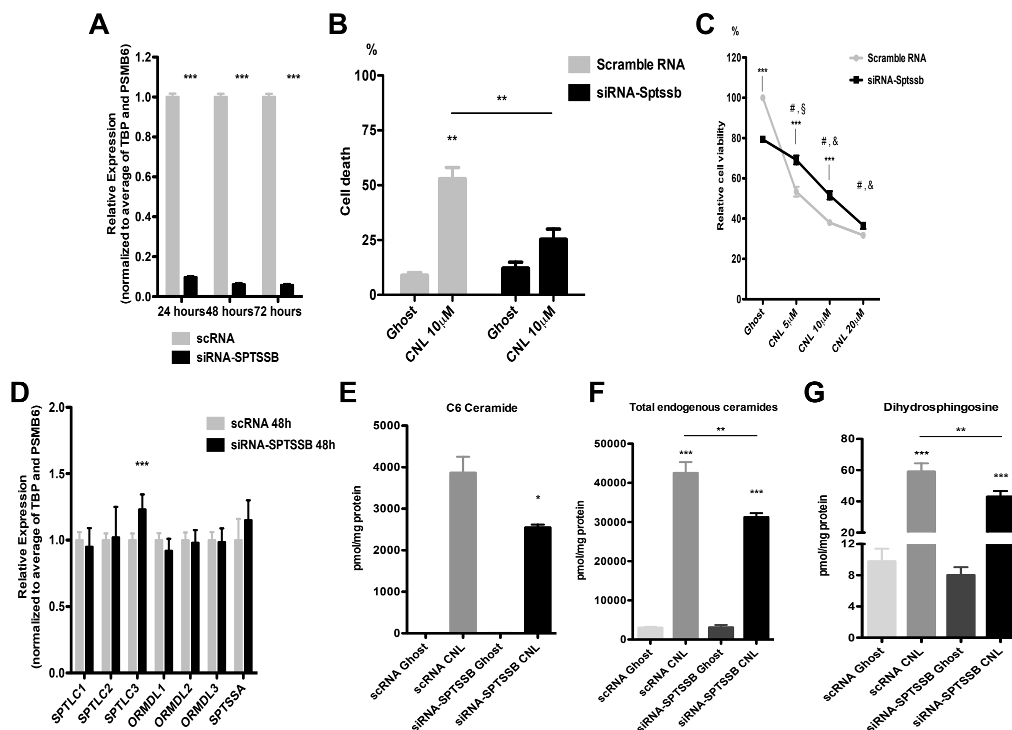


Figure 12 - SPTSSB is essential for CNL efficacy in AR-negative cells.
(A) Expression of SPTSSB after knockdown in PC-3 cells compared to scramble control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.
(B) Cell death of PC-3 cells transfected with scRNA and siRNA-SPTSSB and 24h later treated with CNL or ghost for 48h. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ** represent significance of p-values < 0.01.
(C) Viability in PC-3 cells transfected with scRNA and siRNA-SPTSSB and 24h later treated with CNL or ghost for 48h. Mean ± SEM from three independent experiments is represented. Statistics were obtained using non-parametric Mann–Whitney *U*-test: represented by * in CNL scRNA vs siRNA; § represents p<0.01 in ghost siRNA vs CNL siRNA; & p<0.001 in ghost siRNA vs CNL siRNA; # p<0.001 in ghost scRNA vs CNL scRNA.
(D) Gene expression of SPTLC1-3, ORMDL1-3, SPTSSA in PC-3 cells after knocking down SPTSSB compared to scramble control. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *** represent significance of p-values < 0.001.
(E-G) Mass of (E) C6-ceramide, (F) Total endogenous ceramides, (G) Dihydrosphingosine in PC-3 cells transfected with scRNA and siRNA-SPTSSB for 24h followed by treatment with ghost or CNL for 24h (10μM). Mean ± SEM (n=4) is represented. *P* values obtained using a two-tailed *t* test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

CHAPTER 5: ANDROGEN RECEPTOR AND SPHINGOLIPID ENZYMES

1. RATIONALE

Androgen receptor (AR) is the master regulator of several signaling pathways contributing to the prostate's normal growth and maturation. AR acts mostly as a transcription factor after associating with the transcription machinery, co-activators and co-repressors [136]. Importantly, a ratio between co-activators and co-repressors has been suggested as an important determinant of what kind of regulation AR will impose on gene expression in a tissue- and temporal manner [137]. AR can play multiple roles in cells and in fact has been found to play both an oncogenic and tumor suppressor role [143].

PCa's development and progression are accompanied by higher rates of lipid synthesis and metabolism, mostly coordinated by AR [91,146-149]. Importantly, one of the metabolic hallmarks of PCa is lipid accumulation, a phenomena that has been reported in patients and other models of study [84]. A minimally defined role for AR concerning lipid metabolism is the regulation of exogenous lipid uptake, which can generate a feedback loop: release of fatty acid from adipose cells or cells in tumor microenvironment, and uptake by the prostate cancer cells [147]. PCa is more lipogenic than most tumors, given that it doesn't rely as much on glycolysis [91]. The ability displayed by AR in regulating cell metabolism, and the importance this regulation plays in transition from indolent to aggressive PCa makes it imperative to determine AR regulation of metabolism and lipid metabolism. Taking into consideration the importance that metabolites play in drug resistance and progression after treatment, the interaction between AR and metabolism might lead to better therapeutics for PCa patients.

The importance of sphingolipids in oncology has been established since the early 1990s [345]. In PCa, increased levels of sphingomyelin were observed in cells with higher metastatic potential [402]. Similarly, a study showed that that AR-negative and AR-positive cells differed in their gangliosides content [403]. Moreover, ASAH1 and SPHK1-2 have also been reported as differentially expressed in PCa with relevant impact in the progression of the disease or therapeutic resistance [366,370,414-419]. Recently, a study reported that blocking AR signaling with Abiraterone acetate led to an increase in the mass of ceramide and sphingomyelin [412]. In 2017, the Knudsen laboratory demonstrated that AR regulates the expression of dihydroceramide desaturase (DEGS1), a sphingolipid metabolic enzyme [128]. To our knowledge this is the only established example of AR regulation of sphingolipid metabolism down to

the molecular level. Nonetheless, and considering the vast information available for the role of sphingolipids in tumors other than PCa, these lipids deserve a lot more attention. Understanding sphingolipid metabolism and its regulation by AR is important for a better picture of cancer metabolism in PCa, as well as rendering novel therapeutic targets or approaches in order to combat the current relapse-prone landscape of PCa treatments.

With that goal in mind, in this Chapter we will present data showing AR regulation of the expression of sphingolipid metabolic enzymes by high-throughput techniques. These data can be explored to determine the biological and pathological relevance of the interplay between AR and specific sphingolipid metabolic enzymes. To our knowledge, this is the first study of its kind, where RNA-seq has been used to explore and describe the relationship between AR and sphingolipids in PCa.

2. Results

Androgen receptor regulates expression of sphingolipid metabolic enzymes

To measure the effect of androgen stimulation of the Androgen receptor (AR) on sphingolipid metabolic enzymes expression, we utilized publicly available RNA-seq datasets in different AR-positive PCa cells. We curated a comprehensive list of 99 enzymes involved in the different pathways that comprise sphingolipid metabolism (Figure 13). A cutoff value for the fold change differences after androgen treatment was set at ≥1.5 (up-regulation - red) and ≤0.5 (down-regulation - green). AR was stimulated with the synthetic testosterone analog R1881.

LNCaP: in these cells, treatment with R1881 for 24 hours led to a significant change in the expression of 24 transcripts out of the 99 analyzed (24%). Of these 24 transcripts 7 were down-regulated and 17 up-regulated (Table 1 and 2).

PC-3/AR+: R1881 for 24 hours led to a significant change in the expression of 21 transcripts out of the 99 analyzed (21%). Of these, 9 were down-regulated and 12 were up-regulated (Table 1 and 2).

VCaP: treatment for 24 hours changed the expression of 40 transcripts out of the 99 sphingolipid metabolic enzymes (40.4%). Of these, 13 transcripts were down-regulated and 27 up-regulated (Table 1 and 2).

Regarding down-regulation, one of these transcripts had its expression significantly altered in all 3 cell lines, Serine palmitoyltransferase long chain base subunit 3 (SPTLC3) (Table 1). SPTLC3 plays a critical role in the assembly of

SPTLC/SPTSS complex to generate sphingolipids via the *de novo* pathway (Han, 2009). Another gene involved in the same complex assembly, SPTSSB, was down-regulated in both PC-3/AR+ and VCaP, while undetected in LNCaP (Table 1). The lack of detection of SPTSSB in LNCaP cells point to additional mechanisms of regulation of its expression than just AR. These results point to a negative regulation from AR signaling of the *de novo* synthesis pathway, which in Chapter 3 we showed could be reversed with an anti-androgen, Abiraterone acetate. The concept of negative regulation by transcription factors such as AR, is underexplored and only recently has started to become more evident. This negative regulation is commonly associated with the association of AR with co-repressors that inhibit expression of target genes upon binding to regulatory elements. More studies are necessary to understand what the biological impact of this kind of regulatory effect is.

In the up-regulated category, we found two transcripts to be commonly up-regulated among all three cell lines used. SMPD1 and Beta-1,3-Galactosyltransferase 4 (B3GALT4) were up-regulated in all three cell lines exposed to R1881 (Table 2). SMPD1 is one of four different enzymes responsible for breaking down sphingomyelin into ceramide and mutations in SMPD1 lead to Niemann-Pick type A and B disease, which is characterized by an accumulation of sphingomyelin [273]. There is one publication showing that AR-stimulation leads to up-regulation of SMPD1 in smooth muscle cells [466]. However, in prostate tissues and PCa specifically, it is not known the impact or role of this interaction. B3GALT4 is involved in the synthesis of higher order glycosphingolipids by generating GM1 ganglioside. This enzyme is understudied with currently no publications in PCa or a connection with AR-signaling. More studies are necessary to determine what is the impact, if any, of the positive regulation by AR of these two enzymes. Interestingly, DEGS1, shown by the Knudsen laboratory to be up-regulated by AR in LNCaP cells, showed high levels of up-regulation in both LNCaP and VCaP in this dataset, but not in PC-3/AR+ cells (Table 2). Thus, it is important to note that AR activation or repression of transcription of most sphingolipid metabolic enzymes is cell line dependent suggesting other pathways and expression of either co-activators or co-repressors as crucial for its activity (Figure 14).

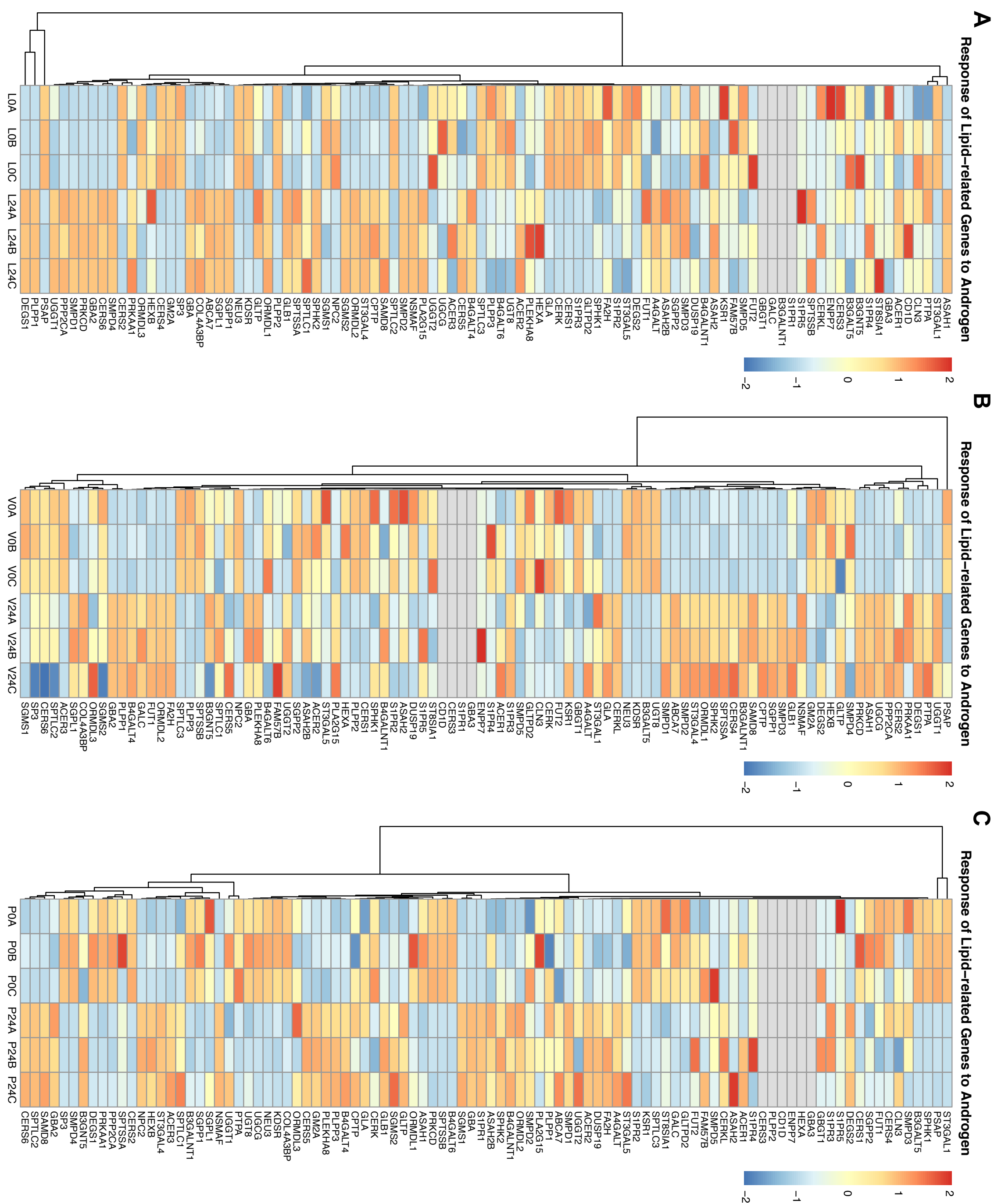


Figure 13 - AR regulates the expression of enzymes involved in sphingolipid metabolism.

Heatmap of sphingolipid metabolic enzymes in prostate cancer cell line models: (A) LNCaP, (B) VCaP, and (C) PC-3/AR+. Data was obtained by RNA-sequencing from untreated and R1881 treated cells with three biological replicates.

Table 2 - List of downregulated sphingolipid enzyme transcripts by AR stimulation in PCa cells lines (red – up-regulation; green down-regulation; fold change to vehicle treated cells).

GENE	FOLD CHANGE		
	LNCAP	VCAP	PC-3/AR+
CERK	0,03	0,15	0,88
SPTLC3	0,24	0,33	0,30
S1PR3	0,25	-	3,20
DEGS2	0,29	-	5,38
CERS1	0,34	0,21	0,64
PLPP2	0,47	0,48	-
FAM57B	0,49	-	-
ACER3	-	0,37	1,45
B3GALT5	-	0,03	0,02
DUSP19	-	0,38	1,88
GBGT1	-	0,25	-
GLTPD2	0,58	0,39	0,42
NEU3	0,65	0,40	0,53
SMPD5	-	0,48	-
B4GALT6	0,71	-	0,38
S1PR2	0,67	0,61	0,40
SPHK1	0,72	-	0,13
ST3GAL1	0,66	-	0,44

Table 3 - List of upregulated sphingolipid enzyme transcripts by AR stimulation in PCa cell lines (red – up-regulation; green down-regulation; fold change to vehicle treated cells).

Gene	Fold Change		
	LNCaP	VCaP	PC-3/AR+
SGPP1	1,50	2,05	0,82
UGGT1	1,53	3,36	-
ASAH2B	1,54	-	-
SMPD4	1,58	0,92	0,80
SGMS2	1,60	0,90	-
ABCA7	1,62	2,36	-
SMPD1	1,67	3,07	2,72
ASAH1	1,78	2,13	-
GBA2	2,10	2,11	1,26
PRKCD	2,17	2,14	0,63
CERS6	2,24	-	1,58
ORMDL2	2,77	3,22	1,32
ST3GAL4	2,97	2,72	1,44
SMPD2	4,65	5,57	-
NSMAF	5,57	1,22	2,73
B3GALT4	6,29	3,55	8,19
PLPP1	7,01	2,07	-
DEGS1	8,76	2,12	0,73
B4GALT4	1,18	1,56	1,49
CERKL	-	14,46	-
CPTP	1,34	1,77	-
FA2H	0,71	3,56	2,19
FUT1	1,30	1,79	-
GBA	1,38	1,57	2,03
GLA	0,56	1,81	-
PLEKHA8	-	1,72	1,22
PPP2CA	1,34	1,53	0,79
SAMD8	1,30	1,64	1,31
SMPD3	-	2,51	-
SPHK2	1,49	1,52	1,25
A4GALT	1,34	-	1,82
ACER2	-	-	3,15
S1PR1	-	-	66,7
SPTLC2	0,79	0,89	1,55

AR regulation of expression fluctuates with timing of stimulation

We then mined the data from an available dataset that had LNCaP cells exposed to dihydrotestosterone for 3, 6, 12, 24, and 48 hours (Figure 15). Unlike the previous dataset analyzed, a time-course analysis of AR stimulation allows us to determine if the regulation is continuous, or if it's time specific. This dataset also serves the purpose of validating the results obtained in LNCaP cells in the previous section. In this dataset we confirmed that SMPD1 was indeed up-regulated after 24 hours of AR stimulation when its expression reached the highest level (Figure 15). Another target that we showed to be up-regulated in all 3 cell lines at 24 hours is B3GALT4. In this experiment, expression of B3GALT4 was increased with time of exposure and in fact reached its peak at 48 hours after treatment (Figure 15). On the other side of the spectrum, SPTLC3 was the main target for down-regulation by AR in all 3 cell lines. In this time-course, SPTLC3 expression reached its lowest point after 24 hours of dihydrotestosterone treatment (Figure 15). This decrease in expression occurred progressively from 6h to 24h, while slightly rebounding from 24h to 48h (Figure 15). This data suggests that AR negatively targets SPTLC3, and that in fact 19 of the enzymes studied had decreased expression after 24 hours of DHT treatment.

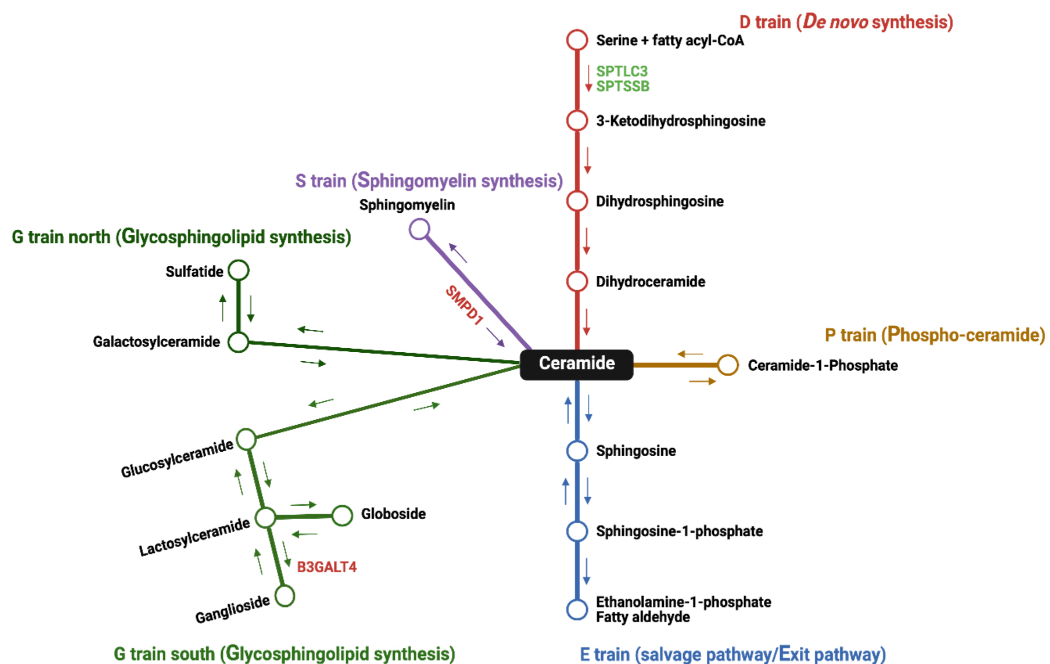


Figure 14 - AR regulation of sphingolipid enzymes' expression: AR stimulation leads to increased expression of B3GALT4 and SMPD1 (red) in 3 different PCa cell lines. These two enzymes are involved in the ganglioside synthesis (B3GALT4) and conversion of sphingomyelin to ceramide (SMPD1). AR stimulation also represses expression of SPTLC3 and SPTSSB (green), which are both involved in the *de novo* synthesis of sphingolipids. Here are only represented the transcripts that are either up- or down-regulated in all three cell lines measured by RNA-seq.

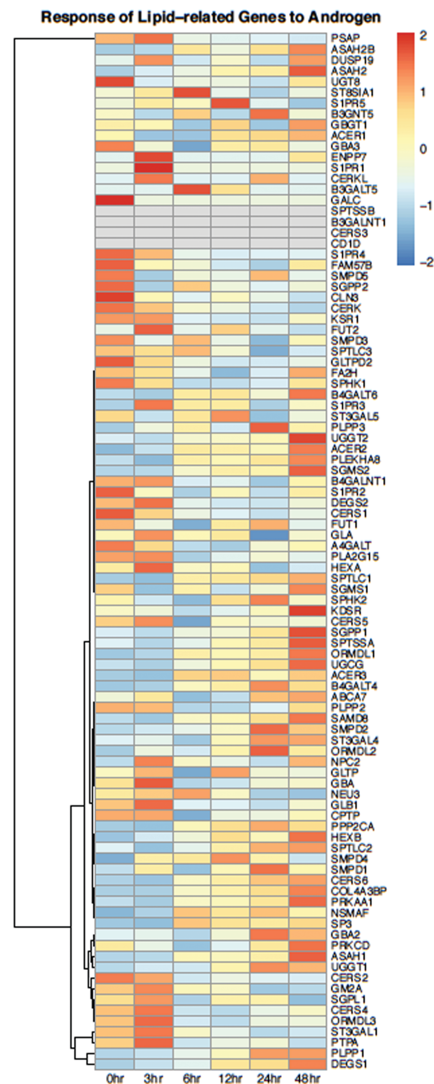


Figure 15 - Regulation of sphingolipid metabolic enzymes by AR stimulation across time.
Heatmap of sphingolipid metabolic enzymes LNCaP after treatment with dihydrotestosterone. RNA-seq data with one replicate per timepoint of stimulation.

In sum these results show that AR is capable of regulating the expression of sphingolipid metabolic enzymes, thus having the potential ability to regulate sphingolipid metabolism in PCa cells, reinforcing another pathway as under control by this powerful regulator in prostate cells. More studies are necessary to ascertain how these regulations occur (direct binding to target promoter *vs* indirect binding via complex formation and co-inhibitor/inducer association), as well as the biological outcome of those. These data also highlight other enzymes that can be explored in the context of PCa and hormonal regulation.

Chapter 6: Ceramide and chirality – the good, the bad and the ugly

1. Rationale

Ceramide is a bioactive lipid that has been described as part of several different death pathways. The first publication tying ceramide with cell death in cancer was published in 1993 [345]. Later studies showed that ceramide levels are increased in cells under stress conditions, and importantly in cancer cells treated with chemotherapeutics and radiation [340,374,375]. To simulate the increase in ceramide levels as a cancer therapeutic, exogenous short-chain ceramides have been developed that are more soluble and can therefore be used in cells and patients [391]. Despite the fact that these exogenous ceramides display similar effects to endogenous ceramides when it comes to cell death and signaling cascades, it is still important to determine the role lipid structure plays.

Given the complex structure of ceramide, researchers have made attempts to determine which components of its structure are essential for its biological role. Initial studies showed that while C2-ceramide was able to induce cell differentiation that was not the case for sphingosine, suggesting a critical role for the amide-linked fatty acid [467]. Natural sphingolipids occur in the D-erythro configuration but three more stereoisomers exist: L-erythro (enantiomer of D-erythro), D-threo, and L-threo. The altered three-dimensional orientation of these lipids might confer different degrees of access by sphingolipid metabolic enzymes. In pharmacology, usually only one enantiomer or stereoisomer of a drug can carry out the purposed effect. Thus, in the sphingolipid field, researchers have tried to determine if the same happens with exogenous short-chain ceramides helping to determine if metabolism of the compound by enzymatic reactions plays a role in the drug effect or if the toxicity arises from only the compound.

