Organoids Model Distinct Vitamin E Effects at Different Stages of Prostate Cancer Evolution

A DISSERTATION

SUBMITTED TO THE GRADUATE SCHOOL
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DOCTOR OF PHILOSOPHY

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By

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ABSTRACT

Prostate cancer (PCa) is the most common non-cutaneous cancer among U.S. men. Lack of effective treatments for advanced disease make it a significant public health concern. However, PCa’s long natural history makes it an excellent target for prevention approaches that reduce overtreatment of indolent disease, treatment related morbidity, and mortality. Oxidative stress has long been linked to prostate carcinogenesis. This fueled interest in the use of antioxidant supplements to inhibit, reverse or slow precancerous events or disease progression (chemoprevention). Analyses of the Alpha-Tocopherol, Beta Carotene (ATBC) prevention and the Nutritional Prevention of Cancer (NPC) trials showed a reduction in risk of PCa as a secondary end point after supplementation with the antioxidants, alpha tocopherol and selenium respectively.

Additionally, epidemiological and preclinical data suggest an anti-tumorigenic role for vitamin E and selenium against PCa. However, the selenium and vitamin E Cancer Prevention Trial (SELECT), testing the efficacy of vitamin E and/or selenium on reducing PCa incidence in 35,533 healthy men found vitamin E to be associated with an increased risk of PCa while selenium was not efficacious. The work in this dissertation focused on understanding SELECT’s negative outcome. We hypothesized that the lack of clinical translation for the preclinical in vitro data was because the latter were derived by testing vitamin E and selenium on advanced PCa cell lines grown in non-physiologic two dimensional cell cultures.

Further, we hypothesized that the more physiologic three-dimensional cultures would yield more clinically relevant data and that the outcome of antioxidant treatment would depend on the stage
of the cancer. To test this, we studied the effects of vitamin E and selenium on a continuum of prostate carcinogenesis from benign, premalignant to malignant cells in three-dimensional organoid cultures which mimic \textit{in vivo} prostate gland architecture. We found that while the supplements decreased proliferation and induced cell death in cancer (LNCaP) organoids, they had no effect on benign organoids derived from normal primary human prostate epithelial cells. This confirms that antioxidants have a different impact on different stages of cancer.

Additionally, vitamin E but not selenium alone or in combination, enhanced cell proliferation and survival in premalignant (RWPE-1) organoids, recapitulating the SELECT results. Relative to vehicle, the vitamin E treated premalignant organoids had low ROS levels and more luminal filling. Furthermore, microarray analysis revealed downregulation in the expression of integrins, glucose transporters and glycolytic enzymes in the vitamin E treated premalignant organoids, suggesting matrix detachment and metabolic alterations. Accordingly, detached RWPE-1 cells treated with vehicle had low ATP levels due to diminished glucose uptake and glycolysis.

However, treating detached RWPE-1 cells with vitamin E rescued ATP by activating fatty acid oxidation (FAO). FAO inhibition abrogated vitamin E’s ATP rescue in detached RWPE-1 cells and diminished survival of the inner matrix deprived cells, restoring the normal hollow lumen morphology in vitamin E treated organoids. Organoid models therefore clarified the paradoxical findings from SELECT. These findings demonstrate that vitamin E promotes tumorigenesis in the early stages of prostate cancer evolution by promoting cell survival in matrix deprived cells by activating FAO.
ACKNOWLEDGEMENTS

I would like to extend my appreciation to my thesis advisor Dr. Sarki Abdulkadir whose advice and feedback helped shape my project. Under Sarki’s mentorship, I have also greatly improved my scientific and research skills. I would also like to thank members of my thesis committee, Drs. Margrit Urbanek, Rex Chisholm, Erik Andersen and David Guis for their time and for helping me think through the project design. Their feedback was invaluable.

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I also wish to acknowledge my collaborators at Northwestern especially Dr. Jonathan C. Zhao who assisted with bioinformatics analysis an expertise I did not have and a pertinent component of the project. I am also very grateful to my friend Dr. Fotini Kouri for her help and for lending an ear in times of doubt. I am also grateful to members of the Chandel lab for assistance with the Seahorse metabolic assays and data interpretation as well as graciously sharing reagents. I am also indebted to labs that allowed me use of their equipment including the Chandel, Guis and Marcus labs.

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I am thankful to Bernice Frederick and Adriana Rosca current and former staff of the pathology core facility respectively. I also received training and help from staff at the Simpson Querrey Institute for BioNanotechnology (SQI) core facility.

My family and friends have also been incredibly supportive during my time in graduate school. I am especially grateful for every sacrifice my parents made enabling me to be where I am today. My dad instilled a passion for education and a love for biology and I hope he would be proud. My siblings and my grandmother are a constant source of encouragement and motivation as are my brother and sister in law as well as my nephews. A special acknowledgement too for my uncle, Dr. Peter Mungai Ngugi my favorite urologist who made me curious about prostate cancer. My friends have all been a great pillar of support too and for the ones in Kenya, they always make my holidays that much more fun.
PERMISSIONS

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<table>
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<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>18F-FDG</td>
<td>18F-fluorodeoxyglucose</td>
</tr>
<tr>
<td>2D</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>2DG</td>
<td>2-Deoxy-D-glucose</td>
</tr>
<tr>
<td>3D</td>
<td>Three-dimensional</td>
</tr>
<tr>
<td>4-HNE</td>
<td>4-Hydroxynonenal</td>
</tr>
<tr>
<td>5ARIs</td>
<td>5α-Reductase inhibitors</td>
</tr>
<tr>
<td>8-OHdG</td>
<td>8-Hydroxy-2-Deoxy Guanosine</td>
</tr>
<tr>
<td>A.U.</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>AA</td>
<td>African-American Men</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP Binding Cassette Subfamily A Member 1</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>ACMG</td>
<td>American College of Medical Genetics and Genomics</td>
</tr>
<tr>
<td>ACOX3</td>
<td>Acyl-CoA Oxidase 3, Pristanoyl</td>
</tr>
<tr>
<td>ACS</td>
<td>Acetyl-CoA Synthase</td>
</tr>
<tr>
<td>ADT</td>
<td>Androgen Deprivation Therapy</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>ALOX5</td>
<td>Arachidonate 5-lipoxygenase</td>
</tr>
<tr>
<td>AMACR</td>
<td>α-methylacyl-CoA racemase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid Leukemia</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>AR-V7</td>
<td>Androgen-receptor splice variant 7</td>
</tr>
<tr>
<td>ASNS</td>
<td>Asparagine Synthetase</td>
</tr>
<tr>
<td>ATBC</td>
<td>Alpha-Tocopherol Beta Carotene Clinical Trial</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUA</td>
<td>American Urological Association</td>
</tr>
<tr>
<td>BPE</td>
<td>Bovine Pituitary Extract</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign Prostatic Hyperplasia</td>
</tr>
<tr>
<td>BrDU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CACT</td>
<td>Carnitine-acylcarnitine translocase</td>
</tr>
<tr>
<td>CAFs</td>
<td>Cancer-associated Fibroblasts</td>
</tr>
<tr>
<td>CAT</td>
<td>Catalase</td>
</tr>
<tr>
<td>CAVs</td>
<td>Caveolins</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazine</td>
</tr>
<tr>
<td>CDEs</td>
<td>CAF-derived exosomes</td>
</tr>
<tr>
<td>CDRs</td>
<td>Cancer Detection Rates</td>
</tr>
<tr>
<td>CNV</td>
<td>Copy Number Variation</td>
</tr>
<tr>
<td>CPT I</td>
<td>Carnitine palmitoyltransferase I</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
</tr>
<tr>
<td>CRPC</td>
<td>Castration Resistant Prostate Cancer</td>
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<tr>
<td>COX</td>
<td>Cyclo-oxygenase</td>
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<tr>
<td>D2HG</td>
<td>D-2-Hydroxyglutarate</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>DCA</td>
<td>Dichloroacetate</td>
</tr>
<tr>
<td>DEC1</td>
<td>Deleted in esophageal cancer 1</td>
</tr>
<tr>
<td>DEGs</td>
<td>Differentially Expressed Genes</td>
</tr>
<tr>
<td>DHAP</td>
<td>Dihydroxyacetone phosphate</td>
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<tr>
<td>DHT</td>
<td>5α-dihydrotestosterone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DRE</td>
<td>Digital Rectal Examination</td>
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<tr>
<td>EA</td>
<td>European-American Men</td>
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<tr>
<td>EAAC1</td>
<td>excitatory amino-acid carrier 1</td>
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<tr>
<td>EBRT</td>
<td>External beam radiation therapy</td>
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<tr>
<td>ECAR</td>
<td>Extracellular Acidification Rate</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>ELOVL7</td>
<td>Elongation of very long chain fatty acids protein 7</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ES</td>
<td>Enrichment Score</td>
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<tr>
<td>ETC</td>
<td>Electron Transport Chain</td>
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<tr>
<td>FA</td>
<td>Fatty Acid</td>
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<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide</td>
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<td>FAO</td>
<td>Fatty Acid Beta Oxidation</td>
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<td>FASN</td>
<td>Fatty Acid Synthase</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FDG-PET</td>
<td>18-Fluoro-deoxyglucose positron emission tomography</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
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<tr>
<td>FPC</td>
<td>Familial Prostate Cancer</td>
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<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
</tr>
<tr>
<td>GATK</td>
<td>Genome Analysis ToolKit</td>
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<tr>
<td>GEMMs</td>
<td>Genetically engineered mouse models</td>
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<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
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<tr>
<td>GLS</td>
<td>Glutaminase</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose Transporter</td>
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<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GPX</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene Set Enrichment Analysis</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione S-transferase</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome-wide Association Studies</td>
</tr>
<tr>
<td>H&amp;E stain</td>
<td>Hematoxylin and eosin stain</td>
</tr>
<tr>
<td>HBP</td>
<td>Hexosamine Biosynthetic Pathway</td>
</tr>
<tr>
<td>HGMD</td>
<td>Human Gene Mutation Database</td>
</tr>
<tr>
<td>HGPIN</td>
<td>High Grade Prostatic Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>HIFU</td>
<td>High-intensity focused ultrasound</td>
</tr>
<tr>
<td>HK2</td>
<td>Hexokinase 2</td>
</tr>
<tr>
<td>HMGCR</td>
<td>HMG-CoA reductase</td>
</tr>
<tr>
<td>HPB</td>
<td>Hexamine Biosynthetic Pathway</td>
</tr>
<tr>
<td>HPC</td>
<td>Hereditary Prostate Cancer</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
</tbody>
</table>
HSP – Heat Shock Proteins
ICAM 1 – Intercellular Adhesion Molecule 1
IDH1/2 – Isocitrate dehydrogenase 1 and 2
IMS – Intermembrane space
IRB – Institutional Review Board
IU – International Units
LBD – Ligand Binding Domain
LD – Lipid Droplets
LGPIN – Low Grade Prostatic Intraepithelial Neoplasia
LH – Luteinizing hormone
LHRH – Luteinizing hormone-releasing hormone
LOA – Loss of Attachment
LOH – Loss of heterozygosity
LP – Lateral Prostate
mAAT – Mitochondrial aspartate aminotransferase
MCM – Minichromosome maintenance protein complex
mCRPC – Metastatic Castration Resistant Prostate Cancer
MCTs – Monocarboxylate transporters
MMPs – Matrix metalloproteinases
MNU – N-methyl N-nitroso Urea
mPCa – Murine Prostate Cancer
MSA – Methylseleninic acid
MSC – Methyl selenocysteine

MSigDB – Molecular Signatures Database
MTHFD2 – Bifunctional methylenetetrahydrofolate dehydrogenase 2/cyclohydrolase
NAC – N-Acetyl-Cysteine
NADH – Nicotinamide adenine dinucleotide
NADPH – Nicotinamide adenine dinucleotide phosphate
NCI – National Cancer Institute
NES – Normalized enrichment score
NMU – N-Nitroso-N-methylurea
NPC – Nutritional Prevention of Cancer Clinical Trial
NSCLC – Non-small cell lung cancer
OCR – Oxygen Consumption Rate
OCT – Organic Cation Transporter
OXPHOS – Oxidative phosphorylation
PCa – Prostate Cancer
PDH – Pyruvate Dehydrogenase
PDK – Pyruvate Dehydrogenase Kinase
PHDGH – Phosphoglycerate dehydrogenase
PHDs - Prolyl hydroxylases
PIA – Proliferative Inflammatory Atrophy
PIN – Prostatic Intraepithelial Neoplasia
PIP3 – Phosphatidylinositol (3,4,5)-trisphosphate
PKC – Protein Kinase C
PKM2 – Pyruvate kinase muscle isozyme M2
PLND – Pelvic lymph node dissection
Poly-HEMA – Poly (2-hydroxyethyl methacrylate)
PPP – Pentose Phosphate Pathway
PrE – Primary Epithelial Cells
PrEC – Prostate Epithelial Cells
PrEGM – Prostate Epithelial Cell Growth Medium
PRX – Perodoxin
PSA – Prostate specific antigen
PUFAs – Polyunsaturated fatty acids
qRT-PCR - Quantitative Reverse Transcription Polymerase Chain Reaction
RB – Retinoblastoma protein
REDUCE – REduction by DUtasteride of prostate Cancer Events
RMA – Robust Microarray Analysis
RNA – Ribonucleic Acid
RNS – Reactive Nitrogen Species
ROS – Reactive Oxygen Species
RP – Radical Prostatectomy
RT – Radiation Therapy
SCD1 – Stearoyl-Coenzyme A desaturase-1
SD – Standard Deviation
SDH – Succinate dehydrogenase
SELECT – Selenium and Vitamin E Cancer Prevention Trial
SeMet – Selenomethionine
SNP – Single Nucleotide Polymorphism
SNV – Single Nucleotide Variant
SOD – Superoxide dismutase
SPC – Sporadic Prostate Cancer
SRE – Sterol Regulatory Element
SREBP – Sterol regulatory element-binding proteins
TBARS – Thiobarbituric acid reactive substances
TCA Cycle – Tricarboxylic Acid Cycle
TET enzymes – Ten-eleven Translocation Enzymes
TOB – RRR-alpha-tocopheryloxybutyric acid
TRAMP – Transgenic adenocarcinoma of the mouse prostate
TRs – Thioredoxin Reductases
TXNIP – Thioredoxin interacting protein
UIC – University of Illinois at Chicago
uPAR – Urokinase-type plasminogen activator receptor
USPSTF – United States Preventive Services Task Force
VEBSA – RRR-alpha-tocopheryloxybutyl sulfonic acid
VES – Vitamin E Succinate
VITAL – VITamins And Lifestyle cohort study
Zip1 – Zinc Transporter 1
DEDICATION

To my mum Pauline Nyachomba Njoroge and the memory of my dad, Joseph Njoroge Kimani. I would not have done this without your unconditional support. I miss you so much dad; your example will forever inspire my life experiences.
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CHAPTER 1: INTRODUCTION

Prostate Cancer Epidemiology

Prostate cancer (PCa) is the most common non-skin cancer in American men which is expected to be diagnosed in the lifetimes of one out of nine American men.\textsuperscript{1,2} In 2018 alone, there were an estimated 164,690 new cases and 29,430 PCa associated deaths.\textsuperscript{3} Age is the most significant risk factor for PCa development. PCa’s prevalence is roughly 35% in men aged 60 – 69 years and 46% in men aged 70 – 81.\textsuperscript{4} The median age of diagnosis is 66 years with the majority of cases being diagnosed between the ages of 65 – 74.\textsuperscript{5}

PCa is a bigger health problem in developed countries where there is a bigger proportion of elderly men with 15% of all male cancers being PCa related compared to developing countries where just 4% is the case.\textsuperscript{6} However, some of the differences in disease epidemiology are attributable to screening, availability of early detection and treatment options.\textsuperscript{1} In the U.S., the incidence of PCa is 1.6 times higher among African American men compared to Caucasian men.\textsuperscript{7}

Prostate Cancer Risk Factors

Risk factors that have inconsistently been associated with PCa include obesity, diet, metabolic syndrome, smoking, physical inactivity and exposure to heavy metals and herbicides.\textsuperscript{8-13} Age, race and a family history of PCa on the other hand are well-established risk factors for developing the disease.\textsuperscript{14,15}

*Age*
PCa has the one of the strongest associations between age of any human cancer.\textsuperscript{16} Though PCa is rarely diagnosed before 40 years of age, the incidence rises sharply thereafter.\textsuperscript{17} Between 2011 and 2015, the PCa incidence rates per 100,000 persons for men aged 35 – 39 years, 60 – 64 years and 65 – 69 years were 0.8, 423.2 and 651.7 respectively.\textsuperscript{17} Elevated oxidative stress and diminished antioxidant defenses with advancing age have been proposed to explain the association between age and PCa.\textsuperscript{18} Inflammation and dysregulation of androgen regulated redox homeostasis are thought to be major contributors of prostatic oxidative stress.\textsuperscript{18} The prostate gland is prone to chronic inflammation probably due to its anatomical proximity to the urinary tract.

Though the exact cause of prostatic inflammation is unknown, waste stimuli from urine reflux, injury from mechanical obstruction by uric acid crystals, as well as infection have been implicated.\textsuperscript{19} Whatever the cause, histological inflammation has been detected in the majority of prostate tissues. In the REDUCE (REduction by DUtasteride of prostate Cancer Events) trial testing the efficacy of dutasteride in reducing the risk of PCa in men with a negative PCa biopsy, 78\% of the enrollees were found to have chronic histologic inflammation.\textsuperscript{20,21}

In fact, oxidative stress from chronic inflammation mediated by cyclo-oxygenase (COX) has led to PCa prevention trials using asprin a nonsteroidal anti-inflammatory drug or COX-2 inhibitors.\textsuperscript{22-24} Other factors that increase PCa risk that are impacted by age include cholesterol metabolism and obesity, levels of sex hormones and increasing accumulation of genetic and epigenetic alterations.\textsuperscript{25} However, the mechanistic links between aging and cancer development in general remain under investigation, it is hoped insights from the former could be leveraged in cancer therapies.\textsuperscript{26}
Race

PCa epidemiology displays significant racial and ethnic differences. The age adjusted PCa incidence rates per 100,000 U.S. males of all ages between 2011 – 2015 were 178.3 for African American men (AA), 105.7 for European-American men (EA), 91.8 for Hispanics, 59.1 for Asian/Pacific Islanders and 54.8 for Native Americans. The corresponding PCa related mortality rates per 100,000 U.S. males of all ages were 39.9 for blacks, 18.2 for white, 16.2 for Hispanics, 14.7 for Native Americans and 8.8 for Asian/Pacific Islanders. Therefore, in the U.S., black men are at 1.7 fold higher risk of a PCa diagnosis and are twice as likely to die from PCa. In addition to the high incidence and mortality rates among AA men, they also have a higher proportion of early onset disease (≤ 55 years at diagnosis) compared to other racial groups.

In one study, (n > 12,000) 8.3% AA men were less than 50 years of age at time of diagnosis compared to 3.3% EA men. In contrast, Native Americans and Asian/Pacific islanders have the lowest incidence rates and PCa mortality rates, respectively among U.S. males. The reasons for these disparities remain unclear. Studies have found that AA men typically present with higher PSA levels and more advanced PCa at diagnosis compared to other racial groups. However, in the Prostate Cancer Outcomes Study, the higher risk for advanced PCa in AA men did not change after adjustment for socio-economic factors and clinical variables. A different study reported that even when diagnosed early and at a similar pathological stage, AA still had a slightly higher risk for biochemical recurrence than EA men. However, because ethnicity is closely linked to genetic factors, it is thought that there is a molecular basis for the racial
differences in incidence and PCa outcomes including mutations, genetic polymorphisms and epigenetics.\textsuperscript{33,34}

For example, the androgen receptor (AR) is important in prostate development but also in prostate carcinogenesis. It has been shown that there are ethnic differences in the distribution of polymorphisms that regulate AR activity.\textsuperscript{35} Polymorphic cytosine, adenine and guanine trinucleotide repeats (CAG)\textit{n} in exon 1 of the AR gene encoding poly-glutamine are thought to regulate AR activity with shorter alleles conferring increased AR activity.\textsuperscript{35} AA men have significantly shorter (CAG)\textit{n} repeat length than CA men with increased androgen sensitivity.\textsuperscript{35}

\textit{Family history}

Compared to men in the general population, first-degree relatives of men with PCa are at twice or thrice the risk of developing the disease, and those with two or more affected first degree relatives have 5 – 11 fold increased risk.\textsuperscript{36-38} The familial PCa risk is compounded in early onset cases with first degree male relatives of men diagnosed at $\leq 60$ years being at a higher risk compared to men whose relatives were diagnosed at $> 60$ years.\textsuperscript{33,39} Familial clustering of PCa can result from inherited susceptibility, lifestyle choices, environmental factors and their interactions.\textsuperscript{40}

The contribution of genetic factors to PCa familial aggregation is evidenced by Nordic twin registries analysis showing that monozygotic twins have 50\% higher risk than dizygotic twins.\textsuperscript{41,42} In fact, genetic factors may be involved in up to 42\% of all PCa cases making it perhaps the most heritable cancer.\textsuperscript{41} Moreover, ethnic and racial differences in PCa whose incidence is highest among African American men and lowest among Americans of Asian
ancestry further support the role of genetic factors in PCa carcinogenesis. However, differences in PCa incidence rates between native and migrant populations suggest that environmental and lifestyle factors play a role in disease risk.

