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DOCOSAHEXAENOIC ACID EFFECTS ON CELL METABOLISM IN PROSTATE CANCER INITIATION AND PROGRESSION

EFEITOS DO ÁCIDO DOCOSAHEXAENÓICO NO METABOLISMO CELULAR NA INICIAÇÃO E PROGRESSÃO TUMORAL PROSTÁTICA

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Tese de Doutorado apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, como parte dos requisitos exigidos para a obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Biologia Celular.

Orientadora: Profª Drª Rejane Maira Góes

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Os membros da Comissão Examinadora acima assinaram a Ata de defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Biologia Celular e Estrutural do Instituto de Biologia.

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RESUMO

A reprogramação metabólica ao longo do processo carcinogênico confere suporte à alta demanda anabólica e energética. Este ajuste também é observado no câncer de próstata (CaP), em especial a desregulação mitocondrial e da lipogênese, vulnerabilidades exploradas como estratégias terapêuticas. O ácido docosahexaenóico (DHA) é um ácido graxo poli-insaturado ômega-3 com propriedade antiproliferativa em diferentes tipos tumorais, inclusive na próstata, cujos mecanismos ainda não estão esclarecidos, mas parecem envolver o metabolismo celular. Este trabalho avaliou a propriedade antitumoral do DHA em diferentes contextos androgênicos do CaP e os mecanismos subjacentes ao metabolismo tumoral. Primeiramente, avaliamos o potencial do DHA em reduzir o crescimento celular associado à via androgênica, de sensores metabólicos que corregulam morte e proliferação celular, como também a via mitocondrial. Para isso, foram utilizadas células epiteliais prostáticas humanas pré-maligna (PNT1A, AR-positiva) e tumorais resistentes à castração (CRPC) 22rv1 (AR-positiva) e PC3 (AR-negativa). O DHA (100µM, 48h) modulou o crescimento celular diferentemente, reduzindo a fase S e induzindo morte celular nas AR-positivas, enquanto provocou parada do ciclo em G2/M na AR-negativa. Nestas linhagens, este ômega-3 induziu estresse oxidativo, acúmulo lipídico e disfunção mitocondrial. A PNT1A mostrou-se mais responsiva quanto à regulação de genes envolvidos com o metabolismo, resposta a hormônios, estresse, como também na fisiologia mitocondrial. Apesar de menor quantidade, o DHA desregulou genes nas células malignas que estão frequentemente alterados de forma oposta no CaP. Na PNT1A, o ômega-3 ainda prejudicou a biogênese mitocondrial e provocou a fragmentação da sua rede. Em seguida, nas mesmas condições experimentais, foi avaliado o metabolismo lipídico em células tumorais malignas AR-positivas LNCaP, andrógeno-responsiva, C4-2 e 22rv1, ambas CRPC. O DHA induziu parada do ciclo celular em todas as linhagens, sendo incorporado em triacilgliceróis e ésteres de colesterol, como também em fosfolipídios com consequente aumento da insaturação na membrana celular, o que sensibiliza células a danos oxidativos. Tal incorporação foi acompanhada de menor regulação da via lipogênica, lipogênese a partir de glicose e redução proteica de FASN. Além disso, o DHA estimulou a oxidação de ácidos graxos, sendo que a linhagem 22rv1 apresentou preferência pelo ômega-3. A redução da lipogênese não ocorreu devido à regulação da via androgênica, mesmo com redução da expressão proteica de AR-FL e AR-V7. Tendo em vista os desafios terapêuticos do CRPC, avaliamos o efeito do DHA sobre o crescimento de organoides MSK-PCa3 e em modelo xenográfico com a linhagem 22rv1 (DHA 10mg/kg/dia/28 dias), o qual teve seu diâmetro reduzido e o crescimento tumoral inibido, respectivamente. Por fim, testamos o efeito simultâneo do DHA e inibidores da lipogênese (FASNi/ACCi-100nM/28 dias) e observamos potencialização na redução do diâmetro dos organoides. A administração de FASNi (100mg/mL/28 dias) e DHA reduziu o crescimento do tumor em mais de 80%. Em conclusão, o DHA é um agente antitumoral multialvo em diferentes contextos androgênicos por vias que culminam na parada do ciclo ou indução de morte celular associadas ao metabolismo, sendo a mitocondrial prevalente em estágio inicial e o metabolismo lipídico em estágios avançados do CaP.

Palavras-chave: Tumores - Metabolismo; Próstata – Câncer; Ácidos graxos ômega-3; ácido docosahexaenóico; lipídios.

ABSTRACT

Metabolic reprogramming during carcinogenesis supports the increased anabolic and energetic demand in proliferating cells. Such adjustment is also observed in the prostate cancer (PCa), mainly the dysregulation of mitochondria and lipogenesis, both vulnerabilities often explored as therapeutic strategies. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid with antiproliferative property in different cancers, including prostate, but the mechanisms are not elucidated yet and seem to be related with cell metabolism regulation. This study evaluated the DHA antitumoral property on distinct androgenic contexts of PCa and the underlying mechanisms with regard to tumor metabolism. Firstly, we tested the DHA potential in decrease cell growth due to regulation of the androgenic pathway, metabolic sensors that also co-regulate death and proliferative pathways, in addition to induce mitochondria dysfunction. For this purpose, we incubated with DHA at 100µM for 48h human epithelial prostate cells from pre-malignant, (PNT1A, AR-positive) and castrated-resistant stage (CRPC) 22rv1 (AR-positive) and PC3 (AR-negative). DHA differently modulated the cell growth by reducing S-phase and inducing cell death in AR-positive while led the AR-negative to cell cycle arrest in G2/M. In these cell lines, the omega-3 raised oxidative stress, lipid accumulation and mitochondrial dysfunction. PNT1A was more responsive regarding to regulation of genes related with metabolism, response to hormones and stress as well as mitochondrial physiology. In 22rv1 and PC3, DHA regulated less genes compared to PNT1A, but they are often altered in PCa samples. In PNT1A, the omega-3 also impaired mitochondrial biogenesis and led to its network fragmentation. Secondly, under same experimental conditions, we evaluated the lipid metabolism in AR-positive malignant cells LNCaP (androgen-responsive), C4-2 and 22rv1, both CRPC. DHA induced cell cycle arrest on these cell lines, being incorporated into triacylglycerol and cholesterol esters as well as phospholipids, which showed increase in unsaturation status hence in the cell membrane, turning cells prone to oxidative damage. Such incorporation was followed by downregulation of the lipogenic pathway, lipogenesis from glucose and reduction of FASN protein levels. Moreover, DHA stimulated fatty acids oxidation, being 22rv1 the cell line with preference for the omega-3 itself. Lipogenesis impairment was not due to androgenic pathway regulation, even though DHA decreased AR-FL and AR-V7 protein levels. Considering the therapeutic challenges of the CRPC stage, we evaluated the DHA effect on MSK-PCa3 organoids growth and 22rv1 xenografts (DHA 10mg/kg/day/28 days). It decreased the organoids diameter and suppressed tumor growth, respectively. Lastly, we tested the concomitant administration of DHA with lipogenesis inhibitors (FASNi/ACCi-100nM/28 days) and observed an enhancement in the reduction of organoids diameters. The FASNi (100mg/mL/28 days) and DHA administration reduced tumor growth in at least 80%. In conclusion, DHA is an antitumor agent at different androgenic contexts and has several targets that culminate in cell cycle arrest and cell death induction. The pathways were associated with the metabolism, being mitochondria the major one at early stages and the lipid metabolism in the advanced PCa.

Key words: Tumor - Metabolism; Prostate – Cancer; Omega-3 fatty acids; Docosahexaenoic acid; Lipids.

LISTA DE ABREVIATURAS E SIGLAS

^{1}H-NMR – Proton Nuclear Magnetic Resonance / Ressonância Magnética Nuclear de Prótons

22rv1 – Linhagem prostática epitelial humana tumoral andrógeno-responsiva

7-AAD – 7-aminoactinomycin D / 7-aminoactinomicina D

ACC – Acetyl-CoA carboxylase / Acetil-CoA carboxilase

ACLY – ATP citrate lyase / ATP citrato liase

AKT – Protein Kinase B / Proteína Quinase B

ALA – Alpha-linolenic Acid / Ácido alfa-Linolênico

AMPK – AMP-activated Protein Kinase / Proteína Quinase Ativada por AMP

APC – Anaphase-Promoting Complex / Complexo Promotor de Anáfase

AR – Androgen Receptor / Receptor de Andrógeno

AREs ou ERAs – Androgen Responsive Elements / Elementos Responsivos a Andrógenos

AR-FL – Androgen Receptor Full-Lenght / Isoforma completa do Receptor de Andrógeno

AR-V7 – Androgen Receptor Variant 7 / Variante 7 do Receptor de Andrógeno

ATCC – American Type Culture Collection

ATM – Ataxia Telangiectasia Mutated kinase / Ataxia Telangiectasia Mutada quinase

ATP – Adenosine Triphosphate / Adenosina Trifosfato

BAD – Bcl-2 Associated Agonist / Agonista Associado a BCL2

BCA – Bicinchoninic Acid Assay / Ensaio do Ácido Bicinconínico

Bcl-2 – B-Cell Lymphoma 2 Protein / Proteína 2 de Células B de Linfoma

BHT – 2,6-di-tert-butyl-4-methylphenol / 2,6-di-tert-butil-4-metilfenol

BPH ou HBP – Benign Prostatic Hyperplasia / Hiperplasia Benigna Prostática

BRCA1 – Breast Cancer Gene 1 / Gene do Câncer de Mama 1

BRCA2 – Breast Cancer Gene 2 / Gene do Câncer de Mama 2

BrDU – 5-Bromo-2´-Deoxyuridine / 5-Bromo-2´-Deoxiuridina

$CaCl_2$ – Calcium Chloride / Cloreto de Cálcio

CAFs – Cancer-associated Fibroblasts / Fibroblastos Associados ao Câncer

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

cDNA – Complementary DNA / DNA complementar

CE – Cholesterol Ester / Éster de Colesteril

CH_3OH – Methanol / Metanol

$CHCl_3$ – Chloroform / Clorofórmio

CHD1 – Chromodomain-Helicase DNA-binding 1 / Cromodomínio-Helicase DNA-ligante 1

CL – Cardiolipin / Cardiolipina

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

CO_2 – Carbon dioxide / Dióxido de carbono

COX-2 – Cyclooxygenase 2 / Ciclooxigenase 2

COX-I – Cyclooxigenase I / Cycloxigenase I

CPM – Counting per Minutes / Contagem por Minuto

CPT1 – Carnitine Palmitoyltransferase 1 / Carnitina palmitoiltransferase 1

CRPC – Castrated Resistant Prostate Cancer / Câncer de Próstata resistente à Castração

DAG – Diacylglycerol / Diacilglicerol

dH_2O – distilled Water / Água destilada

DHA – Docosahexaenoic Acid / Ácido Docosahexaenóico

DMSO – Dimethyl Sulfoxide / Dimetilsulfóxido

DNA – Desoxyribonucleic Acid / Ácido Desoxirribonucleico

DNL – *de novo* Lipogenesis / Lipogênese *de novo*

DoD – Department of Defense

DOX – Doxycyclin / Doxiciclina

DRP-1 – Dynamin-related Protein 1 / Proteína relacionada à Dinamina 1

DU145 – Linhagem prostática epitelial humana tumoral não responsiva a andrógeno

ECACC – European Collection of Authenticated Cell Cultures

ECAR – Extracellular Acidification Rate / Taxa de acidificação extracelular

EDTA – Ethylenediaminetetraacetic Acid / Ácido etilenodiamino tetra-acético

EPA – Eicosapentaenoic Acid / Ácido Eicosapentaenoico

ER – Estrogen Receptor / Receptor de Estrógeno

ER stress – Endoplasmatic Reticulum stress / Estresse de Retículo Endoplasmático

ERF – ETS2 Repressor Fator / Fator de repressão de ETS2

ERG – ETS transcriptional factor / Fator de Transcrição de ETS

ERK1/2 – Extracellular Signal-Regulated Kinase 1/2 / Quinase regulada por sinal extracelular 1/2

ERRα – Estrogen-Related Receptor alpha / Receptor alfa relacionado a estrógeno

ERRγ – Estrogen-Related Receptor gamma / Receptor gama relacionado a estrógeno

ETC ou CTE – Electron Transport Chain / Cadeia Transportadora de Elétrons

FA – Fatty Acid / Ácidos graxos

FAPERP – Fundação de Apoio à Pesquisa e Extensão de São José do Rio Preto

FAPESP – Fundação de Amparo à Pesquisa do Estado de São Paulo

FASN – Fatty Acid Synthase / Ácido graxo sintase

FBS – Fetal Bovine Serum / Soro Fetal Bovino

FCCP – p-triflouromethoxyphenylhydrazone/ p-trifluormetoxifenilhidrazona

FDR – False Discovery Rate / Taxa de descoberta falsa

FFAR4 – Free Fatty Acids Receptor 4 / Receptor de ácidos graxos livres tipo 4

FI – Fluorescence Intensity / Intensidade de fluorescência

FOXA1 – Forkhead box Protein A1 / Proteína A1 de forkhead box

gDNA – genomic DNA / DNA genômico

GLS-1 – Glutaminase 1

GLUT1 – Glucose Receptor type 1 / Receptor de Glicose tipo 1

GLUT3 – Glucose Receptor type 3 / Receptor de Glicose tipo 3

GPR120 – G-Protein Coupled Receptor type 120 / Receptor Acoplado à proteína G tipo 120

GPRs – G-Protein Coupled Receptors / Receptores Acoplados à proteína G

GPX2 – Gluthatione Peroxidase 2 / Glutationa Peroxidase 2

GPX4 – Gluthatione Peroxidase 4 / Glutationa peroxidase 4

GSEA – Gene Set Enrichment Analysis / Análise de Enriquecimento Funcional

GST – Glutathione-S-Transferase / Glutationa-S-Transferase

GSTP1 – Gluthatione-S-Transferase P1 / Glutationa-S-Transferase P1

HCl – Chloridric Acid / Ácido Clorídrico

HIF-1 – Hypoxia-Induced Fator 1 / Fator Induzido por Hipóxia 1

IL-10 – Interleukin type 10 / Interleucina tipo 10

KCl – Potassium Chloride / Cloreto de Potássio

KRAS - Kirsten rat sarcoma virus

LDH – Lactate desidrogenase / Lactato desidrogenase

LNCaP – Linhagem prostática epitelial humana tumoral andrógeno-responsiva

LOX – Lipoxygenases / Lipooxigenases

LXRα – Liver X Receptor alpha / Receptor X hepático alfa

mCRPC – Metastatic Castrated-resistant Prostate Cancer / Câncer de Próstata Resistente à Castração Metastático

MFN-2 – Mitofusin-2 / Mitofusina-2

MMR – Mitochondrial Metabolic Rate / Taxa do metabolismo mitocondrial

mRNA – messenger RNA / RNA mensageiro

mTOR – Mammalian Target Of Rapamycin / Alvo da Rapamicina em mamíferos

MYC – Master regulator of cell cycle entry and proliferative metabolism / Regulador da entrada no ciclo celular e do metabolismo proliferativo

n-3 FA ou ω-3 FA – Omega-3 Fatty Acids / Ácidos graxos ômega-3

Na_2HPO_4 – Disodium phosphate / Fosfato dissódico

NaCl – Sodium chloride / Cloreto de sódio

NADPH – Nicotinamide Adenine Dinucleotide Phosphate / Nicotinamida Adenina Dinucleotídeo Fosfato

NaH_2PO_4 – Monosodium phosphate / Fosfato monossódico

$NaHCO_3$ – Sodium bicarbonate / Bicarbonato de sódio

NEPC – Neuroendocrine Prostate Cancer / Câncer de Próstata Neuroendócrino

NES – Normalized Enrichment Score / Score de Enriquecimento Normalizado

NFkβ – Nuclear Factor kappa beta / Fator Nuclear kappa beta

NIH – National Institute of Health

NMP – 1-methyl-2-pyrrolidinone / 1-metil-2-pirrolidinona

NOX1 – NADPH oxidase 1

NOXA1 – NADPH oxidase Activator 1 / Ativador da NADPH oxidase 1

NR – Nuclear Receptor / Receptor Nuclear

NR0B1 – Nuclear Receptor Subfamily 0 Group B Member 1 / Receptor Nuclear Subfamília 0 Grupo B Membro 1

O_2 – Oxygen / Oxigênio

$O_2^{\bullet-}$ – Superoxide anion / Ânion superóxido

OCR – Oxygen Consumption Rate / Taxa de consumo de oxigênio

OPA – Optic Atrophy-1 protein / Proteína Atrofia óptica mitocondrial

OXPHOS – Oxidative Phosphorylation / Fosforilação Oxidativa

PBS – Phosphate-Buffered Saline / Saline Tamponada com Fosfato

PC – Phosphatidylcholine / Fosfatidilcolina

PC3 – Linhagem prostática epitelial humana tumoral não responsiva a andrógeno

PCa ou CaP ou – Prostate Cancer / Câncer de Próstata

PCF – Prostate Cancer Foundation

PDK-1 – Phosphoinositide-Dependent Kinase-1 / Proteína Quinase Dependente de Fosfatidilinositol 1

PE – Phosphatidylethanolamine / Fosfatidiletanolamina

PFK-1 – Phosphofructokinase-1 / Fosfofrutoquinase-1

PG – Phosphatidylglycerol / Fosfatidilglicerol

PI – Phosphatidylinositol / Fosfatidilinositol

PI3K – Phosphatidylinositol 3-kinase / Fosfatidilinositol-3 quinase

PIN ou NIP – Prostatic Intraepithelial Neoplasia / Neoplasia Intraepitelial Prostática

PIP_3 – Phosphatidylinositol (3,4,5)-trisphosphate / Fosfatidilinositol (3,4,5)-trifosfato

PMSF – Phenylmethylsulfonyl Fluoride / Fluoreto de Fenilmetilsulfonil

PNT1A – Linhagem prostática epitelial humana benigna

PNT2A – Linhagem prostática epitelial humana benigna

PPAR – Peroxisome Proliferator-activated Receptors / Receptor ativado por proliferador de peroxissomo

PPARGC1A – Peroxisome Proliferator-activated Receptor gamma Coactivator 1-alpha / Co-ativador 1-alfa do Receptor Ativado por Proliferador de Peroxissomo

PPARGC1B – Peroxisome Proliferator-activated Receptor gamma Coactivator 1-beta / Co-ativador 1-beta do Receptor Ativado por Proliferador de Peroxissomo

PPARα – Peroxisome Proliferator-activated Receptor alpha / Receptor alfa Ativado por Proliferador de Peroxissomo

PPARγ ou NR1C3 – Peroxisome Proliferator-activated Receptor gamma / Receptor gama Ativado por Proliferador de Peroxissomo

PPARδ – Peroxisome Proliferator-activated Receptor delta / Receptor delta Ativado por Proliferador de Peroxissomo

PrEC – Prostate Epithelial cells / Células epiteliais prostáticas

PSA – Prostate Specific Antigen / Antígeno Específico Prostático

PTEN – Phosphatase and Tensin Homologue / Proteína Homóloga à Fosfatase e Tensina

PUFAs – Polyunsaturated Fatty Acids / Ácidos Graxos poli-insaturado

qRT-PCR – Quantitative Real-Time Polymerase Chain Reaction / Quantificação da Reação da Polimerase em Cadeia em Tempo Real

RB1 – Retinoblastoma Transcriptional Corepressor 1 / Co-repressor Transcricional 1 de Retinoblastoma

RFU – Relative Fluorescence Units / Unidades Relativas de Fluorescência

RIPA – Radioimmunoprecipitation Assay Buffer / Tampão de Ensaio de Radioimunoprecipitação

RNA – Ribonucleic Acid / Ácido Ribonucleico

RORα – Retinoic Acid-related Orphan Receptor α / Receptor Órfão alfa Relacionado ao Ácido Retinóico

ROS ou EROs – Reactive Oxygen Species / Espécies Reativas de Oxigênio

RPM – Rotation per Minutes / Rotação por Minutos

RPMI – Roswell Park Memorial Institute / Memorial Instituto Roswell Park

RT – Room Temperature / Temperatura Ambiente

RWPE-1 – Linhagem prostática epitelial humana benigna

RXR – Retinoid X Receptor X / Receptor X de retinóide

SCD1 – Stearoyl-CoA desaturase-1 / Estearoil Co-A desaturase 1

SDH-A – Succinate Dehydrogenase Complex Subunit A / Complexo da Succinato Desidrogenase Subunidade A

SDS-PAGE – Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis / Eletroforese em Gel de Poliacrilamida com Dodecil- Sulfato de Sódio

SEM – Standard Error of the Mean / Erro Padrão da Média

SFAs – Satured Fatty Acids / Ácidos Graxos Saturados

SM – Sphingomyelin / Esfingomielina

SMAD4 – SMAD Family Member 4 / Membro 4 da Família SMAD

SPOP –Speckle-Type POZ protein / Proteína Speckle-Type POZ

SREBP – Sterol Regulatory Element Binding Proteins / Proteínas de Ligação a Elemento Regulador de Esterol

SV40 – Simian vírus 40 / Vírus símio 40

TAG – Triacylglycerol / Triacilglicerol

TAMs – Tumor-associated Macrophages / Macrófagos Associados a Tumores

TBST – Tris Buffered Saline with Tween 20 / Salina Tamponada com Tris e com Tween 20

TCA – Tricarboxylic Acid Cycle / Ciclo do Ácido Tricarboxílico

TEM – Transmission Electron Microscopy / Microscopia Eletrônica de Transmissão

TFI – Total Fluorescence Intensity / Intensidade de Fluorescência Total por campo

TGFβ – Tumor Growth Factor beta / Fator de Crescimento Tumoral beta

TP53 – Tumor Protein 53 / Proteína Tumoral 53

TRAMP – Transgenic Adenocarcinoma of the Mouse Prostate / Camundongo transgênico para adenocarcinoma prostático

SUMÁRIO

I. INTRODUÇÃO

I.I Aspectos Gerais da Próstata

A próstata é uma glândula acessória do sistema reprodutor masculino localizada abaixo da bexiga e que circunda a uretra, sendo, em humanos, setorizada anatomicamente (Figura 1A) em zona central, transicional e periférica, além de distintas a região fibromuscular e periuretral (VERZE; CAI; LORENZETTI, 2016). Histologicamente, é classificada como uma glândula túbulo-alveolar sendo sua composição dada pelos compartimentos epitelial, luminal e estromal (Figura 1B-C), cujas funções e populações celulares são distintas, mas conservadas entre diferentes espécies de mamíferos como humanos, roedores e morcegos (ALBERNAZ et al., 2021; OLIVEIRA et al., 2016; QUINTAR et al., 2017; VERZE; CAI; L ORENZETTI, 2016). O compartimento epitelial é configurado, em sua maioria (60%), por células luminais secretoras que liberam compostos no lúmen, região de acúmulo do fluido prostático que compõe o sêmen (Figura 1B). Os outros 40% da população celular deste compartimento correspondem a células transientes em amplificação e basais, derivadas das células tronco, que atuam como reservatório do tecido prostático para renovação de células secretoras (Figura 1B), como também as neuroendócrinas (PACKER; MAITLAND, 2016). Já o compartimento estromal é composto por uma maior diversidade celular (Figura 1C), contendo fibroblastos, telócitos, células musculares lisas, células do sistema imunológico, além de vasos e nervos que, em conjunto orquestram a manutenção da glândula prostática (SANCHES et al., 2021a, 2021b).

Portanto, esta organização complexa resulta na principal função da próstata, a produção e secreção de fatores que compõem o fluido seminal (Figura 2), como zinco, citrato, fosfatases e poliaminas, provendo condições adequadas para a sobrevivência e funcionamento de espermatozoides, o que tem impacto direto na fertilização.

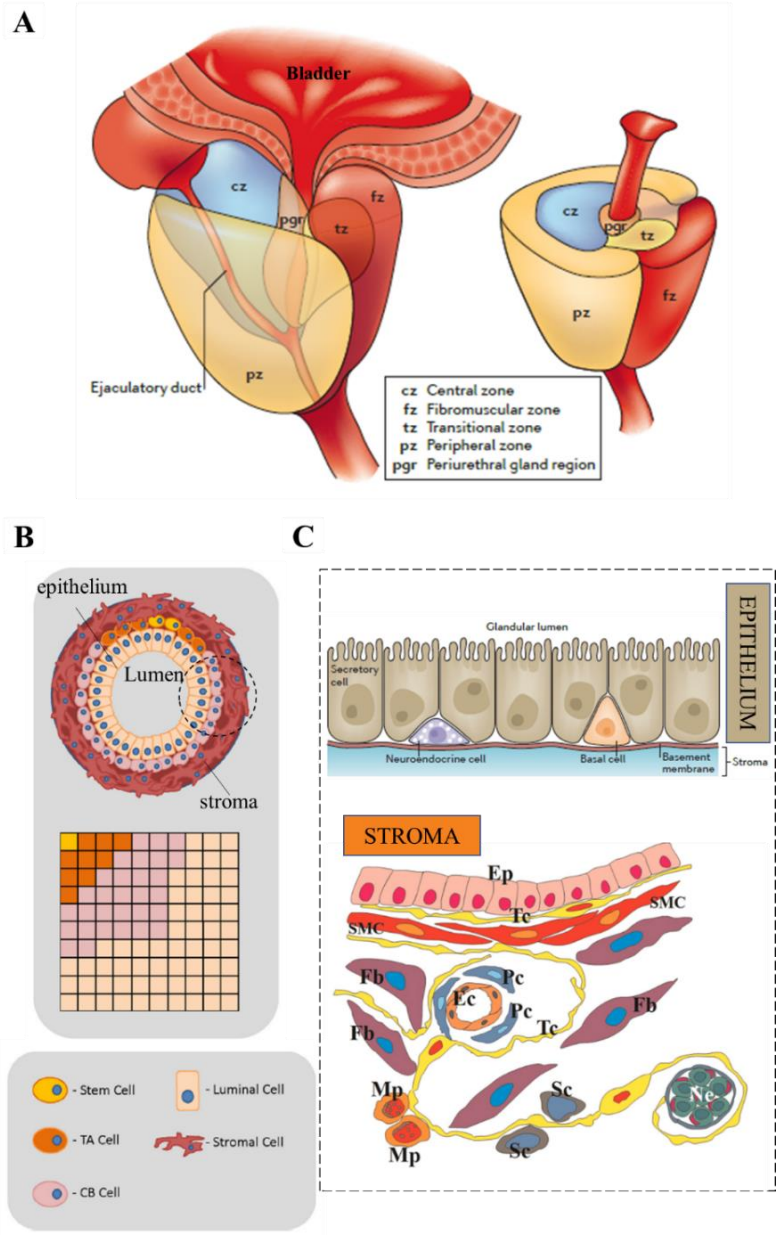

Figura 1. *Anatomia e Histologia da Próstata Humana.* **A)** Posição anatômica e setorização prostática em zonas. Localizada abaixo da bexiga e circundando a uretra, a glândula prostática é dividida em zona central (cz), transicional (tz) e periférica (pz), sendo encontradas também a região fibromuscular (fz) e periuretral (pgr). **B)** Compartimentos histológicos: epitélio, estroma e lúmen. Ilustração da diversidade celular e sua distribuição encontrada nos ácinos, sendo o compartimento epitelial composto por células tronco (amarelas, *stem cell*), células de amplificação transiente (alaranjadas, *TA cell*), basais (roxo, *CB cell)* e luminais (rosa claro, *luminal cell*). **C)** Detalhe da diversidade celular, evidenciando no compartimento epitelial (Ep) células neuroendócrinas e, no estromal, células musculares lisas (SMC), telócitos (Tc), células tronco (Sc), endoteliais (Ec), pericitos (Pc), do sistema nervoso (Ne), do sistema imunológico (Mp) e fibroblastos (Fb). Fonte: Verze et al., 2016; Parker e Maitland, 2016; Sanches et al., 2021, todos modificados.

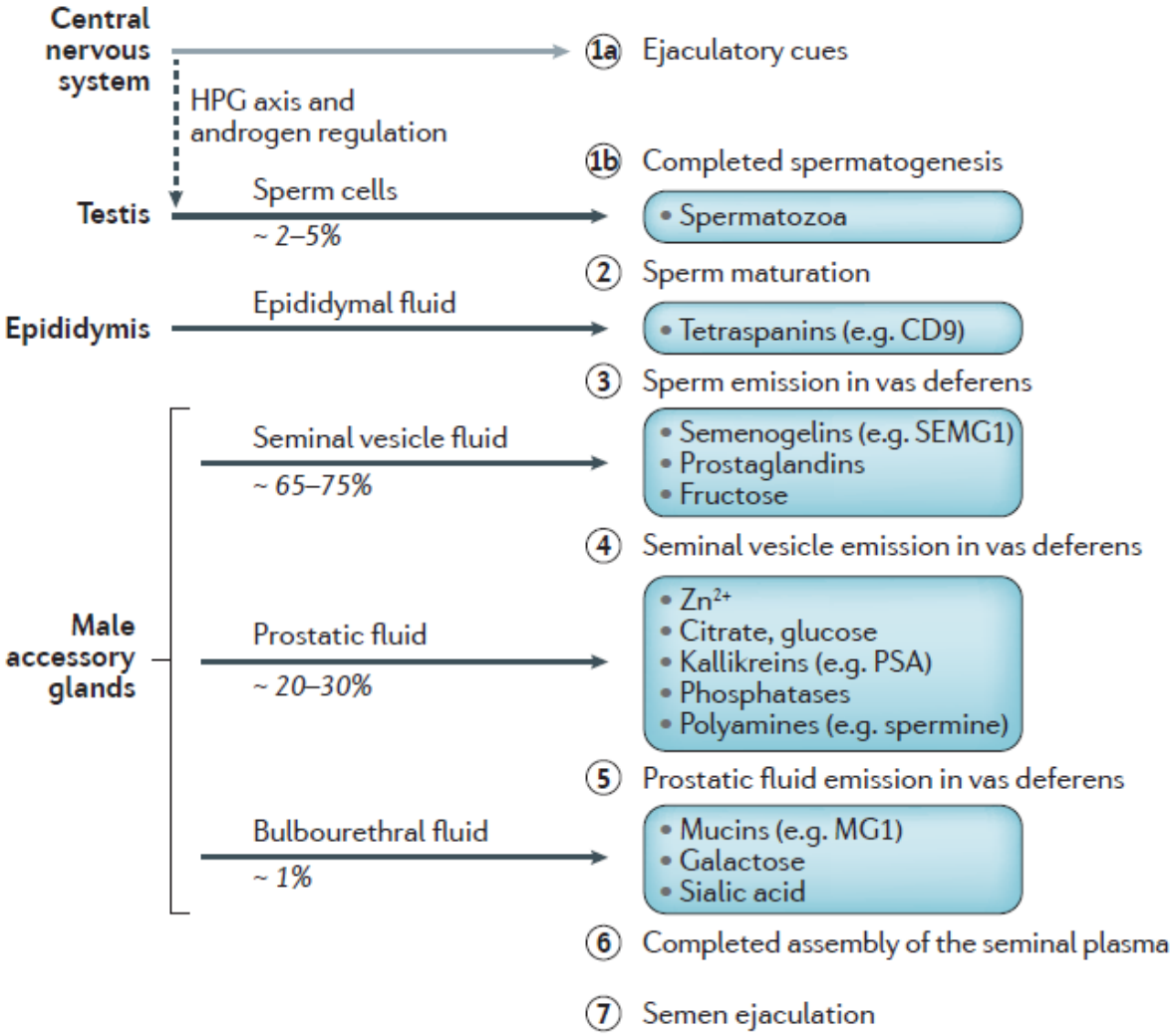

Figura 2. *Processo de produção do sêmen e sua composição.* É possível, nesta figura, destacar a participação da próstata na composição do fluido seminal, a qual contribui via secreção de zinco, citrato, glicose, PSA, fosfatases, poliaminas, entre outros. Fonte: Verze et al., 2016 (adaptado).

A próstata é regulada, entre outros fatores, por esteroides sexuais desde o início do seu desenvolvimento até a vida adulta (MARKER et al., 2003). Estes hormônios atuam via ativação de receptores específicos (receptor de andrógenos – ARs e de estrógenos – ERs) e controlam eventos determinantes na homeostasia glandular, como proliferação, metabolismo e atividade secretora (CUNHA; COOKE; KURITA, 2004; CUNHA, 1996; MARKER et al., 2003). Neste sentido, alterações na resposta à ativação desses receptores e, mesmo a insensibilidade aos seus respectivos ligantes, podem estar relacionadas ao desenvolvimento e agressividade do câncer de próstata (CaP)

(HEBERT et al., 1998; PARSONS et al., 2006; POWERS; MARKER, 2013). Contudo, ainda que a via androgênica e estrogênica sejam considerados principais alvos no combate ao CaP e, atualmente, a linha de frente na estratégia terapêutica, alguns autores têm questionado sua segurança e eficácia por promover a sobrevivência seletiva de células tumorais malignas não responsivas à ablação androgênica, o que resulta na reincidência tumoral em estágio mais agressivo
(CAI et al., 2018; CUI et al., 2017; KARANTANOS; CORN; THOMPSON, 2013; SHAFI; Y EN; WEIGEL, 2013). Portanto, este cenário aponta para necessidade de novas alternativas de tratamento e prevenção da patologia.

I.II Epidemiologia e Etiologia do Câncer de Próstata

O CaP está atualmente entre os cinco tipos com maior incidência na população mundial (Figura 3A), sendo o segundo na população masculina e classificado nesta entre as cinco causas de morte (Figura 3A-B) (SUNG et al., 2020). No Brasil (Figura 3C), é o tipo mais incidente em homens (SUNG et al., 2020). Esta patologia acomete a população masculina principalmente por volta dos 70 anos, sendo uma doença rara em indivíduos abaixo dos 40 anos (CUNNINGHAM; YOU, 2015). A etiologia do CaP ainda não está completamente elucidada, mas estudos populacionais mostram uma distribuição geográfica desigual da sua incidência e agressividade,
(HSING; REICHARDT; STANCZYK, 2002; RAWLA, 2019; SUNG et al., 2021), o que aponta para a influência de características hereditárias e dos hábitos de vida no risco de seu desenvolvimento. Portanto, embora não haja consenso sobre determinados fatores ou sólida informação quanto à sua contribuição individual, o CaP apresenta natureza multifatorial. Por um lado, alterações genômicas como deleções e mutações em genes específicos, tais como *AR*, *APC*, *ATM*, *BRCA1* e *BRCA2*, *CHD1*, *ERF*, *ERG*, *ETS2*, *EZH2*, *FOXA1*, *MYC*, *MYCN*, *SMAD4*, *SPOP*, *RB1* e *TP53*, estão entre as mais comuns e bem descritas (MATEO et al., 2020; WANG et al., 2018). Além disso, a etnia parece ser um fator relevante, uma vez que descendentes afro-americanos apresentam maior risco de desenvolver CaP (HA et al., 2013), o que tem sido associado ao metabolismo diferencial de lipídios (ZHANG et al., 2016). Por outro, fatores ambientais como a dieta, qualidade de lipídios (LABBÉ et al., 2019), histórico gestacional do indivíduo (PYTLOWANCIV et al., 2016) e

frequência de atividade física (CAMPOS et al., 2018; ZASLAU et al., 2012) tem sido reportados como fatores que favorecem o CaP. Em especial, a dieta hiperlipídica tem levantado questões sobre não apenas a quantidade, mas também a qualidade de lipídios. Dados de roedores demonstraram que ácidos graxos saturados favorecem o desenvolvimento do CaP via reprogramação metabólica (LABBÉ et al., 2019), o que também é observado para os insaturados, cuja alta ingestão demonstrou induzir alterações no microambiente tecidual que favorecem o desenvolvimento do CaP, tais como aumento de colágeno, perda da capacidade antioxidante e regulação de PI3K/AKT (RIBEIRO et al., 2012a, 2012b; SILVA et al., 2015; TAMARINDO et al., 2021). É importante mencionar que não somente a ingestão hiperlipídica ao longo da vida pode ser determinante no risco do CaP, mas também o histórico materno, uma vez que tanto a dieta de lipídios durante a gestação, como durante a lactação parecem provocar alterações irreversíveis na próstata da prole na vida adulta (PYTLOWANCIV et al., 2016). Em contrapartida, estudos *in vitro* com ácidos graxos poli-insaturados, em especial os ômega-3, mostraram propriedades preventivas e terapêuticas em estágios iniciais e avançados da doença (BROWN et al., 2006; LIANG et al., 2020; SHIN et al., 2013; TAMARINDO; GÓES, 2020; TAMARINDO et al., 2019), o que será melhor discutido neste trabalho. Apesar destas evidências experimentais, estudos clínicos ainda são escassos ou conflitantes na associação entre os níveis elevados destes diferentes ácidos graxos e o risco de desenvolvimento do CaP.

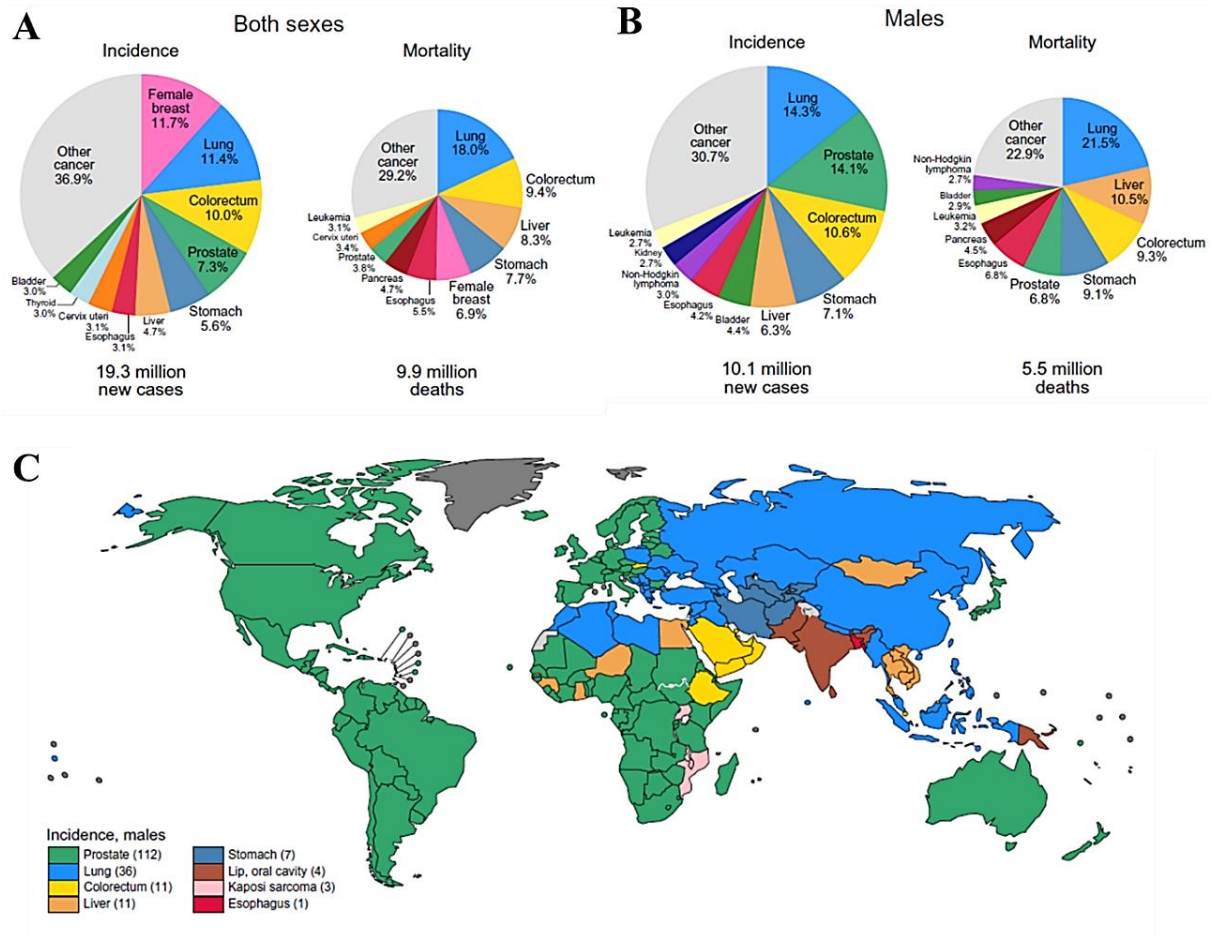

Figura 3. *Distribuição geográfica da incidência e mortalidade do câncer de próstata*. **A)** Proporções na população mundial, independente do gênero biológico. **B)** Proporções na população masculina mundial. **C)** Distribuição por país. Fonte: SUNG et al 2020, modificado.

I.III Progressão Tumoral e Modelos de Estudo *in vitro*

O processo carcinogênico da próstata e sua progressão (Figura 4) envolvem a contribuição de um conjunto de fatores, conforme discutido anteriormente. Anomalias na próstata podem ser benignas ou malignas, de acordo com características da arquitetura tecidual e celular da glândula. De forma simplificada, entre as benignas é comum, em humanos, a ocorrência de neoplasias intraepiteliais prostáticas (NIPs) de baixo e alto grau, de acordo com alterações morfológicas (ZHOU, 2018). A primeira é estabelecida na literatura como uma displasia leve, apresentando um aumento da densidade celular sem perda da polarização ou descaracterização da arquitetura tecidual. A segunda é considerada um precursor do

adenocarcinoma prostático (BRAWER, 2005), com perda da polarização característica de células epiteliais, apresentando alta taxa proliferativa, estratificação e invasão luminal, levando à formação de micro-ácinos que podem ou não ser acompanhados por inflamação e alterações estromais favoráveis à carcinogênese.

A ocorrência do CaP se dá primeiramente na forma dependente de hormônio, na qual as células apresentam, entre outras alterações, dependência da sinalização de andrógenos para manter a alta taxa proliferativa e expressam a isoforma completa do AR (AR-FL). Neste estágio, as principais estratégias terapêuticas têm como alvo as vias de esteroides sexuais, em especial, a de síntese de andrógenos, como por exemplo o uso da abiraterona, além da prostatectomia. Entretanto, eventualmente ocorre reincidência entre 2 e 3 anos após a ablação androgênica, progredindo para estágio mais agressivo e letal (SHEN; ABATE-SHEN, 2010), o câncer de próstata resistente à castração (CRPC). Neste estágio, as células cancerígenas apresentam ajustes moleculares, em especial na via de sinalização de andrógenos (KARANTANOS; CORN; THOMPSON, 2013), que permitem sua proliferação de forma independente da sensibilização por estes hormônios. A amplificação do gene do AR e consequente aumento nos seus níveis proteicos é uma das mais comuns e acomete cerca de 20-25% dos casos de CRPC (BUBENDORF et al., 1999), sendo uma característica rara em tumores primários (MAO et al., 2010). O crescimento tumoral ainda pode depender de mutações no gene do AR, como por exemplo a T877A (VELDSCHOLTE et al., 1990), que permite a sensibilização deste receptor por maior número de hormônios esteroides, como também da expressão de variantes provenientes de *splicing* do AR-FL, gerando isoformas, como a variante 7 (AR-V7). Esta é exclusivamente encontrada no estágio CRPC (DE LAERE et al., 2017; ZHU et al., 2020) e tem sido associada à regulação de diferentes vias, como a do metabolismo de lipídios e ciclo celular (ZADRA et al., 2019). As células cancerígenas prostáticas deste estágio ainda podem apresentar produção intra-tumoral de testosterona e di-hidrotestosterona, mesmo após a terapia androgênica e queda nos níveis séricos de testosterona (KNUUTTILA et al., 2018). Além das alterações na via androgênica, células do CaP apresentam reprogramação metabólica, em especial a superexpressão da ácido graxo sintase (FASN), c-MYC, mudança no funcionamento mitocondrial e quantidade de mitocôndrias, alteração na via PI3K/AKT/mTOR, perda de PTEN, entre outros fatores, o que será discutido adiante. É importante destacar que, embora seja frequente a ocorrência de células que expressam o AR, aqui denominado de fenótipo AR-positivo, alguns tipos de tumores malignos podem progredir para o estágio completamente independente de AR (AR-negativo),

sofrendo trans-diferenciação reversível neuroendócrina (BHAGIRATH et al., 2021), sendo muito raro o seu desenvolvimento a partir de células neuroendócrinas da próstata normal (RICKMAN et al., 2017). Nestes, as células tumorais passam a expressar enolase 2, cromogranina A e sinaptofisina, características moleculares de tumores neuroendócrino de próstata (NEPC). Este estágio da progressão possui agressividade elevada com baixa taxa de sobrevivência (aproximadamente 20%) no período de 5 anos, apresentando metástases com tropismo para pulmões, fígado, ossos e sistema nervoso central em sua maioria (BHAGIRATH et al., 2021). O estágio CRPC não possui estratégias terapêuticas efetivas, muitas vezes levando ao óbito e contribuindo para o estabelecimento do CaP entre as 5 principais causas de morte no mundo.

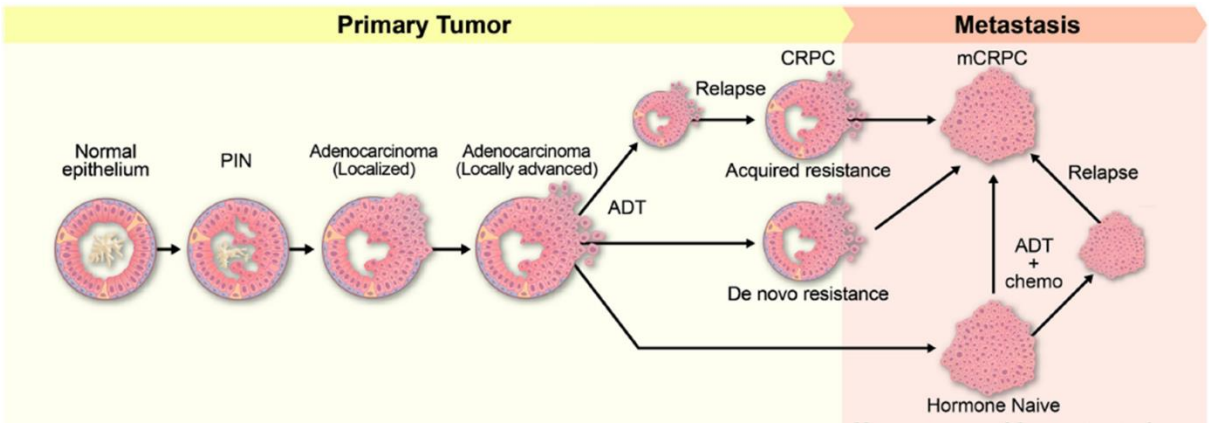

Figura 4. *Progressão Tumoral Prostática*. Esquema do processo carcinogênico a partir do epitélio normal, progredindo para neoplasia intra-epitelial (PIN), adenocarcinoma *in situ*, adenocarcinoma *in situ* avançado, estágio resistente à castração e metástase. Fonte: Wang et al., 2018 (modificado).