The first study concerning the role of different ceramides was published in 1992 where Bielawska *et al.* wanted to determine the structural requirements for ceramide's action using phenylaminoalcohols [467]. These chemicals differ from ceramide by the replacement of sphingosine backbone with a phenyl group. Authors showed that sphingosine was effective at reducing proliferation but not differentiation in HL-60 leukemic cells [467]. The suggested role for the need of the amide linked acyl group was still not sufficiently addressed given the fact that the chemicals used not fully represent sphingolipid structures. In order to address this issue, the same team published another paper in 1993 investigating the effect of

stereoisomers of C2-ceramide [468]. Importantly, the authors observed that D-erythro-C2-dihydroceramide had no activity compared with D-erythro-C2-ceramide [468]. By measuring uptake, the authors were able to disregard lipid uptake as the reason for differential effect of these two lipids [468]. When comparing different chemical structures of C2-ceramide, it was possible to observe that in HL-60, L-erythro-C2-ceramide was equally potent to D-erythro-C2-ceramide; however, L-threo had a bigger impact in cell proliferation than those two structures, while D-threo was the least effective stereoisomer [468]. Similar to dihydroceramides, lipid uptake and metabolism was similar and didn't seem to be related with different biological effects. In fact, a striking 80% of both C2-ceramide and dihydroceramides were still present in the cells after 24h treatment [468]. Interestingly, using labeled C2-ceramide authors couldn't detect any labeled sub-products of ceramide in the form of other sphingolipids [468]. Concerning cellular death, apoptosis was detected only in U937 lymphoma cells but not HL-60 cells showing that the effect might be cell line dependent [468]. The authors suggest that given the biological difference between ceramide and dihydroceramides, this difference might rely on the double bond that distinguishes these two lipids [468]. Another hypothesis proposed by the authors concerning dihydroceramide's inert impact, is that its presence blocks potential detrimental effects of ceramide accumulation. Exogenous ceramide localizes primarily to the Golgi where it can be sequestered, delaying its conversion to endogenous ceramides [468].

Despite all the efforts to validate and confirm the stereospecificity of ceramide's effect, contradicting data has been reported. Hauser *et al.* published that C6-dihydroceramide caused similar effects to C6-ceramide contradicting previous reports [469]. This difference might be originated from testing different cell lines with metabolic molecular pathways that might not be similar. Moreover, it is possible that C2 and C6 exogenous sphingolipids might trigger different effects in cells. One of the most interesting results reported by Hauser and colleagues is the morphological changes in cells treated with C6-ceramide [469]. The authors describe a phenomenon where cells "round up" after 10h of treatment but remain viable, an alteration that can be reversed by removing C6-ceramide from the media up to 18h after starting treatment [469]. Despite not pursuing what molecular changes were behind this morphology alterations, the authors suggest that it might be related with functional Rho proteins and involve lipid inhibition of rhoGAP as reported for phosphatidylinositol 4,5-biphosphate and lysophosphatidic acid, which affect the

cytoskeleton through the actin machinery [469]. This potential relationship is of crucial importance, but still lacks further validation in the literature.

Another publication came out of the Young-Moon Lee laboratory where researchers found that all four isomers of C2-ceramide caused increased sphingosine mass without changes in dihydrosphingosine and equal arrest of cell cycle [470]. A paper from the same year suggests that this effect might be independent of metabolism to other sphingolipid species, as they showed different metabolic outcomes of different isomers [471]. The authors suggest that all enzymes studied (glucosylceramide synthase, sphingomyelin synthase, ceramide synthases) are able to differentiate between the natural (D-erythro) and non-natural (L-erythro) enantiomers of ceramide [471]. It is important to note that for two of the "enzymes" the authors don't specify which enzyme were studied, given that there are 6 ceramide synthases and 2 sphingomyelin synthases.

The Hannun laboratory published another study with the stereoisomer subject in mind in 2002 [472]. The authors of this study showed that only D-erythro-C6-ceramide but not L-erythro-C6-ceramide generated endogenous ceramides after treatment, by recycling of the sphingosine backbone [472]. Strikingly, after blocking endocytosis the authors observed a decrease in the generation of endogenous long-chain ceramides; however, this synthesis was completely abrogated by brefeldin A, a drug that destabilizes the Golgi apparatus [472]. This result is consistent with treatments with fluorescent C6-ceramide showing primarily localization to the Golgi, and tries to address the hypothesis put forth by the same laboratory that the Golgi sequesters and delays conversion to endogenous ceramides [468]. In fact, it seems that complete abrogation of the Golgi apparatus can block conversion to endogenous ceramides, showing more a dual and delayed effect than a all-or-nothing scenario. Moreover, Ogretmen and colleagues also showed that D-erythro inhibited growth of A549 cells while L-erythro did not; this was not the case for HL-60 cells, where both structures caused growth arrest. Interestingly L-erythro-C6-ceramide caused increase in apoptosis (measured by Trypan blue incorporation) without raising levels of endogenous ceramides, suggesting a lipid role independent of conversion to endogenous sphingolipids. Moreover it seems, just like in previous studies, that the effects might be cell and tissue dependent, opening a can of worms when starting to define the role of specific ceramide structures. It is therefore essential to mention in publications concerning this subject that the effect might depend on the tissue of origin.

In this Chapter we explored the effect of different sphingolipids and stereoisomers of C6-ceramide in the viability of Prostate cancer (PCa), as well as its metabolism. To our knowledge this preliminary study is the first one focused on the subject of structure specificity and effect of sphingolipids in PCa. Thus, these data can be further explored to determine the biological relevance of sphingolipid enzymes and their specificity to certain isomers of C6-ceramide. Moreover, understanding the role of sphingolipids such as sphingosine and dihydrosphingosine might hint at other sphingolipid-based therapeutic avenues that can be explored in this disease.

2. RESULTS

Effect of ceramide in Prostate cancer cells depends on stereoisomer structure

To determine the effect of structure requirements for C6-ceramide in reducing PCa viability, two commonly used *in vitro* models of this disease (PC-3 and LNCaP) were selected. We used the same concentration (10μM) of 4 different isomers of C6-ceramide: the natural D-erythro, L-erythro, D-threo, L-threo, plus C6-dihydroceramide and C6-ceramide nanoliposome (CNL). Similar to the results reported in Chapter 3 of this Thesis, PC-3 cells were more responsive to C6-ceramide treatments than LNCaP (Figure 16). This result indicates a cell-specific response to ceramide-based treatments. Moreover, we observed that the effect of C6-ceramide was also dependent on the structure of the lipid.

PC-3 cells were shown to be more susceptible to the C6-treatments, however this was only the case for the D-erythro structure (Figure 16). After 24 hours of treatment both D-erythro-C6 ceramide (free and in nanoliposome) and dihydroceramide significantly reduced cell viability (Figure 16A). Importantly, ceramide was more potent than dihydroceramide showing the importance of the double bond for ceramide's effect. Interestingly, D-threo and L-erythro induced a small increase in viability of PC-3 cells, this effect, however, was not observed at 48 hours post-treatment (Figure 16B). At 48 hours the effect of D-erythro ceramide and dihydroceramide on viability was more pronounced, again with higher impact caused by D-erythro-C6 ceramide (Figure 16B). It is also important to note that in PC-3 cells, the more efficacious formulation in reducing these cells' viability was the D-erythro-C6 ceramide nanoliposome (CNL).

In LNCaP cells at 24 hours after treatment, only D-erythro-C6 ceramide either in its free form or encapsulated in a nanoliposome formulation (CNL) had a significant, albeit limited, effect on cell viability (Figure 16C). At 48 hours, L-erythro

and L-threo, together with both formulations of D-erythro-C6 ceramide significantly reduced viability in these cells (Figure 16D). It is important to note that the reduction in viability in both timepoints was more exacerbated by CNL but didn't surpass 25% of reduction in either timepoint compared with its control.

These results indicate that the effect of C6-ceramide is cell-specific, and also structure specific. In PCa, D-erythro-C6 ceramide is more impactful in reducing viability compared with dihydroceramide, but in the case of PC-3 cells this lipid did cause a reduction in cell viability.

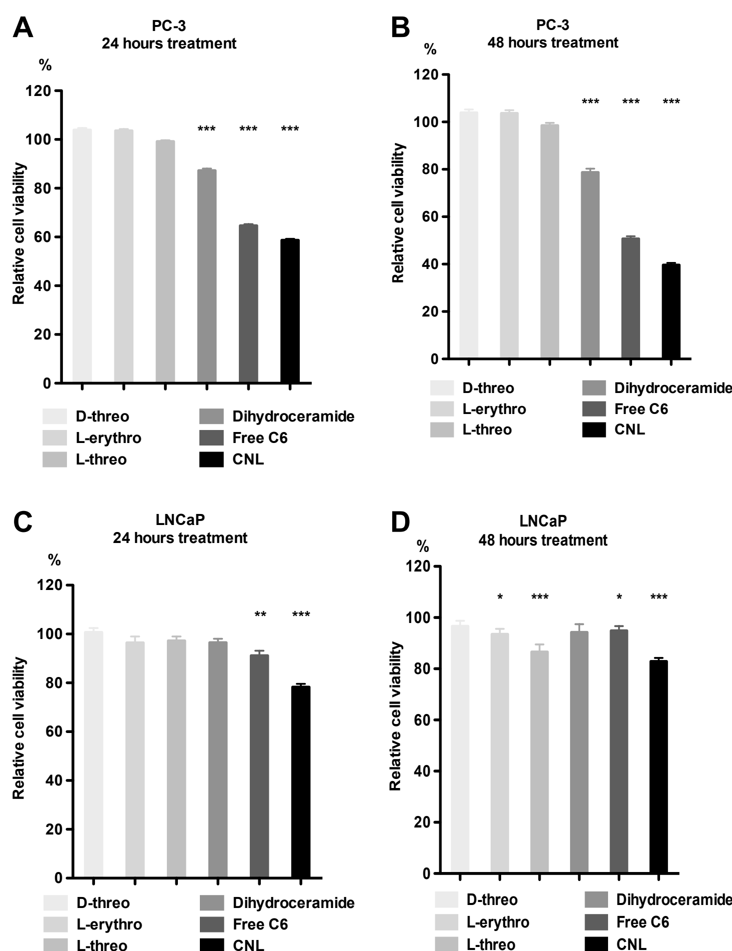


Figure 16 - D-erythro-ceramide is the most effective against PCa cells.

Viability of PC-3 (A,B) and LNCaP (C,D) after treatment with D-threo, L-erythro, L-threo, Dihydroceramide, free C6-ceramide, and CNL (10μM) compared to vehicle or ghost control (20μM) at 24 hours (A,C) and 48 hours (B,D). Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: *, **, and *** represent significance of p-values < 0.05, 0.01, and 0.001 respectively.

Metabolism of C6-ceramide in Prostate cancer depends on lipid structure

Next, we sought to determine the metabolism of the lipids (10μM) above described in LNCaP cells by analyzing the sphingolipid profile of these cells after 1 and 24 hours of treatment, via LC-MS/MS (Figure 17).

D-threo: this lipid was well incorporated by cells and at 1 hour its mass was the highest among all stereoisomers, and D-threo-C6 mass didn't decrease with time as at 24 hours the mass was higher. Interestingly, the levels of endogenous ceramides were not elevated after treatment with D-threo-C6 mass at either timepoint measured. This suggests that D-threo-C6 ceramide presents a challenge to the activity of ceramidases. Importantly, C6-glucosylceramide and C6-sphingomyelin were detected in cells at low levels, again indicating a lower metabolic rate for D-threo-C6 ceramide. This is accompanied by a small increase in the mass of sphingosine at 24 hours indicating lack of activity by ceramidases. Concerning other major classes of sphingolipids measured in this study: glucosylceramide, sphingomyelin, dihydrosphingosine, and S1P, no major increases were detected compared with the control. These data suggest that despite remaining in cells at high concentrations, this structure is not able to trigger the same signaling cascades or allow for the same metabolism as D-erythro-C6, which might be associated with its lack of effect on cell viability.

L-threo: unlike D-threo, L-threo-C6 ceramide was incorporated by LNCaP cells and metabolized between timepoints measured with a drop in about half of the mass. This metabolism resulted in increased levels of endogenous ceramides potentially by recycling of the sphingoid backbone, as there was no increase in the mass of the precursor lipids to ceramide. Again, and unlike D-threo-C6, after 24 hours of treatment the levels of sphingosine were about 10 times higher than in control cells indicating an ability of ceramidases to cleave the fatty acid chain length from the sphingoid backbone. The metabolism of L-threo-C6 ceramide seemed to preferentially lead to an increased mass of C6-sphingomyelin but not endogenous sphingomyelin species. There were also modest increases in both C6-glucosylceramide and endogenous glucosylceramide levels. These data suggest that sphingolipid metabolic enzymes have the ability to metabolize L-threo-C6 ceramide that is higher than D-threo counterpart. The increase in metabolism of this lipid might also be responsible for a significant decrease in cell viability caused by L-threo-C6 ceramide that was not observed with D-threo-C6 ceramide.

L-erythro: we were able to detect significant levels of L-erythro-C6 ceramide at 1 hour of treatment, and the lipid mass increased at 24 hours. This increase was also accompanied by an increase in the levels of sphingosine in the cells. We also were able to detect C6-glucosylceramide and C6-sphingomyelin although not at high concentrations. Despite the increase in the levels of sphingosine, we didn't detect increased levels of endogenous ceramides or glucosylceramides, and only observed a modest increase in sphingomyelin. These results could indicate a diminished ability of ceramide synthases to add fatty acid chain length to the newly generated sphingoid backbone from the reacylation of L-erythro-C6 ceramide. Interestingly, we detected a significant and robust increase in S1P after 24 hours of treatment suggesting that sphingosine kinase activity is not hindered. The accumulation of C6-ceramide and sphingosine can be associated with the modest effect that L-erythro-C6 caused in reducing cell viability.

D-erythro: At 1 hour, the natural structure of ceramide, D-erythro ceramide had the lowest levels of exogenous C6-ceramide of all 4 isomers. This was still the case when lipid mass was measured at 24 hours post-treatment, indicating an ability for cells to metabolize this lipid. At both timepoints, D-erythro-C6 ceramide caused an increase in the sphingosine mass, which suggests that ceramidases have a preferential and higher affinity for the natural occurring D-erythro structure. This increase was also observed for endogenous ceramides, glucosylceramide and S1P. Both C6-glucosylceramide and C6-sphingomyelin were detected at higher levels than other isomers, with the exception of the effect of L-threo on the levels of C6-sphingomyelin at 24 hours. The results for D-erythro-C6 dihydroceramide were similar to the ones observed for D-erythro-C6 ceramide with the exception of a slower increase in sphingosine. Levels of C6-dihydroceramide at the 1 hour timepoint were lower than C6-ceramides suggesting either a more hydrophobic nature of the lipid or more difficulty for cell membranes to take up this lipid in particular. These results combined suggest that the naturally occurring D-erythro ceramide is preferentially metabolized by cells likely due to the programmed action of sphingolipid metabolic enzymes optimized to this structure.

Ceramide NanoLiposome (CNL): this drug delivery vehicle encapsulates D-erythro-C6 ceramide and as previously mentioned has higher permeability and half-life in culture and *in vivo*. Thus, we decided to compare the sphingolipid profile after treatment with CNL to its Ghost control (nanoliposome without the drug payload) and try to understand potential trends comparatively to free C6-ceramide lipids. We

observed that the mass of C6-ceramide in CNL treated cells was higher than what we observed for free D-erythro-C6 ceramide. It is also important to note that this lipid mass was decreased between 1 and 24 hours showing that sphingolipid enzymes have the ability to then metabolize this lipid out of the liposome capsule. There was a concomitant increase in sphingosine levels as well as endogenous ceramide suggesting that both ceramidases and ceramide synthases can reacylate this lipid. Treatment with CNL led to the highest mass of S1P both at 1 and 24 hours, as well as increases in endogenous sphingomyelin and glucosylceramide. We were also able to detect both C6-glucosylceramide and C6-sphingomyelin. Therefore, it is possible to conclude that the biggest difference between free C6-ceramide and CNL when it comes to metabolism originates from different uptake by LNCaP cells.

These results point to the importance of the structure of C6-ceramide for its metabolism and signaling effects that still requires much more investigation. These data also suggest that certain sphingolipid metabolic enzymes might have a more promiscuous ability to play its part and not be restricted to the natural occurring D-erythro ceramides.

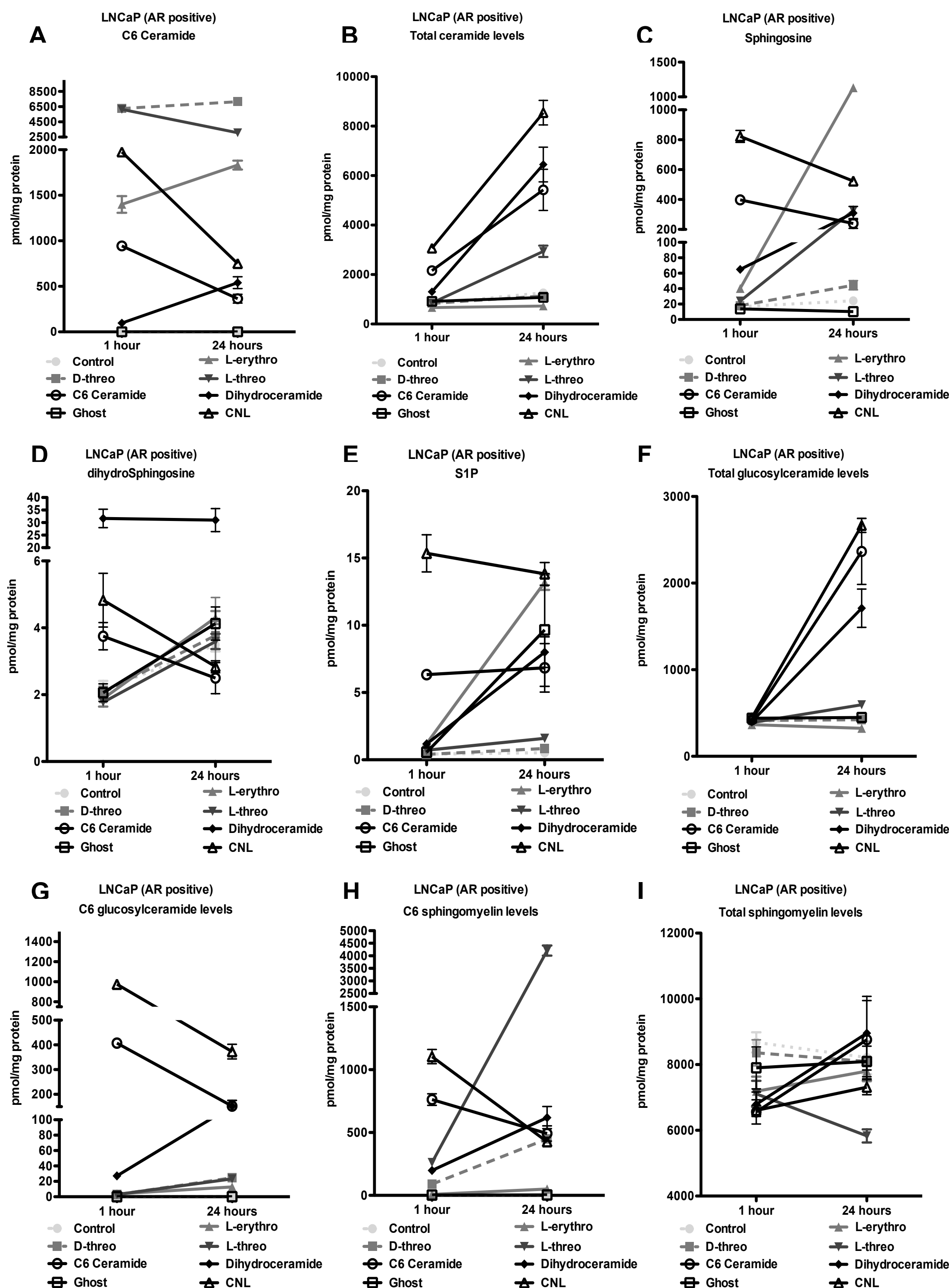


Figure 17 - Different ceramide analogs are metabolized differentially by LNCaP cells.

Mass of (A) C6-ceramide, (B) Total endogenous ceramides, (C) Sphingosine, (D) dihydrosphingosine, (E) S1P, (F), total endogenous glucosylceramide, (G) C6-glucosylceramide, (H) C6-sphingomyelin, (I) total endogenous sphingomyelin in LNCaP cells treated with vehicle, ghost, D-threo, L-erythro, L-threo, Dihydroceramide, free C6-ceramide and CNL (10μM) in LNCaP cells after 1h or 24h. Mean ± SEM (n=5) is represented.

Sphingolipid toxicity in Prostate cancer cells depends on metabolism

Despite the known effects of ceramide accumulation and induction of cell death, the impact of other sphingolipids and their metabolism is not yet fully understood. In fact, there are several key aspects of sphingolipids' structure and their role in cell death that are not fully understood. One of these key issues is the effect of different sphingoid bases that are part of sphingolipid structure. Different SPTLC/SPTSS complex assembly generates sphingoid backbones that range from d14 to d20, with d18 being the most common in human tissues. Defining the altered properties for sphingolipids with different backbones is crucial as human cells can naturally generate them. Another key point is related to the role and effect of atypical sphingolipids (1-deoxysphingolipids), which are generated by condensation of L-alanine or L-glycine and not L-serine. These lack the C1-hydroxyl group present in dihydrosphingosine and are not degraded by the same mechanisms as classical sphingolipids. Thus, we tested a range of sphingoid bases and atypical sphingolipids and their effect in PCa cells' viability.

We observed that treatment with sphingosine (d16:1, d18:1, and d20:1) did not significantly change the viability of PC-3 cells 48 hours after treatment (Figure 18). The same was not observed for dihydrosphingosine; while d20:0 dihydrosphingosine had no significant effect in cell viability, d18:0 dihydrosphingosine caused a significant reduction in PC-3 viability after 48 hours of treatment (Figure 18). In comparison, when 1-deoxysphingolipid counterparts were used the results were striking. Treatment with d18:1 1-deoxysphingosine resulted in a significant decrease of viability, while the classical d18:1 sphingosine had no effect (Figure 18). These deoxysphingolipids can't be metabolized by conventional metabolism, which results in their accumulation in cells leading to imbalance of homeostasis. Even more striking results were observed for d18:0 1-deoxydihydrosphingosine, where the reduction in cell viability reached 80% at 48 hours of treatment compared with 15% caused by d18:0 dihydrosphingosine (Figure 18). A significant reduction in viability was also observed with free C6-ceramide and 1-deoxy-C6 ceramide at similar levels (Figure 18).

These data suggest that in PC-3 cells the toxicity of dihydrosphingosine is higher than that of sphingosine. Moreover, a potential inability of cells to metabolize the atypical lipids results in enhanced effects on cell viability to higher levels in comparison with C6-ceramide. As previously noted, stopping the sphingolipid train system and accumulating specific classes of these lipids is detrimental for cells. Moreover, the data indicates that classical C6-ceramide is as detrimental as 1-deoxy-

C6 ceramide to cell viability, however not ruling out the fact that these two sphingolipids can be differentially metabolized or trafficked.

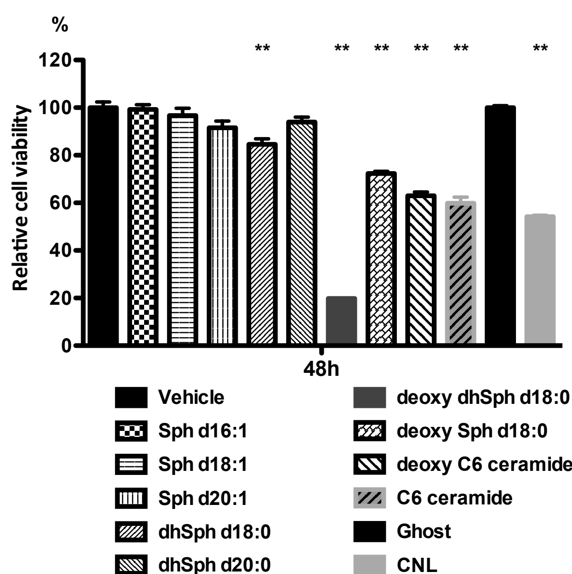


Figure 18 - Impaired metabolism of sphingolipids leads to reduced cell viability.
Viability of PC-3 treated with: vehicle, sphingosine d16:1, sphingosine d18:1, sphingosine d20:1, dihydrosphingosine d18:0, dihydrosphingosine d20:0, deoxy-dihydrosphingosine-d18:0, deoxy-sphingosine-d18:0, deoxy-C6-ceramide, free C6 ceramide, ghost, and CNL (10μM) for 48 hours. Mean ± SEM from three independent experiments is represented.