Based on family history, PCa can be categorized as familial (FPC), sporadic (SPC) or heritable (HPC). SPC cases have no prior family history, and they constitute the majority of all PCa cases at 75 – 85%. Both FPC and HPC have a positive familial history, however, while FPC describes general PCa aggregation in families, HPC specifically describes a subset of FPC that displays a pattern of Mendelian inheritance of susceptibility gene(s). FPC makes up 10-20% of the cases and hereditary prostate cancer (HPC) between 5 – 10%. However, despite these distinctions, sporadic PCa also has a germline genetic component. Moreover, HPC, FPC and SPC are said not to differ clinico-pathologically except for the 6 to 7 years earlier presentation of HPC cases.

**Genetic susceptibility**

Genetic predisposition to a disease can be attributed to rare (highly penetrant) mutations, genetic variants conferring moderate to low risk or a combination of both. Segregation analysis in diverse populations suggest that familial aggregation of PCa follows autosomal dominance, recessive, X-linked or multi-factorial inheritance patterns. However, despite strong evidence for genetic predisposition to PCa, results from linkage studies on families with multiple cases to pinpoint susceptibility genes have not been consistently replicated with the exception of HOXB13. Other debated genes include HPC2/ELAC2, HPC1/RNASEL, MSR1, PCAP,
CAPB, BRCA2 and BRCA1 but their true association with PCa risk needs further validation.\textsuperscript{48,59-68}

The difficulty in identifying consistent susceptibility loci across wide populations has been attributed to genetic heterogeneity and the involvement of multiple incompletely penetrant alleles.\textsuperscript{69} Additionally, genome-wide association studies (GWAS) have identified over 76 Single Nucleotide Polymorphisms (SNPs) associated with PCa.\textsuperscript{48} It is said that 28.4% relative risk of FPC can now be attributed to common variants identified by GWAS.\textsuperscript{70} Another 6% might be explained by rarer but higher risk variants such as those in the BRCA2 and HOXB13 genes.\textsuperscript{70}

**Prostate anatomy**

The prostate is an exocrine gland located near the bladder (Fig 1).\textsuperscript{71} Its secretions are necessary for male fertility and they are enriched in Zn2+, citrate, and Kallikrein-related peptidases.\textsuperscript{72} Prostatic secretions empty through ducts into the prostatic urethra where they make up part of the seminal fluid.\textsuperscript{71} The prostate gland has three distinct anatomical zones that differ in location, histology and disease propensity (Fig 1).\textsuperscript{73} The central zone borders the ejaculatory ducts and it runs from the base of the bladder tapering off at the verumontanum.\textsuperscript{74}
The peripheral zone which makes up the bulk of the prostatic volume contains the prostatic ducts, and it surrounds the central zone stretching down to the prostate apex. The periurethral transition zone is situated next to the proximal urethra, and it borders the central and peripheral zones. While benign prostatic hyperplasia (BPH), a common nonmalignant condition develops in the transition zone, most PCAs develop within the peripheral zone. Histologically, the prostate is a pseudostratified epithelium of luminal and basal epithelial cells and rare neuroendocrine cells. The luminal layer is made of polarized columnar luminal epithelial cells that produce prostatic secretions and highly express AR and low molecular weight cytokeratins 8/18, (CK8+/p63-/AR+). The basal cell epithelial layer separates the luminal cells from the stroma and expresses high molecular weight cytokeratins 5/14, p63 and much lower levels of AR, (CK5+/p63+/AR-).
prostate epithelium also contains cells that express both basal and luminal markers that are termed, transiently amplifying “intermediate cells.” The neuroendocrine cells express synatophysin and chromogranin A and they are thought to secrete factors necessary for luminal cell growth. The prostate epithelium is surrounded by a stromal compartment with several cell types, including smooth muscle cells, myofibroblasts, endothelial cells among others.

**Prostate cancer pathogenesis**

*Precursor lesions of prostate cancer*

Prostatic carcinogenesis is believed to proceed along a pathological and morphological continuum that evolves from benign glands through various premalignant stages and finally invasive disease (Fig. 2). Proliferative inflammatory atrophy (PIA) and high grade prostatic intra-epithelial neoplasia (HGPIN) are some of the histologic lesions considered to be PCa precursors. PCa lesions occur most commonly (80%) in the periphery zone of the gland with the rest occurring in the transition zone located in the periurethral region. PIA, which is associated with chronic inflammation, is found in the peripheral zone and is characterized by focal glandular atrophy and postatrophic hyperplasia and epithelial cell proliferation.

PIA lesions have been proposed to be precursors of prostatic adenocarcinoma directly or indirectly by progressing to PIN. This notion is supported by the common presence of PIA near HGPIN and PCa foci. Like PIA, PIN lesions are also found primarily in the peripheral zone, and they histologically range from the more normal low-grade PIN (LGPIN) to the more abnormal high-grade PIN (HGPIN). Of the PINs, HGPIN, (grade 2–3) is considered to be a precursor for PCA development evidenced by the increased prevalence of HGPIN lesions in
prostates with carcinoma than those without.\textsuperscript{81-83} PIN lesions are characterized by high proliferation rates, and they have some architectural, mutational and cytological alterations in common with early invasive carcinoma.\textsuperscript{80,84,85}

PIN and PCa share mutations like SPOP, NKX3.1 and PTEN deletions, ERG (ETS transcription factor) rearrangements in addition to epigenetic aberrations like GSTP1 silencing.\textsuperscript{86-90} The clonal relationship between HGPIN and PCa seems to depend on their promixity to each other.\textsuperscript{91} HGPIN lesions share more genomic alterations with PCa foci like ERG rearrangement when they are in close proximity while those that lie further away do not harbor such aberrations.\textsuperscript{91} However, HGPIN has low predictive value for subsequent PCa diagnosis.\textsuperscript{92} Clinically, about 40\% of men diagnosed with PCA within three years of HGPIN diagnosis.\textsuperscript{81,93} HGPIN with specific aberrations like ERG overexpression may be more associated with a subsequent cancer diagnosis pointing to HGPIN heterogeneity.\textsuperscript{94}

\textit{Prostate adenocarcinoma}

Localized PCa is typically multifocal with multiple genetically distinct histologic foci.\textsuperscript{73} Multifocal development of PCa is hypothesized to stem from a field effect where cell abnormalities occur beyond regions with morphologically evident tumor facilitating cancer initiation and concurrent development of HGPIN at multiple sites.\textsuperscript{71,95} Studies to identify mechanisms underlying field canerization in PCa are ongoing as these might yield molecular targets for prevention.\textsuperscript{71} Interestingly, despite the phenotypic heterogeneity of metastatic PCas, multiple metastases from the same patient share similar genetic alterations indicating evolution.
from a common clonal origin perhaps through acquisition of a selective advantage or through
drug selection.73

Pathologically, 95% of prostate tumors are classified as adenocarcinomas in spite of the
heterogeneity.73 Histologically, the transition from PIN to adenocarcinoma is accompanied by
several changes. Loss of basal cells is a hallmark of PCa, and most adenocarcinomas stain
positively for α-methylacyl-CoA racemase (AMACR) a luminal marker that is upregulated in
cancer but negatively for basal p63 and cytokeratin 5/14.73 In addition, there is excess prostatic
branching and abnormal cytology with nuclei and nucleoli enlargement.71 However, though
histologic changes can be detected in healthy men as young as 20 suggesting early disease
initiation, PCa is not typically diagnosed until three or four decades later indicating slow
progression.73

*Mutations in Prostate Cancer*

Like other cancers, PCa arises from the accumulation of genetic and epigenetic alterations in the
prostate epithelium which co-evolve with alterations in cells in the extracellular
microenvironment (Fig. 2).96 The somatic alterations in PCa include single nucleotide variants
(SNVs or point mutations), small insertions/deletions (indels) and genome rearrangements which
result in large scale chromosomal structural alterations including deletions, duplications,
inversions, insertions and translocations.97,98 Point mutations result in protein amino acid
changes (missense mutations) or truncations (nonsense mutations) while indels sometimes lead
to changes in protein function (frameshift mutations).99
Genome rearrangements can cause copy number variations (CNVs) which can amplify oncogenes or delete tumor suppressors and gene fusions that can confer gain of function and genome instability.\textsuperscript{100} Relative to other malignancies, PCa has a low mutational frequency of 0.3–5 per Mb; point mutations are thought to play a minor role in prostate tumorigenesis.\textsuperscript{101-104} The mutation frequencies of the top ten mutated genes in PCa are; TP53 (13%), PTEN (7%), SPOP (7%), AR (5%), FOXA1 (5%), LRP1B (4%), KM2TC (4%), KRAS (4%), FAT4 (4%), KMT2D (4%).\textsuperscript{105} Recurrent mutations have also been detected in SPOP, FOXA1, TP53, ATM, PTEN, KDM6A, CDH1, APC, AR, ZFHX3, RB1, and MED12.\textsuperscript{88,106}

In contrast, PCa has a high frequency of CNVs making structural chromosomal alterations the main drivers of PCa tumorigenesis.\textsuperscript{103,104} The CNV burden, which defines the proportion of a tumor genome that is affected by structural changes, correlates with the Gleason score and
clinical outcomes like biochemical recurrence and PCa metastasis. Genes mapped to chromosomal regions with CNVs in primary PCa include the oncogene MYC (8q24.21), and the tumor suppressors PTEN (10q23.31), NKX3.1 (8p21.2), RB1 (13q14.2), CDKN1B (12p13.1) and TP53 (17p13.1). Overall, losses exceed gains and these can be either mono or bi-allelic, they could also be focal or affect large areas.

PTEN (~15%) and CHD1 (~10%) are the most frequent homozygous deletions in PCa. Others include BNIP3L, LRP1B (2q22.1), RB1, USP10, HTR3A, RYPB, MAP3K7, TP53 among others. Per Knudson’s “two-hit” hypothesis, loss-of-heterozygosity (LOH) of a tumor suppressor gene is required for tumor formation. However, there is now ample evidence that loss of function is not always recessive, and the inactivation of a single allele can lead to tumor development through the reduction of protein dosage (haploinsufficiency) or in concert with other genetic and/or epigenetic changes. In PCa, haploinsufficiency has been demonstrated for PTEN (10q23.31), NKX3.1 (8p21.2) and KLF5 (13q21).

In the PCa genome, most gains tend to be hemizygous, though amplifications with more than two extra chromosomal copies have been reported primarily on 8q24 where MYC is located. Additionally, analysis of clonal and subclonal genomic aberrations have allowed the temporal ordering of somatic genetic events in PCa. Early events tend to be clonal, and they are associated with cancer initiation and they can serve as disease biomarkers for early diagnosis or intervention. Later events are subclonal, and they are associated with disease progression and cancer aggressiveness. Early, clonal, events in PCa carcinogenesis include: NKX3-1 and FOXP1 deletions, TMPRSS2-ETS fusions, SPOP and FOXA1 mutations.
However, ETS arrangements and SPOP mutations appear to be mutually exclusive, a trend that has been used for molecular subtyping of prostate tumors.\textsuperscript{88,118} Late, sub-clonal events in PCa include CDKN1B and PTEN deletions and lesions in TP53, AR, and RB1.\textsuperscript{86,117} In addition to genetic alterations, epigenetic alterations play an important role in PCa initiation and progression. For PCa, epigenetic alterations are an early event manifesting earlier than genetic changes and retained throughout the malignant progression.\textsuperscript{90,119,120} Epigenetics refers to the different mechanisms that affect gene function and regulation without affecting the underlying DNA sequence and can be inherited transgenerationally.\textsuperscript{121}

They include, DNA methylation, histone modifications, expression of non-coding RNAs, genomic imprinting among others. Some of the earliest epigenetic changes seen in PCa include DNA hypermethylation that silences GSTP1, APC and RASSF1A.\textsuperscript{119,120} In contrast, DNA hypomethylation is associated with advanced disease, especially in metastasis.\textsuperscript{122} DNA hypomethylation opens up chromatin and unmasks retrotransposable elements promoting genome instability.\textsuperscript{121} The repertoire of epigenetic changes in PCa has recently been reviewed.\textsuperscript{121}

**Prostate cancer etiology**

Though the mechanisms driving PCa development and progression are not well understood mechanisms under investigation include inflammation, androgen driven mechanisms, metabolism and oxidative stress.

*Inflammation*

Epidemiological, pathological, and molecular data support a causal role of chronic inflammation in prostate carcinogenesis.\textsuperscript{73} Sources of prostatic inflammation include altered sex hormone
levels (androgenic activation of AR can increase the transcription of pro-inflammatory cytokines), bacterial and viral infections, physical trauma and dietary factors. Prostatic inflammation is said to cause inflammatory cell infiltration and prostatic atrophy creating putative PCa precursor lesions termed proliferative inflammatory atrophy (PIA). Immune cells in the prostate microenvironment increase the levels of inflammatory mediators like cytokines and reactive oxygen species (ROS), which damage DNA and increase cell proliferation. PIA lesions, which proliferate probably in an attempt to repair cell damage, are characterized by oxidative stress and are hypothesized to progress to PCa through PIN.

PIA and PIN lesions share some molecular alterations including loss of NKX3.1 and GSTP1 promoter methylation. This suggests that inflammation from the prostate microenvironment may be involved in PCa initiation. However, atrophic prostate epithelial cells might also secrete inflammatory mediators generating an inflammatory microenvironment causing neoplastic transformation. Likewise, tumor cells support their growth by inducing an inflammatory microenvironment explaining how inflammation might play a role in PCa progression. Prostatic inflammation has been linked mostly to lymphocytes and macrophages, and less often plasma cells and eosinophils. Maspin inhibition and IKKa activation by prostate tumor infiltrating T cells, B cells and monocytic cells might promote metastasis. Leukocyte and B cell prostate tumor infiltration after ADT therapy leads to CRPC by activating IKKa and STAT3.

Suppression of T cell responses by myeloid-derived suppressor cells, which are recruited in response to increased IL6, has been linked to PCa progression and correlates with disease stage. ROS, growth factors, chemokines and cytokines from infiltrating macrophages have
been shown to remodel tissue and lead to PCa progression in experimental models.\textsuperscript{125} Tumor promoting pro-inflammatory cytokines in the prostate include IL8, CCL2, CXCL12 and IL6 through the regulation of the transcription factors, NF\(\kappa\)B, HIF1\(\alpha\) and STAT3.\textsuperscript{125} In addition, mutations and genetic polymorphisms of genes in inflammatory pathways have also been described.\textsuperscript{126} The involvement of inflammation in PCa initiation and progression makes it a target in PCa prevention. Studies show that use of nonsteroidal anti-inflammatory drugs is associated with reduced PCa risk.\textsuperscript{127-130}

\textit{Androgen-driven mechanisms}

The normal development, growth and function of the prostate gland is dependent on androgens that exert their effects by binding to the androgen receptor (AR).\textsuperscript{131} In the absence of androgen ligand, AR a nuclear receptor transcription factor, localizes to the cytoplasm where it is bound by heat shock proteins (HSP).\textsuperscript{131} Upon androgen binding, the HSPs dissociate allowing AR to dimerize and translocate in to the nucleus where it mediates gene transcription.\textsuperscript{131} Canonical global AR target genes are necessary for prostate growth and luminal differentiation.\textsuperscript{132} Treatment with androgens promotes cell proliferation, survival and differentiation \textit{in vitro}.\textsuperscript{133,134} However, AR signaling also plays a significant role in PCa development and progression.

Recently, it has been suggested that switches in AR DNA binding activate non-canonical AR target genes overrepresented with genes involved in luminal epithelial de-differentiation and cell cycle genes enhancing tumorigenesis.\textsuperscript{132} AR is also known to trigger the expression of the common PCa oncogenic gene fusionTMPRSS2-ERG.\textsuperscript{131} The central role of AR in PCa makes it an attractive therapeutic target. Initially, the majority of prostate tumors are androgen dependent
and regress upon androgen deprivation with improvement of cancer related symptoms.\textsuperscript{135}

Inevitably however, the tumors recur and become castration resistant, with a median survival of 18-24 months.\textsuperscript{131} The concurrent rise in serum PSA levels in CRPC indicates AR reactivation.\textsuperscript{131}

Mechanisms mediating CRPC include; AR mutations that alter binding specificity, AR amplifications which increase androgen sensitivity, AR splice variants with a truncated ligand binding domain (LBD) allowing androgen-independent AR activation, coactivator and corepressor mutations and intratumoral androgen synthesis.\textsuperscript{136} Efforts to find therapies for CRPC are ongoing.

\textit{Metabolic alterations in prostate cancer}

\textbf{Cellular metabolism and its reprogramming in cancer}

Low levels of growth factors limit nutrient uptake in differentiated, quiescent cells which, depend on catabolic metabolism, mostly through oxidative phosphorylation (OXPHOS) to maximize ATP generation for normal cellular maintenance.\textsuperscript{137} However, in the presence of abundant growth factors, proliferating cells couple catabolic metabolism to generate energy for replicative cell division with anabolic metabolism for biomass synthesis for the newly created cells and to maintain redox homeostasis.\textsuperscript{137} By definition, cancer is uncontrolled cell proliferation and tumor metabolism parallels that of proliferating normal cells.\textsuperscript{137} Altered metabolism to support rapid cell growth often in poorly vascularized, nutrient poor microenvironments is actively selected for in tumorigenesis, establishing it as a hallmark of cancer (Fig. 3).\textsuperscript{137-139}
Figure 3. Main pathways in cancer metabolism. Explanations can be found in the text.

Mammalian cells mainly depend on glucose, glutamine and lipids for biosynthesis and cell survival. Glucose can be metabolized through glycolysis to pyruvate, which under anaerobic conditions is converted to lactate and two moles of ATP per mole of glucose in the cytoplasm. In aerobic conditions, pyruvate is converted to Acetyl-CoA through oxidative decarboxylation by pyruvate dehydrogenase (PDH) in the mitochondria. Oxidative phosphorylation (OXPHOS) of Acetyl-CoA in the mitochondria’s TCA cycle produces the reducing equivalents NADH and FADH2, which mediate electron transfer in the electron transport chain (ETC) to generate the proton motive force that drives ATP synthesis. OXPHOS yields 36 moles of ATP per mole of glucose.

One of the earliest identified metabolic derangements in cancer cells is the increased glucose uptake for glycolysis even in normoxic conditions - the Warburg effect. Proliferating cells are known to select for glycolysis because it generates intermediates for use in anabolic pathways. For example, dihydroxyacetone phosphate (DHAP) is a precursor in the synthesis of structural cell membrane lipids triacylglyceride and phospholipids. DHAP is also a precursor for cardiolipin a mitochondrial membrane lipid. 3-phosphoglycerate is a precursor of sphingolipids a membrane component as well as the synthesis of the amino-acids serine, cysteine, and glycine. Pyruvate can also be used to synthesize malate and alanine.