A busca por terapias efetivas levou ao estabelecimento de diferentes modelos experimentais, tanto *in vitro* quanto *in vivo*, desenvolvidos ao longo dos últimos 20 anos com o objetivo de coletar evidências pré-clínicas, como também elucidar mecanismos envolvidos na carcinogênese da glândula. Tendo em vista o foco deste trabalho, serão enfatizados os sistemas *in vitro*. A cultura de células 2D é um modelo experimental estabelecido há décadas, sendo hoje uma das importantes ferramentas para compreender os mecanismos envolvidos (PHILIPPEOS et al., 2012). É um método que possui a vantagem de estabelecer o crescimento populacional celular de forma rápida a partir de material retirado do paciente, ser adaptável a

diferentes condições de trabalho e de baixo custo comparado à experimentação animal. Além disso, permite distinguir os mecanismos que ocorrem em um tipo celular específico sem a interferência da diversidade celular presente no tumor, o que proporciona fortes evidências para a proposição de novas alternativas terapêuticas. Por outro lado, também apresenta a desvantagem de eliminar a arquitetura tumoral e a comunicação entre outros tipos celulares, o que influencia diretamente na sinalização intra e inter-celular. As linhagens humanas mais comuns e estabelecidas no estudo do CaP são epiteliais (CUNNINGHAM; YOU, 2015), compartimento onde frequentemente a carcinogênese se inicia, derivadas de pacientes e posteriormente cultivadas em laboratório. Pode-se classificá-las, de forma generalizada, em células responsivas a andrógenos e não responsivas ou CRPC. É importante ressaltar que, embora o principal critério de classificação seja a dependência da via de andrógenos extra prostático para a sobrevivência, existem inúmeras alterações no contexto molecular e genômico que contribuem para o fenótipo neoplásico, sendo as principais detalhadas na Tabela 1. Portanto, é possível perceber, mesmo em modelos *in vitro*, a heterogeneidade do CaP e necessidade de estratégias mais assertivas. Para suprir essa necessidade, têm sido utilizados modelos de cultivo 3D, os quais podem ser esferoides ou organoides. Nestes sistemas, o cultivo ocorre com componentes da matriz extracelular, principalmente colágeno, o que oferece uma arquitetura favorável à formação de massas tumorais de pequeno volume, como também a possibilidade de co-cultivo com outros tipos celulares, em especial células estromais, como fibroblastos, macrófagos e células do sistema imunológico (KUNZ-SCHUGHART; KREUTZ; KNUECHEL, 1998; LIN; LIN; CHANG, 2008). No caso dos esferoides, a massa tumoral, benigna ou maligna, é composta exclusivamente por um tipo celular, o que não reproduz características originais encontradas no paciente (SGOUROS; YANG; ENMON, 2003). Esta falha metodológica levou ao desenvolvimento de organoides, os quais remontam às características histológicas, genéticas e moleculares originais do paciente, incluindo a diversidade celular, e permitem o cultivo e armazenamento a longo prazo. Na próstata, os organoides têm sido adotados como modelos pré-clínicos, servindo como uma ferramenta translacional no teste de compostos antitumorais (GAO et al., 2014; RICHARDS et al., 2019; ZADRA et al., 2019).

Além das linhagens tumorais malignas também existem as benignas, as quais sofreram o processo de imortalização por SV40, como a PNT1A, PNT12B e RWPE-1, para manutenção a longo prazo (Tabela 1). Ainda, há células primárias não-imortalizadas, que são extraídas de doadores jovens, como a PrEC, e utilizadas para o estudo da condição normal da

próstata. Vale destacar que estas linhagens imortalizadas, ainda que benignas, apresentam elevado potencial proliferativo e frequentemente alterações pré-malignas, como a PNT1A, dadas pela superexpressão de c-MYC e modificações genômicas frequentemente encontradas no adenocarcinoma prostático (DEGEORGES et al., 1995). Portanto, elas servem como um excelente modelo de estudo *in vitro* para estágios iniciais da carcinogênese, sendo amplamente utilizadas (GOBBO et al., 2015; TAMARINDO et al., 2019, 2021).

Linhagem	***Patologia***	***AR status***	***AR-Vs***	***PTEN***	***Identificador***
LNCaP	Tumoral	AR^+ (T877A mutante)	-	$PTEN^-$	CRL-1740 [1]
C4-2	Tumoral	AR^+ (T877A mutante)	AR-V8	$PTEN^-$	CRL-3314 [1]
22rv1	Tumoral	AR^+ (H874Y mutante)	AR-V1-V9, V12-14	$PTEN^+$	CRL-2505 [1]
PC3	Tumoral	AR^-	-	$PTEN^-$	CRL-1435 [1]
DU145	Tumoral	AR^-	-	$PTEN^+$	HTB-81 [1]
PNT1A	Benigna	AR^+	-	$PTEN^+$	95012614 [2]
RWPE-1	Benigna	AR^+	-	$PTEN^+$	CRL-11609 [1]
PrEC	Normal	AR^+	-	$PTEN^+$	CC-2555 [3]

Tabela 1. *Linhagens celulares epiteliais prostáticas humanas.* Células frequentemente utilizadas na cultura celular para compreensão de mecanismos. Estão listadas linhagens tumorais, benignas (alto potencial proliferativo e anomalias genômicas, mas não-tumorais) e normais. Legenda: AR – receptor de andrógeno; AR-V – variante do receptor de andrógeno; PTEN – fosfatase homóloga à tensina; [1] – ATCC; [2] – ECACC; [3] – Lonza. Fonte: Cunningham e You, 2015 (modificado) e fornecedores.

I. IV Metabolismo Prostático

Próstata Normal

As células epiteliais secretoras prostáticas saudáveis apresentam metabolismo particular devido à fisiologia mitocondrial diferir de outros tecidos. Isto ocorre devido ao seu papel na síntese de citrato, um componente da secreção prostática (Figura 2) e também intermediário da via bioenergética

(COSTELLO; FRANKLIN, 2016; COSTELLO et al., 1997; TWUM-AMPOFO et al., 2016). Na maioria das células de outros tecidos, como músculo, fígado e coração, o citrato é oxidado no ciclo do ácido Tricarboxílico (TCA) para produção de coenzimas reduzidas que sustentam a cadeira transportadora de elétrons (CTE), tendo como resultado a síntese de ATP acoplada à redução do oxigênio a água. Na próstata normal, esta oxidação ocorre em uma taxa muito reduzida devido à diminuição da atividade da aconitase mitocondrial, o que se dá pela regulação dos Elementos Responsivos a Andrógenos (ERAs), como também pelas altas concentrações de zinco e testosterona na mitocôndria (COSTELLO; FRANKLIN, 2016; EIDELMAN et al., 2017). Como consequência, ocorre maior produção e acúmulo de citrato, o qual é exportado da mitocôndria e então secretado para o compartimento luminal, não sendo utilizado majoritariamente no processo bioenergético. Assim, o atendimento à demanda energética da próstata depende principalmente de outras rotas, como a glicólise, a qual possui um rendimento de ATP inferior à fosforilação oxidativa (OXPHOS).

Câncer de Próstata

As células tumorais passam por uma reprogramação metabólica, o que supre as demandas anabólicas e energéticas necessárias à manutenção da alta taxa proliferativa. Este processo de ajuste metabólico foi primeiramente descrito por Otto Warburg (1956), o qual observou a maior captação de glicose e sua metabolização preferencialmente por fermentação, conforme ilustrado na Figura 5A, sendo o prejuízo da OXPHOS uma das causas do processo tumoral (WARBURG, 1956). Ao longo dos anos, este conceito tem sido discutido, sendo proposta sua caracterização como um desbalanço entre a quantidade de glicose captada e oxidada via respiração aeróbica, mesmo com a manutenção do funcionamento mitocondrial (DEBERARDINIS; CHANDEL, 2020). Desta forma, células tumorais apresentariam maior captação de glicose e sua utilização majoritariamente na via anaeróbica mesmo em condições de disponibilidade de oxigênio, aumentando a liberação de lactato no meio extracelular (Figura 5B). Embora a predominância da via glicolítica ofereça rendimento energético inferior à OXPHOS, este processo permite o redirecionamento de intermediários do metabolismo para processos anabólicos, cujas vias estão frequentemente mais ativas em resposta à sinalização oncogênica (DEBERARDINIS; CHANDEL, 2020). Isto inclui a via das pentose-fosfato, a qual fornece substratos para a síntese de nucleotídeos, como também NADPH; a via de hexoaminas,

necessária para a glicosilação de proteínas; o metabolismo de aminoácidos associado à síntese de glutationa, nucleotídeos e reações de metilação; além de disponibilização de glicerol para a produção de lipídios complexos, necessários no processo de síntese membranas (SCAGLIA et al., 2014) e sinalização celular (DEBERARDINIS; CHANDEL, 2020). Além disso, neste processo, ainda são produzidos metabólitos que, em condições saudáveis, seriam eliminados pela célula ou metabolizados em níveis reduzidos, como amônia e corpos cetônicos (SUN et al., 2018).

Ainda que a glicose tenha papel central na reprogramação metabólica, estudos também têm reportado a glutamina, o aminoácido mais abundante na circulação, como fonte principal que alimenta o TCA em diferentes tipos tumorais. Sendo um nutriente anaplerótico, o esqueleto carbônico da glutamina é incorporado em α-cetoglutarato via glutaminase 1 (GSL-1), podendo ocorrer carboxilação redutiva, processo no qual o α-cetoglutarato é retroconvertido em citrato na presença de NADPH por isocitrato desidrogenases (ALTMAN; STINE; DANG, 2016). Este processo é frequente no contexto tumoral, sendo uma das principais rotas envolvidas na síntese de ácidos graxos, especialmente quando há defeitos mitocondriais ou hipóxia (GAMEIRO et al., 2013; METALLO et al., 2011; MULLEN et al., 2011; WARD et al., 2010) , funcionando como um mecanismo de resistência. Além dos lipídios, o metabolismo da glutamina fornece suporte à síntese de aminoácidos, como também atua como um reservatório de nitrogênio para a produção de purinas e pirimidinas (ALTMAN; STINE; DANG, 2016). A glutamina ainda pode ativar mTOR, suprimir estresse de retículo e promover a síntese de proteínas, eventos que favorecem a carcinogênese (ALTMAN; STINE; DANG, 2016). Neste contexto, o metabolismo da glutamina ainda pode ser ativado por oncogenes, como KRAS e MYC, listado entre três genes mais desregulados em diferentes tipos de câncer humano (ZACK et al., 2013). Como resultado, há maior expressão dos transportadores de glutamina e glutaminase (GAO et al., 2009; WISE et al., 2008), o que indica a dependência da glutamina em células tumorais. Esse conjunto de dados tem posicionado o metabolismo da glutamina como oportunidade terapêutica.

Embora a reprogramação metabólica ainda não esteja completamente elucidada para o CaP, sabe-se que ela não segue estritamente o fenótipo observado no efeito Warburg. Apesar de identificar dados discrepantes entre estudos, meta-análises dos principais achados metabolômicos presentes na literatura (KELLY et al., 2016; MCDUNN et al., 2013) apontam para o papel chave do metabolismo energético na iniciação e progressão tumoral prostática.

Diferentemente de outros tipos tumorais, nos estágios avançados do CaP parece ocorrer a reativação mitocondrial e predominância do fenótipo de OXPHOS, como também maior captação de glicose pelas células tumorais em relação à condição benigna (VAZ et al., 2012), utilização de glutamina e aumento da síntese e oxidação de lipídios, ajustes metabólicos que serão discutidos adiante.

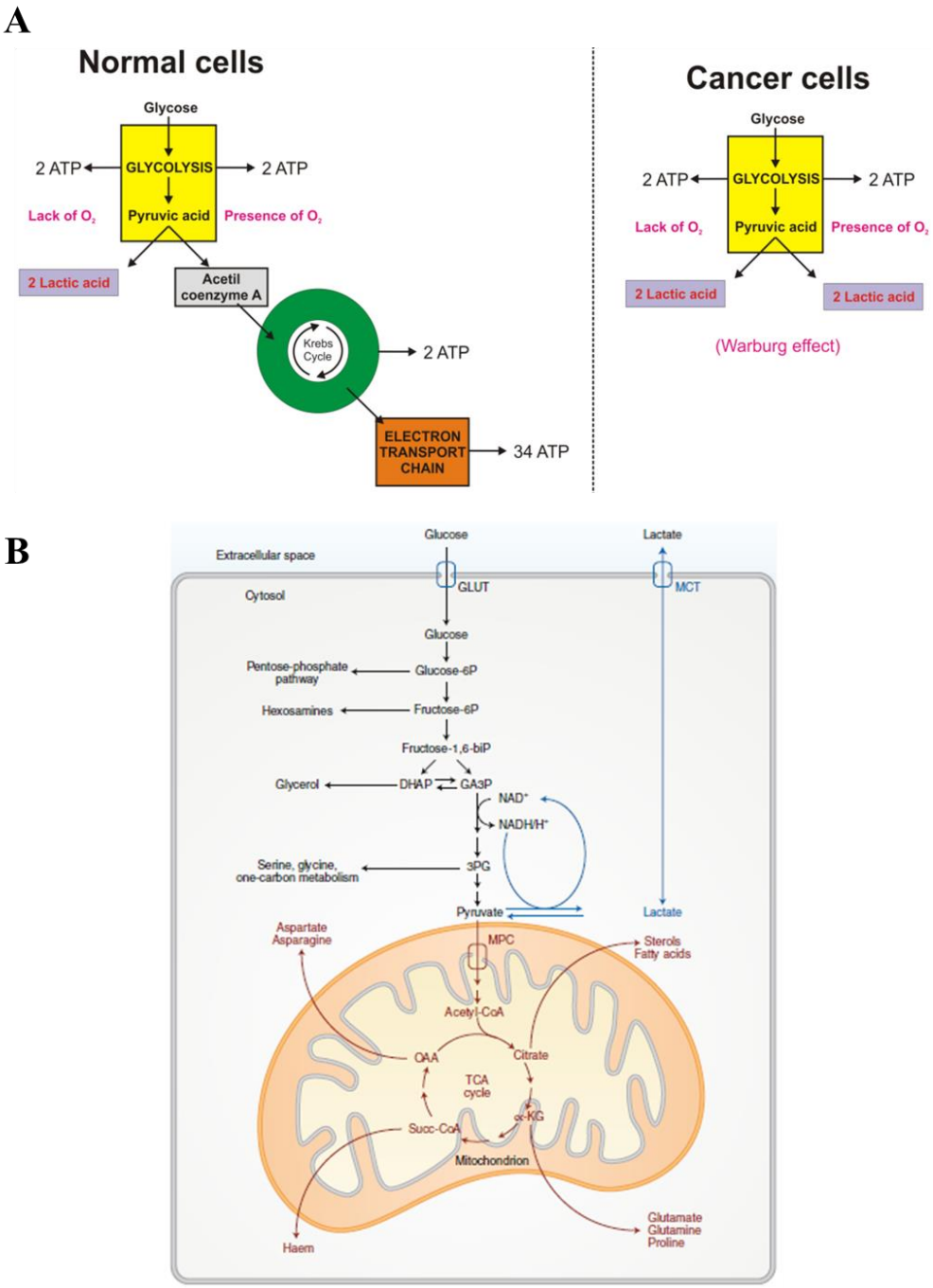

Figura 5. *Reprogramação Metabólica no Câncer*. Células tumorais sofrem reprogramação metabólica de forma que a via glicolítica se torne mais ativa para suprir a demanda energética e compostos intermediários para vias anabólicas. **A)** Proposta clássica elaborada por Otto Warburg. **B)** Proposta reformulada, apontando a utilização de intermediários metabólicos necessários ao suprimento da demanda tumoral. Legenda: ATP – adenosina trifosfato; O_2 – oxigênio; GLUT – transportador de glicose; MCT – transportador de moléculas de um carbono; Glucose-6P – glicose-6-fosfato; Frustose-6P – frutose-6-fosfato; Fructose-1,6-bP – frutose-1,6-bifosfato; DHAP – dihidroacetona-fosfato; GA3P – gliceroaldeído-3-fosfato; MPC – transportador de piruvato; OAA – oxaloacetato; TCA – ciclo do ácido tricarboxílico; α-KG – alfa-cetoglutarato; Succ-CoA – succinato-CoA; NAD^+ - Dinucleótido de nicotinamida e adenina. (Devic, 2016, adaptado).

O metabolismo celular se torna mais complexo no CaP devido à sua regulação pelo AR (MASSIE et al., 2011), o qual influencia diferentes rotas metabólicas, como a glicolítica, síntese de lipídios, o metabolismo mitocondrial e da glutamina (BAJPAI et al., 2019; WHITE et al., 2017). A presença de andrógenos estimula da captação de glicose e a glicólise aeróbica no CaP hormônio responsivo, mesmo em condições de normóxia (MASSIE et al., 2011). Isto é corroborado por evidências que mostram maior atividade da fosfofrutoquinase 1 (PFK-1) em células andrógeno-responsivas (VAZ et al., 2012), como também a maior expressão de receptores GLUT1 e GLUT3 após administração de di-hidrotestosterona em células LNCaP (VAZ et al., 2016), de forma que estas alterações não são observadas em células AR-negativas, PC3 e DU145 (VAZ et al., 2012, 2016). A utilização da glicose e sua completa oxidação também parecem diferir em células AR-negativas, uma vez que há elevada atividade da lactato desidrogenase (LDH) na PC3, essencial ao processo de respiração anaeróbica, resultando na maior produção e exportação de lactato, o que não ocorre com a LNCaP, AR-positiva (VAZ et al., 2012). A linhagem PC3 é derivada do estágio mais agressivo do CaP e o transporte de lactato teria a função de acidificar o meio extracelular, o que tem sido correlacionado à metástase em outros tipos de tumores (ALBERS et al., 2008; SCHWICKERT et al., 1995; TEAHAN et al., 2011). Além da via glicolítica, os andrógenos ainda promovem a proliferação de células LNCaP mediada pela maior captação de glutamina, além de induzir a expressão de seus receptores SLC1A4 e SLC1A5, ambos super expressos no CaP (WHITE et al., 2017). Por outro lado, a privação androgênica suprime a expressão de KGA (*kidney-type glutaminase*), uma isoforma da GSL-1 dependente de AR, promovendo a expressão de glutaminase C (Xu et al 2021 PNAS), a qual independe de AR. Em células PC3M, linhagem metastática derivada da PC3 em camundongos nude, a glutamina é mais utilizada em comparação à sua isogênica, sugerindo o uso preferencial deste aminoácido na metástase e estágios mais agressivos do CaP (ZACHARIAS et al., 2017). No CaP resistente à terapia, as células dependem da glutamina, sendo que seu metabolismo promove a síntese de pirimidinas favorecendo a sobrevivência celular (XU et al., 2021, 2022). Portanto, estas evidências indicam que, ainda que o AR tenha papel central no metabolismo, a sua regulação pode levar a ajustes metabólicos que favorecem a progressão tumoral e a ocorrência de estágios mais agressivos.

Este conjunto de achados levantam questões quanto à participação do metabolismo mitocondrial na iniciação e progressão tumoral. Essa questão frequentemente apresenta controvérsias devido à heterogeneidade molecular encontrada no CaP. Comparado à condição

normal, este apresenta elevado pleomorfismo mitocondrial (MAO; NAKAO; ANGRIST, 1996), como também aumento na quantidade dessas organelas (GRUPP et al., 2013; PANOV; ORYNBAYEVA, 2013), tanto em amostras de pacientes quanto *in vitro,* de forma que a linhagem LNCaP apresenta 4,6 vezes mais mitocôndrias que as normais, enquanto que na PC3 e DU145, este índice reduz à metade (PANOV; ORYNBAYEVA, 2013). As alterações mitocondriais ainda se estendem à composição da sua membrana pela redução dos níveis de ácido graxos saturados e aumento de cardiolipina (ZICHRI et al., 2021), como também a superexpressão de genes relacionados à biogênese, bioenergética e apoptose (BURCH; RHIM; NYALWIDHE, 2016). Apesar destas alterações estruturais, as diferenças no funcionamento mitocondrial durante a progressão tumoral não estão esclarecidas. Por um lado é possível destacar, independentemente do contexto androgênico, adaptações no TCA na condição tumoral o qual funciona como dois ciclos acoplados, sendo um que converte α-cetoglutarato em oxaloacetato e outro de oxaloacetato a α-cetoglutarato que inclui a reação de síntese de citrato (PANOV; ORYNBAYEVA, 2013). Por outro, a fosforilação oxidativa é remodelada no CaP avançado ocorrendo aumento da oxidação de succinato e redução da capacidade respiratória a partir de glutamato e malato. Interessantemente, os ajustes metabólicos ainda envolvem o diálogo entre componentes epiteliais e estromais, como fibroblastos associados ao câncer (CAFs). Estes induzem a ativação de OXPHOS via liberação de lactato e troca de mitocôndrias com as células tumorais (IPPOLITO et al., 2019). As discrepâncias no número de mitocôndrias entre linhagens responsivas ou não a andrógenos apontam para o envolvimento da dinâmica mitocondrial na progressão tumoral (GRANDEMANGE; HERZIG; MARTINOU, 2009), a qual está associada à reprogramação do metabolismo celular. Sabe-se que a alta taxa de OXPHOS está frequentemente acompanhada de expansão da rede mitocondrial e expressão das proteínas OPA e Mfn2, envolvidas na fusão mitocondrial, enquanto em células com predominância da via glicolítica, observa-se maior expressão de proteínas envolvidas na fissão, como Drp-1, e um fenótipo mais fragmentado das mitocôndrias (ALIROL; MARTINOU, 2006). Alguns autores reportaram que o AR regula a transcrição da Drp-1, de tal forma que sua expressão é maior em células andrógeno-responsivas e menor nas não-responsivas (CHOUDHARY et al., 2011). Contudo, esta relação ainda é controversa, tendo em vista que outros estudos reportaram aumento da fusão e da biogênese mitocondrial (BURCH; RHIM; NYALWIDHE, 2016; PHILLEY et al., 2016), como também comprometimento da CTE associada à progressão tumoral em oposição a estudos que apontam

integridade da OXPHOS (CAINO; ALTIERI, 2016; FANTIN; ST-PIERRE; LEDER, 2006; MORENO-SÁNCHEZ et al., 2007; PHILLEY et al., 2016; WEINHOUSE, 1976). É importante mencionar, ainda, que disfunções na OXPHOS podem elevar a geração de Espécies Reativas de Oxigênio (EROs), que atuam como iniciadores do processo tumoral por estimular a ativação de pro-oncogenes e inibir outros supressores de tumor (ALIROL; MARTINOU, 2006). Em conjunto, estas evidências demonstram que existem alterações metabólicas progressivas na carcinogênese prostática, ainda pouco compreendidas, as quais podem ser moduladas pela via androgênica, inclusive a função mitocondrial.

Estritamente associado às mitocôndrias, o metabolismo de lipídios exibe alterações durante a progressão tumoral prostática (ZADRA; LODA, 2018). Sabe-se que a oxidação de lipídios é uma das principais fontes de ATP para as células tumorais (LIU, 2006), corroborando a hipótese de que, embora a captação de glicose seja alta, seu catabolismo anaeróbico fornece principalmente intermediários para vias anabólicas. Entretanto, a taxa de oxidação de lipídios parece diferir entre células de diferentes contextos androgênicos, tendo em vista que células LNCaP tem maior expressão da carnitina palmitoil-CoA transferase 1 (CPT1), enzima responsável pela entrada de ácidos graxos na mitocôndria, o que ocorre de forma atenuada com a PC3 (SADEGHI; KARAMI-TEHRANI; SALAMI, 2015). Assim como o processo de lipólise, células tumorais prostáticas possuem maior expressão de enzimas lipogênicas, como ATP citrato liase (ACLY), acetil-CoA carboxilase (ACC) (GALBRAITH; LEUNG; AHMAD, 2018) e FASN (GALBRAITH; LEUNG; AHMAD, 2018; PIZER et al., 2001; SINGH; SINGH, 2017; ZADRA et al., 2019). Em especial, a superexpressão de FASN é um evento molecular inicial na carcinogênese da próstata e se torna mais elevada no quadro de metástase e resistência à castração (ZADRA; PHOTOPOULOS; LODA, 2013; ZADRA et al., 2019). Esta enzima é regulada pelo AR e está envolvida na síntese *de novo* de ácidos graxos, apresentando expressão reduzida em células saudáveis (SWINNEN et al., 1997a, 2002). O processo de síntese endógena de lipídios está relacionado à produção de membranas durante a divisão celular, sendo essencial para a progressão no ciclo celular (SCAGLIA et al., 2014). Isto complementa evidências de que a FASN é regulada por vias de proliferação e sobrevivência, como AKT/PI3K/PTEN, e sua maior expressão estimula proliferação celular *in vivo* e *in vitro* enquanto que a sua supressão tem efeito inibitório (MIGITA et al., 2009). Apesar de sua atuação no citosol, a FASN pode apresentar localização nuclear, o que está associada à

agressividade, de forma que sua expressão muito baixa ocorre na hiperplasia benigna prostática (HBP), lesões pré-malignas de alto grau e tumores de baixo grau (MADIGAN et al., 2014). Apesar do seu papel não estar completamente elucidado, a superexpressão de FASN está relacionada à resistência à quimioterapia (WU et al., 2016b) e recentemente tem sido reportada sua co-localização com o AR-V7 em linhagens CRPC (ZADRA et al., 2019).

O metabolismo celular é composto por complexas vias metabólicas que respondem a estímulos (ou ausência deles), o que ocorre por meio de sensores metabólicos. Estes, em parte, são receptores nucleares (NR), os quais constituem uma superfamília de fatores de transcrição cuja estrutura é parcialmente conservada, e regulam elementos responsivos via associação direta ou interação com outras proteínas já associadas ao DNA (WU et al., 2016a). Estes receptores, incluindo o AR, podem atuar por diferentes mecanismos afetando a proliferação, morte celular e vias metabólicas (CHAWLA et al., 2001). Entre os que mais se destacam, estão o ERRα (NR3B1) e ERRγ (NR3B3), ambos relacionados ao ER, e associados à manutenção da homeostase energética, incluindo o metabolismo glicolítico e o mitocondrial (GIGUÈRE, 2008). Na condição tumoral, estes receptores estão diretamente relacionados à progressão e proliferação celular devido à sua participação na reprogramação metabólica, o que parece envolver HIF-1 e PPARGC1A, associado à modulação do metabolismo de lipídios. Além destas, alterações no metabolismo celular podem envolver outros NRs, como a ativação específica do Receptor X Hepático alfa (LXRα), a qual está associada à disfunção e estresse oxidativo mitocondrial (HE et al., 2014). Este receptor também está envolvido na proliferação e morte celular e, na próstata, pode estabelecer *cross-talk* com a via androgênica. O AR participa da regulação metabólica também por estimular a expressão de enzimas lipogênicas (BROWN; GOLDSTEIN, 1997; SWINNEN et al., 1997a, 1997b) via fatores de transcrição como o de Proteínas Ligantes de Elementos Reguladores de Esteróis (SREBPs), os quais são controlados pelo LXRα, essencial para o status proliferativo na próstata (KIM et al., 2009). Em estudos com roedores submetidos à dieta hiperlipídica insaturada, nosso grupo de pesquisa reportou uma associação entre a redução da frequência de células positivas para o LXRα no estroma e AR-positivas epiteliais com hiperplasia prostática, o que indica uma complexa sinalização, ainda pouco estudada, entre estas vias (PYTLOWANCIV et al., 2016) .

O Receptor Ativador de Proliferação do Peroxissomos (PPAR), NR de grande relevância para a próstata, também tem papel chave na reprogramação metabólica durante a carcinogênese. A ativação do PPARγ/α está relacionada à maior expressão de enzimas responsáveis pela entrada de ácidos graxos na mitocôndria, maior atividade mitocondrial, assim

como a biogênese desta organela em células HepG2, o que influencia a proliferação celular (PIEMONTESE et al., 2017). O PPARγ (NR1C3) ainda coordena alterações conformacionais do AR, influenciando no crescimento tumoral e resposta diferencial em células responsivas ou não a andrógenos (SCHAUFELE et al., 2005) e sua maior ativação promove inibição da expressão do Antígeno Específico Prostático (PSA) em células tumorais (HISATAKE et al., 2000). Estudos também têm descrito uma superexpressão da isoforma α em carcinoma prostático com índice Gleason alto, mas células LNCaP reduzem sua expressão em 40% na presença de testosterona, (COLLETT et al., 2000), sugerindo seu papel na progressão tumoral prostática. Estudos conduzidos em nosso laboratório revelaram que em células benignas PNT1A o PPARγ, de grande relevância para a próstata, apresenta disposição predominantemente nuclear (dados não publicados), enquanto em células tumorais sua localização é citoplasmática (LEE et al., 2013). Esses dados, em conjunto com a literatura, mostram sua potencial associação com o processo tumoral, via regulação do metabolismo e da sinalização androgênica.

Este conjunto de dados corroboram o papel fundamental do metabolismo celular no CaP, sendo regulado por uma rede de fatores complexos que inclui a sinalização androgênica e ajustes metabólicos que podem responder diferentemente a estímulos externos via diferentes famílias de NR. Portanto, isto indica que fatores externos podem influenciar a proliferação e sobrevivência celular, oferecendo novas oportunidades de intervenção preventiva e terapêutica.

I.V Lipídios e o Câncer de Próstata

As evidências apresentadas permitem levantar questionamentos sobre o potencial da qualidade da dieta em modular o metabolismo celular e influenciar positiva ou negativamente o desenvolvimento tumoral. Os lipídios são necessários aos seres vivos e o seu consumo tem aumentado na população mundial (MICHA et al., 2014). Existem dados de estudos experimentais *in vivo*, inclusive do nosso grupo de pesquisa, que comprovam seus efeitos no tecido prostático, provocando remodelação estromal e alterações na proliferação/apoptose das células epiteliais, muitas vezes levando ao desenvolvimento de PINs e até mesmo adenocarcinoma (DE JESUS et al., 2015; DI SEBASTIANO; MOURTZAKIS, 2014; PYTLOWANCIV et al., 2016; RIBEIRO et al., 2012a; SILVA et al., 2015; TAMARINDO et al., 2021). É importante a

ressalva de que não somente a quantidade, mas também o tipo de lipídio parece ser relevante. Estudos com ácidos graxos saturados tem mostrado que a dieta rica nestes lipídios estimula a carcinogênese e progressão prostática via reprogramação metabólica por MYC (LABBÉ et al., 2019), além de apresentarem efeitos diferentes dos insaturados, inclusive na expressão do AR (ESCOBAR; GOMES-MARCONDES; CARVALHO, 2009; FURRIEL et al., 2014). Por outro lado, o papel dos ácidos graxos poli-insaturados (PUFAs) na carcinogênese ainda é controverso, tendo em vista que estudos populacionais mostram uma correlação positiva entre sua alta concentração plasmática e a incidência de tumores sólidos, inclusive o prostático (SAUER; DAUCHY; BLASK, 2001; SORONGON-LEGASPI et al., 2013; WANG et al., 2016; ZHAO et al., 2014), o que é contraposto a estudos experimentais que apontam o efeito contrário (FRIEDRICHS et al., 2011; LIANG et al., 2016, 2020; TAMARINDO; GÓES, 2020). Isto indica que lipídios pertencentes à mesma classificação de insaturação podem exercer efeitos diferentes, o que exige cautela na generalização dos seus efeitos e estudos mais aprofundados de ácidos graxos específicos.

Os PUFAs apresentam insaturações na sua molécula e podem ser classificados de acordo com a posição da primeira dupla ligação em relação ao grupo metil terminal do esqueleto de carbono (CALDER, 2016). Entre aqueles que apresentam significativa atividade biológica estão os que possuem a primeira instauração no terceiro ou sexto carbono, denominado de ômega, caracterizando os PUFAS ômega-3 e ômega-6, respectivamente (ANDER et al., 2003; ESER et al., 2013). Esta importância biológica é dada pela incorporação em membranas, mas também atribuída aos seus metabólitos, os quais exercem diferentes efeitos dependendo do contexto molecular, envolvendo propriedades que afetam desde o metabolismo energético a mediadores inflamatórios de forma tecido específico. Devido a esta multiplicidade de funções, diversos estudos têm buscado associações entre tais PUFAs e a carcinogênese prostática. Berquin e colaboradores (BERQUIN et al., 2007) em estudos populacionais reportaram haver uma correlação positiva entre o risco de CaP e o elevado consumo de ácidos graxo ômega-6, cujo perfil nutricional se assemelha à dieta ocidental. Entretanto, análises com populações orientais em que há maior ingestão de ômegas-3, o risco de desenvolvimento do CaP parece ser reduzido, o que também foi associado ao aumento das concentrações séricas destes ácidos graxos. Esta relação é corroborada pela baixa razão ômega-6/ômega-3 que mostrou-se capaz de atrasar a progressão tumoral prostática via indução da apoptose

(APTE et al., 2013). Ainda, outros estudos têm proposto o aumento de processos inflamatórios como um potencial microambiente que favorece a iniciação e progressão do CaP (GUCALP et al., 2017; STARK; LIVAS; KYPRIANOU, 2015; TORIOLA et al., 2013).
Neste sentido, a propriedade anti ou pró-tumoral se deve, também, à ação anti-inflamatória dos ômega-3 e pró-inflamatória dos ômega-6, respectivamente, mas ainda não está estabelecido um consenso (CALDER, 2011). Portanto, este panorama corrobora a relação entre a qualidade nutricional e a carcinogênese prostática, permitindo novas estratégias na prevenção e tratamento.

O Ácido Docosahexaenóico

Entre os ácidos graxos ômega-3 de relevância biológica está o ácido docosahexaenóico (DHA), também conhecido como ácido cervônico, e quimicamente descrito como *cis*-4,7,10,13,16,19-ácido docosahexaenóico, onde os números indicam sucessivamente a posição de cada umas das 6 ligações duplas a partir da extremidade da carboxila (CALDER, 2016) (Fig.6A). Este ácido graxo recebe a nomenclatura C22:6n-3 ou C22:6ω-3, indicando a quantidade de carbonos no esqueleto da molécula, de insaturações e a posição da primeira ligação dupla a partir do grupo metila (carbono ômega, ω), extremidade oposta à carboxila. Devido à sua alta insaturação, o DHA apresenta baixa temperatura de fusão (-44°C) e propriedades biológicas únicas, a serem discutidas adiante (CALDER, 2016). Sua síntese ocorre principalmente a partir do ácido alfa-linolênico (ALA, 18:3n-3), um ácido graxo essencial, o qual passa por uma sequência de elongação e desaturação no retículo endoplasmático e nos peroxissomos até a síntese completa deste ômega-3 (Fig.6B). Este processo encontra-se reduzido em mamíferos devido à baixa expressão ou ausência de enzimas da sua via de biossíntese, sendo menor em indivíduos biologicamente do sexo masculino em comparação ao feminino (GILTAY et al., 2004). Apesar desta condição, este ômega-3 é detectado no músculo cardíaco e esquelético, fígado, hemácias, plaquetas, neutrófilos, tecido adiposo e espermatozoides, sendo sua maior concentração no cérebro e nos olhos (LACOMBE; CHOUINARD-WATKINS; BAZINET, 2018). Assim, sua principal obtenção se dá pela dieta, em especial pelo consumo de animais marinhos de águas frias, em sua maioria peixes, mas também encontrado em quantidades modestas em algas e ovos (CALDER, 2016), além da suplementação alimentar na sua forma concentrada. Semelhante a outros lipídios, após

sua ingestão, o DHA é transportado para a corrente sanguínea como constituinte de lipoproteínas, sendo também encontrado como ácidos graxos não esterificados devido à sua liberação por adipócitos, onde é esterificado como triacilglicerol e éster de colesterol.

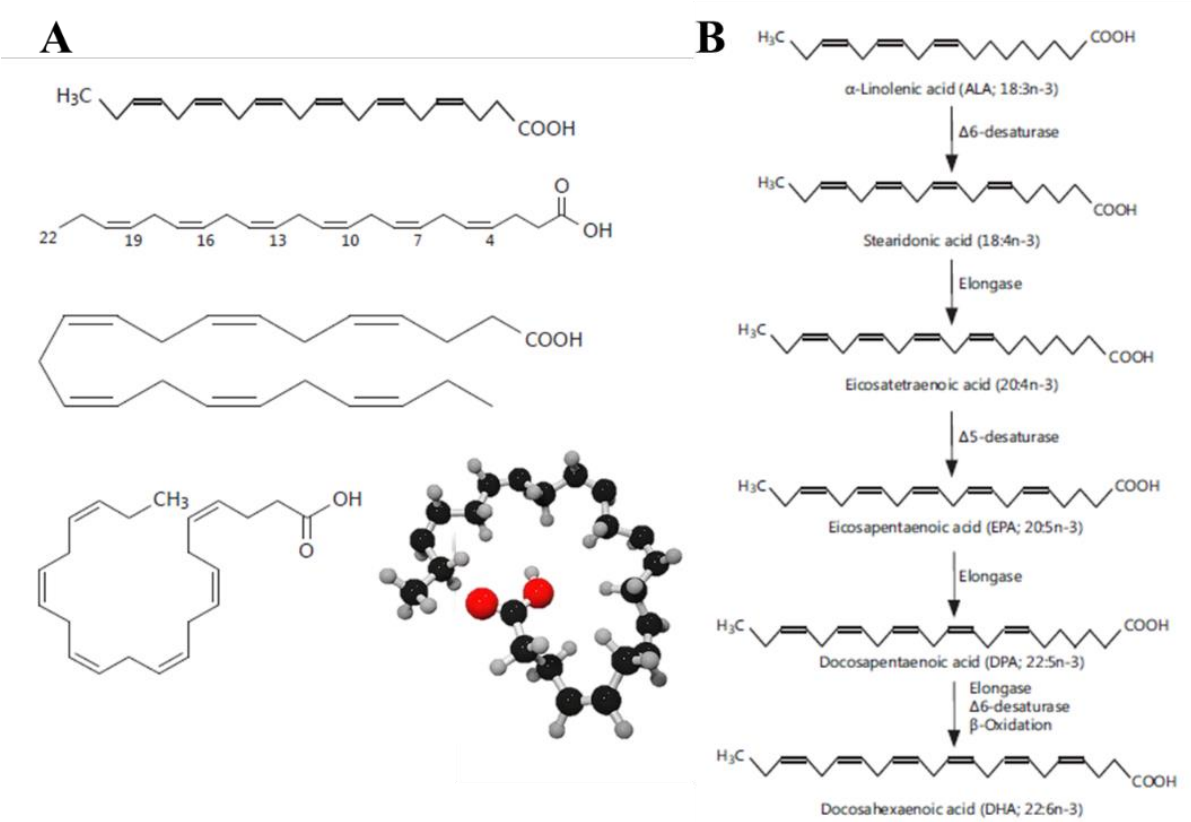

Figura 6. *Estrutura e Síntese do Ácido Docosahexaenóico.* **(A)** Estrutura Molecular em diferentes representações e conformações. Esferas em preto representam átomos de carbono, cinza de hidrogênio e vermelho oxigênio, evidenciando o grupo carboxila em uma das extremidades. **(B)** Via de síntese. (Calder, 2016, modificado).

Nas células, este ômega-3 compõe glicerofosfolipídios em sistemas de membrana, incluindo fosfatidilcolina, fosfatidiletanolamina, fosfatidilserina e fosfatidilinositol, sendo também complexado a outros lipídios (HISHIKAWA et al., 2017). Na membrana celular, em função da sua estrutura única, o aumento da concentração de DHA afeta diretamente propriedades físico-químicas da bicamada lipídica. Devido às suas insaturações, o DHA dificulta a organização destes fosfolipídios na membrana e agregação de proteínas, influenciando diretamente na fluidez, flexibilidade, formação e sinalização de jangadas lipídicas, transmissão de impulsos elétricos em neurônios, como também aumentando a susceptibilidade à oxidação. Entre os seus mecanismos de ação mais conhecidos em condições saudáveis, está a sensibilização de sensores metabólicos, de receptores acoplados à proteína G

(GPRs) e produção de metabólitos bioativos, os quais desencadeiam diferentes respostas celulares a depender do contexto. O DHA é um conhecido ligante não-covalente de PPARs, de forma que pode ativar PPARα e PPARγ ou induzir a sua expressão gênica, cuja relevância para o metabolismo, proliferação e sobrevivência celular já foram anteriormente discutidos. Além destes NRs, o DHA, juntamente com outros ômega-3, pode sensibilizar o receptor de membrana GPR120 (ou FFAR4), o qual atua como transportador de ácidos graxos livres, e ser um dos mecanismos pelo qual o DHA induz alterações metabólicas. Isto porque o knockout para GPR120 não promove o perfil de expressão gênica observada na presença de DHA, em especial da via anti-inflamatória, como também alterações no metabolismo de glicose (CALDER, 2016; OH et al., 2010). Especialmente em macrófagos, esse receptor está relacionado à ação anti-inflamatória do ômega-3, além de ter sido observada a polarização em direção ao tipo 2 via p38 e ativação de padrão específico de citocinas, tais como a maior produção de interleucina 10 (IL-10) e fator beta de crescimento tumoral (TGFβ) (KAWANO et al., 2019). Por fim, o DHA ainda é metabolizado gerando lipídios ativos. Em humanos, as enzimas envolvidas no seu metabolismo são a cicloxigenase 2 (COX2 ou prostaglandina-endoperóxido sintase) (SERHAN et al., 2002), e a 5-, 12- e 15-Lipoxigenase (LOX), as quais produzem hidroperóxidos de DHA que servem como substratos para hidrolases, glutationa-S-transferase (GST) e membros da superfamília do citocromo P450 (SERHAN et al., 2015; SPITE; CLÀRIA; SERHAN, 2014). Os produtos do seu metabolismo envolvem metabólitos oxigenados, conjugados de DHA e proveniente de oxidação enzimática e não-enzimática. Os principais metabólitos gerados a partir do DHA e sua via de síntese estão detalhados na Figura 7. Entre eles, estão as maresinas, protectinas e resolvinas da série D que apresentam relevância biológica devido à sua propriedade anti-inflamatória e cicatrizante, as quais serão melhor discutidas adiante no Capítulo 1.

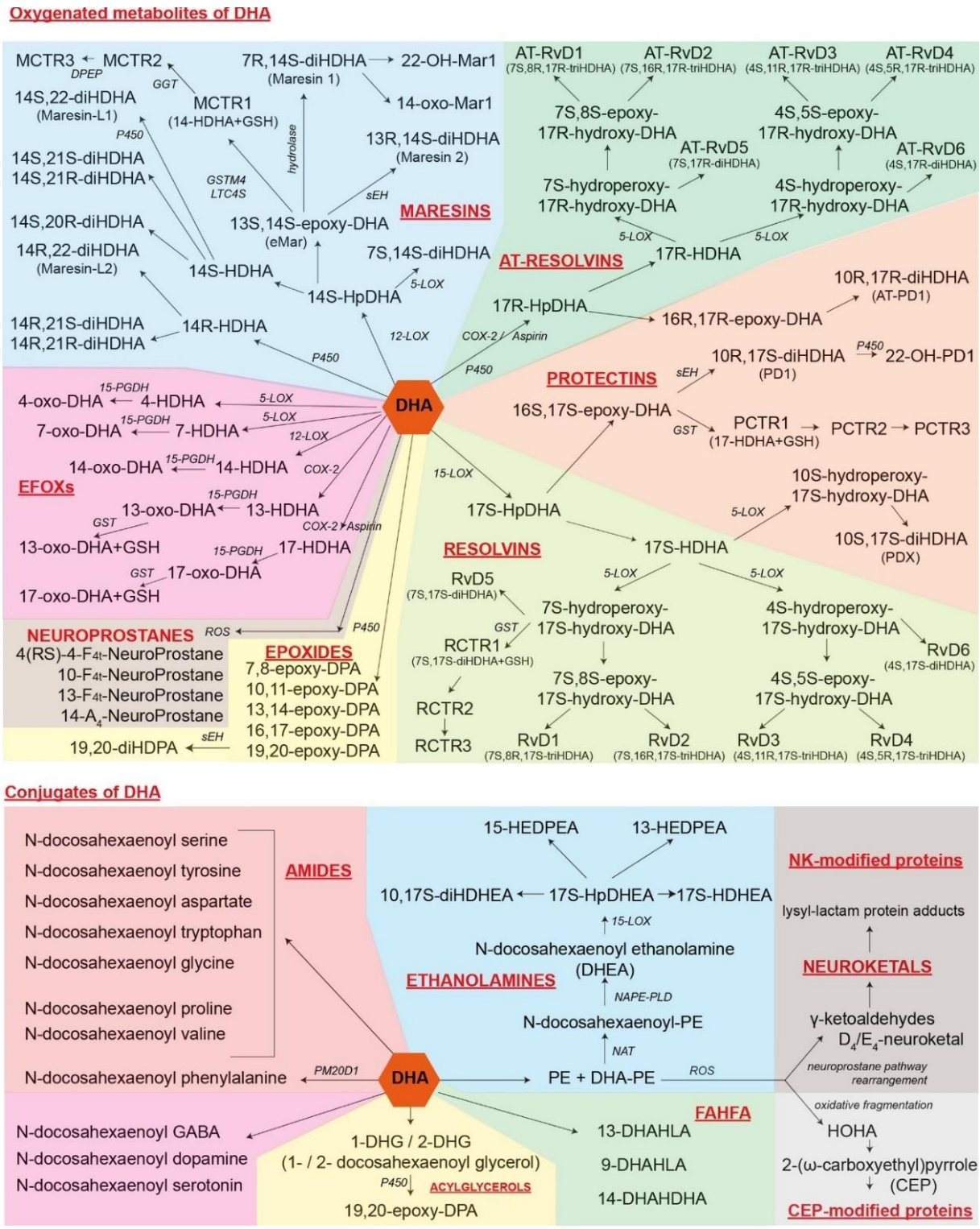

Figura 7. *Metabolismo do ácido docosahexaenóico (DHA).* Esquema representativo dos possíveis produtos bioativos do metabolismo do DHA. Fonte: Kuda 2017.

O consumo deste ômega-3, ainda que a dose e a frequência sejam variáveis na literatura, é recomendado com respaldo de estudos clínicos que mostram normalização do perfil lipídico sérico e menor risco de cardiopatias, melhora funcional do sistema nervoso, inclusive durante a gestação no período de formação do sistema neural. Apesar destes benefícios para a saúde humana, sua propriedade antitumoral tem sido pouco explorada. A primeira publicação associada ao DHA no câncer e indexada no buscador PubMed (NIH) data em 1979, sendo observado um aumento nesta temática a partir dos últimos dez anos com ápice em 2016 (Figura 8). Embora esta tendência indique a contínua busca em compreender o papel deste ômega-3 no câncer, ainda há diversas lacunas a serem preenchidas, em especial o efeito atribuído ao DHA em distinção a outros ácidos graxos, como EPA, como também ensaios clínicos, uma vez que a maioria das evidências ainda são experimentais.

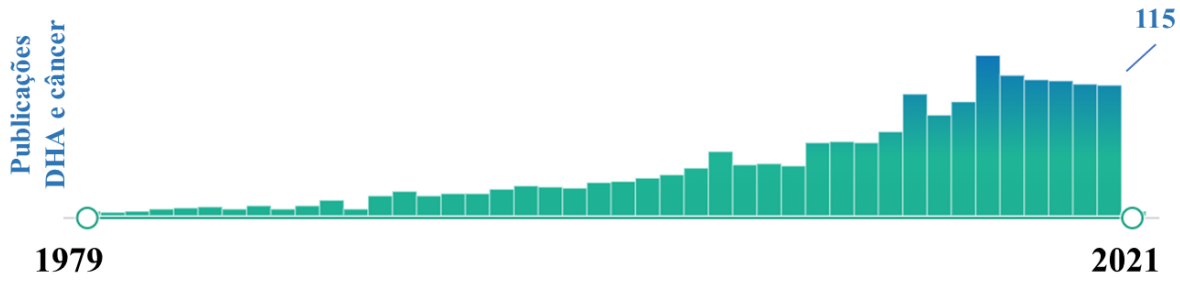

Figura 8. *Cronologia de publicações indexadas na base de dados MEDLINE sobre o tema DHA e câncer.* A primeira publicação data em 1979, sendo observado um aumento nesta temática a partir dos últimos dez anos com ápice em 2016. Fonte: PubMED, acesso em 14 de Dezembro de 2021.