Removing C6-ceramide from cell culture impedes drug effect

As previously mentioned, in 1994 Hauser and colleagues observed morphological changes in cells that were treated with C6-ceramide [469]. The change in cell shape was described, "rounding up", and occurred after 10h of treatment while the cells were still viable [469]. No molecular mechanism to fully explain the phenomena was described. This change, however, was reversed if the drug was removed from the media up to 18h after treating [469]. As part of the studies in this Thesis, we treated PC-3 cells with 10μM of CNL for 6 hours and 12 hours before removing the drug from the media allowing for a total of 24 hours before measuring viability (Figure 19). As a control, we treated PC-3 cells with the same concentration for 24 hours without drug removal. We observed that all three time-points of drug exposure were able to reduce PC-3 cell viability, albeit with different effects (Figure 19). In fact, there was a time of exposure-dependent effect on the viability of PC-3 cells caused by CNL: the higher the amount of time exposed the more pronounced effect (Figure 19). There was a significant difference between all three different exposure times. These results suggest that the maximum effect of CNL in cells occurs

from continuous uptake of the drug and accumulation of sphingolipids in cells. It is possible that the addition of new complete media could boost cells metabolism and favor survival, however there were clear differences in morphology similar to the ones described by Hauser *et al.* [469]. Our data adds to this previous work that hasn't been followed since, to the best of our knowledge. In sum, the results in this section highlight that both C6-ceramide in the nanoliposome and its metabolism by cells is important for the anti-cancer effect of CNL.

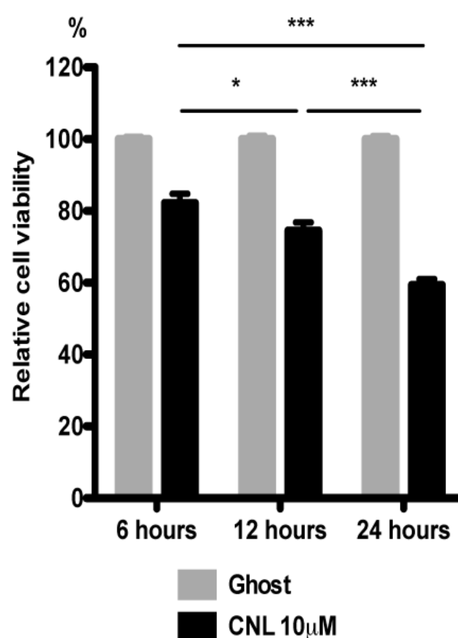


Figure 19 - Removing CNL from cell culture media hinders its efficacy. PC-3 cells treated with CNL (10μM) or Ghost for 6h, 12h followed by media removal and fresh media added to cells. Treatment for 24h with CNL and Ghost without media removal used as a reference point. Cell viability measured 24h after the start of the treatment. Mean ± SEM from three independent experiments is represented. *P* values obtained using non-parametric Mann–Whitney *U*-test: ***, * represent significance of p-values < 0.001 and <0.05 respectively.

In sum, this Chapter shows that different conformations of C6-ceramide lead to different outcomes when the readout is cell viability, highlighting that C6-ceramide itself is not the only culprit of the anti-cancer properties of CNL. A better appreciation of metabolic patterns shows that the effect of CNL is most likely a compounding effect between the metabolism of C6-ceramide to other sphingolipids combined with C6-ceramide itself. Moreover, accumulation of atypical sphingolipids (e.g. 1-deoxydihydrosphingosine) has been shown to lead to neurodegeneration [245]. In this Thesis, we show that exogenous short-chain versions of these sphingolipids are attractive candidates for a CNL 2.0 as these can only be metabolized by a small group of enzymes.

CHAPTER 7: DISCUSSION AND FUTURE PERSPECTIVES

1. DISCUSSION

Prostate cancer (PCa) is a disease that encompasses major sociological and financial concerns worldwide despite all the efforts put forth by researchers and clinicians. This disease is characterized by a high degree of molecular heterogeneity that has made it hard to stratify according to risk levels. The heterogeneity behind PCa has made it hard to develop robust biomarkers, as well as treatments that can help reduce the lethality of this disease among men. Thus, understanding the disease at its molecular core is important to allow better epidemiological and pharmacological tools to be developed to help patients in the clinic. The Department of Defense (DoD) through its Prostate Cancer Research Program (PCRP) has identified the following four major challenges designed to help bridge the gap between clinical care and knowledge of this disease:

- Improve the quality of life for survivors of prostate cancer;
- Develop new therapeutic modalities that improves the outcome of patients with lethal PCa;
- Reduce incidence of lethal PCa in populations at higher risk (e.g. African Americans, Veterans);
- Define the biology of lethal PCa to help reducing death rates.

These overarching goals are crucial for the direction of PCa research and helps set the blueprint for what remains to be done. The Thesis here presented is an attempt to address 2 of those goals: **to better define the biology of lethal PCa** and **develop new therapeutic modalities** that can bring some better results to patients.

In this work, we found that Ceramide NanoLiposomes (CNL) is a promising novel therapeutic for highly aggressive neuroendocrine models of PCa. Currently these cases have no viable treatment options available and ultimately result in death from PCa. This sphingolipid-based drug also enhanced the efficacy of FDA-approved anti-androgens in models that represent less aggressive PCa disease. Moreover, we also report that Androgen receptor (AR) signaling blockade leads to altered sphingolipid metabolism by induction of *de novo* synthesis of sphingolipids via novel tracing technique. We uncover *SPTSSB*, a subunit in the *de novo* synthesis pathway, as critical for maximum efficacy of CNL in AR-negative models. Additionally, *SPTSSB*

is negatively regulated by AR-signaling, which shows that AR is able to regulate sphingolipid metabolism at the transcriptional level. The importance of sphingolipids in the context of normal as well as disease development is striking, but more studies need to be performed to bridge the gap between theoretical and effective pathobiological roles.

Androgen receptor regulates expression of sphingolipid enzymes

Androgen receptor (AR) is a transcription factor that regulates several key pathways contributing to prostate cells normal function. Importantly, recent evidence suggests that AR can increase and decrease expression of its targets depending on the ratio between co-activators and co-repressors [138,142]. This gives AR a higher level of flexibility when it comes to regulating different pathways, depending on cell context. Recent data from the Knudsen laboratory showing that AR triggers different targets and pathways depending on cell cycle highlights the versatility of AR [128]. Hormones and AR play a critical role in the onset and progression of PCa, one of the key aspects of this regulation is through metabolism adaptation [142].

As previously stated, many enzymes in the lipid synthesis pathway have been shown to be upregulated by AR in PCa, where lipids play an important role in sustaining continued growth [91]. Nonetheless, the connection between AR and lipid synthesis is still not fully understood, with different lipid classes having been scarcely studied. In this Thesis, we focus our studies on a better understanding of sphingolipids in the context of PCa. Here, we report that stimulation of AR with synthetic androgens in PCa cell lines leads to altered expression of several sphingolipid enzymes. Some studies in the past have shown dysregulation of sphingolipids in PCa, but these have been mostly concerned with levels of sphingolipid species in *in vitro* models [401-403]. Thus, there aren’t a lot of studies directly tying sphingolipid metabolic enzymes to AR signaling.

The only established connection between AR and a sphingolipid enzyme was recently published by the Knudsen laboratory [128]. DEGS1 is an enzyme responsible for the conversion from dihydroceramide to ceramides, and in this study was associated with increased migration and worse prognosis [128]. However, the authors of this study didn’t measure the impact of this AR-related upregulation of DEGS1 on the dysfunction of global sphingolipid metabolites in PCa. The increased expression of an enzyme responsible for generating more ceramides is counterintuitive to the principle that more ceramide species equates to higher cell death levels. This phenomenon is not novel and has been reported in breast cancer where tumor

samples had higher levels of ceramide when compared with normal cells [473]. In this Thesis, we have confirmed that stimulation of AR signaling leads to increased expression of DEGS1 in PCa cells. We suggest two different hypotheses for why increase in ceramide species might be beneficial for cancer cells: (1) sustained proliferation and growth of cancer cells demands elevated synthesis of lipid species for cell structure and cell signaling – two roles that ceramides are able to perform; (2) upregulation of ceramide synthesizing enzymes is not enough to lead to accumulation of ceramides in the cells due to conversion to other sphingolipid species that contribute to cell survival. The data presented in this Thesis suggests that the second suggested hypothesis is more likely to play a bigger role, but more biophysical studies and organelle quantification are necessary to address specific increases in ceramides in different cellular compartments. We also show that AR stimulation blocks generation of ceramides via the *de novo* pathway, both by decreasing expression of enzymes involved in this pathway as well as sphingolipid mass generated, suggesting that this transcription factor's regulation of sphingolipid metabolism is very intricate. Our study also highlights that transcription factors such as AR can decrease expression of target genes. In this Thesis, we fall short of showing specific binding of AR to the promoters of these sphingolipid enzymes by Chromatin Immunoprecipitation sequencing (ChIP-seq), which we suggest as a logical next step. Nonetheless, our studies are the first to fully explore AR signaling effect on a wide array of sphingolipid metabolic enzymes something that has been lacking in the sphingolipid and PCa fields. It is our assessment that these data will guide further studies that will allow for a better understanding of the role played by sphingolipids in PCa. We have only begun to scratch the surface and the constant advancement of mass spectrometry allows increased sensitivity and specificity of metabolites' detection and quantification will contribute to a better understanding of diseases, such as PCa.

Ceramide-based therapeutics: valuable options for Prostate cancer

Due to the dependence of prostate cells, both normal and cancer, on hormones such as androgens it is not surprising that targeting hormonal signaling is the main non-surgical treatment option for PCa. There are two main strategies deployed to attack this dependence: block the synthesis of male hormones, or block AR the main player downstream of those hormones. However, these two strategies end up in the same narrow path for patients: resistance and development of castration resistant PCa (CRPCa), the most aggressive form of the disease. Thus, one

of the main incentives in PCa research is to find novel therapeutics or to repurpose older drugs for better treating PCa patients.

In this Thesis, we sought to determine the potential of Ceramide NanoLiposomes (CNL) as a treatment option for PCa. Here, we report that this sphingolipid-based therapeutic, has the potential to be an effective treatment against neuroendocrine-CRPCa. Most importantly, when used in combination with FDA-approved anti-androgens, CNLs may provide benefit for virtually all PCa patients. One of the limitations of these studies is the lack of *in vivo* data, thus, the next step is to validate the findings in *in vivo* models of PCa. Nonetheless, these preclinical findings are important especially because CNL just finished a Phase I clinical trial (NCT02834611) with minimal toxicity and with 50% of stage 4 solid tumor cancer patients showing stable disease. The low toxicity results are exciting because it might allow clinicians to use CNL combined with conventional chemotherapeutics already administered to patients to maximize the effect of both drugs.

The rationale for the development of ceramide-based drugs started in the mid-90s where researchers found that radiotherapy and chemotherapy used in cancer patients lead to increased ceramide generation [348,354]. The increase in ceramide levels associates with cell death in tumor cells, and ceramide has been described as part of several different cell death pathways. Most of the initial data on cell death connected ceramide with apoptosis [198]. In this Thesis however, we didn't observe increases in apoptotic markers upon C6-ceramide treatment of PCa cells. It is likely that the cell death pathway of choice upon ceramide treatment is dependent on the cell type being tested. In agreement with this hypothesis, several other studies have shown that increases in ceramide induce other forms of cell death and not just apoptosis [379,385,386]. One of the limitations of our study is that, despite showing increased cell death from C6-ceramide in PCa cells, we didn't reach a conclusion on the specific mechanism behind this cell death. From the preliminary data we generated, we speculate that more than one cell death pathway is activated at the same time from this treatment. It is noteworthy that the effect we observed is specific to cancer cells and that normal prostate cells are mostly protected, suggesting the low toxicity of C6-ceramide.

The Reader might wonder what is the need or advantage of delivering C6-ceramide through a nanoparticle. Ceramide is a very complex and hydrophobic lipid, which makes it hard to deliver this drug. Endogenous ceramides in most tissues range in the size of their fatty acid tail from 14-26 carbons [202] The increase in the size of the fatty acid tail is accompanied by a decrease in solubility of this lipid [388]. Thus,

this led to the synthesis of membrane-permeable exogenous ceramides: C2-, C6, and C8 ceramide, that circumvent the low solubility allowing for its delivery to cells, animals and people. A cell can recognize and metabolize these exogenous ceramides as if they were its own endogenous counterparts, giving researchers and clinicians a substitute to study and manipulate sphingolipid metabolism [391,392]. Even with increased solubility provided by these exogenous short-chain ceramides, their delivery is still impaired. Therefore, in the early 2000s the Kester lab developed a C6-ceramide NanoLiposome (CNL) [393]. These nanoparticles have enhanced solubility allowing for ceramide to be administered in an aqueous buffer, higher half-life *in vivo* and less toxicity than administration of free-ceramide [396]. The CNL were our delivery vehicle of choice for this work.

In the past, laboratories have shown C2- and C6-ceramide treatments to be effective against different PCa cell lines independent of their AR status [407,409,410]. In our studies, we were not able to validate these results, and instead found for the first time a dichotomy in the response to ceramide treatment based on AR status. It is important to note that this difference could be attributed to the nanoliposomal C6-ceramide versus non-liposomal C6-ceramide formulation. However, we conducted side-by-side studies with both formulations and found that to not be the case, reporting in this Thesis that non-liposomal or nanoliposomal had the same effect in PCa cells. These findings show the importance of conducting foundational experiments, even if those have already been reported, in order to build a stronger and elegant project, instead of relying in literature without further validation.

Ceramide species vs sphingolipid metabolism, who is the culprit?

Despite several scientific publications reporting that ceramides have anti-tumor properties, it is still not fully understood what the mechanisms behind the cell death effect of this sphingolipid are. This is one of the gaps in the field that this Thesis attempted to answer, but much more work remains to be done. One of the main advancements provided by this work is the "tracking" of sphingolipid metabolism after CNL treatment. Most studies in the literature have not provided a picture of sphingolipid metabolism in cells across time, essentially opting to select a unique timepoint of choice. In this work, we were able to determine the sphingolipid metabolism of PCa cells upon addition of C6 ceramide across time. It was this experiment that pointed us towards a more careful study of the *de novo* synthesis of sphingolipids. The importance of providing a more complete picture of the metabolism upon ceramide treatments is important, as several enzymes that convert

ceramides into other sphingolipid species have been shown to be up-regulated in drug resistance models and could result in evasion to ceramide treatments [361,363-366,368,369]. Thus, the full sphingolipidome can point out key differences in degrees of sensitivity in cells.

Another key phenomenon is chirality in sphingolipids with altered three-dimensional orientation. Many studies have attempted to determine the importance of this three-dimensional orientation for the metabolism of sphingolipids explained by differential access to the lipid structure by the sphingolipid metabolic enzymes. Most of the studies that looked at the different effect of different stereoisomers of ceramide were performed more than two decades ago, which raises the need to revisit this literature and findings with the novel techniques that have been made available since. In this Thesis, we show that in PCa cells different stereoisomers of C6-ceramide have different effects in cell viability, as well as in sphingolipid metabolism. Our findings open up new avenues for studying specific enzymes that might metabolize these isomers differently according to their conformation. Given these data, we are thus inclined to postulate that the effect of C6-ceramide is not unique to the lipid itself, but more likely to its metabolism and the compounding overload of other sphingolipid species it causes. Another piece of data that we generated concerned removal of CNL from the media of cells at different timepoints. We observed that by removing the drug and not allowing it to stay in culture for more than 12 hours, the effect on cell viability was drastically impaired. In fact, a study in 1994 by Hauser and colleagues reported that cells treated with C6-ceramide rounded up after 10h of treatment but were still viable [469]. This change in morphology was reversed by removing C6-ceramide from the media up to 18 hours after first adding the treatment [469]. These results agree with our findings in PCa cells and again suggest that the continuous metabolism of C6-ceramide is the most likely culprit for the effect in cancer cells. However, despite our validation of these findings much is left to be known, including to profile the sphingolipidome of cells where C6-ceramide is added and subsequently removed at different timepoints. Our laboratory intends to perform these experiments in PCa cells, albeit those were not performed in time for this Thesis. The results from this experiment will help to zoom in on the metabolism hypothesis and again might suggest specifically what pathways are key to the effect of ceramide, or if the overall accumulation of lipid mass is the responsible for the cell death.

Cell response to CNL treatment needs to be fully characterized

One of the main takeaways for this body of work is that sphingolipids deserve more credit and more attention in cancer biology and therapeutics. The same applies within the sphingolipid field to the only pathway known for the synthesis of these lipids *de novo*. *De novo* synthesis of sphingolipids occurs via condensation of L-serine with an acyl-CoA substrate, and is mediated by the serine palmitoyltransferase complex [226]. This complex consists of two different enzymatic subunits (SPTLC 1-3) and a cofactor (SPTSSA-B). When this complex assemblies it leads to generation of dihydrosphingosine, a precursor to ceramide, a bioactive lipid that influences cell signaling and plays a role in neurodegeneration [220]. The ORMDL family (ORMDL1-3) have been described as negative regulators of this complex, a way for cells to keep the synthesis of sphingolipids in check [202,211,460,461]. In 2012, Siow and Wattenberg published a report showing that addition of exogenous ceramides to cells would inhibit *de novo* synthesis of sphingolipids via ORMDL [224]. This has been an established and dogmatic perspective of this understudied pathway. However, in our studies we show that, after being treated with exogenous ceramides, AR-negative PCa cells up-regulate *de novo* synthesis of sphingolipids leading to accumulation of endogenous ceramides. To the best of our knowledge, this is the first report showing this phenomenon. Additionally, we report that blocking AR-signaling with Abiraterone acetate led to increased expression of enzymes involved in the *de novo* pathway and activity. It is important to note that expression of ORMDLs was elevated upon CNL treatment in AR-negative cells but this didn't stop the cells from elevating the synthesis of dihydrosphingosine. Thus, our data highlights the need for more investigation into the response to exogenous ceramides and the role played by the *de novo* synthesis pathway in this response. In the technical aspect, our work established a new method that can be used by researchers to determine the levels of *de novo* synthesis of sphingolipids without the need for radioactive compounds like the ones used in the past. We have developed a novel methodology that solely requires cell culture media without serine, where stable isotope labeled serine is added for tracing. Any novel generated sphingolipids can then be traced via mass spectrometry across time without the burden of radiation playing an effect. This method is also being optimized to better distinguish between *de novo* synthesis of sphingolipids from re-acylation. This can be accomplished by treating cells with a labelled C6-ceramide where its sphingosine chain can be traced by mass spectrometry. It is our goal to establish this technique as the gold standard for the study of sphingolipid synthesis in the laboratory setting.

Classically, the trigger of cell death in response to CNL treatment has been proposed to fit the rheostat model where the ratio between sphingosine-1-phosphate (S1P) and ceramides determines cellular fate: higher S1P equals more survival, while higher ceramides more cell death [474]. In 2018, our laboratory proposed a novel way to think about sphingolipid metabolism and cell death that encompasses more sphingolipid pathways and enzymes than the rheostat [327]. We postulated that each cell has a pool of ceramide and several escape routes to avoid cell death by ceramide accumulation [327]. In AR-negative PCa cells, we suggest that the administration of exogenous ceramides overloads the ceramide pool by increasing re-acylation of the exogenous ceramide, and increasing *de novo* lipid synthesis leading to cell death. This phenomenon was not observed in AR-positive cells, where cells displayed a similar behavior to the data previously published by decreasing *de novo* synthesis in response to CNL treatment. We postulate that AR-negative cells have unchecked *de novo* synthesis of sphingolipids that can then be further increased by ceramide-based therapeutics leading to cell death. We observed that the increase in dihydrosphingosine after CNL treatment is accompanied by increased transcription of multiple enzymes involved in the *de novo* synthesis of sphingolipids. These data point out a gap in the sphingolipid literature: how do cells recognize ceramide/sphingolipid content? What are the molecular players responsible for this recognition? What happens post-recognition of ceramide/sphingolipid pool? Taking into consideration our data in both AR-negative and positive cells, it is possible that by answering these questions, researchers will be able to bypass a potential ceramide sensing pathway and pharmacologically explore that mechanism to induce cell death in cancer cells by ceramide accumulation. At this moment in time, we are not fully aware of why AR-negative cells up-regulate *de novo* synthesis of sphingolipids even in the presence of exogenous ceramides in the cells. We hypothesize that (1) mutations in the enzymes responsible for the SPTLC complex assembly or ORMDLs would maximize the complex formation or stop ORMDLs from blocking its assembly; (2) biophysical properties of the membranes in AR-negative cells promotes the assembly of SPTLC complex evading ORMDL regulation; or (3) that other lipid sensors that have not been described yet might be involved in the recognition of ceramides. One of the major limitations of this work is that we didn’t reach a conclusion on which of these alternatives explains why AR-negative cells evade the pattern of response to CNL. Moreover, with the advancement of mass spectrometry equipment and with the emergence of new protocols to study *de novo* synthesis of sphingolipids like the one we developed, it is possible that other cell types will be

found to evade the current dogma. We postulate that cells that are very responsive to CNL, such as AR-negative PCa, will up-regulate *de novo* synthesis of sphingolipids inundating themselves with sphingolipids, which will ultimately result in cell death.

SPTSSB: a small molecule for man, a giant co-factor for sphingolipids

In our work, we ended up zooming in on one particular molecule involved in the synthesis of sphingolipids. Serine palmitoyltransferase small subunit B (*SPTSSB*) is a 9kDa protein identified in 2005 [240]. *SPTSSB* was discovered in a mouse model that can't produce testicular testosterone in the mouse's prostate [240,241]. This is the first, and only, report showing a connection between expression of SPTSSB and AR signaling. The authors further showed that treating these mice with testosterone reduced expression of SPTSSB in the prostate [240]. Therefore, it indicates a potential connection between SPTSSB expression and the development of hormone refractory prostate cancer. Nonetheless, the relevance of SPTSSB in this process is still unknown and two hypotheses might be suggested: as a biomarker for this progression from hormone dependence to independence, or an active player by regulating signaling pathways. Interestingly, the authors of this study showed that SPTSSB was more expressed in the brain of normal mice and that this expression was decreased in *hpg* (hypogonadal) mice [240]. This suggests an androgen regulation that is tissue-dependent and could be explained by the presence of co-factors that differently impact transcription. Moreover, activating mutations in SPTSSB have been described as leading to neurodegenerative phenotype [220]. The connection between levels of SPTSSB in the prostate and the brain and its regulation by AR pointed us towards a relationship between neuroendocrine differentiation after castration.

SPTSSB is a crucial co-factor that plays a role in the assembly of the SPTLC complex. The presence of SPTSSB in the complex maximizes activity and synthesis of dihydrosphingosine that then can be progressively converted to ceramides [211]. Our data support the importance of this small subunit as we report significant differences between AR-positive and AR-negative cells in terms of response to exogenous ceramides and regulation of *de novo* synthesis. SPTSSB is overexpressed in AR-negative cells compared with AR-positive, a stark difference that is then amplified in the presence of CNL (Figure 20). This is one of the more interesting questions arising from our work: how does unchecked *de novo* sphingolipid synthesis potentially benefit aggressive PCa cell survival? Speculations around ceramide localization, signaling cascades, and metabolism to less toxic lipid species have all been

considered. One potential explanation to this question is the increased synthesis of pro-mitogenic gangliosides in response to ceramide, an observation previously documented [403]. In fact, our studies do not sufficiently answer this intriguing observation. Despite uncertainty around the oncological benefits of increased/unregulated *de novo* synthesis in AR-negative PCa, our studies identify this pathway as an avenue to be exploited by ceramide-centric therapeutics.