Additionally, while glycolysis is less efficient for ATP generation relative to OXPHOS per mole of glucose, it allows for faster ATP generation in comparison. Warburg’s hypothesis that impaired mitochondrial respiration leads to the increase in glycolysis in cancer cells fueled the erroneous belief that cancer cells depend primarily on glycolysis for ATP production. While 18F-fluorodeoxyglucose (18F-FDG) positron emission tomography validates that most human
tumors have increased glucose uptake compared to normal tissue, many tumors also concurrently retain oxidative metabolism.\textsuperscript{143,145,146} Besides, diminishing ATP generation from glycolysis by inhibiting pyruvate kinase does not halt tumorigenesis.\textsuperscript{147} Moreover, mitochondrial dysfunction impedes cell proliferation and tumorigenesis.\textsuperscript{148-150}

Cancer cells therefore use OXPHOS for ATP generation in spite of high glycolytic flux unless their mitochondrial metabolic enzymes suffer disabling mutations.\textsuperscript{151} Rather than damage to mitochondrial respiration, cancer cells have dysregulated glycolysis due to activation of oncogenes or loss of tumor suppressors.\textsuperscript{141,151} In fact, rapidly proliferating cells like cancer cells can also obtain biosynthetic precursors from the TCA cycle for \textit{de novo} macromolecule biosynthesis (cataplerosis).\textsuperscript{152} Citrate can be used to generate acetyl-CoA and oxaloacetate.\textsuperscript{153} Acetyl-CoA is a precursor for fatty acid synthesis.\textsuperscript{153} Oxaloacetate can be converted to malate and then pyruvate generating NAD$^+$ a glycolysis co-factor and NADPH a reducing equivalent.\textsuperscript{153}

Oxaloacetate and α-Ketoglutarate can be converted into the amino acids aspartate and glutamate respectively, which then serve as precursors for purine synthesis.\textsuperscript{153} Succinyl-CoA is important in the synthesis of porphyrin and heme, which are upregulated in breast and non-small-cell lung (NSCLC) cancers.\textsuperscript{153} Besides ATP generation however, glucose has several other fates in the cell. Through one carbon metabolism, glucose is used for nucleotide metabolism, redox homeostasis, lipid biosynthesis, amino acid homeostasis and methylation metabolism.\textsuperscript{154} Glucose can also be shunted through the Pentose Phosphate Pathway (PPP) to generate pentose phosphates, ribose-5-phosphates a precursor for ribonucleotides and NADPH a cofactor used in anabolic reactions.\textsuperscript{155}
NADPH is a required electron donor for reductive steps in lipid synthesis, nucleotide metabolism and in maintaining GSH in its reduced state.\textsuperscript{156} Glucose can also be metabolized through the hexamine biosynthetic pathway (HBP) to produce UDP-N-acetylglucosamine (UDP-N-GlcNAc).\textsuperscript{157} UDP-N-GlcNAc is needed for \textit{O}-linked posttranslational protein glycosylation and for the synthesis of glycosylphosphatidylinositol (GPI) anchors which secure proteins to membranes.\textsuperscript{157} When TCA cycle precursors from glucose catabolism are withdrawn for biosynthesis, they are replenished using glutamine-derived α-ketoglutarate to maintain mitochondrial integrity- \textit{anapleurosis}.\textsuperscript{158}

Glutamine is also an important source of reduced nitrogen for the biosynthesis of purine and pyrimidine nucleotides, essential amino-acids, glutathione and lipid synthesis via reductive carboxylation.\textsuperscript{159} In addition the conversion of glutamine-derived malate to pyruvate by malic enzyme is a source of NADPH.\textsuperscript{160} Many cancers and proliferating cells metabolize more glutamine than is needed for protein and nucleotide synthesis, and some cancer cells depend on glutamine for survival in what is termed glutamine addiction.\textsuperscript{161} In addition to glucose and glutamine, many cellular functions depend on lipids, and alterations in lipid and cholesterol metabolism are often seen in tumor cells.\textsuperscript{162} Lipids are water insoluble molecules, comprising triacylglycerols, phosphoglycerides, sterols and sphingolipids.\textsuperscript{163}

Since triacylglycerols are highly reduced and anhydrous, fatty acids provide twice as much ATP as carbohydrates or proteins and six times more ATP than glycogen, relative to their dry mass.\textsuperscript{164} Fatty acids therefore preferentially serve as metabolic energy reservoirs under conditions of nutrient abundance in the form of lipid droplets (LD).\textsuperscript{164} Lipids are critical components of biological membranes and cholesterol regulates membrane fluidity.\textsuperscript{165,166} Cholesterol and
sphingolipids form membrane microdomains called lipid rafts, which concentrate receptors acting as hubs for signal transduction.\textsuperscript{167} Cholesterol also participates in signaling as a precursor of steroid hormones including androgens, progestagens, glucocorticoids, mineralocorticoids, and estrogens.\textsuperscript{168}

Additionally, intermediates of cholesterol biosynthesis, farnesyl pyrophosphate (FPP) or geranylgeranyl pyrophosphate (GGPP) activate Ras and Rho GTPases through posttranslational prenylation.\textsuperscript{169} These small GTPases perform numerous cellular functions relevant to cancer, including control of cytoskeleton dynamics, migration, cell cycle progression, generation of ROS and gene expression.\textsuperscript{170} Intermediates of \textit{de novo} lipogenesis such as diacylglycerol (DAG) also serve as second messengers in cellular signal transduction.\textsuperscript{166} Lipid mediated post translational modification regulates the localization and function of various signaling proteins like GPI (glycosylphosphatidylinositol) anchored proteins some of which are associated with cancer, like the urokinase-type plasminogen activator (uPA)-receptor (uPAR).\textsuperscript{166}

Protein S-palmitoylation, involving the formation of a thioester bond between a cysteine thiol side chain with the 16-carbon fatty acid palmitate, is a widespread lipid modification crucial for regulating protein subcellular localization, stability among other functions.\textsuperscript{166} The oncogenic activation of $\beta$-catenin through Wnt palmitoylation, which is mediated by fatty acid synthase (FASN) might play a role in PCa.\textsuperscript{166} Protein N-myristoylation a lipid modification by N-myristoyltransferase (NMT) that attaches myristate, a 14-carbon fatty acid, to protein N-terminal glycine residues regulates cellular localization and signal transduction.\textsuperscript{171} Myristoylation activates the serine threonine kinase Akt leading to neoplastic transformation.\textsuperscript{166}
Cancer cells display a great demand for lipids and cholesterol. With the exception of the liver, adipose tissue and mammary cells which synthesize fats \textit{de novo}, most adult normal cells preferentially use circulating fatty acids (FAs) or those complexed with proteins \textit{e.g.} low-density lipoproteins from dietary sources or from lipogenic tissues.\textsuperscript{165} To provide lipids for membrane production, a lot of cancers synthesize lipids and cholesterol \textit{de novo} and to a lesser extent increase the uptake of exogenous fats; LD and cholesterol levels correlate with tumor aggressiveness.\textsuperscript{162,165} Lipid synthesis (lipogenesis) starts with the conversion of citrate from the TCA cycle in to acetyl-CoA and oxaloacetate by ATP-citrate lyase.\textsuperscript{165} Glucose and glutamine can both contribute Acetyl-CoA for fatty acid synthesis.\textsuperscript{172}

Pyruvate dehydrogenase (PDH) converts glycolysis-derived pyruvate to Acetyl-CoA.\textsuperscript{172} Glutamine-derived $\alpha$-ketoglutarate is reductively carboxylated to isocitrate (by isocitrate dehydrogenase 1, IDH1) then citrate (by aconitase) which is cleaved to oxaloacetate and Acetyl-CoA.\textsuperscript{172} Acetyl-CoA is converted to Malonyl-CoA by acetyl-CoA carboxylase (ACC) which is rate limiting in lipid synthesis.\textsuperscript{166} Subsequently, fatty-acid synthase enzyme (FASN) synthesizes the saturated fatty acid palmitate (16:0) with Acetyl-CoA as a primer, Malonyl-CoA as a two-carbon donor and NADPH the reducing equivalent.\textsuperscript{166} To generate NADPH, oxaloacetate can be converted to malate then pyruvate by malate dehydrogenase and malic enzyme respectively.\textsuperscript{166} Alternatively, NADPH could be derived from the PPP pathway.

Saturated FAs are elongated or desaturated by elongase and desaturase enzymes (like Stearoyl-CoA desaturase, SCD1), respectively, to generate various long chain saturated or desaturated fatty acids.\textsuperscript{165} Cholesterol on the other hand is synthesized from Acetyl-CoA in the mevalonate pathway.\textsuperscript{165} The process begins with the condensation of three Acetyl-CoA molecules by
acetoacetyl-CoA thiolase and HMG-CoA synthase to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). In the subsequent rate-limiting step, HMG-CoA is reduced to mevalonate in the presence of NADPH by HMG-CoA reductase (HMGCR) an enzyme under complex regulatory control. When sterol isoprenoids are in short supply, sterol regulatory element binding proteins (SREBP) bind to sterol regulatory elements (SREs) on the HMGCR promoter increasing its transcription.

When sterol levels are sufficient, the HMGCR sterol-sensing domain mediates its ubiquitin mediated degradation. During metabolic stress however, AMP-activated protein kinase (AMPK) phosphorylates and deactivates HMGCR lowering sterol metabolism. Mevalonate is then converted to isoprenoids including cholesterol by various enzymes in the melavonate pathway. During starvation and in certain situations like loss of matrix attachment however, cancer cells activate fatty acid β-oxidation (FAO) to generate acetyl-coA and ATP. While short and medium chain fatty acids are able to diffuse in to the mitochondria for FAO, the mitochondrial membranes are impermeable to long chain fatty acids.

Prior to transportation in to the mitochondria, long chain fatty acids are first be activated in to fatty acyl-CoA by acyl-CoA synthetase (ACS) in the cytosol. In FAO’s rate limiting step, carnitine palmitoyltransferase-1 (CPT1) on the outer mitochondrial membrane conjugates fatty acyl-CoAs to carnitine forming acylcarnitines for transportation into the mitochondrial inter membrane space (IMS). Transport from the IMS to the mitochondrial matrix is facilitated by carnitine–acyl carnitine translocase (CACT). CPT2 located on matrix side of the inner mitochondrial membrane regenerates fatty acyl-CoA from the fatty acyl carnitine for β-oxidation and the free carnitine is transported back in to the cytosol by CACT.
In FAO, repeated cycles of oxidation, hydration, another oxidation and thiolytic cleavage shorten even-chain-length fatty acyl-CoA molecules two carbons per cycle to generate acetyl CoA, NADH and FADH2. The shortened fatty acyl-CoA enters the \( \beta \)-oxidation cycle until a four-carbon fatty acyl molecule is reduced to two acetyl-CoA molecules that are fed into the TCA cycle and the electron transport chain for ATP synthesis. Odd-chain-length fatty acyl-CoA molecules, a rare species, also undergo the same oxidation pathway but they yield both acetyl-CoA and propionyl-CoA as end products.

The last \( \beta \)-oxidation cycle of odd-chain-length fatty acyl-CoA cleaves five carbon acyl-CoA molecules into a three carbon molecule (propionyl-CoA) and a two carbon molecule (Acetyl-CoA). The propionyl-CoA is converted into succinyl-CoA before entry into the TCA cycle.

**Mechanisms of metabolic reprogramming in cancer cells**

Metabolic reprogramming in cancer cells is mainly driven by oncogenes, loss of tumor suppressors and to a lesser extent, mutations in metabolic enzymes. MYC, an oncogene often upregulated or amplified in cancer, transcriptionally upregulates nutrient transporters, glycolytic and glutaminolytic enzymes as well as mediating glutamine addiction in some cancers. The tumor suppressor p53 induces TIGAR (TP53-induced glycolysis regulator) that converts fructose-2,6-bisphosphate an allosteric activator of the glycolysis rate limiting enzyme PFK1 to fructose-6-bisphosphate downregulating glycolysis and increasing PPP flux. Loss of p53 as seen in half of human cancers therefore results in increased glycolytic flux and ROS accumulation that promotes tumorigenesis.
The PI3K/AKT/mTOR pathway, which is abnormally activated in many cancers, increases glycolysis and \textit{de novo} lipogenesis via the hypoxia-inducible factor (HIF1) and SREBP respectively.\textsuperscript{181-183} BRAF and KRAS oncogenes also enhance glycolysis by activating AKT.\textsuperscript{155} HIF1 increases GLUT1 and hexokinase 2 (HK2) expression upregulating glucose uptake and glycolysis respectively.\textsuperscript{184,185} Besides the hypoxic conditions found in most tumors activate HIF1 increasing glycolysis.\textsuperscript{151}

However, some tumors can constitutively activate HIF1 even under normoxic conditions through several mechanisms, including loss of von Hippel-Lindau (pVHL) which targets hydroxylated HIF for ubiquitin-mediated degradation, accumulation of ROS and metabolic signaling.\textsuperscript{151} Inactivating mutations in succinate dehydrogenase (SDH) and fumarate hydratase (FH) lead to mitochondrial accumulation of their substrates succinate and fumarate, respectively, which inhibit HIF prolyl-4-hydroxylases (PHDs) when they leak into the cytosol preventing HIF1 degradation.\textsuperscript{186} Other mutational mechanisms leading to metabolic reprogramming have also been described.

Phosphoglycerate dehydrogenase (PHDGH) diverts 3-phosphoglycerate, a glycolytic metabolite, into the serine biosynthetic pathway, which contributes one-carbon units for methylation reactions, nucleotide and NADPH biosynthesis.\textsuperscript{151} The PHDGH gene is amplified in approximately 6% of breast cancers and 40% of melanomas, and tumors that overexpress it are dependent on it for growth.\textsuperscript{187} In gliomas and acute myeloid leukemia (AML), recurrent mutations lead isocitrate dehydrogenase 1 and 2 (IDH1/2) to lose their normal ability to interconvert isocitrate to \(\alpha\)-ketoglutarate.\textsuperscript{137} Instead, mutated IDH1/2 reduce \(\alpha\)-ketoglutarate to D-2-hydroxyglutarate (D2HG) a rare metabolite.\textsuperscript{137}
Increased concentration of D2HG in tumors inhibits several α-ketoglutarate-dependent dioxygenase enzymes including the TET family of enzymes which methylate DNA altering gene expression.137 Besides mutations, tumors preferentially express specific isoforms of metabolic enzymes.137 Cancer cells preferentially express the M2 isoform of the glycolytic enzyme pyruvate kinase (PKM2) that favors anabolic metabolism over the M1 isoform that is predominant in differentiated, quiescent tissue.137

Metabolic cancer therapies

Though metabolic reprogramming contributes to tumorigenesis, it also offers metabolic vulnerabilities that can be exploited for cancer therapy.188 However, normal proliferating cells in bone marrow, intestinal epithelium and hair follicles also reprogram their metabolism in a manner similar to tumor cells reducing the therapeutic window for anti-proliferative metabolic therapies.188 The feasibility of anti-metabolic therapies depends on how well they can be tolerated because disrupting the function of metabolic enzymes in normal tissue can result in systemic toxicity.151 However, for decades, antifolates (like methotrexate, aminopterin and pemetrexed), which target nucleotide generating one-carbon metabolism, have been crucial components in chemotherapies.188

The clinical success of antifolates for cancer therapy is predicated on the increased demand for nucleotide synthesis and DNA replication in neoplastic cells.188 Although antifolates do cause toxicity in rapidly proliferating tissue, they are integral in many chemotherapy regimens that increase patient survival.188 As amino-acids drive protein synthesis, mitochondria metabolism and form intermediate metabolites for biosynthetic pathways, several therapies target amino-acid
metabolism. The FDA has approved L-asparaginase which depletes plasma asparagine as an anti-cancer treatment in patients with acute lymphoblastic leukemia (ALL).

Whereas asparagine synthetase (ASNS) can synthesize asparagine *de novo* in normal cells, low ASNS expression sensitizes leukemic lymphoblasts to asparagine withdrawal, which diminishes asparagine-dependent protein synthesis and induces cell death. Also, given that cancer cells upregulate lipid and cholesterol synthesis for membrane synthesis and for lipid signaling intermediates, inhibiting enzymes in these pathways could be an effective anti-cancer strategy. Several clinical trials are evaluating the efficacy of statins, which inhibit cholesterol synthesis through HMGCR and are approved for treating cardiovascular disease, as anti-cancer therapy.

Mitochondrial metabolism is another emerging therapeutic target because it is frequently upregulated in cancer to meet increased demand for ATP and biosynthetic precursors. Metformin, an antiguconeogenic drug approved to treat type 2 diabetes also inhibits mitochondrial complex I in cancer cells decreasing ATP generation and inducing apoptosis in glucose limiting conditions. Epidemiologic data also suggest that metformin lowers the risk of cancer development in diabetic patients who are cancer naïve and increases survival in diabetic patients with cancer. Moreover, the selective uptake of metformin by organic cation transporters (OCTs) expressed in some tumors and in a few tissues like the liver makes it well tolerable.

Metformin’s efficacy against cancer is currently being assessed in numerous clinical trials in non-diabetic patients. Additionally, the FDA has approved mitochondrial complex III inhibiting arsenic trioxide for the treatment of relapsed/refractory acute promyelocytic leukemia
(APL) and it is also being tested on other cancers.\textsuperscript{140} Another potential therapeutic strategy against mitochondrial metabolism would be the inhibition of mitochondrial enzymes.\textsuperscript{140} There are ongoing trials to test inhibitors of mutated IDH1/2 in glioma and AML patients which convert $\alpha$-ketoglutarate to the oncometabolite D2HG, a unique feature of cancer metabolism.\textsuperscript{140} By inhibiting pyruvate dehydrogenase kinase (PDK), dichloroacetate (DCA) increases the conversion of Acetyl-CoA from pyruvate by pyruvate dehydrogenase (PDH) decreasing glycolytic flux, and inducing apoptosis in cancer cells with deficient mitochondrial function.\textsuperscript{140}

Several clinical trials have evaluated DCA in cancer patients though its clinical efficacy may depend on a tumor’s metabolic profile.\textsuperscript{140,190} As mitochondrial metabolism is linked to oxidative stress, focal and metastatic cancer cells increase their antioxidant capacity to counter ROS production.\textsuperscript{151} Alternative therapeutic strategies target redox metabolism by inhibiting antioxidants; the resulting accumulation in ROS causes apoptosis.\textsuperscript{151} MTHFD2 an enzyme in the folate pathway that generates NADPH needed to maintain several antioxidant systems is overexpressed in 19 different cancers but not in normal cells making it a potential therapeutic target.\textsuperscript{191}

\textit{In vitro}, suppression of MTHFD2 in cancer cells alters multiple metabolic pathways and increases sensitivity to oxidant-induced apoptosis.\textsuperscript{192-194} In cells, the oxidized form of Vitamin C, dehydroascorbate (DHA), preferentially transported by the glucose transporter GLUT1 which is often upregulated in tumors, is reduced to vitamin C using glutathione (GSH), and NADPH.\textsuperscript{195} High doses of Vitamin C administered to mice harboring KRAS and BRAF mutant colorectal cancers is oxidized to DHA in the bloodstream and its uptake depletes NADPH and GSH, leads to metabolic collapse and induces cell death.\textsuperscript{196} As next generation sequencing enables tumor
molecular profiling there is interest in personalized cancer therapy where drugs are tailored against driver mutations in individual patients as opposed to depending solely on clinical factors.197

These efforts are however hampered by intertumoral and intratumoral genomic heterogeneity.197 Some argue that despite diverse mutations in tumor suppressors and oncogenes, they drive common metabolic phenotypes and so metabolism might be a better target for cancer therapy.140 However plastic metabolic phenotypes exist within and among tumors under influence from the tumor microenvironment.198 Understanding the metabolic reciprocity among tumor cells and between tumor and stromal cells will help in the development of effective metabolic therapies.151

Metabolic reprogramming in prostate cancer

Prostate secretory epithelial cells in the peripheral zone are metabolically adapted for the specialized function of producing prostatic fluid.199 In the most mammalian cells, glycolysis-derived pyruvate is decarboxylated to Acetyl-CoA, which is condensed with oxaloacetate forming citrate which is oxidized for ATP generation in the TCA cycle.200 In contrast, high expression of ZIP1 and ZIP3 zinc transporters in secretory prostate epithelial cells leads to high zinc accumulation, which inhibits mitochondrial aconitase (m-aconitase) blocking citrate oxidation.201-205 Attenuation of the TCA cycle and additional synthesis of citrate by prostate cells lead to net citrate accumulation in the prostatic fluid for subsequent secretion into the seminal fluid.204

Seminal fluid contains up to 12-fold higher levels of citrate compared to blood plasma.204 Citrate is believed to act as a buffering agent and a chelator of calcium and zinc.206-208 The use of
glycolysis-derived pyruvate to generate Acetyl CoA for citrate synthesis in the prostate is impeded by the slow rate of glycolysis in normal prostate cells. In fact, slow glucose uptake by prostate cells attributed to low expression of glucose transporter 1 (GLUT1), has precluded the use of FDG-PET for the detection and staging of PCa. Diminished glycolysis in the normal prostate has also been linked to the inhibitory effect of high levels of citrate on phosphofructokinase a key glycolytic enzyme.

Unlike most other solid tumors, prostate tumors only exhibit a glycolytic phenotype in metastatic or castration resistant stages as evidenced by the accumulation of 18F-FDG and the expression of glycolytic markers and monocarboxylate transporters. In the face of limited glycolysis, FAO has been proposed to be the major source of Acetyl-CoA for prostate citrate production. Benign prostate cells preferentially use fatty acids over glucose to meet their bioenergetics needs, and they also contain a higher than usual intracellular cholesterol content. In the prostate, oxaloacetate for citrate production is derived from the transamination of aspartate by mitochondrial aspartate aminotransferase (mAAT). The prostate epithelium reportedly uses the excitatory amino acid carrier 1 (EAAC1) as a high affinity aspartate transporter.