Nestas publicações, entre os tipos tumorais mais estudados está o câncer de mama, no qual o DHA foi capaz de induzir piroptose em células triplo-negativas (PIZATO et al., 2018), modular a atividade de macrófagos associados a tumores (TAMs) via inibição na secreção de citocinas pró-inflamatórias (GIONFRIDDO et al., 2020), exercer função anti-angiogênica (ASLAN et al., 2020), promover efeito pró-apoptótico e parada do ciclo celular (CHÉNAIS et al., 2020; NEWELL; BRUN; FIELD, 2019; XUE et al., 2017), reduzir expressão de genes envolvidos na via de colesterol e de lipídios (CHÉNAIS et al., 2020), prejudicar a bioenergética celular (MOURADIAN et al., 2015), inibir transição epitélio-mesenquimal (SUNG et al., 2020) e metástase (JAVADIAN et al., 2020), inclusive com maior eficiência em comparação ao EPA (RAHMAN et al., 2013). Além do câncer de mama, partes desses efeitos já foi observada em outras neoplasias, como o câncer de

cólon (SKENDER; VACULOVA; HOFMANOVA, 2012), pulmão (BAI et al., 2019; JIANG et al., 2018), ovário (BILYK et al., 2021; WAN; FU; ABABAIKELI, 2016), gástrico (ORTEGA et al., 2021; WU et al., 2012) e de fígado (JUMP et al., 2015). Este conjunto de evidências mostra a versatilidade do DHA como potencial agente terapêutico, mas também a complexidade de seus mecanismos de atuação. Neste contexto, é importante mencionar que o aumento no número de estudos que abordam o DHA em diferentes tipos tumorais se dá pelo baixo custo e tempo reduzido de fabricação, fácil comercialização como suplementação nutricional, além de efeitos colaterais insignificantes em comparação a quimioterapias tradicionais.

Assim como em outros tumores, o DHA tem sido estudado na próstata, conforme detalhado no Capítulo 1. Estudos do nosso grupo de pesquisa que precederam o presente trabalho mostraram que na linhagem pré-maligna PNT1A (TAMARINDO et al., 2019), o ômega-3 reduziu a proliferação celular na incubação de 100µM por 48 horas associada ao acúmulo de lipídio intracelular concomitantemente ao comprometimento da função mitocondrial, indução do estresse oxidativo, em especial a produção do ânion superóxido, como também via regulação de vias de sobrevivência e proliferação celular. É válido mencionar que estes efeitos não foram dependentes do AR, tendo em vista que sua expressão não foi alterada. Estes resultados indicaram que o DHA pode servir como agente preventivo, mas também levantaram novos questionamentos sobre seu potencial antiproliferativo em células prostáticas de estágios distintos da progressão tumoral. Além disso, permitiram observar que este ômega-3 foi capaz de desregular fatores centrais envolvidos na iniciação e progressão do CaP, nomeadamente o metabolismo mitocondrial e de lipídios, os quais não foram investigados até momento na próstata. Desta forma, nesta Tese foi avaliada a hipótese de que o *DHA regula a sobrevivência e proliferação de células tumorais com diferentes contextos androgênicos via modulação do metabolismo celular.*

II. OBJETIVOS

Tendo como pressuposto que o metabolismo tem um papel preponderante, embora ainda pouco compreendido, na carcinogênese prostática, esse estudo teve como objetivo principal avaliar os efeitos do DHA na sua modulação durante a progressão tumoral e as implicações para a proliferação e sobrevivência celular. Para isso, foram estabelecidos os seguintes objetivos específicos:

1) Estabelecer as condições em que o DHA exerce seu efeito antitumoral e os impactos sobre a modulação do ciclo celular, tanto em células epiteliais prostáticas humanas pré-malignas como em tumorais responsivas a andrógenos e resistentes à castração;
2) Avaliar os efeitos do DHA na regulação de genes envolvidos no metabolismo e na via androgênica;
3) Testar se o DHA induz alterações em parâmetros mitocondriais (função, produção de EROs, morfologia e expressão gênica) e morte celular;
4) Avaliar como o DHA afeta o metabolismo de lipídios;
5) Validar *in vivo* sua a propriedade antitumoral.

III. RESULTADOS

Os resultados foram apresentados em quatro capítulos:

Capítulo 1 – *Separating the tares from the wheat: differential effects of docosahexaenoic acid among the others omega-3 in prostate cancer.*

Capítulo 2 – *Docosahexaenoic acid differentially modulates the cell cycle and metabolism-related genes in tumor and pre-malignant prostate cells.*

Capítulo 3 – *Mitochondria is a potential target of docosahexaenoic acid in pre-malignant and prostate cancer cells.*

Capítulo 4 – *Docosahexaenoic acid deregulates lipid metabolism and decreases proliferation of androgen sensitive and castrated-resistant prostate cancer phenotypes.*

CAPÍTULO 1

Separating the tares from the wheat: differential effects of docosahexaenoic acid among the others omega-3 in prostate cancer

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ABSTRACT

The polyunsaturated fatty acids of the omega-3 class have been widely investigated due to its antitumor properties, including in the prostate cancer (PCa). Among them, is docosahexaenoic acid (DHA, C22:*n*-3) which biological activity is higher than others omegas-3, exhibiting stronger impact on PCa cell proliferation. Most studies has focused on general omega-3 effects which may blur the specific mechanisms triggered either by eicosapentanoic acid (EPA, C20:*n*-3) or DHA, delaying the understating of their biological role hence alternative therapeutic approaches. DHA is differentially processed between normal and malignant epithelial PCa cells and exert its effects by triggering several signaling pathways, which often occurs simultaneously and are not independent. At cell-specific level, it downregulates two key pathways in PCa, the androgen signaling and lipid metabolism, but also changes in membrane composition by disrupting phospholipids balance and increasing unsaturation status, arrests the cell cycle, induces apoptosis and ROS overproduction. At tissue level, DHA seems to influence stromal components, such as inhibition of cancer-associated fibroblasts differentiation, and to promote the resolution of inflammation which generates a microenvironment often favorable to PCa initiation and progression. Considering that such effects are commonly misunderstood and assigned to omega-3 in a distorted general manner, the aim of this review was to discuss the specific effects of DHA on PCa based on evidence retrieved from *in vitro* and *in vivo* experiments.

Key words: prostate cancer; PUFAs; lipids; DHA, omega-3; fish-oil.

INTRODUCTION

In the last decade, the polyunsaturated fatty acids (PUFAs) belonging to the omega-3 class have been in the spotlight due to their properties in several healthy conditions, such as brain function improvement [1], serum lipid profile normalization [2], liver injury mitigation [3], control of cardiac pathologies [4] and suppression of tumor growth in different tissues [5–9]. These highly unsaturated long-chain fatty acids have the first double bond located on the third carbon (omega), being the first (alpha) in the carboxyl group [10]. The most abundant omega-3 is the alpha-linolenic acid (ALA, C18: 3ω−3), found in edible plants, particularly in some seeds, being the precursor for longer and more unsaturated fatty acids such as eicosatetraenoic acid (EPA, C20:5ω -3) and the docosahexaenoic acid (DHA, C22:6ω -3) [11]. This conversion is very low in mammals, including humans and especially in men [10,12], becoming the diet their main source through the intake of marine cold fishes, eggs, algae or nutritional supplementation [10,13]. Such condition imposes that omegas-3 are mostly obtained as a mixture and not as isolated compounds, reflecting many experimental and epidemiological studies that investigated DHA and EPA together, even though the ratio were emphasized [14–16]. Despite of these fatty acids' beneficial effects, raising evidence has shown that each of the omega-3 seems to play different roles in health and disease, becoming difficult to determine their individual contribution on a given outcome.

Compared to EPA, DHA has many distinct effects in health, such as on heart function, protein synthesis, inflammation, lipoprotein metabolism, hemodynamics, vascular function and insulin signaling [17,18]. In human body, DHA is found mostly in cell membranes and is crucial to many organs' function, including heart, eyes, skeletal muscles, testis and liver [19,2011,21]. Specially in the brain, DHA is required for its optimum development, featuring 10-20% of brain total fatty acids and 90% of its omega-3 content, which is closely related with synapses functioning and transmission of electric pulses [20]. In the male reproductive system there is a specific DHA requirement in the testis for normal spermatogenesis and it is essential for sperm motility [22] due to acrosome biogenesis via modulation of protein traffic from Golgi complex, necessary for proacrosomal granule fusion [23]. However, DHA function in other tissues, such as prostate gland under healthy and pathological conditions is not fully elucidated. In prostate cancer (PCa), DHA has been suggested to exert a protective effect, given that normal cells exhibit increased concentration of the omega-3 compared to malignant [24]. This is in line with studies focusing DHA isolated function to better understand the underlying mechanisms

behind cell proliferation inhibition in a broad spectrum of PCa stages compared to others omega-3 [24,25]. This is of particular interest since PCa is among the five leading causes of death in men worldwide [26] and the available therapies may lead to recurrence to a more aggressive stages, such as the castration resistant phenotype [27], a scenario that urges for alternative approaches. Therefore, in light of the growing evidence on DHA antitumor effects and its unclear role, we aimed to review its specific associations with PCa, retrieved from epidemiologic and experimental studies performed in both cell culture and animal models.

OVERVIEW OF MOLECULAR FEATURES, METABOLISM AND SOURCES

DHA, rarely referred as cervonic acid, is formally known as *cis*-4,7,10,13,16,19-docosahexaenoic acid and has 22 carbon chain with 6 *cis* double bonds starting at the third omega carbon [10]. Despite it has been considered an essential fatty acid in the past, DHA may be synthesized from ALA at low rates in humans through elongations and desaturations that occur in the endoplasmic reticulum and peroxisomes [10,12,20]. This is supported by evidence that increased ALA intake did not exert strong effect on DHA serum levels, corroborating that its main source is from diet [20,28]. Once ingested, DHA is processed by the gastrointestinal tract and carried out to the bloodstream as a component of lipoproteins, being accumulated into adipocytes or used by other tissues, as already mentioned [10]. In the cells, DHA can enter through GPR120, CD36 and Mfsd2a, a protein G-coupled protein, a translocase and a sodium-dependent transporter, respectively, but others have been also investigated [29–34]. Specially in GPR120, it triggers several downstream signaling, including glucose uptake, inhibition of TLR2/3/4 and TNF-α pro-inflammatory cascade in addition to TAK1 through a β-arrestin2/TAB1 [31,32]. DHA is also a known ligand of the peroxisome proliferator-activated receptors (PPARs) which are transcription factors that regulate cell proliferation, apoptosis, adipogenesis, lipid metabolism enzymes, mitochondria biogenesis and function, and other metabolism-related genes [35–38]. Moreover, DHA is incorporated into glycerophospholipids, such as phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in addition to alter membranes systems, including mitochondria and endoplasmic reticulum [39,40].

Although this review did not aim to discuss the PUFAs and DHA metabolism itself since it has already been done [41,42], is important to mention that derivatives from DHA are formed via enzymatic pathways that oxidize it into mono-, di- and tri-hydroxy-DHA, epoxy-

and oxo-DHA or modify it by a free radical non-enzymatic mechanism to neuroprostanes [41]. DHA catabolism is ruled out by cyclooxynage 2 (COX2) as well as 5-, 12- and 15-lipoxygenase (LOX) forming hydroperoxides that serve as substrates for hydrolases, glutathione S-transferase and members of cytochrome P450 superfamily (epoxydases, ω-hydrolases) [10,43–45]. Also, its metabolites degradation may be mediated by oxidation, conjugation with glutathione (GSH) or hydrolysis by fatty acid amide hydrolase as well as epoxide hydrolase [45–47]. DHA metabolism involves formation of several potent signaling lipids, as maresins, protectins, epoxides, neuroprostanes related to biological outcomes like antiproliferative, inflammatory modulation, lipoperoxidation, cardiac function and antitumor [41]. Indeed, DHA has many other mechanisms described in literature (Fig. 1) which varies according to cell type and between normal and pathological conditions. This states that this is a complex fatty acid which has gotten recent attention and we will limit to discuss the mechanisms reported only in the prostate.

DHA ASSOCIATION WITH PCa RISK: EPIDEMIOLOGIC FINDINGS

Despite of raising evidence concerning the DHA antiproliferative property, studies have suggested its increase in serum levels with high risk of PCa while others reported this correlation as negative or at least inconsistent. On one hand, elevated DHA levels were observed in lipid extract from red blood cells from men undergoing to radical prostatectomy greater than 35 years old, Gleason sum ≥6, clinical, ≥T1c and PSA ≥ 4ng/ml and free of 5-alpha reductase inhibitors treatment, suggesting an association with high-grade PCa [48]. Such association was also described in men from the SELECT Trail [49] as well as patients from the Prostate Cancer Prevention Trial (55–84 years old) which showed a positive, but not linear, association between the DHA levels in the serum and the risk of high-grade PCa [50]. In a meta-analysis study, it was reported a non-linear positive association of non-aggressive PCa risk with DHA, but not the aggressive phenotype which was linked to EPA [51]. The serum DHA levels were positively correlated to PSA, but when combined to others fatty acids may be associated to increase or decrease of tumor volume, as linoleic acid and arachidonic acid, respectively [52]. However, the association between serum DHA or total omega-3 and PSA was either nonexistent or quite weak in another clinical study [53]. Although epidemiologic and systemic analyses have indicated a positive association between increased DHA levels and PCa risk, this is grossly inconsistent in literature and need careful interpretations [54]. On the opposite side, Chua and colleagues [55] did not find association between DHA intake and PCa risk in their meta-analysis

from clinical studies. Norrish and colleagues [56] reported high DHA levels in erythrocyte phosphatidylcholine associated to PCa risk whereas the inverse correlation was described by Chavarro and colleagues [57]. Evidence from study with Jamaican men which showed one of the highest PCa incidence in the world, also revealed the DHA tumor suppressor action [52]. In a meta-analysis study, Brouwer [58] did not find a strong association of DHA and PCa risk. The lack of concordance among literature regarding the DHA link to PCa may rely on different analytical approaches, as small number of assessed samples, technical limitation on fatty acid detection, and biased interpretations due to patient background like lifestyle, nutritional profile and others. Moreover, the epidemiological data that suggests a positive association between high levels of serum DHA and PCa does not reflect most of *in vitro* and *in vivo* studies that support the antiproliferative role of this omega-3 or even its ability to serve as co-adjuvant in chemotherapy, as further discussed. Therefore, DHA role defined from epidemiologic evidence on prostate carcinogenesis remains inconclusive and still an open question that require refinement and appropriate analysis.

ANDROGEN SIGNALING

Prostate is an accessory gland of male reproductive tract regulated by steroid hormones, mainly androgens, through specific receptors that under normal conditions control proliferation, cell death, metabolism and secretory function [59,60]. This regulation by androgens is a pitfall that has driven therapeutic strategies in PCa [61,62]. In this line, DHA has been reported to affect androgen receptor signaling via different mechanisms. Mitsuhashi and colleagues [63] published that unsaturated fatty acids, including DHA, decreased binding to the synthetic androgen R1881. Such regulation was further supported by evidence that the omega-3 repressed androgen-regulated gene expression as *PSA, ODC, TMPRSS2, NKX3-1* and *FKBP51* [64]. In the same study, the authors showed that DHA decreased cell proliferation associated to proteasome-mediated AR degradation in androgen-responsive LNCaP cells whereas unchanged *AR* gene transcription. This is in line with previous report that DHA prevented PCa progression to androgen independent stage due to AR expression decrease associated to AKT/mTOR modulation [65]. AR regulation by DHA was also described for RWPE-1 benign cells that showed decreased proliferation linked to AR and estrogen receptor alpha (ERα) reduction [66]. However, its effects at early stages of PCa are controversial since others have reported unchanged alterations on AR expression, such as the pre-malignant PNT1A cell line [67]. It is important to

mention that other mechanisms might be triggered by DHA since its antiproliferative effect is observed also in androgen-independent cells that do not produce AR (PC3) [9], suggesting that AR signaling depletion is not the major DHA mechanism in PCa.

DHA effects on androgen signaling were elucidated based on *in vitro* studies. Up to date, there was any study concerning DHA free of additional fatty acids, since its main source is fish oil-derived supplements composed by a combination of lipids. Therefore, animal investigations did not isolate the influence of DHA but consider its combination to EPA at different ratios. This experimental approach is tricky because on one hand is closer to human nutritional scenario, since those fatty acids are not ingested alone, but on the other does not allow elucidate the isolated role of each omega-3 in PCa. Omega-3 consumption (5%) reduced estradiol, testosterone and AR levels, promoting apoptosis and suppressing cell proliferation in C3(1)Tag mice [68]. Also, administration of seal oil (mixtures of 12% DHA; 18% EPA) to rats decreased benign prostatic hyperplasia (BPH) and AR expression in tissue which was related to DHA effect also observed in assays with RWPE-1 cells [66]. The AR degradation mediated by proteasome activity was also observed in castrated-resistant PTEN-null PCa mouse models [69]. Results from our research group also showed that DHA-enriched diet delayed PCa progression at early stages by decreasing high grade lesions, proliferation rate and frequency of epithelial AR-positive cells in TRAMP mice (data not published). It was not our aim to review the omega-3 effects themselves in PCa, but the findings described above are similar to the *in vitro*, suggesting a specific DHA property.

Taken together, the literature supports androgen pathway suppression by DHA at different conditions which are also observed in animal studies with omega-3 administration, even though the experimental design was not appropriate to solid conclude the DHA contribution. In addition, since DHA also decreased cell proliferation and invasion of AR-negative cells, it is reasonable to suggest that androgen signaling is not the major mechanism of this fatty acid but additive in the AR-positive context instead.

NUCLEAR RECEPTORS

The well-known association between DHA and nuclear receptors, including AR, is on the PPARs signaling. This omega-3 is a natural ligand of PPARγ. PPARδ and PPARα by which it triggers oligodendrocyte differentiation [70], pro-apoptotic signaling [71–73], decrease cell

invasion [74] and attenuation of inflammation [75,76] in different cell types. In PCa, the role of PPARs is still under debate, mainly the PPARγ because it may either favor PCa or act as a suppressor. At early stages, PPARγ overexpression was associated with the gland protection against cancer [77] whereas in advanced stages it contributes to *de novo* lipogenesis [78] which is one of the key pathways on PCa carcinogenesis [79,80]. O'Flaherty and colleagues [81] showed that DHA itself or its 15-LOX-metabolites elicited pro-apoptotic effect on PC3, LNCaP, and DU145 cells through PPARγ/syndecan-1 axis. Recently, we have reported that in both pre-malignant and tumor cells, regardless of their androgen status (responsive or castrated-resistant either AR-positive or AR-negative), DHA was able to modulate nuclear receptors and their co-regulators expression at 100μM incubated for 48 hours [9]. These findings also suggested that the DHA ability to modulate such nuclear receptors expression decreases along PCa progression because, in pre-malignant PNT1A, a higher number of regulated genes was found compared to castrated-resistant 22rv1 and PC3, being the latter the less responsive. In AR- and PTEN-positive cells such omega-3 decreased *PPARG1A* and *PPARG1B* (PNT1A and 22rv1, respectively) whereas upregulated *PPARG1A* in AR- and PTEN-negative [9]. In addition, it increased *PPARG* in pre-malignant but not in tumor cells [9]. Lack of *PPARG1A* and *PPARG1B* was related to tumor suppression due to lipid and mitochondrial metabolism impairment and *PPARG* upregulation in benign conditions was linked to its protective function [77]. DHA is known to bound endogenously to other nuclear receptors such as retinoid X receptor (RXR) and liver X receptor (LXR) [82–84], but the modulation of signaling pathways of these receptors by DHA in normal and cancer condition is largely unknown in the prostate, representing an opportunity for further exploration and therapeutic strategies.

METABOLISM IN PROSTATE CANCER AND DHA

PCa has complex etiology with contributions of genetic background as well as environmental factors, food consumption included [85,86]. In the past decades, several studies revealed the role of high-fat diet in PCa initiation and progression which pointed diabetes and obesity as potential risk factors [86–92]. In this context, investigators have observed that not only the amount but also the type of lipids ingested may exert different effects on the prostate [93]. This is of particular interest for PCa, since metabolic reprogramming is tightly related to neoplastic transformation, mainly the lipid metabolism [79]. The lipid synthesis, also known as *de novo* lipogenesis (DNL), is the process whereby cells synthesize fatty acids from acetyl-CoA

exported from mitochondria and is an early hallmark of prostate carcinogenesis [79], often due to fatty acid synthase (FASN) overexpression [94,95]. The increased fatty acid synthesis is required by several cell functions, mainly membrane production [96], cell cycle progression [96] and intracellular signaling [97]. In PCa, lipogenic enzyme genes (such as FASN; Acetil-CoA, Carboxilase, ACC; ATP citrate lyase, ACLY) have their expression regulated by AR via Sterol Regulatory-Element Binding Protein (SREBP) [98,99]. Moreover, FASN was reported to co-localize with AR-V7 [80], a variant of AR linked to resistance to castration [100], and lipogenesis inhibition decreased cell and tumor growth [80]. In addition to their increased synthesis, fatty acids are also rapidly oxidized compared to glucose in PCa and palmitate uptake is approximately 20 times higher than glucose, being the β-oxidation the main source of energy in PCa [101,102]. This is supported by evidence of its block by Etomoxir, an inhibitor of long-chain fatty acids transport into mitochondria via carnitine palmitoyltransferase 1 (CPT-1) that led to cell death [101]. Interestingly, lipid uptake is controlled by androgens reflecting in the different landscapes of fatty acid membrane transporters among the androgenic backgrounds found in PCa [103]. Androgens enhanced lipid uptake in AR-positive cells, especially fatty acids, cholesterol and low-density lipoprotein particles [103]. Taken together, this collective evidence indicates that molecular adjustments in lipid metabolism occur and are crucial for PCa, being fatty acids differentially used in each stage of disease progression. Furthermore, these findings also showed that lipid metabolism may be the Achilles heel in the PCa and have been explored as therapeutic approach [104].

Given that PCa cells can uptake DHA, is reasonable to assume lipid deregulation at some extent. DHA incubation affected palmitic acid uptake in PCa which is essential to support the lipid metabolism in tumor [105] and may be one of the multiple mechanism whereby such omega-3 decreases cell proliferation. It also downregulated lipogenic genes such as *FASN*, *P-SREB*P and *M-SREBP* in breast [106] and prostate cancer cells [24]. Findings from our research group revealed that DHA at 100μM after 48h decreased proliferation of PNT1A, 22rv1 and PC3 cells associated to lipid accumulation, ROS overexpression, mitochondrial damage and cell cycle arrest [9]. These effects were followed by regulation of several genes related with metabolism, response to lipids, stress and hormones but in distinct expression pattern according to androgenic background and pathological stage [9]. Both androgen responsive and castrated-resistant AR-positive cells showed DHA accumulation into lipid droplets, mostly as triacylglycerol and cholesterol esters at low ratio, suggesting its structural function (data not published). In the same study, the omega-3 downregulated the gene activation by SREBPs,

which reflected on reduced expression of FASN. These findings were validated by decreased DNL from glucose without compensation by palmitate uptake. DHA not only deregulated lipid metabolism but also increased glucose complete oxidation, suggesting a metabolic switch from lipid toward glucose metabolism (data not published). Importantly, these DHA effects on DNL were not due to androgen signaling even though AR expression decreased. This is a very promising outcome because the omega-3 effects on one of the hallmarks of PCa does not rely on AR, a target in current therapeutic strategies that often lead to tumor recurrency. Despite of this effects, DNL from glucose increased in PC3 and DU145, even though cell proliferation decreased, showing that DHA metabolism in AR-negative cells differs from the positive, mainly on these neuroendocrine-like lineages (data not published). A study with PC3 sub-population expressing markers of epithelial-mesenchymal-transition (EMT) showed that cells with high invasiveness and low metastatic potential have DHA accumulation associated with decreased proliferation rate whereas others with increased proliferation, low invasiveness and high metastatic potential did not [107]. This difference was related with increase of CPT-1 expression as well as β-oxidation, probably a mechanism to supply cell demands and eliminate antiproliferative molecules, like DHA. Taken together, this body of evidence suggests that DHA may assume different fates according to the molecular context, mainly the androgenic. Most importantly, regardless of the background DHA was able to decrease cell proliferation but seems that in AR-positive cells DNL might have a stronger impact whereas other pathways are triggered in AR-negative, being required further investigation to better clarify this issue.

As lipid metabolism, mitochondria are altered in PCa. It shows increased pleomorphism [108], yield [109,110], bioenergetics and biogenesis gene overexpression [111], tricarboxylic acid adjustments [109] and remodeling of oxidative phosphorylation (OXPHOS) [112], being also related with progression to androgen independency [113]. Therefore, mitochondria has been investigated as a potential target in prostate but also in many other cancers [114,115]. In an *in vitro* study, DHA exerted preventive property in pre-malignant epithelial prostate cells PNT1A via bioenergetics modulation [67]. It increased mitochondria area and impaired the bioenergetic reserve capacity, enhancing the antiproliferative effect of melatonin [67]. In addition, DHA induced membrane hyperpolarization, superoxide anion overproduction, dysregulation of mitochondria biogenesis protein expression, downregulated plenty of mitochondrial genes related with transport, organization and homeostasis, changed the organelle membrane composition and triggered cell death (data not published). The ability to reduce the cell ability to respond to further stress (also known as bioenergetic reserve capacity or spare capacity) via

mitochondria dysfunction is of particular interest in cancer because might be a strategy to sensitize cells to additional compounds. Indeed, DHA was reported to improve the antiproliferative effects of chemotherapeutic compounds in several cancers [116–118]. Such mitochondria impairment and decrease in bioenergetic reserve capacity was also observed in castrated-resistant cells 22rv1 and PC3, associated with depletion of ATP production, increase in superoxide anion production, membrane hyperpolarization and decrease in glycolytic reserve (data not published).

ANTITUMOR MECHANISMS BEYOND ANDROGEN SIGNALING AND METABOLISM REGULATION

Regardless of the cancer type, most evidence of DHA antitumoral property was described in cell culture in a range of time of incubation (24h and 48h) and concentrations belonging to the micromolar scale. In PCa, DHA at 100µM incubated at 48h decreased cell growth through cell cycle modulation whereby in PNT1A and 22rv1 it decreased S-phase whereas in PC3 it arrested in G2/M [9], pointing out that the mechanisms behind its antiproliferative property are stage-dependent. Regarding the AR-negative cells, DHA was reported to inhibit proliferation and survival pathways, as AKT/mTOR mediated by ROS inducing apoptosis in PC3 and DU145, both metastatic cells expressing mutant p53 [119]. Sun and colleagues [120] reported in DU145 that at 50µmol/L for 24h, DHA modulated several apoptosis-related genes involved in p53 pathway as well as TNF signaling, AKT and mitochondrial-related pathway, suggesting the modulation of the both extrinsic and intrinsic apoptosis. The DHA-induced apoptosis in both PC3 and DU145 was also related with YAP phosphorylation and cytoplasm translocation via the FFAR1/FFAR4-Gαs-PKA-Hippo pathways[33]. In the same study this was not observed for androgen-sensitive LNCaP cells which strengthens the different mechanisms triggered by this omega-3 in different molecular contexts. However, authors reported that DHA selectively inhibited AKT^{T308} but not AKT^{S473} phosphorylation, altered PIP3 and AKT protein localization and induced apoptosis through AKT downstream BAD inhibition regardless of the androgenic background [121]. In addition to apoptosis induction, DHA at different concentrations led to decreased migration, invasiveness and proliferation of PC3 cells [122].

The differences in naturally occurring DHA in prostate cells are also one of the possible mechanisms whereby the omega-3 supplementation may either suppress proliferation or trigger apoptosis at distinct levels among them. Without DHA supplementation in the medium, RWPE-1 benign cells naturally exhibit its intracellular levels higher than those found in malignant PC3, 2.58 ± 0.49 and 0.71 ± 0.03%, respectively [25]. Once supplemented in the medium, DHA levels increase in both non-tumor and PCa cells fatty acid milieu [25] and can change the *sn*-2 position of the glycerol backbone, thereby changing the species of phospholipids [121]. PUFAs are known to induce membrane lipoperoxidation due to their highly unsaturation levels which lead to two scenarios. First, benign cells have low tolerance for PUFAs, DHA included, because they already have their own pool in addition to show a glycolytic phenotype over fatty acid oxidation. Since DHA is incorporated into phospholipids, its supplementation may disrupt lipid membrane status by increasing unsaturation hence susceptibility to oxidative stress. This would be in line with studies showing the stronger effect of DHA on pre-malignant cells growth [9,67]. Secondly, PCa cells uptake fatty acids differently along disease progression, as previously discussed, and already have increased oxidative stress [123,124]. Therefore, is reasonable to consider that PCa cells avoid PUFAs which may sensitize them to oxidative damage. Indeed, DHA has been shown to increase sensitivity to oxidative stress involving NFKβ [125] and this matches observations that prostate cells accumulate DHA into lipid droplets which would serve as a buffer (data not published). The lower DHA concentration can also be an indication that such fatty acid is a tumor suppressor, given its higher levels at benign stage and lower in PCa. Therefore, we cannot discard any hypothesis and further studies are required to better elucidate this issue.

Among the main features associated with PCa are stromal alterations which have been widely related with carcinogenesis. High-fat diet is investigated as a relevant factor that engages PCa initiation or promotes its progression due to generation of a favorable microenvironment [87–92]. However, a body of evidence showed that omega-3 fatty acids may exert the opposite effect [126–128], suppressing tumor growth instead. Regarding DHA, it inhibited the differentiation of fibroblasts into myofibroblasts, the active form of the former and usually referred as cancer-associated fibroblasts (CAFs), which decreased PC3 cells invasiveness [129]. In addition, DHA is known by its anti-inflammatory function hence antitumor mechanism given that increase in inflammation is frequently linked to PCa development [130,131]. Its anti-inflammatory effect is mainly produced along its oxygenation and, in PCa, resolvins from class D (RvD1, RvD2, RvD3 and RvD4) have caught attention. RvD1 and RvD2 inhibited tumor-

associated macrophages (TAMs) and macrophages polarization into M1 whereas stimulated M2a, a phenotype that attenuate inflammation [132].

The mechanisms underlying the DHA antitumor effects have been investigated in combination with other compounds to potentiate the outcome. In cells, DHA was reported to amplify the melatonin antiproliferative effect in pre-malignant cells [67]. Also, it synergistically enhanced the cytotoxic effect of docetaxel in PCa cells through increased apoptosis by suppression of genes involved in the NFkβ pathway [133]. In animal models, DHA-only experiments are scarce due to its availability as a mixture with EPA. However, the omega-3 administration was shown to sensitize several tumors like breast, lung, prostate and lymphoma to different chemotherapeutic agents [67,134]. Clinical studies that assessed chemotherapy combined to fish oil administration (2.2 g EPA + 240 mg DHA or 2.2 g EPA + 500 mg DHA/day during 4 cycles) revealed that it may improve the outcome in advanced non-small cell lung cancer, including survival rate [135]. Regarding DHA administration only, this omega-3 at 1.8 g/day previous to chemotherapy (1-7 days) and along it (5 months) increased its plasma levels associated to improved outcome and overall survival in metastatic breast cancer patients [136]. It is important also to mention that these studies confirmed non-toxic effects of DHA or fish oil administration [135,136]. As the chemotherapeutic agent, DHA is systematically distributed when administrated, but its selective action in tumors rather normal cells was reported and revised [134]. Despite evidence has suggested its safety, there is a lack of evidence concerning PCa and required further investigations.

CONCLUDING REMARKS

Taken together, we concluded that in PCa DHA has antiproliferative function and may trigger a plethora of mechanisms which cannot be considered alone but seem to work in an orchestrated manner towards the decrease of cell or tumor growth (Fig. 2). The underlying mechanisms include oxidative stress induction, metabolism deregulation, stromal alterations, and changes in membrane composition. Specially, the androgenic signaling, which is often a target in the current therapies, seems to be additive being a promising outcome since its depletion may lead to recurrence in a more aggressive phenotype. Moreover, it is a PUFA with accessible cost obtained through diet or nutritional supplementation with insignificant side effects compared with conventional therapy, being often recommended due to its health benefits

in cancer-free patients. In this context, even if not fully efficient by itself in suppress tumor growth, DHA has shown to improve chemotherapy as seen by the effects of the omega-3 mixture in rodents. Although its promising effects, one limitation for solid conclusions on PCa was the scarce *in vivo* evidence of DHA-only experiments which must be addressed in future investigations to assign its specific effects.

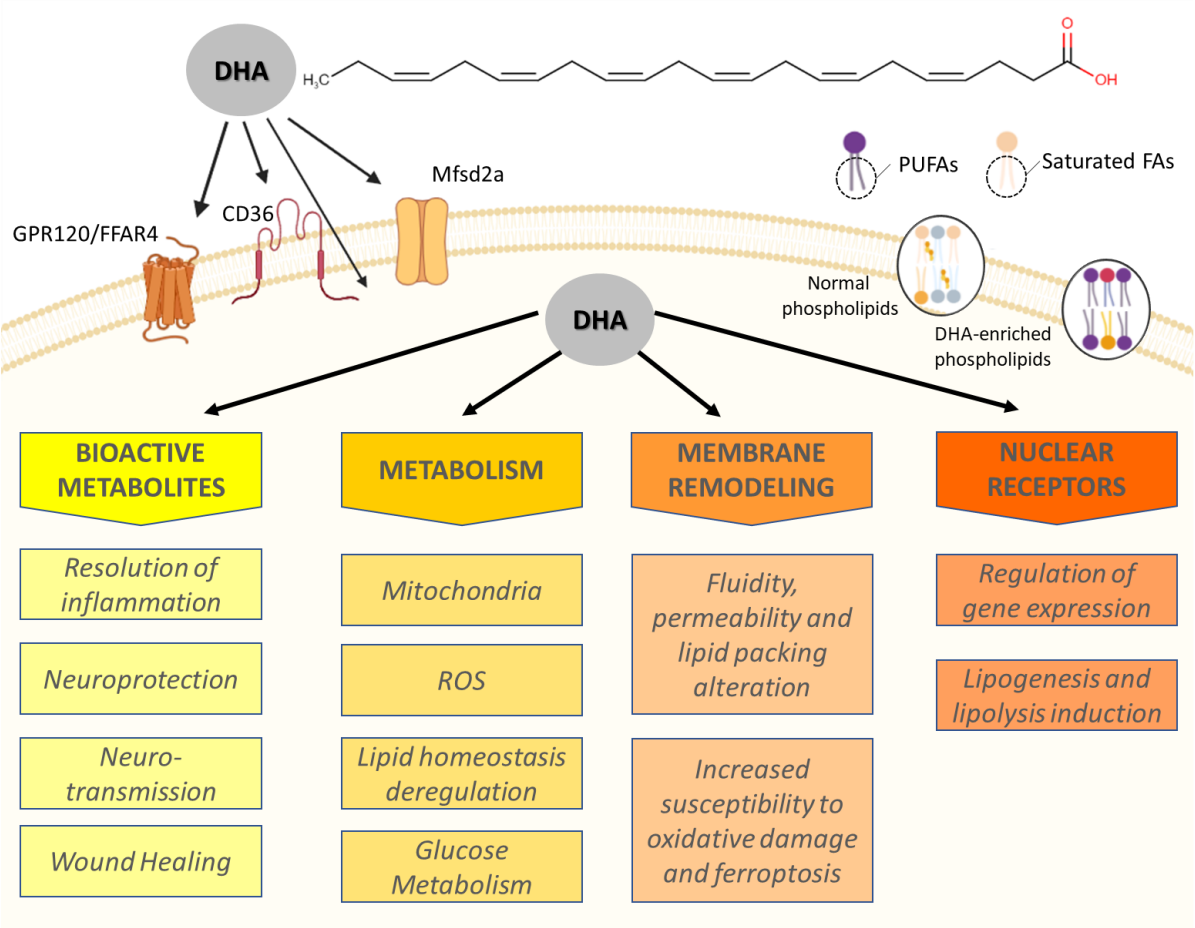

Figure 1. *DHA general mechanisms*. DHA may enter into the cells by diffusion or through membrane receptors GPR120/FFAR4, CD36 and Mfsd2a. Once inside the cells, the omega-3 is likely to trigger downstream biological events, affecting many pathways whose outcome depends on the cell type, its molecular context and DHA concentration. Legend: DHA – docosahexaenoic acid; ROS – Reactive oxygen species; FA – fatty acids; PUFAs – Poly-unsaturated fatty acids. Figure partially created with Biorender tool.

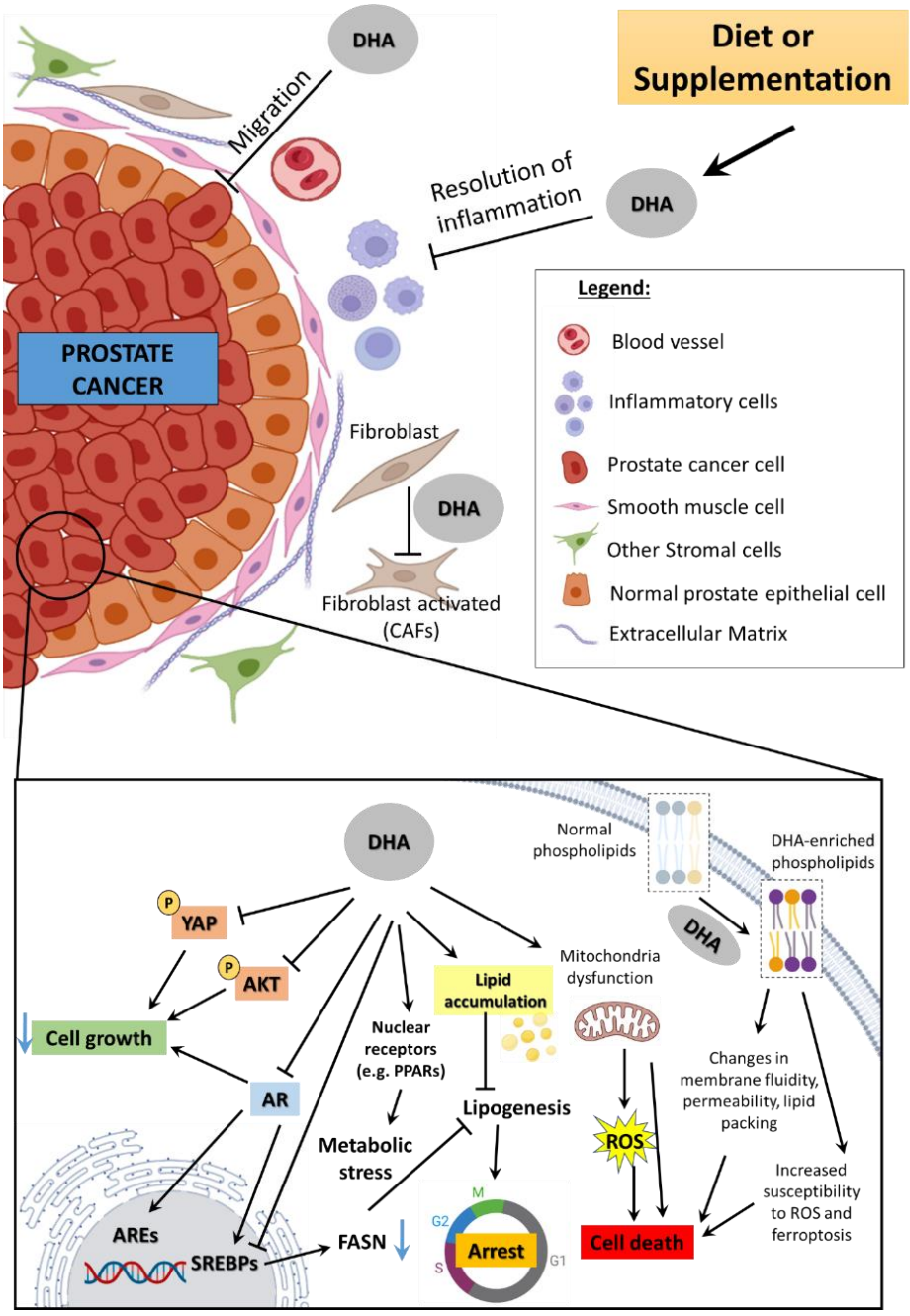

Figure 2. *DHA specific effects on prostate cancer.* DHA obtained through diet or supplementation reaches prostate cancer and may act at tissue level by decreasing cell migration, resolution of inflammation or inhibiting fibroblast activation. At cell level, the omega-3 suppresses cell growth via different pathways depending on the androgenic background. In AR-positive cells, it leads to AR degradation through proteasome which downregulates AREs and SREBPs, affecting lipid metabolism decreasing FASN hence lipogenesis. Also, DHA dysregulates mitochondria and induces ROS overproduction which is tightly related to cell death. Increase in DHA uptake is harmful to the cells because the raise in unsaturation status in cell membrane sensitize cells to oxidative damage, but also change membrane properties related to cell signaling, lipid packing, fluidity and permeability. Legend: DHA – docosahexaenoic acid; AR – androgen receptor; ROS – reactive oxygen species; AREs – androgen responsive elements; SREBPs – sterol regulatory elements binding proteins; FASN – fatty acid synthase. Figure partially created with Biorender tool.

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CAPÍTULO 2

Docosahexaenoic acid differentially modulates the cell cycle and metabolism-related genes in tumor and pre-malignant prostate cells

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ABSTRACT

Prostate cancer (PCa) has different molecular features along progression, including androgen profile, which is associated to therapy inefficiency leading to more aggressive phenotype. Docosahexaenoic acid (DHA) has antiproliferative and pro-apoptotic properties in different cancers associated to cell metabolism modulation. The latter is of particular interest since metabolic reprogramming is one of PCa hallmarks, but is not clear how this occurs among disease progression. Therefore, we evaluated DHA antiproliferative potential in distinct androgenic backgrounds associated to metabolism modulation and androgen-regulated genes. For this purpose, premalignant PNT1A and tumor AR-positive 22rv1, and AR-negative PC3 cells were incubated with DHA at 100 μM–48 h. DHA reduced at least 26% cell number for all lineages due to S-phase decrease in AR-positive and G2/M arrest in AR-negative. Mitochondrial metabolic rate decreased in PNT1A (~38%) and increased in tumor cells (at least 40%). This was associated with ROS overproduction (1.6-fold PNT1A; 2.1 22rv1; 2.2 PC3), lipid accumulation (3-fold PNT1A; 1.8 22rv1; 3.6 PC3) and mitochondria damage in all cell lines. AKT, AMPK and PTEN were not activated in any cell line, but p-ERK1/2 increased (1.5-fold) in PNT1A. Expression of androgen-regulated and nuclear receptors genes showed that DHA affected them in a distinct pattern in each cell line, but most converged to metabolism regulation, response to hormones, lipids and stress. In conclusion, regardless of androgenic or PTEN background DHA exerted antiproliferative effect associated to cell cycle impairment, lipid deregulation and oxidative stress, but differentially regulated gene expression probably due to distinct molecular features of each pathologic stage.

Key-words: prostate cancer; DHA; lipids; metabolism.

I. INTRODUCTION

Most of available therapies for prostate cancer (PCa) are primary focused on ablation of tumor-stimulating hormones, as androgens. These therapies may drive to castrated-resistant phenotypes which are related to reoccurrence with increased aggressiveness and poor prognosis [1,2]. This lack of inefficient therapeutic strategies urges for new approaches as PCa still remains among the most frequent malignancy in men worldwide [3]. Metabolic reprogramming is a well-known event described in cancer and supports increased energy demand and anabolic processes required for high proliferative rates [4]. In PCa, deregulation of lipid metabolism is an early hallmark of this metabolic switch and each pathological stage of disease shows distinct patterns of overexpression for several enzymes [5] [6], such as fatty acid synthase (FASN) [6–9] that is linked to castrated-resistant phenotype [7,10,11]. Also, lipolysis is the main catabolic pathway in PCa [12] which according to androgenic background exhibit different fatty acid transporters [13]. This evidence suggests that tumor metabolism is differently regulated during disease progression and that external lipids may play a distinct role among androgenic profiles found in the pathology spectrum.

This is of particular interest with regard to PCa etiology since dietary factors have been correlated with demographic differences in its incidence, particularly the amount and type of lipids [14–18]. On one hand, saturated fatty acids (SFAs) have been associated to prostate malignancy as well as polyunsatured fatty acids (PUFAs) omega-6 (ω-6) [19,20]. On the other, PUFAs of omega-3 (ω-3) class were reported to decrease PCa risk serving as preventive, therapeutic or even coadjuvant agents [21,22]. This is supported by evidence that lower ω-6/ω-3 ratio decreased tumor growth in xenograft models [23,24], delayed PCa progression [25] and impaired cell migration [26] whereas higher ratio increased the risk of PCa development [27]. The mechanism related to the protective property of lower ω-6/ω-3 ratio is not fully elucidated, but has been linked to the omega-3 fatty acids due to down-modulation of Akt/mTOR/NFκB axis, cell cycle arrest and anti-inflammatory mediators [23,25]. Moreover, preclinical studies suggest that oil fish-derived omega-3 have good outcome in androgen sensitive and castrated-resistant PCa [28,29], including in the absence of PTEN [30]. However, PUFAs' role in the gland carcinogenesis still remains under debate [18] and have been shown their increased levels in the blood [31].

Docosahexaenoic acid (DHA) is a PUFA omega-3 that shows much reduced synthesis in humans, especially in men, and is obtained mainly from nutritional

supplementation or diet with higher levels found in marine cold fishes [32]. Along the past years, studies have reported DHA antitumor properties in several tissues which raised discussions about its role in PCa initiation and progression [32]. Compared to others omega-3 with biological activity, DHA shows higher effectiveness in decrease cell viability and induce apoptosis compared to others, as EPA (eicosapentanoic acid) and ALA (α-linoleic acid) in PCa cells [33]. The underlying mechanisms in prostate are partially known and involves the reduction in cell migration [34] and proliferation through ROS-mediated AKT/mTOR suppression [35], downregulation of lipid metabolism enzymes [36], modulation of cell death or survival pathways as AKT/PIP3 [37] and PDK1/AKT/Bad [38], which are most tightly related to cell metabolism. Recently, we reported the preventive potential of DHA by decreasing proliferation of PNT1A pre-malignant cells due to mitochondria bioenergetics modulation, decreasing their capacity to respond to stress stimuli and enhancing antiproliferative effect of melatonin [22]. Several studies have reported that such omega-3 regulated metabolic sensors as PPARγ, PPARδ and RXRs [39–41]. These nuclear receptors together with others were described to play a role in several cancers [42] and may respond to extracellular compounds, as hormones and factors modulating cell proliferation, bioenergetics [41], mitochondrial dynamics and lipid metabolism.

PCa metabolic vulnerabilities have been investigated as therapeutic strategy [7]. Taken together, these evidence supports that metabolism regulation may be a potential mechanism of DHA, but is not clear how this omega-3 affects different molecular contexts found among disease progression. In the present study, we tested DHA potential to decrease proliferation of pre-malignant (benign) and castrated-resistant tumor cell lines with different androgenic backgrounds associated to metabolism regulation and androgen signaling. For this purpose we used three different cell lines: PNT1A, which maintain androgen sensibility, PTEN expression, normal secretory phenotype but also exhibit non-malignant alterations [43], as observed in benign prostatic hyperplasia (BPH) and early stages of carcinogenesis; castrated-resistant tumor 22rv1 (express full-length AR and variants with constitutional activation similar to hormone refractory phenotype; PTEN-positive) and, more aggressive, PC3 (AR-negative, neuroendocrine-like adenocarcinoma; PTEN-negative) [44]. Our findings revealed that although DHA led to lipid accumulation, ROS overproduction and reduced cell number, it differently modulated cell cycle in each line by decreasing S-phase in AR-positive and arresting AR-negative cells in G2/M. In addition, this omega-3 differently regulated gene expression in

distinct pattern in each pathological stage, despite most of them converged to metabolism regulation and response to stress and hormones.

II. MATERIAL AND METHODS

2.1 Cell Culture

PNT1A (#95012614—Health Protection Agency, England, UK), 22rv1 and PC3 (CRL1435) cells were cultured in RPMI 1640 medium (#R6504—Sigma-Aldrich, St. Louis, Missouri, EUA) enriched with 10% fetal bovine serum (FBS) (#S0011—Vitrocell, Campinas, São Paulo, Brazil), 1% of penicillin, streptomycin, and amphotericin B (#15240062—Life Technologies, Paisley, UK) and kept in a wet incubator with 5% of CO_2 at 37°C. For cell maintenance, the medium was replaced every 2–3 days and subculture was done when 70–85% confluence was reached. For the experiments, the cells were seeded at the desired density and allowed to attach during 24 hours.