Translating the results obtained by Singh *et al.* (downregulation of SPTSSB in the prostate by exposure to testosterone) to the human prostate, we show that blocking androgen-signaling led to increased expression of *SPTSSB* in AR-positive cells while AR-stimulation reduced its expression. Additionally, *in vitro* and *in silico* analysis of *SPTSSB* expression suggest that this enzyme is up-regulated in more aggressive ADT-treated PCa tumors and that *SPTSSB* levels can potentially serve as a biomarker for clinical outcome. Importantly, in a study published by Li and colleagues, castration-resistant prostate cancer xenografts of LNCaP cells significantly upregulated SPTSSB, both in response to castration as well as in the context of resistance to Enzalutamide [465]. These findings identify a possible dysregulation of the *de novo* pathway under AR-negative conditions in PCa and an “Achilles’ heel” that could be exploited by ceramide-centric therapeutics. Of particular interest, our studies document that CNL can target this pathway by driving SPTSSB-dependent generation of dihydrosphingosine that can lead to further accumulation of pro-death ceramide species. We hypothesize that this arises from remodeling of sphingolipid profile, where cells in the presence of an exogenous sphingolipid instead of shutting down the production of new sphingolipids instead elevate its production resulting in imbalanced homeostasis. Our study also provides insight into the expression of *SPTSSB* in datasets of PCa patients. Strikingly, in a neuroendocrine dataset, *SPTSSB* was significantly up-regulated in almost a quarter of the cases. This supports our hypothesis that SPTSSB expression increases in cells that resist AR signaling inhibition. More molecular studies are necessary to determine at what point in the PCa progression SPTSSB activity increases and if this enzyme can be used as a predictive biomarker for patients who can benefit from ceramide-based therapeutics. Importantly, it is necessary to expand the datasets currently made available by scientific consortiums to include lipidomics in order to obtain a more complete picture of tumor pathogenesis. In 2017, studies on two cohorts of PCa patients showed that of 19 plasma lipid signatures important for prognostic evaluation, 12 were sphingolipid species (gangliosides, ceramides and

sphingomyelins) [413]. This groundbreaking study identifies sphingolipid changes as biomarkers for prognosis but lacks depth in number of sphingolipid species analyzed.

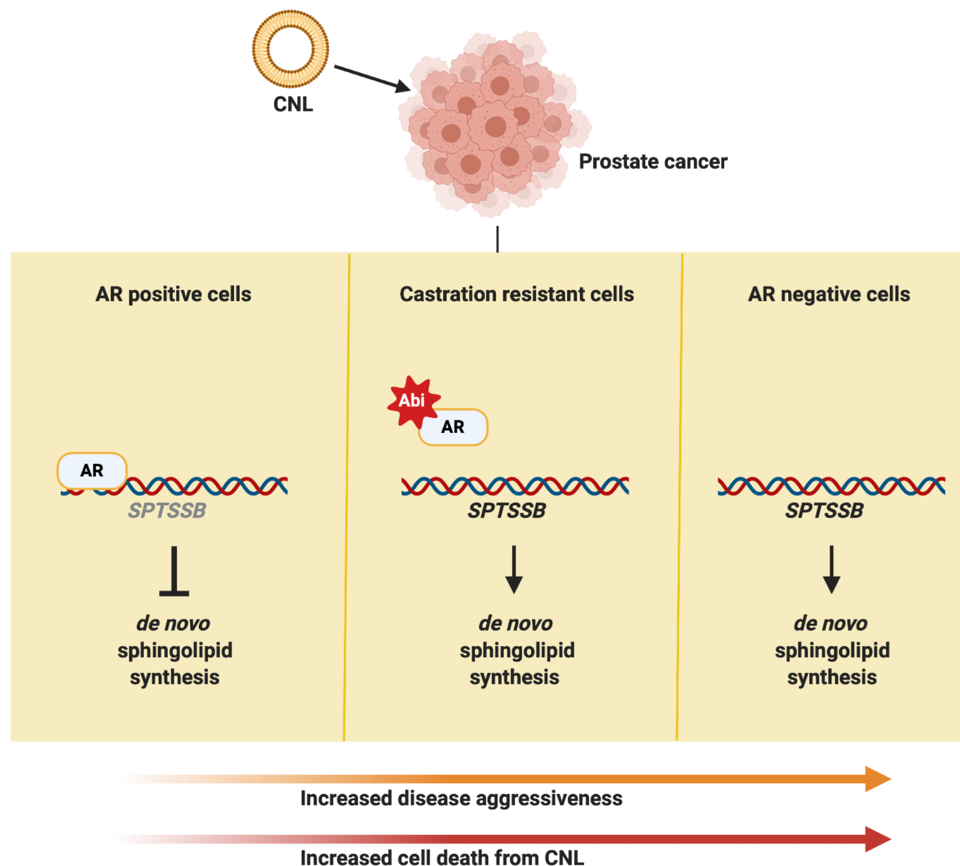


Figure 20 – *SPTSSB* is an Achilles' heel in PCa: while negatively regulated by AR signaling in less aggressive tumors, its expression increases with the progression of the disease and renders susceptibility to CNL in more aggressive PCa cells.

Our results point to a potential role of *de novo* sphingolipid synthesis in the progression of the disease and suggest evaluating sphingolipids involved in this process would improve our understanding of PCa and its development. It is our understanding that a whole lot is left to be known and characterize regarding most of the sphingolipid metabolic enzymes and the cellular processes these enzymes participate in. Our studies further advance the knowledge of SPTSSB, but more research is necessary to determine SPTSSB role in normal cells and disease.

2. LIMITATIONS OF THESE STUDIES AND CHALLENGES REMAINING

In this Thesis, we have showed that CNL is a promising therapeutic option for all PCa patients. We postulate that the combination of CNL with already FDA-approved anti-androgens will have enhanced effect against prostate tumors. These results were validated in several *in vitro* models of PCa. Notwithstanding, this important finding needs to be validated in more translationally-relevant *in vivo* models of the disease. The use of *in vivo* models of PCa to test the combination treatment can vary from xenografts, patient-derived xenografts, genetically engineered mouse models, or orthotopic models. Moreover, the use of anti-androgens and hormonal therapies to develop castration resistant tumors *in vivo,* following similar strategies employed by Zou *et al.* can determine the impact of CNL in cells that have escaped the effect of these already-approved treatments for PCa [25].

In addition, the AR-dependent negative regulation of sphingolipid *de novo* synthesis modulated by SPTSSB is a novel molecular pathway that can be explored for improved PCa therapeutics. One of the most important pieces of information that is lacking from this work is how this regulation occurs. It is well established that AR regulates transcription of downstream targets, but its negative impact in transcription is still not fully characterized. Despite the *in silico* prediction of binding sites of AR in the promoter region of SPTSSB, the biological meaning of these AREs have not been validated. In this Thesis, we did not characterize the importance of AR binding to the promoter of SPTSSB and regulate its expression. Several key experiments can be performed in order to determine this regulation: (1) ChIP-seq would identify the binding of AR to the SPTSSB promoter; (2) a luciferase assay with a plasmid encoding the SPTSSB promoter region followed by activation of AR to measure the impact on SPTSSB expression; (3) mutation of the AREs predicted to exist in the SPTSSB promoter and determine with luciferase assay if the impact of stimulating AR activity still occurs; (4) using a plasmid encoding the promoter of SPTSSB, perform a pull-down of DNA and extract proteins to determine which proteins were binding to the promoter of SPTSSB. To determine the mechanisms behind the regulation of SPTSSB expression in PCa is essential to further our understanding of sphingolipid metabolism in the context of normal prostate, PCa, and CRPCa.

Importantly, it is still not fully clear how cells respond to exogenous and endogenous ceramides to regulate this putative negative feedback loop. Despite showing the importance of SPTSSB in the response to CNL in AR-negative cells, this

work falls short of proving how CNL promotes or enhances the complex formation of SPTLC-SPTSS in these cells. One approach that can help understand this phenomenon is to mutate the specific enzymes and co-factors involved in *de* novo synthesis of sphingolipids. This would stop the assembly of specific complexes and allow us to understand how these protein interactions impact the response to CNL. However, further research is necessary to define how human cells sense exogenous ceramides.

There are other questions that when addressed will help shape our understanding of the role sphingolipids play in PCa, and how the interaction with AR and steroid synthesis impact the pathophysiology of this disease. The main questions that I believe need to be addressed but fall out of the scope of this Thesis are the following:

- What are the molecular processes that regulate and potentiate the metabolic switch that occurs in PCa?
- How to target the metabolic switch in PCa to starve the cancer cells?
- What are the main molecular changes responsible for the castration-resistant and neuroendocrine phenotypes?
- What is the specific role of sphingolipid metabolic enzymes depending on their cellular localization and tissue?
- How does AR regulate sphingolipid metabolism?
- What is the role of sphingolipids and their metabolic enzymes in the development of castration and neuroendocrine phenotype after AR inhibition?
- Are the synthesis of male steroids and sphingolipids connected? Can this intersection be used to further PCa therapeutics?

3. Future Perspectives

Just like most scientific studies, the work here sheds some light on the field of sphingolipids in prostate cancer but raises more questions that require further investigation. One of the most important of those questions is the translational capability of Ceramide NanoLiposomes (CNL) in prostate cancer *in vivo* models. Factors like the low toxicity observed in tumor patients during a Phase 1 clinical trial and the synergistic effect when combined with anti-androgens makes CNL into a promising therapeutic agent for PCa patients. However, these data need to be shown in more complex and heterogeneous models so these combination treatments can move from the cell culture dish to help patients in the clinic. Specifically, the use of *in vivo* genetic models as well as xenografts that reflect the heterogeneity of PCa (AR-negative, AR-positive naïve, AR-positive resistant to inhibition) is crucial for a better understanding of the translational potential of CNL. It is important to note that these liposomes can also be targeted with prostate-specific antibodies (such as prostate-specific membrane antigen, PSMA, and PSA) and to facilitate the delivery of CNL specifically to the prostate increasing the likelihood of incorporation in the tumors and better treatment efficacy. All these approaches should be considered moving forward with these studies.

The sphingolipid in cancer field has mostly revolved around the opposite roles played by ceramide and sphingosine-1-phosphate; nowadays it has become clear that the picture is way more complex. It is crucial to have a better understanding, and this starts with a more complete characterization of the enzymes involved in the sphingolipid pathways. With the advent of CRISPR technology, it is important that the sphingolipid field shifts to a better comprehension of what the enzymes that generate the metabolites actually do. Too little is known about the vast majority of these enzymes, and while their role in the sphingolipid machinery might be well established, compensatory mechanisms and other biological roles have been described, but information remains scarce. Another important limitation in the field is the lack of specific and selective antibodies that recognize the enzymes that compose the sphingolipid pathway. The ability to measure the actual products of these enzymes compensates for this absence but leaves cellular architecture and protein localization behind. More studies are absolutely necessary to quantify sphingolipids in the different cellular compartments, especially knowing that these lipids can interact with proteins and other lipids triggering a multitude of biological pathways. One example that fits this problem is SPTSSB the co-factor we studied in

this Thesis. There is only one commercially available antibody for SPTSSB and antibody validation immunofluorescence show SPTSSB in nuclear speckles, which are areas of regulation of transcription in cells. This is surprising given that all reports so far only show *de novo* synthesis of sphingolipids occurring in the endoplasmic reticulum. Thus, it is possible that sphingolipid machinery is present in more organelles than the ones currently established. Moreover, it is possible that SPTSSB has different roles in cells that have not been described in the literature yet and that are independent of sphingolipid synthesis, yet again differentiating SPTSSB from the other small subunit, SPTSSA. More molecular studies with knockout cells and mutations in the protein are necessary to address this important question.

An important finding that is important to retain from this Thesis is that sphingolipids are underappreciated but play a key role in diseases such as PCa. For decades the focus has centered in genomic mutations and protein kinases; however, it is clear that lipid and non-lipid cellular metabolites are phenotype-drivers and thus require more attention. Lipid and non-lipid metabolites are involved in virtually every cell pathway in mammalian cells, and technological advances will only uncover more roles for these under-appreciated small-molecules. One of the "new frontiers" that I am personally most intrigued about is the connection between epigenetic regulation and lipid metabolism and metabolites. These two dynamic regulatory machineries (epigenetics and lipid metabolomics) in cells are inter-dependent as they regulate each other contributing to normal homeostasis and to disease when that homeostasis is disturbed. Taking into consideration that neither lipids and non-lipid metabolites nor epigenetic changes are definite like DNA mutations, these are attractive drug targets that can benefit patients when this relationship is further established. This is, to me, the most exciting topic in cancer biology and cancer therapeutics nowadays, especially with all the new techniques available for measuring epigenetic marks as well as quantification of lipids and metabolites by mass spectrometry.

REFERENCES

1 Kumar, A. *et al.* Substantial interindividual and limited intraindividual genomic diversity among tumors from men with metastatic prostate cancer. *Nature medicine* **22**, 369-378, doi:10.1038/nm.4053 (2016).

2 Beltran, H. *et al.* Divergent clonal evolution of castration-resistant neuroendocrine prostate cancer. *Nature medicine* **22**, 298-305, doi:10.1038/nm.4045 (2016).

3 DeVita, V. T., Lawrence, T. S. & Rosenberg, S. A. *Cancer: Principles & Practice of Oncology*. (Wolters Kluwer/Lippincott Williams & Wilkins, 2008).

4 Lee, C. H., Akin-Olugbade, O. & Kirschenbaum, A. Overview of prostate anatomy, histology, and pathology. *Endocrinol Metab Clin North Am* **40**, 565-575, viii-ix, doi:10.1016/j.ecl.2011.05.012 (2011).

5 Shen, M. M. & Abate-Shen, C. Molecular genetics of prostate cancer: new prospects for old challenges. *Genes & development* **24**, 1967-2000, doi:10.1101/gad.1965810 (2010).

6 Hricak, H. & Scardino, P. T. *Prostate cancer*. (Cambridge University Press, 2009).

7 McNeal, J. E. Regional morphology and pathology of the prostate. *Am J Clin Pathol* **49**, 347-357, doi:10.1093/ajcp/49.3.347 (1968).

8 Selman, S. H. The McNeal prostate: a review. *Urology* **78**, 1224-1228, doi:10.1016/j.urology.2011.07.1395 (2011).

9 McNeal, J. E. Normal histology of the prostate. *The American journal of surgical pathology* **12**, 619-633, doi:10.1097/00000478-198808000-00003 (1988).

10 Cohen, R. J. *et al.* Central zone carcinoma of the prostate gland: a distinct tumor type with poor prognostic features. *The Journal of urology* **179**, 1762-1767; discussion 1767, doi:10.1016/j.juro.2008.01.017 (2008).

11 Davies, A. H., Beltran, H. & Zoubeidi, A. Cellular plasticity and the neuroendocrine phenotype in prostate cancer. *Nature reviews. Urology* **15**, 271-286, doi:10.1038/nrurol.2018.22 (2018).

12 Alcaraz, A., Hammerer, P., Tubaro, A., Schroder, F. H. & Castro, R. Is there evidence of a relationship between benign prostatic hyperplasia and prostate cancer? Findings of a literature review. *Eur Urol* **55**, 864-873, doi:10.1016/j.eururo.2008.11.011 (2009).

13 Young, R. H. & Eble, J. N. The history of urologic pathology: an overview. *Histopathology* **74**, 184-212, doi:10.1111/his.13753 (2019).

14 Schenk, J. M. *et al.* Serum adiponectin, C-peptide and leptin and risk of symptomatic benign prostatic hyperplasia: results from the Prostate Cancer Prevention Trial. *The Prostate* **69**, 1303-1311, doi:10.1002/pros.20974 (2009).

15 Briganti, A. *et al.* Benign Prostatic Hyperplasia and Its Aetiologies. *European Urology Supplements* **8**, 865-871 (2009).

16 Vasto, S., Italiano, E., Miceli, V. & Carruba, G. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 115-122 (Springer London, 2013).

17 De Marzo, A. M., Haffner, M. C., Lotan, T. L., Yegnasubramanian, S. & Nelson, W. G. Premalignancy in Prostate Cancer: Rethinking What we Know. *Cancer Prev Res (Phila)* **9**, 648-656, doi:10.1158/1940-6207.CAPR-15-0431 (2016).

18 Ramon, J. & Denis, L. *Prostate Cancer*. (Springer, Berlin, 2007).

19 Epstein, J. I. Precursor lesions to prostatic adenocarcinoma. *Virchows Archiv : an international journal of pathology* **454**, 1-16, doi:10.1007/s00428-008-0707-5 (2009).

20 Bostwick, D. G. & Cheng, L. Precursors of prostate cancer. *Histopathology* **60**, 4-27, doi:10.1111/j.1365-2559.2011.04007.x (2012).

21 Trabzonlu, L. *et al.* Molecular Pathology of High-Grade Prostatic Intraepithelial Neoplasia: Challenges and Opportunities. *Cold Spring Harb Perspect Med* **9**, doi:10.1101/cshperspect.a030403 (2019).

22 Boyd, L. K., Mao, X. & Lu, Y. J. The complexity of prostate cancer: genomic alterations and heterogeneity. *Nature reviews. Urology* **9**, 652-664, doi:10.1038/nrurol.2012.185 (2012).

23 Humphrey, P. A., Moch, H., Cubilla, A. L., Ulbright, T. M. & Reuter, V. E. The 2016 WHO Classification of Tumours of the Urinary System and Male Genital Organs-Part B: Prostate and Bladder Tumours. *Eur Urol* **70**, 106-119, doi:10.1016/j.eururo.2016.02.028 (2016).

24 Nadal, R., Schweizer, M., Kryvenko, O. N., Epstein, J. I. & Eisenberger, M. A. Small cell carcinoma of the prostate. *Nature reviews. Urology* **11**, 213-219, doi:10.1038/nrurol.2014.21 (2014).

25 Zou, M. *et al.* Transdifferentiation as a Mechanism of Treatment Resistance in a Mouse Model of Castration-Resistant Prostate Cancer. *Cancer discovery* **7**, 736-749, doi:10.1158/2159-8290.CD-16-1174 (2017).

26 Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians* **68**, 394-424, doi:10.3322/caac.21492 (2018).

27 Bray, F. *et al.* Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: a cancer journal for clinicians* **68**, 394-424 (2018).

28 Siegel, R. L., Miller, K. D. & Jemal, A. Cancer statistics, 2020. *CA: a cancer journal for clinicians* **70**, 7-30, doi:10.3322/caac.21590 (2020).

29 Mariotto, A. B., Yabroff, K. R., Shao, Y., Feuer, E. J. & Brown, M. L. Projections of the cost of cancer care in the United States: 2010-2020. *Journal of the National Cancer Institute* **103**, 117-128, doi:10.1093/jnci/djq495 (2011).

30 Ilic, D., Neuberger, M. M., Djulbegovic, M. & Dahm, P. Screening for prostate cancer. *Cochrane Database Syst Rev*, CD004720, doi:10.1002/14651858.CD004720.pub3 (2013).

31 Heidenreich, A. *et al.* EAU guidelines on prostate cancer. Part II: Treatment of advanced, relapsing, and castration-resistant prostate cancer. *Eur Urol* **65**, 467-479, doi:10.1016/j.eururo.2013.11.002 (2014).

32 Wolf, A. M. *et al.* American Cancer Society guideline for the early detection of prostate cancer: update 2010. *CA: a cancer journal for clinicians* **60**, 70-98, doi:10.3322/caac.20066 (2010).

33 Kirby, R. S., Fitzpatrick, J. M. & Irani, J. Prostate cancer diagnosis in the new millennium: strengths and weaknesses of prostate-specific antigen and the discovery and clinical evaluation of prostate cancer gene 3 (PCA3). *BJU international* **103**, 441-445, doi:10.1111/j.1464-410X.2008.08280.x (2009).

34 Yates, D. R. & Anderson, J. B. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 333-346 (Springer London, 2013).

35 Thompson, I. M. *et al.* Prevalence of prostate cancer among men with a prostate-specific antigen level < or =4.0 ng per milliliter. *The New England journal of medicine* **350**, 2239-2246, doi:10.1056/NEJMoa031918 (2004).

36 Martin, N. E. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 207-213 (Springer London, 2013).

37 Punnen, S., Pavan, N. & Parekh, D. J. Finding the Wolf in Sheep's Clothing: The 4Kscore Is a Novel Blood Test That Can Accurately Identify the Risk of Aggressive Prostate Cancer. *Rev Urol* **17**, 3-13 (2015).

38 Singh, P. B., Moore, C. M. & Emberton, M. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 511-524 (Springer London, 2013).

39 Gleason, D. F. & Mellinger, G. T. Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. *The Journal of urology* **111**, 58-64, doi:10.1016/s0022-5347(17)59889-4 (1974).

40 Lotan, T. L. & Epstein, J. I. Clinical implications of changing definitions within the Gleason grading system. *Nature reviews. Urology* **7**, 136-142, doi:10.1038/nrurol.2010.9 (2010).

41 Epstein, J. I. An update of the Gleason grading system. *The Journal of urology* **183**, 433-440, doi:10.1016/j.juro.2009.10.046 (2010).

42 Braunhut, B. L., Punnen, S. & Kryvenko, O. N. Updates on Grading and Staging of Prostate Cancer. *Surg Pathol Clin* **11**, 759-774, doi:10.1016/j.path.2018.07.003 (2018).

43 Epstein, J. I. *et al.* The 2014 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma: Definition of Grading Patterns and Proposal for a New Grading System. *The American journal of surgical pathology* **40**, 244-252, doi:10.1097/PAS.0000000000000530 (2016).

44 Buyyounouski, M. K. *et al.* Prostate cancer - major changes in the American Joint Committee on Cancer eighth edition cancer staging manual. *CA: a cancer journal for clinicians* **67**, 245-253, doi:10.3322/caac.21391 (2017).

45 Cheng, L., Montironi, R., Bostwick, D. G., Lopez-Beltran, A. & Berney, D. M. Staging of prostate cancer. *Histopathology* **60**, 87-117, doi:10.1111/j.1365-2559.2011.04025.x (2012).

46 Edge, S. B. & Compton, C. C. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Annals of surgical oncology* **17**, 1471-1474, doi:10.1245/s10434-010-0985-4 (2010).

47 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674, doi:10.1016/j.cell.2011.02.013 (2011).

48 Ittmann, M. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 147-155 (Springer London, 2013).

49 Schrecengost, R. & Knudsen, K. E. Molecular pathogenesis and progression of prostate cancer. *Semin Oncol* **40**, 244-258, doi:10.1053/j.seminoncol.2013.04.001 (2013).

50 Shaw, G. L. & Neal, D. E. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 19-34 (Springer London, 2013).

51 Augello, M. A., Hickey, T. E. & Knudsen, K. E. FOXA1: master of steroid receptor function in cancer. *EMBO J* **30**, 3885-3894, doi:10.1038/emboj.2011.340 (2011).

52 Sahu, B. *et al.* Dual role of FoxA1 in androgen receptor binding to chromatin, androgen signalling and prostate cancer. *EMBO J* **30**, 3962-3976, doi:10.1038/emboj.2011.328 (2011).

53 Gerhardt, J. *et al.* FOXA1 promotes tumor progression in prostate cancer and represents a novel hallmark of castration-resistant prostate cancer. *The American journal of pathology* **180**, 848-861, doi:10.1016/j.ajpath.2011.10.021 (2012).

54 Blando, J. *et al.* PTEN deficiency is fully penetrant for prostate adenocarcinoma in C57BL/6 mice via mTOR-dependent growth. *The American journal of pathology* **174**, 1869-1879, doi:10.2353/ajpath.2009.080055 (2009).

55 Tomlins, S. A. *et al.* Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. *Science* **310**, 644-648, doi:10.1126/science.1117679 (2005).

56 Arora, K. & Barbieri, C. E. Molecular Subtypes of Prostate Cancer. *Curr Oncol Rep* **20**, 58, doi:10.1007/s11912-018-0707-9 (2018).

57 Tomlins, S. A. *et al.* Role of the TMPRSS2-ERG gene fusion in prostate cancer. *Neoplasia* **10**, 177-188, doi:10.1593/neo.07822 (2008).

58 Gupta, S. *et al.* FZD4 as a mediator of ERG oncogene-induced WNT signaling and epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer research* **70**, 6735-6745, doi:10.1158/0008-5472.CAN-10-0244 (2010).

59 Smith, J. A. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 609-614 (Springer London, 2013).

60 Heidenreich, A. *et al.* EAU guidelines on prostate cancer. part 1: screening, diagnosis, and local treatment with curative intent-update 2013. *Eur Urol* **65**, 124-137, doi:10.1016/j.eururo.2013.09.046 (2014).