The transformation of prostate epithelial is accompanied by the loss of zinc accumulation due to decreased expression of zinc transporters. Downregulation of Zinc transporters in PCa cells occurs partly through epigenetic silencing of the ZIP1 and ZIP3 transcription regulator, AP-2alpha, through promoter hypermethylation. DNA demethylation using 5-aza-2'-deoxycytidine increased the expression of ZIP1 and ZIP3 and Zinc uptake in DU-145 and LNCaP PCa cell lines. Loss of zinc accumulation also inhibits its non-metabolic anti-tumorigeneic effects aiding prostate tumorigenesis. For example, supplementation of PCa cell lines with physiological
amounts of zinc induces apoptosis by stimulating Cytochrome C release and suppression of the antiapoptotic protein c-IAP2 downstream of NF-κB.\textsuperscript{219,220}

Zinc supplementation also reduces invasiveness by downregulating ICAM-1 (Intercellular Adhesion Molecule 1) and inhibits NF-κB pro-angiogenic and pro-metastatic effectors.\textsuperscript{221} More importantly however, low zinc reactivates m-aconitase leading to citrate oxidation to meet the cells’ growing energetic needs.\textsuperscript{199,222,223} The switch to citrate oxidation is thought to be an early event in the progression of PCa preceding detectable histological changes.\textsuperscript{223-225} However, citrate oxidation is necessary but insufficient for full transformation.\textsuperscript{200} PCa cells need additional metabolites for anabolic and energy needs some of which they obtain from cells in the tumor microenvironment.\textsuperscript{200} Stromal cells and prostate epithelial cells have been shown to mutually reprogram each other’s metabolism to enhance tumorigenesis.\textsuperscript{226,227}

Fiaschi \textit{et al.}, reported that physical contact with PCa cells activates stromal fibroblasts triggering a glycolytic phenotype with increased GLUT-1 expression, glucose metabolism and oxidative stress.\textsuperscript{226} The activated fibroblasts increased lactate production and efflux through newly expressed monocarboxylate transporter-4 (MCT4).\textsuperscript{226} Conversely, fibroblast contact increased OXPHOS and lactate uptake via the MCT1 lactate transporter but downregulated GLUT-1 expression in PCa cells.\textsuperscript{226} Eventually, the PCa cells ceased metabolizing glucose relying instead on lactate demonstrating how PCa cells could use cancer associated fibroblasts (CAFs) to adapt to low glucose environments.\textsuperscript{226} In this case, the reciprocal metabolic programming between stromal and PCa epithelial cells was regulated by HIF-1, which was stabilized by Sirtuin-3 even under normoxic conditions.\textsuperscript{226}
Valencia et al., showed that a decrease in stromal p62 downregulates mTORC1 and MYC reducing GSH and NADPH synthesis from glucose and glutamine. Diminished redox metabolism in p62 deficient prostate stromal fibroblasts led to oxidative stress which stimulated IL6 secretion triggering TGFβ synthesis. TGFβ activated the fibroblasts, which enhanced prostate tumor growth and invasion in vivo. CAFs also mediate post-translational modifications important for pyruvate kinase M2 (PKM2) nuclear localization where it complexes with HIF1 and the transcriptional repressor DEC1 (Differentially Expressed in Chondrocytes-1). The trimeric complex downregulated miR205 expression, which switched the metabolism of PCa cells to OXPHOS during PCa progression.

PCa also displays increased glutamine metabolism in the more aggressive metastatic stages primarily for TCA cycle anaplerosis. Glutaminase (GLS) converts glutamine to glutamate, a source of the TCA cycle intermediate α-ketoglutarate (α-KG). PCa cells overexpress GLS1 and in patients GLS1 expression correlates highly with PCa tumor stage and disease progression. In PCa cells, MYC promotes glutaminolysis by upregulating the expression of GLS1 through repression of miRNA-23a/b. Withdrawing glutamine or knocking down GLS in glutamine addicted PC3 PCa cells decreased ATP, cell proliferation and GSH levels causing ROS-induced apoptosis. In addition to glutaminolysis, GLS1 downregulates the glycolytic repressor, thioredoxin interacting protein (TXNIP) increasing glucose utilization in PCa cells.

Coordination of glutamine and glucose metabolism is thought to constitute a metabolic checkpoint that restricts cell growth when levels of either nutrient is limiting. Glutamine is imported in to cells by the solute carrier (SLC) group of transporters. PCa cells overexpress glutamine transporters ASCT2 (or SLC1A5) and SLC1A4 due to multiple oncogenic
AR mediated signaling drives glutamine dependent proliferation of prostate cells by upregulating the expression of SLC1A4 and SLC1A5 glutamine transporters through mTORC1. MYC also regulates AR mediated glutamine uptake but only in PTEN null prostate cells. Additionally, the constitutively active androgen-receptor splice variant 7 (AR-V7) expressed in CRPC increases dependence on glutaminolysis for TCA cycle anaplerosis as well as glutamine reductive carboxylation.

Inhibition of ASCT2 in prostate cells decreased glutamine uptake and fatty acid synthesis reducing the growth of cancer cells and tumor xenografts as well as metastasis. On a molecular level, ASCT2 inhibition downregulated E2F cell cycle proteins and mTORC1 activation. Glutamine metabolism in PCa cells can also be affected by exosomes secreted by cancer-associated fibroblasts (CAFs). Addition of CAF-derived exosomes (CDEs) to PCa cells increased ready-made metabolites, decreased OXPHOS and increased glycolysis as well as reductive carboxylation of glutamine. These findings further demonstrate how tumors might use stromal CDEs to reprogram their metabolism in nutrient deficient conditions.

An aberrant increase in de novo lipogenesis is another common occurrence in early clinical PCa. The peripheral zone which shows the highest propensity for PCa development has increased transcription of lipid biosynthesis enzymes with a concomitant rise in fatty acid metabolites. Paradoxically, this zone also displays increased fatty acid catabolism indicating an increased capacity for lipid metabolism in general. Overexpression of the lipogenic enzyme FASN occurs early in PCa tumorigenesis with both mRNA and protein being detected in increasing amounts from PIN to metastases. FASN overexpression is associated with PCa progression and it has been linked to poor prognosis and reduced disease free survival.
addition, nuclear localization of FASN in a subset of PCa cells has been associated with a more aggressive phenotype.\textsuperscript{244}

Moreover, overexpression of FASN transforms immortalized, benign prostate epithelial cells and FASN transgenic mice develop PIN.\textsuperscript{245} FASN oncogenesis in PCa has been linked to Wnt1 palmitoylation which results in cytoplasmic accumulation of $\beta$-catenin, inhibition of apoptosis, protection from ER stress and production of lipids for membrane synthesis.\textsuperscript{245-247} In addition, relative to matching normal tissue, FASN expressing prostate tumors have elevated levels of saturated and mono-unsaturated fatty acids and decreased polyunsaturated fatty acids.\textsuperscript{248} The switch to more saturated fatty acid species protects cells from oxidative stress and doxorubicin-induced cell death.\textsuperscript{248} In addition, as saturated phospholipids are preferentially incorporated in to lipid fats, increasing their synthesis would significantly impact the lipid raft signaling landscape.\textsuperscript{249}

Inhibition of FASN decreased cell viability, proliferation, migration and clonogenic survival while increasing the selective apoptosis of different human cancer cells including prostate.\textsuperscript{245,250-253} \textit{In vivo}, FASN inhibition reduced the growth of LNCaP tumors pointing to the importance of a lipogenic phenotype for PCa growth and survival.\textsuperscript{254} FASN overexpression in the prostate has been linked to several mechanisms. FASN copy number alterations have been detected in some PCa cells lines as well as adenocarcinoma and metastatic cancers implicating gene amplification in increased FASN expression.\textsuperscript{255} Increased H3K27Ac marks in the FASN gene promoter by P300 upregulated FASN expression leading to lipid accumulation \textit{in vitro} and in the prostates of mice with a PTEN knockout.\textsuperscript{256}
Growth factors and their receptors as well as hormones can also upregulate FASN expression. In prostate cells, androgens and EGF can stimulate SREBP-1 which in turn regulates the expression of AR in a feedback loop that further drives the expression of lipogenic enzymes. In PTEN null, LNCaP as well as human prostate tissues, FASN expression was linked to the activation of the PI3K/Akt pathway. Accordingly, inhibition of PI3K/AKT in PCa cells reduces FASN expression. Post-translational modifications that stabilize FASN in prostate cells have also been described. The deubiquitinating enzyme USP2a prevents FASN proteasomal degradation with its knockdown decreasing levels of FASN.

Besides FASN, inhibition of ACYL, another lipid synthesis enzyme, reduces PCa cell viability in the absence of exogenous lipid supplies. Concomitant inhibition of ACYL and AR in CRPC cells induced energetic stress, activated AMPK and synergistically suppressed AR decreasing cell proliferation and increasing apoptosis. There are conflicting reports on the expression of SCD1 in PCa. One study reported frequent loss of SCD1 transcripts and protein in human PCa tumors compared to benign tissue. Different studies report that SCD1 is upregulated in PCa increasing the ratio of monounsaturated to saturated fatty acids. High intracellular accumulation of saturated fatty acids has been linked to lipotoxicity so SCD1 overexpression in cancers might protect cells from such toxicity.

Overexpression of SCD1 increased tumorigenicity in androgen sensitive LNCaP cells and tumor growth in nude mice. Inhibiting SCD1 decreased lipid synthesis, cell proliferation and in vivo tumor growth in nude mice and prostate orthografts while increasing survival. SCD1 inhibition downregulated the AKT pathway possibly through decreased levels of PIP3, (phosphatidylinositol-3,4,5-trisphosphate (PI (3,4,5) P3)). In addition, SCD1 inhibition
activated AMPK and decreased β-catenin, which activated glycogen synthase α/β (GSK3 α/β).\textsuperscript{268} Furthermore, SCD1 activity appears to be important in Ras oncogene cell transformation.\textsuperscript{268} SCD1 has also been implicated in AR activation in LNCaP cells.\textsuperscript{270}

High-grade PCas also overexpress the fatty acid elongation enzyme ELOVL7 through the activation of SREBP1 by the androgen pathway.\textsuperscript{272} Knockdown of ELOVL7 altered phospholipids and neutral lipids like cholesterol a precursor for androgen synthesis.\textsuperscript{272} Consequently, knockdown of ELOVL7 decreased androgen synthesis and attenuated PCa cell proliferation.\textsuperscript{272} Consumption of a high fatty diet on the other hand increased tumor growth in ELOVL7 expressing tumors linking diet and PCa.\textsuperscript{272} As NADPH and acetyl-CoA from glucose and glutamine metabolism can drive lipid biosynthesis, increased lipogenesis in cancer cells could result from the upregulation of these two pathways and not necessarily the dysregulation of lipid synthesis enzymes.\textsuperscript{166}

In advanced PCa, upregulation of SREBP2 that transcriptionally regulates enzymes involved in cholesterol biosynthesis and loss of sterol feedback inhibition increases androgen synthesis.\textsuperscript{273,274} In PCa cells, promoter hypermethylation of the cholesterol efflux protein ABCA1 downregulates its expression altering cholesterol homeostasis.\textsuperscript{275} ABCA1 downregulation correlates inversely with Gleason grade implicating high cholesterol levels to cancer progression.\textsuperscript{275} Besides promoting androgen synthesis, cholesterol in lipid rafts also promotes PCa progression by activating AKT signaling.\textsuperscript{276,277} Metabolic intermediates in cholesterol synthesis are used to prenylate small GTPases like RhoC GTPase, increasing proliferation and metastasis in PCa.\textsuperscript{278}
Additionally, major structural components of the cholesterol-enriched invaginations of the plasma membrane caveolae called caveolins (CAV) are upregulated in PCa, and they are linked to disease progression.\textsuperscript{279,280} CAV-1 is suggested to have a role in the modulation of cholesterol in the mitochondria as well as mediating androgen receptor activity.\textsuperscript{281,282} Deletion of Cav-1 decreased tumor burden in the prostates of TRAMP mice as well as lymph node metastases.\textsuperscript{283} Epidemiologic data indicates that the use of statins (HMG-CoA reductase inhibitors) is associated with a lower risk of developing advanced PCa.\textsuperscript{284} Statins have therefore been proposed for secondary and tertiary PCa chemoprevention.\textsuperscript{284}

Dysregulation in fatty acid catabolism has also been demonstrated in human cancers, including PCa. AMACR (alpha-methylacyl-CoA racemase), which controls $\beta$-oxidation of dietary branched chain fatty acids in peroxisomes is frequently upregulated in PIN and PCa and its expression is associated with an increased risk of disease.\textsuperscript{285-287} In addition, treatment of PCa cells \textit{in vitro} with branched fatty acids from dairy and beef products increased the expression AMACR.\textsuperscript{288} Increased expression of pristanoyl-CoA oxidase (ACOX3), which is downstream of AMACR, further points to the importance of perosisomal $\beta$-oxidation of branched-chain fatty acids in PCa.\textsuperscript{289} Peroxisomal $\beta$-oxidation has been linked to the production of reactive oxygen species (ROS), which might contribute to carcinogenesis through DNA damage.\textsuperscript{290}

\textit{Oxidative stress driven mechanisms}

Reactive oxygen species (ROS) are reactive molecules derived from the partial reduction of molecular oxygen ($O_2$). The source of intracellular ROS can be exogenous or endogenous from normal cellular processes.\textsuperscript{291} Endogenous ROS are by-products of aerobic respiration and protein
folding or produced by NADPH oxidase (NOX) complexes and inflammation. ROS include free radicals such as the oxygen radical (O$_2^-$), hydroxyl (OH$^-$), superoxide anion (O$_2$-) and peroxide radicals (ROO$^-$) but also non-radicals like hydrogen peroxide (H$_2$O$_2$) and singlet molecular oxygen (¹O$_2$). Non-radicals can give rise to free radicals for example in the presence of transition metals.

In small quantities ROS take part in signal transduction (i.e. redox signaling) by reversibly oxidizing protein thiol groups affecting numerous physiological processes including hypoxia, gene transcription through redox sensitive transcription factors, cell proliferation and differentiation. However, high levels of ROS result in the indiscriminate damage of cellular macromolecules; lipids, proteins, and DNA. Oxidative DNA damage is thought to play a critical role in all stages of carcinogenesis. ROS can modify DNA bases, form DNA adducts, induce DNA cross-linking and cause DNA strand breaks. Oxidative DNA lesions that are not removed prior to DNA replication can lead to replication errors, mutations and genome instability, increasing the risk of carcinogenesis.

ROS also indirectly increase cancer risk if they cause lesions on tumor suppressor genes or inactivate negative regulators of oncogenes and DNA repair proteins. Under physiological conditions, ROS accumulation is regulated by endogenous enzymatic and non-enzymatic antioxidant defense systems. Antioxidant enzymes include glutathione peroxidase (GPX), Gltathione S-transferase (GST), catalase (CAT) and superoxide dismutase (SOD). Non-enzymatic antioxidants include vitamin C and E, flavonoids and low molecular weight co-factors and peptides like glutathione (GSH), NADPH and peroxiredoxin (PRX). Oxidative stress
occurs when ROS levels outstrip these cellular antioxidant defenses. Epidemiological, experimental and clinical data link oxidative stress to PCa development and progression.

In Nkx3.1 prostate specific knockout (KO) mice, dysregulation in the expression of antioxidant and prooxidant enzymes led to oxidative stress and development of HGPIN while Nkx3.1/Pten double heterozygous mice sustained more oxidative damage and progressed to adenocarcinoma. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of PCa, products of oxidative damage; 8-hydroxy-2'-deoxyguanosine (8-OHdG), 4-hydroxynonenal (4-HNE)-protein-adducts and nitrotyrosine (Ntyr), could be detected in early prostatic tumorigenesis. Administration of testosterone and β-estradiol triggered the expression of prooxidant enzymes and oxidative damage, which induced stromal inflammation and dysplasia in the lateral prostate (LP) of the noble (Nb) rat hormonal carcinogenesis model. These studies implicate oxidative stress from genetic perturbations or sex hormones in PCa initiation and progression.

Relative to benign prostate cells, human PCa cell lines have higher oxidative stress which is associated with a more aggressive phenotype. Besides, transcription factors important for PCa like NK-κB, AP-1, HIF-1 and p53 are redox sensitive. Several PCa risk factors, including age, diet, inflammation and androgens, are also associated with oxidative stress. Moreover, key genetic and epigenetic changes in PCa have been shown to decrease the expression of genes important for prostatic redox homeostasis like GSTP1, NRF2, NKX3.1 and NADPH oxidases. Accordingly, PCa patients show increasing oxidative biomarkers, including thiobarbituric acid reactive substances (TBARS), 8-OHdG and 4HNE-modified proteins, concomitant with loss of antioxidant defenses with disease progression. These data are the
basis of the belief that antioxidants, by inhibiting oxidative stress, could be chemopreventive against PCa.

**Prostate cancer diagnosis and treatment**

**Screening**

In the U.S., the prostate-specific antigen (PSA) is used for PCa screening and early detection followed by a confirmatory biopsy. Digital rectal examination (DRE) was used prior to PSA testing, but its use for primary PCa screening is limited by poor sensitivity and specificity and high inter-observer variability. A normal PSA has a common arbitrarily threshold of \( \leq 4.0 \) ng/mL, but this only has a 30% positive predictive value in men \( \geq 50 \) years and 85% negative predictive value in men with a median age of 69 years at biopsy. Widespread PSA screening in the U.S. shifted the diagnosis stage away from metastases in the 1980s and ‘90s and increased detection of curable early-stage cancers.

PCa specific mortality also decreased by 4% by 1992 (5 years after the introduction of PSA screening) though it remains contentious whether the decrease is directly attributable to PSA testing. Besides, PSA is not a PCa specific marker. Serum PSA can increase due to disruption of normal prostate anatomy by other prostatic diseases, including benign prostatic hyperplasia (BPH), prostatitis and prostatic trauma. PSA also has low specificity with 75% men with a PSA \( > 3 \)ng/mL having a negative biopsy while 7 – 56% of those with a positive biopsy would have died without clinical disease and therefore considered “overdetected”.
Lack of PSA specificity leads to unnecessary biopsies which carry the risk of bleeding and infection, detection and overtreatment of clinically insignificant PCa with accompanying treatment-related morbidities. On the flip side, high-grade cancers can cause deceptively low serum PSA levels that would be missed under the PSA guidelines for a biopsy. The inability to distinguish between indolent and lethal PCas remains a major clinical challenge. Assessing prostate morphology combined with function and physiology using multiparametric magnetic resonance imaging (mpMRI) might decrease the risks associated with PCa screening and treatment.

**Biopsy**

The need for a prostate biopsy is determined by PSA levels, a suspicious DRE, patient’s age, and comorbidities. Multifocality in PCa is common so to increase cancer detection rates, (CDRs), the American Urological Association (AUA) recommends surveying 10–12 cores of prostate tissue including far lateral and apical samples, in the initial biopsy. Pathological evaluation is based on the Gleason grade, which is a five-tier grade system representing a continuum of histological and differentiation of the prostate. The Gleason score (ranging from 2 to 10) comprises the most prevalent pattern or the primary grade and the second most prevalent pattern, the secondary grade. For biopsies with cancer, Gleason score is a major determinant of treatment options as well as the tumor volume, which is estimated from the number of cores with cancer.

**Treatment**
The therapeutic management of PCa depends on a patient’s age and life expectancy, presence of comorbidities, patient’s preferences and the probability of recurrence and metastasis after treatment (risk stratification). Depending on PSA level, Gleason score, tumor grade and the degree of prostate involvement (number of biopsy cores with cancer) patients are divided into several prognostic groups as very low-risk, low risk, intermediate risk, high-risk, very high risk and for those with lymph node involvement, stage IV disease. Patients with very low risk (PSA $\leq 10$ µg/L, Gleason score $\leq 6$ on biopsy, clinical stage T1c, fewer than three positive biopsies and $\leq 50\%$ PCa in any core) and low risk PCa, (PSA $\leq 10$ µg/L, Gleason score $\leq 6$ on biopsy and clinical stage T1 – T2a) may opt for “active surveillance,” radiation therapy or radical prostatectomy.