For all analysis, to ensure that the observed effects were mainly due to DHA, control assays were incubated with same vehicle volume. Also, the concentration of vehicle in the medium never exceeded 0.5% which did not exert alteration on cell proliferation, as previously tested (data not shown).

2.2 Mitochondrial Metabolic Rate

5×10^4 cells/well were incubated with DHA (#D2534—Sigma-Aldrich, St. Louis, Missouri, EUA) at concentrations 10 μM, 20 μM, 50 μM, and 100 μM for 24 h and 48 h, freshly prepared in RPMI 1640 culture medium from a stock solution of 20 mM DHA in anhydrous ethanol (vehicle). These concentrations were chosen according to our previous study [22]. Mitochondrial metabolic rate (MMR) was performed with commercial colorimetric kit CellTiter 96® AQueous One Solution Cell Proliferation Assay (#G3580—Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. This is a tetrazolium salt-based assay that measures mitochondrial dehydrogenases activity [45] and based on that principle, we adopted it to evaluate the MMR rather than cell proliferation to avoid biased interpretations. Absorbance was determined at 490 nm in an Epoch microplate reader (BioTek Instruments Inc., Winooski, VT, US). DHA concentration and time of

incubation that altered MMR most significantly and with distinct pattern between cell lines were chosen to perform all forward assays (100µM-48h). Three independent experiments in triplicate were performed for statistical analysis. Values were shown in terms of fold-change compared to control and SEM.

2.3 Cell Number and Cell Cycle Analysis

To evaluate the effects on cell proliferation, 10^3 cells were seeded in culture flask and incubated with DHA at 100µM for 48h. Then, cells were treated with trypsin-EDTA 0.25% for 10 minutes in wet incubator at 37°C and added medium enriched with FBS 10%. Population growth was assessed by manual counting in Neubauer chamber. Three independent experiments were performed for statistical analysis (n=7). Values were shown as mean of cell number ($x10^6$) and SEM was plotted.

For flow cytometry analyses, 10^6 cells were seeded in culture flask and, before the end of experiment, incubated with bromodeoxyuridine (BrdU) for 2 hours in wet incubator at 37°C with 5% CO_2 using BD Pharmingen™ FITC BrdU Flow Kit (#559619 – BD Biosciences, San Jose, CA, US). Samples were fixed and prepared according to manufacturer's instructions. DNA amount was assessed with 7-AAD dye. Data was acquired for at least 10^4 events with BD FACS Canto II using BD FACS Diva™ software. Cell populations were gated into G1, G2/M and S-phase and analyzed with Flowing 2.5.1 software. Values were shown as mean of events and SEM for three independent experiments and a representative histogram was plotted for each tested condition.

2.4 Lipid accumulation

Intracellular neutral lipids were assessed after incubation (10^4 cells/well) with 5 µM of BODIPY™ Lipid Probes 493/503 (#D3922—Molecular Probes®, Invitrogen) prepared in serum-free medium for 5 minutes in a wet incubator with 5% of CO_2 at 37°C, according to the manufacturer's instructions. Immediately, images were captured at 20x amplification with an inverted fluorescence microscope (Axio Vert.A1 Carl Zeiss AG, DE) with the same time of exposure (280 ms). Quantification of total fluorescence intensity (TFI) was assessed with NIH ImajeJ (1.39) software [46] and at least 5300 cells per incubation from three consecutive

passages in triplicate were considered. Values were shown as Fluorescence Intensity (FI), assessed after TFI normalized by nuclei number per field and SEM.

2.5 ROS determination

Superoxide anion production ($O_2^{\bullet -}$) was assessed after incubation with MitoSOX® Red dye (#M36008—Molecular Probes, Thermo Fisher Inc.), according to the manufacturer's instructions. After treatments, the medium was removed and cells (10^4/well) were washed twice with warmed KH buffer (NaCl 120 mM, $NaHCO_3$ 15 mM, KCl 5 mM, NaH_2PO_4 1.5 mM, and Na_2HPO_4 0.7 mM) and incubated with dye at 3 μM for 15 minutes at 37°C in incubator with 5% CO_2. Then, cells were washed twice with KH buffer to remove the dye that did not enter into mitochondria. A total of fifteen images from wells containing cells of consecutive passages in triplicate were captured at the same exposure time (1200 ms) with 20x objective in inverted fluorescence microscope (Axio Vert.A1 Carl Zeiss AG, DE). TFI was measured with NIH ImajeJ (1.39) software [46] and at least 2000 cells per incubation from three consecutive passages in triplicate were considered. Values were shown as FI, assessed after normalization of TFI by nuclei number per field and SEM.

2.6 Transmission Electron Microscopy

For ultrastructural analysis, 2 x 10^6 cells were seeded in culture flask and, after incubations, pelleted and immersed in a fixation solution (2% paraformaldehyde, 2.5% glutaraldehyde and 0,25% $CaCl_2$ in 0.1M cacodylate buffer – pH 7.4) with 10x of fragment volume for 2 hours at room temperature (RT). Pellets were washed twice with buffer, post-fixed in 2% osmium tetroxide in 0.1M cacodylate for 1 hour at RT. Then, samples were immersed in 0.5 uranyl acetate for 1 hour, dehydrated in acetone and embedded in araldite resin. Ultrathin sections were placed onto properly grid and stained with 2% alcoholic uranyl acetate and 2% lead citrate in 1N sodium hydroxide for 10 minutes. Transmission Electron Microscopy (TEM) was performed in JEOL 1400Plus microscope.

2.7 Western Blotting

After incubations, 10^6 cells were lysed in RIPA buffer in the presence of 10% protease inhibitor cocktail, sodium orthovanadate (1 : 100), and PMSF (1 : 1000). All reagents were obtained from Sigma-Aldrich (St. Louis, Missouri, EUA). Total protein was determined with Bradford method [47] and measured in Epoch microplate reader (BioTek Instruments Inc., Winooski, VT, EUA) at 595 nm. After electrophoresis in SDS-PAGE, proteins (50μg) were transferred to nitrocellulose membrane for 2 hours in a semi-dry system. Nonspecific bindings were blocked with non-fat milk at 5% in TBST for 30-60 minutes at RT in a shaker and membranes were incubated overnight with primary antibody at 4°C. The following primary antibodies were used: anti-phospho-AMPK Thr172 (#4060S; rabbit polyclonal, Cell Signaling, 1 : 1200), anti-AMPK (#2532; rabbit polyclonal, Cell Signaling, 1 : 1200), anti-phospho-PTEN (#9554S – rabbit polyclonal, Cell Signaling, 1 : 500), anti-phospho-AKT (rabbit polyclonal, Santa Cruz Biotechnology, 1 : 400), anti-AKT (rabbit polyclonal, Immuny, 1:400); anti-phospho-ERK1/2 (#4370S – rabbit polyclonal, Cell Signaling, 1 : 500), anti-ERK1/2 (#9102 – rabbit polyclonal, Cell Signaling, 1 : 1200), anti-GSTP1 (#3369S – mouse polyclonal, Cell Signaling, 1 : 1000) and anti-β-actin (#SC47778 – mouse monoclonal, Santa Cruz Biotechnology, 1 : 600). Secondary antibodies were prepared at 10x dilution of the respective primary antibody and incubated at 4°C for 1 h. ECL system was adopted for band visualization and image capture. Protein quantification was performed for all samples in the same electrophoresis, one protein per gel in addition to loading control. Relative densitometry was assessed using NIH ImajeJ (1.39) software [46] after normalization with β-actin (loading control) or the total form of protein of interest.

2.8 qRT-PCR array

All qRT-PCR arrays were performed with Qiagen (Hilden, GE) workflow according to manufacturer's instructions. Briefly, 2 × 106 cells were seeded in culture flask and, after incubation, total RNA were obtained with RNAeasy Plus Mini kit (#74134). RNA quantification and purity was determined in NanoDrop spectrophotometer ND-1000. Prior to qRT-PCR arrays, RNA integrity was checked in agarose gel 1% and cDNA was obtained with First Strand Kit (#330404) in a thermocycler (Multigene – Labnet International Inc.). Then, cDNA quality, reaction efficiency and gDNA contamination was assessed with QC Human

array (PAHS-999ZC) prior to qRT-PCR array. RT^2 Profiler for Human Androgen Signaling Pathway (PAHS-142ZC) and Nuclear Receptors & Coregulators (PAHS-056YC) were performed (5 μg) for three pooled samples from consecutive passages. All qRT-PCR arrays were performed with SYBR Green® ROX/MASTERMIX (#330523) in StepOnePlus PCR Real Time System (Applied Biosystems). Gene expression changes were analyzed by ΔCt (cycle threshold) method [48], subtracting the average Ct values obtained from the housekeeping genes (*ACTB, B2M, GAPDH, HPRT1 AND RPLP0*) provided in the array from those of each gene of interest present in the same array. Levels of gene expression were plotted as a chart and the upregulated genes were represented in zero-left side and downregulated in the right. Those genes that reached at least 50% of upregulation or halved compared to control in both arrays were submitted to STRING online tool for molecular interaction estimation. Biological processes were retrieved from GeneOntology annotations at STRING platform and Venn diagrams built with InteractVenn [49].

2.9 Statistical Analyses

Statistical analyses were performed with GraphPad Prism® software (GraphPad Prism software, v.5.0). First, distribution of samples was analyzed with Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Parametric distributions were submitted to unpaired t-test or one-way ANOVA followed by Tukey test (post hoc) and non-parametric distributions to Mann-Whitney or Kruskal-Wallis test followed by Dunn test (post hoc). $p < 0.05$ was considered statistically different.

III. RESULTS

3.1 DHA conversely modulates MMR from benign and malignant prostate cells

After 24h, DHA did not affect MMR of PNT1A at any concentration used here, however, decreased of PC3 at 10 and 20μM (22% and 37%, respectively) and increased about 40% of 22rv1 at 100μM (Fig.1A). Regarding the longer incubation (48h), DHA stimulated PNT1A mitochondrial metabolism at 10μM (35%) and 50μM (40%) and reduced about 38% at 100μM (Fig.1B). The omega-3 increased mitochondrial metabolism of both PCa lines PC3 and

22rv1 at 100µM when incubated for 48h (Fig.1B). Then, this condition was selected to perform all forward assays, since mostly affected mitochondria and in a different pattern between benign and malignant cells.

3.2 DHA decreases population growth and affects cell cycle of all cell lines

In order to determine if MMR modulation affected cell proliferation, we assessed population growth and cell cycle. Incubation with DHA at 100µM for 48h reduced cell number of PNT1A (Fig.1C), 22rv1 (Fig.1D) and PC3 lines (Fig.1E) in 27%, 26% and 34%, respectively. After incubation, were observed sharp alterations in each line under contrast phase microscopy (Fig.1F-K), as changes in cell morphology as well as large lipid inclusions (confirmed thereafter, as described in the next topic). Cell cycle analysis (Fig.2) showed decreased proliferation (S-phase) only in AR-positive cells PNT1A (10%; Fig.2A-D) and 22rv1 (26%; Fig.2E-H) whereas did not change G1 and G2/M. In AR-negative PC3 (Fig.2I-L) the omega-3 slightly decreased G1 (4%) while increased 30% in G2/M, but unchanged in S-phase.

3.3 DHA increases intracellular lipid accumulation, lead to ROS overproduction and mitochondrial damage

Given the morphological alterations observed in the phase contrast microscopy and our previous work [22], we evaluated if DHA affected lipid storage and mitochondrial ROS production, both metabolic parameters related to cell proliferation. Lipid accumulation reached different levels between cell lines after DHA incubation with the highest in PC3 (267%), the lower in 22rv1 (82%) and PNT1A in the middle (194%) (Fig.3). Such increase was confirmed by TEM (Fig.5). In addition, ultrastructural analysis showed that DHA seems have affected lipid droplets content which showed higher eletrondensity in PNT1A and 22rv1, but lower in PC3 cells. Regarding $O_2^{\bullet -}$ production, tumor cells were more sensitive to DHA exhibiting higher levels then PNT1A. The omega-3 raised it to 57% in PNT1A while 118% in 22rv1 and 120% in PC3 cells (Fig.4). TEM analysis indicated that cells incubated with vehicle only (Fig.5A-B; 5F-G; 5K-L) exhibited mitochondria with thin and parallel-oriented cristae and DHA incubation led to in cristae disorganization and misfolding in tumor lines (Fig.5H-I; 5M-N). PNT1A showed mitophagy and mitochondria disorganization (Fig.5D-E). Regardless of

cell type, such organelle was localized near to lipid droplets, mainly after lipid accumulation. DHA also induced the presence of myelin bodies (Fig.5O).

3.4 DHA effects were not mainly due to proliferation and survival pathways regulation

Due to decrease of cell proliferation and cell metabolism impairment, we interrogated if the omega-3 affected activation of proliferation or survival pathways. Interestingly, DHA did not change the AKT, PTEN and AMPK phosphorylation in any cell line (Fig.6). Phospho-ERK 1/2 increased 1.5-fold in PNT1A, but remained unchanged in 22rv1 and PC3 (Fig.6B). GSTP1 expression did not alter in PNT1A and PC3 cells after incubation with DHA. No expression of PTEN and ERK1/2 was found for PC3 as well as GSTP1 for 22rv1.

3.5 DHA differentially regulates gene expression between benign and tumor prostate cells and PNT1A is the most affected

In addition to functional assays, we investigated if DHA also led to alterations in the expression of metabolism and androgen-related genes. We found a different pattern of androgen and nuclear receptors genes regulation by DHA between cell lines, being 21 altered in PNT1A, 16 in 22rv1 and 12 in PC3 (Fig. 7). The functional enrichment indicated that for all three cell lines the most affected were involved in metabolism as well as response to lipids, hormones and stress (Fig.8A-C). In terms of percentage, 85.7% of these genes were related to metabolism regulation in PNT1A, 68.7% in 22rv1 and 83.3% in PC3; 57.1% were associated to response to lipids in PNT1A, 56.2% in 22rv1 and 58.3% in PC3; 42.8% to stress in PNT1A, 43.7% in 22rv1 and 66.6% in PC3; and 23% to steroid hormone response in PNT1A, 56.2% in 22rv1 and 50% in PC3. This analysis showed that *Nr0b1*, *Pparg* and *Errfi1* (upregulated) as well as *Ppargc1a* and *Fos* (downregulated) matched to all of these functions in PNT1A; *Hdac5*, *Fos, Nr0b1, Ppatgc1b* and *Rora* (upregulated) in 22rv1; and *Ppargc1a* (upregulated) and *Nr0b1* (downregulated) in PC3 cells, as illustrated in Fig.8D-F. The molecular interaction analysis pointed that for PNT1A and PC3 most of them belong to a cluster that may regulate network activity while this was not clear for 22rv1 (Fig.8G-I).

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IV. DISCUSSION

Despite DHA antiproliferative potential has already been described in several cancers [34–38], its protective effects against PCa progression have been discussed and the underlying mechanisms remain to be elucidated. To the best of our knowledge, this is the first study to compare the ability of DHA to decrease cell proliferation in pre-malignant and PCa castrated-resistant cells with different androgenic backgrounds and the association with metabolism regulation. Our results revealed that in AR-positive cells, DHA at 100µM-48h decreased S-phase while arrested in G2/M the AR-negative. It also induced ROS production with higher levels in tumor cells than PNT1A. The omega-3 led to lipid accumulation and, according to androgenic background, the malignant lines showed distinct levels. Despite of omega-3 differently has affected expression of androgen-regulated genes or nuclear receptors in each line, metabolism regulation, response to lipids, stress or hormones were the major modulated pathways. Moreover, most of them were regulated in opposite manner than those found in prostate cancer samples.

Lipid metabolism reprogramming is one of early hallmarks of prostate carcinogenesis and were reported that fatty acids oxidation is the main energy source in PCa cells as well as lipogenesis is over-stimulated [12,50]. Then, disruption of this rewriting, essential to fuel cell proliferation, has been investigated as therapeutic approach [7]. Our results revealed that DHA led to lipid accumulation which suggests deregulation of lipid metabolism. This omega-3 has been reported to impair lipogenesis via *Fasn*, *m-Srebp-1* and *p-Srebp-1* downregulation in human breast cancer cells [36] associated to reduction of cell proliferation which may indicate that lipolysis was mostly affected than lipogenesis herein. Also, studies reported unsaturated fatty acids accumulation, including PUFAs, into lipid droplets during apoptosis, suggesting that DHA may play a role in cell death [51–53]. Despite we could not determine the species, our methodology allow us to assume that neutral lipids were mainly stored. In addition, qualitative data from ^{1}H-NMR (not shown) assay revealed peaks in the spectrum that are present in the lipophilic extract from DHA-incubated cells but not in the control condition, indicating that DHA or at least its metabolites were accumulated. Despite not fully elucidated, our findings revealed that lipid accumulation occurred in all pathological stages and that was not strictly dependent of androgen receptor or its variants, since occurs independent of presence or lack of receptor. However, this may play a role in the lipid accumulation levels given that it was different in each cell line. Fatty acid transporters are

differently expressed in distinct androgenic backgrounds in addition to androgens may orchestrate lipid uptake and regulate expression of genes involved with metabolic processes [13].

As lipid accumulation, ROS production and mitochondrial damage are related to metabolism and was also either observed at different levels among cell lines. ROS generation was already described for DHA at different conditions [22,35,54,55] and associated to increased lipid accumulation [22], cell cycle arrest as well as decrease of proliferation [54]. However, this is the first evidence to describe that DHA differently modulate them in each androgenic backgrounds and pathological status. The omega-3 led to decrease of S-phase in PNT1A, depletion of MMR, increase of ROS and activation of p-ERK1/2 which occurs under oxidative stress [56]. Mitochondria morphology is sharply related to its activity [57–59] and cristae shape is under remodeling in several situations, like mitochondria malfunction, damage, OXPHOS impairment and cell growth. In a previous work [22], we reported that DHA at same conditions used here raised ROS to lethal levels in PNT1A, increased mitochondria membrane potential, negatively affected cell capacity to respond to stress and amplified the antiproliferative effect of melatonin. This is in line with the mitochondria disorganization observed for PNT1A as well as decrease of cell population. However, on both tumor cells the omega-3 increased MMR, induced higher ROS production and led to mitochondrial alterations without change p-ERK1/2, while differently modulated cell cycle. PCa cells show elevated ROS levels among disease progression and its overproduction by DHA may be associated to sharp morphological alterations in the mitochondria and mitophagy induction. Although, MMR increased in these cells and it was probably associated to metabolism stimulation, given that fatty acids are the main energy source in PCa cells [10,11], which would be correlated to the lipid droplets near to mitochondria observed in TEM. Despite speculative, this may also be explained by alterations in mitochondria network to support its function under oxidative damage. We reported previously that DHA was able to induce mitochondrial network rearrangements under ROS overproduction without change OXPHOS, but depleting mitochondrial capacity to respond to stress leading to chemosensitization [22]. Taken together, this evidence revealed that DHA induced oxidative damage via mitochondrial impairment probably leading to cell cycle arrest and decrease of proliferation in benign and malignant phenotypes.

Our findings also revealed that the omega-3 differently regulated gene expression in each cell line and part of them supports the functional assays (Fig.9). PPARGC1A may

induce upregulation of genes related to oxidative phosphorylation, Kreb's cycle and *de novo* lipogenesis [60–63]. In cancer cells its loss showed protective function due to mitochondrial and fatty acid metabolism modulation associated to suppression of tumor growth [64]. This evidence supports the imbalance in lipid metabolism and mitochondrial damage in PNT1A because *Ppargc1a* was downregulated on it which may be a mechanism of the omega-3 protective role. This is also corroborated by *Pparg* upregulation in the benign cells since PPARγ knockout in mice led to low-grade PIN development [65], suggesting that its expression in normal prostate serves as tumor suppressor. Regarding PCa cancer cells, DHA upregulated *Ppargc1a* in AR-negative while *Ppargc1b* in AR-positive. These upregulations may be linked to increased MMR observed in cancer lines since both co-regulators stimulate mitochondria biogenesis [66]. However, *Pparg* expression was unchanged in PCa cells, indicating DHA selective property in malignant stages. Differently from healthy prostate that show low expression [67], PPARγ is overexpressed in all PCa stages and may favor tumor growth depending on molecular context [68], like in association to PTEN loss it enhanced prostate tumorigenesis [69]. Therefore, the selective *Pparg* modulation was a good outcome, mainly for PC3. In 22rv1, DHA upregulated *Rora* which were reported as tumor suppressor in breast cancer [70], showed decreased expression in PCa and related to cell stress response [71,72]. RORα activation affected the mitogenic activity of fatty acids via 5- lipoxygenase downregulation and cell cycle arrest [73,74]. In this cell line, DHA also upregulated *Hdac5*, a class II histone deacetylase that inhibits lipogenic genes expression through suppression of LXR activity [75]. Therefore, our data revealed that DHA modulated nuclear receptors expression which control metabolism and that are deregulated in PCa.

Regarding the androgen signaling, *Errfi1* was upregulated in PNT1A and its overexpression have been described to cell cycle arrest at G1/S [76] as well as may act as molecular brake for proliferation pathways [77]. In addition, *Fos*, a well-known proto-oncogene coupled to androgenic response in PCa associated to cell proliferation and differentiation [78], was downregulated in PNT1A whereas upregulated in 22rv1. *Fos* upregulation was described in carcinomas with Gleason score ≤ 7 [79] and androgen-independent tumor [80], suggesting that its increase may play a role in PCa development. However, FOS activity depends on heterodimeric formation with other proteins, as Jun family (unchanged herein), [81] which was not assessed [82]. The androgen signaling expression profile also revealed that *Nr0b1* was upregulated in both AR-positive which is in aligned with the decrease of both cell population since it restrict the AR transcriptional activity [83]. *Nr0b1* (or *Dax1*) is highly expressed in

steroidogenic tissues and known as negative regulator of steroid production [84]. Nakamura and colleagues [84] reported that decreased expression of NR0B1 is correlated to Gleason score 8-10, indicating that such nuclear receptor is lost among PCa progression and may be linked to androgen-independent state. However, DHA downregulated it in AR-negative, but was not clear how DHA differently modulate the nuclear receptor expression in each cell line. These findings showed that androgen-regulated genes were affected by DHA even in the absence of AR.

Together, our results suggest a plenty of pathways regulated by DHA in each cell line and at different levels. The differences were related to distinct metabolic and molecular contexts as PTEN loss as well as lack (PC3) or presence of AR (PNT1A and 22rv1) and its variants (22rv1) which may activate different gene modulation. Likewise, as mentioned before, lipid uptake is not similar between AR-positive and AR-negative cells, and the different effects may be related to the amount of DHA that entered into the cell [13].

V. CONCLUSION

The present study provided evidence that DHA regardless of PTEN status and androgenic background was able to decrease cell proliferation associated to metabolism regulation, but via different mechanisms. However, for most of genes, the omega-3 regulated them in an opposite manner that those observed in PCa samples which supports its antitumor property at least *in vitro*. Especially for castrated-resistant cells that lack clinical efficient treatment, DHA may be an alternative therapeutic strategy in different molecular backgrounds and should be considered in combination to other compounds, especially those that target metabolism and induce mitochondrial damage, to improve therapeutic outcome. In pre-malignant phenotype, DHA was able to delay or reduce cell proliferation and suggests its protective role at early stages of PCa progression.

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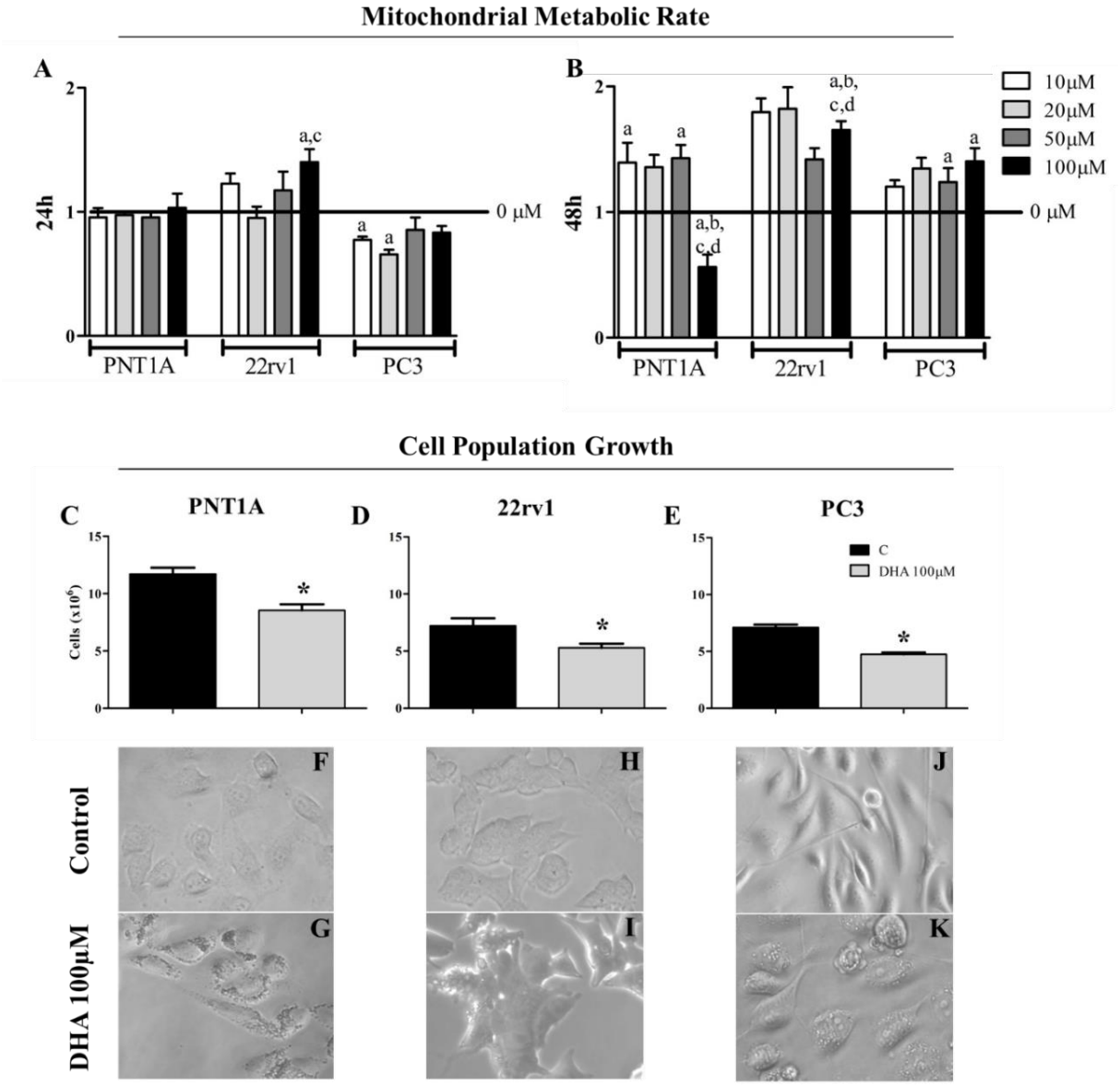

Fig. 1. (A) Mitochondrial metabolic rate. DHA modulated the mitochondrial metabolic activity in a concentration and time of incubation dependent manner. (A) 24 h and (B) 48 h after incubation at 10, 20, 50 and 100 µM. Values were show in terms of fold-change compared to control (0 µM, vehicle only) and SEM determined by MTS assay. Statistical analysis of panels A–B: a – different from 0 µM; b – different from 10 µM; c –different from 20 µM; d – different from 50 µM; data showed parametric distribution and was submitted to one-way ANOVA followed by Tukey test. (C–E) Cell number. Effects on cell number in (C) PNT1A, (D) 22rv1 and (E) PC3 cells. DHA decreased cell number in all lines. It was estimated by manual counting. Values show total number of cells and SEM. (F–K) Morphological changes in PNT1A (F–G), 22rv1 (H–I) and (J–K) PC3 cells after incubation. Images match to cell line identified in each column and were captured with contrast phase inverted microscope with 40× objective. Legend: C – control (vehicle); DHA – docosahexaenoic acid. Statistical analysis of panels C–E: * - different from C; PNT1A and PC3 data were parametric (unpaired t-test) and 22rv1 non-parametric (Mann-Whitney test); $p < 0.05$ was determined as statistically different for both.

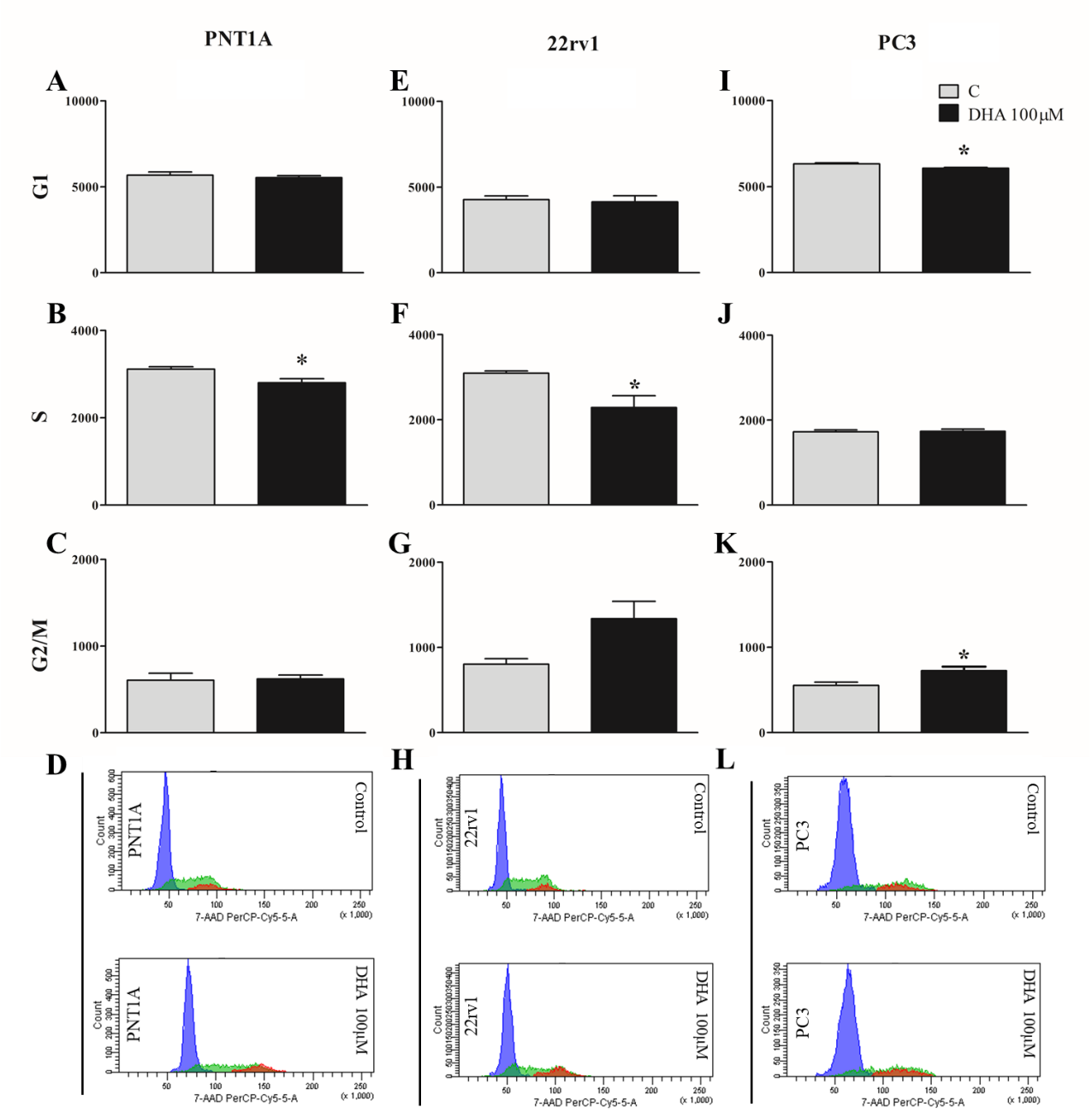

Fig. 2. Cell cycle. Columns show PNT1A (A–D), 22rv1 (E–H) and PC3 (I–L) cell cycle analysis. Representative histograms show G1 (blue), S (green) and G2/M (red) phase of the cell cycle for Control and 100 µM DHA after 48 h of incubation. At least 104 events were acquired in each analysis. Statistical analysis: three independent experiments were considered for statistical analysis; all data were submitted to parametric unpaired t-test; $p < 0.05$ was determined as statistically different (*).

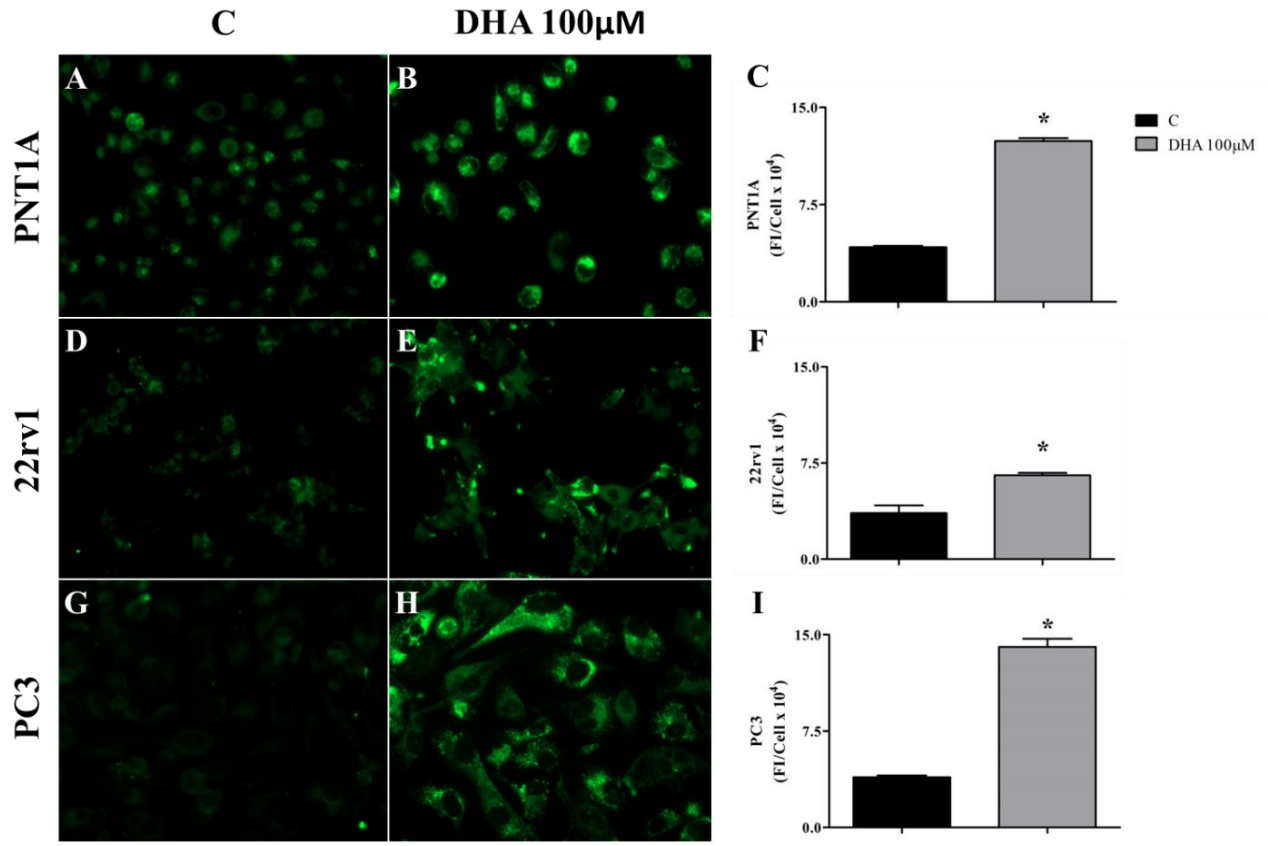

Fig. 3. Lipid accumulation. DHA increased intracellular lipids (green) in PNT1A (A–C), 22rv1 (D–F) and PC3 (G–I) cells as evidenced by BODIPY® dye. Images were captured with 20× objective. Values show fluorescence intensity (FI) per cell (×104) and SEM. Legend: C – control (vehicle); DHA – docosahexaenoic acid. Statistical analysis: * - different from C; at least 5300 cells per incubation from three consecutive passages in triplicate were considered; all data showed non-parametric distribution (Mann-Whitney test); $p < 0.05$ was determined as statistically different.

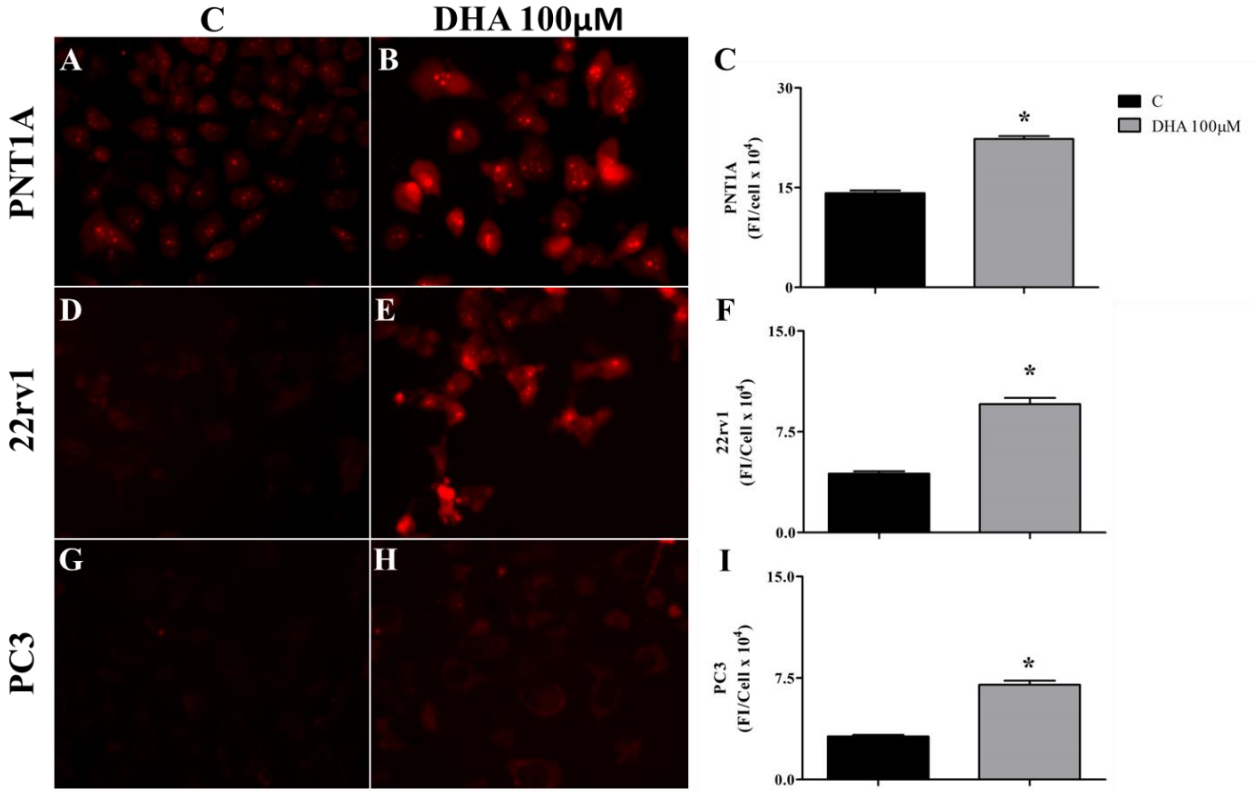

Fig. 4. ROS production. Superoxide anion generation (red) in PNT1A (A–C), 22rv1 (D–F) and PC3 (G–I) cells determined by MitoSOX® Red dye. DHA induced ROS production in all cell lines. Images were captured with 20× objective. Values show fluorescence intensity (FI) per cell (×104) and SEM. Legend: C – control (vehicle); DHA – docosahexaenoic acid. Statistical analysis: * - different from C; at least 2000 cells per incubation from three consecutive passages in triplicate were considered; all data showed non-parametric distribution (Mann-Whitney test); $p < 0.05$ was determined as statistically different.

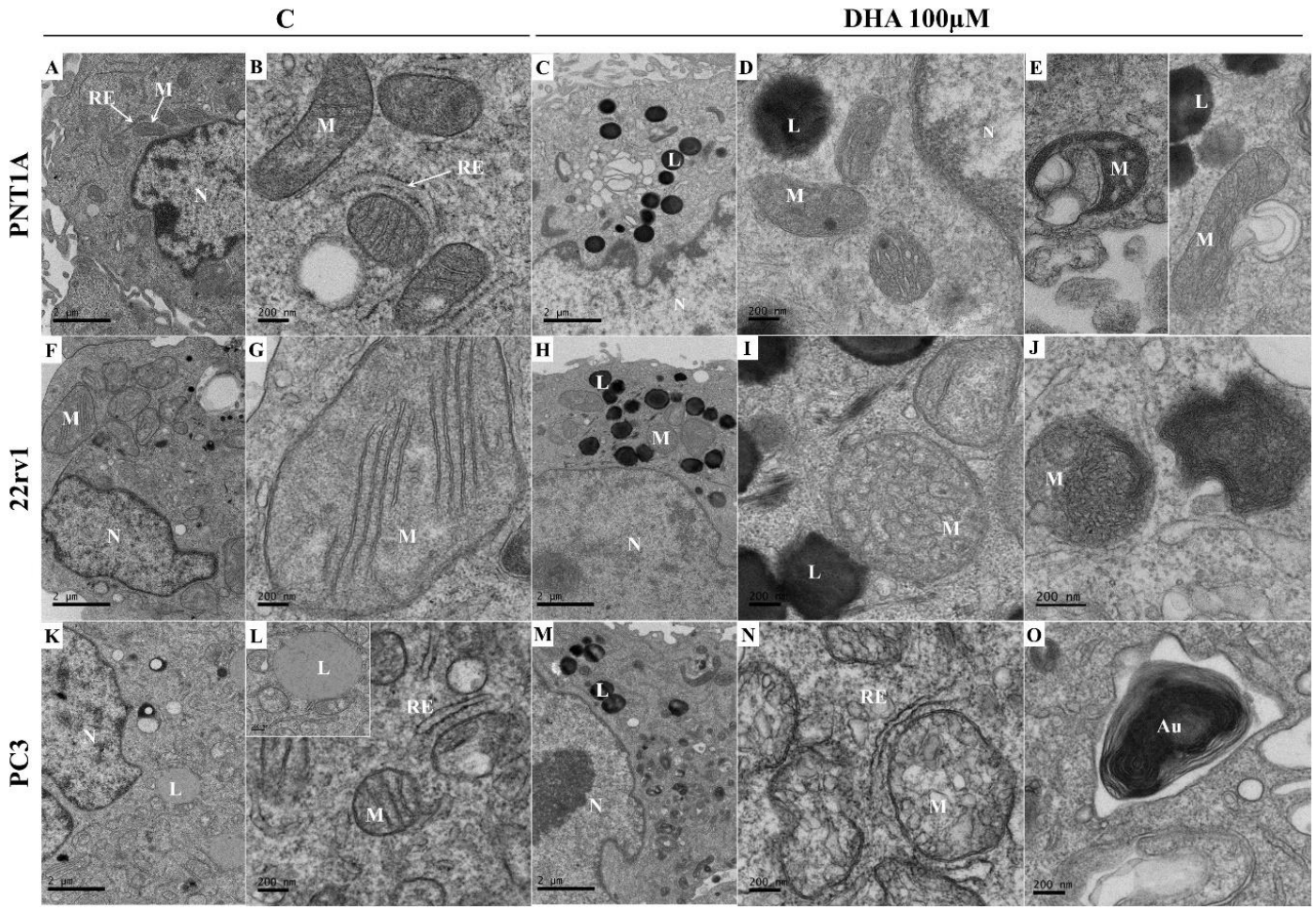

Fig. 5. Ultrastructure. TEM analysis of PNT1A (A–E), 22rv1 (F–J) and PC3 (K–O) cells evidencing mitochondria damage (cristae misfolding), lipid droplets and traces of autophagy (O), suggesting metabolic impairment after DHA incubation. Legend: C – control (vehicle); DHA – docosahexaenoic acid; M – mitochondria; N – nucleus; L – lipid droplets; RE – endoplasmic reticulum; Au – autophagy.

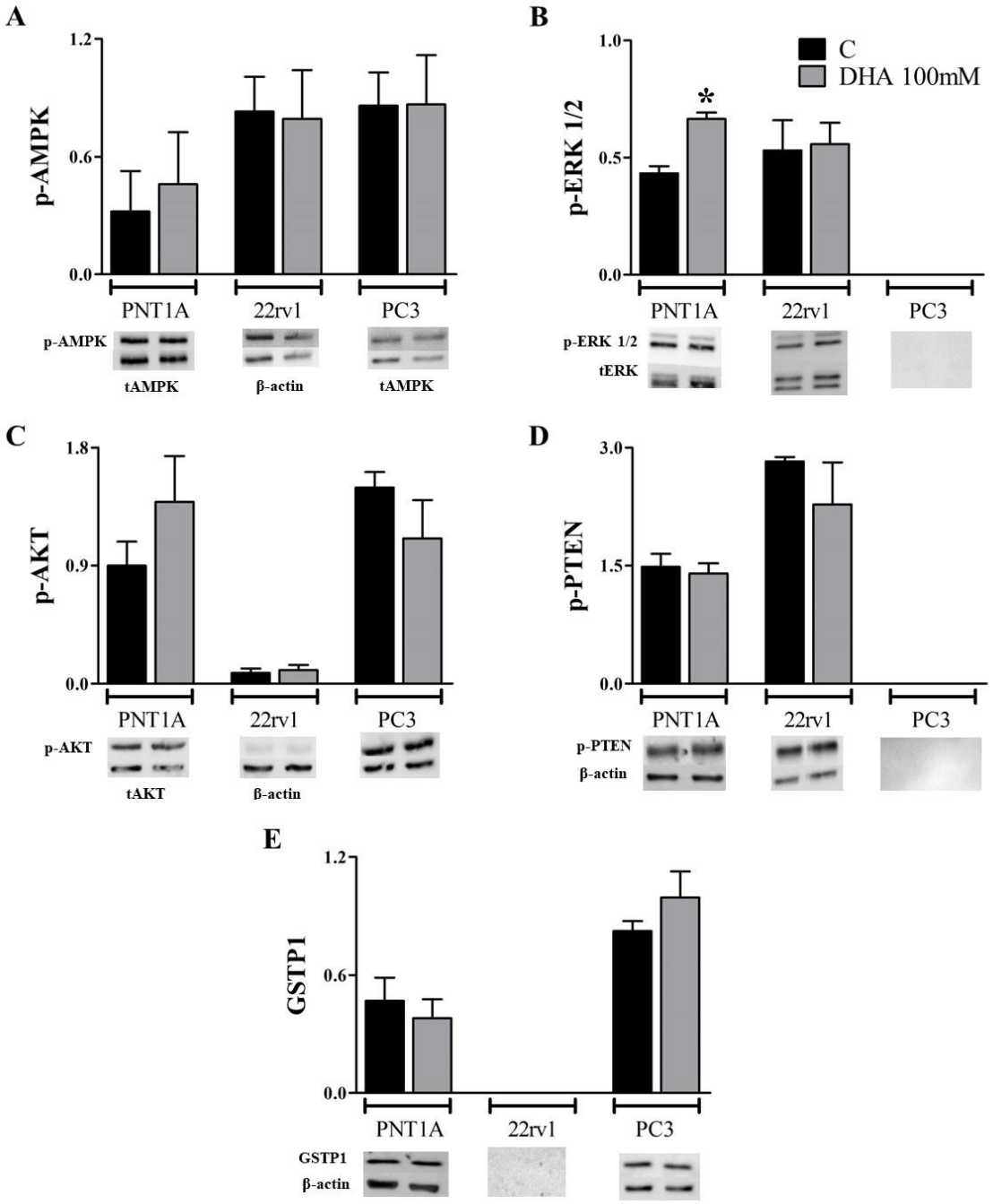

Fig. 6. Proliferation and survival pathways. DHA did not affect the phosphorylation of AMPK (A), AKT (C) or PTEN (D) in all cell lines which was also observed for GSTP1 expression (E). Increase of ERK 1/2 activation (B) was observed only in PNT1A. Values show the relative density normalized to β-actin or total form of the tested protein and SEM. For each blot, the samples were run in the same electrophoresis. Lack of bars or bands means no expression detected. Statistical analysis: * - different from C; samples from consecutive passages were considered (n = 4); all data showed non-parametric distribution (Mann-Whitney test); $p < 0.05$ was determined as statistically different.