61 Filson, C. P., Marks, L. S. & Litwin, M. S. Expectant management for men with early stage prostate cancer. *CA: a cancer journal for clinicians* **65**, 264-282 (2015).

62 Hammerer, P. & Madersbacher, S. Landmarks in hormonal therapy for prostate cancer. *BJU international* **110 Suppl 1**, 23-29, doi:10.1111/j.1464-410X.2012.11431.x (2012).

63 Damber, J. E. & Aus, G. Prostate cancer. *Lancet* **371**, 1710-1721, doi:10.1016/S0140-6736(08)60729-1 (2008).

64 Carceles-Cordon, M. *et al.* Cellular rewiring in lethal prostate cancer: the architect of drug resistance. *Nature reviews. Urology*, doi:10.1038/s41585-020-0298-8 (2020).

65 Felici, A., Pino, M. S. & Carlini, P. A changing landscape in castration-resistant prostate cancer treatment. *Frontiers in endocrinology* **3**, 85, doi:10.3389/fendo.2012.00085 (2012).

66 Gleave, A. M., Ci, X., Lin, D. & Wang, Y. A synopsis of prostate organoid methodologies, applications, and limitations. *The Prostate* **80**, 518-526, doi:10.1002/pros.23966 (2020).

67 Wu, X., Gong, S., Roy-Burman, P., Lee, P. & Culig, Z. Current mouse and cell models in prostate cancer research. *Endocrine-related cancer* **20**, R155-170, doi:10.1530/ERC-12-0285 (2013).

68 Cunningham, D. & You, Z. In vitro and in vivo model systems used in prostate cancer research. *J Biol Methods* **2**, doi:10.14440/jbm.2015.63 (2015).

69 Berish, R. B., Ali, A. N., Telmer, P. G., Ronald, J. A. & Leong, H. S. Translational models of prostate cancer bone metastasis. *Nature reviews. Urology* **15**, 403-421, doi:10.1038/s41585-018-0020-2 (2018).

70 Horoszewicz, J. S. *et al.* LNCaP model of human prostatic carcinoma. *Cancer research* **43**, 1809-1818 (1983).

71 Thalmann, G. N. *et al.* Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer research* **54**, 2577-2581 (1994).

72 Kaighn, M. E., Narayan, K. S., Ohnuki, Y., Lechner, J. F. & Jones, L. W. Establishment and characterization of a human prostatic carcinoma cell line (PC-3). *Invest Urol* **17**, 16-23 (1979).

73 Stone, K. R., Mickey, D. D., Wunderli, H., Mickey, G. H. & Paulson, D. F. Isolation of a human prostate carcinoma cell line (DU 145). *International journal of cancer. Journal international du cancer* **21**, 274-281, doi:10.1002/ijc.2910210305 (1978).

74 Sobel, R. E. & Sadar, M. D. Cell lines used in prostate cancer research: a compendium of old and new lines--part 2. *The Journal of urology* **173**, 360-372, doi:10.1097/01.ju.0000149989.01263.dc (2005).

75 Sobel, R. E. & Sadar, M. D. Cell lines used in prostate cancer research: a compendium of old and new lines--part 1. *The Journal of urology* **173**, 342-359, doi:10.1097/01.ju.0000141580.30910.57 (2005).

76 Rea, D. *et al.* Mouse Models in Prostate Cancer Translational Research: From Xenograft to PDX. *Biomed Res Int* **2016**, 9750795, doi:10.1155/2016/9750795 (2016).

77 Weeber, F., Ooft, S. N., Dijkstra, K. K. & Voest, E. E. Tumor Organoids as a Pre-clinical Cancer Model for Drug Discovery. *Cell Chem Biol* **24**, 1092-1100, doi:10.1016/j.chembiol.2017.06.012 (2017).

78 Grabowska, M. M. *et al.* Mouse models of prostate cancer: picking the best model for the question. *Cancer metastasis reviews* **33**, 377-397, doi:10.1007/s10555-013-9487-8 (2014).

79 Civenni, G., Carbone, G. M. & Catapano, C. V. Overview of Genetically Engineered Mouse Models of Prostate Cancer and Their Applications in Drug Discovery. *Curr Protoc Pharmacol* **81**, e39, doi:10.1002/cpph.39 (2018).

80 Wang, S. *et al.* Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer cell* **4**, 209-221, doi:10.1016/s1535-6108(03)00215-0 (2003).

81 Ellwood-Yen, K. *et al.* Myc-driven murine prostate cancer shares molecular features with human prostate tumors. *Cancer cell* **4**, 223-238, doi:10.1016/s1535-6108(03)00197-1 (2003).

82 Warburg, O. On respiratory impairment in cancer cells. *Science* **124**, 269-270 (1956).

83 Warburg, O. On the origin of cancer cells. *Science* **123**, 309-314, doi:10.1126/science.123.3191.309 (1956).

84 Bader, D. A. & McGuire, S. E. Tumour metabolism and its unique properties in prostate adenocarcinoma. *Nature reviews. Urology*, doi:10.1038/s41585-020-0288-x (2020).

85 Barron, E. S. G. & Huggins, C. The Metabolism of Isolated Prostatic Tissue[*]. *Journal of Urology* **51**, 630-634, doi:doi:10.1016/S0022-5347(17)70403-X (1944).

86 Costello, L. C. & Franklin, R. B. Aconitase activity, citrate oxidation, and zinc inhibition in rat ventral prostate. *Enzyme* **26**, 281-287, doi:10.1159/000459195 (1981).

87 Costello, L. C., Akuffo, V. & Franklin, R. B. Testosterone stimulates net citrate production from aspartate by prostate epithelial cells. *Horm Metab Res* **20**, 252-253, doi:10.1055/s-2007-1010807 (1988).

88 Costello, L. C. & Franklin, R. B. Concepts of citrate production and secretion by prostate: 2. Hormonal relationships in normal and neoplastic prostate. *The Prostate* **19**, 181-205, doi:10.1002/pros.2990190302 (1991).

89 Costello, L. C. & Franklin, R. B. The clinical relevance of the metabolism of prostate cancer; zinc and tumor suppression: connecting the dots. *Molecular cancer* **5**, 17, doi:10.1186/1476-4598-5-17 (2006).

90 Zadra, G., Photopoulos, C. & Loda, M. The fat side of prostate cancer. *Biochimica et biophysica acta* **1831**, 1518-1532, doi:10.1016/j.bbalip.2013.03.010 (2013).

91 Giunchi, F., Fiorentino, M. & Loda, M. The Metabolic Landscape of Prostate Cancer. *Eur Urol Oncol* **2**, 28-36, doi:10.1016/j.euo.2018.06.010 (2019).

92 Spratt, D. E. *et al.* Utility of FDG-PET in clinical neuroendocrine prostate cancer. *The Prostate* **74**, 1153-1159, doi:10.1002/pros.22831 (2014).

93 Sonveaux, P. *et al.* Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *The Journal of clinical investigation* **118**, 3930-3942, doi:10.1172/JCI36843 (2008).

94 Dhup, S., Dadhich, R. K., Porporato, P. E. & Sonveaux, P. Multiple biological activities of lactic acid in cancer: influences on tumor growth, angiogenesis and metastasis. *Current pharmaceutical design* **18**, 1319-1330, doi:10.2174/138161212799504902 (2012).

95 Cutruzzolà, F. *et al.* Glucose metabolism in the progression of prostate cancer. *Frontiers in physiology* **8**, 97 (2017).

96 Rehfeld, A., Nylander, M. & Karnov, K. in *Compendium of Histology: A Theoretical and Practical Guide* 517-539 (Springer International Publishing, 2017).

97 Falkenstein, E. & Wehling, M. Nongenomically initiated steroid actions. *European journal of clinical investigation* **30**, 51-54 (2000).

98 McEwan, I. J. The Nuclear Receptor Superfamily at Thirty. *Methods Mol Biol* **1443**, 3-9, doi:10.1007/978-1-4939-3724-0_1 (2016).

99 Miller, W. L. & Auchus, R. J. The molecular biology, biochemistry, and physiology of human steroidogenesis and its disorders. *Endocr Rev* **32**, 81-151, doi:10.1210/er.2010-0013 (2011).

100 Miller, W. L. & Auchus, R. J. The "backdoor pathway" of androgen synthesis in human male sexual development. *PLoS Biol* **17**, e3000198, doi:10.1371/journal.pbio.3000198 (2019).

101 Swerdloff, R. S., Dudley, R. E., Page, S. T., Wang, C. & Salameh, W. A. Dihydrotestosterone: Biochemistry, Physiology, and Clinical Implications of Elevated Blood Levels. *Endocr Rev* **38**, 220-254, doi:10.1210/er.2016-1067 (2017).

102 Brinkmann, A. O. Molecular mechanisms of androgen action--a historical perspective. *Methods Mol Biol* **776**, 3-24, doi:10.1007/978-1-61779-243-4_1 (2011).

103 Porter, B. A., Ortiz, M. A., Bratslavsky, G. & Kotula, L. Structure and Function of the Nuclear Receptor Superfamily and Current Targeted Therapies of Prostate Cancer. *Cancers (Basel)* **11**, doi:10.3390/cancers11121852 (2019).

104 Weikum, E. R., Liu, X. & Ortlund, E. A. The nuclear receptor superfamily: A structural perspective. *Protein Sci* **27**, 1876-1892, doi:10.1002/pro.3496 (2018).

105 Chang, C. S., Kokontis, J. & Liao, S. T. Molecular cloning of human and rat complementary DNA encoding androgen receptors. *Science* **240**, 324-326, doi:10.1126/science.3353726 (1988).

106 Lubahn, D. B. *et al.* Cloning of human androgen receptor complementary DNA and localization to the X chromosome. *Science* **240**, 327-330, doi:10.1126/science.3353727 (1988).

107 Trapman, J. *et al.* Cloning, structure and expression of a cDNA encoding the human androgen receptor. *Biochemical and biophysical research communications* **153**, 241-248, doi:10.1016/s0006-291x(88)81214-2 (1988).

108 Tilley, W. D., Marcelli, M., Wilson, J. D. & McPhaul, M. J. Characterization and expression of a cDNA encoding the human androgen receptor. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 327-331, doi:10.1073/pnas.86.1.327 (1989).

109 Simental, J. A., Sar, M., Lane, M. V., French, F. S. & Wilson, E. M. Transcriptional activation and nuclear targeting signals of the human androgen receptor. *The Journal of biological chemistry* **266**, 510-518 (1991).

110 Jenster, G., van der Korput, H. A., Trapman, J. & Brinkmann, A. O. Identification of two transcription activation units in the N-terminal domain of the human androgen receptor. *The Journal of biological chemistry* **270**, 7341-7346, doi:10.1074/jbc.270.13.7341 (1995).

111 Luisi, B. F. *et al.* Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* **352**, 497-505, doi:10.1038/352497a0 (1991).

112 Claessens, F. *et al.* Selective DNA binding by the androgen receptor as a mechanism for hormone-specific gene regulation. *J Steroid Biochem Mol Biol* **76**, 23-30, doi:10.1016/s0960-0760(00)00154-0 (2001).

113 Roche, P. J., Hoare, S. A. & Parker, M. G. A consensus DNA-binding site for the androgen receptor. *Molecular endocrinology* **6**, 2229-2235 (1992).

114 Khorasanizadeh, S. & Rastinejad, F. Nuclear-receptor interactions on DNA-response elements. *Trends Biochem Sci* **26**, 384-390, doi:10.1016/s0968-0004(01)01800-x (2001).

115 Moehren, U., Denayer, S., Podvinec, M., Verrijdt, G. & Claessens, F. Identification of androgen-selective androgen-response elements in the human aquaporin-5 and Rad9 genes. *Biochem J* **411**, 679-686, doi:10.1042/BJ20071352 (2008).

116 Bennett, N. C., Gardiner, R. A., Hooper, J. D., Johnson, D. W. & Gobe, G. C. Molecular cell biology of androgen receptor signalling. *Int J Biochem Cell Biol* **42**, 813-827, doi:10.1016/j.biocel.2009.11.013 (2010).

117 Jenster, G., Trapman, J. & Brinkmann, A. O. Nuclear import of the human androgen receptor. *Biochem J* **293 (Pt 3)**, 761-768, doi:10.1042/bj2930761 (1993).

118 Kumar, R. & McEwan, I. J. Allosteric modulators of steroid hormone receptors: structural dynamics and gene regulation. *Endocr Rev* **33**, 271-299, doi:10.1210/er.2011-1033 (2012).

119 Cutress, M. L., Whitaker, H. C., Mills, I. G., Stewart, M. & Neal, D. E. Structural basis for the nuclear import of the human androgen receptor. *Journal of cell science* **121**, 957-968, doi:10.1242/jcs.022103 (2008).

120 Clinckemalie, L., Vanderschueren, D., Boonen, S. & Claessens, F. The hinge region in androgen receptor control. *Mol Cell Endocrinol* **358**, 1-8, doi:10.1016/j.mce.2012.02.019 (2012).

121 Matias, P. M. *et al.* Structural evidence for ligand specificity in the binding domain of the human androgen receptor. Implications for pathogenic gene mutations. *The Journal of biological chemistry* **275**, 26164-26171, doi:10.1074/jbc.M004571200 (2000).

122 Li, J. & Al-Azzawi, F. Mechanism of androgen receptor action. *Maturitas* **63**, 142-148, doi:10.1016/j.maturitas.2009.03.008 (2009).

123 Paschalis, A. *et al.* Alternative splicing in prostate cancer. *Nat Rev Clin Oncol* **15**, 663-675, doi:10.1038/s41571-018-0085-0 (2018).

124 Lamb, A. D., Massie, C. E. & Neal, D. E. The transcriptional programme of the androgen receptor (AR) in prostate cancer. *BJU international* **113**, 358-366, doi:10.1111/bju.12415 (2014).

125 Wilson, S., Qi, J. & Filipp, F. V. Refinement of the androgen response element based on ChIP-Seq in androgen-insensitive and androgen-responsive prostate cancer cell lines. *Scientific reports* **6**, 32611, doi:10.1038/srep32611 (2016).

126 Massie, C. E. *et al.* The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis. *EMBO J* **30**, 2719-2733, doi:10.1038/emboj.2011.158 (2011).

127 Toropainen, S. *et al.* Global analysis of transcription in castration-resistant prostate cancer cells uncovers active enhancers and direct androgen receptor targets. *Scientific reports* **6**, 33510, doi:10.1038/srep33510 (2016).

128 McNair, C. *et al.* Cell cycle-coupled expansion of AR activity promotes cancer progression. *Oncogene* **36**, 1655-1668, doi:10.1038/onc.2016.334 (2017).

129 Young, C. Y. *et al.* Hormonal regulation of prostate-specific antigen messenger RNA in human prostatic adenocarcinoma cell line LNCaP. *Cancer research* **51**, 3748-3752 (1991).

130 Lin, B. *et al.* Prostate-localized and androgen-regulated expression of the membrane-bound serine protease TMPRSS2. *Cancer research* **59**, 4180-4184 (1999).

131 Zamagni, A., Cortesi, M., Zanoni, M. & Tesei, A. Non-nuclear AR signaling in prostate cancer. *Frontiers in chemistry* 7, 651 (2019).

132 Foradori, C. D., Weiser, M. J. & Handa, R. J. Non-genomic actions of androgens. *Front Neuroendocrinol* **29**, 169-181, doi:10.1016/j.yfrne.2007.10.005 (2008).

133 Heinlein, C. A. & Chang, C. The roles of androgen receptors and androgen-binding proteins in nongenomic androgen actions. *Mol Endocrinol* **16**, 2181-2187, doi:10.1210/me.2002-0070 (2002).

134 Grosse, A., Bartsch, S. & Baniahmad, A. Androgen receptor-mediated gene repression. *Mol Cell Endocrinol* **352**, 46-56, doi:10.1016/j.mce.2011.06.032 (2012).

135 Freeman, M. R., Cinar, B. & Lu, M. L. Membrane rafts as potential sites of nongenomic hormonal signaling in prostate cancer. *Trends Endocrinol Metab* **16**, 273-279, doi:10.1016/j.tem.2005.06.002 (2005).

136 Heemers, H. V. & Tindall, D. J. Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex. *Endocr Rev* **28**, 778-808, doi:10.1210/er.2007-0019 (2007).

137 Gerlach, C., Roell, D. & Baniahmad, A. Analysis of ligand-specific co-repressor binding to the androgen receptor. *Methods Mol Biol* **776**, 199-223, doi:10.1007/978-1-61779-243-4_13 (2011).

138 Burd, C. J., Morey, L. M. & Knudsen, K. E. Androgen receptor corepressors and prostate cancer. *Endocrine-related cancer* **13**, 979-994 (2006).

139 Xu, J. *et al.* Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* **279**, 1922-1925, doi:10.1126/science.279.5358.1922 (1998).

140 Gehin, M. *et al.* The function of TIF2/GRIP1 in mouse reproduction is distinct from those of SRC-1 and p/CIP. *Molecular and cellular biology* **22**, 5923-5937, doi:10.1128/mcb.22.16.5923-5937.2002 (2002).

141 Khan, O. Y. *et al.* Multifunction steroid receptor coactivator, E6-associated protein, is involved in development of the prostate gland. *Mol Endocrinol* **20**, 544-559, doi:10.1210/me.2005-0110 (2006).

142 Gritsina, G., Gao, W. Q. & Yu, J. Transcriptional repression by androgen receptor: roles in castration-resistant prostate cancer. *Asian J Androl* **21**, 215-223, doi:10.4103/aja.aja_19_19 (2019).

143 Niu, Y. *et al.* Androgen receptor is a tumor suppressor and proliferator in prostate cancer. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 12182-12187, doi:10.1073/pnas.0804700105 (2008).

144 Galani, A., Kitsiou-Tzeli, S., Sofokleous, C., Kanavakis, E. & Kalpini-Mavrou, A. Androgen insensitivity syndrome: clinical features and molecular defects. *Hormones (Athens)* 7, 217-229, doi:10.14310/horm.2002.1201 (2008).

145 Knudsen, K. E. & Penning, T. M. Partners in crime: deregulation of AR activity and androgen synthesis in prostate cancer. *Trends Endocrinol Metab* **21**, 315-324, doi:10.1016/j.tem.2010.01.002 (2010).

146 Mills, I. G. Maintaining and reprogramming genomic androgen receptor activity in prostate cancer. *Nature reviews. Cancer* **14**, 187-198, doi:10.1038/nrc3678 (2014).

147 Butler, L. M., Centenera, M. M. & Swinnen, J. V. Androgen control of lipid metabolism in prostate cancer: novel insights and future applications. *Endocrine-related cancer* **23**, R219-227, doi:10.1530/ERC-15-0556 (2016).

148 Swinnen, J. V., Van Veldhoven, P. P., Esquenet, M., Heyns, W. & Verhoeven, G. Androgens markedly stimulate the accumulation of neutral lipids in the human prostatic adenocarcinoma cell line LNCaP. *Endocrinology* **137**, 4468-4474, doi:10.1210/endo.137.10.8828509 (1996).

149 Audet-Walsh, E. *et al.* Androgen-Dependent Repression of ERRgamma Reprograms Metabolism in Prostate Cancer. *Cancer research* **77**, 378-389, doi:10.1158/0008-5472.CAN-16-1204 (2017).

150 Swinnen, J. V. *et al.* Androgens, lipogenesis and prostate cancer. *J Steroid Biochem Mol Biol* **92**, 273-279, doi:10.1016/j.jsbmb.2004.10.013 (2004).

151 Migita, T. *et al.* Fatty acid synthase: a metabolic enzyme and candidate oncogene in prostate cancer. *Journal of the National Cancer Institute* **101**, 519-532, doi:10.1093/jnci/djp030 (2009).

152 Heemers, H. *et al.* Androgens stimulate lipogenic gene expression in prostate cancer cells by activation of the sterol regulatory element-binding protein cleavage activating protein/sterol regulatory element-binding protein pathway. *Mol Endocrinol* **15**, 1817-1828, doi:10.1210/mend.15.10.0703 (2001).

153 Shi, Y. *et al.* Androgens promote prostate cancer cell growth through induction of autophagy. *Mol Endocrinol* **27**, 280-295, doi:10.1210/me.2012-1260 (2013).

154 Abate-Shen, C. Prostate Cancer Metastasis - Fueled by Fat? *The New England journal of medicine* **378**, 1643-1645, doi:10.1056/NEJMcibr1800808 (2018).

155 Chen, M. *et al.* An aberrant SREBP-dependent lipogenic program promotes metastatic prostate cancer. *Nature genetics* **50**, 206-218, doi:10.1038/s41588-017-0027-2 (2018).

156 Sacca, P. A. *et al.* Human periprostatic adipose tissue: its influence on prostate cancer cells. *Cell Physiol Biochem* **30**, 113-122, doi:10.1159/000339051 (2012).

157 Nieman, K. M., Romero, I. L., Van Houten, B. & Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochimica et biophysica acta* **1831**, 1533-1541, doi:10.1016/j.bbalip.2013.02.010 (2013).

158 Wang, Q. *et al.* Targeting amino acid transport in metastatic castration-resistant prostate cancer: effects on cell cycle, cell growth, and tumor development. *Journal of the National Cancer Institute* **105**, 1463-1473, doi:10.1093/jnci/djt241 (2013).

159 Watson, P. A., Arora, V. K. & Sawyers, C. L. Emerging mechanisms of resistance to androgen receptor inhibitors in prostate cancer. *Nature reviews. Cancer* **15**, 701-711, doi:10.1038/nrc4016 (2015).

160 Chen, Y., Clegg, N. J. & Scher, H. I. Anti-androgens and androgen-depleting therapies in prostate cancer: new agents for an established target. *Lancet Oncol* **10**, 981-991, doi:10.1016/S1470-2045(09)70229-3 (2009).

161 Rice, M. A., Malhotra, S. V. & Stoyanova, T. Second-Generation Antiandrogens: From Discovery to Standard of Care in Castration Resistant Prostate Cancer. *Front Oncol* **9**, 801, doi:10.3389/fonc.2019.00801 (2019).

162 Stein, M. N., Patel, N., Bershadskiy, A., Sokoloff, A. & Singer, E. A. Androgen synthesis inhibitors in the treatment of castration-resistant prostate cancer. *Asian J Androl* **16**, 387-400, doi:10.4103/1008-682X.129133 (2014).

163 Pinto-Bazurco Mendieta, M. A. *et al.* CYP17 inhibitors. Annulations of additional rings in methylene imidazole substituted biphenyls: synthesis, biological evaluation and molecular modelling. *Arch Pharm (Weinheim)* **341**, 597-609, doi:10.1002/ardp.200700251 (2008).

164 Bryce, A. & Ryan, C. J. Development and clinical utility of abiraterone acetate as an androgen synthesis inhibitor. *Clin Pharmacol Ther* **91**, 101-108, doi:10.1038/clpt.2011.275 (2012).

165 Richards, J. *et al.* Interactions of abiraterone, eplerenone, and prednisolone with wild-type and mutant androgen receptor: a rationale for increasing abiraterone exposure or combining with MDV3100. *Cancer research* **72**, 2176-2182, doi:10.1158/0008-5472.CAN-11-3980 (2012).

166 Yin, L. & Hu, Q. CYP17 inhibitors--abiraterone, C17,20-lyase inhibitors and multi-targeting agents. *Nature reviews. Urology* **11**, 32-42, doi:10.1038/nrurol.2013.274 (2014).

167 Norris, J. D. *et al.* Androgen receptor antagonism drives cytochrome P450 17A1 inhibitor efficacy in prostate cancer. *The Journal of clinical investigation* **127**, 2326-2338, doi:10.1172/JCI87328 (2017).

168 Snow, O. *et al.* Androgen receptor plasticity and its implications for prostate cancer therapy. *Cancer treatment reviews* **81**, 101871, doi:10.1016/j.ctrv.2019.05.001 (2019).

169 Tan, M. H., Li, J., Xu, H. E., Melcher, K. & Yong, E. L. Androgen receptor: structure, role in prostate cancer and drug discovery. *Acta pharmacologica Sinica* **36**, 3-23, doi:10.1038/aps.2014.18 (2015).

170 Tran, C. *et al.* Development of a second-generation antiandrogen for treatment of advanced prostate cancer. *Science* **324**, 787-790, doi:10.1126/science.1168175 (2009).

171 Ito, Y. & Sadar, M. D. Enzalutamide and blocking androgen receptor in advanced prostate cancer: lessons learnt from the history of drug development of antiandrogens. *Res Rep Urol* **10**, 23-32, doi:10.2147/RRU.S157116 (2018).