Active surveillance involves postponing treatment with annual or biannual PSA and DRE monitoring. In active surveillance, treatment with curative intent is done on sign of progression and this is meant to decrease treatment related morbidities in low risk men who might never develop lethal PCa. In contrast, monitoring of disease symptoms termed, “watchful waiting” is recommended for patients who do not have very long to live (per the American Urological Association (AUA), this would be less than or equal to five years), those with significant co-morbidities or those who do not wish to have curative treatments. For men with local or regional disease at diagnosis, 81% and 12% respectively, single modality with radical prostatectomy (RP) or radiation therapy (RT; external beam or brachytherapy) are effective treatments with a five-year survival rate of 100%. However, treatment related morbidity including urinary incontinence, erectile dysfunction or declining bowel function result in a poorer quality of life. Some of the side effects have
been ameliorated by use of nerve-sparing radical prostatectomy.\textsuperscript{343} Other approaches for localized PCa include focal ablation therapy where cryotherapy, high-intensity focused ultrasound (HIFU), photodynamic therapy, or laser ablation are used to destroy a targeted region harboring PCa.\textsuperscript{344} While focal ablation therapy is associated with diminished treatment related morbidity, its utility for long-term treatment remains to be seen.\textsuperscript{344} Early PCa progression is driven by androgens making androgen ablation therapy (ADT) a mainstay treatment.\textsuperscript{345,346}

ADT can be achieved by the surgical removal of both testicles (bilateral orchiectomy) which make testosterone or pharmacologically.\textsuperscript{136} Testicular testosterone production is under the regulation of the hypothalamic–pituitary–gonadal (HPG) axis.\textsuperscript{347} Hypothalamic Gonadotrophin-releasing hormone (GnRH) stimulates the pituitary gland to secrete Luteinizing hormone (LH) which triggers testicular testosterone production and in a negative feedback loop, testosterone dose dependently suppresses LH secretion.\textsuperscript{347} Pharmacologically therefore, ADT can be achieved using luteinizing hormone-releasing hormone (LHRH) agonists and antagonists which block LH secretion in the pituitary via negative feedback or competitive inhibition and subsequently shut down testosterone production.\textsuperscript{136}

Together with anti-androgens (\textit{e.g.} bicalutamide and ARN-509) that competitively inhibit androgens from binding to AR and androgen synthesis inhibitors, (\textit{e.g.} Abiraterone acetate and ketoconazole) that inhibit androgen production in the adrenal and prostate glands, these agents can completely suppress androgen signaling.\textsuperscript{136,348} Treatments recommended for men with intermediate risk disease, (PSA of $\geq 10 - \leq 20$ µg/L, Gleason score = 7 and clinical stage T2b – T2c), include RT with either external beam radiation therapy (EBRT) or brachytherapy alone or combined with androgen deprivation therapy.\textsuperscript{337} Alternatively, men with intermediate risk can
have an RP and a Pelvic Lymph Node Dissection (PLND) for initial therapy since metastases often involve lymph nodes and then adjuvant therapy with EBRT (plus or minus ADT).337

Men with high risk (PSA of ≥ 20 µg/L, Gleason score = 8 – 10 and clinical stage T3a) and very high risk disease (PSA of ≥ 20 µg/L, Gleason score = 8 – 10 and clinical stage T3b – T4) receive EBRT (alone or combined with a brachytherapy boost) and ADT or docetaxal chemotherapy.337 For men with locally advanced disease without fixation to local tissues can receive radical prostatectomy with PLND.337 Regional disease is treated with EBRT and ADT while metastatic disease is treated with ADT.337

PSA testing is used to monitor for recurrence after local therapy with biochemical recurrence characterized as a rise of 0.2ng/mL after RP or 2ng/mL after RT.349 However, 20 – 40% RP and 30 – 50% RT patients experience biochemical recurrence within 10 years.350-352 Depending on the initial local therapy administered and the whether the recurrence is local or metastatic, recurrent PCa is treated with salvage radiation therapy, salvage prostatectomy or ADT.353 Eventually, however, most tumors become castration resistant (CRPC). Non-metastatic CRPC, is however still treated with ADT therapy because the AR pathway is found to be still active in these patients.354 First-generation therapies include anti-androgens; flutamide, nilutamide and bicalutamide or inhibitors of androgen synthesis like ketoconazole with steroid.354

Enzalutamide, a second-generation androgen receptor antagonist approved for metastatic CRPC (mCRPC) is also used to treat non-metastatic CRPC.354 Enzalutamide inhibits androgenic signaling by inhibiting AR nuclear translocation, chromatin binding, and coregulator binding.355 Treatments for mCRPC treatment include androgen synthesis inhibitors, (Abiraterone acetate
plus prednisone or ketoconazole), first (bicalutamide, nilutamide, flutamide) and second
generation AR blockers (enzalutamide), and chemotherapy (Docetaxel or Cabazitaxel).\textsuperscript{354}
Additionally, in 2010, Food and Drug Administration (FDA) approved an immunotherapy,
Sipuleucel-T, for the treatment of asymptomatic or minimally symptomatic mCRPC.\textsuperscript{356}
Sipuleucel-T uses a patient’s autologous antigen presenting cells activated against a common
PCa antigen (prostatic acid phosphatase) and linked to granulocyte-macrophage colony-
stimulating factor (an adjuvant) thereby activating the host’s PCa specific T cell response.\textsuperscript{356}
Unfortunately, CRPC treatments confer a median overall survival benefit of less than five
months.\textsuperscript{357-363}

**Prostate cancer prevention**

Given the drawbacks of screening, treatment associated morbidity, lack of effective treatment for
advanced disease and several non-modifiable risk factors like age, ethnicity and genetics,
effective PCa prevention strategies are imperative. Chemoprevention describes the use of
natural, synthetic or biological substances to reverse, retard or inhibit the initiation of
carcinogenesis or the progression of neoplasia to malignancy.\textsuperscript{364} PCa has a slow progression;
although early pathological changes like PIN are evident in men younger than 30 at autopsy, the
median age of diagnosis with clinical disease is 66.\textsuperscript{340,365} Similarly, PCa has a long latency
within the gland before progressing through several stages of locally invasive, metastatic, and
castration resistant disease.

The long natural-history of PCa offers a long window for preventive interventions. Like other
carcinomas, PCa proceeds through the sequential accumulation of genetic and epigenetic
changes to the epithelium and the surrounding microenvironment transforming normal cells to malignancy and eventually metastasis.\textsuperscript{366} As the disease progresses therefore, therapies would need to reverse many more molecular aberrations compared to early-stage disease, supporting the rationale for early prevention.\textsuperscript{367} Additionally, late-stage interventions are bound to be compounded by co-morbidities which are more common in old age.\textsuperscript{368} PCa chemoprevention is also an attractive public health strategy since reduction in disease incidence averts treatment associated morbidities along with the linked costs.\textsuperscript{369}

\textit{Shortfalls of Pre-clinical Models of Prostate Cancer Chemoprevention}

Two-dimensional tissue culture and organ-specific animal models are typically used to identify and test the efficacy, mechanism of action, dosing and safety of agents for the chemoprevention of specific forms of cancers.\textsuperscript{370} Quantitative data gleaned from short-term mechanistic and biochemical \textit{in vitro} assays serve as early indicators for chemoprevention efficacy for further evaluation in longer term assays and in whole animal models.\textsuperscript{370} The efficacy, dose response, toxicity and pharmacokinetic data of promising agents are then tested in well established chemically induced, spontaneous or genetically engineered PCa models with the best candidates proceeding to clinical chemoprevention trials.\textsuperscript{370}

However, although conventional tissue culture has advanced the conceptual understanding of PCa biology, it fails to recapitulate \textit{in vivo} three-dimensional cell-cell and cell-matrix interactions, whose disruption plays a key role in cancer initiation and progression.\textsuperscript{371} Unlike in 2D cultures, \textit{in vivo}, cells in the tumor microenvironment (immune cells, fibroblasts, myofibroblasts, and endothelial cells), nutrient and oxygen gradients all regulate tumor
growth.\textsuperscript{371} Furthermore, compared to other common cancers, there is a shortage of PCa cell lines as they are difficult to establish.\textsuperscript{371} Moreover, majority of the \textit{in vitro} studies prescreening primary chemopreventive agents utilized immortalized human PCa cell lines, like DU-145, PC-3 and LNCaP, which model advanced disease.

Preclinical models should however recapitulate the phase of disease progression under study and these studies should have used models of disease initiation or benign prostate epithelial cells instead. Ideal animal models for PCa chemoprevention also ought to reflect human PCa tumor biology as much as possible in terms of precursor lesions, course of progression, histology and molecular aberrations.\textsuperscript{370} Additionally, the carcinogen or mutations used to generate the cancer should be relevant to the human disease and should generate a consistent tumor burden in a reasonable time frame.\textsuperscript{370} In addition, the predictive accuracy value of the animal model for the clinical trial outcome should be $\geq 80\%$.\textsuperscript{370} So ideally, murine PCa (mPCa) would originate from epithelial cells progressing to invasive adenocarcinoma through PIN.\textsuperscript{372}

mPCa should also be androgen responsive like most human PCas and regress with ADT with recurrent tumors progressing to CRPC.\textsuperscript{372} Metastases in mPCa tumors should also show the same tropism as human PCa which mostly metastasize to the bone.\textsuperscript{372} However, no single model encompasses all these characteristics, and most murine models just recapitulate one or more stages of PCa progression.\textsuperscript{372} Besides, considerable differences exist between human and murine prostates, which are some of the most popular PCa animal models. Whilst PCa is a disease of old men with slow progression, these features cannot be modeled in to mice without compromising the experimental design.\textsuperscript{372} Furthermore, mice rarely spontaneously develop PCa unlike human
males who have a one in nine (1/9) lifetime risk of developing PCa pointing to differences in human and mouse prostate biology and tumorigenesis. Additionally, unlike single-organ human prostates, mice prostates have four distinct lobes calling into question which lobe is most representative of the human prostate. Moreover, differences also exist between the mouse and human stroma. The most commonly used animal models for PCa chemoprevention studies include; the transgenic adenocarcinoma of the mouse prostate (TRAMP), the LADY and PTEN-deficient mouse models and the Dunning rat model. In the TRAMP model, prostatic adenocarcinoma is driven by expression of the SV40 small and large T antigens under the androgen regulated rat probasin (PB) promoter, which inactivates the p53 and Rb tumor suppressors. This model rapidly progresses to prostatic neoplasia at 28 weeks with 100% and 67% of the animals harboring lymph node and pulmonary metastases respectively.

In the TRAMP model, 80% of the mice were castration resistant a phenotype mediated by differentiation to neuroendocrine tumors. In the LADY, the large PB promoter drives the expression of the SV40 large T antigen with a truncated small T antigen distinguishing it from TRAMP. Prostatic changes in LADY mice progress somewhat slower than in the TRAMP with mice PIN (mPIN) with limited adenocarcinoma developing by 15 – 22 weeks without metastasis to other organs. Mouse models bearing similar genetic lesions as human PCa have also been used in chemoprevention studies. Loss of the PTEN tumor suppressor is a common event in PCa leading to preclinical chemoprevention studies using mice with Pten heterozygous deletion often in combination with other genetic common PCa lesions like Nkx3.1 haploinsufficiency. However the Nkx3.1; Pten double heterozygous mice mimic key features of advanced PCa.
Criticisms against the use of these mice models for primary chemoprevention include: The quick progression of mPCa in the TRAMP mice compared to slow progressing human PCa. In the TRAMP and LADY models, mPCa is driven by viral oncogenes irrelevant to human PCa. TRAMP mice mPCas also develop a neuroendocrine phenotype typically observed in a small subset of advanced PCAs making it a poor model for PCa initiation that is also irrelevant for the majority of patients. Additionally, the genetic changes driving prostate tumor formation in all three models, loss of p53, Rb and PTEN typically occur in advanced human PCAs. Primary chemoprevention models should ideally only contain genetic lesions that occur early in PCa development and have slow progression like that seen in human PCAs.

Ironically, while several early-stage tumorigenesis models have been developed, they have not been popular in chemoprevention studies. Other chemoprevention models include the Dunning rat model whose spontaneous and slow-growing prostate tumors have been used to generate cell lines for use in orthotopic xenograft models to test inhibition of disease progression. Chemoprevention studies have also utilized rat models whose prostate tumors were induced using hormones, like the NBL (or Noble) steroid hormone rat model, or carcinogens, like the N-Nitroso-N-methylurea (NMU) rat model. As dogs develop spontaneous prostate tumors with aged dogs exhibiting HGPIN lesions and adenocarcinoma, canine models could be relevant for PCa chemoprevention studies. However, the development of canine chemoprevention models has been hampered by the high cost and genetic heterogeneity.

Chemoprevention strategies

Targeting the AR axis
Since PCa is linked to androgen signaling, two chemoprevention trials tested 5α-reductase inhibitors (5ARIs) finasteride and dutasteride, which decrease 5α-dihydrotestosterone (DHT), the most active form of androgen. Finasteride and dutasteride reduced the risk of low grade PCa by 25% and 23% in the Prostate Cancer Prevention Trial (PCPT) and the Dutasteride of Prostate Cancer Events (REDUCE) trials respectively. These drugs were not approved by the FDA for PCa prevention however due to a slight increase in the risk of high-grade PCa.

Targeting oxidative stress

Oxidative stress has been linked to prostate carcinogenesis, propelling the use of dietary antioxidant supplements including vitamin E and selenium in PCa chemoprevention studies.

Basis for Selenium and vitamin E supplementation

*Vitamin E preclinical findings:*

Vitamin E refers collectively to four different isomers of tocopherols and tocotrienols, α, β, γ and δ that differ in the degree of methyl substitution on their chroman rings and often with differing biological activity. Naturally occurring α-tocopherol has an RRR-configuration at the 2’, 4’, and 8’ chiral carbons (RRR-α-tocopherol). Synthetic α-tocopherol esters (acetate or succinate) contain an equimolar mix of all the eight different stereoisomers arising at the three chiral centers (all-racemic, or all-rac-α-tocopherol) and are used to fortify food and in supplements. RRR-α-tocopherol is said to be more biopotent than all-rac-α-tocopherol probably because some of the isomers in the latter are inactive.
Interestingly, even different esters are shown to have different bioactivities with α-tocopherol-succinate having a distinct anti-prostaglandin effect relative to the acetate ester or (RRR-α-tocopherol) in human lung epithelial cells.\textsuperscript{393} Both α and γ tocopherol exhibit anti-tumorigenic effects but due to its ability to scavenge both ROS and reactive nitrogen species (RNS), γ-tocopherol is said to be superior to α-tocopherol.\textsuperscript{394-396} In vitro, α-tocopherol is anti-proliferative and apoptotic whereas α-tocopherol-succinate (Vitamin E succinate- VES) induces G1 cell-cycle arrest and decreases expression of anti-apoptotic proteins XIAP, Bcl-XL and Bcl2 in PCa cells.\textsuperscript{397-400}

Besides, overexpression of the alpha tocopherol transfer protein (α-TTP), which specifically facilitates α-tocopherol transportation in to plasma membranes, sensitizes PCa cells to the anti-proliferative effects of α-tocopherol by suppressing intracellular ROS.\textsuperscript{401,402} The analog RRR-alpha-tocopherylloxybutyric acid (TOB) and VES reduce cell viability, induce apoptosis and disrupt AR signaling, which is indicated by the decreased expression of the AR target, PSA in PCa cell lines.\textsuperscript{403,404} VES and α-tocopherol acetate trigger JNK signaling and upregulate the expression of Fas and FasL, which activates extrinsic and JNK cell death pathways in PCa cell lines but not in normal human prostate epithelial cells (PrEC).\textsuperscript{405,406}

VES also induces the antiproliferative and proapoptotic insulin-like growth factor binding protein-3 (IGFBP-3), reducing growth of PCa xenografts \textit{in vivo} and slowing tumor progression in TRAMP mice.\textsuperscript{407} VES inhibits NFκB and cell adhesion molecules suppressing a metastatic phenotype in PCa cell lines.\textsuperscript{398} However, a study in chicken broilers showed that α-tocopherol acetate is better for use \textit{in vivo} as hydrolysis and poor absorption led to lower tissue concentrations of VES in tissues.\textsuperscript{408} Additionally, whereas a different vitamin E analogue, RRR-
alpha-tocopheryloxybutyl sulfonic acid (VEBSA), had similar antiproliferative and apoptotic activities on PCa cells \textit{in vitro}, it had a much higher activity than VES \textit{in vivo} against tumor xenografts and disease progression in TRAMP mice.\textsuperscript{409}

Oral administration of all-rac-\(\alpha\)-tocopherol also inhibited growth of prostate tumor xenografts in nude mice on a high-fat but not low-fat diet implicating dietary fats and oxidative stress on disease progression.\textsuperscript{410} However, intraperitoneal injections of VES significantly reduced the growth of prostate tumor xenografts in nude mice on both low and high-fat diets suggesting that the route of administrating affects vitamin E activity.\textsuperscript{411} Oral administration of \(\gamma\)-tocopherol induced apoptosis and reduced PCa progression in a dose-dependent manner in the Transgenic Rat for Adenocarcinoma of Prostate (TRAP) model.\textsuperscript{412}

In TRAMP mice, a tocopherol mixture enriched in \(\gamma\)-tocopherol significantly reduced tumor growth and reduced the incidence of HGPIN.\textsuperscript{413} Supplementation with a \(\gamma\)-T-rich mixture of tocopherols reduced the development of mPIN lesions and DNA damage in CYP1A-humanized (hCYP1A) mice with chemically induced PCa using PhIP, a dietary carcinogen.\textsuperscript{414} \(\alpha\) and \(\gamma\)-tocopherol specifically dephosphorylate Akt at Ser473 by co-recruiting Akt and the phosphatase PHLPP1 to the plasma membrane through PH domain recognition.\textsuperscript{415} \(\alpha\) and \(\gamma\)-tocopherols that were structurally optimized for Akt and PHLPP1 recruitment, \(\alpha\)-VE5 and \(\gamma\)-VE5 respectively, had increased Akt Ser473 dephosphorylation activity causing more apoptosis to PCa cells \textit{in vitro} and anti-tumorigenic effects to PCa xenografts.\textsuperscript{415}

\(\gamma\)-tocopherol but not \(\alpha\)-tocopherol synergized with \(\delta\)-tocopherol to inhibit PCa but not normal cell growth \textit{in vitro} and induced apoptosis in androgen sensitive PCa cells by interrupting \textit{de}
novo sphingolipid synthesis. δ-tocopherol induced apoptosis more efficiently in human PCa cells in vitro than α-tocopherol and was antitumorigenic against tumor xenografts in vivo. In prostate specific Pten−/− mice, δ-tocopherol but not α-tocopherol inhibited pAKT(T308) which slowed PCa progression by reducing proliferation and inducing apoptosis. Interestingly, the Pten−/− mice did not have oxidative stress in the course of PCa progression.

Reduction in the incidence of epithelial dysplasia in rat ventral prostates of N-methyl-N-nitrosourea (MNU) induced PCa by γ-tocopherol was attributed to suppression of cell proliferation, MMP-9 activation as well as (GST-pi) and Cox-2 immunoexpression.

Selenium preclinical findings

Selenium (Se), an essential trace element, is incorporated in to more than 25 selenoproteins a special tRNA. Some selenoproteins like glutathione peroxidases (GPx) and Thioredoxin reductases (TRs) have antioxidant activity. Therefore, inorganic (selenite and selenite) and organic forms (Methylseleninic acid (MSA) and selenomethionine (SeMet)) of selenium have been tested for anti-cancer effects. High doses of sodium selenite paradoxically induced oxidative stress in LNCaP cells with short-term and long-term treatments causing apoptosis and cell cycle arrest respectively. In DU145 PCa cells, selenium slowed cell growth and induced extrinsic and intrinsic apoptotic pathways by increasing; death receptor 5 (DR5) expression, caspase 8 activation and Bid cleavage.

In several cancer cell lines, including DU-145, SeMet caused dose-dependent growth inhibition, aberrant mitosis and apoptosis. High local concentrations of MSA inactivated mitogenic protein kinase C isoenzymes (PKC) whose cysteine sulfhydryls in the catalytic domain
underwent catalytic oxidation inhibiting growth and inducing apoptosis in DU145 cells an effect that is reversed by the selenoprotein Thioredoxin reductase (TR).\textsuperscript{426} High TR expression in PCa cells correlated with MSA resistance and auranofin, a TR specific inhibitor sensitized PCa cells to MSA indicating that levels of PKCε and TR might modulate the effects of selenium.\textsuperscript{426,427}

Sodium selenite and MSA prevent NFκB activation induced by inflammatory stimuli by inhibiting IκB-α degradation reducing cell growth and inducing apoptosis in DU145 and JCA1 PCa cells.\textsuperscript{428} In LNCaP cells, high concentrations of MSA decreased the expression of NFκB and disrupted its DNA binding, which decreased the expression of antiapoptotic and pro-inflammatory genes NFκB target genes.\textsuperscript{429} MSC (Methyl selenocysteine) inhibited proliferation, colony formation and induced apoptosis in DU145 cells by upregulating connexin 43 (Cx43), downregulating Bcl-2 and up-regulating Bad expression.\textsuperscript{430} MSA led to detachment of DU145 cells and loss of attachment (LOA) cell death (anoikis) partly through Caspace 8 activation whereas sodium selenite led to apoptosis through JNK and p38.\textsuperscript{431}

Sodium selenite and SeMet caused dose-dependent inhibition of proliferation and anchorage independent growth and induced apoptosis in PCa cells but not normal primary prostate cells with sodium selenite being more potent.\textsuperscript{432} SeMet increased the phosphorylation of Tyr15 in the p34cdc2 (cdk1) kinase, causing G2-M cell cycle arrest.\textsuperscript{432} Low concentrations of MSA increased the expression of p27kip1 and p21cip1 by upregulating the zinc-finger transcription factor Krüppel-like factor 4 (KLF4) causing G1 cell cycle arrest in PCa cells; higher MSA concentrations also induced apoptosis attributed to reduction in pAKT and pERK1/2.\textsuperscript{433-435} In contrast, sodium selenite decreased p27kip1 and p21cip1 expression and induced caspase-independent cell death by increasing JNK1/2, and p38MAPK phosphorylation.\textsuperscript{433}
SeMet increased the expression of Cip1/p21 and Kip1/p27 leading to a G1 cell cycle arrest in androgen sensitive cells but not in the AR-null cell line, PC3. AR expression in PC3 cells led to a G2/M cell cycle arrest indicating that the effects of SeMet were dependent on a functional AR. In LNCaP cells, MSA and methylselenol enhanced the recruitment of AR corepressors reducing the expression of AR target genes, decreased AR mRNA stability and nuclear localization. In contrast, sodium selenite inhibits AR expression through a superoxide mediated redox mechanism, and by inhibiting the DNA binding of the AR transcription factor SP1, it also inhibits IL6 mediated AR activation by upregulating c-Jun.