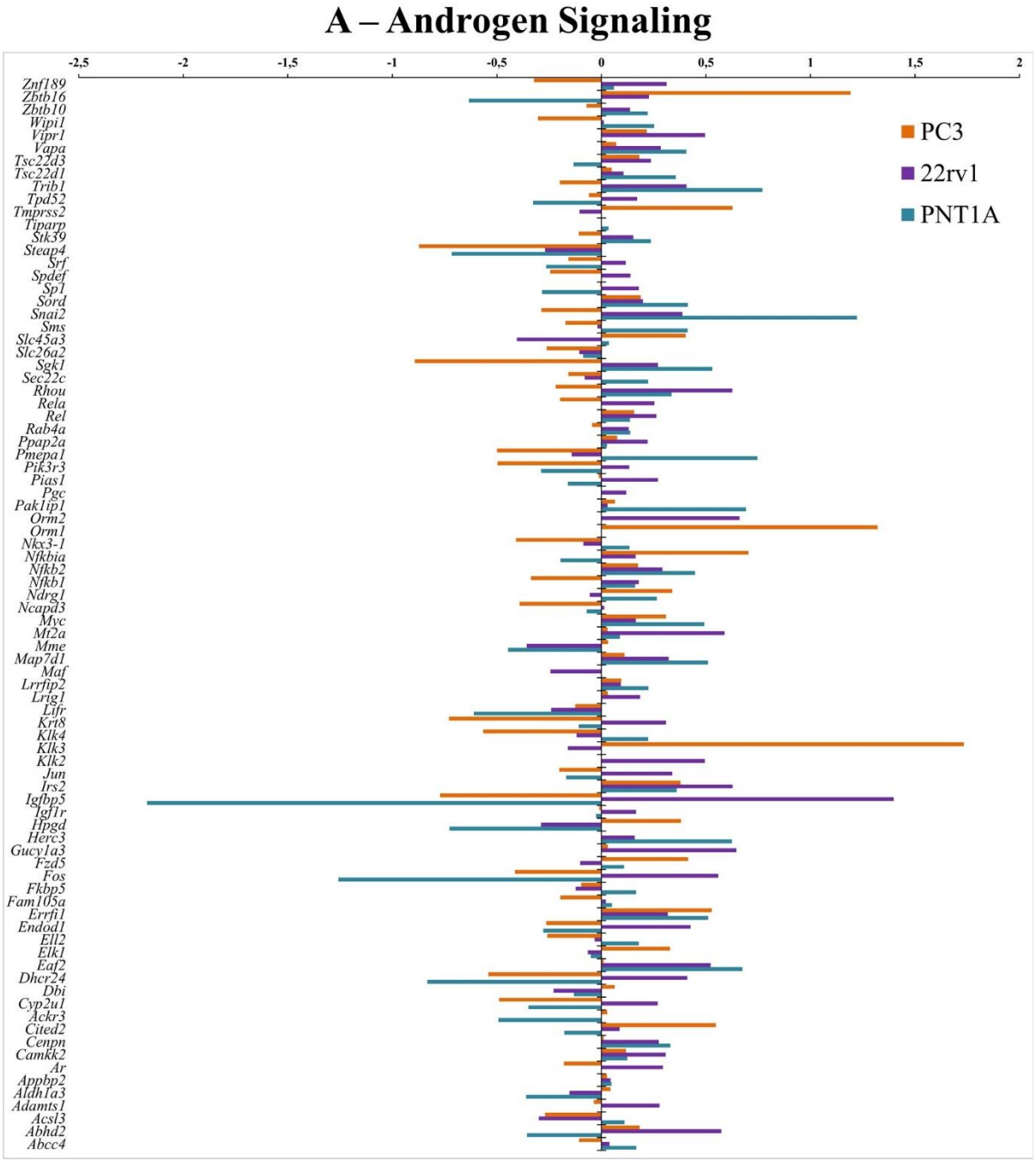

Fig. 7. (continued next page)

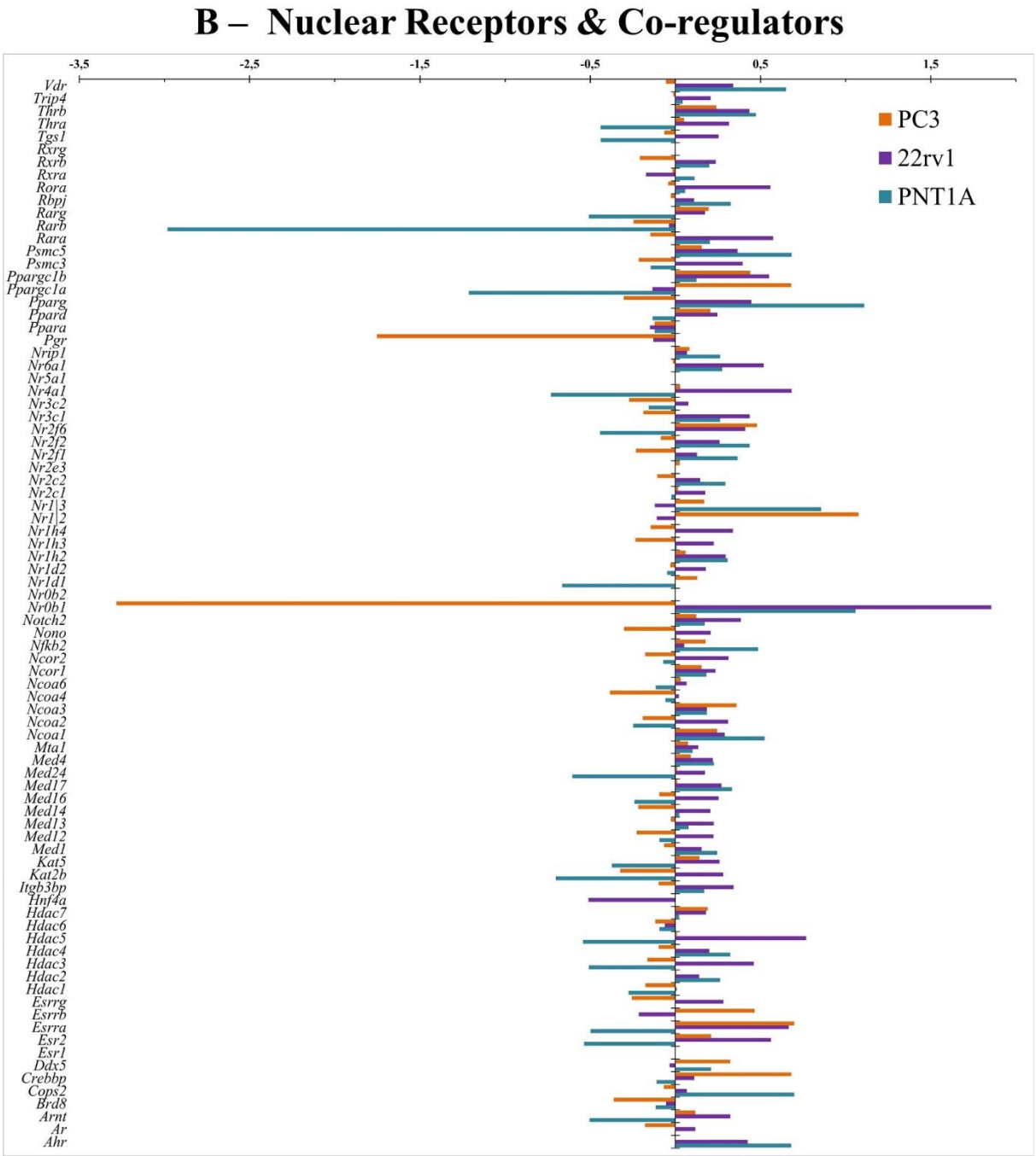

Fig. 7. Gene expression profile. (A) Androgen signaling gene expression (rows). Continue in the next page. (B) Nuclear receptors and co-regulators expression after DHA incubation for PNT1A (blue), 22rv1 (purple) and PC3 (orange) in a distinct pattern, as represented in the chart. Zero left bars indicate downregulated and right bars the upregulated genes (rows) whereas zero or lack the unchanged or undetermined. Three samples from three consecutive passages were pooled and analyzed by qRT-PCR array.

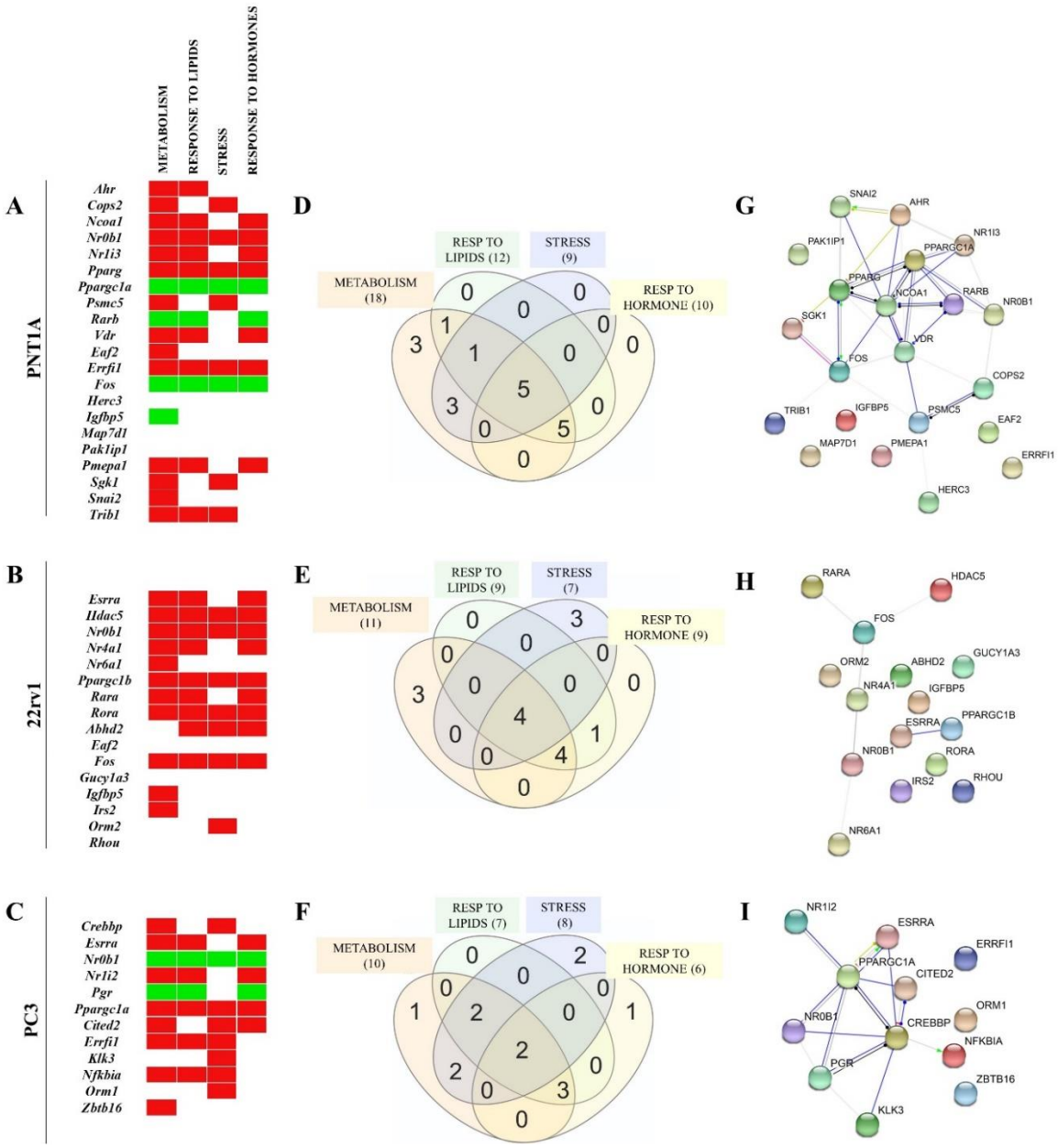

Fig. 8. Gene set functional analysis. DHA regulated mostly the genes associate to cell metabolism, stress and response to lipids or hormones. (A–C) Columns show functional category retrieved by GeneOntology database and squares the gene match. Red squares represent the upregulated genes and green the downregulated. (D–F) Venn diagrams highlighted the number of altered genes that matched to all functional categories for each cell line. (G–I) Estimated molecular interactions (STRING database). Legend: blue – binding; grey – undetermined; yellow – transcriptional regulation; pink - posttranslational modification; black – reaction; arrow – positive regulation; dot – unspecified; straight – negative regulation.

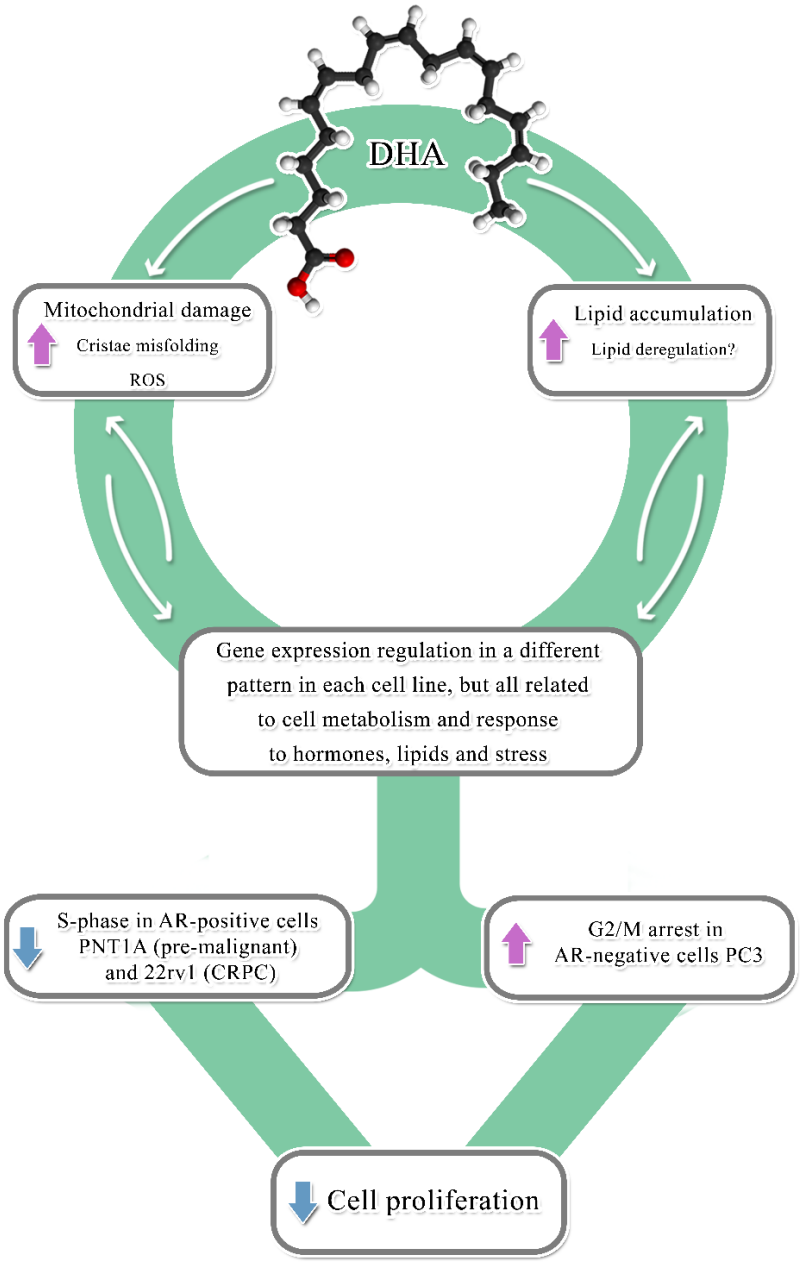

Fig. 9. Summary. DHA effects on pre-malignant and castrated-resistant cells with different androgenic backgrounds. Incubation with DHA at 100μM for 48 h led to lipid accumulation in addition to mitochondrial damage. The omega-3 alterations also regulated expression of several genes in different pattern in each cell line, but most was related to metabolism and response to hormone, lipids or stress. However, is not clear if this modulation was a cause or a consequence of mitochondrial alterations or lipid accumulation which still need to be addressed. As endpoint, DHA led to cell proliferation decrease which was related cell cycle modulation like delay (or reduction) in S-phase in AR-positive cells whereas G2/M arrest in AR-negative. Legend: DHA – docosahexaenoic acid; AR – androgen receptor; ROS – reactive oxygen species.

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CAPÍTULO 3

Mitochondria is a potential target of docosahexaenoic acid in pre-malignant and prostate cancer cells

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ABSTRACT

Prostate cancer (PCa) has metabolic vulnerabilities that may be adopted as therapeutic strategy, including mitochondria which undergoes to metabolic reprogramming among disease progression. Docosahexaenoic acid (DHA) is an omega-3 polyunsaturated fatty acid that shows antiproliferative properties due to metabolism modulation, but the underlying mechanisms are not clear. Here we investigated DHA potential to impair mitochondria function, membrane composition and biogenesis triggering apoptosis in pre-malignant and castrated-resistant cells. For this purpose, we incubated PNT1A, 22rv1 and PC3 cells with DHA at 100μM for 48h. DHA led to mitochondria dysfunction by decreasing its basal activity (54% 22rv1; 20% PNT1A and PC3), the ATP production (at least 25%) and strongly impaired bioenergetic reserve capacity (70% PNT1A; 51% 22rv1; 38% PC3), especially in PNT1A. The omega-3 seemed to increase glycolysis rate in PNT1A (23%) but decreased it in tumor cells which was followed by glucose complete oxidation (at least 1.2-fold), suggesting a metabolic shift. Such alterations were followed by mitochondrial hyperpolarization, ROS overproduction and changes in membrane phosphatidylglycerol composition, specially the DHA incorporation which led to increase in unsaturation index in PNT1A and a decrease in tumor cells. Compared to 22rv1 and PC3, DHA exerted stronger effect on PNT1A in terms of biogenesis impairment and mitochondria fragmentation. In addition, DHA downregulated 58% and upregulated 3.5% of mitochondrial genes tested in PNT1A solely, most of them with clinical relevance and related with the organelle homeostasis, influx and efflux from the matrix and organization. These mitochondria alterations reflected on apoptosis, increasing its levels in PNT1A (2.2-fold) and 22rv1 (1.5-fold), but not in PC3. Taken together, our findings showed that DHA, regardless of cell line, impaired mitochondria function and composition and may sensitize cells to other compounds due to decrease in bioenergetic reserve capacity. Also, it showed a stronger effect on pre-malignant cells, suggesting its preventive role.

Key words: prostate cancer, omega-3, polyunsaturated fatty acids, lipid metabolism.

INTRODUCTION

Mitochondria orchestrates many key functions in cell maintenance, as energy supply, Ca^{+2} concentration and availability, sexual steroids synthesis, reactive oxygen species (ROS) production and cell death. During carcinogenesis, its metabolism is reprogrammed and it mostly channels catabolic intermediates towards anabolic pathways, among others, substrates for *de novo* lipogenesis that is required for membrane synthesis and cell cycle progression (1), localized ATP production for enhanced invasion (2), apoptosis evasion and ROS production at levels that serve as mitogenic stimuli (3). In addition to this evidence, mitochondria depletion induces cell death in cancer cells which indicates that it plays a key role in cancer initiation and progression, becoming a potential therapeutic target (4,5)

Differently from most tumors, prostate cancer (PCa) undergoes to mitochondria reactivation from glycolytic profile, maintained by androgens and high zinc concentration, towards an oxidative phenotype (6). Compared to normal condition, PCa cells have increased mitochondria yield (7,8) and pleomorphism (9), alterations on its membrane composition due to reduction in saturated fatty acids and higher levels of cardiolipin (10) and overexpression of several genes related with biogenesis, bioenergetics and apoptosis (11). From a metabolic perspective, oxidative phosphorylation (OXPHOS) is remodeled in high-grade PCa with reduced respiratory capacity of glutamate and malate in the expense of higher succinate oxidation (12). Strikingly, the metabolic reprogramming in PCa seems also involve stromal components, as cancer-associated fibroblasts (CAFs) (13). CAFs lead to shift to OXPHOS through lactate release and mitochondria exchange with tumor cells (13). In addition, mitochondria seem to be involved in PCa progression via androgen receptor (AR) signaling. Androgen-responsive LNCaP cells show 4-fold more mitochondria than normal cells and 2-fold more than PC3, suggesting that androgen signaling might influence the organelle response (7). Indeed, AR is a central modulator of cell metabolism (14) and is imported into mitochondria (15) where it regulates the organelle dynamics (16) and the expression of electron transport chain (ETC) complexes subunits (15). Interestingly, loss and depletion of mitochondrial DNA seem to be required for androgen independency (17). Therefore, this body of evidence suggests that a spectrum of mitochondrial alteration is involved in PCa progression.

Mitochondria has been suggested as a target in PCa (18–20), including by fatty acids (20,21). Recently, we reported that the docosahexaenoic acid (DHA), an omega-3 poly-unsaturated fatty acid (PUFA), is capable of decrease cell growth via a differential modulation of cell cycle and metabolism between pre-malignant and castrated-resistant PCa cells (22). Compared to the pre-malignant PNT1A cells, we found that in 22rv1 and PC3 DHA induced higher superoxide anion ($O_2^{\bullet-}$) production and metabolic activity in addition to upregulate the expression of *Ppargc1a* and *Ppargc1b*, suggesting a potential regulation of mitochondrial biogenesis and function (22). Several studies reported apoptosis induction by DHA via mitochondria-mediated pathways, such as ROS production (21), Bcl-2/caspase axis (23) and organelle perturbation (24). Despite these findings regarding the negative effect on mitochondria, other studies reported the property of DHA to enhance the organelle function and increase cell survival (25,26). However, the data on mitochondria in PCa cells is scarce and remains elusive. Our studies regarding the pre-malignant prostate cells showed that such omega-3 decreased bioenergetic reserve, a mitochondria capacity to respond to insults (27). Indeed, DHA capacity to sensitize tumor cells has been reported in breast and colorectal cancers (28–30), but it is not clear for PCa although evidence suggests that it occurs through mitochondria.

PCa is among the main cause of death in men worldwide and, up to date, most of available strategies targets the AR signaling which can lead to the lethal castration-resistant phenotype (31,32). It has metabolic vulnerabilities involving mitochondrial metabolism (19,33), but is still poorly explored, mainly the role of fatty acids which seem to be distinctly metabolized between normal and tumor cells (34). Among others omega-3, as eicosapentanoic acid (EPA) and α-linoleic acid (ALA), DHA is the one with highest effectiveness in decrease cell viability and induce apoptosis (35). In addition, it is easily obtained from diet-enriched cold water marine fish and offered as nutritional supplementation with many benefits in health (36,37). In the present study, we tested the DHA capability to modulate mitochondrial parameters in the pre-malignant PNT1A cell line and two castrated-resistant, 22rv1 (AR-positive) and PC3 (AR-negative, neuroendocrine-like). Here we investigated whether DHA affected mitochondria function, biogenesis and the

metabolic profile, in addition to the expression of mitochondrial genes associated with cell death, homeostasis, dynamics and transport with clinical relevance.

MATERIAL AND METHODS

Cell culture and Incubations

Prostate epithelial human cell lines PNT1A (#95012614 – Health Protection Agency, England, UK), 22rv1 and PC3 (CRL1435) were cultured in RPMI 1640 medium (#R6504 – Sigma-Aldrich, USA; #11835030 – ThermoFischer Scientific, USA) supplemented with 10% fetal bovine serum (FBS) (#S0011—Vitrocell, Campinas, São Paulo, Brazil), 1% of penicillin, streptomycin, and amphotericin B (#15240062 – Life Technologies, UK) and kept in 5% of CO_2 at 37°C. For cell maintenance, the medium was replaced every 2–3 days and subculture done when confluence reached 80–90%. For experiments, cells were seeded at the desired density and attachment allowed for 24 hours.

Cells were incubated with DHA (#D2534—Sigma-Aldrich, USA) at 100µM for 48h, freshly prepared in the culture medium from a 20mM stock solution in anhydrous ethanol (vehicle). Both concentration and time of incubation were chosen based on our previous study showing different mitochondrial metabolic rate between benign and tumor cells with a decrease in the former and stimulation in the last, associated with reduction of cell proliferation (22). To ensure that the observed effects were mainly due to DHA, control assays were incubated with same vehicle volume and used reagents from same brand and condition.

Extracellular Flux Analysis

Two 100mm dishes were seeded with $2x10^6$ cells each and incubated with DHA or vehicle. After 24h of incubation, cells were collected with trypsin-EDTA 0.25%, counted, and reseeded at $2x10^4$ density in Seahorse XF96 V3 PS Cell Culture Microplates (#101085-004, Agilent Technologies Inc, USA) with the same medium to avoid changes in the DHA

concentration. Then, the Seahorse XF Cell Mito Stress Test assay (#103015-100, Agilent Technologies Inc, USA) was performed according to the manufactures' instructions. Briefly, Oligomycin (1µM), FCCP (2µM for PNT1A and 22rv1; 0.5µM for PC3) and Rotenone/Antimycin A (0.5µM) were loaded at the cartridge ports. FCCP best concentration and cell density per well were determined by prior titration experiments. Medium was removed from the microplate with the cells and replaced with warm and freshly prepared Seahorse XF Base Medium (#103681-100, Agilent Technologies Inc, USA), containing 1mM pyruvate, 2 mM glutamine, 10 mM glucose (pH 7.4). Microplate coupled to the cartridge was placed at 37°C non-CO_2 incubator for 45 minutes, followed by determination of oxygen consumption (OCR) and Extracellular Acidification rates (ECAR) with Seahorse XFe96 Analyzer (Agilent Technologies Inc, USA). At the end of readings, cells in each well were lysed with RIPA buffer and total protein determined by BCA assay for normalization. For statistical purpose, two independent experiments were performed with at least 10 replicates each (n=20). Values were shown as mean of pmolO_2/minute/protein for OCR or mpH/minute/protein for ECAR plus SEM.

ATP Content

$3x10^4$ cells per well were seeded on white clear bottom microplates and, at the end of experiment, 100µL of CellTiter-Glo® 2.0 Assay (#G9241, Promega, USA) at room temperature (RT) were added to each well and incubated for 10 minutes under shaking. Then, luminescence was determined with SpectraMax iD5 microplate reader (Molecular Devices) and 25µL of samples collected from each well to perform BCA assay for normalization. Three independent experiments were performed with at least 3 replicates each (n=9). Values were shown as mean of RFU and SEM.

Glucose oxidation

Cells were seeded at $0.5x10^6$ density in a 60mm dish and incubated with DHA as previously described. Glucose complete oxidation was determined by [^{14}C]-CO_2 release after

incubation with [^{14}C]-Glucose at 0.5 µCi/µl (ARC-0122D). Briefly, at the end of incubation 100µL of 70% perchloric acid were added to dish and immediately closed with a trapped Whatman filter grade 3 circle saturated with 200µL of phenethylamine. [^{14}C]-CO_2 capture was allowed for 2h at RT under slow shaking and circles collected for quantification. Radioactivity was determined by liquid scintillation in vials containing 10mL of UltraGold™ (#6013326 – PerkinElmer) and assessed with β-counter (Tri-carb 2910 TR Liquid Scintillation Counter, PerkinElmer). Counts per minute (CPM) were normalized to viable cells. Values were shown as fold-change to control and SEM. At least three independent experiments were performed for statistical analysis (n=6).

Phosphatidylglycerol quantification and unsaturation profile

Cells were seeded at 10^6 density and, after DHA incubations, collected with trypsin-EDTA 0.25%, centrifuged at 300g for 5 min at RT and pellets stored at -80ºC. Samples were shipped out and processed by Lipometrix (Leuven, Belgium). Briefly, cells homogenized in water (volume equivalent to 20 µg of protein) were mixed with 800 µl 1 N HCl:CH3OH 1:8 (v/v), 900 µl $CHCl_3$, 200 µg/ml of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT; Sigma Aldrich) and 3 cl of SPLASH® LIPIDOMIX® Mass Spec Standard (#330707, Avanti Polar Lipids). After vortexing and centrifugation, the lower organic fraction was collected and evaporated using a Savant Speedvac spd111v (Thermo Fisher Scientific) at RT and the remaining lipid pellet used for analysis after reconstitution in absolute ethanol. Lipid species were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) on a Nexera X2 UHPLC system (Shimadzu) coupled with hybrid triple quadrupole/linear ion trap mass spectrometer (6500+ QTRAP system; AB SCIEX). Chromatographic separation was performed on a XBridge amide column (150 mm × 4.6 mm, 3.5 µm; Waters) maintained at 35°C using mobile phase A [1 mM ammonium acetate in water-acetonitrile 5:95 (v/v)] and mobile phase B [1 mM ammonium acetate in water-acetonitrile 50:50 (v/v)]. Phosphatidylglycerol (PG) was measured in negative ion mode by fatty acyl fragment ions. Lipid quantification was performed by scheduled multiple reactions monitoring (MRM). Peak integration was determined with the MultiQuant™ software version 3.0.3. Signals were corrected for isotopic

contributions (calculated with Python Molmass 2019.1.1), quantified based on internal standard signals and adheres to the guidelines of the Lipidomics Standards Initiative (LSI) (level 2 type quantification as defined by the LSI). Three independent experiments were performed (n=3) for each condition and cell line.

Mitochondrial Membrane Potential and Superoxide anion

To determine the mitochondrial polarization, we performed a ratiometric assay with JC-1 dye (#ab113850, Abcam, USA). Cells were seeded at $3x10^4$ density per well in a black microplate with clear bottom and, at the end of experiment, warmed JC-1 was added to reach 10 µM in the wells. After incubation for 20 min at 37ºC, the dye was removed, and wells washed twice with 1X dilution buffer provided by the manufacturer. Fluorescence intensity (FI) was assessed at 535/590nm and 475/530nm to quantify aggregated and monomeric forms, respectively. Mitochondrial membrane potential was determined by the ratio of FI aggregates to FI monomers. FCCP at 100µM was added 4h prior to JC-1 in one well and used as reference for mitochondrial membrane depolarization. Values were shown as mean and SEM.

Superoxide anion production was assessed by MitoSOX™ Red dye (#M36008, ThermoFischer). Cells were incubated at $3x10^4$ density per well in a black microplate with clear bottom and, at end of DHA incubation, medium replaced with 5 µM of the dye followed by 20 minutes incubation at 37ºC. Then, wells were washed in warm PBS and FI assessed at 510/580nm. At the end of readings, RIPA buffer was added to each well and protein content determined by BCA assay for normalization. Values were shown as fold-change and SEM. For both assays described in this section, three independent experiments were performed (n=7 at least) and FI collected with SpectraMax iD5 microplate reader (Molecular Devices).

Mitochondria Biogenesis

To assess mitochondria morphology and distribution, cells at 10^4 density/well were seeded in CELL^{view} slide (#543999 – Greiner Bio-one). Mitochondria and nuclei visualization were done after staining with MitoTracker Orange CMTMRos (#M7510 Molecular Probes, Invitrogen) at 50 nM and Hoechst 33342, respectively. After 15 minutes incubation at 37°C and 5% CO_2, staining solution was removed, cells fixed with 4% paraformaldehyde, washed twice in PBS and slide covered with mounting media. Mitochondria network was assessed at 554/576 nm with inverted super-resolution microscope AiryScan LSM880 (Carl Zeiss AG, Germany) and DNA at 350/461 nm at 63x objective. Maximum projection image was adopted for analysis.

Biogenesis was determined by MitoBiogenesis™ In-Cell ELISA Kit (#ab140359, Abcam) to determine the mitochondrial-encoded and nuclei-encoded gene expression ratio. For this purpose, cells at $3x10^4$ density per well were seeded in a black clear bottom microplate. FI of succinate dehydrogenase A (SDH-A) and subunit I of Complex IV (COX-I) were determined according to manufactures' instructions and collected with SpectraMax iD5 microplate reader (Molecular Devices). Three independent experiments were performed with at least 3 replicates each (n=9).

Mitochondrial Gene Expression Analyses

RT-qPCR arrays were performed with Qiagen (Hilden, GE) supplies as described by Tamarindo and Góes (2020) (22). Briefly, 2 x 10^6 cells were seeded in culture flask, and, after incubation, total RNA were obtained with RNAeasy Plus Mini kit (#74134). RNA quantification and purity were determined in NanoDrop spectrophotometer ND-1000. Prior to qRT-PCR arrays, RNA was checked for integrity in agarose gel 1% and cDNA obtained with First Strand Kit (#330404) in a thermocycler (Multigene – Labnet International Inc.). Then, cDNA quality, RT-qPCR efficiency and gDNA contamination were tested with QC Human array (PAHS-999ZC). RT² Profiler for Human Mitochondria (PAHS-087Z) arrays were performed with SYBR Green® ROX/MASTERMIX (#330523) in StepOnePlus PCR

Real Time System (Applied Biosystems) for pooled samples (5µg) from three consecutive experiments that passed the quality test. Gene expression changes were analyzed by ΔCt (cycle threshold) (38), subtracting the average Ct values obtained from the housekeeping genes (*Actb*, *B2m*, *Gapdh*, *Hprt1* and *Rplp0*) provided in the array from those of each gene of interest. Genes that doubled or halved their expression compared to vehicle were considered as potentially regulated and their biological function previously determined by the array manufacturer. Gene set enrichment analysis (GSEA) for those that passed the cut-off was performed with GSEA_4.1.0 software (39) in a pre-Ranked module using fold-change as input. Hallmarks (v.7.4), curated genes (v.7.4) and Gene Ontology (v.7.4) molecular signatures data base were run as references. Those output with false discovery rate (FDR) ≤ 0.20 were considered enriched.

To determine whether the tested genes are relevant in PCa human samples, we submitted them to cBioPortal platform (40). No overlapping cohorts with at least 100 samples were chosen and retrieved both overall and disease-free survival. In the present study, our aim was to understand the contribution of those mitochondrial genes on PCa onset and to avoid biased interpretations, *Tp53* (p53) was excluded from this analysis because it is already frequently altered.

Annexin V assay

Cell death was assessed by flow cytometry using the BD Pharmingen™ Annexin V Apoptosis Detection Kit I (#559763, BD Biosciences). Briefly, cells were seeded at 10^6 density and, at the end of DHA incubation, collected with trypsin-EDTA 0.25%, centrifuged at 1500 RPM for 5 minutes at RT and stained according to the manufacturer's instructions. Cell populations were immediately gated as viable (Annexin V-/7-AAD-), early apoptosis (Annexin V+/7-AAD-), late apoptosis (Annexin V+/7-AAD+) and potentially necrosis (Annexin V-/7-AAD+). Data was acquired for 10^4 events with BD FACS Canto II using BD FACS Diva™ software and analyzed with Flowing 2.5.1 software. Values were shown as fold-change from three independent experiments and a representative flow cytometry chart was plotted for each tested condition.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism® software (v.9.2.0). First, distribution of samples was analyzed with Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Parametric distributions were submitted to unpaired *t*-test and non-parametric distributions to Mann-Whitney. $p<0.05$ was considered statistically different.

RESULTS

DHA impairs mitochondria function, ATP production and bioenergetic reserve

The first step to investigate whether DHA affects mitochondria was to determine the organelle function. Figure 1A and 1B show the OCR and ECAR profile, respectively, after incubation with DHA or vehicle for each cell line. The omega-3 decreased the basal OCR in all cell lines (Fig.1C), with strongest effect on 22rv1 (54%) and similar level on PNT1A and PC3 (around 20%). The OCR required for ATP production also decreased in the same manner (Fig.1D). Therefore, we assessed the ATP content (Fig.1E) to determine whether cells use less oxygen to generate ATP or its production is reduced. We found out that DHA reduced it in all cell lines (39% in PNT1A, 25% in 22rv1 and 32% in PC3), confirming that ATP production was impaired. Then, we investigated the mitochondrial ability to respond to stress. We observed that in all cell lines, DHA decreased both maximal respiration (Fig.1F) and the spare capacity (Fig.1G). In both parameters, the strongest effect was on PNT1A with a decrease in 70% of the spare capacity, compared to 51% and 38% in 22rv1 and PC3, respectively. Mitochondria dysfunction and ROS production are related with proton leak. It increased in PNT1A but decreased in 22rv1 while remained unchanged in PC3. Then, to understand whether cells rely on glycolysis we interrogated the basal (Fig.1I) and glycolytic (Fig.1J) ECAR in addition to CO_2 production from glucose (Fig.1K). In the absence of any perturbation, DHA induced 23% the extracellular acidification in PNT1A and

reduced it 40% in 22rv1 (Fig.1I). Under mitochondrial ATP synthesis inhibition (Fig.1J), it reduced only in tumor cells (40% 22rv1 and 20% PC3). Glucose complete oxidation (Fig.1K) increased in tumor cells at least 1.2-fold, but unchanged in PNT1A.

DHA induces ROS, mitochondrial membrane hyperpolarization and changes PG unsaturation status

To better understand changes in the energetic profile we looked at mitochondrial membrane polarization (Fig.2A) because it is directly associated with mitochondrial function and ROS production. We found out that DHA induced mitochondrial membrane hyperpolarization in all cell lines (PNT1A 27%; 22rv1 92%; PC3 23%). Since such alteration may induce ROS overproduction, we checked superoxide anion levels (Fig.2B), an indicator of mitochondrial dysfunction. Our data showed that the omega-3 increased its production at least 1.5-fold compared to vehicle in all cell lines. Changes in mitochondria function are closely related with its membrane composition. Thus, we checked the main phospholipid found in mitochondria and precursor of cardiolipin (CL), PG (Fig.2C-F). PG content decreased 65% in PNT1A followed by increase in the fatty acid unsaturation (3,4,5 and 6 double bonds) profile and a reduction in the saturated fraction. Also, PG fatty acids with 14-18 carbons decreased while increased those with 20 and 22, including DHA (20:6) (Fig.2D). Regarding the tumor cells, it exerted an opposite effect since we found 40% more PG but reduction on the unsaturation status, mainly due to increase in the saturated fatty acids and decrease in the monosaturated ones. Additionally, DHA decreased fatty acids with 2 and 4 double bonds in PC3 but increased those with 5 in 22rv1. The PG fatty acid composition showed the same PNT1A pattern for 22rv1 (Fig.2E), but PC3 seemed to be less responsive (Fig.2F). In both tumor cells, 22:6 fatty acid increased indicating DHA incorporation into PG (Fig.2E-F). Also, in PNT1A and 22rv1 20:5 fatty acids (EPA) increased in PG composition.

DHA regulates mitochondrial gene expression profile and impairs mitochondria biogenesis in pre-malignant but not in tumor cells

To determine whether mitochondrial dysfunction by DHA impacts the biogenesis, we examined mitochondrial network. DHA clearly induced mitochondria fragmentation in PNT1A cells evidenced by round mitochondria under super-resolution microscopy (Fig.3A). However, this was not observed on tumor cells since they displayed elongated mitochondria organized as a network, as in control incubation. To determine whether such fragmentation is due to impaired biogenesis, we assessed the nuclei- and mitochondria-encoded proteins expression. DHA induced asymmetrical SDH-A (Fig.3B) and COX-I expression (Fig.3C) leading to the ratio increase (Fig.3C), mainly due to increase in the last.

To further investigate DHA effects on mitochondria, we examined its gene expression profile (Fig.4A-B) related with homeostasis, organization, transport and cell death. Our data revealed that for PNT1A 58% of the evaluated genes were downregulated and 3.5% upregulated. The GSEA analysis showed that the potentially regulated genes in PNT1A were intrinsic components of the mitochondrial membrane with negative NES of 1.6 (Fig.4A-inset). Regarding the tumor cells, 99% remained unchanged, except for *SLC25A20* which was upregulated on 22rv1 and *SH3GLB1* on PC3. Then, we inquired their relevance on PCa onset. Data retrieved from several cohorts showed that once altered, the deregulated genes observed on PNT1A cells decreased both overall (Fig.4C) and disease-free survival (Fig.4D).

DHA induces cell death in AR-positive cells

Given that mitochondria dysfunction is strictly related with cell death, we estimated early and late apoptosis (Fig.5). Regarding PNT1A cells, we found that on average DHA induced 2.2-fold more late apoptosis (Fig.5A-C) events than vehicle. For tumor cells, apoptosis was clearly observed only in 22rv1, being 1.65- more early events and 2.2-fold the

late apoptosis (Fig.5D-F). However, cell death seemed not be triggered by DHA on PC3 (Fig.5g-I).

DISCUSSION

Mitochondria vulnerabilities have been explored as a therapeutic alternative in PCa (33), but the role of fatty acids, mainly DHA remains unclear. In the present study, we observed that DHA impaired mitochondria function, induced ROS overproduction and led to changes in mitochondria membrane composition either in pre-malignant or tumor cells. However, DHA seemed to exert different effects between them, being stronger on the pre-malignant, since it induced proton leak, had the most impaired spare capacity, downregulated several mitochondrial genes related with membrane components and disrupted the organelle biogenesis which reflected on its fragmentation.

PCa metabolism is very different from most solid tumors, since it exhibits increased OXPHOS (19,41), *de novo* lipogenesis (42,43) and rely on fatty acids β-oxidation for energy supply (44) while most of cancers are glycolytic (6). We have reported that DHA at same conditions used herein induced lipid accumulation, changes in mitochondria cristae and decreased cell population growth associated with metabolism-related genes deregulation, including mitochondrial at some extent (22). Thus, it is in line with mitochondria dysfunction and depletion of ATP production found in all cell lines, and together with our previous data, indicates that DHA targets PCa vulnerabilities. It was reported previously that it lowered ETC complexes in cardiac tissue (45) and here we found that in addition to dysfunction, DHA led to different metabolic adjustment between PNT1A and tumor cells. The former seemed to compensate the mitochondrial malfunction with increase in glycolysis rate reflected on higher basal ECAR, but not the glycolytic reserve. This was expected, since prostate benign cells already rely on anaerobic metabolism (6) and DHA was reported to upregulate glycolysis pathways genes concurrent to lactate production in normal tissue (46). However, the opposite effect was observed for tumor cells, even though there was only a trend ($p=0.09$) for PC3 basal ECAR. This suggests that DHA impairs not only mitochondria but also

glycolysis as energy source which is a relevant outcome for castrated-resistant cells. Glycolysis increase correlates with very-high risk for resistance to castration in PCa (47), stimulates cell proliferation and migration (48) and acts as anabolic pathway in cancer (49,50). Despite we did not evaluate the mechanisms behind ECAR, we can speculate that DHA shifted glucose towards its complete oxidation. This is supported by increase in [^{14}C]-CO_2 release after radio-labeled glucose incubation in 22rv1 and PC3, but not PNT1A. In addition, *SLC25A20* upregulation in 22rv1 and PNT1A suggests increased fatty acid utilization as energy source (51), specially DHA. This gene product is a carnitine-acylcarnitine translocase embedded in the inner mitochondrial membrane that mediates the long-chain fatty acid transport for oxidation (52). *SLC25A20* is naturally upregulated in PC3 (51) which points out that both tumor and pre-malignant cells have metabolic flexibility and suggests a mechanism of their resistance, but not stronger than DHA effect. Taken together, our findings showed the omega-3 led to mitochondria dysfunction in both pre-malignant and PCa cells, changing glucose utilization in the last which are crucial for cell proliferation and survival.

A supportive finding of mitochondria dysfunction is the $O_2^{\bullet-}$ overproduction, which has even been used as indicator (22,27). DHA induces mitochondrial ROS (21,22,27,53) which is closely related with membrane hyperpolarization (54–56) and is maintained by reduced activity of ETC complexes (57). Such increase in membrane polarization is an early event on apoptosis and independent from caspases (58) and matches the apoptosis observed in AR-positive cells. However, this was not observed in PC3 cells which had lower hyperpolarization, downregulation of *SH3GLB1*, a pro-apoptotic gene, and seemed to evade apoptosis via cytochrome c degradation (data not shown) (59). ROS production was demonstrated to induce apoptosis (21,53) and decrease the bioenergetic reserve capacity (60) as well as the oxidized lipids (61). Once the bioenergetic reserve capacity is impaired, the potential for drug resistance is dramatically weakened. This is of particular interest for castrated-resistant tumor cells given that several studies have reported the DHA property to sensitize cancer cells to other compounds (27) and increase the efficacy of chemotherapy (28–30). Taken together, our data suggests that DHA could be used as co-adjuvant in chemotherapy, but further studies are required.

An interesting finding of our work was the distinct effect of DHA on PG amount, fatty acid composition and unsaturation index between pre-malignant and tumor cells. PG is a precursor of CL (62) which represents 15-20% of inner mitochondrial membrane, stabilizes the ETC complexes (63) and is determinant on the cristae curvature due to its unique structure (64). The increase of 20:6 FA composition indicates that DHA is incorporated into PG hence likely into CL, which is susceptible to oxidation by ROS (65), leading to ATP production depletion and cell death. Indeed, *n*-3 fatty acids enriched diet was reported to increase the unsaturation index of mitochondrial fatty acid in normal tissue (66) and low PUFAs on its phospholipids associated with resistance to ROS (67). This is of particular interest for PNT1A because of its remarkable increase in PG unsaturation index and early apoptosis, but also *TAZ* downregulation, a CL remodeling protein required for mitochondria quality control and mitophagy (68). Despite changes in fatty acid composition were less responsive in the tumor cells, they showed an increase in saturated fatty acids, a pattern opposite to observed in PCa samples (10). Increase in the saturation to unsaturation ratio are related with more fluid mitochondrial membrane which affects the organelle function. Therefore, this data indicates that CL-enriched DHA increased in PNT1A given that PG is reduced, leading to higher unsaturation index, increased susceptibility to ROS and mitochondria dysfunction. On the other hand, tumor cells showed PG excess which is harmful to mitochondria function (69) and suggests that CL synthesis is impaired.

We proposed previously that mitochondria impairment is a potential preventive strategy in pre-malignant cells (27) and here we described further the impact of DHA on them. Plenty of the deregulated genes in PNT1A govern mitochondria homeostasis, influx and efflux from the matrix and impact the organelle metabolism. In addition to them, *COX18* is a key protein in cytochrome oxidase assembly (70) hence its downregulation likely impairs OXPHOS. The fission-like shape and network fragmentation reflect organelle dysfunction (71), the glycolytic phenotype (72) and ROS overproduction (54,72). In this context, the fusion-related *MFN1* and *MFN2* as well as the fission-related *DNM1L* and *FIS1* proteins, all key genes involved in mitochondria dynamics, were downregulated. Despite we expected upregulation in the two last genes, other proteins may regulate mitochondria dynamics (73). Indeed, we showed that at same conditions, DHA downregulated *PPARGC1A* in PNT1A but

not in tumor cells and it coordinates mitochondria biogenesis (22). Therefore, together these findings could explain the stronger reduction in bioenergetic reserve and proton leak. Also, we reported here that DHA downregulated a plethora of genes with clinical relevance, being part of them overexpressed in PCa, including *TSPO* (74) and *UCP2* (75).

CONCLUSION

In conclusion, DHA induced mitochondrial dysfunction and cell capability to respond to insults in both pre-malignant and castrated-resistant PCa cells, but also impaired glucose metabolism in the last. The omega-3 seemed to decrease cell growth through different mechanisms between AR-positive and AR-negative tumor cells, but regardless of pathway, it sensitizes cells to other compounds and should be considered alone or in combination with chemotherapy. Also, the omega-3 led to stronger effects on pre-malignant cells affecting not only function, but also plenty of mitochondrial gene expression and biogenesis, triggering apoptosis. Thus, our findings provide evidence of the preventive potential of DHA at early stages of carcinogenesis and support for *in vivo* studies.