172 Moilanen, A. M. *et al.* Discovery of ODM-201, a new-generation androgen receptor inhibitor targeting resistance mechanisms to androgen signaling-directed prostate cancer therapies. *Scientific reports* **5**, 12007, doi:10.1038/srep12007 (2015).

173 Clegg, N. J. *et al.* ARN-509: a novel antiandrogen for prostate cancer treatment. *Cancer research* **72**, 1494-1503, doi:10.1158/0008-5472.CAN-11-3948 (2012).

174 Leone, G. *et al.* Antiandrogen withdrawal syndrome (AAWS) in the treatment of patients with prostate cancer. *Endocrine-related cancer* **25**, R1-R9, doi:10.1530/ERC-17-0355 (2018).

175 Fizazi, K. *et al.* Abiraterone plus Prednisone in Metastatic, Castration-Sensitive Prostate Cancer. *The New England journal of medicine* **377**, 352-360, doi:10.1056/NEJMoa1704174 (2017).

176 Heldin, C. H., Rubin, K., Pietras, K. & Ostman, A. High interstitial fluid pressure - an obstacle in cancer therapy. *Nature reviews. Cancer* **4**, 806-813, doi:10.1038/nrc1456 (2004).

177 Mattheolabakis, G. & Mikelis, C. M. Nanoparticle Delivery and Tumor Vascular Normalization: The Chicken or The Egg? *Front Oncol* **9**, 1227, doi:10.3389/fonc.2019.01227 (2019).

178 Jeong, C. W. *et al.* The role of hypoxia-inducible factor-1alpha and -2alpha in androgen insensitive prostate cancer cells. *Urologic oncology* **31**, 1448-1456, doi:10.1016/j.urolonc.2012.03.022 (2013).

179 Alshaker, H. *et al.* Everolimus (RAD001) sensitizes prostate cancer cells to docetaxel by down-regulation of HIF-1alpha and sphingosine kinase 1. *Oncotarget* **7**, 80943-80956, doi:10.18632/oncotarget.13115 (2016).

180 Zhu, P. *et al.* Macrophage/cancer cell interactions mediate hormone resistance by a nuclear receptor derepression pathway. *Cell* **124**, 615-629, doi:10.1016/j.cell.2005.12.032 (2006).

181 Gabrilovich, D. I. & Nagaraj, S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews. Immunology* **9**, 162-174, doi:10.1038/nri2506 (2009).

182 Meads, M. B., Gatenby, R. A. & Dalton, W. S. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nature reviews. Cancer* **9**, 665-674, doi:10.1038/nrc2714 (2009).

183 Di Lorenzo, G., Buonerba, C. & Kantoff, P. W. Immunotherapy for the treatment of prostate cancer. *Nat Rev Clin Oncol* **8**, 551-561, doi:10.1038/nrclinonc.2011.72 (2011).

184 Dehm, S. M., Schmidt, L. J., Heemers, H. V., Vessella, R. L. & Tindall, D. J. Splicing of a novel androgen receptor exon generates a constitutively active androgen receptor that mediates prostate cancer therapy resistance. *Cancer research* **68**, 5469-5477, doi:10.1158/0008-5472.CAN-08-0594 (2008).

185 Hornberg, E. *et al.* Expression of androgen receptor splice variants in prostate cancer bone metastases is associated with castration-resistance and short survival. *PloS one* **6**, e19059, doi:10.1371/journal.pone.0019059 (2011).

186 Antonarakis, E. S. *et al.* AR-V7 and resistance to enzalutamide and abiraterone in prostate cancer. *The New England journal of medicine* **371**, 1028-1038, doi:10.1056/NEJMoa1315815 (2014).

187 Liu, L. L. *et al.* Mechanisms of the androgen receptor splicing in prostate cancer cells. *Oncogene* **33**, 3140-3150, doi:10.1038/onc.2013.284 (2014).

188 Arora, V. K. *et al.* Glucocorticoid receptor confers resistance to antiandrogens by bypassing androgen receptor blockade. *Cell* **155**, 1309-1322, doi:10.1016/j.cell.2013.11.012 (2013).

189 Taylor, B. S. *et al.* Integrative genomic profiling of human prostate cancer. *Cancer cell* **18**, 11-22, doi:10.1016/j.ccr.2010.05.026 (2010).

190 Lamoureux, F., Zoubeidi, A. & Gleave, M. E. in *Prostate Cancer: A Comprehensive Perspective* (ed Ashutosh Tewari) 43-64 (Springer London, 2013).

191 Beltran, H. *et al.* Molecular characterization of neuroendocrine prostate cancer and identification of new drug targets. *Cancer discovery* **1**, 487-495, doi:10.1158/2159-8290.CD-11-0130 (2011).

192 Dardenne, E. *et al.* N-Myc Induces an EZH2-Mediated Transcriptional Program Driving Neuroendocrine Prostate Cancer. *Cancer cell* **30**, 563-577, doi:10.1016/j.ccell.2016.09.005 (2016).

193 Li, X. *et al.* SOX2 promotes tumor metastasis by stimulating epithelial-to-mesenchymal transition via regulation of WNT/beta-catenin signal network. *Cancer letters* **336**, 379-389, doi:10.1016/j.canlet.2013.03.027 (2013).

194 Russo, M. V. *et al.* SOX2 boosts major tumor progression genes in prostate cancer and is a functional biomarker of lymph node metastasis. *Oncotarget* **7**, 12372-12385, doi:10.18632/oncotarget.6029 (2016).

195 Ku, S. Y. *et al.* Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and antiandrogen resistance. *Science* **355**, 78-83, doi:10.1126/science.aah4199 (2017).

196 Mu, P. *et al.* SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer. *Science* **355**, 84-88, doi:10.1126/science.aah4307 (2017).

197 Hannun, Y. A. & Obeid, L. M. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nature reviews. Molecular cell biology* **9**, 139-150, doi:10.1038/nrm2329 (2008).

198 Hannun, Y. A. & Obeid, L. M. Sphingolipids and their metabolism in physiology and disease. *Nature reviews. Molecular cell biology* **19**, 175-191, doi:10.1038/nrm.2017.107 (2018).

199 Carter, H. E., Haines, W. J. & et al. Biochemistry of the sphingolipides; preparation of sphingolipides from beef brain and spinal cord. *The Journal of biological chemistry* **169**, 77-82 (1947).

200 Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr. & Bell, R. M. Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *The Journal of biological chemistry* **261**, 12604-12609 (1986).

201 Merrill, A. H., Jr. *et al.* Inhibition of phorbol ester-dependent differentiation of human promyelocytic leukemic (HL-60) cells by sphinganine and other long-chain bases. *The Journal of biological chemistry* **261**, 12610-12615 (1986).

202 Ogretmen, B. Sphingolipid metabolism in cancer signalling and therapy. *Nature reviews. Cancer* **18**, 33-50, doi:10.1038/nrc.2017.96 (2018).

203 Merrill, A. H., Jr. Sphingolipid and glycosphingolipid metabolic pathways in the era of sphingolipidomics. *Chem Rev* **111**, 6387-6422, doi:10.1021/cr2002917 (2011).

204 Morad, S. A. & Cabot, M. C. Ceramide-orchestrated signalling in cancer cells. *Nature reviews. Cancer* **13**, 51-65, doi:10.1038/nrc3398 (2013).

205 Astudillo, L. *et al.* Human genetic disorders of sphingolipid biosynthesis. *J Inherit Metab Dis* **38**, 65-76, doi:10.1007/s10545-014-9736-1 (2015).

206 Wei, J. *et al.* in *Sphingolipid Biology* (eds Yoshio Hirabayashi, Yasuyuki Igarashi, & Alfred H. Merrill) 25-47 (Springer Japan, 2006).

207 Braun, P. E. & Snell, E. E. The biosynthesis of dihydrosphingosine in cell-free preparations of Hansenula ciferri. *Proceedings of the National Academy of Sciences of the United States of America* **58**, 298-303, doi:10.1073/pnas.58.1.298 (1967).

208 Stoffel, W., LeKim, D. & Sticht, G. Biosynthesis of dihydrosphingosine in vitro. *Hoppe Seylers Z Physiol Chem* **349**, 664-670, doi:10.1515/bchm2.1968.349.1.664 (1968).

209 Stoffel, W., Sticht, G. & LeKim, D. Metabolism of sphingosine bases. IX. Degradation in vitro of dihydrospingosine and dihydrospingosine phosphate to palmitaldehyde and ethanolamine phosphate. *Hoppe Seylers Z Physiol Chem* **349**, 1745-1748, doi:10.1515/bchm2.1968.349.2.1745 (1968).

210 Colacios, C., Sabourdy, F., Andrieu-Abadie, N., Ségui, B. & Levade, T. in *Bioactive Sphingolipids in Cancer Biology and Therapy* (eds Yusuf A. Hannun, Chiara Luberto, Cungui Mao, & Lina Marie Obeid) 1-20 (Springer International Publishing, 2015).

211 Han, G. *et al.* Identification of small subunits of mammalian serine palmitoyltransferase that confer distinct acyl-CoA substrate specificities. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 8186-8191, doi:10.1073/pnas.0811269106 (2009).

212 Hornemann, T. *et al.* The SPTLC3 subunit of serine palmitoyltransferase generates short chain sphingoid bases. *The Journal of biological chemistry* **284**, 26322-26330, doi:10.1074/jbc.M109.023192 (2009).

213 Duan, J. & Merrill Jr, A. H. 1-Deoxysphingolipids encountered exogenously and made de novo: dangerous mysteries inside an enigma. *Journal of Biological Chemistry* **290**, 15380-15389 (2015).

214 Hanada, K., Hara, T. & Nishijima, M. D-Serine inhibits serine palmitoyltransferase, the enzyme catalyzing the initial step of sphingolipid biosynthesis. *FEBS letters* **474**, 63-65, doi:10.1016/s0014-5793(00)01579-9 (2000).

215 MANDON, E. C., van ECHTEN, G., BIRK, R., SCHMIDT, R. R. & SANDHOFF, K. Sphingolipid biosynthesis in cultured neurons: Down‐regulation of serine palmitoyltransferase by sphingoid bases. *European journal of biochemistry* **198**, 667-674 (1991).

216 Nicholson, G. A. *et al.* The gene for hereditary sensory neuropathy type I (HSN-I) maps to chromosome 9q22.1-q22.3. *Nature genetics* **13**, 101-104, doi:10.1038/ng0596-101 (1996).

217 Dawkins, J. L., Hulme, D. J., Brahmbhatt, S. B., Auer-Grumbach, M. & Nicholson, G. A. Mutations in SPTLC1, encoding serine palmitoyltransferase, long chain base subunit-1, cause hereditary sensory neuropathy type I. *Nature genetics* **27**, 309-312, doi:10.1038/85879 (2001).

218 Gable, K. *et al.* A disease-causing mutation in the active site of serine palmitoyltransferase causes catalytic promiscuity. *The Journal of biological chemistry* **285**, 22846-22852, doi:10.1074/jbc.M110.122259 (2010).

219 Penno, A. *et al.* Hereditary sensory neuropathy type 1 is caused by the accumulation of two neurotoxic sphingolipids. *The Journal of biological chemistry* **285**, 11178-11187, doi:10.1074/jbc.M109.092973 (2010).

220 Zhao, L. *et al.* Elevation of 20-carbon long chain bases due to a mutation in serine palmitoyltransferase small subunit b results in neurodegeneration. *Proceedings of the National Academy of Sciences of the United States of America* **112**, 12962-12967, doi:10.1073/pnas.1516733112 (2015).

221 Davis, D. L. *et al.* Dynamics of sphingolipids and the serine-palmitoyltransferase complex in rat oligodendrocytes during myelination. *J Lipid Res*, doi:10.1194/jlr.RA120000627 (2020).

222 Hjelmqvist, L. *et al.* ORMDL proteins are a conserved new family of endoplasmic reticulum membrane proteins. *Genome Biol* **3**, RESEARCH0027, doi:10.1186/gb-2002-3-6-research0027 (2002).

223 Breslow, D. K. *et al.* Orm family proteins mediate sphingolipid homeostasis. *Nature* **463**, 1048-1053, doi:10.1038/nature08787 (2010).

224 Siow, D. L. & Wattenberg, B. W. Mammalian ORMDL proteins mediate the feedback response in ceramide biosynthesis. *The Journal of biological chemistry* **287**, 40198-40204, doi:10.1074/jbc.C112.404012 (2012).

225 Kiefer, K. *et al.* Coordinated regulation of the orosomucoid-like gene family expression controls de novo ceramide synthesis in mammalian cells. *The Journal of biological chemistry* **290**, 2822-2830, doi:10.1074/jbc.M114.595116 (2015).

226 Davis, D., Kannan, M. & Wattenberg, B. Orm/ORMDL proteins: Gate guardians and master regulators. *Adv Biol Regul* **70**, 3-18, doi:10.1016/j.jbior.2018.08.002 (2018).

227 Kihara, A. & Igarashi, Y. FVT-1 is a mammalian 3-ketodihydrosphingosine reductase with an active site that faces the cytosolic side of the endoplasmic reticulum membrane. *The Journal of biological chemistry* **279**, 49243-49250, doi:10.1074/jbc.M405915200 (2004).

228 Wigger, D., Gulbins, E., Kleuser, B. & Schumacher, F. Monitoring the sphingolipid de novo synthesis by stable-isotope labeling and liquid chromatography-mass spectrometry. *Frontiers in cell and developmental biology* **7**, 210 (2019).

229 Gault, C. R., Obeid, L. M. & Hannun, Y. A. An overview of sphingolipid metabolism: from synthesis to breakdown. *Advances in experimental medicine and biology* **688**, 1-23, doi:10.1007/978-1-4419-6741-1_1 (2010).

230 Egilmez, N. K., Chen, J. B. & Jazwinski, S. M. Specific alterations in transcript prevalence during the yeast life span. *The Journal of biological chemistry* **264**, 14312-14317 (1989).

231 Venkataraman, K. *et al.* Upstream of growth and differentiation factor 1 (uog1), a mammalian homolog of the yeast longevity assurance gene 1 (LAG1), regulates N-stearoyl-sphinganine (C18-(dihydro)ceramide) synthesis in a fumonisin B1-independent manner in mammalian cells. *The Journal of biological chemistry* **277**, 35642-35649, doi:10.1074/jbc.M205211200 (2002).

232 Riebeling, C., Allegood, J. C., Wang, E., Merrill, A. H., Jr. & Futerman, A. H. Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *The Journal of biological chemistry* **278**, 43452-43459, doi:10.1074/jbc.M307104200 (2003).

233 Weinmann, A., Galle, P. R. & Teufel, A. LASS6, an additional member of the longevity assurance gene family. *International journal of molecular medicine* **16**, 905-910 (2005).

234 Mizutani, Y., Kihara, A. & Igarashi, Y. LASS3 (longevity assurance homologue 3) is a mainly testis-specific (dihydro)ceramide synthase with relatively broad substrate specificity. *Biochem J* **398**, 531-538, doi:10.1042/BJ20060379 (2006).

235 Mullen, T. D., Hannun, Y. A. & Obeid, L. M. Ceramide synthases at the centre of sphingolipid metabolism and biology. *Biochemical Journal* **441**, 789-802 (2012).

236 Levy, M. & Futerman, A. H. Mammalian ceramide synthases. *IUBMB life* **62**, 347-356, doi:10.1002/iub.319 (2010).

237 Laviad, E. L., Kelly, S., Merrill, A. H., Jr. & Futerman, A. H. Modulation of ceramide synthase activity via dimerization. *The Journal of biological chemistry* **287**, 21025-21033, doi:10.1074/jbc.M112.363580 (2012).

238 Yamaji, T. & Hanada, K. Sphingolipid metabolism and interorganellar transport: localization of sphingolipid enzymes and lipid transfer proteins. *Traffic* **16**, 101-122, doi:10.1111/tra.12239 (2015).

239 Omae, F. *et al.* DES2 protein is responsible for phytoceramide biosynthesis in the mouse small intestine. *Biochem J* **379**, 687-695, doi:10.1042/BJ20031425 (2004).

240 Singh, J., Young, L., Handelsman, D. J. & Dong, Q. Molecular cloning and characterization of a novel androgen repressible gene expressed in the prostate epithelium. *Gene* **348**, 55-63, doi:10.1016/j.gene.2004.12.047 (2005).

241 Bianco, J. J., Handelsman, D. J., Pedersen, J. S. & Risbridger, G. P. Direct response of the murine prostate gland and seminal vesicles to estradiol. *Endocrinology* **143**, 4922-4933, doi:10.1210/en.2002-220493 (2002).

242 Duan, J. & Merrill, A. H., Jr. 1-Deoxysphingolipids Encountered Exogenously and Made de Novo: Dangerous Mysteries inside an Enigma. *The Journal of biological chemistry* **290**, 15380-15389, doi:10.1074/jbc.R115.658823 (2015).

243 Lone, M. A., Santos, T., Alecu, I., Silva, L. C. & Hornemann, T. 1-Deoxysphingolipids. *Biochim Biophys Acta Mol Cell Biol Lipids* **1864**, 512-521, doi:10.1016/j.bbalip.2018.12.013 (2019).

244 Zitomer, N. C. *et al.* Ceramide synthase inhibition by fumonisin B1 causes accumulation of 1-deoxysphinganine: a novel category of bioactive 1-deoxysphingoid bases and 1-deoxydihydroceramides biosynthesized by mammalian cell lines and animals. *The Journal of biological chemistry* **284**, 4786-4795, doi:10.1074/jbc.M808798200 (2009).

245 Alecu, I. *et al.* Localization of 1-deoxysphingolipids to mitochondria induces mitochondrial dysfunction. *J Lipid Res* **58**, 42-59, doi:10.1194/jlr.M068676 (2017).

246 Guntert, T. *et al.* 1-Deoxysphingolipid-induced neurotoxicity involves N-methyl-d-aspartate receptor signaling. *Neuropharmacology* **110**, 211-222, doi:10.1016/j.neuropharm.2016.03.033 (2016).

247 Sugiura, M. *et al.* Ceramide kinase, a novel lipid kinase. Molecular cloning and functional characterization. *The Journal of biological chemistry* **277**, 23294-23300, doi:10.1074/jbc.M201535200 (2002).

248 Bajjalieh, S. M., Martin, T. F. & Floor, E. Synaptic vesicle ceramide kinase. A calcium-stimulated lipid kinase that co-purifies with brain synaptic vesicles. *The Journal of biological chemistry* **264**, 14354-14360 (1989).

249 Dressler, K. A. & Kolesnick, R. N. Ceramide 1-phosphate, a novel phospholipid in human leukemia (HL-60) cells. Synthesis via ceramide from sphingomyelin. *The Journal of biological chemistry* **265**, 14917-14921 (1990).

250 Simanshu, D. K. *et al.* Non-vesicular trafficking by a ceramide-1-phosphate transfer protein regulates eicosanoids. *Nature* **500**, 463-467 (2013).

251 Wijesinghe, D. S. *et al.* Ceramide kinase is required for a normal eicosanoid response and the subsequent orderly migration of fibroblasts. *J Lipid Res* **55**, 1298-1309, doi:10.1194/jlr.M048207 (2014).

252 Hogback, S. *et al.* Ceramide 1-phosphate increases intracellular free calcium concentrations in thyroid FRTL-5 cells: evidence for an effect mediated by inositol 1,4,5-trisphosphate and intracellular sphingosine 1-phosphate. *Biochem J* **370**, 111-119, doi:10.1042/BJ20020970 (2003).

253 Gomez-Munoz, A., Kong, J. Y., Salh, B. & Steinbrecher, U. P. Ceramide-1-phosphate blocks apoptosis through inhibition of acid sphingomyelinase in macrophages. *J Lipid Res* **45**, 99-105, doi:10.1194/jlr.M300158-JLR200 (2004).

254 Gomez-Munoz, A. *et al.* Ceramide-1-phosphate promotes cell survival through activation of the phosphatidylinositol 3-kinase/protein kinase B pathway. *FEBS letters* **579**, 3744-3750, doi:10.1016/j.febslet.2005.05.067 (2005).

255 Hinkovska-Galcheva, V. *et al.* Ceramide 1-phosphate, a mediator of phagocytosis. *The Journal of biological chemistry* **280**, 26612-26621, doi:10.1074/jbc.M501359200 (2005).

256 Boudker, O. & Futerman, A. H. Detection and characterization of ceramide-1-phosphate phosphatase activity in rat liver plasma membrane. *The Journal of biological chemistry* **268**, 22150-22155 (1993).

257 Shinghal, R., Scheller, R. H. & Bajjalieh, S. M. Ceramide 1-phosphate phosphatase activity in brain. *J Neurochem* **61**, 2279-2285, doi:10.1111/j.1471-4159.1993.tb07470.x (1993).

258 Waggoner, D. W., Gómez-Muñoz, A., Dewald, J. & Brindley, D. N. Phosphatidate phosphohydrolase catalyzes the hydrolysis of ceramide 1-phosphate, lysophosphatidate, and sphingosine 1-phosphate. *Journal of Biological Chemistry* **271**, 16506-16509 (1996).

259 Brindley, D. N., Xu, J., Jasinska, R. & Waggoner, D. W. Analysis of ceramide 1-phosphate and sphingosine-1-phosphate phosphatase activities. *Methods in enzymology* **311**, 233-244, doi:10.1016/s0076-6879(00)11086-9 (2000).

260 Tang, X., Benesch, M. G. & Brindley, D. N. Lipid phosphate phosphatases and their roles in mammalian physiology and pathology. *J Lipid Res* **56**, 2048-2060, doi:10.1194/jlr.R058362 (2015).

261 Presa, N., Gomez-Larrauri, A., Dominguez-Herrera, A., Trueba, M. & Gomez-Munoz, A. Novel signaling aspects of ceramide 1-phosphate. *Biochim Biophys Acta Mol Cell Biol Lipids* **1865**, 158630, doi:10.1016/j.bbalip.2020.158630 (2020).

262 Bienias, K., Fiedorowicz, A., Sadowska, A., Prokopiuk, S. & Car, H. Regulation of sphingomyelin metabolism. *Pharmacol Rep* **68**, 570-581, doi:10.1016/j.pharep.2015.12.008 (2016).

263 Diringer, H., Marggraf, W. D., Koch, M. A. & Anderer, F. A. Evidence for a new biosynthetic pathway of sphingomyelin in SV 40 transformed mouse cells. *Biochemical and biophysical research communications* **47**, 1345-1352, doi:10.1016/0006-291x(72)90220-3 (1972).

264 Ullman, M. D. & Radin, N. S. The enzymatic formation of sphingomyelin from ceramide and lecithin in mouse liver. *The Journal of biological chemistry* **249**, 1506-1512 (1974).

265 D'Angelo, G., Moorthi, S. & Luberto, C. Role and Function of Sphingomyelin Biosynthesis in the Development of Cancer. *Advances in cancer research* **140**, 61-96, doi:10.1016/bs.acr.2018.04.009 (2018).

266 Huitema, K., van den Dikkenberg, J., Brouwers, J. F. & Holthuis, J. C. Identification of a family of animal sphingomyelin synthases. *The EMBO journal* **23**, 33-44 (2004).

267 Vacaru, A. M. *et al.* Sphingomyelin synthase-related protein SMSr controls ceramide homeostasis in the ER. *J Cell Biol* **185**, 1013-1027, doi:10.1083/jcb.200903152 (2009).

268 Tafesse, F. G. *et al.* Sphingomyelin synthase-related protein SMSr is a suppressor of ceramide-induced mitochondrial apoptosis. *Journal of cell science* **127**, 445-454, doi:10.1242/jcs.138933 (2014).

269 Ridgway, N. D. Interactions between metabolism and intracellular distribution of cholesterol and sphingomyelin. *Biochimica et biophysica acta* **1484**, 129-141, doi:10.1016/s1388-1981(00)00006-8 (2000).

270 Micheli, M., Albi, E., Leray, C. & Magni, M. V. Nuclear sphingomyelin protects RNA from RNase action. *FEBS letters* **431**, 443-447, doi:10.1016/s0014-5793(98)00810-2 (1998).

271 Scassellati, C. *et al.* Intranuclear sphingomyelin is associated with transcriptionally active chromatin and plays a role in nuclear integrity. *Biol Cell* **102**, 361-375, doi:10.1042/BC20090139 (2010).