SeMet decreases proliferation of tumor adjacent ‘normal’ primary prostate cells with a concurrent decrease in AR signaling. Sodium selenite selectively inhibits proliferation and induces p53 dependent and independent apoptosis of human PCa but not normal cells. Metabolites of the polyunsaturated fatty acids (PUFAs) metabolic enzyme, arachidonate 5-lipoxygenase (ALOX5), or their precursor arachidonic acid could rescue sodium selenite’s apoptotic phenotype. Sodium selenite therefore induces apoptosis partly by disrupting the metabolism of arachidonic acid a common PUFA in high fat Western-style diets that is associated with PCa cell proliferation.

PCa cells differed in their sensitivity to the effects of different forms of selenium depending on their PTEN status due to differential effects on pAKT activity and distinct effects on pERK1/2 activity. MSA induced ER stress and an unfolded protein response (UPR) in p53-null PC-3 cells by disrupting oxidative protein folding culminating in p21(WAF) dependent growth arrest and apoptosis. Sodium selenite increased p53 activity and p53 dependent superoxide generation from sodium selenite metabolism, which coupled with decreased expression of
dismutase enzymes MnSOD and SOD selectively induced apoptosis in human prostate tumors but not the matching normal tissue and sensitized p53 wild type PCa cells to apoptosis.\textsuperscript{452-455}

In PTEN deficient PC3 cells, selenium decreased PI3K activity attenuating PIP3 levels and membrane recruitment of AKT and its activating kinase PDK1 decreasing pAKT (Thr308).\textsuperscript{456} Selenium also decreased pAKT (Ser473) in a calcineurin (a calcium-dependent phosphatase) dependent manner.\textsuperscript{456} SeMet and MSA activated overlapping and distinct gene transcription programs involved in cell cycle regulation, apoptosis and androgen signaling in LNCaP cells suggesting different forms of selenium have unique cellular effects.\textsuperscript{457} Selenite decreased levels of the DNA methyltransferase 1 (DNMT1) causing partial promoter demethylation leading to a dose- and time-dependent re-expression of the detoxifying enzyme, π-class glutathione-S-transferase (GSTP1) in LNCaP cells.\textsuperscript{458}

In DU145 cells, sodium selenite increased PTEN phosphorylation at Ser370 in a dose dependent manner probably by increasing the activity of casein kinase-2 (CK2) which decreased p-AKT(Ser473).\textsuperscript{459} In PCa cells, MSC but not selenite decreased levels of several types of collagen including collagen type I alpha 1 (COL1A1), COL1A2, COL7A1, COL6A1 and COL4A5 indicating that anticancer cancer effects of MSC might result from extracellular matrix modulation.\textsuperscript{460} MSA inhibited the expression of the antiapoptotic protein survivin in LNCaP cells by preventing SP1 promoter binding, while MSA did not alter survivin levels in castration resistant PCa cells, its knockdown and MSA treatment had a synergistic antiproliferative and apoptotic effect.\textsuperscript{461,462}
In LPS-stimulated PC3 cells, sodium selenite inhibited NFκB nuclear translocation, reducing the expression of TGFβ1 an immunosuppressive cytokine, VEGF an angiogenic factor and the pro-inflammatory factor IL-6. In hypoxic conditions, MSA induced apoptosis and inhibited growth in castration resistant PCa cells by decreasing the expression of HIF-1α and increasing the expression of REDD1 (regulated in development and DNA damage response-1), which is a negative regulator of mTOR. In nude mice, sodium selenite retarded the growth and progression of castration resistant PC3 orthotopic tumors and lymph node metastases associated with anti-angiogenic activity. In vivo, MSA and MSC had a superior dose-dependent growth inhibition of DU145 and PC-3 human PCa xenografts compared to SeMet and selenite in spite of lower tumor retention.

MSA and MSC induced apoptosis, inhibited proliferation, decreased circulatory levels of insulin-like growth factor 1 (IGF1), delayed PCa progression and increased survival in the TRAMP mouse model. MSC reduced tumor growth in the Dunning model of rat prostate cancer through unknown mechanism(s) while Vitamin E did not. MSA reduced the incidence of HGPIN and PCa progression in a Pten prostate-specific knockout (KO) mice by inducing senescence through P53 activation and attenuation of pAkt and AR signaling. In LADY transgenic mice, a mix of vitamin E, selenium, and lycopene decreased proliferation and inhibited development of PCa with increased survival. Sodium selenite and MSA decreased the growth of androgen-dependent LAPC-4 and LNCaP tumor xenografts respectively with a concomitant decrease in AR and PSA expression.

However, some studies have found null activity for selenium and vitamin E in vivo against PCa. Neither SeMet nor alpha-tocopherol inhibited PCa development in the testosterone plus
estradiol-treated NBL rat model where prostate tumorigenesis is driven by sex hormone-induced oxidative stress and inflammation.\textsuperscript{474,475} Supplementation with SeMet and/or \( \alpha \)-tocopherol did not decrease PCa incidence in Wistar-Unilever rats with N-methyl-N-nitrosourea (MNU) induced PCa followed by chronic stimulation with androgen.\textsuperscript{476} However, some studies have indicated that there is a synergistic effect for selenium and vitamin E. A combination of MSA and \( \gamma \)-Tocopherol decreased growth of tumor xenografts by upregulating the expression of proapoptotic proteins Bax and Bad and downregulating pro-survival Bcl2.\textsuperscript{477}

MSA and VES (but not \( \alpha \)-tocopherol acetate) activated separate cell death pathways in PCa cells suggesting synergistic chemoprevention.\textsuperscript{478} Flow cytometry shows that vitamin E and selenium reduce the number of cycling LNCaP cells presumably by leveraging distinct mechanistic pathways.\textsuperscript{479}

\textit{Vitamin E and Selenium clinical trial findings:}

Support for use of vitamin E in a large PCa chemoprevention trial came from secondary analysis of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Trial (ATBC).\textsuperscript{480} ATBC tested the efficacy of 50 mg daily of \( \alpha \)-tocopheryl acetate and/or 20 mg daily of \( \beta \)-carotene supplementation for a median of 6.1 years on prevention of lung and other cancers in 29,133 male smokers aged 50–69 years in Finland.\textsuperscript{481} Unexpectedly, \( \beta \)-carotene increased risk of lung cancer and total mortality whereas \( \alpha \)-tocopherol had a null effect.\textsuperscript{481} However, there was a 32\% and 41\% reduction in PCa incidence and mortality respectively among men on \( \alpha \)-tocopherol which was inversely correlated with serum \( \alpha \)-tocopherol levels especially in advanced disease.\textsuperscript{482,483}
Nevertheless, this beneficial $\alpha$-tocopherol effect disappeared post-intervention.\textsuperscript{484} In the VITamins And Lifestyle (VITAL) cohort study, long term vitamin E or selenium supplementation had a null effect on overall PCa risk though there was a 57% reduction in risk of advanced PCa among men on vitamin E.\textsuperscript{485} An earlier study on 47,780 healthy men in the U.S found that at least 100 IU supplemental vitamin E daily lowered risk of metastatic and fatal PCa but only in smokers or recent quitters.\textsuperscript{486} Analysis of 72,704 men in the Cancer Prevention Study II Nutrition Cohort found no association between regular vitamin E supplementation and overall risk of PCa or advanced PCa, although there was a slight risk reduction in smokers.\textsuperscript{487}

Selenium was first demonstrated to be protective against liver, stomach and esophageal cancers in large randomized trials in Qidong and Linxian, China.\textsuperscript{488} A general population interventional trial with selenized table salt for eight years in Qidong County ($n=130,471$) where hepatitis B virus (HBV) infection and liver cancer are prevalent reduced the incidence of liver cancer by 35% an effect that reversed by ceasing supplementation.\textsuperscript{489} A smaller interventional trial ($n=226$) in the same region found that supplementation with selenized yeast for four years reduced liver cancer cases among people positive for Hepatitis B Surface Antigen compared to the placebo an effect that reversed upon cessation of supplementation.\textsuperscript{489}

The Linxian Nutritional Intervention Trials tested whether supplementation with multiple vitamins and minerals would decrease the risk of cancer and cancer mortality.\textsuperscript{490,491} The first trial found significantly reduced overall mortality among individuals on carotene, inorganic selenium and vitamin E attributed to a decline in cancer incidence in the general population.\textsuperscript{490} However, a second trial found that $\beta$-carotene, inorganic selenium and vitamin E had a null effect on esophageal cancer incidence and mortality in people with esophageal dysplasia.\textsuperscript{491} Enthusiasm
for use of selenium in a PCa chemoprevention trial came from findings of the Nutritional Prevention of Cancer (NPC) study.\textsuperscript{480}

In this trial, 1,312 participants (74\%) male with a prior history of non-melanoma skin cancer were randomized to receive 200 μg daily dose of selenium from selenized yeast or placebo for 4.5 ± 2.8 years and followed for 6.4 ± 2.0 years for the development of non-melanoma skin cancer and other cancers.\textsuperscript{492} Although the brewer’s yeast had no effect on the primary endpoint, there was a 63\% reduction in risk of PCa as a secondary end point.\textsuperscript{492} Additional stratified analyses found the reduction in PCa risk to be greater in men with low baseline selenium levels.\textsuperscript{493,494}

\textit{The Selenium and Vitamin E Cancer Prevention Trial (SELECT)}

In 2001, the NCI initiated the SELECT trial, which enrolled 35,533 men over the age of 50 years, to test the chemopreventive benefit of selenium and vitamin E on prostate cancer (PCa) with an intended follow up of 12 years.\textsuperscript{480,495} As part of the inclusion criteria, the men had to be free of a prior PCa diagnosis, have a non-suspicious digital rectal examination (DRE) and a PSA \(\leq 4\) ng/mL.\textsuperscript{480} SELECT, a phase III, double-blinded, prospective, 2x2 factorial clinical trial randomized participants to a daily dose of 200 μg L-selenomethionine, 400 IU α-tocopheryl acetate, a combination of both agents at the same dosage or placebo.\textsuperscript{480,495}

The intended 12-year study was powered to detect a 25\% decline in PCa incidence on single agent therapy and an additional 25\% reduction with the combined therapy compared to the placebo.\textsuperscript{480} However, an interim analysis after 5.5 years median follow up found no benefit for selenium or the combined therapies and a marginal increased risk of PCa in the vitamin E group.
leading to early termination of the trial. An additional 54,464 person-years of follow-up showed a 17% increased risk of PCa with vitamin E (P = 0.008). A recent case-cohort study on SELECT participants tested whether the outcome of selenium and vitamin E supplementation on PCa risk depended on baseline selenium status.

In the placebo group, toenail selenium, a reflection of long-term selenium exposure, was not associated with PCa risk. Additionally, selenium supplementation, alone or in combination with vitamin E did not affect the PCa risk in men with low baseline selenium status. However, in men with higher baseline selenium status, Selenium increased the risk of high-grade PCa by 91% (P = 0.007). On the other hand, vitamin E supplementation (alone) had no effect on PCa risk in men with high selenium status but it increased the risk of total (63%, P = 0.02), low-grade (46%, P = .09), and high-grade PCa (111%, P = .008) in men with lower selenium status.

Thus, whereas there was no benefit of selenium supplementation in men with low baseline selenium status, those with high baseline status had increased risk of high-grade disease. Besides, vitamin E increased PCa risk in men with low baseline selenium status. A separate phase III clinical trial in 699 men found selenized yeast supplementation to have no effect on the incidence of PCa in men at high risk for PCa (PSA > 4 ng/ml and/or suspicious DRE and/or PSA velocity > 0.75 ng/ml/year but negative PCa biopsy).

Summary

The outcome of the SELECT trial remains a puzzle. As detailed above, numerous in vitro studies suggest that vitamin E and selenium have anticancer effects by inhibiting proliferation, altering redox homeostasis, inducing apoptosis and blocking inflammatory signaling. The lack of
efficacy and even harmful effects of vitamin E and selenium in large randomized clinical trials points to the low predictive value of PCa chemoprevention outcomes for in vitro and in vivo models referenced in the design of SELECT. Although primary prevention studies call for models of disease initiation, most of the preclinical studies described above used advanced PCa models.

Interestingly, several studies summarized above reported a null effect for vitamin E and selenium on normal prostate cells. Furthermore, the VITAL study found that vitamin E and selenium supplementation had no effect on the risk of latent or early PCa but long-term vitamin E supplementation reduced the risk of advanced PCa. Therefore, we hypothesized that prostate cells at different stages of cancer evolution process may respond differently to antioxidants. In addition, some have attributed the lack of efficacy in SELECT to the doses and formulations of vitamin E/selenium used. SELECT tested a daily dose of vitamin E, (α-tocopheryl acetate; 400 mg), and/or selenium (L-selenomethionine; 200 μg).

The ATBC trial had however demonstrated efficacy for a much lower dose of 50 mg α-tocopheryl acetate whilst the NPC trial had used 200 μg selenized yeast. Furthermore, vitamin-E isomers have different bioactivities. Due to its ability to scavenge both ROS and reactive nitrogen species (RNS), γ-tocopherol is superior to α-tocopherol. We tested the hypothesis that different vitamin E isomers and even an unrelated, water soluble antioxidant would have different effects in vitro. Moreover, the vitamin E and selenium in vitro studies prior to SELECT utilized conventional 2D culture.
Although 2D tissue culture has been useful in unravelling PCa biology, important limitations restrict its utility. Cells in 2D lack physiological cell and matrix interactions and attachment to artificial surfaces affects cell morphology and signaling.\textsuperscript{502} Additionally, lack of oxygen and nutrient gradients in 2D cultures makes the environment non-physiologically uniform.\textsuperscript{503} As much as the use of animal models overcomes some of these limitations, the systemic and physiologic differences between mouse and human prostates can affect phenotype.\textsuperscript{371}

In addition, genetically engineered mouse (GEM) models are expensive and also take long to generate whereas xenograft models are limited by the small number of available PCa cell lines.\textsuperscript{371} Moreover, the intractability of whole animal models makes them less ideal for investigating molecular mechanisms at the cellular level necessitating cell cultures.\textsuperscript{504} As detailed previously, vitamin E and selenium supplementation studies in animal models produced mixed results with several studies showing no effect.

Given the shortcomings of 2D cell cultures and the difficulty of modeling the spectrum of human prostate tumorigenesis \textit{in vivo}, we modeled different stages of PCa progression in 3D organoids and tested the effects of the SELECT supplements on PCa tumorigenesis. In three-dimensional (3D) cultures, cells form cell-cell and cell-matrix attachments mimicking an \textit{in vivo} environment.\textsuperscript{505} Additionally; growth factor, nutrients and oxygen gradients in 3D cultures yield heterogeneous cell populations like \textit{in vivo}.\textsuperscript{505}
CHAPTER 2: MATERIALS AND METHODS

Ethics statement

As previously published, all human subject research adhered to ethical standards, and all experiments on human samples were performed in accordance with stipulated guidelines and regulations.\textsuperscript{506,507} Benign human primary prostate epithelial cells were acquired after approval from the University of Illinois at Chicago (UIC) Institutional Review Board (IRB) and informed consent obtained from all patients.\textsuperscript{506,507}

Prostate tissue collection and cell preparation

Prostate tissue was obtained from radical prostatectomy PCa patients at the UIC Medical Center with informed consent and in accordance to IRB guidelines as previously described.\textsuperscript{506,507} Benign tissue from the peripheral zone was resected, and a final H&E histological assessment done on a thin slice of tissue by a pathologist.\textsuperscript{506,507} Only tissue confirmed to be 100% benign was utilized.\textsuperscript{506,507} Prostate epithelial cells were isolated using a method developed by Donna Peehl.\textsuperscript{508} In brief, tissue was digested with collagenase and primary epithelial cells (PrE) isolated by plating on collagen-coated dishes in serum-free prostate epithelial cell growth medium (PrEGM) media (Lonza no. CC-3166 & CC-4177).\textsuperscript{506,507} qRT-PCR for expression of known basal epithelial cell markers (CK5+/p63+/AR-) confirmed the cell type.\textsuperscript{506,507}

Cell culture
In preparation for organoid culture, 50,000 PrE cells from two patients were plated in 10 cm dishes in primocin (Invitrogen no. ant-pm-1) containing PrEGM media as described by Unno et al. To expand them, the cells were passaged once at 50-70% confluence and used for organoid culture on the second passage at 80% confluence. LNCaP cells were cultured in RPMI 1640 media (Gibco Life Technologies no. 11875-093) with 10% fetal bovine serum (FBS) - (Life Technologies no. 10437-028) and 1% Pen Strep antibiotic solution (Life Technologies no. 15140-122). RWPE-1 cells were cultured in keratinocyte serum-free media with 0.05 mg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor (Thermo Fischer Scientific no. 17005042) and 1% Pen Strep antibiotic solution. Cells were tested and treated for mycoplasma and cell lines genetically authenticated by short-tandem repeat (STR) profiling.

**Organoid culture and treatments**

After expansion in their optimum media, the different cell types were trypsinized and transferred to organoid media as previously described. Briefly; 5000 cells were resuspended in organoid media containing low percentage matrigel (5%) then plated in to 96-well ultralow attachment plates (Corning no. 3474). A hundred microliters of fresh media was added to the cultures every four days or every two days once the treatments commenced. Treatments were added to the following final concentrations; 40 μM DL-αTocopherol-Acetate (Sigma no. T3376), 40 μM RRR-γ-Tocopherol (Sigma no. T1782) and/or 1.3 μM Seleno-L-methionine (Sigma no. S3132) representing the mean concentrations attained in the blood plasma of the SELECT subjects. N-acetyl cysteine (NAC; Sigma no. A9165) and Etomoxir sodium salt hydrate (Eto; Sigma no. E1905) were used at various concentrations as indicated in the figures. Organoid growth was
captured by brightfield microscopy using Zeiss Axioskop/Nuance microscope (Carl Zeiss Inc. Oberkochen, Germany).

2D Cell Proliferation Assay

To monitor the effect of the SELECT supplements on cell proliferation in 2D, replicates of pretreated RWPE-1 (n = 6 – 9) were plated in 96-well plates at densities of 2000 cells/well. The plates were scanned with the IncuCyte ZOOM™ live cell imaging system (Essen BioScience) with continued treatments. Images were captured every four hours for the durations indicated using the 10x objective. Cell confluence was calculated with the IncuCyte ZOOM™ software (version 2015A).

High Throughput Sequencing

A high throughput strategy targeting 222 cancer related genes was used to sequence DNA extracted from human prostate epithelial cells used for organoid culture to ascertain that they did not contain PCa relevant mutations. DNA Probes for capturing exon regions in these genes were manufactured by Roche NimbleGen. SeqCap EZ Library SR User’s Guide (Roche, Pleasanton, CA) was followed for library preparation and capture of targeted sequences. Paired-end sequencing of 2 x 150 bp was performed on a MiSeq platform (Illumina, San Diego, USA). Twelve individual libraries were multiplexed for a MiSeq flow cell. The mean sequencing depth of coverage was 135x.
Bioinformatics analysis

Paired-end reads were aligned to the GRCh37 version of the human genome using Burrows-Wheeler Aligner v0.7 to generate BAM files. The BAM files were sorted with samtools, PCR duplicates marked using Picard and realignment around putative gaps performed using the Genome Analysis Toolkit (GATK) v3.2-2. The GATK Haplotype caller was used for variant calling. ANNOVAR (http://annovar.openbioinformatics.org/en/latest) was used to annotate variants as well as retrieving variant information from population-based studies such as the 1000 Genomes Project (www.1000genomes.org), NHLBI-ESP 6500 exomes or ExAC (http://exac.broadinstitute.org/), and clinical databases like the Human Gene Mutation Database (HGMD) and ClinVar. Variant pathogenicity was defined based on the American College of Medical Genetics and Genomics (ACMG) criteria. Specifically, pathogenic and likely pathogenic mutations are defined as 1) all protein-truncating mutations unless their allele frequency is 5% or higher in any racial group in population databases or is reported as benign or likely benign in the ClinVar, and 2) nonsynonymous changes if their allele frequency is less than 5% and reported as pathogenic and likely pathogenic mutations in the ClinVar. The high throughput sequencing and analysis were carried out by the Genomics Core Facility at the NorthShore University Health System (Chicago, IL).