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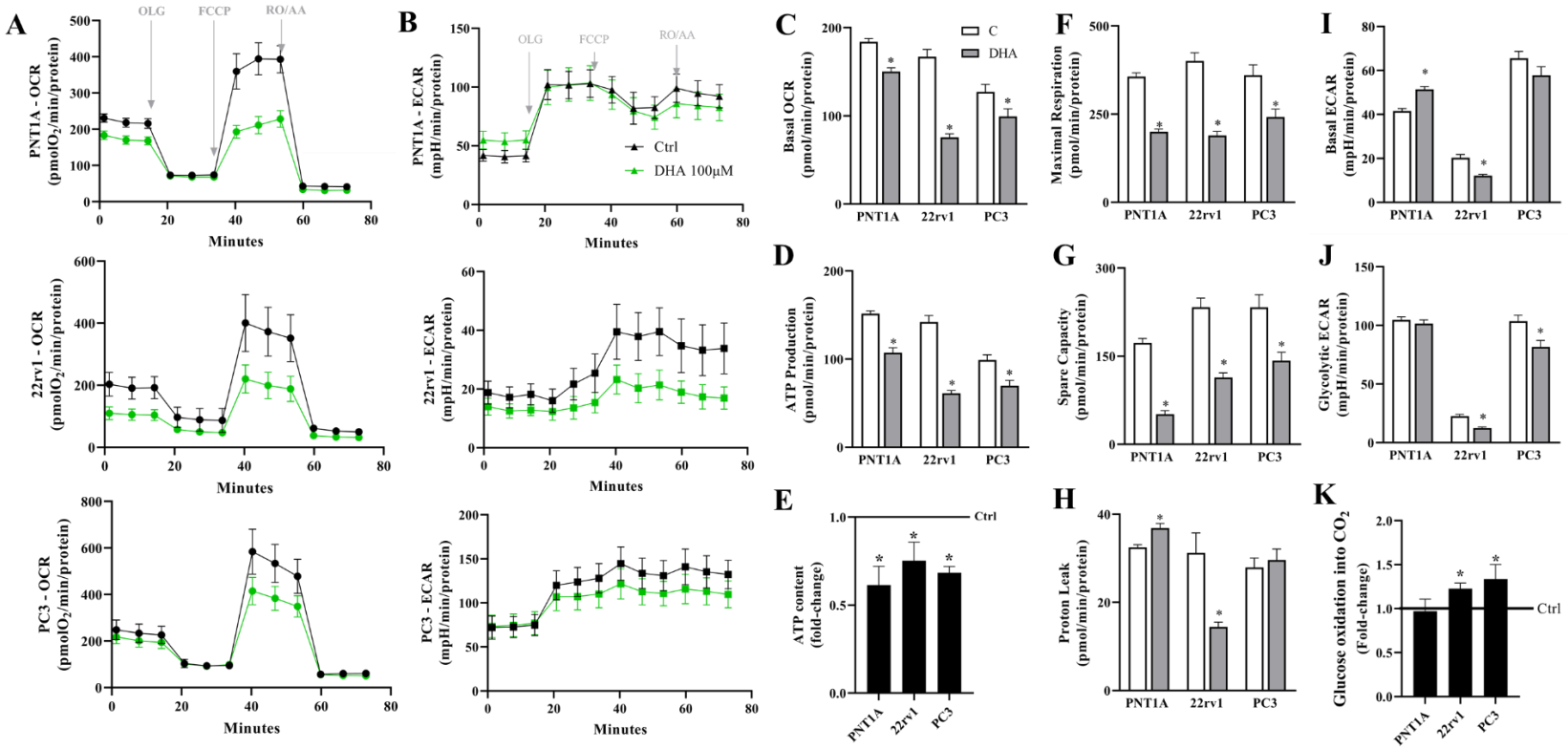

Figure 1. *DHA induces mitochondria dysfunction in pre-malignant and castrated-resistant prostate cells.* **A)** Oxygen Consumption Rate (OCR) and **B)** Extracellular Acidification Rate (ECAR) representative profiles for each cell line. Black lines show control while green the DHA incubation. **C)** Basal OCR without any inhibitors indicated mitochondria activity reduction. **D)** OCR required for ATP production and **E)** ATP content, meaning decrease on its production. **F)** Maximum Respiration and **G)** Spare capacity showed DHA bioenergetic reserve decreased. **H)** Proton Leak. OCR values show mean and SEM of pmolO_2/minute/protein (n=20, two independent experiments) while ATP content indicates fold-change relative to control (n=9, three independent experiments). **I)** Basal ECAR and **J)** ECAR under oligomycin inhibition, suggesting glycolytic rate. Values show mpH/minute/protein and SEM (n=20, two independent experiments). **K)** Glucose complete oxidation into CO_2. Values show fold-change to vehicle and SEM (n=6, three independent experiments). Legend: Ctrl – vehicle incubation; DHA – docosahexaenoic acid; * - statistically different from control ($p < 0.05$) after unpaired *t*-test.

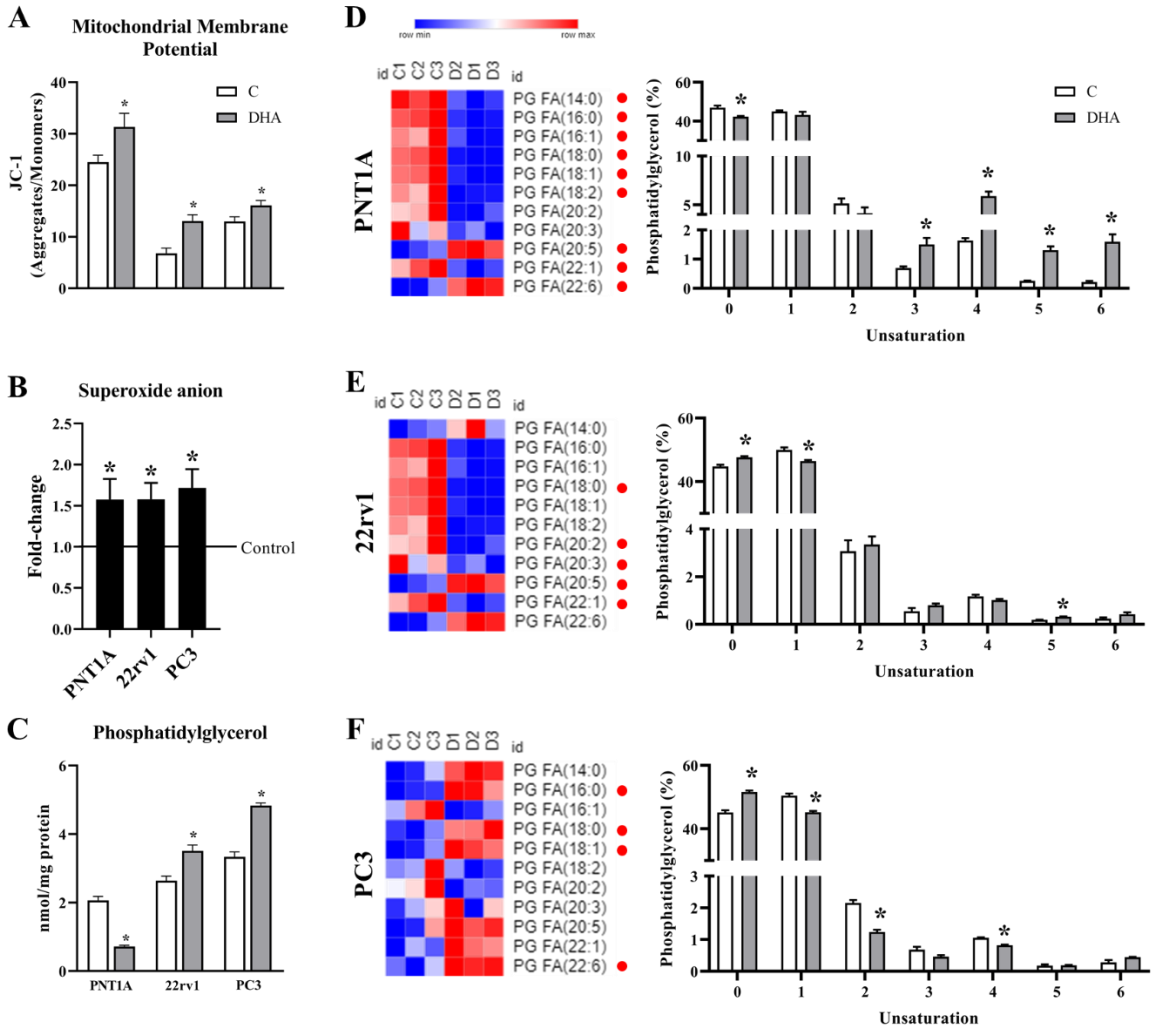

Figure 2. *DHA leads to mitochondria hyperpolarization, $O_2^{\bullet-}$ overproduction and changes in phosphatidylglycerol composition.* **A)** Increase in mitochondria membrane potential after DHA incubation, assessed with JC-1 dye. Values show mean of J-aggregates to monomers ratio and SEM (n=9). **B)** $O_2^{\bullet-}$ overproduction determined by MitoSOX Red. Values show mean of fold-change relative to control and SEM (n=9). **C)** Phosphatidylglycerol concentrations in each cell line after DHA or vehicle incubation. Values show mean of nmol/mg of protein and SEM (n=3). **D-F)** Heat maps on left display the most significant phosphatidylglycerol fatty acid composition determined by mass spectrometry. Red squares in each row mean increase and blue decrease in concentration scaled to z-score. Red dots showed statistically different compared to control. Graphs on the right side indicate the fatty acid unsaturation index. Values show percentage and SEM (n=3). Three independent experiments were performed for statistical analysis. Legend: FA – fatty acid; PG – phosphatidylglycerol; * - statistically different from control ($p < 0.05$) after unpaired *t*-test.

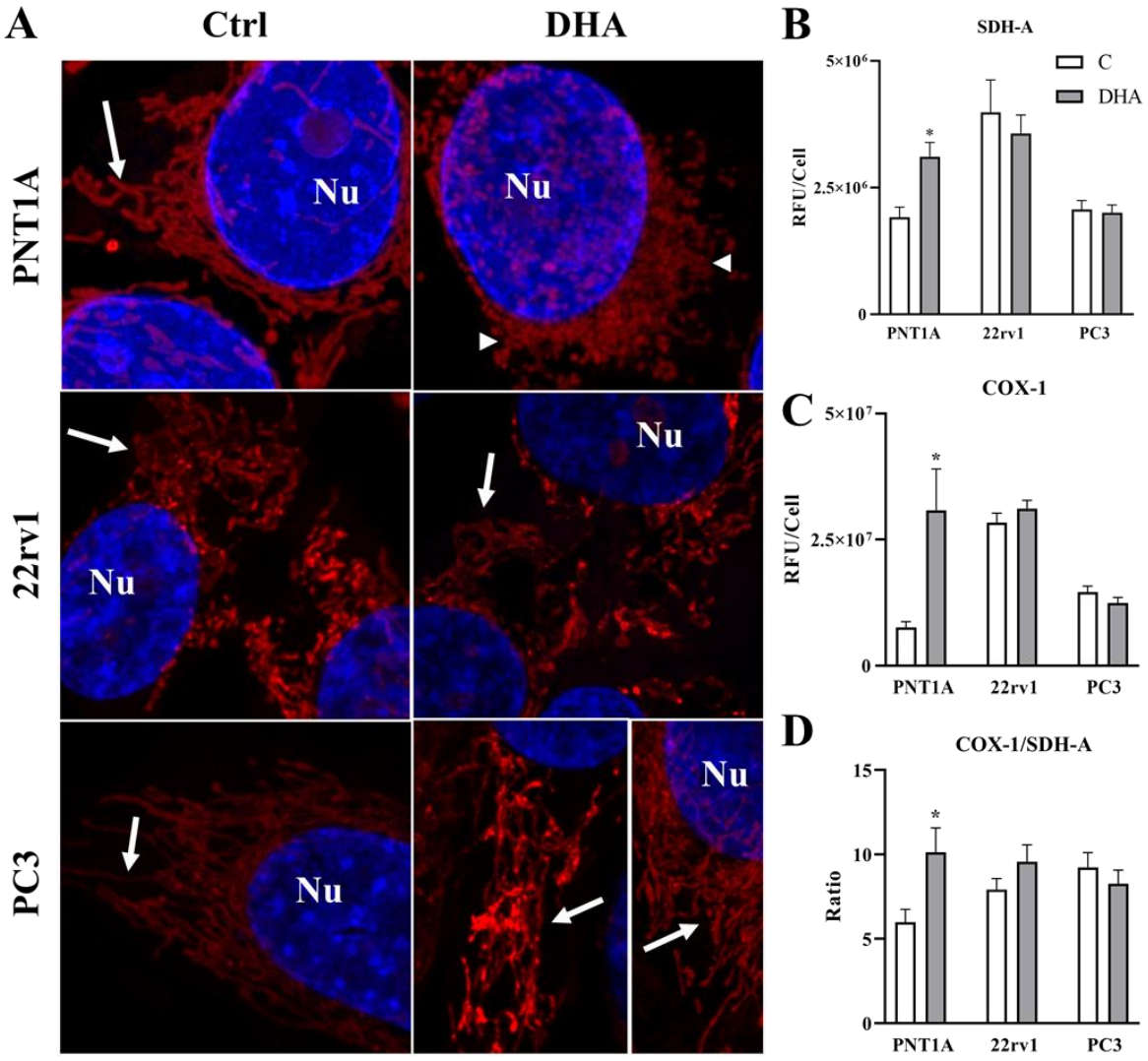

Figure 3. *DHA impairs biogenesis and induces mitochondria fragmentation in pre-malignant cells.* **A)** Mitochondria network in red and nuclei in blue (Nu) evidenced by MitoTracker Orange CMTMRos dye and Hoechst 33342, respectively. Arrows points to elongated mitochondria whereas arrow head to the fragmented. Images were captured at 400x magnification. **B)** Expression of Succinate dehydrogenase A (SDH-A), a mitochondria-encoded protein, **C)** Subunit I of Complex IV (COX-I), a nuclei-encoded, and **D)** COX-I to SDH-A ratio. Values show mean of Relative Fluorescence Units (RFU) per cell and SEM. Three independent experiments were performed for statistical analysis. Legend: C or Ctrl – control (vehicle); DHA – docosahexaenoic acid; * – statistically different from control ($p < 0.05$) after unpaired *t*-test.

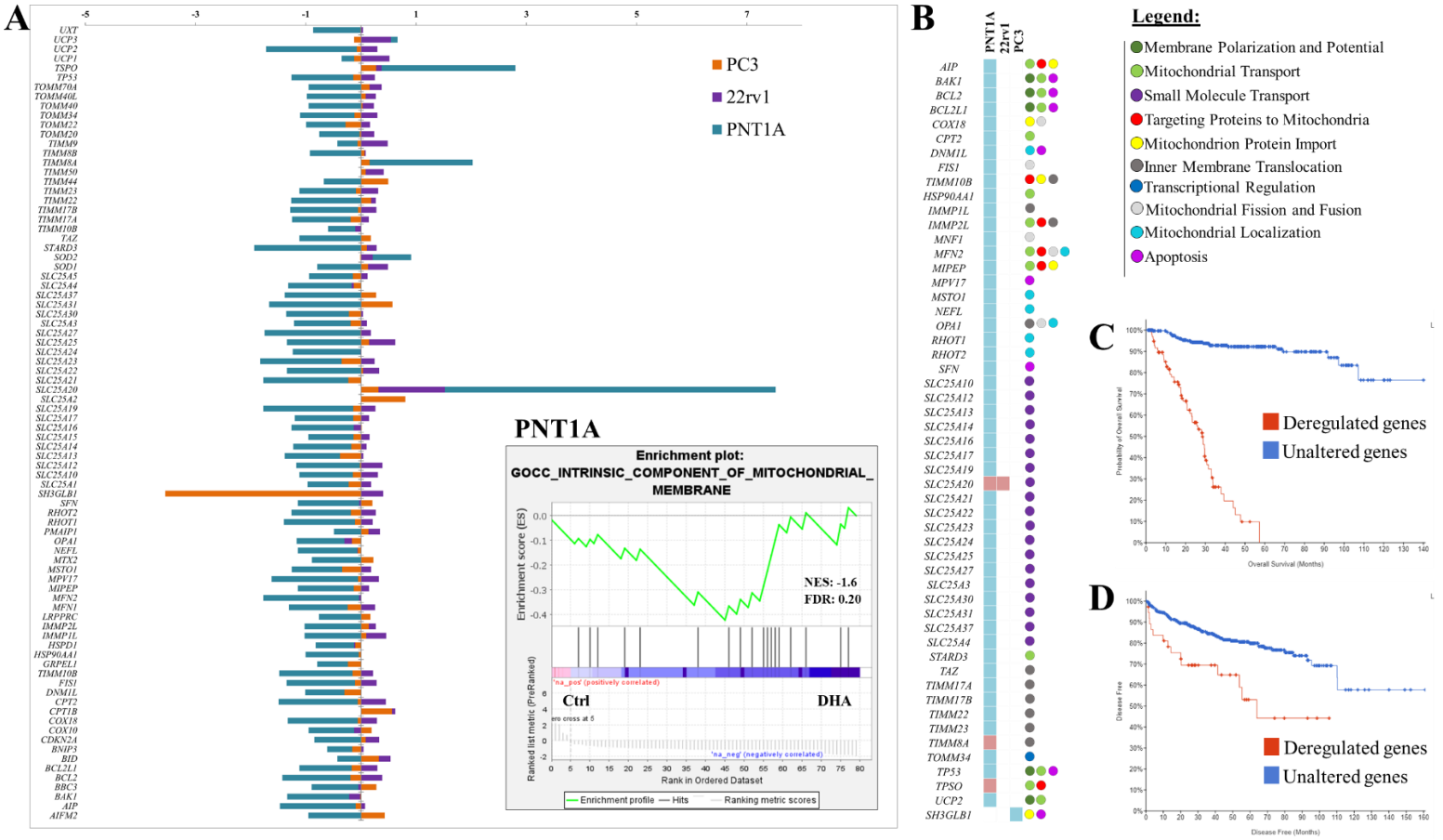

Figure 4. *DHA downregulates mitochondria membrane genes.* **A)** Mitochondrial gene expression profile for PNT1A (blue), 22rv1 (purple) and PC3 (orange) determined by RT-qPCR array. Values above 1 or lower than -1 were considered as potentially regulated. **Inset)** Gene set enrichment analysis indicates a downregulation of genes related with membrane components (NES -1.6 and FDR 0.20). **B)** Potentially affected genes (rows) by DHA for each cell line (columns). Blue squares mean downregulation, pink upregulation and white unchanged. Biological function was labeled in a color system (circles) aside. **C)** Disease-free and **D)** Overall survival of PCa patients with alterations in the mitochondrial genes modulated by DHA (red line), showing the clinical relevance of the omega-3.

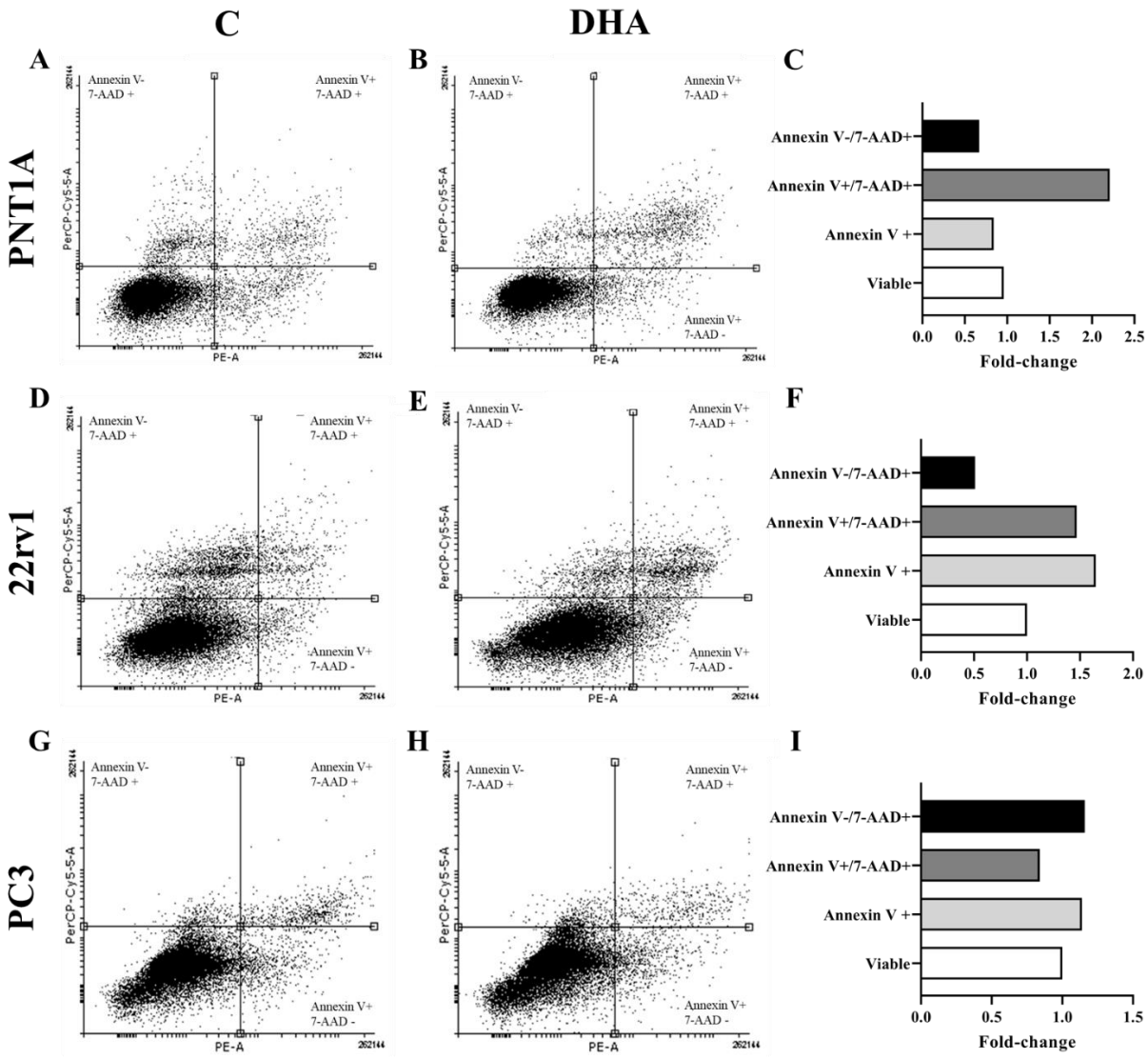

Figure 5. *DHA triggers apoptosis in AR-positive cells, but not AR-negative.* Representative flow cytometry charts for **A-C)** PNTA, **D-F)** 22rv1 and **G-I)** PC3 populations. DHA induced apoptosis in 22rv1 and PNT1A which was not clear for PC3. Legend: Annexin V-/7-AAD- – viable population; Annexin V-/7-AAD+ - potentially necrosis; Annexin V+/7-AAD- – early apoptosis; Annexin V+/7-AAD+ – late apoptosis. Three independent experiments were performed (n=3) and acquired 10^4 events.

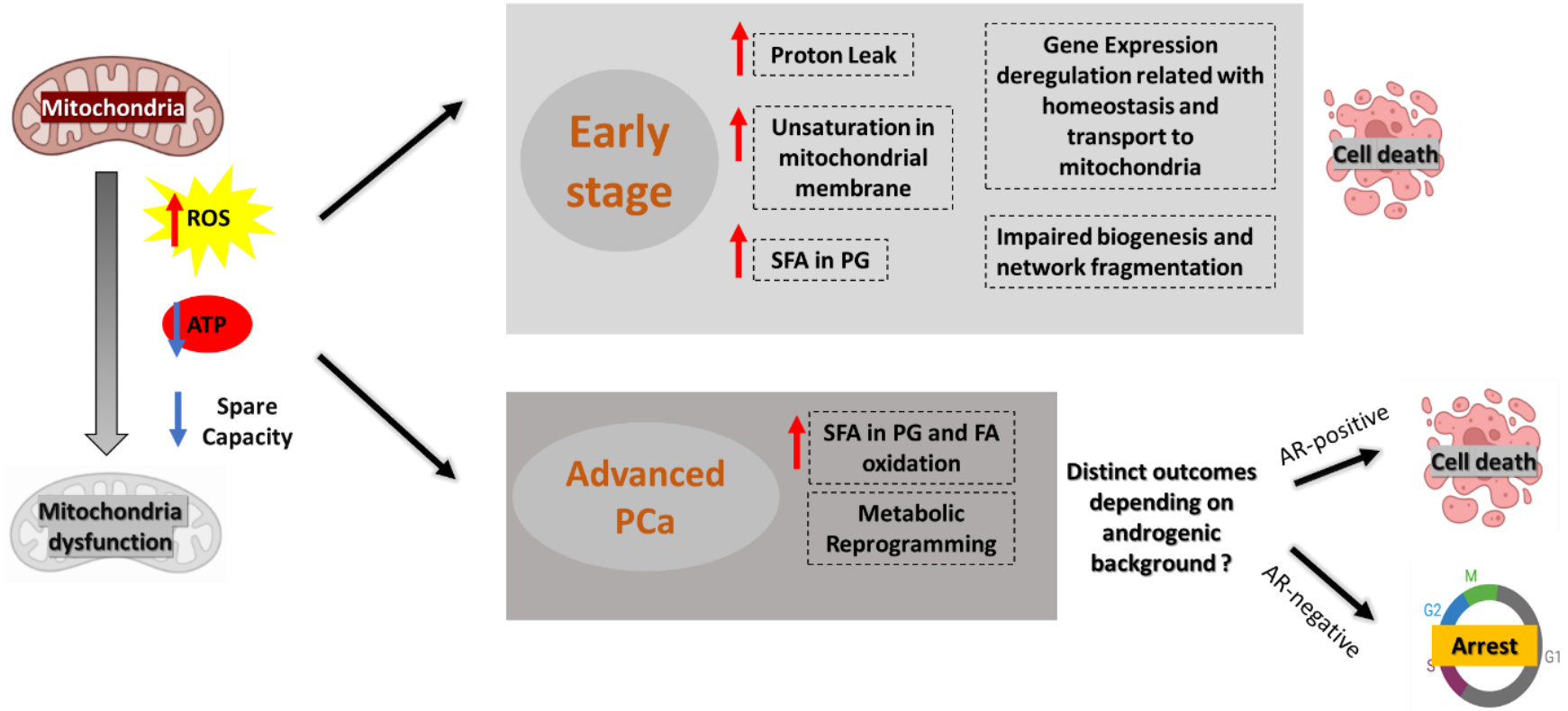

Figure 6. *Summary*. DHA led to mitochondria dysfunction in pre-malignant and castrated-resistant prostate cell lines by inducing ROS, impairment of ATP production and spare capacity. Such effect led to distinct outcomes depending on the molecular background. At early stages, DHA increased proton leak and changes in the mitochondrial membrane composition that raised the unsaturation status turning PNT1A more susceptible to oxidative damage. Also, the omega-3 induced remarkable mitochondrial gene deregulation affecting homeostasis, influx and efflux between mitochondrial matrix and the cytosol, in addition to biogenesis impairment and network fragmentation. At advanced stage, DHA induced metabolic reprogramming, specially by decreasing glycolysis capacity, a crucial route for biosynthesis in proliferating cells, and shifting glucose towards its complete oxidation. However, the androgenic background seemed to determine the outcome of these alterations, being cell death triggered in AR-positive and cell cycle arrest in AR-negative.

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CAPÍTULO 4

Docosahexaenoic acid deregulates lipid metabolism and decreases proliferation of androgen sensitive and castrated-resistant prostate cancer phenotypes

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ABSTRACT

Background: Docosahexaenoic acid (DHA) is a polyunsaturated fatty acid omega-3 obtained by food intake and at reduced levels in prostate cancer (PCa) cells, suggesting its protective property. It has been shown to modulate PCa metabolism, but its role in lipid metabolism is not fully understood. *De novo* lipogenesis (DNL) increase is an early hallmark in prostate carcinogenesis due to fatty acid synthase (FASN) overexpression. It has been suggested as a target, but its modulation through diet is not elucidated. Therefore, we evaluated the potential of DHA in regulate lipid metabolism and inhibit growth of cells with distinct androgen backgrounds. Methods: LNCaP, C4-2 and 22rv1 cells were incubated with DHA at 100μM for 48h and evaluated the cell growth. The effects on lipid metabolism were determined mainly by lipidomics, RNA-seq, GSEA and metabolic trace with [^{14}C]-labeled molecules. The contribution of androgenic pathway was assessed by protein levels and rescue experiments with AR overexpression. For validation, we incubated MSK-PCa3 organoids with DHA and also conducted 22rv1 xenografts (DHA at 10mg/kg/day). Results: DHA decreased population of all cell lines tested associated with cell cycle arrest, with strongest impact on 22rv1. The omega-3 was incorporated into glycerophospholipids increasing their unsaturation status and unbalancing their proportion mainly due to augmentation of phosphatidylcholine levels. DHA was accumulated in triacylglycerol and cholesterol esters into lipid droplets in addition to increase ROS production, mitochondrial included. Also, it downregulated activation of genes through activation of SREBP pathway leading to decreased expression of FASN which effect reflected on reduced lipogenesis from glucose. DHA stimulated fatty acid β-oxidation, being the omega-3 preferably oxidized by 22rv1 over palmitate, but also glucose complete oxidation, suggesting a metabolic reprogramming. Moreover, DHA effects on lipogenesis were not due to androgen signaling. Similarly to 22rv1, DHA decreased CRPC organoids as well as tumor growth around 80%. Conclusions: Collectively, these findings showed that DHA deregulated lipid metabolism, mainly DNL regardless of androgen signaling. Most importantly, it sharply decreased growth of CRPC models either *in vitro* or *in vivo*, offering a new diet-based approach as therapeutic strategy.

Key words: omega-3; prostate cancer; lipids; diet; DHA.

INTRODUCTION

Metabolic reprogramming is a phenomenon observed in cancer that provides several intermediates for biosynthetic pathways to supply demands of rapid proliferating cells (1). In prostate cancer (PCa), one of the metabolic hallmarks is *de novo* lipogenesis (DNL) that switches progressively from low levels in normal phenotype towards high in the tumor (2–4). Indeed, normal prostate cells mostly rely on diet-derived lipids whereas in cancer condition they show increased fatty acid synthesis regardless of lipid availability (5,6). Although other lipogenic enzymes are deregulated in PCa (6), such as ATP citrate lyase (ACLY) and Acetyl-CoA carboxylase (ACC), DNL increase is often associated with fatty acid synthase (FASN) overexpression (2,3,7). It ultimately renders palmitate that is used by many cellular processes, including membrane production (8), cell cycle progression (8,9) and post-translational modification of proteins (10). In PCa, the lipogenic enzyme genes are regulated by androgens (11,12) and FASN increased levels has been related with aggressiveness, being often overexpressed in high-grade PCa (7,9) while is reduced in benign hyperplasia, pre-malignant lesions and low-grade tumors (13). Moreover, it is associated with resistance to chemotherapy (14) whereas its inhibition induces to cell cycle arrest and apoptosis (4,9). In addition to lipid metabolism, FASN has oncogenic signaling through AKT (15,16) , PI3K (17) and colocalizes with the variant seven of androgen receptor (AR-V7) expressed in castrated-resistant cells (9). DNL has been suggested as a target through different approaches (5,18), such as FASN pharmacological inhibition which decreased growth of castrated-resistant cells and xenograft tumors (9). Therefore, this evidence points out the potential of lipid metabolism in PCa treatment.

Despite of this body of evidence showing that endogenously synthesized fatty acids are strongly associated with PCa, several studies reported the correlation of dietary fatty acids on tumor progression and initiation which seems to depend on lipid nature. On one hand, saturated fatty acids (SFAs) were correlated with increased PCa risk (19) and described to induce metabolic reprogramming through MYC driving PCa progression (20), migration (21), cell proliferation as well as several stromal alterations (22). On the other, polyunsaturated fatty acids (PUFAs), especially the omega-3 class (PUFAs ω-3), were shown to exert antiproliferative effect in different cancers (23), such as breast (24), multiple myeloma (25), non-melanoma skin cancer (26), colorectal (27) and prostate (28–30). Among them is the docosahexaenoic acid (DHA, C22:*n*-3) and eicosapentaenoic acid (EPA; C20:*n*-3), being the

former the one with increased biological activity compared to EPA (31,32). DHA is synthesized from alfa-linoleic acid with reduced synthesis in humans, being even lower in men (33,34). Therefore, it is mainly obtained through diet by the intake of marine cold fishes, eggs, algae and supplemental nutrition (34). Interestingly, normal prostate cells RWPE-1 have higher levels of DHA compared with malignant (35) suggesting that such fatty acid has a protective role against PCa. This is supported by several evidence of its antitumoral effect, such as decreased androgen receptor (AR) expression via proteasome degradation (36), induced apoptosis (37), cell cycle arrest (28), impaired mitochondria function (38), ROS overproduction (28,30), decreased cell migration (39) and anti-inflammatory response in the tumor microenvironment (40). Recently, we described the DHA property in stimulate neutral lipids accumulation (28,38) and regulate several metabolism-related genes in pre-malignant and castrated-resistant prostate cells (28). These previous findings support that DHA may affect lipid metabolism which is strengthened by evidence from prostate and breast cancer that showed *FASN* downregulation after incubation with the omega-3 (31). However, lipid metabolism regulation by DHA was not elucidated in PCa yet, mainly the DNL in cells with distinct androgenic backgrounds.

Despite the tremendous research efforts, PCa remains among the five most incident cancers in men and cause of death worldwide (41). These higher rates are probably due to inefficient therapies which have as a target the androgen signaling (42,43), given that prostate cells proliferation and survival are androgen-driven beginning at developmental stage. This approach usually involves the androgen deprivation therapy which may select androgen independent cells, leading to recurrence in a lethal and more aggressive phenotype (42,43). Therefore, new approaches are needed, mainly those that do not focus exclusively on AR, inhibit growth of castrated-resistant tumors or share targets with all pathological stages. Given the properties aforementioned, DHA is a potential candidate with safe ingestion and irrelevant side effects compared to conventional chemotherapy, being even recommended in cancer-free conditions due to its benefits in health, like brain function improvement (44) and decrease in cardiac pathologies risk (45). In the present study, we evaluated the DHA metabolism in androgen responsive and castrated-resistant cells and its property in deregulate lipid metabolism regardless of AR signaling. Also, we assessed if its combination with lipogenesis inhibitors enhanced the antiproliferative effect and validated it in castrated-resistant organoids and xenograft model.

MATERIAL AND METHODS

Cell Culture and DHA incubation

LNCaP, C4-2 and 22rv1 cells were cultured in RPMI 1640 medium (#11835030 – Gibco, ThermoScientific, US) enriched with 10% fetal bovine serum (#F4135 – Sigma Aldrich, US), 1% of penicillin and streptomycin (#15140-122 – Gibco, ThermoFischer, US) and kept in a wet incubator with 5% of CO_2 at 37°C. For cell maintenance, the medium was replaced every 2–3 days and subculture done when 70–85% confluence was reached. Mycoplasma was monitored periodically (3-4 months) with MycoAlert™ assay (#LT07-118 – Lonza, US). For experiments, cells never exceeded 15 passages after thawing. Unless otherwise stated, cells were seeded at the desired density and allowed to attach during 24h (48h for LNCaP) before incubations. For all analysis, control assays were incubated with vehicle (anhydrous ethanol or DMSO) at same volume and never exceeded 0.5% in the medium.

Cell Growth Assay

Cells were seeded at 10^6 density in 100mm dishes and incubated with DHA (#D2534—Sigma-Aldrich, US) at 100µM freshly prepared in the culture medium from a sterile stock solution at 20mM in anhydrous ethanol. This length of incubation and work concentration were previously determined due to decrease in cell population growth (28) and also be safely reachable in the human serum (46). At the end of incubation, cells were harvested with trypsin-EDTA 0.25% and number of viable cells immediately determined with the automated counter Vi-Cell Blue XR (Beckman Coulter, USA) based on trypan blue exclusion method. Values were shown as percentage of cell growth (n=9) compared to vehicle (100%) and SEM.

Lipid accumulation

Neutral Lipids accumulation was assessed by fluorescent probe BODIPY™ 490/593 (#D3922, ThermoFischer). Briefly, $3x10^4$ cells/well were seeded and at the end of experiment, medium replaced by pre-warmed PBS with 5µM of the dye, followed by 20 min of incubation at 37°C (28,38). Then, PBS was replaced probe-free. Fluorescence intensity (FI) was measured in live cells at 490/593nm using SpectraMax iD5 (Molecular Devices) and normalized to protein content from respective well using by Pierce™ BCA Protein Assay Kit (#23225, ThermoFishcer, USA) following the manufacturer's instructions. Values were shown

as mean of FI and SEM (n=8). For imaging, cells were grown at same density in bottom glass dishes and stained with BODIPY™ 490/593 and Hoechst 33358 (500ng/mL) for lipid droplets and nuclei visualization, respectively, as already described. Then, cells were washed twice in PBS and fixed with formalin 4% for 15 minutes at room temperature (RT). Images were taken with THUNDER Imager Live Cell & 3D Cell Culture & 3D Assay microscope (Leyca Microsystems, USA).

Lipidomics

Cells were seeded at 10^6 density and collected at the end of incubation with trypsin-EDTA 0.25%, centrifuged at 300g for 5 min at RT and pellets stored at -80ºC. Samples (n=3 per condition) were shipped out and processed by Lipometrix (Leuven, Belgium). Briefly, 700 µl of cells (20 µg of protein) were mixed with 800µl of 1 N HCl:CH_3OH 1:8 (v/v), 900µl of $CHCl_3$, 200 µg/ml of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT; Sigma Aldrich) and 3cl of SPLASH® LIPIDOMIX® Mass Spec Standard (#330707, Avanti Polar Lipids). After vortexing and centrifugation, the lower organic fraction was collected and evaporated using a Savant Speedvac spd111v (Thermo Fisher Scientific) at RT and the remaining lipid pellet stored at - 20°C under argon. Just before mass spectrometry analysis, lipid pellets were reconstituted in 100% ethanol. Lipid species were analyzed by liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI/MS/MS) on a Nexera X2 UHPLC system (Shimadzu) coupled with hybrid triple quadrupole/linear ion trap mass spectrometer (6500+ QTRAP system; AB SCIEX). Chromatographic separation was performed on a XBridge amide column (150 mm × 4.6 mm, 3.5 µm; Waters) maintained at 35°C using mobile phase A [1 mM ammonium acetate in water-acetonitrile 5:95 (v/v)] and mobile phase B [1 mM ammonium acetate in water-acetonitrile 50:50 (v/v)]. Sphingomyelin (SM) was measured in positive ion mode with a precursor scan of 184.1, 369.4, 264.4, 266.4, 264.4 and 264.4 respectively. Triacylglycerol (TAG) and diacylglycerol (DAG) were measured in positive ion mode with a neutral loss scan for one of the fatty acyl moieties. Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), Phosphatidylinositol (PI) and Phosphatidylserine (PS) were measured in negative ion mode by fatty acyl fragment ions. Lipid quantification was performed by scheduled multiple reactions monitoring (MRM), the transitions being based on the neutral losses or the typical product ions as described above. Peak integration was performed with the MultiQuant™ software version 3.0.3. Lipid species signals were corrected for isotopic contributions (calculated with Python Molmass 2019.1.1) and were quantified based on internal

standard signals and adheres to the guidelines of the Lipidomics Standards Initiative (LSI) (level 2 type quantification as defined by the LSI).

Metabolic Flux Assays

For all assays, cells were seeded at $0.5x10^6$ density in a 60mm dish or a 6 wells plate and incubated with DHA at conditions previous described. To determine lipogenesis, [^{14}C]-Glucose (ARC-0122D; 1µCi/well) or [^{14}C]-Glutamine (ARC-0196; 0.1µCi/well) were added 24h before the end of experiment (9). Briefly, cells were harvested with trypsin-EDTA 0.25%, centrifuged at 300g for 5 minutes at 4°C, washed with cold PBS and pelleted down. Then, cells were lysed with cold extraction solution (2 mL methanol, 1mL chloroform and 0.5mL of dH_2O) in glass tubes and incubated overnight at 4°C. Samples were centrifuged at 400g for 10 minutes at 4°C and supernatant collected for lipophilic fraction extraction with cold chloroform (1mL) and dH_2O (1mL). After centrifugation, the lipophilic phase was collected and dried at 50°C under nitrogen flux to avoid oxidation. Lyophilized samples were ressuspended in 500µL of cold Bligh & Dyer solution (2:1 volume of methanol:chloroform) and radioactivity assessed. [^{14}C]-DHA (ARC-0380; 0.2µCi/well) was added to the medium to assess its incorporation into cell lipids following same protocol.

Fatty acid β-oxidation rate was determined by [^{14}C]-Palmitic acid (ARC-01762A; 0.25µCi/well) or [^{14}C]-DHA (0.2µCi/well) incubation and measured the [^{14}C]-CO_2 released. Radiolabeled molecules were added to the medium 3 hours before the end of each experiment (9), except for DHA that were kept for 24h. Briefly, 100µL of 70% perchloric acid were added to wells and immediately closed with a trapped Whatman filter grade 3 circle saturated with 200µL of phenethylamine. Released [^{14}C]-CO_2 was captured for 2h at RT under slow shaking. Then, circles were collected and used for radioactivity measurement. Same protocol was performed for determination of glucose complete oxidation by incubating cells with [^{14}C]-Glucose (ARC-0122D; 1µCi/well). Lipid uptake was assessed by [^{14}C]-Palmitate uptake (0.25µCi/well) added 3h before the end of experiment. For this purpose, cells were harvested and lysed with RIPA buffer for 15 minutes on ice, centrifuged at 14000 RPM for 20 minutes at 4°C and the supernatant used for radioactivity counting. [^{14}C]-Palmitate assays were performed in a conjugated form with BSA-fatty acid free.

Radioactivity was determined by liquid scintillation vials containing 10mL of UltraGold™ (#6013326 – PerkinElmer) and assessed with β-counter (Tri-carb 2910 TR Liquid

Scintillation Counter, PerkinElmer). Counts per minute (CPM) were normalized to protein content and values plotted as percentage compared to control condition (n=6 at least) plus SEM.

Transcriptomics

Gene expression regulation by DHA was investigated by RNA-seq analyses (n=3 per condition) and performed by the external facility Genewiz Inc (South Plainfield, NJ, US). Briefly, cells were seeded at 10^6 density and incubated with DHA at 100µM for 48h. Then, cells were collected with trypsin-EDTA 0.25%, centrifuged at 300g for 5 minutes at 4°C, washed once with cold PBS and pelleted down. PBS was discarded and samples immediately frozen at -80°C. RNA samples were quantified using Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and RNA integrity was checked using Agilent TapeStation 4200 (Agilent Technologies, Palo Alto, CA, USA). RNA sequencing libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit for Illumina following manufacturer's instructions (NEB, Ipswich, MA, USA). Briefly, mRNAs were first enriched with Oligo(dT) beads. Enriched mRNAs were fragmented for 15 minutes at 94 °C. First strand and second strand cDNAs were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapters were ligated to cDNA fragments, followed by index addition and library enrichment by limited-cycle PCR. The sequencing libraries were validated on the Agilent TapeStation (Agilent Technologies, Palo Alto, CA, USA), and quantified using MiSeq Nano or Qubit 4.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (KAPA Biosystems, Wilmington, MA, USA). The sequencing libraries were pooled and clustered on three lanes of a flowcell. After clustering, the flowcell was loaded on the Illumina HiSeq instrument (4000 or equivalent) according to manufacturer's instructions. The samples were sequenced using a 2x150bp Paired End configuration. Image analysis and base calling were conducted by the HiSeq Control Software. Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mismatch was allowed for index sequence identification.

After investigating the quality of the raw data, sequence reads were trimmed to remove possible adapter sequences and nucleotides with poor quality using Trimmomatic v.0.36. The trimmed reads were mapped to the *Homo sapiens* reference genome available on ENSEMBL using the STAR aligner v.2.5.2b. The STAR aligner is a splice aligner that detects splice junctions and incorporates them to help align the entire read sequences. BAM files were generated as a result of this step. Unique gene hit counts were calculated by using feature Counts

from the Subread package v.1.5.2. Only unique reads that fell within exon regions were counted. After extraction of gene hit counts, the gene hit counts table was used for downstream differential expression analysis. Using DESeq2, a comparison of gene expression between the groups of samples was performed. The Wald test was used to generate p-values and Log2 fold changes. Genes with adjusted p-values < 0.05 and absolute log2 fold changes > 1 were called as differentially expressed genes for each comparison.

DSEq2 output from RNA-seq data were submitted to Gene Set Enrichment Analysis (GSEA_4.1.0, Broad Institute) software. Pre-Ranked module was run using {fold change x [-log10(p value)]} from the DSEq-2 output. Hallmarks (v.7.4), Reactome (v.7.4) and KEGG (v.7.4) molecular signatures data bases were run as references. Output with false discovery rate (FDR) ≤ 0.20 were considered enriched. Data was shown as enrichment plots with normalized enrichment score (NES).

ROS determination

For this set of experiments, cells were seeded at $3x10^4$ density per well. General ROS production was determined with the Cellular Reactive Oxygen Species Detection Assay Kit (#ab186027, Abcam, USA), following manufactures' instructions. Briefly, at the end of experiment probe was added to the medium and reached the working concentration, followed by incubation for 20 minutes at 37ºC in 5% CO_2 and fluorescence assessed at 520/605nm. Superoxide anion ($O_2^{\bullet-}$) production was determined by MitoSOX Red (#M36008 ThermoFischer Scientific) probe at 5µM in PBS for 20 minutes at 37°C. Then, medium was replaced by pre-warmed probe-free PBS and FI measured at 510/580nm. Both assays had FI assessed in live cells with SpectraMax iD5 (Molecular Devices) microplate reader and normalized to protein content from the respective well, determined with Pierce™ BCA Protein Assay Kit (#23225, ThermoFishcer, USA). Values were shown as mean of fold-change to control (n=6) and SEM.

Western blotting

Cells were incubated at $0.5x10^6$ density per dish and, at the end of incubation, collected with trypsin-EDTA 0.25% and pelleted down at 300g for 5 min at 4˚C. They were lysed with RIPA buffer supplemented with 1X SigmaFAST™ protease cocktail inhibitors (#S8830, Sigma Aldrich, USA) and phosphoSTOP (#4906845001, Sigma Aldrich, USA) as

well. Total protein was determined by Pierce™ BCA Protein Assay Kit (#23225, ThermoFishcer, USA) following the manufacturer's instructions and absorbance read at 562nm with SpectraMax iD5 (Molecular Devices) microplate reader. Samples (20µg protein) were loaded in Novex™ WedgeWell™ 10% Tris-Glycine gels (#XP00105BOX, ThermoFischer, USA), and electrophoresis performed. Then, proteins were transferred to a nitrocellulose membrane for 2h in a wet system. Nonspecific bindings were blocked with skimmed milk at 5% in TBST for 30–60 min at RT in a shaker and membranes incubated overnight with primary antibody at 4 °C. The following primary antibodies were used: anti-Cyclin A2 (#4656S; Cell Signaling, 1:500), anti-Cyclin B1 (#4135S; Cell Signaling, 1:500), anti-Cyclin D1 (#2922S/ Cell Signaling, 1:500), anti-c-Myc (#5605S; Cell Signaling, 1:500), anti-FASN (#610963; BD Biosciences, 1:1000); anti-PSA (#A0562; Dako, 1:500), anti-AR-FL/AR-V7 (#ab108341; Abcam, 1:1000), anti-GPx4 (#52455; Cell Signaling, 1:1000) and anti-Vinculin (#V9264; Sigma Aldrich, 1:1000). Secondary antibodies were prepared at 1:5000 dilution and incubated at 4 °C for 1 h. ECL system was adopted for band visualization and image capture. Protein quantification was performed for all samples in the same electrophoresis. Relative densitometry was assessed using NIH ImajeJ (1.39) software after normalization to vinculin (loading control). Values were shown as mean and SEM (n=6).