272 Tafesse, F. G., Ternes, P. & Holthuis, J. C. The multigenic sphingomyelin synthase family. *The Journal of biological chemistry* **281**, 29421-29425, doi:10.1074/jbc.R600021200 (2006).

273 Schuchman, E. H. & Desnick, R. J. Types A and B Niemann-Pick disease. *Mol Genet Metab* **120**, 27-33, doi:10.1016/j.ymgme.2016.12.008 (2017).

274 Duan, R.-D. Alkaline sphingomyelinase: an old enzyme with novel implications. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids* **1761**, 281-291 (2006).

275 Selzner, M. *et al.* Induction of apoptotic cell death and prevention of tumor growth by ceramide analogues in metastatic human colon cancer. *Cancer research* **61**, 1233-1240 (2001).

276 Airola, M. V. & Hannun, Y. A. Sphingolipid metabolism and neutral sphingomyelinases. *Handbook of experimental pharmacology*, 57-76, doi:10.1007/978-3-7091-1368-4_3 (2013).

277 Morell, P. & Radin, N. S. Synthesis of cerebroside by brain from uridine diphosphate galactose and ceramide containing hydroxy fatty acid. *Biochemistry* **8**, 506-512, doi:10.1021/bi00830a008 (1969).

278 Basu, S., Kaufman, B. & Roseman, S. Enzymatic synthesis of glucocerebroside by a glucosyltransferase from embryonic chicken brain. *The Journal of biological chemistry* **248**, 1388-1394 (1973).

279 Shayman, J. A. Targeting Glucosylceramide Synthesis in the Treatment of Rare and Common Renal Disease. *Semin Nephrol* **38**, 183-192, doi:10.1016/j.semnephrol.2018.01.007 (2018).

280 Zoller, I., Bussow, H., Gieselmann, V. & Eckhardt, M. Oligodendrocyte-specific ceramide galactosyltransferase (CGT) expression phenotypically rescues CGT-deficient mice and demonstrates that CGT activity does not limit brain galactosylceramide level. *Glia* **52**, 190-198, doi:10.1002/glia.20230 (2005).

281 Xiao, S., Finkielstein, C. V. & Capelluto, D. G. The enigmatic role of sulfatides: new insights into cellular functions and mechanisms of protein recognition. *Advances in experimental medicine and biology* **991**, 27-40, doi:10.1007/978-94-007-6331-9_3 (2013).

282 Han, X. The pathogenic implication of abnormal interaction between apolipoprotein E isoforms, amyloid-beta peptides, and sulfatides in Alzheimer's disease. *Mol Neurobiol* **41**, 97-106, doi:10.1007/s12035-009-8092-x (2010).

283 Uemura, S., Go, S., Shishido, F. & Inokuchi, J.-i. Expression machinery of GM4: the excess amounts of GM3/GM4S synthase (ST3GAL5) are necessary for GM4 synthesis in mammalian cells. *Glycoconjugate journal* **31**, 101-108 (2014).

284 Yamashita, T. *et al.* A vital role for glycosphingolipid synthesis during development and differentiation. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 9142-9147, doi:10.1073/pnas.96.16.9142 (1999).

285 Platt, F. M. Sphingolipid lysosomal storage disorders. *Nature* **510**, 68-75, doi:10.1038/nature13476 (2014).

286 Messner, M. C. & Cabot, M. C. Glucosylceramide in humans. *Advances in experimental medicine and biology* **688**, 156-164, doi:10.1007/978-1-4419-6741-1_11 (2010).

287 Futerman, A. H. & Platt, F. M. The metabolism of glucocerebrosides - From 1965 to the present. *Mol Genet Metab* **120**, 22-26, doi:10.1016/j.ymgme.2016.11.390 (2017).

288 Grabowski, G. A. Gaucher disease and other storage disorders. *Hematology Am Soc Hematol Educ Program* **2012**, 13-18, doi:10.1182/asheducation-2012.1.13 (2012).

289 Aerts, J. *et al.* Glycosphingolipids and lysosomal storage disorders as illustrated by gaucher disease. *Curr Opin Chem Biol* **53**, 204-215, doi:10.1016/j.cbpa.2019.10.006 (2019).

290 Liu, Y. Y., Han, T. Y., Giuliano, A. E. & Cabot, M. C. Expression of glucosylceramide synthase, converting ceramide to glucosylceramide, confers adriamycin resistance in human breast cancer cells. *The Journal of biological chemistry* **274**, 1140-1146, doi:10.1074/jbc.274.2.1140 (1999).

291 Liu, Y. Y., Han, T. Y., Giuliano, A. E., Hansen, N. & Cabot, M. C. Uncoupling ceramide glycosylation by transfection of glucosylceramide synthase antisense reverses adriamycin resistance. *The Journal of biological chemistry* **275**, 7138-7143, doi:10.1074/jbc.275.10.7138 (2000).

292 Liu, Y. Y., Han, T. Y., Giuliano, A. E. & Cabot, M. C. Ceramide glycosylation potentiates cellular multidrug resistance. *FASEB J* **15**, 719-730, doi:10.1096/fj.00-0223com (2001).

293 Gouaze, V. *et al.* Overexpression of glucosylceramide synthase and P-glycoprotein in cancer cells selected for resistance to natural product chemotherapy. *Molecular cancer therapeutics* **3**, 633-639 (2004).

294 Groux-Degroote, S., Guerardel, Y. & Delannoy, P. Gangliosides: Structures, Biosynthesis, Analysis, and Roles in Cancer. *Chembiochem* **18**, 1146-1154, doi:10.1002/cbic.201600705 (2017).

295 Hakomori, S. Glycosylation defining cancer malignancy: new wine in an old bottle. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 10231-10233, doi:10.1073/pnas.172380699 (2002).

296 Schnaar, R. L. The Biology of Gangliosides. *Adv Carbohydr Chem Biochem* **76**, 113-148, doi:10.1016/bs.accb.2018.09.002 (2019).

297 Breiden, B. & Sandhoff, K. Mechanism of Secondary Ganglioside and Lipid Accumulation in Lysosomal Disease. *International journal of molecular sciences* **21**, doi:10.3390/ijms21072566 (2020).

298 Krengel, U. & Bousquet, P. A. Molecular recognition of gangliosides and their potential for cancer immunotherapies. *Front Immunol* **5**, 325, doi:10.3389/fimmu.2014.00325 (2014).

299 Hakomori, S. I. Structure and function of glycosphingolipids and sphingolipids: recollections and future trends. *Biochimica et biophysica acta* **1780**, 325-346, doi:10.1016/j.bbagen.2007.08.015 (2008).

300 Felis, A., Whitlow, M., Kraus, A., Warnock, D. G. & Wallace, E. Current and investigational therapeutics for Fabry disease. *Kidney international reports* **5**, 407-413 (2020).

301 Li, C. M. *et al.* Insertional mutagenesis of the mouse acid ceramidase gene leads to early embryonic lethality in homozygotes and progressive lipid storage disease in heterozygotes. *Genomics* **79**, 218-224, doi:10.1006/geno.2002.6686 (2002).

302 Bernardo, K. *et al.* Purification, characterization, and biosynthesis of human acid ceramidase. *The Journal of biological chemistry* **270**, 11098-11102, doi:10.1074/jbc.270.19.11098 (1995).

303 Tani, M., Igarashi, Y. & Ito, M. Involvement of neutral ceramidase in ceramide metabolism at the plasma membrane and in extracellular milieu. *The Journal of biological chemistry* **280**, 36592-36600, doi:10.1074/jbc.M506827200 (2005).

304 Kono, M. *et al.* Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. *The Journal of biological chemistry* **281**, 7324-7331, doi:10.1074/jbc.M508382200 (2006).

305 Mao, C. *et al.* Cloning and characterization of a novel human alkaline ceramidase. A mammalian enzyme that hydrolyzes phytoceramide. *The Journal of biological chemistry* **276**, 26577-26588, doi:10.1074/jbc.M102818200 (2001).

306 Mao, C. *et al.* Cloning and characterization of a mouse endoplasmic reticulum alkaline ceramidase: an enzyme that preferentially regulates metabolism of very long chain ceramides. *The Journal of biological chemistry* **278**, 31184-31191, doi:10.1074/jbc.M303875200 (2003).

307 Xu, R. *et al.* Golgi alkaline ceramidase regulates cell proliferation and survival by controlling levels of sphingosine and S1P. *FASEB J* **20**, 1813-1825, doi:10.1096/fj.05-5689com (2006).

308 Sun, W. *et al.* Upregulation of the human alkaline ceramidase 1 and acid ceramidase mediates calcium-induced differentiation of epidermal keratinocytes. *J Invest Dermatol* **128**, 389-397, doi:10.1038/sj.jid.5701025 (2008).

309 Wang, K. *et al.* Alkaline ceramidase 3 deficiency aggravates colitis and colitis-associated tumorigenesis in mice by hyperactivating the innate immune system. *Cell death & disease* 7, e2124, doi:10.1038/cddis.2016.36 (2016).

310 Maceyka, M. & Spiegel, S. Sphingolipid metabolites in inflammatory disease. *Nature* **510**, 58-67, doi:10.1038/nature13475 (2014).

311 Xiong, Y., Yang, P., Proia, R. L. & Hla, T. Erythrocyte-derived sphingosine 1-phosphate is essential for vascular development. *The Journal of clinical investigation* **124**, 4823-4828, doi:10.1172/JCI77685 (2014).

312 Pappu, R. *et al.* Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* **316**, 295-298, doi:10.1126/science.1139221 (2007).

313 Pham, T. H. *et al.* Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med* **207**, 17-27, doi:10.1084/jem.20091619 (2010).

314 Snider, A. J. *et al.* A role for sphingosine kinase 1 in dextran sulfate sodium‐induced colitis. *The FASEB Journal* **23**, 143-152 (2009).

315 Mizugishi, K. *et al.* Essential role for sphingosine kinases in neural and vascular development. *Molecular and cellular biology* **25**, 11113-11121, doi:10.1128/MCB.25.24.11113-11121.2005 (2005).

316 Bryan, A. M. & Del Poeta, M. Sphingosine‐1‐phosphate receptors and innate immunity. *Cellular microbiology* **20**, e12836 (2018).

317 Xiong, Y. *et al.* CD4 T cell sphingosine 1-phosphate receptor (S1PR)1 and S1PR4 and endothelial S1PR2 regulate afferent lymphatic migration. *Sci Immunol* **4**, doi:10.1126/sciimmunol.aav1263 (2019).

318 Hait, N. C. *et al.* Regulation of histone acetylation in the nucleus by sphingosine-1-phosphate. *Science* **325**, 1254-1257, doi:10.1126/science.1176709 (2009).

319 Mandala, S. M. *et al.* Sphingoid base 1-phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 150-155, doi:10.1073/pnas.95.1.150 (1998).

320 Ogawa, C., Kihara, A., Gokoh, M. & Igarashi, Y. Identification and characterization of a novel human sphingosine-1-phosphate phosphohydrolase, hSPP2. *The Journal of biological chemistry* **278**, 1268-1272, doi:10.1074/jbc.M209514200 (2003).

321 Le Stunff, H. *et al.* Recycling of sphingosine is regulated by the concerted actions of sphingosine-1-phosphate phosphohydrolase 1 and sphingosine kinase 2. *The Journal of biological chemistry* **282**, 34372-34380, doi:10.1074/jbc.M703329200 (2007).

322 Johnson, K. R. *et al.* Role of human sphingosine-1-phosphate phosphatase 1 in the regulation of intra- and extracellular sphingosine-1-phosphate levels and cell viability. *The Journal of biological chemistry* **278**, 34541-34547, doi:10.1074/jbc.M301741200 (2003).

323 Cartier, A. & Hla, T. Sphingosine 1-phosphate: Lipid signaling in pathology and therapy. *Science* **366**, doi:10.1126/science.aar5551 (2019).

324 Stoffel, W., LeKim, D. & Sticht, G. Distribution and properties of dihydrosphingosine-1-phosphate aldolase (sphinganine-1-phosphate alkanal-

lyase). *Hoppe Seylers Z Physiol Chem* **350**, 1233-1241, doi:10.1515/bchm2.1969.350.2.1233 (1969).

325 Ikeda, M., Kihara, A. & Igarashi, Y. Sphingosine-1-phosphate lyase SPL is an endoplasmic reticulum-resident, integral membrane protein with the pyridoxal 5'-phosphate binding domain exposed to the cytosol. *Biochemical and biophysical research communications* **325**, 338-343, doi:10.1016/j.bbrc.2004.10.036 (2004).

326 Schmahl, J., Raymond, C. S. & Soriano, P. PDGF signaling specificity is mediated through multiple immediate early genes. *Nature genetics* **39**, 52-60, doi:10.1038/ng1922 (2007).

327 Shaw, J. *et al.* Novel Sphingolipid-Based Cancer Therapeutics in the Personalized Medicine Era. *Advances in cancer research* **140**, 327-366, doi:10.1016/bs.acr.2018.04.016 (2018).

328 Maula, T., Al Sazzad, M. A. & Slotte, J. P. Influence of hydroxylation, chain length, and chain unsaturation on bilayer properties of ceramides. *Biophysical journal* **109**, 1639-1651 (2015).

329 Fahy, E. *et al.* A comprehensive classification system for lipids. *J Lipid Res* **46**, 839-861, doi:10.1194/jlr.E400004-JLR200 (2005).

330 Castro, B. M., Prieto, M. & Silva, L. C. Ceramide: a simple sphingolipid with unique biophysical properties. *Progress in lipid research* **54**, 53-67, doi:10.1016/j.plipres.2014.01.004 (2014).

331 Basanez, G. *et al.* Morphological changes induced by phospholipase C and by sphingomyelinase on large unilamellar vesicles: a cryo-transmission electron microscopy study of liposome fusion. *Biophys J* **72**, 2630-2637, doi:10.1016/S0006-3495(97)78906-9 (1997).

332 Montes, L. R., Ruiz-Arguello, M. B., Goni, F. M. & Alonso, A. Membrane restructuring via ceramide results in enhanced solute efflux. *The Journal of biological chemistry* **277**, 11788-11794, doi:10.1074/jbc.M111568200 (2002).

333 Dobrowsky, R. T., Kamibayashi, C., Mumby, M. C. & Hannun, Y. A. Ceramide activates heterotrimeric protein phosphatase 2A. *The Journal of biological chemistry* **268**, 15523-15530 (1993).

334 Bourbon, N. A., Yun, J. & Kester, M. Ceramide directly activates protein kinase C zeta to regulate a stress-activated protein kinase signaling complex. *The Journal of biological chemistry* **275**, 35617-35623, doi:10.1074/jbc.M007346200 (2000).

335 Ruvolo, P. P., Clark, W., Mumby, M., Gao, F. & May, W. S. A functional role for the B56 alpha-subunit of protein phosphatase 2A in ceramide-mediated regulation of Bcl2 phosphorylation status and function. *The Journal of biological chemistry* **277**, 22847-22852, doi:10.1074/jbc.M201830200 (2002).

336 Fox, T. E. *et al.* Ceramide recruits and activates protein kinase C zeta (PKC zeta) within structured membrane microdomains. *The Journal of biological chemistry* **282**, 12450-12457, doi:10.1074/jbc.M700082200 (2007).

337 Dadsena, S. *et al.* Ceramides bind VDAC2 to trigger mitochondrial apoptosis. *Nat Commun* **10**, 1832, doi:10.1038/s41467-019-09654-4 (2019).

338 Stiban, J. & Perera, M. Very long chain ceramides interfere with C16-ceramide-induced channel formation: A plausible mechanism for regulating the initiation of intrinsic apoptosis. *Biochimica et biophysica acta* **1848**, 561-567, doi:10.1016/j.bbamem.2014.11.018 (2015).

339 Kroesen, B. J. *et al.* BcR-induced apoptosis involves differential regulation of C16 and C24-ceramide formation and sphingolipid-dependent activation of the proteasome. *The Journal of biological chemistry* **278**, 14723-14731, doi:10.1074/jbc.M210756200 (2003).

340 Siskind, L. J. *et al.* Anti-apoptotic Bcl-2 Family Proteins Disassemble Ceramide Channels. *The Journal of biological chemistry* **283**, 6622-6630, doi:10.1074/jbc.M706115200 (2008).

341 Siskind, L. J. *et al.* The BCL-2 protein BAK is required for long-chain ceramide generation during apoptosis. *The Journal of biological chemistry* **285**, 11818-11826, doi:10.1074/jbc.M109.078121 (2010).

342 Hartmann, D. *et al.* Long chain ceramides and very long chain ceramides have opposite effects on human breast and colon cancer cell growth. *Int J Biochem Cell Biol* **44**, 620-628, doi:10.1016/j.biocel.2011.12.019 (2012).

343 Oleinik, N. *et al.* Mitochondrial protein import is regulated by p17/PERMIT to mediate lipid metabolism and cellular stress. *Sci Adv* **5**, eaax1978, doi:10.1126/sciadv.aax1978 (2019).

344 Colombini, M. Ceramide Channels. *Advances in experimental medicine and biology* **1159**, 33-48, doi:10.1007/978-3-030-21162-2_3 (2019).

345 Obeid, L. M., Linardic, C. M., Karolak, L. A. & Hannun, Y. A. Programmed cell death induced by ceramide. *Science* **259**, 1769-1771, doi:10.1126/science.8456305 (1993).

346 Cowart, L. A. & Obeid, L. M. Yeast sphingolipids: recent developments in understanding biosynthesis, regulation, and function. *Biochimica et biophysica acta* **1771**, 421-431, doi:10.1016/j.bbalip.2006.08.005 (2007).

347 Beckham, T. H., Cheng, J. C., Marrison, S. T., Norris, J. S. & Liu, X. Interdiction of sphingolipid metabolism to improve standard cancer therapies. *Advances in cancer research* **117**, 1-36, doi:10.1016/B978-0-12-394274-6.00001-7 (2013).

348 Bose, R. *et al.* Ceramide synthase mediates daunorubicin-induced apoptosis: an alternative mechanism for generating death signals. *Cell* **82**, 405-414, doi:10.1016/0092-8674(95)90429-8 (1995).

349 Perry, D. K. *et al.* Serine palmitoyltransferase regulates de novo ceramide generation during etoposide-induced apoptosis. *The Journal of biological chemistry* **275**, 9078-9084, doi:10.1074/jbc.275.12.9078 (2000).

350 Chalfant, C. E. *et al.* De novo ceramide regulates the alternative splicing of caspase 9 and Bcl-x in A549 lung adenocarcinoma cells. Dependence on protein

phosphatase-1. *The Journal of biological chemistry* **277**, 12587-12595, doi:10.1074/jbc.M112010200 (2002).

351 Chauvier, D., Morjani, H. & Manfait, M. Ceramide involvement in homocamptothecin- and camptothecin-induced cytotoxicity and apoptosis in colon HT29 cells. *International journal of oncology* **20**, 855-863 (2002).

352 Strum, J. C., Small, G. W., Pauig, S. B. & Daniel, L. W. 1-beta-D-Arabinofuranosylcytosine stimulates ceramide and diglyceride formation in HL-60 cells. *The Journal of biological chemistry* **269**, 15493-15497 (1994).

353 Jaffrezou, J. P. *et al.* Daunorubicin-induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis. *EMBO J* **15**, 2417-2424 (1996).

354 Haimovitz-Friedman, A. *et al.* Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. *J Exp Med* **180**, 525-535 (1994).

355 Santana, P. *et al.* Acid sphingomyelinase-deficient human lymphoblasts and mice are defective in radiation-induced apoptosis. *Cell* **86**, 189-199 (1996).

356 Lozano, J. *et al.* Cell autonomous apoptosis defects in acid sphingomyelinase knockout fibroblasts. *The Journal of biological chemistry* **276**, 442-448, doi:10.1074/jbc.M006353200 (2001).

357 Vit, J. P. & Rosselli, F. Role of the ceramide-signaling pathways in ionizing radiation-induced apoptosis. *Oncogene* **22**, 8645-8652, doi:10.1038/sj.onc.1207087 (2003).

358 Scarlatti, F. *et al.* Resveratrol sensitization of DU145 prostate cancer cells to ionizing radiation is associated to ceramide increase. *Cancer letters* **253**, 124-130, doi:10.1016/j.canlet.2007.01.014 (2007).

359 Mesicek, J. *et al.* Ceramide synthases 2, 5, and 6 confer distinct roles in radiation-induced apoptosis in HeLa cells. *Cell Signal* **22**, 1300-1307, doi:10.1016/j.cellsig.2010.04.006 (2010).

360 Galadari, S., Rahman, A., Pallichankandy, S. & Thayyullathil, F. Tumor suppressive functions of ceramide: evidence and mechanisms. *Apoptosis : an international journal on programmed cell death* **20**, 689-711, doi:10.1007/s10495-015-1109-1 (2015).

361 Hara, S. *et al.* p53-Independent ceramide formation in human glioma cells during gamma-radiation-induced apoptosis. *Cell death and differentiation* **11**, 853-861, doi:10.1038/sj.cdd.4401428 (2004).

362 Mahdy, A. E. *et al.* Acid ceramidase upregulation in prostate cancer cells confers resistance to radiation: AC inhibition, a potential radiosensitizer. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**, 430-438, doi:10.1038/mt.2008.281 (2009).

363 Cheng, J. C. *et al.* Radiation-induced acid ceramidase confers prostate cancer resistance and tumor relapse. *The Journal of clinical investigation* **123**, 4344-4358, doi:10.1172/JCI64791 (2013).

364 Tan, S. F. *et al.* Acid ceramidase promotes drug resistance in acute myeloid leukemia through NF-kappaB-dependent P-glycoprotein upregulation. *J Lipid Res* **60**, 1078-1086, doi:10.1194/jlr.M091876 (2019).

365 Pearson, J. M. *et al.* Ceramide Analogue SACLAC Modulates Sphingolipid Levels and MCL-1 Splicing to Induce Apoptosis in Acute Myeloid Leukemia. *Molecular cancer research : MCR* **18**, 352-363, doi:10.1158/1541-7786.MCR-19-0619 (2020).

366 White-Gilbertson, S. *et al.* Tamoxifen is a candidate first-in-class inhibitor of acid ceramidase that reduces amitotic division in polyploid giant cancer cells-Unrecognized players in tumorigenesis. *Cancer Med*, doi:10.1002/cam4.2960 (2020).

367 Lavie, Y. *et al.* Agents that reverse multidrug resistance, tamoxifen, verapamil, and cyclosporin A, block glycosphingolipid metabolism by inhibiting ceramide glycosylation in human cancer cells. *The Journal of biological chemistry* **272**, 1682-1687, doi:10.1074/jbc.272.3.1682 (1997).

368 Liu, Y. Y. *et al.* Glucosylceramide synthase, a factor in modulating drug resistance, is overexpressed in metastatic breast carcinoma. *International journal of oncology* **39**, 425-431, doi:10.3892/ijo.2011.1052 (2011).

369 Madigan, J. P. *et al.* A role for ceramide glycosylation in resistance to oxaliplatin in colorectal cancer. *Experimental cell research* **388**, 111860 (2020).

370 Beckham, T. H. *et al.* Acid ceramidase induces sphingosine kinase 1/S1P receptor 2-mediated activation of oncogenic Akt signaling. *Oncogenesis* **2**, e49, doi:10.1038/oncsis.2013.14 (2013).

371 Akao, Y. *et al.* High expression of sphingosine kinase 1 and S1P receptors in chemotherapy-resistant prostate cancer PC3 cells and their camptothecin-induced up-regulation. *Biochemical and biophysical research communications* **342**, 1284-1290, doi:10.1016/j.bbrc.2006.02.070 (2006).

372 Wang, F. & Wu, Z. Sphingosine kinase 1 overexpression is associated with poor prognosis and oxaliplatin resistance in hepatocellular carcinoma. *Experimental and therapeutic medicine* **15**, 5371-5376 (2018).

373 Oskouian, B. *et al.* Sphingosine-1-phosphate lyase potentiates apoptosis via p53- and p38-dependent pathways and is down-regulated in colon cancer. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 17384-17389, doi:10.1073/pnas.0600050103 (2006).

374 Lee, H. *et al.* Mitochondrial ceramide-rich macrodomains functionalize Bax upon irradiation. *PloS one* **6**, e19783, doi:10.1371/journal.pone.0019783 (2011).

375 Chang, K. T. *et al.* Ceramide channels: destabilization by Bcl-xL and role in apoptosis. *Biochimica et biophysica acta* **1848**, 2374-2384, doi:10.1016/j.bbamem.2015.07.013 (2015).