Histology and immunostaining

Fixation, processing, H&E and immunofluorescence staining were done as previously described. The following primary antibodies were used: Ki-67 (1:100; eBioscience no.14-
5698-80), BrDU (1:100; Abcam no. ab6326), CK8 (1:400; Covance no. MMS-162P) and CK14 (1:500; Covance no. PRB-155P). The secondary antibodies (Life Technologies) used at 1:400 each were; goat anti-rabbit Alexa Fluor 647 (no. A21244), goat anti-rat Alexa Fluor 488 (no. A11006) and goat anti-mouse Alexa Fluor 568 (no. A11004). Sections were counterstained with 0.5 mg/ml DAPI (Sigma no. D-9542) and mounted in ProLong Diamond Antifade reagent (Molecular Probes no. P36961). When appropriate, BrDU (Invitrogen no. 00–0103) was added into the organoid medium at 1:100 dilution (3 μg/mL) overnight. BrdU was detected using a rat anti-BrdU antibody (1:100; Abcam no. ab6326). Imaging was performed using a Nikon A1R (A) Spectral laser scanning confocal microscopy (Nikon Instruments Inc. Yokohama, Japan, Japan).

**Detection of Reactive Oxygen Species (ROS)**

Mitochondrial ROS were detected using CellROX Green (Thermo Scientific C10444) according to the manufacturer’s instructions. In brief, on day 21 of organoid culture, the probe was added to a final probe concentration of 5 μM. Staining was done in the dark for 1 hour at 37°C. Organoids were washed in PBS and placed in chamber slides for imaging using the Nikon A1R (A) Spectral laser confocal microscope. The mean fluorescence intensity per image was determined using the Fiji (ImageJ) software.

**Microarray analysis of antioxidant treated organoids**

RNA was extracted using Trizol (Life Technologies No. 15596-026) from triplicates of organoids pooled from eight 96-plate wells. The RNA was cleaned up using an RNeasy Mini Kit
(Qiagen no. 74104) with DNase (Qiagen no. 79254) on column treatment. The RNA was hybridized to Affymetrix HTA 2.0 transcriptome arrays and analyzed with the Affymetrix AGCC suite at the University of Illinois at Chicago (UIC) Genomics core (Chicago, IL). The CEL files were imported into R (windows version 3.1.1) using the Bioconductor (version 3.3) oligo package. Raw intensity scores for probes were normalized by quantiles and background corrected with RMA. Differentially expressed genes (DEGs) were identified by the Bioconductor limma package. For functional analysis, the C2 (curated) gene sets of MSigDB (version 5.1) were queried using the Gene Set Enrichment Analysis (GSEA) algorithm.\textsuperscript{514} Results were visualized with the Enrichment Map plug-in [version 2.0.1] on Cytoscape [version 3.2.1] using a p-value cutoff of 0.005, an FDR cutoff of 0.25, and an overlap coefficient cutoff of 0.5.\textsuperscript{515,516}

**Poly-HEMA coating and suspension cell cultures**

We made a 1.5% solution of the anti-adhesive polymer, poly 2-hydroxyethyl methacrylate (Poly-HEMA; Sigma no. P3932), in 95% ethanol which we dissolved by rotating for several hours at 65\(^\circ\)C. We used 4 ml, 700 \(\mu\)L/well and 60 \(\mu\)L/well of the Poly-HEMA solution to coat 10 cm, 6-well and 96 well plastic culture plates respectively. The plates were left open overnight to dry and sterilized by UV for 45 minutes before use. Poly-HEMA reduces the adhesiveness of plastic surfaces in a concentration dependent manner.\textsuperscript{517} While adherent cells in 2D plastic tissue culture are flat and stretched, cells cannot attach on poly-HEMA coated plates, keeping them in a suspension of spherically shaped cells.\textsuperscript{517}
**Glucose absorption assay**

We plated 11,000 cells per well in 96-well plates with or without a 1.5% poly-HEMA coating (Sigma no. P3932). Media was collected after 24 h and diluted 1:2000 in water. The amount of glucose was measured using the Amplex Red Glucose Assay Kit (Thermo Fisher Scientific no. A22189) per the manufacturer’s instructions.

**ATP assay**

Cells were plated in poly-HEMA coated or uncoated 96-well plates at a density of 11,000 cells per well. After 24 h, ATP was measured using the ATPlite Luminescence kit (PerkinElmer no. 6016943) per the manufacturer’s protocol.

**Oxygen consumption (OCR) and extracellular acidification rate (ECAR) measurements.**

To measure the effects of the SELECT supplements on the energy metabolism of RWPE-1 cells, treatments were done for 5 days and switched to non-adherent conditions for 24 hours. We plated replicates of 30,000 cells/well (n = 15) in Celltak (Corning no. 354240) coated XF96 well Seahorse cell culture plates (Agilent no. 101085-004). An XF96 extracellular flux analyzer (Seahorse Bioscience) was then used to measure oxygen consumption and extracellular acidification rates. Oligomycin, Carbonyl cyanide 3-chlorophenylhydrazone (CCCP), Antimycin, Rotenone and 2-Deoxy-D-glucose (2DG) (Sigma) were injected to final concentrations of 5 μM, 0.75 μM, 2 μM, 2 μM, and 40 mM respectively. Experiments were performed in the DMEM based, XF-Base medium (Agilent Technologies no. 103335-100) without phenol red, bicarbonate, glucose or glutamine.
The medium was supplemented with 10 mM glucose (Sigma), 2 mM glutamine (Sigma), 5 mM HEPES (Sigma no. H0887), 2.5 µg human recombinant Epidermal Growth Factor and 25 mg Bovine Pituitary Extract (BPE) (Thermo Fisher Scientific no. 17005042). Basal OCR is the OCR value before the injection of any drugs and after the subtraction of the OCR values after the injection of antimycin A and rotenone (A/R) to discount non-mitochondrial OCR. Maximal OCR is the OCR value after the induction of respiration with CCCP subtracting the non-mitochondrial OCR. Basal and maximal ECAR are the sensitivity of the extracellular acidification rate before the injection of any drugs and the injection of oligomycin respectively. The ECAR value after glycolysis inhibition with 2DG is subtracted from both the basal and maximal rates to discount non-glycolytic ECAR.

**Statistical analysis**

Statistical analyses were performed using a two tailed Student’s t-test, one-way or two-way Analysis of Variance (ANOVA) with Tukey’s correction for multiple comparisons. All results are presented as mean ± Standard Deviation or Standard Error. P values ≤ 0.05 were considered significant.
CHAPTER 3: RESULTS

The SELECT supplements decrease proliferation and induce cell death in LNCaP cancer organoids

Laboratory studies showing anti-tumorigenicity of vitamin E or selenium in the prostate mostly utilized LNCaP, PC-3, and DU145 tumor cells. However, the failure of SELECT led us to hypothesize that the response to antioxidants may depend on disease stage. To test this, we first evaluated the effects of the SELECT agents on LNCaP prostate cancer cell organoids (Fig. 4A). The human prostate epithelium contains basal, luminal and neuroendocrine cells distinguishable by the expression of specific markers. From the CK8+ staining and absence of basal cell markers, LNCaP yielded purely luminal organoids (Fig. 4B).

In agreement with previous reports, the antioxidants strongly decreased BrDU incorporation relative to the vehicle control (Figs. 4B & C). Furthermore, whereas the cycling cells in the vehicle were dispersed throughout the organoids, indicating anchorage independent growth, those in the antioxidant treated organoids especially vitamin E were restricted to the outermost layer proximate to the ECM (Fig. 4B). Additionally, the antioxidants induced cell death especially in the inner, extra-cellular matrix (ECM) deprived cells (Fig. 4B) consistent with previous reports.
Figure 4. The SELECT antioxidants decrease proliferation and increase cell death in LNCaP organoids. LNCaP cells were grown in organoid media, treated with antioxidants then immunostained with anti-CK8, anti-BrdU antibodies and a DAPI counterstain (A) Time line of the culture and treatment of organoids (B) LNCaP cells gave rise to luminal organoids displaying a significant decrease in proliferation and increased cell death when treated with antioxidants. (C) Quantification of BrDU incorporating cells as a percentage of total cells shows that the antioxidants significantly reduced the number of actively dividing cells. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey’s correction for multiple comparisons). *p ≤ 0.0001; error bars represent SD.
The SELECT supplements do not increase cell proliferation in benign primary prostate organoids

Next, we tested the effects of the supplements on benign primary human prostate epithelial cell organoids. We generated organoids using benign primary prostate epithelial cells from two African American subjects as previously reported.\textsuperscript{509} The absence of prostate cancer related alterations after the targeted sequencing of 222 cancer genes confirmed the cells to be benign (Table 1). The primary organoids were heterogeneous in size, morphology and expression of basal or luminal markers (Fig. 5A). Notably, antioxidant monotherapies did not affect the proliferation of these organoids whereas the combination treatment decreased Ki67 staining in the first subject (Figs. 5B & C).

Vitamin E significantly increased cell proliferation in premalignant RWPE-1 prostate organoids but not in 2D culture

The SELECT trial revealed a deleterious effect of vitamin E on a fraction of individuals without prior evidence of prostate cancer.\textsuperscript{496} Therefore we reasoned that these individuals might have harbored “initiated” cells in a pre-malignant state that were pushed to malignancy by chronic antioxidant treatment. We tested this on organoids generated from the immortalized but non-tumorigenic RWPE-1 human prostate epithelial cell line. RWPE-1 cells are immortalized with the E7 oncoprotein from HPV18 which modulates the activity of the retinoblastoma (Rb) tumor suppressor.\textsuperscript{518,519} Whereas most RWPE-1 organoids treated with the vehicle or selenium had hollow lumens, those treated with vitamin E or the combination displayed marked luminal filling (Fig. 6A).
Vitamin E induced a near two fold increase in proliferation with 35% Ki67 positivity compared to the vehicle at 19% (Fig. 6B). In contrast, selenium had no impact on proliferation whereas the combination treatment had an intermediate effect at 18% and 25% Ki67 positivity respectively (Fig. 6B). Confirming these results, vitamin E had the highest number of BrDU incorporating cells at 37%, however, Selenium (24%) and not the vehicle (27%) had the lowest (Fig. 6C). These in vitro RWPE-1 organoid findings where vitamin E enhances proliferation whereas selenium counteracts vitamin E, are highly reminiscent of the clinical trial data from SELECT. In contrast, vitamin E had no significant effect on the growth rate of RWPE-1 cells grown in 2D (Fig. 7). In this condition, the combination of vitamin E and selenium significantly increased cell growth (Fig. 7) indicating that 2D culture could not recapitulate the SELECT results.
Figure 5. Vitamin E has no significant effect on the proliferation of healthy primary organoids. To model the SELECT trial, organoids from normal primary prostate cells of two subjects were treated with the SELECT agents. (A) Bright-field view showing several phenotypes of organoids from one of the subjects. The H&E stain shows the formation of large organoids with hollow lumens while the immuno-staining shows that these organoids expressed both luminal CK8 and basal CK14 epithelial markers. (B) Ki67 quantification showed no significant impact for vitamin E or selenium but their combination decreased organoid proliferation in the first subject. (C) Quantification of Ki67 in organoids from the second subject showed no significant difference in proliferation between vehicle and vitamin E treatments. Each data point represents a single field of view. Scale bars represent 100 μm for Brightfield and IF images and 50 μm H&E. Asterisks represent statistical significance (One way ANOVA with Tukey’s correction for multiple comparisons or a two-tailed t-test). * $p \leq 0.05$; error bars represent SD.
Figure 6. Vitamin E alone drives proliferation in organoids from the non-tumorigenic prostate cell line, RWPE-1, recapitulating the SELECT trial. Antioxidant-treated RWPE-1 organoids were sectioned and stained with H&E or anti-CK8, CK14 and Ki67 antibodies (A) H&E staining showed that organoids treated with vehicle or selenium had mostly hollow lumens (arrows) compared to the filled morphology in vitamin E treated organoids. The confocal images show basal and luminal staining and increased Ki67 detection in vitamin E treated organoids (B) Quantification of the percentage of Ki-67 positive cells showed a highly significant increase in proliferation in organoids treated with vitamin E alone. (C) Quantification of the percentage of BrDU incorporating cells in RWPE-1 organoids after two weeks of culture showed that vitamin E alone significantly increased the number of dividing cells. Each data point represents a single field of view. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey’s correction for multiple comparisons). **p ≤ 0.005, ****p ≤ 0.0001; error bars represent SD.
Proliferation in the premalignant organoids is independent of antioxidant structure or mechanism of action

To test the effect of other antioxidants, we treated RWPE-1 organoids with a different vitamin E isomer, γ-Tocopherol or NAC (Fig. 8). Organoids treated with γ-Tocopherol alone or in combination with selenium had a filled lumen morphology (Fig 8A). Further, γ-Tocopherol increased proliferation to 34% Ki67 positivity compared to vehicle at 19% (Fig. 8C). The combination of γ-Tocopherol and selenium also had a higher proliferation rate compared to

Figure 7. RWPE-1 cells in 2D do not recapitulate the results of the SELECT trial. Percent confluence over time of RWPE-1 cells with SELECT supplement treatment. Asterisks represent statistical significance (Two way ANOVA with Tukey’s correction for multiple comparisons). *p ≤ 0.05, ****p ≤ 0.0001; error bars represent SD (n = 6 – 9).
vehicle at 29% (Fig. 8C). Therefore, unlike α-Tocopherol (Fig. 6B), the addition of selenium did not greatly attenuate the effect of γ-Tocopherol (Fig. 8C).

Whereas vitamin E isomers are lipophilic antioxidants that prevent lipid peroxidation, NAC is a precursor in the synthesis of the intracellular antioxidant glutathione.\textsuperscript{520,521} NAC-treated organoids had a dose-dependent proliferation increase and filled lumens (Figs. 8B & D). Further, to determine whether the SELECT supplements affect the levels of ROS in RWPE-1 organoids, we quantified fluorescence in treated organoids stained with mitochondrial CellROX probes. The SELECT supplements significantly lowered mitochondrial ROS (Figs. 9A & B).
Figure 8. The proliferative phenotype in RWPE-1 organoids is independent of the Vitamin E isomer or antioxidant structure. (A) H&E staining showing hollow lumens (arrows) in vehicle and selenium and filled lumens for γ-Tocopherol or its combination with selenium. The confocal images show Ki67 immunostaining on sections of RWPE-1 organoids treated with the vehicle, γ-Tocopherol, its combination with selenium or selenium alone (B) H&E images showing hollow and filled morphology in vehicle and NAC treated organoids respectively and Ki67 immunostaining on sections of RWPE-1 organoids treated with vehicle or increasing concentrations of NAC (C) Quantification of Ki-67 positive cells from (A) showed a significant increase in the proliferation of organoids treated with γ-Tocopherol or its combination with selenium but not selenium alone. (D) Quantification of Ki-67 positive cells from (B) showed a dose-dependent increase in proliferation in organoids treated with NAC. Each data point represents a single field of view. Scale bars represent 50 μm. Asterisks represent statistical significance (One way ANOVA with Tukey’s correction for multiple comparisons). *p ≤ 0.05, **p ≤ 0.01, ****p ≤ 0.0001; error bars represent SD.
Microarray analysis revealed opposing effects of vitamin E and selenium on cell proliferation in the premalignant organoids

To gain further insight into the effects of SELECT supplements on RWPE-1 organoids, we performed gene expression profiling using microarrays followed by gene-set enrichment analysis (GSEA). Vitamin E upregulated cancer and cell cycle related gene sets that were suppressed by selenium and the combination treatments (Figs. 10 – 12). The leading-edge subsets describe
the genes that contribute most to a gene set’s enrichment score and thus have the highest
correlation with the phenotype of interest.

The leading-edge analysis showed an upregulation of cell cycle and genome replication genes
including cyclins and mini-chromosome maintenance proteins (MCM) by vitamin E but
downregulated by selenium and the combination treatments (Table 2). The GSEA results were
visualized with the Cytoscape Enrichment Map plug-in, which groups significant gene sets into
functional networks based on annotation similarity and gene overlap.\textsuperscript{515,516} The key network
affected by all the treatments was cell proliferation which was upregulated by vitamin E (Figs.
13A & B), but suppressed by selenium (Figs. 13C & D) and the combination treatment (Figs.
14A & B).
Figure 10. Top 30 gene sets that are significantly enriched by vitamin E identified by gene-set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005)
Figure 11. Top 30 gene sets that are significantly enriched by selenium identified by gene set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005).
Figure 12. Top 30 gene sets that are significantly enriched by vitamin E and selenium combined identified by gene set enrichment analysis (GSEA) of differentially expressed genes (p value ≤ 0.005).
Figure 13. In RWPE-1 organoids, Vitamin E upregulates a cell proliferation gene network which is suppressed by Selenium. We used Cytoscape to cluster together functionally related gene sets found to be significantly enriched after querying our microarray data with MSigDB’s C2 curated gene sets using GSEA. We show networks containing ≥ 5 gene sets (False Discovery Rate q value < 0.25). Node size corresponds to gene set size. Red circles represent up-regulation and blue circles represent down-regulation of the gene set. Colour intensity represents significance by enrichment p value. Line thickness connecting the gene set nodes represents the degree of gene overlap between the two sets. (A) Vitamin E upregulated a gene set network associated with cell proliferation (B) Example GSEA enrichment plots for selected gene sets from the vitamin E network. The Enrichment Score (ES; y-axis) reflects the degree to which a gene set was upregulated (cumulative positive score) or down-regulated (cumulative negative score) in the treatment group. Each vertical line in the ‘bar code’ represents a single gene in a gene set. Hue designates the direction in which the genes are altered (red = enriched in vitamin E, blue = depleted in Vitamin E). Nominal p value (statistical significance of the enrichment) and the FDR are indicated. (C) Selenium downregulated cell proliferation, glycosis gene networks among others (D) Example GSEA enrichment plots for selected gene sets from the selenium affected networks.
Suppression of glucose uptake leads to a drop in ATP generation in detached premalignant cells

From the gene expression analysis, we observed a downregulation of glucose transporters and glycolytic enzymes to varying degrees among the treatments (Fig. 15A). Several integrins which mediate ECM cell attachment were also downregulated pointing to loss of matrix attachment (Fig. 15A). Studies in mammary organoids have demonstrated differences in the glycolytic rates between the outer ECM attached and the inner detached cells. We therefore tested whether suppression of the glycolysis pathway was associated with differences in glucose absorption and glycolysis. We used adherent and nonadherent cells on poly-HEMA coated plates to mimic the
attached matrix proximal cells (outer) and detached matrix distal (inner) organoid cells respectively.

The non-ionic nature of poly-HEMA prevents ECM deposition on tissue culture plates, poly-HEMA coated tissue culture plates prevent cellular attachment and spreading.\textsuperscript{523} \textit{In vitro} growth in soft agar, the gold standard for measuring anchorage-independent growth, correlates with growth of human epithelial cancer cells on poly-HEMA coated plates and tumorigenicity.\textsuperscript{524,525} Therefore, growth of human epithelial cancer cells on poly-HEMA is also an indicator of tumorigenicity.\textsuperscript{525} As such, poly-HEMA adhesion deprived cell cultures provide a model for studying the regulation of anchorage-independent cell survival and growth especially for studies that are difficult to perform on cells embedded in soft agar or matrigel like in organoids.\textsuperscript{526}

With this approach, several studies have elucidated alterations in various cellular metabolic pathways under matrix nonadherent conditions.\textsuperscript{522,527-529} We found that, adhesion deprived RWPE-1 cells significantly reduced glucose absorption, and this was not rescued by the addition of antioxidants (Fig. 15B). Consequently, detached RWPE-1 cells had significantly lower levels of basal and maximal ECAR, a measure of lactate production from glycolysis, compared to attached cells which was not rescued by the addition of vitamin E (Figs. 16A – C).

Next, we measured ATP levels under the same conditions to determine the effect of reduced glycolysis and antioxidants on cell energetics. The detached cells had significantly lower ATP levels compared to attached cells (Fig. 16D). Though vitamin E did not rescue glycolysis, it did rescue the diminished ATP levels in detached cells (Fig. 16D) consistent with findings in
mammary cells. One caveat of the poly-HEMA suspension assay is that it can lead to death by anoikis. To minimize this effect, all assays were conducted within 24 hours.