AR-FL overexpression

LNCaP cells carrying pcDH-EF1-CymR-T2A-Puro-ARFL vector were seeded at $0.5x10^6$ density and, after attachment, incubated with doxycycline (DOX) at 50 ng/mL and puromycin at 1 µg/mL to switch on AR-FL expression and to select cells carrying vector, respectively. Both were added at same time of DHA or vehicle. At the end of 48h, cells were collected with trypsin-EDTA 0.25% and viability determined as described previously. Lipogenesis was determined as described for metabolic flux assays. AR-FL overexpression was confirmed by western blotting as already mentioned. Values were shown as percentage of cell growth (n=6) compared to control condition and SEM.

Membrane lipid oxidation

For lipoperoxidation analysis we performed BODIPY™ 581/591 C11 Lipid sensor (#D3861, ThermoFischer) ratiometric assay. Briefly, cells were seeded at $0.2x10^6$ density in bottom glass dish and the dye added at 5µM to each well freshly prepared in pre-warmed

medium, followed by incubation for 30 minutes at 37˚C. Then, medium was replaced by probe-free PBS and cells analyzed under AiryScan Super-resolution microscopy (Zeiss). Set up was done for one channel at 581/591nm to determine the reduced form, and another for 488/510nm to assess the oxidized one. Red to green FI ratio was adopted to determine the lipid oxidation levels. At least fifteen images (n=15) from cells at consecutive passages were taken at 400x magnification. FI for each channel was extracted using NIH ImageJ software. Values were plotted as mean of non-oxidized to oxidized lipids and SEM.

***In vivo* and *in vitro* experiments with lipogenesis inhibitors**

For *in vitro* lipogenesis inhibitors we used 2D and 3D culture. Briefly, $0.4x10^6$ LNCaP, C4-2 and 22rv1 cells were seeded for the 3 days assay and $0.2x10^6$ for 6 days. After 24h of attachment, IPI-9119 (non-commercially available) or ND646, a FASN and ACC1/2 inhibitor respectively, at 100nM were added alone or combined with DHA (100µM) in both lengths of incubation. These conditions for lipogenesis inhibitors were determined previously based on the time required for metabolic alterations (9) . At the end of incubations, cells were collected with trypsin-EDTA 0.25% and performed the cell growth protocol. Values were shown as percentage compared to control condition (n=6) and SEM. For 3D experiments, MSK-PCa3 mCRPC organoids (47), kindly provided by Yu Chen (Memorial Sloan-Kettering Cancer Center, New York, NY, USA), were gently dissociated to single cells with TrypLE™ Express Enzyme (#12604013, ThermoFischer, USA) for 15 minutes at 37˚C. Then, $2x10^3$ cells were mixed with 40µL of growth factor-reduced Matrigel and seeded in a 24-well plate. Organoids were cultured in the special medium (47) in the presence of DHA and inhibitors at same concentrations described for 2D assays in this section. Culture was done for 25 days and medium replaced every 5 days. At the end of experiment, images were captured with Zeiss Axio inverted microscope and organoids diameter determined with NIH ImageJ. Values were shown as fold-change and SEM (n=6).

For *in vivo* experiments, CRPC xenografts were performed with male Balb/c nude (C.Cg/AnNTac-Foxn1^{nu} NE9) castrated mice, purchased from Taconic. Animals were castrated at 6 weeks of age and at 9th weeks, $7x10^6$ 22rv1 cells were mixed with Matrigel (1:1) and injected into their flank. After tumors reached $100mm^3$, mice were randomly assigned into Control, DHA, IPI-9119 and DHA+IPI-9119 groups (n=12/group at least). DHA was provided by oral gavage (100µL final volume) at 10mg/kg/day (Naturelo DHA Omega-3 from Algae, Kroger, USA) for 5 days per week and DHA-free groups received same procedure with glycerol

20% (vehicle). IPI-9119 at 100mg/ml was delivered by ALZET® 2002 osmotic pumps (DURECT Corporation, USA) at release rate of 0.5µL/h along 14 days and then replaced surgically for a second round till the end of treatment. Osmotic pumps were seeded subcutaneously in the dorsum. DHA and Control groups received pumps with vehicle at same volume (1-methyl-2-pyrrolidinone, NMP). Isoflurane 4% was used as anesthesia in all surgical procedures. Tumor size was measured three times a week along treatment, using a caliper and volume calculated as V = (length x width x 0.5). Mice were kept in polyethylene cages with water and food (#1811751, PicoLab® with 0.12% amoxicillin) *ad libitum* and health monitored every two days or for three days after surgery. At 28th day of treatment, mice were euthanized by CO_2 inhalation followed by cervical dislocation and Tumor Growth rate calculated as TG = average of tumor volume of the last week/tumor volume of the first day of pump implantation. Values were plotted as mean of fold-change and SD. All animal experiments were approved and performed in compliance to guidelines from Weill Cornell Medicine Institutional Animal Care & Use Committee (Protocol 2019-0031).

Statistical Analysis

For statistical purpose, we performed three independent experiments and data analyzed with GraphPad Prism® software (GraphPad Prism software, v.8.0). First, distribution of samples was analyzed with Kolmogorov-Smirnov and Shapiro-Wilk normality tests. Parametric distributions were submitted to unpaired t-test, one-way or two-way ANOVA, followed by Tukey test (post hoc) and non-parametric distributions to Mann-Whitney or Kruskal-Wallis test followed by Dunn test (post hoc). $p < 0.05$ was considered statistically different.

RESULTS

DHA decreases cell growth and arrests cell cycle in AR-positive prostate cancer cells

The first step was to determine whether DHA affects cell growth and whether this is different between androgen-responsive and castrated-resistant phenotypes. We found that DHA decreased LNCaP and C4-2 populations around 22% whereas the omega-3 had stronger impact (38%) on 22rv1 (Fig.1A). Such decrease was followed by morphological changes in all

of them, like accumulations on the cytosol (Fig.1B). To better understand the cell biology behind cell growth reduction, we checked GSEA outputs. We observed that DHA led to downregulation of cell cycle pathways in LNCaP, C4-2 and 22rv1 with NES of -1.71, -1.48 and -1.95, respectively (Fig.1C). Therefore, we checked cyclins expressions (Fig.1D-F) and observed a decrease in cyclin D1 in LNCaP, but an increase in A2 and B1 in C4-2 which decreased in 22rv1.

DHA is accumulated into lipid droplets

To better understand how DHA affects lipid metabolism we first investigated how PCa cells metabolize it. The omega-3 induced neutral lipids accumulation, respectively, 1.72-, 1.83- and 2.51-fold change in LNCaP, C4-2 and 22rv1 (Fig.2A-B). Then, we interrogated if such accumulation was due to DHA uptake and observed that it was incorporated into lipids by all cells lines, being the lowest rate in 22rv1 (Fig.2C). To evaluate if DHA changed lipid droplets we assessed their main two components, TAG and CE amount and fatty acid profile. The TAG to CE ratio increased in all cell lines (LNCaP Ctrl 0.46± 0.24, DHA 2± 0.96, $p=0.054$; C4-2 Ctrl 0.35±0.08, DHA 1.33± 0.30; 22rv1 Ctrl 0.08± 0.01, DHA 0.6± 0.05, $p < 0.05$), indicating that the omega-3 induced mainly structural lipids production (Fig.2D). Levels of TAG (Fig.2E) remarkably increased in all cell lines (at least 20-fold) while CE levels (Fig.2F) only increased in 22rv1 (7.65-fold). Then, FA profile confirmed that the main component of lipid droplets was DHA either in TAG or CE (Fig.2G-H).

DHA deregulates lipid metabolism and stimulates glucose complete oxidation

Given that DHA increased lipid droplets content, we assessed whether it affects lipolysis and lipogenesis. Our transcriptome data showed that in all cell lines DHA downregulated activation of gene expression by SREBF and SREBP (Fig.3A first row) in addition to the synthesis of very long chain fatty acid in LNCaP and C4-2 (Fig.3A second row). To validate this data, we performed both protein expression and metabolic flux analysis. DHA decreased lipogenesis from glucose (45%, 17% and 22% in LNCaP, C4-2 and 22rv1, respectively) and FASN expression (around 50%) in all cell lines (Fig.3B-C) whereas slightly increased lipogenesis from glutamine (Fig.3D) only in castrated resistant cells (13% and 20%

in C4-2 and 22rv1, respectively). Regarding lipolysis, DHA stimulated palmitate β-oxidation in LNCaP (32%) and C4-2 (75%) while reduced it 24% in 22rv1 (Fig.3E). In addition, all cell lines were able to oxidize DHA, being 22rv1 the one with highest rate (Fig.3F). None of these changes affected palmitate uptake (Fig.3G). Then, we checked if lipid metabolism deregulation led to changes in glucose complete oxidation and observed that all cells tested raised its oxidation towards CO_2 around 1.2-fold (Fig.3H).

DHA disturbs phospholipids ratio, increases their unsaturation status and induces ROS overproduction

Given that DHA deregulated lipid synthesis and it might be a requirement for phospholipids production (8), we interrogated PC, PE, PI, PS and SM levels, unsaturation status and ratio and data was detailed in Figure 4. Lipidomics analyses showed PC was the most abundant specie among the phospholipids and that DHA increased its concentration around 1.5-fold in all cell lines (Fig.4A). However, any changes were observed for the others except for an increase in PE in C4-2 (Fig.4E,I,M,Q). Regarding the unsaturation status, DHA induced fatty acids with 6 bonds in all phospholipids, except in SM (Fig.4R-T), and 5 bonds in PC (Fig.4B-D), PE (Fig.4F-G) and PI (Fig.4J-L) in all cell lines. Also, the omega-3 increased SFAs followed by a decrease in fatty acids with 1 and 2 bonds in all cell lines in the most abundant species, PC (Fig.4B-D), PE (Fig.4F-G) and PI (Fig.4J-L). Then, we calculated the phospholipids ratio and found that DHA imbalanced it by leading to PC/PI and PC/PS increase in all cell lines (Fig.5A-C), whereas it did for PC/PE only in the castrated-resistant lines (Fig.4B-C).

Since PUFAs in cell membrane are closely related with oxidative stress (48), we checked ROS overproduction either in general or superoxide anion. DHA increased ROS general production in 1.4-fold in LNCaP and 1.2-fold in C4-2 and 22rv1 (Fig.5D). The same effect was observed for superoxide anion levels which raised around 1-5-fold in all cell lines (Fig.5E). Then, we investigated if such increase led to lipid membrane oxidation and potentially ferroptosis by GPx4 levels. LNCaP cells showed decreased membrane lipoperoxidation whereas it increased in C4-2 but remained unchanged in 22rv1 (Fig.5G). Any change was observed for GPx4 protein expression (Fig.5F).

AR signaling impairment by DHA is not related with decrease in lipogenesis

Since AR may regulate lipogenic enzymes expression (11,12), we interrogated whether DHA affects the receptor signaling and whether this is related with the lipogenesis effects observed. Gene set enrichment analysis showed that response to androgen pathway was downregulated in both LNCaP and C4-2, NES -1.68 and -1.80, respectively (Fig.6A). In LNCaP and 22rv1, DHA decreased AR-FL expression around 20% (Fig.6B) and its targets PSA (Fig.6C) and c-Myc (Fig.6D), supporting downregulation in AR signaling. However, this was not observed for C4-2. In addition, DHA reduced AR-V7 expression at same proportion in 22rv1 (Fig.6E). Importantly, we also showed that alterations in protein levels were not due to decrease in protein synthesis capacity (Supplementary Material S1). To confirm the AR role in cell growth by DHA, we used LNCaP cells overexpressing AR-FL to overcome the omega-3 effects. As expected, it was not able to decrease cell population growth (Fig.6F) neither AR-FL expression (Fig.6G). Then, we assessed the lipogenesis from glucose in these cells and observed that even under AR-FL overexpression DHA decreased fatty acid synthesis (Fig.6H).

DHA effects on cell growth is potentiated by lipogenesis inhibitors in castrated-resistant models in vitro and in vivo

Given that DHA deregulated lipogenesis, we tested whether its combination with lipogenesis inhibitors IPI-9119 (FASNi) and ND646 (ACCi) would enhance the fatty acid effects (Fig.7A). DHA decreased cell growth in both length of incubations, remaining similar after 3 and 6 days. Remarkably, DHA combined with lipogenesis inhibitors potentiated cell growth suppression in both lengths. In LNCaP, after 3 days of incubation, DHA+ND646 decreased 65% cell growth and DHA+IPI-9119 did 53% whereas after 6 days these rates elevated to 90% and 80%, respectively. In C4-2, DHA+ND646 reduced cell growth in 70% and DHA+IPI-9119 did 55% after 3 days, whereas these raised to 92% and 85%, respectively, after 6 days. In 22rv1, DHA+ND646 inhibited cell growth in 59% whereas DHA+IPI-9119 did 51% after 3 days, being these rates increased to 92% and 85%, respectively, after 6 days.

As the castrated-resistant cells had a better outcome, we moved forward to test the combinatory effects on CRPC organoids and xenograft model. We observed that either DHA or lipogenesis inhibitors alone decreased organoids diameter, being IPI-9119 40%, ND646 50% and 20% the omega-3 (Fig.7B-C). When combined to DHA, it decreased to 65% with IPI-9119

and 82% with ND656 (Fig.7B-C). Regarding the tumor growth in the xenografts, there were large variations among samples, but DHA was able to avoid tumor growth by reducing in 80% the volume whereas the combination with IPI-9119 did 90%, both compared to Control (Fig.7D).

DISCUSSION

The omega-3 antitumoral property has been reported by several studies (23–26), including in PCa (28–30), but the individual contribution of each fatty acid within the class is not fully elucidated. Here we described the DHA antiproliferative effect on PCa cells from distinct androgenic backgrounds via cell cycle modulation, but in distinct pattern in each cell line. We observed that such fatty acid is mostly accumulated in lipid droplets as TAG and CE but is also oxidized and incorporated into phospholipids, changing their ratio, unsaturation status and inducing ROS overproduction. We showed that DHA downregulated fatty acid biosynthesis pathways reflecting on reduction of DNL from glucose and FASN expression which did not affect palmitate uptake. Such alterations were followed by increase in fatty acid oxidation, but also glucose complete oxidation towards CO_2. Importantly, DHA was able to decrease AR-FL expression in most of cell lines as well as AR-V7, but this was not a mechanism whereby lipogenesis was reduced. Moreover, we validated DHA antitumoral effects in castrated-resistant organoids and 22rv1 xenografts, but also showed that enhancing it with lipogenesis inhibitors strengths DHA therapeutic potential in PCa.

Cancer cells have been reported to preferably uptake PUFAs (49), including DHA (50), and as shown here, it assumes plenty of metabolic fates. DHA incorporation into phospholipids by tumor cells was previously described (51,52) and associated with apoptosis in breast cancer (53). Increase in membrane PUFAs are known to favor lipid peroxidation due to higher unsaturation levels and sensitize cells to ferroptosis (48), which can also occur due to uptake from exogenous sources (54). Therefore, we would expect that by increasing the phospholipid unsaturation status, DHA would exert same effects. Instead, we observed a different response in each cell line and no ferroptosis which can be related with the antioxidant response to the omega-3 and buffering by lipid droplets. In LNCaP, lipid peroxidation decreased concomitantly with *GPX2* upregulation (Supplementary Material S2), an antioxidant enzyme with phospholipid hydroperoxidase activity responsive to ROS and protective against

oxidative stress-induced apoptosis (55,56). On the other direction, DHA increased lipid peroxidation in C4-2 and upregulated pro-oxidant enzymes, such as *NOX1* and *NOXA1* (57,58), while unchanged in 22rv1. In the last, DHA upregulated *GPX3* which exhibits phospholipid hydroperoxidase activity, but also showed the highest lipid accumulation levels (2.5-fold) in the presence of DHA. Oxidative stress has been reported to induce lipid droplets formation (59) and cancer cells store PUFAs into them (60) as an response to oxidative stress, especially lipid oxidation in membranes (61,62). In addition to their antioxidant role, they may serve as a buffer to avoid sharp changes biological membranes properties. DHA-enriched membranes have increased fluidity, flexibility and permeability due to the omega-3 unique structure (48,63). These features impair lipid packing and phospholipids containing long-chain fatty acids with *cis* configuration are thinner which increase permeability to small polar molecules (63). Also, DHA has strong aversion to cholesterol which alters lipid rafts composition forming a highly disordered membrane (64) compared to SFAs (65). These alterations in membrane properties affect membrane proteins signaling and possibly contributed to the cell growth decrease, as observed in breast cancer cells after DHA incorporation (66). Collectively, this evidence suggests that DHA-enriched microenvironment is harmful for PCa cells which trigger "buffering" responses to avoid damage, but the mechanisms may differ due to their intrinsic molecular context.

Increase in lipid uptake via cargo selective and non-selective transport was reported to down-stimulate lipid synthesis (48). This is of particular interest because DNL is a hallmark in several cancers (67) and a key factor in PCa progression since it is increased in CRPC compared with hormone-sensitive phenotype (5). Its suppression through different targets, such as FASN, ACC and Stearoyl CoA Desaturase 1 (SCD1) has been shown to decrease PCa cell growth and improve enzalutamide effects (5,9). Here we reported that DHA decreased SREBP signaling, a master regulator of lipogenesis (68,69), changed several lipogenic genes expression (Supplementary Material S5), and validated it by decrease of FASN expression and lipogenesis from glucose. FASN overexpression in an early event in PCa carcinogenesis (3) related with migration (70,71), cell proliferation and apoptosis prevention (4), being associated with disease progression (2,7,72) and castrated-resistant phenotype (9). Its pharmacological inhibition or genetic ablation has been shown to decrease cell and tumor growth and revert most of its oncogenic properties (9,73,74,75) as well as suggested in combination with other therapies, such as radiotherapy and enzalutamide (9,76). FASN expression is sensitive to diet (77) and its mRNA levels were downregulated by plant and marine-derived omega-3 in PC3 and LNCaP

cells, including DHA (31). Despite of decreased DNL from glucose, DHA increased it when glutamine was the source in castrated-resistant cells. Glutamine usage for lipid synthesis via reductive carboxylation is observed in PCa (78,79) and is triggered under oxidative stress and mitochondrial impairment (80,81). This is in line with our findings, because increased of DNL from glutamine correlates with ROS overproduction, either general or mitochondrial, in the castrated-resistant cell lines. Despite ROS levels also increase in LNCaP, lipids synthesized from glutamine did not which would be expected given that these cells showed upregulation of the antioxidant system, reflected also in lipoperoxidation decrease. Taken together, these findings suggest that DHA accumulation into neutral lipids to avoid further damage triggered DNL reduction, partially due to decrease in FASN expression, a key enzyme in PCa.

DNL provides fatty acids for phospholipids and membrane production to overcome the lipogenic checkpoint and move forward on cell cycle (8). PC is the most abundant glycerophospholipid in membranes and its synthesis is impaired when DNL is inhibited (8). However, it cannot be rescued by exogenous fatty acids, been the palmitate endogenously synthesized required for cell cycle progression (8). Here we observed that in addition to DNL decrease, DHA was incorporated into PC and increased its levels through the Kennedy's pathway, but also due to remodeling via Land's cycle in 22rv1 (Supplementary Material S3). Indeed, we incubated cells with palmitate concomitantly with DHA (Supplementary Material S4), but cell growth was not rescued as expected, given that palmitate uptake unchanged and cannot overcome the cell cycle arrest. This evidence showed that like SFAs, PUFAs do not replace endogenously produced palmitate when DNL is impaired. This explains the decreased cell growth observed as well as the cell cycle-related pathways modulation. The cyclin D1 decrease suggests that DHA impaired S-phase in LNCaP whereas deregulation of cyclin A2 and B1 led to cell cycle arrest in C4-2 and 22rv1, most likely in G2/M. Indeed, this is in line with previous results concerning the DHA property in distinctly modulate cell cycle in PCa according to cells' molecular context (28).

To the best of our knowledge, this is the first study to show metabolic flux changes by DHA in PCa and to demonstrate that its effects on DNL were not AR-dependent. In PCa, lipogenic genes expression is regulated by androgens via SREBP , including FASN (11,12) which is supported by its decreased activity after AR inhibition (12,82). Indeed, DHA decreased AR-FL in LNCaP and 22rv1 impairing the androgen signaling, but its overexpression did not rescue lipogenesis from glucose, showing that AR degradation is not the mechanism whereby

the omega-3 decreased DNL. Interestingly, DHA did not affect AR-FL expression in C4-2 (as also observed in AR-overexpressing cells), a cell line with AR amplification, suggesting a threshold for DHA effects on androgen signaling. However, it decreased AR-V7 in 22rv1, a variant known for drive the CRPC phenotype (83). This is an important outcome since most of available therapies aim to target the androgenic pathway which may lead to recurrence in a more aggressive stage. Also, indicates that DHA may be considered in combination with current available chemotherapies, especially in the castrated-resistant stage which corresponding cell lines showed more sensitive to DHA compared to androgen-responsive (84), as observed herein. It is worth mentioning that despite of DNL effects, DHA stimulated fatty acid and glucose oxidation. Our findings showed that AR-V7-driven cells 22rv1 preferably oxidize PUFAs rather palmitate whereas LNCaP and C4-2 use mostly the last. Despite speculative, this suggests a metabolic adjustment in 22rv1 whereby cells accumulate high amounts of PUFAs and use them as energy source. In addition to increase DNL from glutamine, this could indicate a mechanism of resistance in CRPC cells. Therefore, we combined DHA with lipogenesis inhibitors. FASN inhibitors have been proposed as strategy in PCa therapy based on *in vivo* and *in vitro* evidence, but this is the first study to combine them with DHA. We observed a potentiated growth suppression in time dependent manner in cell lines. Also, DHA alone was able to suppress in both CRPC organoids and 22rv1 xenografts which was remarkably potentiated by lipogenesis inhibitors.

CONCLUSION

Firstly, our findings demonstrated that DHA uptake led to its accumulation and incorporation into membrane phospholipids, increasing sensibility to ROS and membrane properties that directly affect its organization and signaling. Then, this scenario led to metabolic adjustments, including decrease in DNL regardless of AR signaling, even though AR-FL and AR-V7 were additive to the cell growth suppression. These findings suggested that DHA is a multitarget fatty acid that arrested cell cycle reflecting in the decreased cell growth. Secondly, we provided evidence of its therapeutic potential by validating it on organoids and xenograft model, pointing out that DHA supplementation itself was able to suppress tumor growth. Finally, we showed that lipogenesis inhibitors either *in vitro* or *in vivo* were able to potentiate DHA antitumor effects, opening new approaches for PCa treatment.

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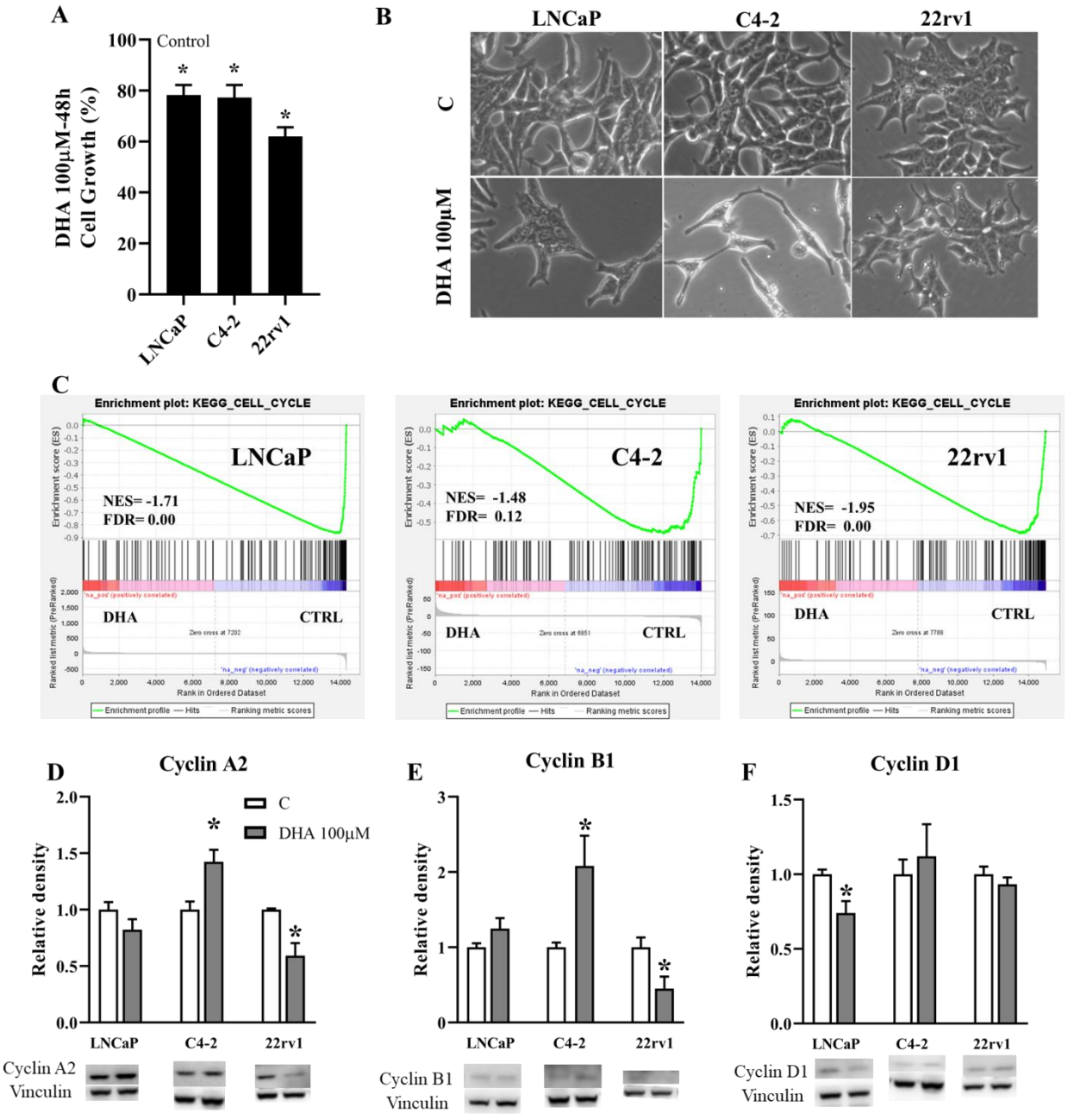

Figure 1. *DHA decreases cell growth via cell cycle modulation in androgen-responsive and castrated resistant prostate cancer cells.* **A)** Cell growth after DHA incubation at 100µM for 48h. Values shown as percentage compared to vehicle (control, 100%) and SD (n=9). **B)** Images at 400x magnification obtained with phase contrast microscope showing morphological alterations due to DHA. **C)** Enrichment plots from GSEA (FDR≤0.20) indicating downregulation of cell cycle pathways in LNCaP, C4-2 and 22rv1 cells. **D-F)** Cyclins expression indicating cell cycle arrest in G2/M for C4-2 and 22rv1 whereas G1/S for LNCAP. Values shown as mean and SEM of relative density (n=4 at least) to vinculin (loading control). Statistical analysis: Student t-test; * - different from control condition; three independent experiments were performed.

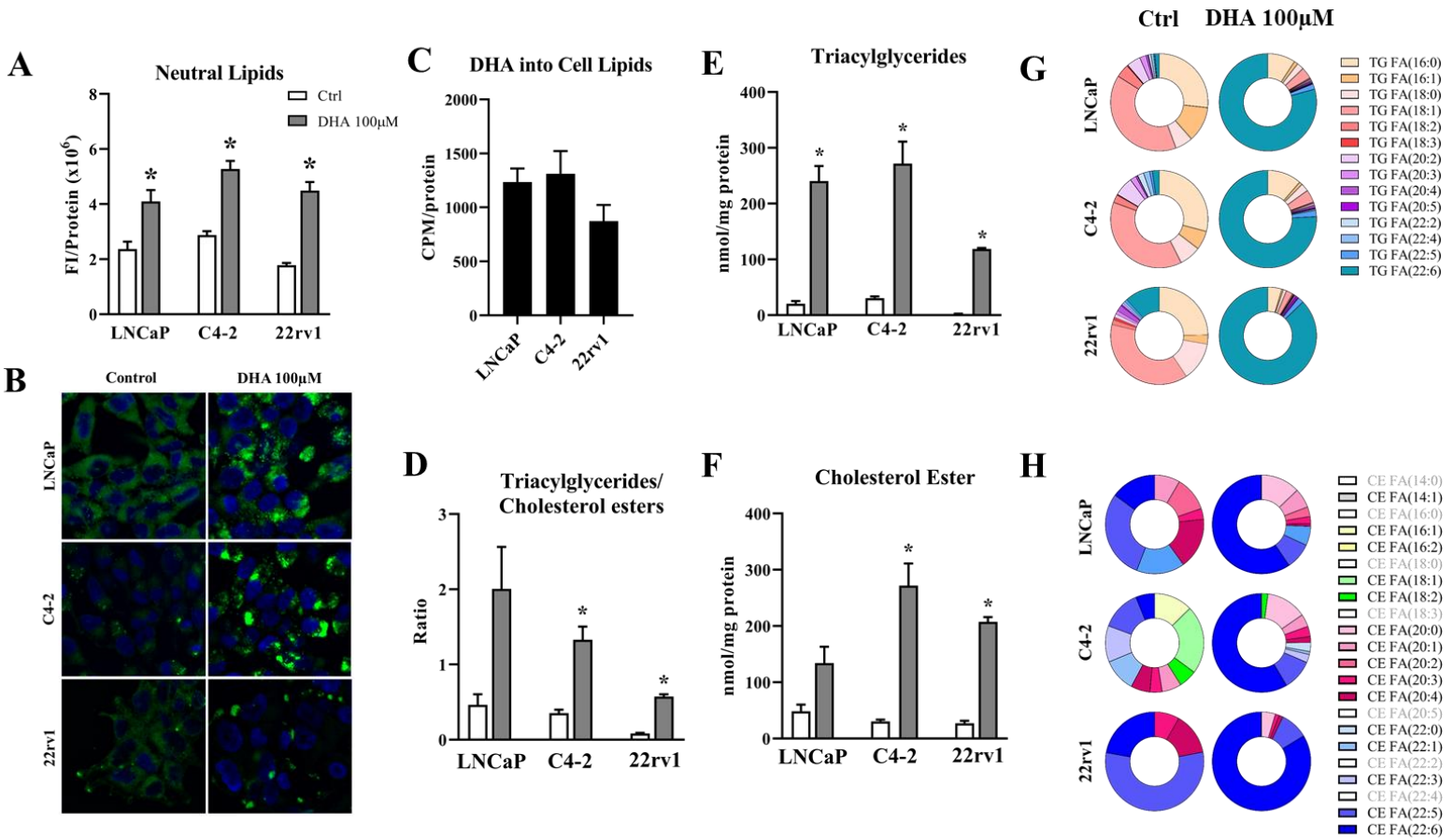

Figure 2. *DHA is accumulated into lipid droplets*. **A)** Neutral Lipids quantification after staining with BODIPY fluorescent dye. Values show average of fluorescence intensity to protein content (n=12). **B)** Images at 400x magnification captured with THUNDER imaging system showing neutral lipids in green and DNA in blue. **C)** [^{14}C]-DHA incorporation into cell lipids. Values show CPM in hydrophobic extract to protein (n=3 at least). **D)** Triacylglycerides to Cholesterol esters ratio, **E)** triacylglycerol levels, **F)** Cholesterol ester levels and their fatty acid profile **(G-H)**. Values shown as average of nmol/mg protein and SD and percentage for each fatty acid (n=3) obtained by mass spectrometry. Clear CE FA writing means not detected. Statistical analysis: Student t-test; * - different from control condition; three independent experiments were performed.

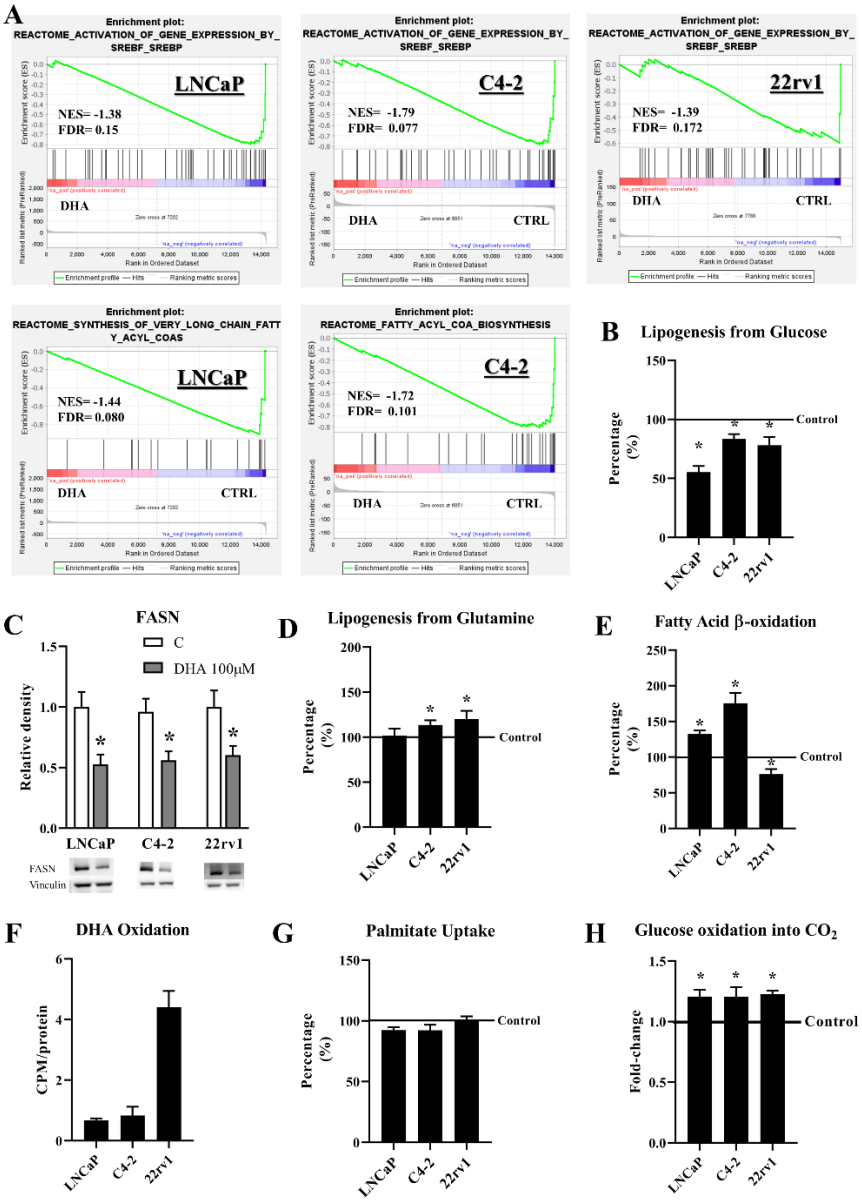

Figure 3. *DHA is oxidized by prostate cancer cells and deregulates lipid metabolism.* **A)** Enrichment plots from GSEA (n=3, FDR≤0.20) showing downregulation of SREBP1 and SREBP2 pathway (first row) in all cell lines and additional pathways related with decreased FA synthesis in LNCaP and C4-2 (second row). **B)** Lipogenesis from glucose and **C)** FASN expression. **D)** Lipogenesis from glutamine. Values shown as percentage compared to vehicle (control, 100%) and SD (n=6 at least) and relative density to vinculin (n=6), respectively. **E)** Palmitate β-oxidation. Values shown as percentage compared to vehicle (control, 100%) and SD (n=6 at least). **F)** DHA oxidation. Values shown as average of CPM to protein content and SD. **G)** Palmitate uptake and **H)** Glucose complete oxidation into CO_2. Values shown as fold-change compared to vehicle and SD (n=6 at least). Statistical analysis: Student t-test; * - different from control condition; all analyzes were performed after three independent assays.

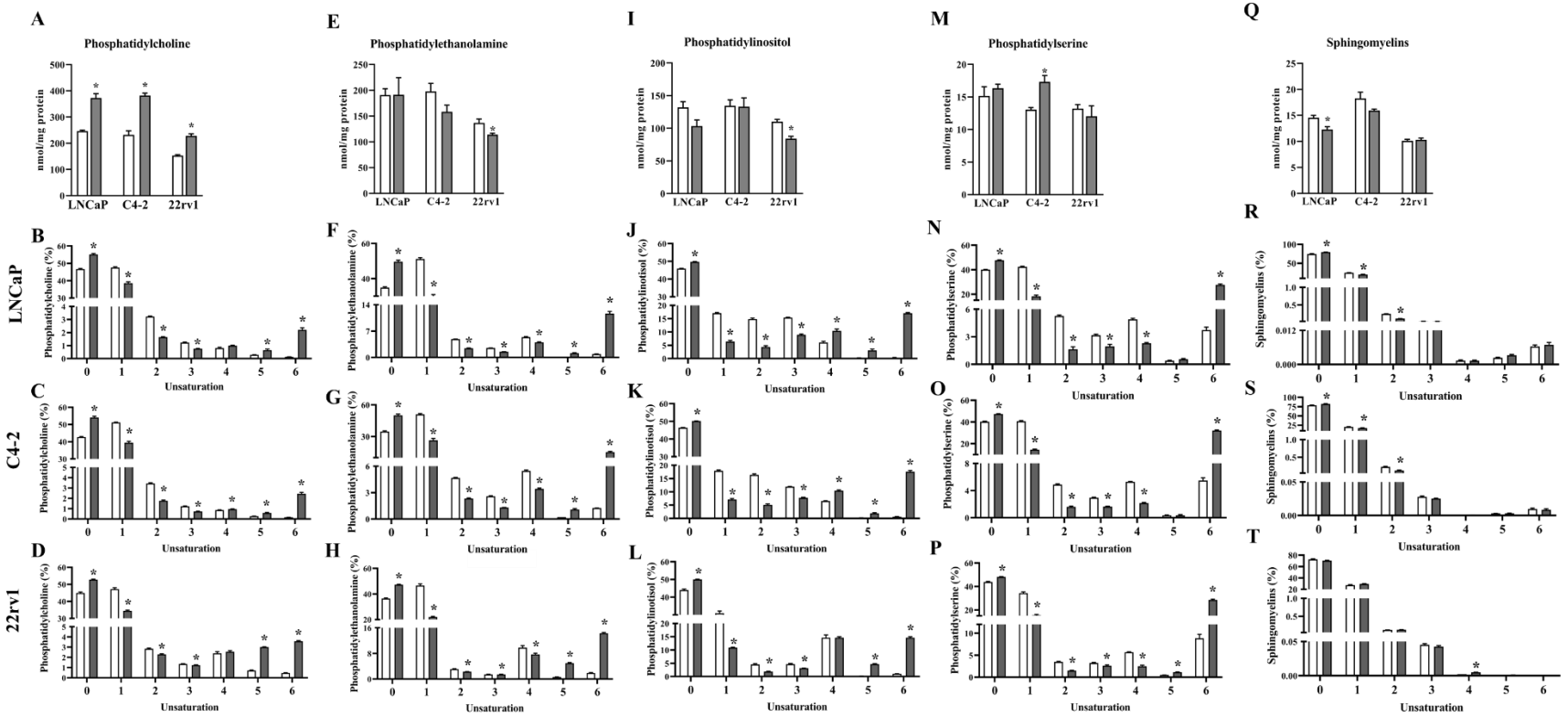

Figure 4. *DHA increased phospholipids unsaturation status*. **A-D)** Phosphatidylcholine, **E-H)** Phosphatidylethanolamine, **I-L)** Phosphatidylinositol, **M-P)** Phosphatidylserine and **Q-T)** Sphingomyelin levels and their unsaturation profile for fatty acids from 0 to 6 double bonds. White bars show control values whereas grey bars show DHA. Values show average of nmol/mg protein or percentage and SD (n=3). Statistical analysis: Student t-test; * - different from control condition; three independent experiments were performed.

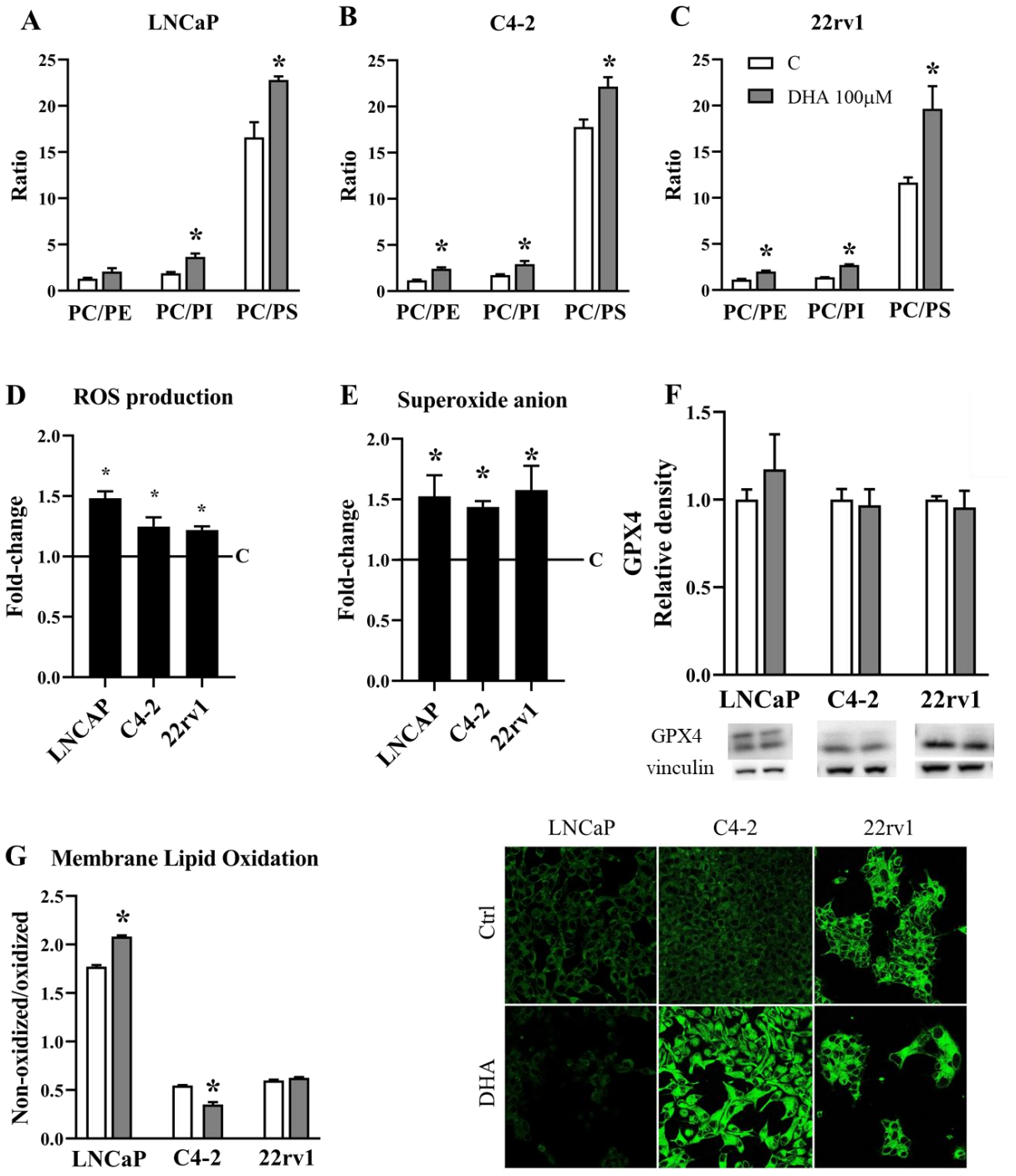

Figure 5. *Membrane composition is imbalanced by DHA affecting phospholipids ratio and induces ROS production.* **A-C)** The omega-3 increased the ratio of PC to PE, PI, or PS in each cell line (n=3) determined by mass spectrometry. **D)** General ROS and **E)** Superoxide anion production after staining with selective probes. Values show average of fold-change to control (1.0) and SD (n=9). **F)** GPx4 protein levels, a ferroptosis marker. Values show relative density to vinculin and SD (n=6). **G)** Membrane lipid oxidation assessed with C11 BODIPY ratiometric assay. Images show oxidized lipids in membrane (green) and were taken at 400x magnification with super-resolution microscope. Values shown as mean of Non-oxidized/oxidized lipids and SEM (n=15 microscopic fields/condition). Statistical analysis: Student t-test; * - different from control condition; all analyzes were performed after three independent assays.

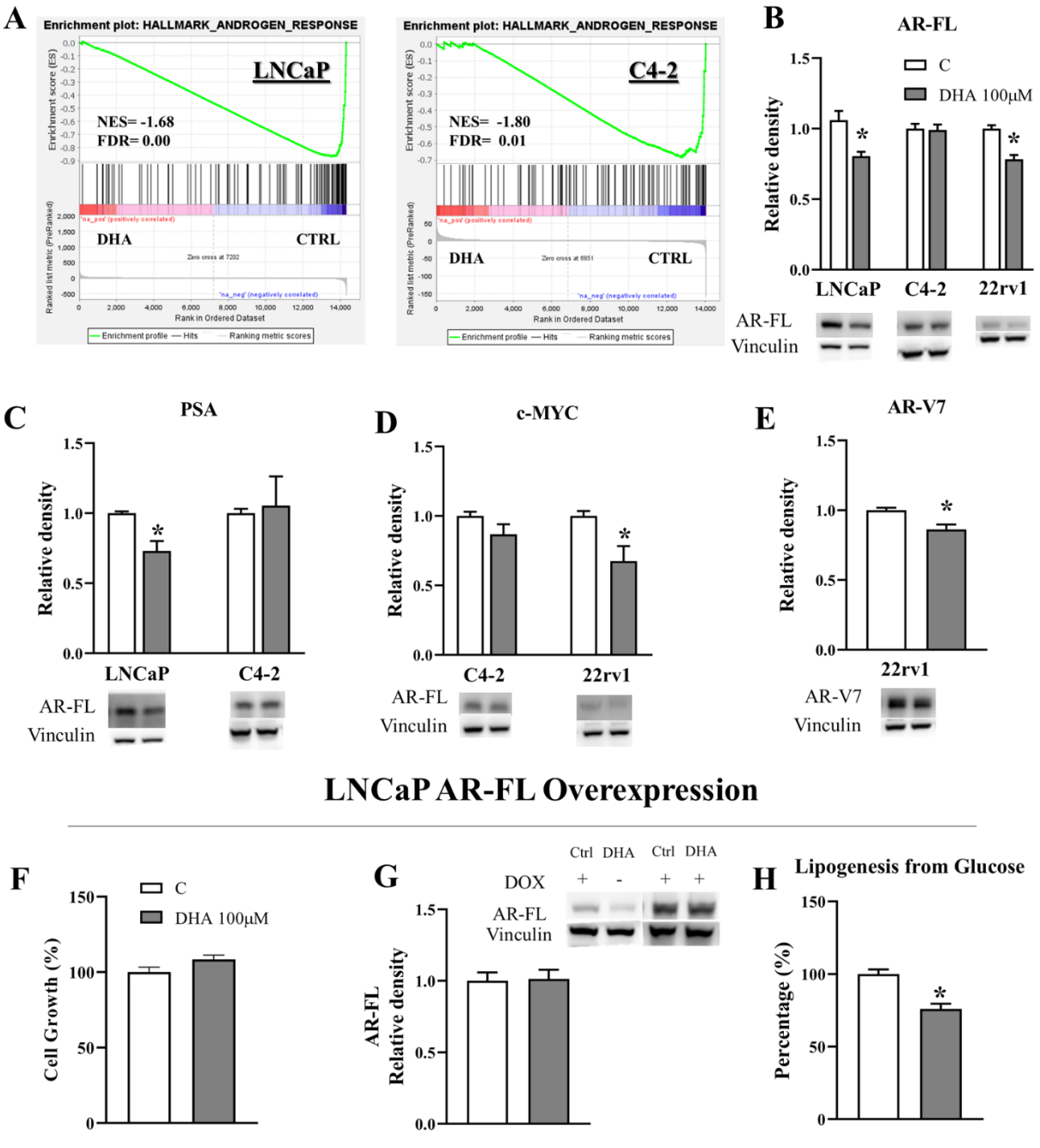

Figure 6. *DHA impairs androgen receptor signaling, but this is not related with decrease in lipogenesis.* **A)** Enrichment plots from GSEA showing downregulation of pathways in response to androgens (n=3, FDR≤0.20) in LNCaP and C4-2. **B)** AR-FL, **C)** PSA, **D)** c-Myc and **E)** AR-V7 expression. Values for protein expression show relative density to vinculin and SD (n=6). **F)** Cell growth in LNCaP cells overexpressing AR-FL and **G)** its protein level after DHA incubation. Values shown as mean and SD for percentage (%) compared to control for cell growth data whereas mean of relative density to vinculin was plotted for protein expression (n=6). **H)** Lipogenesis from glucose in LNCaP cells overexpressing AR-FL. Values shown as percentage (%) to control and SD (n=6). Statistical analysis: Student t-test; * - different from control condition; all analyzes were performed after three independent assays (n=4 at least).