376 Chipuk, J. E. *et al.* Sphingolipid metabolism cooperates with BAK and BAX to promote the mitochondrial pathway of apoptosis. *Cell* **148**, 988-1000, doi:10.1016/j.cell.2012.01.038 (2012).

377 Blom, T. *et al.* LAPTM4B facilitates late endosomal ceramide export to control cell death pathways. *Nat Chem Biol* **11**, 799-806, doi:10.1038/nchembio.1889 (2015).

378 Chalfant, C. E. *et al.* FAS activation induces dephosphorylation of SR proteins; dependence on the de novo generation of ceramide and activation of protein phosphatase 1. *The Journal of biological chemistry* **276**, 44848-44855, doi:10.1074/jbc.M106291200 (2001).

379 Zhang, X., Matsuda, M., Yaegashi, N., Nabe, T. & Kitatani, K. Regulation of Necroptosis by Phospholipids and Sphingolipids. *Cells* **9**, doi:10.3390/cells9030627 (2020).

380 Saddoughi, S. A. & Ogretmen, B. Diverse functions of ceramide in cancer cell death and proliferation. *Advances in cancer research* **117**, 37-58, doi:10.1016/B978-0-12-394274-6.00002-9 (2013).

381 Zhu, W., Wang, X., Zhou, Y. & Wang, H. C2-ceramide induces cell death and protective autophagy in head and neck squamous cell carcinoma cells. *International journal of molecular sciences* **15**, 3336-3355, doi:10.3390/ijms15023336 (2014).

382 Zhang, X. *et al.* Ceramide Nanoliposomes as a MLKL-Dependent, Necroptosis-Inducing, Chemotherapeutic Reagent in Ovarian Cancer. *Molecular cancer therapeutics* **17**, 50-59, doi:10.1158/1535-7163.MCT-17-0173 (2018).

383 Nganga, R. *et al.* Receptor-interacting Ser/Thr kinase 1 (RIPK1) and myosin IIA-dependent ceramidosomes form membrane pores that mediate blebbing and necroptosis. *The Journal of biological chemistry* **294**, 502-519, doi:10.1074/jbc.RA118.005865 (2019).

384 Young, M. M. & Wang, H. G. Sphingolipids as Regulators of Autophagy and Endocytic Trafficking. *Advances in cancer research* **140**, 27-60, doi:10.1016/bs.acr.2018.04.008 (2018).

385 Jiang, W. & Ogretmen, B. Autophagy paradox and ceramide. *Biochimica et biophysica acta* **1841**, 783-792, doi:10.1016/j.bbalip.2013.09.005 (2014).

386 Sentelle, R. D. *et al.* Ceramide targets autophagosomes to mitochondria and induces lethal mitophagy. *Nat Chem Biol* **8**, 831-838, doi:10.1038/nchembio.1059 (2012).

387 Dany, M. *et al.* Targeting FLT3-ITD signaling mediates ceramide-dependent mitophagy and attenuates drug resistance in AML. *Blood* **128**, 1944-1958, doi:10.1182/blood-2016-04-708750 (2016).

388 Grosch, S., Schiffmann, S. & Geisslinger, G. Chain length-specific properties of ceramides. *Progress in lipid research* **51**, 50-62, doi:10.1016/j.plipres.2011.11.001 (2012).

389 Nybond, S., Bjorkqvist, Y. J., Ramstedt, B. & Slotte, J. P. Acyl chain length affects ceramide action on sterol/sphingomyelin-rich domains. *Biochimica et biophysica acta* **1718**, 61-66, doi:10.1016/j.bbamem.2005.10.009 (2005).

390 Megha, Sawatzki, P., Kolter, T., Bittman, R. & London, E. Effect of ceramide N-acyl chain and polar headgroup structure on the properties of ordered lipid domains (lipid rafts). *Biochimica et biophysica acta* **1768**, 2205-2212, doi:10.1016/j.bbamem.2007.05.007 (2007).

391 Barth, B. M., Cabot, M. C. & Kester, M. Ceramide-based therapeutics for the treatment of cancer. *Anticancer Agents Med Chem* **11**, 911-919 (2011).

392 Chapman, J. V. *et al.* Metabolism of short-chain ceramide by human cancer cells--implications for therapeutic approaches. *Biochemical pharmacology* **80**, 308-315, doi:10.1016/j.bcp.2010.04.001 (2010).

393 Stover, T. & Kester, M. Liposomal delivery enhances short-chain ceramide-induced apoptosis of breast cancer cells. *J Pharmacol Exp Ther* **307**, 468-475, doi:10.1124/jpet.103.054056 (2003).

394 Stover, T. C., Sharma, A., Robertson, G. P. & Kester, M. Systemic delivery of liposomal short-chain ceramide limits solid tumor growth in murine models of breast adenocarcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research* **11**, 3465-3474, doi:10.1158/1078-0432.CCR-04-1770 (2005).

395 Zolnik, B. S. *et al.* Rapid distribution of liposomal short-chain ceramide in vitro and in vivo. *Drug Metab Dispos* **36**, 1709-1715, doi:10.1124/dmd.107.019679 (2008).

396 Kester, M. *et al.* Preclinical development of a C6-ceramide NanoLiposome, a novel sphingolipid therapeutic. *Biological chemistry* **396**, 737-747, doi:10.1515/hsz-2015-0129 (2015).

397 Klibanov, A. L., Maruyama, K., Torchilin, V. P. & Huang, L. Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes. *FEBS letters* **268**, 235-237, doi:10.1016/0014-5793(90)81016-h (1990).

398 Hankins, J. L. *et al.* The therapeutic potential of nanoscale sphingolipid technologies. *Handbook of experimental pharmacology*, 197-210, doi:10.1007/978-3-7091-1368-4_11 (2013).

399 Ryland, L. K. *et al.* C6-ceramide nanoliposomes target the Warburg effect in chronic lymphocytic leukemia. *PloS one* **8**, e84648, doi:10.1371/journal.pone.0084648 (2013).

400 Watters, R. J., Kester, M., Tran, M. A., Loughran, T. P., Jr. & Liu, X. Development and use of ceramide nanoliposomes in cancer. *Methods in enzymology* **508**, 89-108, doi:10.1016/B978-0-12-391860-4.00005-7 (2012).

401 Narayan, P. & Dahiya, R. Alterations in sphingomyelin and fatty acids in human benign prostatic hyperplasia and prostatic cancer. *Biomed Biochim Acta* **50**, 1099-1108 (1991).

402 Dahiya, R. *et al.* Metastasis-associated alterations in phospholipids and fatty acids of human prostatic adenocarcinoma cell lines. *Biochem Cell Biol* **70**, 548-554, doi:10.1139/o92-085 (1992).

403 Ravindranath, M. H. *et al.* Gangliosides of organ-confined versus metastatic androgen-receptor-negative prostate cancer. *Biochemical and biophysical research communications* **324**, 154-165, doi:10.1016/j.bbrc.2004.09.029 (2004).

404 Sosnowski, J., Stetter-Neel, C., Cole, D., Durham, J. P. & Mawhinney, M. G. Protein kinase C mediated anti-proliferative glucocorticoid-sphinganine synergism in cultured Pollard III prostate tumor cells. *The Journal of urology* **158**, 269-274, doi:10.1097/00005392-199707000-00084 (1997).

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

406 Nava, V. E. *et al.* Sphingosine enhances apoptosis of radiation-resistant prostate cancer cells. *Cancer research* **60**, 4468-4474 (2000).

407 Wang, X. Z., Beebe, J. R., Pwiti, L., Bielawska, A. & Smyth, M. J. Aberrant sphingolipid signaling is involved in the resistance of prostate cancer cell lines to chemotherapy. *Cancer research* **59**, 5842-5848 (1999).

408 Akao, Y. *et al.* Ceramide accumulation is independent of camptothecin-induced apoptosis in prostate cancer LNCaP cells. *Biochemical and biophysical research communications* **294**, 363-370, doi:10.1016/S0006-291X(02)00462-X (2002).

409 Gewies, A., Rokhlin, O. W. & Cohen, M. B. Ceramide induces cell death in the human prostatic carcinoma cell lines PC3 and DU145 but does not seem to be involved in Fas-mediated apoptosis. *Lab Invest* **80**, 671-676, doi:10.1038/labinvest.3780070 (2000).

410 Engedal, N. & Saatcioglu, F. Ceramide-induced cell death in the prostate cancer cell line LNCaP has both necrotic and apoptotic features. *The Prostate* **46**, 289-297, doi:10.1002/1097-0045(20010301)46:4<289::aid-pros1035>3.0.co;2-k (2001).

411 Eto, M. *et al.* C16 ceramide accumulates following androgen ablation in LNCaP prostate cancer cells. *The Prostate* **57**, 66-79, doi:10.1002/pros.10275 (2003).

412 Murdica, V. *et al.* Abiraterone and Ionizing Radiation Alter the Sphingolipid Homeostasis in Prostate Cancer Cells. *Advances in experimental medicine and biology* **1112**, 293-307, doi:10.1007/978-981-13-3065-0_20 (2018).

413 Lin, H. M. *et al.* A distinct plasma lipid signature associated with poor prognosis in castration-resistant prostate cancer. *International journal of cancer. Journal international du cancer* **141**, 2112-2120, doi:10.1002/ijc.30903 (2017).

414 Seelan, R. S. *et al.* Human acid ceramidase is overexpressed but not mutated in prostate cancer. *Genes, chromosomes & cancer* **29**, 137-146, doi:10.1002/1098-2264(2000)9999:9999<::aid-gcc1018>3.0.co;2-e (2000).

415 Camacho, L. *et al.* Acid ceramidase as a therapeutic target in metastatic prostate cancer. *J Lipid Res* **54**, 1207-1220, doi:10.1194/jlr.M032375 (2013).

416 Saad, A. F. *et al.* The functional effects of acid ceramidase overexpression in prostate cancer progression and resistance to chemotherapy. *Cancer biology & therapy* **6**, 1455-1460 (2007).

417 Turner, L. S. *et al.* Autophagy is increased in prostate cancer cells overexpressing acid ceramidase and enhances resistance to C6 ceramide. *Prostate cancer and prostatic diseases* **14**, 30-37, doi:10.1038/pcan.2010.47 (2011).

418 Malavaud, B. *et al.* Sphingosine kinase-1 activity and expression in human prostate cancer resection specimens. *Eur J Cancer* **46**, 3417-3424, doi:10.1016/j.ejca.2010.07.053 (2010).

419 Voelkel-Johnson, C., Norris, J. S. & White-Gilbertson, S. Interdiction of Sphingolipid Metabolism Revisited: Focus on Prostate Cancer. *Advances in cancer research* **140**, 265-293, doi:10.1016/bs.acr.2018.04.014 (2018).

420 Faustino, R. S. *et al.* Ceramide regulation of nuclear protein import. *J Lipid Res* **49**, 654-662, doi:10.1194/jlr.M700464-JLR200 (2008).

421 Zhu, Q. Y. *et al.* C6-ceramide synergistically potentiates the anti-tumor effects of histone deacetylase inhibitors via AKT dephosphorylation and alpha-tubulin hyperacetylation both in vitro and in vivo. *Cell death & disease* **2**, e117, doi:10.1038/cddis.2010.96 (2011).

422 Yu, C., Alterman, M. & Dobrowsky, R. T. Ceramide displaces cholesterol from lipid rafts and decreases the association of the cholesterol binding protein caveolin-1. *J Lipid Res* **46**, 1678-1691, doi:10.1194/jlr.M500060-JLR200 (2005).

423 Blouin, C. M. *et al.* Plasma membrane subdomain compartmentalization contributes to distinct mechanisms of ceramide action on insulin signaling. *Diabetes* **59**, 600-610, doi:10.2337/db09-0897 (2010).

424 Chen, C. L. *et al.* Ceramide induces p38 MAPK and JNK activation through a mechanism involving a thioredoxin-interacting protein-mediated pathway. *Blood* **111**, 4365-4374, doi:10.1182/blood-2007-08-106336 (2008).

425 Kajiwara, K. *et al.* c-Src-induced activation of ceramide metabolism impairs membrane microdomains and promotes malignant progression by facilitating the translocation of c-Src to focal adhesions. *Biochem J* **458**, 81-93, doi:10.1042/BJ20130527 (2014).

426 Pulli, I., Asghar, M. Y., Kemppainen, K. & Tornquist, K. Sphingolipid-mediated calcium signaling and its pathological effects. *Biochim Biophys Acta Mol Cell Res* **1865**, 1668-1677, doi:10.1016/j.bbamcr.2018.04.012 (2018).

427 Sharma, N. L. *et al.* The androgen receptor induces a distinct transcriptional program in castration-resistant prostate cancer in man. *Cancer cell* **23**, 35-47, doi:10.1016/j.ccr.2012.11.010 (2013).

428 Barfeld, S. J., Itkonen, H. M., Urbanucci, A. & Mills, I. G. Androgen-regulated metabolism and biosynthesis in prostate cancer. *Endocrine-related cancer* **21**, T57-66, doi:10.1530/ERC-13-0515 (2014).

429 Bravo, R. *et al.* Endoplasmic reticulum and the unfolded protein response: dynamics and metabolic integration. *Int Rev Cell Mol Biol* **301**, 215-290, doi:10.1016/B978-0-12-407704-1.00005-1 (2013).

430 Grummt, I. The nucleolus-guardian of cellular homeostasis and genome integrity. *Chromosoma* **122**, 487-497, doi:10.1007/s00412-013-0430-0 (2013).

431 Segawa, T. *et al.* Androgen-induced expression of endoplasmic reticulum (ER) stress response genes in prostate cancer cells. *Oncogene* **21**, 8749-8758, doi:10.1038/sj.onc.1205992 (2002).

432 Li, S. *et al.* Angiogenin mediates androgen-stimulated prostate cancer growth and enables castration resistance. *Molecular cancer research : MCR* **11**, 1203-1214, doi:10.1158/1541-7786.MCR-13-0072 (2013).

433 Zaidi, N., Swinnen, J. V. & Smans, K. ATP-citrate lyase: a key player in cancer metabolism. *Cancer research* **72**, 3709-3714, doi:10.1158/0008-5472.CAN-11-4112 (2012).

434 Wellen, K. E. *et al.* ATP-citrate lyase links cellular metabolism to histone acetylation. *Science* **324**, 1076-1080, doi:10.1126/science.1164097 (2009).

435 Santana, P. *et al.* Interleukin-1 beta stimulates sphingomyelin hydrolysis in cultured granulosa cells: evidence for a regulatory role of ceramide on progesterone and prostaglandin biosynthesis. *Endocrinology* **137**, 2480-2489, doi:10.1210/endo.137.6.8641202 (1996).

436 Budnik, L. T., Jahner, D. & Mukhopadhyay, A. K. Inhibitory effects of TNF alpha on mouse tumor Leydig cells: possible role of ceramide in the mechanism of action. *Mol Cell Endocrinol* **150**, 39-46, doi:10.1016/s0303-7207(99)00029-5 (1999).

437 Meroni, S. B., Pellizzari, E. H., Canepa, D. F. & Cigorraga, S. B. Possible involvement of ceramide in the regulation of rat Leydig cell function. *J Steroid Biochem Mol Biol* **75**, 307-313, doi:10.1016/s0960-0760(00)00188-6 (2000).

438 Li, Q. L. *et al.* Inhibition of steroidogenesis and induction of apoptosis in rat luteal cells by cell-permeable ceramide in vitro. *Sheng Li Xue Bao* **53**, 142-146 (2001).

439 Morales, V. *et al.* Intratesticular delivery of tumor necrosis factor-alpha and ceramide directly abrogates steroidogenic acute regulatory protein expression and Leydig cell steroidogenesis in adult rats. *Endocrinology* **144**, 4763-4772, doi:10.1210/en.2003-0569 (2003).

440 Kwun, C. *et al.* Ceramide increases steroid hormone production in MA-10 Leydig cells. *Steroids* **64**, 499-509, doi:10.1016/s0039-128x(99)00013-6 (1999).

441 Soboloff, J., Sorisky, A., Desilets, M. & Tsang, B. K. Acyl chain length-specific ceramide-induced changes in intracellular Ca2+ concentration and progesterone production are not regulated by tumor necrosis factor alpha in hen granulosa cells. *Biol Reprod* **60**, 262-271, doi:10.1095/biolreprod60.2.262 (1999).

442 Arai, N. *et al.* Ceramide and adenosine 5'-monophosphate-activated protein kinase are two novel regulators of 11beta-hydroxysteroid dehydrogenase type 1 expression and activity in cultured preadipocytes. *Endocrinology* **148**, 5268-5277, doi:10.1210/en.2007-0349 (2007).

443 Ruckhaberle, E. *et al.* Acid ceramidase 1 expression correlates with a better prognosis in ER-positive breast cancer. *Climacteric* **12**, 502-513, doi:10.3109/13697130902939913 (2009).

444 Marchetti, M. C., Di Marco, B., Cifone, G., Migliorati, G. & Riccardi, C. Dexamethasone-induced apoptosis of thymocytes: role of glucocorticoid receptor-associated Src kinase and caspase-8 activation. *Blood* **101**, 585-593, doi:10.1182/blood-2002-06-1779 (2003).

445 Lepine, S. *et al.* Sphingosine contributes to glucocorticoid-induced apoptosis of thymocytes independently of the mitochondrial pathway. *J Immunol* **173**, 3783-3790, doi:10.4049/jimmunol.173.6.3783 (2004).

446 Dirks-Naylor, A. J. & Griffiths, C. L. Glucocorticoid-induced apoptosis and cellular mechanisms of myopathy. *J Steroid Biochem Mol Biol* **117**, 1-7, doi:10.1016/j.jsbmb.2009.05.014 (2009).

447 Urs, A. N., Dammer, E. & Sewer, M. B. Sphingosine regulates the transcription of CYP17 by binding to steroidogenic factor-1. *Endocrinology* **147**, 5249-5258, doi:10.1210/en.2006-0355 (2006).

448 Jividen, K. *et al.* Genomic analysis of DNA repair genes and androgen signaling in prostate cancer. *BMC cancer* **18**, 960, doi:10.1186/s12885-018-4848-x (2018).

449 Schmittgen, T. D. & Livak, K. J. Analyzing real-time PCR data by the comparative C(T) method. *Nature protocols* **3**, 1101-1108, doi:10.1038/nprot.2008.73 (2008).

450 Cerami, E. *et al.* The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer discovery* **2**, 401-404, doi:10.1158/2159-8290.CD-12-0095 (2012).

451 Narayanan, S., Srinivas, S. & Feldman, D. Androgen-glucocorticoid interactions in the era of novel prostate cancer therapy. *Nature reviews. Urology* **13**, 47-60, doi:10.1038/nrurol.2015.254 (2016).

452 Nadiminty, N. *et al.* NF-kappaB2/p52 induces resistance to enzalutamide in prostate cancer: role of androgen receptor and its variants. *Molecular cancer therapeutics* **12**, 1629-1637, doi:10.1158/1535-7163.MCT-13-0027 (2013).

453 Zong, Y. & Goldstein, A. S. Adaptation or selection--mechanisms of castration-resistant prostate cancer. *Nature reviews. Urology* **10**, 90-98, doi:10.1038/nrurol.2012.237 (2013).

454 Aggarwal, R., Zhang, T., Small, E. J. & Armstrong, A. J. Neuroendocrine prostate cancer: subtypes, biology, and clinical outcomes. *J Natl Compr Canc Netw* **12**, 719-726, doi:10.6004/jnccn.2014.0073 (2014).

455 Kolesnick, R. & Fuks, Z. Radiation and ceramide-induced apoptosis. *Oncogene* **22**, 5897-5906, doi:10.1038/sj.onc.1206702 (2003).

456 Modrak, D. E., Gold, D. V. & Goldenberg, D. M. Sphingolipid targets in cancer therapy. *Molecular cancer therapeutics* **5**, 200-208, doi:10.1158/1535-7163.MCT-05-0420 (2006).

457 Beckham, T. H. *et al.* Acid ceramidase-mediated production of sphingosine 1-phosphate promotes prostate cancer invasion through upregulation of cathepsin B. *International journal of cancer. Journal international du cancer* **131**, 2034-2043, doi:10.1002/ijc.27480 (2012).

458 Ryland, L. K., Fox, T. E., Liu, X., Loughran, T. P. & Kester, M. Dysregulation of sphingolipid metabolism in cancer. *Cancer biology & therapy* **11**, 138-149 (2011).

459 Taha, T. A., Mullen, T. D. & Obeid, L. M. A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochimica et biophysica acta* **1758**, 2027-2036, doi:10.1016/j.bbamem.2006.10.018 (2006).

460 Di Pardo, A. *et al.* De novo Synthesis of Sphingolipids Is Defective in Experimental Models of Huntington's Disease. *Front Neurosci* **11**, 698, doi:10.3389/fnins.2017.00698 (2017).

461 Dolgachev, V. *et al.* De novo ceramide accumulation due to inhibition of its conversion to complex sphingolipids in apoptotic photosensitized cells. *The Journal of biological chemistry* **279**, 23238-23249, doi:10.1074/jbc.M311974200 (2004).

462 de Launoit, Y., Veilleux, R., Dufour, M., Simard, J. & Labrie, F. Characteristics of the biphasic action of androgens and of the potent antiproliferative effects of the new pure antiestrogen EM-139 on cell cycle kinetic parameters in LNCaP human prostatic cancer cells. *Cancer research* **51**, 5165-5170 (1991).

463 Lee, C. *et al.* Regulation of proliferation and production of prostate-specific antigen in androgen-sensitive prostatic cancer cells, LNCaP, by dihydrotestosterone. *Endocrinology* **136**, 796-803, doi:10.1210/endo.136.2.7530653 (1995).

464 Tsihlias, J. *et al.* Involvement of p27Kip1 in G1 arrest by high dose 5 alpha-dihydrotestosterone in LNCaP human prostate cancer cells. *Oncogene* **19**, 670-679, doi:10.1038/sj.onc.1203369 (2000).

465 Li, Q. *et al.* Linking prostate cancer cell AR heterogeneity to distinct castration and enzalutamide responses. *Nature communications* **9**, 1-17 (2018).

466 Durham, J. P. & Mawhinney, M. G. Down-regulation of neutral sphingomyelinase in androgen-dependent smooth muscle. *Journal of Surgical Research* **130**, 136-139 (2006).

467 Bielawska, A., Linardic, C. M. & Hannun, Y. A. Ceramide-mediated biology. Determination of structural and stereospecific requirements through the use of N-acyl-phenylaminoalcohol analogs. *The Journal of biological chemistry* **267**, 18493-18497 (1992).

468 Bielawska, A., Crane, H. M., Liotta, D., Obeid, L. M. & Hannun, Y. A. Selectivity of ceramide-mediated biology. Lack of activity of erythro-dihydroceramide. *The Journal of biological chemistry* **268**, 26226-26232 (1993).

469 Hauser, J. M., Buehrer, B. M. & Bell, R. M. Role of ceramide in mitogenesis induced by exogenous sphingoid bases. *The Journal of biological chemistry* **269**, 6803-6809 (1994).

470 Baek, M. Y., Yoo, H. S., Nakaya, K., Moon, D. C. & Lee, Y. M. Sphingolipid metabolic changes during chiral C2-ceramides induced apoptosis in human leukemia cells. *Arch Pharm Res* **24**, 144-149, doi:10.1007/BF02976482 (2001).

471 Venkataraman, K. & Futerman, A. H. Comparison of the metabolism of L-erythro- and L-threo-sphinganines and ceramides in cultured cells and in subcellular fractions. *Biochimica et biophysica acta* **1530**, 219-226, doi:10.1016/s1388-1981(01)00085-3 (2001).

472 Ogretmen, B. *et al.* Biochemical mechanisms of the generation of endogenous long chain ceramide in response to exogenous short chain ceramide in the A549 human lung adenocarcinoma cell line. Role for endogenous ceramide in mediating the action of exogenous ceramide. *The Journal of biological chemistry* **277**, 12960-12969, doi:10.1074/jbc.M110699200 (2002).

473 Schiffmann, S. *et al.* Ceramide synthases and ceramide levels are increased in breast cancer tissue. *Carcinogenesis* **30**, 745-752, doi:10.1093/carcin/bgp061 (2009).

474 Newton, J., Lima, S., Maceyka, M. & Spiegel, S. Revisiting the sphingolipid rheostat: Evolving concepts in cancer therapy. *Exp Cell Res* **333**, 195-200, doi:10.1016/j.yexcr.2015.02.025 (2015).