Figure 15. Antioxidant treated organoids display ECM detachment and downregulation of glucose transporters lowering glucose uptake (A) Heat map of differentially expressed genes in the glycolysis and cell attachment pathways identified to be affected by the gene set analysis showed a downregulation of both pathways to varying degrees by antioxidant treatment (B) Measurement of glucose uptake in adherent or non-adherent, (poly-HEMA coated plates), RWPE-1 cells showed a significant decrease in the detached cells that was not rescued by the antioxidants after 24h.
Vitamin E increases the survival of detached premalignant cells by stimulating fatty acid oxidation

Next, we sought to determine whether this ATP rescue despite the decreased glycolytic flux was through increased oxidative phosphorylation, the other major pathway for energy generation.
Figure 17. Vitamin E restores ATP levels in detached cells by stimulating fatty acid oxidation leading to organoid luminal filling. (A) OCAR analysis of RWPE-1 cells grown in adherent and non-adherent conditions and treated with the SELECT supplements for 24h, n = 15. (B) Basal OCAR was calculated by subtracting non-mitochondrial OCAR from the basal OCAR measurements. (C) Maximal OCAR was calculated by subtracting non-mitochondrial OCAR from the OCAR measurements after the injection of CCCP. (D) Detached RWPE-1 cells were treated with vehicle, vitamin E or vitamin E and an FAO inhibitor, Etomoxir (Eto, 25μM) for 24h; ATP measurement showed that FAO inhibition abrogated the ATP rescue by vitamin E. (E) Immunostained sections of RWPE-1 organoids from (D), showed that the vitamin E treated organoids had filled lumens while those co-treated with Vitamin E and Etomoxir or vehicle had hollow lumens (arrows). (F) Cell density of organoids from (E) measured by dividing the number of total cells per organoid by its area showed that the vitamin E organoids had the highest cell densities while those co-treated with vitamin E and Etomoxir had the lowest cell densities. Scale bars represent 100 μm. Asterisks represent statistical significance (One way ANOVA with Tukey’s correction for multiple comparisons). **p ≤ 0.05, ***p ≤ 0.01, ****p ≤ 0.0001, error bars represent SD.
To this end, we measured changes in oxygen consumption rate (OCR), which is linked to mitochondrial oxidative phosphorylation. Whereas vehicle-treated detached cells had low basal and maximum OCR, those treated with vitamin E had significantly higher OCR levels that were comparable to the attached cells (Figs. 17A – C). When glucose is depleted, cells can derive energy from fats through fatty acid oxidation (FAO). Since vitamin E did not rescue glucose uptake, we tested whether it might stimulate FAO. ATP levels were measured in non-adhering, antioxidant treated RWPE-1 cells with or without Etomoxir, an FAO inhibitor. FAO inhibition abrogated the ATP rescue by antioxidants (Fig. 17D). To move these findings to a more physiologically relevant model, we tested the effect of FAO inhibition in vitamin E-treated RWPE-1 organoids.

FAO inhibition in vitamin E treated organoids selectively killed the inner, ECM detached cells, reverting the filled lumen morphology back to a normal hollow morphology (Fig. 17E). Consequently, vitamin E treated organoids had the highest cell densities while those co-treated with etomoxir had the lowest cell densities pointing to increased cell survival (Fig. 17F).
CHAPTER 4: DISCUSSION

The role of antioxidants in cancer chemoprevention has been controversial. The surprising findings from the SELECT trial showing an increased risk of prostate cancer with vitamin E supplementation have been the subject of much discussion.\textsuperscript{496} The discordance between the \textit{in vitro} studies that informed SELECT’s design and the trial’s outcome is partly due to the predominant use of conventional 2D tissue culture which bears little resemblance to the \textit{in vivo} environment. For mechanical support, the cell monolayer in 2D cultures adheres to a flat surface typically glass or polystyrene, giving the cells an abnormally flattened and stretched morphology, which impacts cellular behavior, growth and function.\textsuperscript{505,530}

In addition, 2D cell epithelial cultures lack native tissue architecture due to loss of apical-basal polarity and lack of the natural three-dimensional (3D) environment in native tissue where cells are surrounded by other cells and the extracellular matrix (ECM).\textsuperscript{505,531} Furthermore, 2D cultures also fail to model pertinent aspects of human tumors. Unlike in human tumors, 2D monolayers have equal access to nutrients and growth factors in the culture media resulting in homogenous cell proliferation.\textsuperscript{505} In contrast, epithelial cells embedded in matrices like collagen or reconstituted ECM (matrigel) aggregate in three dimensions (3D) forming native like cell-cell and cell-matrix interactions bridging the gap between artificial 2D \textit{in vitro} and \textit{in vivo} conditions.\textsuperscript{531}

Moreover, nutrient, growth factor and oxygen gradients in 3D cultures gives rise to heterogeneous cell populations of proliferating, quiescent, apoptotic or necrotic and hypoxic cells similar to poorly vascularized tumors.\textsuperscript{505} In addition to providing physical support and
serving as a scaffold for tissue organization, ECM attachment also provides biochemical and biomechanical cues necessary for anchorage-dependent cell growth, proliferation, migration and differentiation.\textsuperscript{532} During normal epithelial glandular development, ECM–cell interactions align the mitotic spindle so that cells divide parallel and not perpendicular to the epithelial plane, and balance proliferation and cell death for tissue homeostasis and lumen morphogenesis.\textsuperscript{533}

The normal prostate gland is a psuedostratified polarized epithelium made of basal cells, rare neuroendocrine cells and luminal cells, which secrete prostatic fluid in to a central lumen (Fig. 2).\textsuperscript{534} In 3D culture, prostate epithelial cells induce apical-basal polarity generating lumen-containing acini resembling \textit{in vivo} prostate gland architecture with restored tissue-specific prostatic fluid secretion hence, they are termed organoids.\textsuperscript{535} Tissue architecture is said to be critical for epithelial tissue function.\textsuperscript{536} Indeed, the malignant transformation of the prostate epithelium is marked by the gradual loss of cell adhesion and glandular architecture.\textsuperscript{537-539} Therefore, 3D cultures lend themselves well to studies of both gland morphogenesis and effects of tissue architecture disruption in carcinogenesis.

We showed that premalignant but not benign or malignant prostate epithelial cells grown as 3D organoids respond to antioxidant treatment in a manner that recapitulates the findings of the SELECT trial. The supplements decreased proliferation and increased cell death in malignant LNCaP organoids, consistent with reports that show antioxidant efficacy in established cancer cell lines.\textsuperscript{599,400} The LNCaP organoid data therefore support the concept that moderate levels of ROS damage DNA leading to mutations that can aggravate cancer.\textsuperscript{540} In contrast, the agents with the exception of selenium significantly increased the proliferation of premalignant RWPE-1 cell organoids.
As cells at different stages of tumorigenesis experience different levels of ROS, it is reasonable to expect the antioxidant effect to be dependent on a cell’s position in the tumor progression spectrum. In fact, the SELECT supplements did not affect the proliferation of benign organoids derived from primary prostate epithelial cells. These results are consistent with SELECT where just a fraction more of the men on vitamin E developed PCa compared to those on the placebo. We suggest that these individuals might have harbored initiated, pre-malignant cells with molecular aberrations that were pushed in to malignancy by vitamin E.

In support of this hypothesis, Martinez et al., demonstrated that the antioxidant NAC causes prostatic epithelial hyperplasia in mice with prostate specific deletion of the Nkx3.1 tumor suppressor but not in wild-type mice. In addition, polymorphisms in NKX3.1 were found to modulate PCa risk in men on the interventional arms of the SELECT trial. This points to the importance of the underlying genetic background of prostate cells in modifying the response to antioxidant supplementation. However, the methods used in SELECT’s inclusion criteria, a PSA count and a non-suspicious DRE, could not have ruled out the existence of molecular aberrations.

Our benign organoids had proper glandular structure with well formed lumens a morphology that was not affected by vitamin E and/or selenium treatment. As organoids are avascular, this allowed us to mimic the in vivo spatial constraints in cells in often poorly vascularized solid tumor cells. Similar to tumors, cells at the center of large organoids are under various stresses including loss of ECM attachment and limits in the diffusion of nutrients and oxygen. In benign organoids, these cells die off since nutrient uptake depends on the ECM attachment status and growth factor signaling (Fig. 18).
In contrast, the malignant LNCaP cells formed organoids without lumens (filled morphology) a phenotype consistent with the acquisition of anchorage-independent survival and loss of glandular differentiation. On the other hand, the vehicle treated premalignant RWPE-1 organoids had more differentiated acini structures and predominantly hollow lumens. When treated with vitamin E but not selenium however, the premalignant organoids developed a predominantly filled lumen morphology indicating increased cell survival in a low matrix environment. This further demonstrates that the premalignant RWPE-1 organoids best recapitulated results of the SELECT trial.

Microarray analysis of RNA extracted from vitamin E treated RWPE-1 organoids displayed significant downregulation of several integrins. Because integrins mediate ECM-cell attachments these results confirmed the loss of matrix attachment. The microarray data also revealed decreased expression of glucose transporters 1 and 3 (GLUT 1 and 3) and several glycolytic enzymes in the vitamin E treated RWPE-1 organoids. These results imply dysregulation of glucose metabolism in spite of increased cell growth and survival in these organoids. In mammary organoids, loss of matrix attachment rapidly induced autophagy a lysosomal degradation pathway of proteins and organelles that promotes cell survival under metabolic stress.\textsuperscript{545,546}

In MCF-10A breast acini, measurement of native NADPH fluorescence as a metabolic read out showed that only the inner matrix deprived cells but not the outer ECM attached cells were metabolically compromised.\textsuperscript{522} These findings confirm that loss of matrix attachment (LOA) jeopardizes cellular metabolism. Indeed, when cultured in non-adherent conditions to mimic the matrix deprived inner organoid cells, RWPE-1 prostate benign epithelial cells had significantly
reduced ATP levels and glucose uptake a phenomenal also observed in MCF-10A benign epithelial cells. Altered metabolism after cell detachment has been attributed to the loss of integrin activation of the PI3K/AKT pathway, a crucial regulator of glucose and glutamine uptake and metabolism (Fig. 19).

ECM-integrin contact leads to the recruitment of adapter proteins like talin and paxillin as well as signaling molecules like focal adhesion kinase (Fak) and small GTPases to form large macromolecular structures termed focal adhesions connecting the ECM and the actin cytoskeleton. Autophosphorylation of FAK (Y397) downstream of integrin signaling activates its kinase function leading to the activation of the SRC/MAPK and PI3K/AKT pathways, which are crucial for progression through the G1/S checkpoint, cell survival and proliferation. Cross talk between integrins and receptor protein tyrosine kinases (RPTKs) also activates the PI3K/AKT pathway downstream of the epidermal growth factor receptor (EGFR). In contrast, matrix deprived cells in the vehicle treated malignant LNCaP organoids continued to survive and proliferate leading to the filled lumen morphology.

The accumulation of multiple alterations allows cancer cells to circumvent extracellular regulation enabling them to uptake nutrients constitutively. In contrast, the PI3K pathway is important in normal epithelial cells for survival, and its inactivation by loss of ECM attachment leads to a form of cell death termed anoikis which is Greek for “homelessness.” In vivo, anoikis prevents re-adhesion of detached cells to matrices in the wrong location and their abnormal growth. Autophagy activation in these cells is thought to be short-term metabolic safeguard against anoikis that presumably allows them to survive in case they are able to reattach.
to the matrix.\textsuperscript{552} Whereas prolonged autophagy can lead to cell death, a mechanism used for luminal clearance in normal mammary acini, short-term, autophagy increases cell viability.\textsuperscript{553}

As well as diminished glucose uptake which lowered ATP generation, detached benign mammary epithelial cells also had elevated levels of reactive oxygen species (ROS).\textsuperscript{522} This was shown to result from the decreased glucose flux through the Pentose Phosphate Pathway (PPP) for NADPH generation.\textsuperscript{522} Interestingly, treatment of premalignant prostate organoids with the SELECT agents not only decreased ROS levels but also rescued ATP but not glucose uptake. Similar findings have been reported in detached mammary cells treated with NAC or trolox, a soluble form of vitamin E.\textsuperscript{522} In the absence of glucose, cells can catabolize fatty acids for ATP generation. In Akt transformed glioblastoma, cells activate fatty acid oxidation (FAO) to survive upon glucose withdrawal.\textsuperscript{181}

We showed that vitamin E treatment in detached RWPE-1 cells rescued the ATP deficiency in a FAO-dependent manner which increased premalignant cell survival and filled lumens in organoids (summarized in Figs. 18 & 19). Consequently, FAO inhibition decreased cell survival and enhanced organoid luminal clearance indicating that antioxidants are necessary for anchorage independent cell survival. It has been postulated that ROS inhibits FAO and hydrogen peroxide a form of ROS, has been shown to inhibit peroxisomal FAO.\textsuperscript{554-556} Though the mechanism remains undefined, this implies that antioxidants activate FAO by eliminating ROS.

Finally in this study, just like in SELECT, selenium had more complex effects. In addition to decreasing ROS, selenium affected the expression of more genes in RWPE-1 organoids compared to vitamin E. Selenium is incorporated in to various selenoproteins with a broad range
of activities besides redox homeostasis.\textsuperscript{421,557,558} It is possible that any protumorigenic effects of selenium’s antioxidant function are counteracted by its effect on other anti-tumorigenic pathways. However, although selenium treatment induced an anti-proliferative gene signature, it did not lower the proliferation index in RWPE-1 organoids probably due to alternative mechanisms that override transcriptional regulation.

This study was limited by the difficulty of establishing cultures of pre-malignant prostate epithelial cells \textit{in vitro} so we used the RWPE-1 cell line instead. Additionally, we did not directly measure FAO but used the more general mitochondrial respiration readout, OCR.

\textbf{CONCLUSIONS}

This study highlights the importance of neutralizing ROS for anchorage independent cell growth and survival implying that ROS can imperil detached cells. In support of this notion, other antioxidant-driven metabolic rescue mechanisms following loss of attachment have also been described.\textsuperscript{559} In lung cancer spheroids, LOA upregulates glutamine reductive metabolism by cytosolic isocitrate dehydrogenase-1 (IDH1) to generate NADPH which is shuttled to inhibit mitochondrial ROS enhancing cell growth.\textsuperscript{559}

Anchorage independence facilitates cell growth and survival in ectopic environments for example in metastasis.\textsuperscript{560} Therefore, identifying mechanisms that enable anchorage independence in normal and premalignant cells offers clues on how this might be impeded to eradicate metastatic cancer cells.\textsuperscript{560} Moreover, inactivating mutations and deletions in PTEN are frequent events seen in 20\% primary PCa and 50\% CRPC respectively.\textsuperscript{561} These events lead to
activation of the PI3K pathway which is associated with metabolic alterations that enable anchorage-independent survival.

Our data suggest that antioxidants could be effective against malignant PCa but they promote tumorigenesis in premalignant cells. Moreover, these results show that the use of preclinical models that better mimic in vivo conditions and disease stage yield data that is more relevant for clinical translation. Finally, given the central role that metabolism plays in cancer initiation and progression, preclinical studies ought to include metabolic endpoints when assessing potential chemopreventive agents.

**FUTURE DIRECTIONS**

ROS are said to inhibit FAO, in particular, hydrogen peroxide has been shown to inhibit FAO in peroxisomes. This implies that antioxidants activate FAO by eliminating ROS. However, the mechanism through which this happens remains undefined. It has been proposed that this might be through an indirect mechanism, by the inhibition of TCA cycle enzymes like mitochondrial aconitase, or directly by inhibiting FAO enzymes. In addition, though we observed an increase in the proliferation index and in the expression of genes involved in cell proliferation in premalignant organoids treated with vitamin E, the downstream signaling pathways effecting this phenotype also remain undetermined.

For these questions, A CRISPR–Cas9-based genome-wide screen can be used to identify genes whose loss decreases FAO, cell survival and cell proliferation in low-attachment conditions. Not only would this uncover the mechanisms through which antioxidants increase proliferation, cell
survival and correct metabolic deficiencies after ECM detachment, but they might also unveil new therapeutic targets. Our study also did not determine what kind of ROS-induced cell death was responsible for luminal clearance in the vehicle treated organoids or how it might be regulated. In mammary acini, inhibition of apoptosis and autophagy merely delays cell death implicating alternative cell death mechanisms besides apoptosis and autophagic cell death.\textsuperscript{526}

Uncovering the mechanism(s) responsible for the non-apoptotic cell death of prostate epithelial cells after ECM detachment could unveil therapeutic targets for the clearance of metastatic cells which are often in non-native matrix environments.
Figure 18. Model of anchorage-dependent regulation of cell survival and glucose metabolism.

Adhesion of cells to the ECM activates integrins and receptor tyrosine kinase (RTK) signaling which triggers different pro-survival and proliferation pathways like AKT and MAPK. AKT inhibits BIM, a pro-apoptotic protein preventing anoikis and increases glucose uptake and glycolysis by upregulating the transcription of glucose transporters (GLUT1) and hexokinase 2 (HK2) respectively. Oxidative phosphorylation (OxPHOS) of pyruvate derived from glucose yields ATP for cellular function. Glucose shunted through the PPP pathway generates NADPH which prevents ROS induced cell death.
Due to loss of integrin and PI3K signaling, cell detachment from the ECM results in the reduced expression of glucose transporters and low glucose uptake (shown in broken lines and grey boxes). This decreases ATP production and cell survival. Moreover, low NADPH generation through attenuated PPP flux leads to ROS accumulation which can induce cell death. ROS also inhibits fatty acid beta-oxidation (FAO) an alternative ATP generation pathway under glucose limiting conditions. However, treating detached cells with exogenous antioxidants like vitamin E neutralizes ROS which activates FAO increasing cell survival. Attenuating FAO using etomoxir which inhibits carnitine palmitoyltransferase (CPT1), FAO’s rate limiting enzyme, abrogates vitamin E’s ATP rescue ultimately diminishing cell survival.
Table 1. Summary of non-synonymous mutations found from the targeted sequencing of 222 cancer genes in the primary cells from the two subjects used to generate benign organoids.

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<th>Subject 1</th>
<th>Gene</th>
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Table 2. Selected leading edge genes from a sample of gene sets significantly enriched in the SELECT supplement treated premalignant organoids

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<td>Reactome Cyclin E</td>
<td>CCNH CCNE2 PSMA3 CCNA1 RK1B PSMA4 CDK7 PSME2 PSMA5 PSMB3 PSMD11 PSMD12 PSMA7 MNTA1 RPS27A PSMA6 PSMC2 PSMA1 PSM7 PSMA2 PSM3 PSM4 PSM5 CSM6 PSMB9 SKP1 MAX PSMB7 PSMB5 PSM6 PSMC5 PSMB1</td>
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<td>Elvidge Hypoxia Up AHN42K DTTA CYP1B1 CSRP2 BXX ATXN1 ALDOCG IGFBP3 DVP3L GSY1 MTS BI3NK ANDK1 YEAT5 MXI1 NDRG1 SORL1 G1B1 JUN DSC2 FMALL3 SAMM4A SLC2A1I SRD3A TNAPF1 C9DF7 TRA7A EGFR SFXN3 CAD14 CDC4 CSM4 SLGOL2A GOLGAB2 LOC383983 ANKRD36 CYHL1</td>
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<td>Kobayashi EGFR Signaling 24HR Down</td>
<td>Kobayashi EGFR Signaling 24HR Down KIF2C NACPD3 ABC1E1 KIF4A CDK1 FRM1 GPM2 RACGAP1 POL2 GINSI RRM2 NAA15 CDK2 NUDT15 RAD51 EXO1 MET RAD54B DUSP6 BRCa1 VRK1 DONSON ZCHAVI1 NEK2 TUSP1 USC MCM6 SHCBP1 NACPD2 MADD2L1 DFRH GJB3 RCR1 KIF15 BUB1 DNAJC9 DSN1 D2K1 TPX1 CCNA2 ITGA5 BCLDL2 ECT2 STEAP1 MELK TTP2 TMEM194A SLC40A1I FAM11A1 ELL2 MBD47 DEPDC1 FANC CEP55 STIL SPC25 SMC4 ATAD2 NOC3L KIF3 MYB1 PNIN ERC4C4L EON2 KIF14 WULCH DTL DUSP4 NSUPA1 NRGI PKL4 DLGAP5 LMB11 CCM2 SRF5 F8 PBK POLA1 DCSI NACPD4 HMMR TOP2A HELLS SMCC2 HMG2A ASPM STC1</td>
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<td>Pujana BRCA Centered Network</td>
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Selected Leading Edge Genes Down-regulated by Combination Treatment

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<td>Rosty Cervical Cancer Proliferation Cluster TACC3 DNA2 MAD2L1 ACA4A KIAA1598 RMI1 UBL3 KDELC1 CALM2 SVIP MSL1 LRP1B NAB1 MBD4 GPR126 NT5DC1</td>
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Uncertainty in significance
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