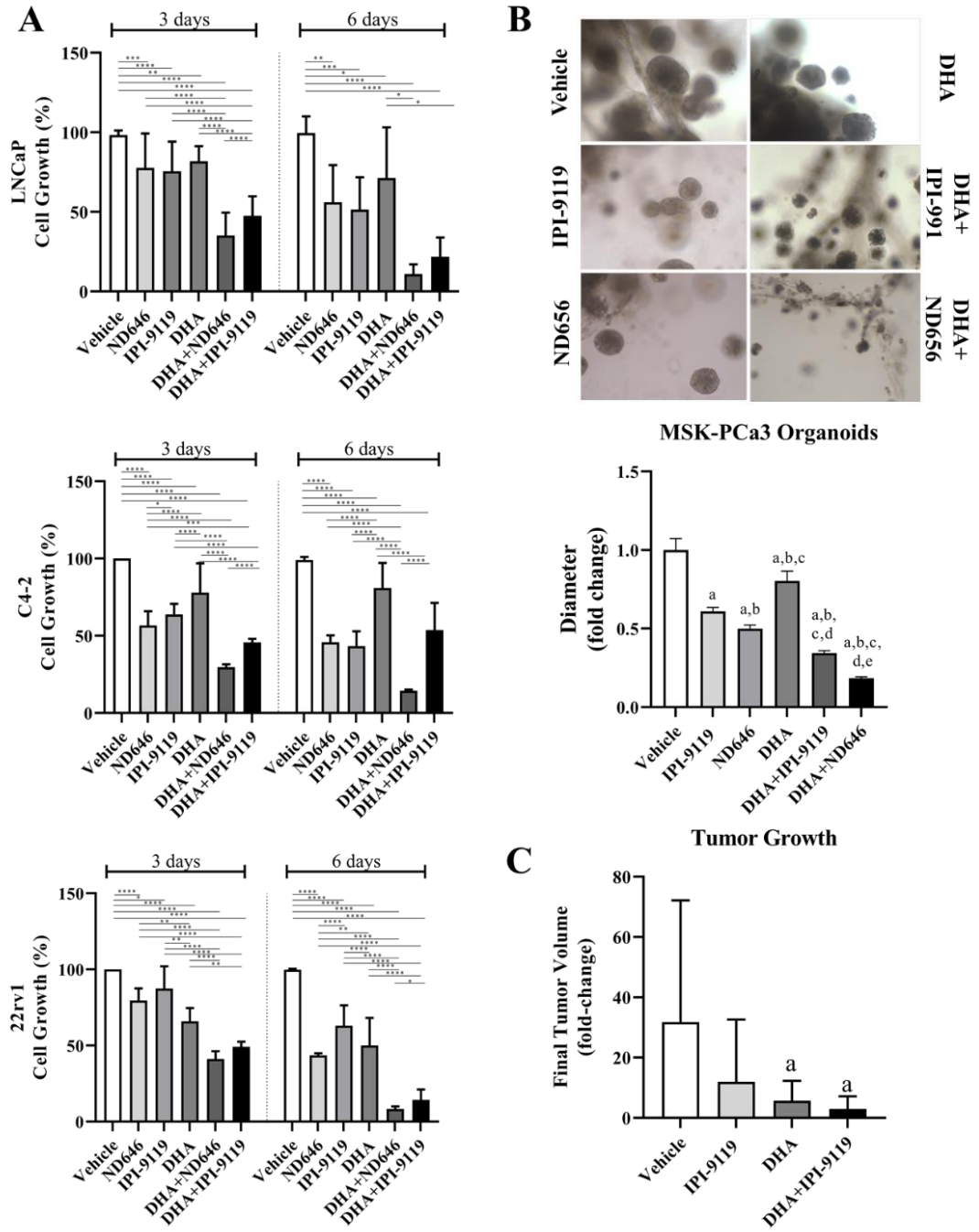

Figure 7. *DHA effect on cell growth is potentiated by lipogenesis inhibitors in vitro and in castrated-resistant xenografts.* **A)** LNCaP, C4-2 and 22rv1 cell growth after 3 and 6 days of incubation with IPI-9119 (FASNi), ND656 (ACC1/2i), DHA 100µM and their combination. Values shown as percentage (%) to control and SEM (n=9). **B)** CRPC organoids MSK-PCa3 diameter after 28 days of incubation with same combination described (n=6). **C)** Tumor growth assessed by final volume compared to initial in 22rv1 xenografts treated for 28 days with IPI-9119 (100mg/ml), DHA (10mg/kg/day) and their combination. Values shown as fold-change and SD (n=12). Statistical analysis: two-way ANOVA; * - $p<0.01$;** - $p<0.001$;*** - $p=0.0001$ **** - $p<0.0001$; a – different from vehicle; b – different from IPI-9119; c – different from ND646; d – different from DHA; e – different from DHA+IPI-9119.

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SUPPLEMENTARY MATERIAL

S1. SUnSET assay

To determine the protein synthesis, we performed the SUnSET assay. Briefly, cells were seeded at $0.5x10^6$ density and, 30 minutes prior to the end of experiment, added 1 µg/mL of puromycin to the medium. Then, cells were collected processed for western blotting. Total newly synthetized protein detected with the antibody anti-Puromycin (#MABE343; Millipore, 1:1000) and normalized to vinculin. Three independent experiments were performed in duplicate (n=6). Mean of relative density was plotted plus SD.

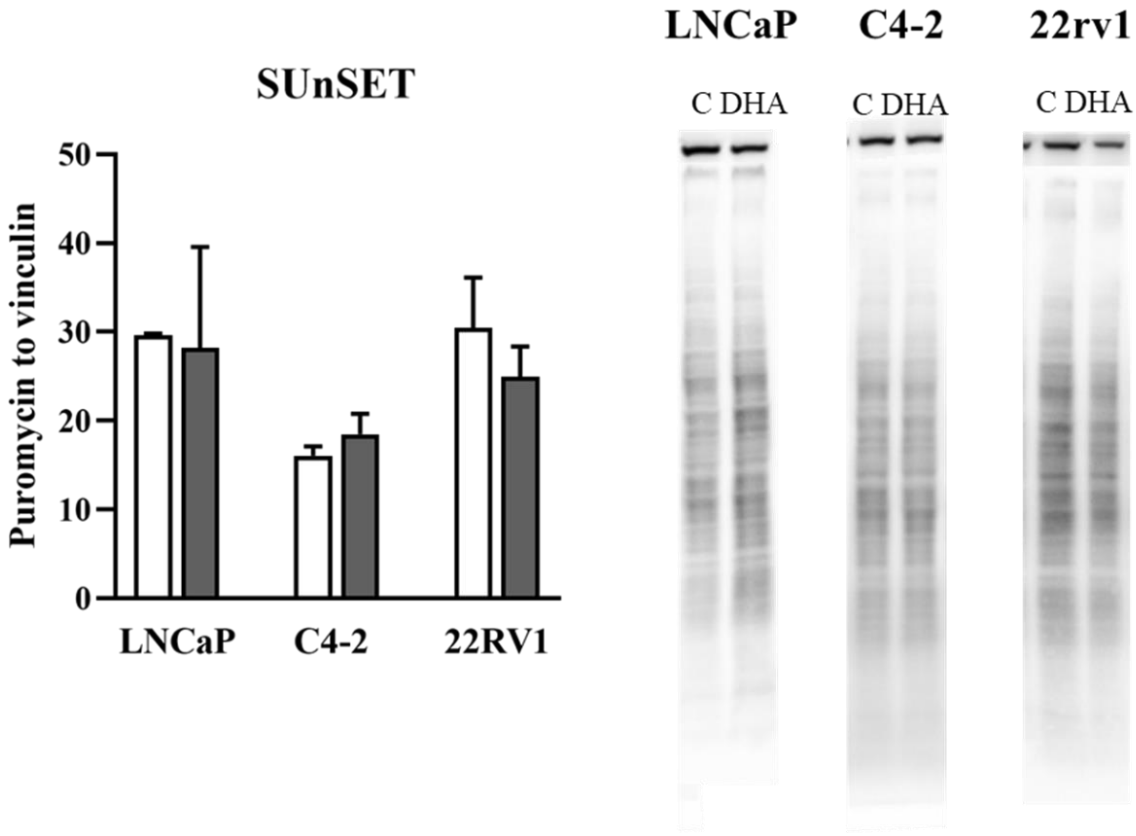

Figure S1. *SUnSET assay.* Newly protein synthesized under vehicle (ethanol) or DHA incubation. DHA did not change protein synthesis. On the left, graph shows relative density of puromycin incorporation to vinculin. Values show mean and SD. On the right, western blotting membrane with anti-puromycin antibody.

S2. Antioxidant genes expression

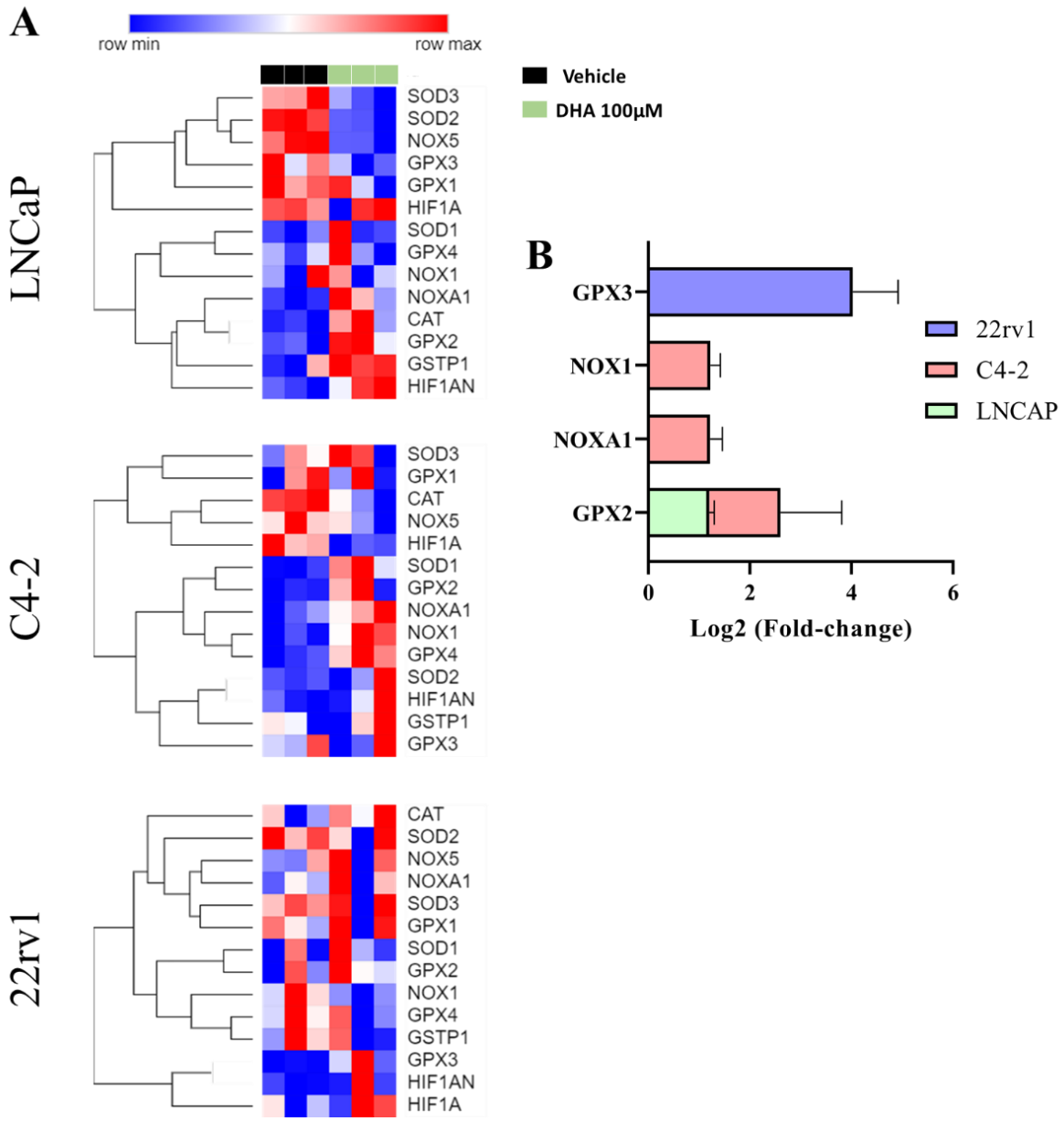

Figure S2. *Antioxidant gene expression.* A) Heat maps for gene expression profile from RNA-seq data. B) Statistically significant gene expression for LNCaP, C4-2 and 22rv1. Three independent experiments were performed (n=3).

S3. Phosphatidylcholine metabolism

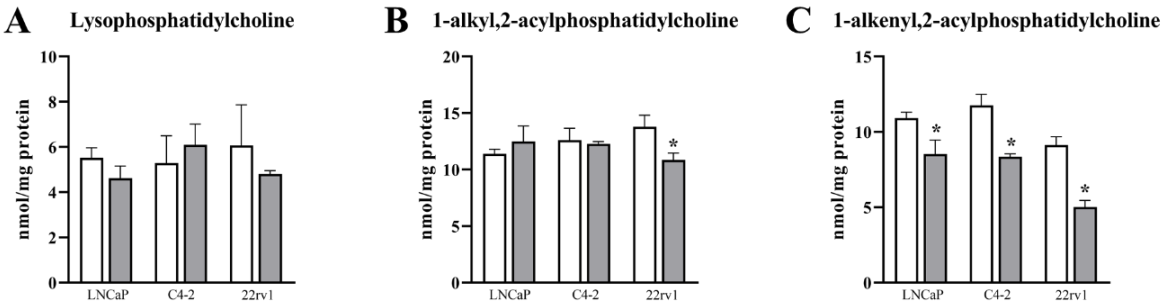

Figure S3. A) Lysophosphatidylcholine**, B)** 1-alkyl,2-acylphosphatidylcholine and **C)** 1-alkenyl,2-acylphosphatidylcholine. Values shown as mean of nmol/mg protein and SD. Three independent experiments were performed (n=3).

S.4 Palmitate Rescue

To determine whether DHA effects were exclusively from DNL, we performed the Palmitate Rescue experiment. Briefly, cells were seeded at $0.5x10^6$ density and after 24h incubated with DHA at 100µM and Palmitate (#102720-100, Seahorse XF Palmitate-BSA FAO Substrate, Agilent) at 50µM for 48h. Then, cells were collected and growth determined by trypan blue method exclusion using Vi-Cell Blue XR (Beckman Coulter, USA). Values were shown as percentage of cell growth (n=6 at least) compared to vehicle (100%) and SD.

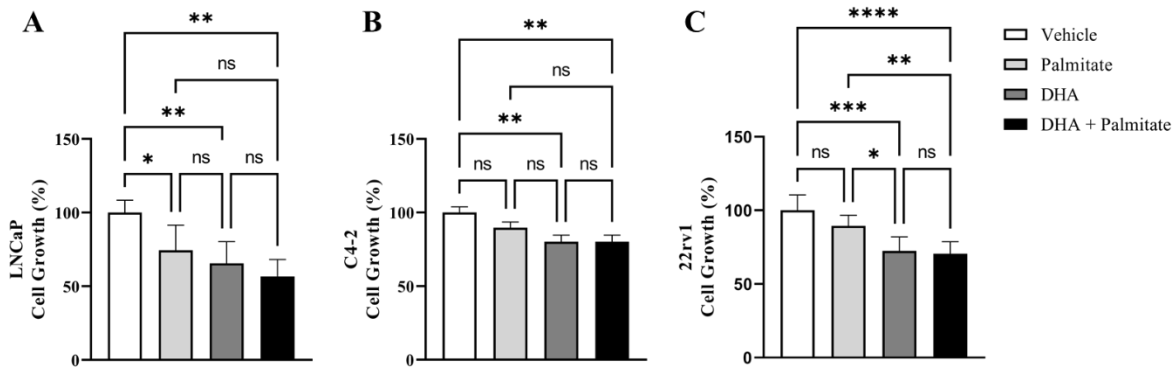

Figure S4. *Palmitate Rescue experiment*. **A)** LNCaP**, B)** C4-2 and **C)** 22rv1 cell growth after DHA and Palmitate incubation for 48h. Values shown as mean of percentage and SD. Three independent experiments were performed.

S.5 Lipogenic Gene Expression Profile

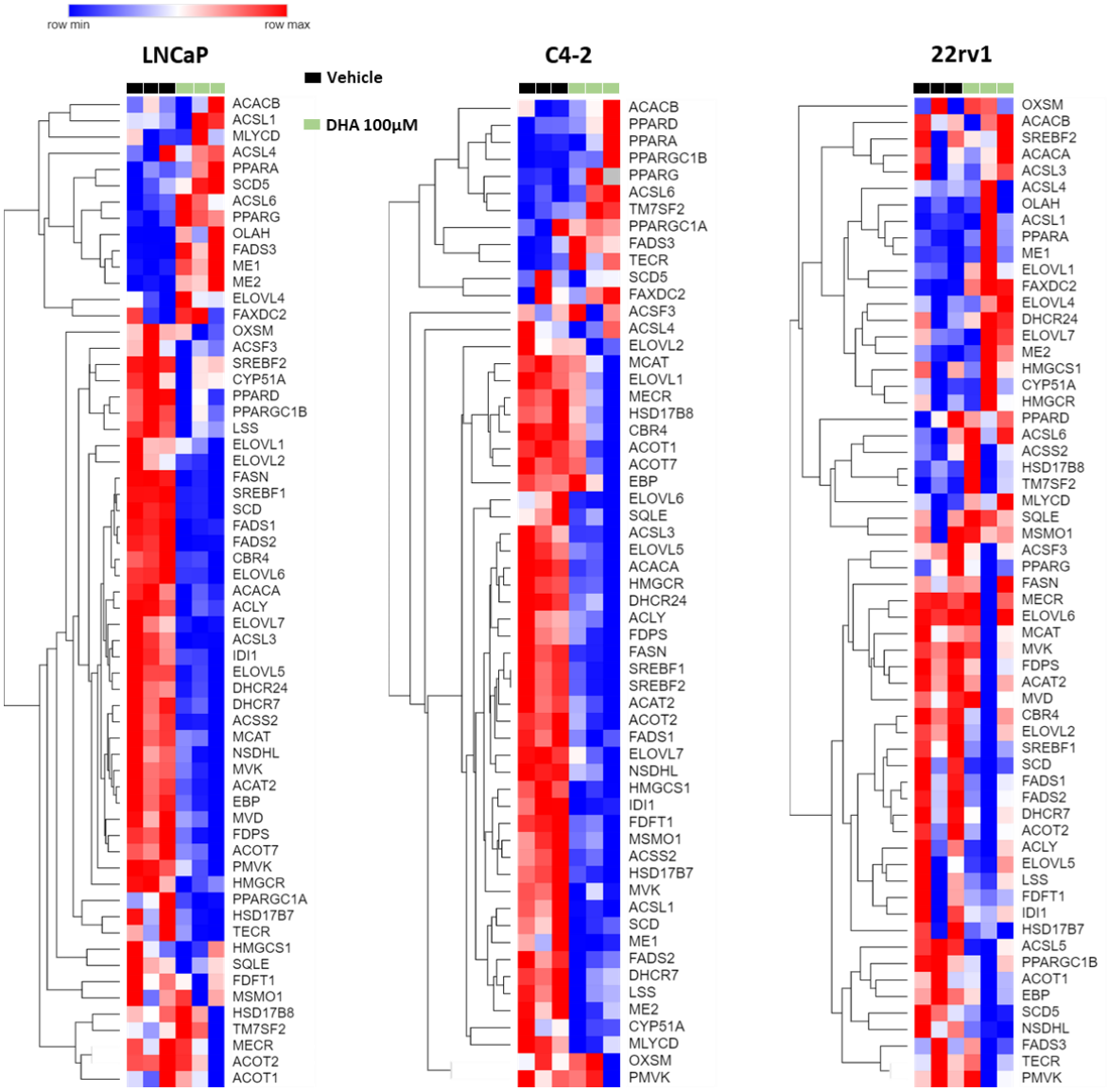

Figure S5. *Lipogenic Genes Expression Profile*. Gene Expression profile from RNA-seq output for LNCaP, C4-2 and 22rv1 after DHA incubation for 48h at 100µM. Genes were clustered with Euclidean algorithm using Morpheus tool (https://software.broadinstitute.org/morpheus/).

IV. DISCUSSÃO

As alterações no metabolismo celular no CaP são cruciais para as etapas de iniciação e progressão, sendo fortemente relacionados à agressividade e atividade metastática (AHMAD; CHERUKURI; CHOYKE, 2021; DEEP; SCHLAEPFER, 2016; SCHÖPF et al., 2020; ZADRA; PHOTOPOULOS; LODA, 2013). Neste contexto, fatores externos podem influenciar na modulação do metabolismo tumoral, em especial a dieta, o que tem sido associada tanto positivamente (LABBÉ et al., 2019; SILVA et al., 2015; TAMARINDO et al., 2019) quando negativamente (LIANG et al., 2016; TAMARINDO; GÓES, 2020; TAMARINDO et al., 2019) ao desenvolvimento do CaP. Nesta Tese, mostramos que o DHA, um ácido graxo de baixa síntese em humanos (GILTAY et al., 2004) e facilmente obtido pela dieta, é capaz de modular o metabolismo de células prostáticas em diferentes frentes mecanísticas, promovendo a redução do crescimento celular (Figura 8).

Primeiramente, observamos que a 100µM por 48 horas o DHA reduziu o crescimento tanto em células pré-malignas quanto tumorais independentemente do seu contexto molecular (TAMARINDO; GÓES, 2020). É importante mencionar que esta concentração escolhida se aproxima da encontrada no plasma humano ou após 28 dias de suplementação com ácidos graxos ômega-3 (MANN et al., 2006; RUSCA et al., 2009; WANG et al., 2008), de forma que os achados neste trabalho apresentam potencial aplicação. Neste estudo, descrevemos o acúmulo lipídico em células prostáticas após a incubação com DHA, como também o aumento na geração do ânion superóxido em todas as linhagens, porém em níveis diferentes em cada uma delas. Estas alterações foram acompanhadas de alterações na morfologia mitocondrial que indicam potenciais danos no funcionamento desta organela, sugerindo modulação do metabolismo celular pelo DHA. Para comprovar esta hipótese, foi realizada uma análise direcionada de vias específicas, em especial de receptores nucleares que atuam como sensores metabólicos, como também de genes envolvidos com a via androgênica. Observamos a propriedade do DHA em regular genes associados à resposta metabólica, estresse e da via de hormônios, conforme esperado, em todas as linhagens. Entretanto, a resposta na regulação do perfil de expressão gênica apresentou maior intensidade na linhagem pré-maligna PNT1A em comparação com as tumorais. Estes achados ainda mostraram que genes que tiveram sua expressão alterada pelo DHA, em sua maioria, estão desregulados em amostras de CaP de pacientes e de forma contrária à regulação exercida por esse ômega-3.

Além de reduzir o crescimento celular, o DHA na concentração e tempo de incubação utilizados, mostrou um efeito diferencial no metabolismo mitocondrial, de forma que na linhagem pré-maligna houve redução e, nas tumorais, aumento deste parâmetro, ainda que o crescimento celular tenha sido suprimido. Este achado, somado à menor expressão de genes relacionados à via mitocondrial, como o *PPARGC1A*, levantaram questões sobre o envolvimento das mitocôndrias no efeito antitumoral do DHA. Desta forma, avaliamos parâmetros envolvidos na função, biogênese, composição de membrana e expressão de genes mitocondriais, além da morte celular. Observamos que o ômega-3 prejudicou a função mitocondrial, a síntese de ATP e a capacidade celular de responder a insultos tanto na linhagem pré-maligna quanto nas resistentes à castração. Além disso, o DHA pareceu estimular a glicólise ao seu máximo na PNT1A, enquanto nas tumorais reduziu a reserva glicolítica associado ao aumento da oxidação completa da glicose. Em conjunto, estes achados indicam que cada linhagem responde de forma diferente ao comprometimento mitocondrial, de forma que na PNT1A parece haver uma resposta compensatória da via respiração anaeróbica, enquanto nas tumorais ocorre o desvio de glicose para uso mitocondrial. Apesar destes efeitos necessitarem de experimentos adicionais para melhor elucidação dos mecanismos, estes resultados também indicam que o DHA sensibiliza as células, o que tem sido descrito em estudos que mostraram o efeito potencializado da combinação do ômega-3 a outros compostos, inclusive de nosso laboratório (TAMARINDO et al., 2019). Portanto, estes achados se alinham aos resultados de redução na atividade mitocondrial anteriormente descritos (TAMARINDO et al., 2019). Além disso, em especial na linhagem pré-maligna, a incubação com DHA induziu drasticamente à menor expressão de genes mitocondriais codificados no núcleo relacionados à homeostase, tráfego de moléculas e organização, o que não ocorreu para as linhagens tumorais. Ainda, este ômega-3 foi capaz de desregular a biogênese mitocondrial levando à fragmentação da rede desta organela. Desta forma, estes dados indicam que, assim como demonstrado anteriormente (TAMARINDO et al., 2019), o DHA apresenta um potencial preventivo no CaP por induzir resposta mais intensa nas células PNT1A, em especial via mitocondrial, o que leva à morte celular e, portanto, à redução do crescimento celular.

Células benignas apresentam naturalmente maiores níveis de DHA, enquanto nas tumorais estes níveis estão reduzidos (ESER et al., 2013). Este cenário indica que o ômega-3 detém propriedade na próstata saudável ou com distúrbios proliferativos iniciais, o que pode

ser perdida durante a carcinogênese de forma que células prostáticas malignas passam a metabolizar estes PUFAs ou a eliminá-los, uma vez que estes ácidos graxos induzem à maior susceptibilidade ao estresse oxidativo (TOUSIGNANT et al., 2020), como também a β-oxidação de lipídios é a principal fonte de energia no CaP (LIU, 2006). Então, na etapa seguinte deste trabalho, avaliamos o efeito do DHA no metabolismo de lipídios, assim como o potencial de células tumorais prostáticas em metabolizá-lo, o que não foi descrito ainda na literatura. Células andrógeno responsivas (LNCaP) e resistente à castração (C4-2 e 22rv1) de diferentes contextos androgênicos captaram o ômega-3, acumulando-o como lipídios neutros devido à sua incorporação em triacilgliceróis (TAG) e ésteres de colesterol (CE). A baixa razão TAG/CE encontrada para todas as células testadas demonstra que o DHA apresenta principalmente função estrutural, embora seja também utilizado como fonte de energia. Nossa hipótese é que, por ser um PUFA, seu acúmulo pode sensibilizar células e induzir a morte celular, uma vez que o elevado número de insaturações nesses lipídios de membrana favorecem a lipoperoxidação (TOUSIGNANT et al., 2020). Isto é corroborado por nossos dados que mostram o aumento nos níveis de insaturações nos glicerofosfolipídios, em especial fosfatidilcolina, fosfatidiletanolamina, fosfatidilserina, fosfatidilinositol e esfingomielina. Além disso, a deposição de PUFAs em gotículas lipídicas pode constituir um mecanismo de proteção antioxidante, evitando morte celular por ferroptose (BAILEY et al., 2015). O acúmulo de lipídios intracelulares está associado à menor síntese de lipídios (TOUSIGNANT et al., 2020), fenômeno também observado em nosso trabalho. A incubação com DHA modulou negativamente a via de ativação gênica via SREBP, mecanismo de regulação da expressão de genes lipogênicos, como *FASN* (SWINNEN et al., 1997b). Isto refletiu na menor expressão proteica de FASN, como também na reduzida lipogênese a partir de glicose, o que reduz a disponibilidade de palmitato utilizado na síntese de membranas, sendo um requerimento para progressão do ciclo celular (SCAGLIA et al., 2014). Apesar de reduzir a lipogênese a partir de glicose, o DHA aumentou a síntese de lipídios a partir da glutamina nas células resistentes à castração, o que pode indicar um mecanismo de resistência, em especial em condições de estresse oxidativo (HUANG et al., 2016). Além da lipogênese, o ômega-3 ainda induziu à maior oxidação de ácidos graxos, sendo na 22rv1 preferencialmente o próprio DHA. Isto indica que células correspondentes a diferentes estágios tumorais metabolizam de forma distinta os ácidos graxos, o que tem sido sugerido entre células benignas e malignas (DUEREGGER et al., 2015), e pode estar associado à progressão e agressividade tumoral. É valido mencionar que a linhagem 22rv1 apresentou maior redução do crescimento celular, o

que está alinhado a outro estudo que apontou o maior efeito do DHA em linhagens não responsivas a andrógenos comparado a responsivas (BRATTON; MALY; HOFMANN, 2019), como também à hipótese de que células tumorais apresentam, sem suplementação, baixa concentração do ômega-3 por este apresentar propriedade antitumoral.

Um dos principais desafios atualmente é a busca por estratégias terapêuticas que não possuam como alvo principal a via androgênica, uma vez que pode ocorrer reincidência e em estágio mais agressivo (CAI et al., 2018; KARANTANOS; CORN; THOMPSON, 2013; SHAFI; YEN; WEIGEL, 2013). De forma inédita, nossos dados mostraram que o AR está envolvido na redução do crescimento celular, mas não é o mecanismo pelo qual a via lipogênica está suprimida. O DHA reduziu a expressão dos níveis proteicos de AR, tanto a isoforma completa, como o AR-V7, associado exclusivamente à resistência à castração (ZHU et al., 2020). Entretanto, este efeito do DHA parece obedecer a um limite, uma vez que em células com amplificação do gene do AR, assim como naquelas que o superexpressam de forma induzida, o seu efeito nos níveis de AR é nulo. A redução de AR em células incubadas com DHA foi descrita previamente e ocorre via degradação proteassomal (HU et al., 2015), o que não foi avaliado em nosso estudo. Portanto, nossos dados são promissores por indicarem que a via androgênica pode ser aditiva, mas não majoritária, na inibição do crescimento celular pelo DHA, e não está envolvida na redução da lipogênese, um dos principais marcos da carcinogênese na próstata, associado à iniciação e progressão tumoral.

Tendo em vista falta de estratégias efetivas no estágio resistente à castração e o efeito mais expressivo do DHA na 22rv1, testamos sua validade em organoides humanos e em modelo xenográfico. Observamos que o DHA reduziu o diâmetro e o crescimento tumoral, o que foi potencializado por inibidores da lipogênese, em especial da FASN. A inibição farmacológica desta enzima tem sido proposta como alternativa terapêutica no estágio resistente à castração, induzindo a parada do ciclo celular e comprometimento da sinalização de AR-V7 (BASTOS et al., 2021; ZADRA et al., 2019). Desta forma, nossa proposta em combinar fatores da dieta e a administração destes inibidores mostram-se potencializados quando praticados concomitantemente. Porém, estudos mais aprofundados precisam ser realizados para melhor compreender os mecanismos moleculares envolvidos.

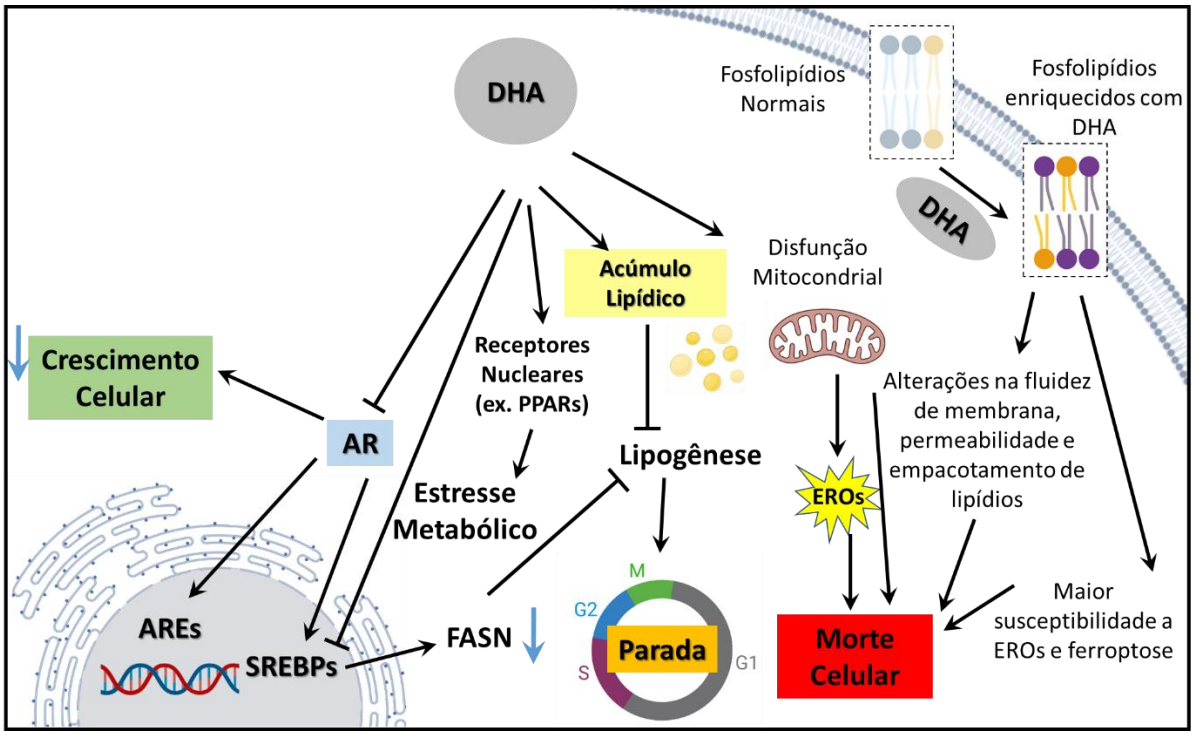

Figura 8. *Resumo*. O DHA é uma molécula multialvo tendo como desfecho a redução do crescimento celular via modulação do metabolismo. Os mecanismos envolvidos diferem a depender do contexto androgênio e estágio da progressão tumoral prostática, mas é possível afirmar que o DHA tem propriedade de alterar a composição de membranas aumentado o índice de insaturação e, portanto, a susceptibilidade a danos oxidativos e induzindo alterações nas propriedades físico-químicas que afetam a sinalização e permeabilidade. O microambiente rico em DHA pode ser danoso às células, o que induz ao acúmulo de DHA em gotículas lipídicas, funcionando como um tampão para alívio do estresse metabólico. Esta condição inibe a lipogênese *de novo*, como também vias de sinalização correguladoras da proliferação e sobrevivência, fatores essenciais para a progressão do ciclo celular. O DHA ainda suprimiu a via androgênica, cujo desfecho foi aditivo na redução do crescimento celular, mas a menor taxa de síntese de lipídios não dependeu desta via. Além destas alterações, o ômega-3 ainda causou disfunção mitocondrial provocando aumento na produção de EROs, como também comprometendo a capacidade da célula em responder a insultos adicionais, o que indica sua propriedade de sensibilização a outros compostos antitumorais. A via mitocondrial parece ser o principal mecanismo da ação preventiva do DHA no estágio pré-maligno causando a morte celular, enquanto o metabolismo de lipídios e parada do ciclo celular parecem ser os mecanismos predominantes no contexto tumoral.

V. CONCLUSÃO

Em conclusão, o DHA mostrou propriedade antitumoral pela modulação do metabolismo celular via diferentes alvos, tanto em células pré-malignas como nas tumorais andrógeno responsivas e resistentes à castração, sendo estas AR expressivas ou AR não-expressivas. Este ômega-3 induziu maior produção de ROS, como também o acúmulo de lipídios neutros em todas as linhagens, o que foi acompanhado de diferentes cenários a depender do tipo celular. No estágio pré-maligno, o DHA reduziu a proliferação e induziu a morte celular, modulando principalmente a expressão gênica da via de andrógenos, de receptores nucleares e seus regulares, como também induziu ao comprometimento da função, biogênese e fisiologia mitocondrial. Nas linhagens tumorais, a propriedade antitumoral foi associada à parada do ciclo celular e morte celular a depender da linhagem considerada, principalmente devido à modulação da reprogramação metabólica, com foco na redução da síntese *de novo* de lipídios, uns dos principais eventos da carcinogênese prostática, como também na alteração de propriedades da membrana celular. Além destes achados, o DHA ainda reduziu a capacidade de reserva bioenergética mitocondrial em linhagens resistentes à castração, indicando seu potencial uso terapêutico quando associado a outros compostos para amplificar o efeito antitumoral. Por fim, a propriedade antitumoral do DHA foi validada em organoides e animais xenográficos resistentes à castração, oferecendo novas perspectivas no tratamento do câncer de próstata.

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VII. APÊNDICE

ATIVIDADES COMPLEMENTARES

Artigos publicados não relacionados à Tese

SANCHES, B.D.A.; TAMARINDO, G.H.; MALDARINE, J.D.S.; DA SILVA, A.D.T.; DOS SANTOS, V.A.; GÓES, R.M.; TABOGA, S.R.; CARVALHO, H.F. Telocytes of the male urogenital system: Interrelationships, possible functions, and pathological implications. CELL BIOLOGY INTERNATIONAL. doi: 10.1002/cbin.11612, 2021.

TAMARINDO, G.H.; GOBBO, M.G. ; TABOGA, S.R.; ALMEIDA, E.A. ; GÓES, R.M. . Melatonin ameliorates degenerative alterations caused by age in the rat prostate and mitigates high-fat diet damages. CELL BIOLOGY INTERNATIONAL, v. 1, p. 1-15, 2020.

SANCHES, B.D.A. ; MALDARINE, J.D.S.; TAMARINDO, G.H.; DA SILVA, A.D.T. ; LIMA, M.L.D. ; RAHAL, P. ; GÓES, R.M. ; TABOGA, S.R. ; CARVALHO, H.F . Explant culture: A relevant tool for the study of telocytes. CELL BIOLOGY INTERNATIONAL, v. 2020, p. 1-14, 2020.

SANCHES, B.D.A.; LEONEL, E.C.R. ; MALDARINE, J.S. ; TAMARINDO, G.H. ; BARQUILHA, C.N.; FELISBINO, S.L. ; GOÉS, R.M. ; VILAMAIOR, P.S.L ; TABOGA, S.R. . Telocytes are associated with tissue remodeling and angiogenesis during the post lactational involution of mammary gland in gerbils. CELL BIOLOGY INTERNATIONAL, v. 44, p. 2512-2523, 2020.

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SANCHES, B.D.A ; MALDARINE, J. S. ; ZANI, B.C. ; TAMARINDO, G.H.; BIANCARDI, M.F. ; SANTOS, F.C A. ; RAHAL, P. ; GÓES, R.M. ; FELISBINO, S. L. ; VILAMAIOR, P.S.L. ; TABOGA, S.R. Telocytes play a key role in prostate tissue organisation during the gland morphogenesis. JOURNAL OF CELLULAR AND MOLECULAR MEDICINE (PRINT), v. XX, p. 1-13, 2017.

GOBBO, M.G.; TAMARINDO, G.H.; RIBEIRO, D.L. ; DE CAMPOS, S.G.P.; TABOGA, S.R.; GÓES, R.M. Pathological lesions and global DNA methylation in rat prostate under streptozotocin-induced diabetes and melatonin supplementation. CELL BIOLOGY INTERNATIONAL, v. -, p. 1-18, 2017.

Capítulo de livro publicado

GOBBO, M.G.; TAMARINDO, G.H.; TABOGA, S.R.; GOES, R.M. The Protective Role of Exogen Melatonin on the Prostate Gland under Experimental Models. In: Lore Correia e Germaine Mayers. Melatonin: Medical Uses and Role in Health and Disease. Nova Science Publishers, 2018. ISBN: 978-1-53612-987-8.

Apresentações em eventos científicos relacionadas à Tese

- “Docosahexaenoic acid decrease proliferation of benign and malignant cells via metabolic stress induction and dysregulation of androgen pathway, de autoria de Guilherme Henrique Tamarindo e Rejane Maira Goes, foi apresentado no XXXIV FeSBE Annual Metting, em Campos do Jordão, São Paulo, 2019.

- “Docosahexaenoic Acid Differentially Modulates the Cell Cycle and Metabolism-Related Genes in Prostate Cells and Delays Tumor Progression in TRAMP Mice in the session on Mitochondria and Cellular Metabolism”, de autoria de Guilherme Henrique Tamarindo; Alana Della Torre Silva; Gustavo Matheus Amaro; Celina de Almeida Lamas, Valéria Helena Alves Cagnon Quitete e Rejane Maira Góes, foi apresentado no Cell Bio Virtual 2021 da American Society for Cell Biology, Estados Unidos, 2020.

- “Docosahexaenoic acid anti-tumor property is associated with mitochondrial impairment in prostate pre-malignant cells and distinct growth suppression in castrated-resistant phenotypes”, de autoria de Guilherme Henrique Tamarindo, Caroline Fidalgo Ribeiro, Fernanda Ramos Gadelha, Massimo Loda e Rejane Maira Góes, foi apresentado no XX Congress of the Brazilian Society for Cell Biology, Virtual, Brasil, de 27 a 20 de Janeiro de 2021.

- “Docosahexaenoic acid deregulates lipid metabolism in androgen sensitive and castrated-resistant prostate cancer models", de autoria de Guilherme Henrique Tamarindo, Caroline Fidalgo Ribeiro, Massimo Loda, Rejane Maira Góes, foi apresentado XXXV Reunião Anual da Federação de Sociedades de Biologia Experimental - FeSBE, realizada online, no período de 8 a 11 de setembro de 2021.

Participação em mesa-redonda e palestras

- “O grande escape: modificações na sobrevivência, proliferação e metabolismo de células tumorais”, no I Simpósio de Biociências e Microbiologia, no Instituto de Biociências, Letras e Ciências Exatas, IBILCE – UNESP, em São José do Rio Preto, São Paulo, no dia 23 de novembro de 2017.

- “Mesa Redonda: Biólogo no Exterior”, organizado pela Empresa Junior BUDJr da Universidade Federal de São Paulo, Virtual, em 2021.

“A Biologia Celular e o Câncer”, organizado pelo ENEM Conectado do Sesi Pedro Leopoldo (MG), Virtual, em 2021.

Cursos realizados

- “São Paulo School For Advanced Science In Cell Biology”, ESPCA-FAPESP, na Faculdade de Ciências Farmacêuticas da Universidade de São Paulo (FCF-USP), em São Paulo, São Paulo, de 3 a 13 de março de 2018.

- “Câncer, regeneração e envelhecimento: da cromatina ao metabolismo celular”, curso pré-congresso do XIX Congress of the Brazilian Society for Cell Biology, em Ribeirão Preto, São Paulo, em 18 de julho 2018.

Organização de evento

- Membro Comissão Organizadora da II Curso de Verão em Biologia Celular e Estrutural, Virtual, Campinas, São Paulo, realizada pelo Instituto de Biologia da Universidade Estadual de Campinas, de Janeiro a Fevereiro de 2021.

VIII. ANEXOS

VIII.I Termo de aprovação da pesquisa pela Comissão de Bioética e/ou Biossegurança pertinente

Weill Cornell Medical College

Institutional Animal Care and Use Committee

To:	Loda, Massimo
From:	Dr. Andrew Nicholson, Chairman Institutional Animal Care and Use Committee (IACUC)
Date:	February 24, 2021
Subject:	IACUC Approval Notification
Project Title:	FASN inhibitor effects on prostate cancer

Your protocol has been approved.

Approval Date:	February 24, 2021
Protocol Number:	2019-0031
Request:	Amendment
Personnel Listed:	Loda, Massimo; Babich, John; Jere, Madhavi; Kelly, James; Pakula, Hubert; Ribeiro, Caroline; Tamarindo, Guilherme; Williams, Clarence

The following grant/sponsored project application(s) associated with this IACUC protocol was/were reviewed and the IACUC confirms that the research involving animals specified in the application(s) listed below is approved.

Grant sponsored Project PI:	Loda, Massimo
Grant sponsored Project Title:	Pharmacologic Inhibition of Lipogenesis Suppresses AR and Its Splice Variants to Inhibit Progression of Castration-Resistant Prostate Cancer
Grant sponsored Project Number:	W81XWH-17-1-0-0483
Grant Agency/Sponsor:	United States Department of Defense (United States Department of Defense)

Species Use	Category C	Category D	Category E
Experimental Plan for Mice	930	1260	0

Biologic hazards(s):	Human Xenograft
Chemical hazard(s):	Abiraterone, Docetaxel, Enzalutamide, Etomoxir, Galaterone, IPI-9119, Olaparib, Paclitaxel
Radionuclide hazard(s):	11C-acetate, 11C-DHA, 11C-palmitate, 18-F-FDG

INFORMAÇÃO CEUA nº 3/2022

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CEUA/Unicamp

INFORMAÇÃO

A Comissão de Ética no Uso de Animais da UNICAMP – CEUA/UNICAMP – esclarece que não há necessidade de submeter o projeto de pesquisa **"Efeitos do ácido docosahexaenóico no metabolismo celular na iniciação e progressão tumoral prostática"**, de responsabilidade do Profa. Dra. Rejane Maira Góes e do executor: Guilherme Henrique Tamarindo, para análise desta comissão.

Justifica-se por se tratar de projeto que foi realizado durante o período de estágio no exterior. Os experimentos com animais (modelo xenográfico em camundongos Nude) foram realizados durante o Estágio Sanduíche do aluno Guilherme Henrique Tamarindo no âmbito do Programa CAPES/Print em colaboração com o Prof. Dr. Massimo Loda, pesquisador na Weill Cornell Medicine em Nova York, Estados Unidos. O projeto foi submetido e aprovado em Fevereiro de 2021 pela CEUA da referida instituição estrangeira (Institutional Animal Care and Use Committee, IACUC), Protocolo N° 2019-0031. O camundongo Nude é geneticamente modificado, mas não se enquadra na categoria transgênico, por apresentar mutação espontânea no gene Forkhead box N1 no cromossomo 11, por isso, não houve necessidade de aprovação do Institutional Biosafety Committee (IBC). Não haverá manipulação *in vivo* na Unicamp.

Campinas, 12 de janeiro de 2022.

Prof. Dr. Wagner José Fávaro
Presidente da CEUA/UNICAMP

Rosangela dos Santos
Secretária Executiva

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VIII.II Direitos Autorais

1. Declaração

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Efeitos do ácido docosahexaenóico no metabolismo celular na iniciação e progressão tumoral prostática**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 03 de Janeiro de 2022

Assinatura : _____
Nome do(a) autor(a): **Guilherme Henrique Tamarindo**
RG n.° 48.200.498-8

Assinatura : _____
Nome do(a) orientador(a): **Rejane Maira Góes**
RG n.° 17404810

2. Autorização de inserção do artigo *Docosahexaenoic acid differentially modulates the cell cycle and metabolism- related genes in tumor and pre-malignant prostate cells* (Capítulo 2).