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Ácidos graxos poli-insaturados da série marinha na progressão do câncer de próstata: Avaliação do microambiente tumoral e da resposta inflamatória

São José do Rio Preto
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RESUMO

O câncer de próstata (CaP) é uma das neoplasias mais incidentes em homens em todo mundo e a segunda em número de óbitos por câncer, no Brasil. Os processos inflamatórios assumem importância preponderante no desenvolvimento e progressão do CaP. Os ácidos graxos poli-insaturados omêga-3 possuem diversas propriedades anti-tumorais e apesar das evidências que o seu consumo mitiga o risco e gravidade do CaP, o impacto sobre o microambiente inflamatório precisa ser melhor compreendido. O presente trabalho avaliou os efeitos do consumo do omêga-3 ácido docosahexaenoico (DHA) sobre a progressão tumoral de camundongos transgênicos para adenocarcinoma de próstata (TRAMP), em estágio inicial e tardio da doença. Os resultados obtidos enfatizaram a resposta do microambiente tumoral e a modulação da população de linfócitos e macrófagos, o processo de transição epitélio-mesenquimal, a proteína anti-inflamatória Anexina A1 (ANXA1) e os processos de morte celular regulada. Os animais foram alimentados com uma dieta rica em DHA (DHA-d) por quatro (8ª-12ª semana de vida) ou por dez semanas (8ª-18ª semana de vida). Os lobos ventrais da próstata foram processados para análises morfológicas, imuno-histoquímicas e de Western Blotting, e amostras de sangue foram coletadas para dosagem sistêmica de mediadores inflamatórios. O consumo da DHA-d reduziu a incidência de lesões pré-malignas e malignas, efeitos associados à redução da proliferação celular e aumento da apoptose. Mecanisticamente, esses efeitos se deram pela ativação da via ERK1/2 no estágio inicial da doença e inativação da via da Akt no estágio tardio. Ainda, a DHA-d reduziu a piroptose em ambos os estágios e aumentou a necroptose no estágio avançado. Independentemente do período, também reduziu os linfócitos $CD4^+$ e macrófagos M2, levando ao aumento de linfócitos $CD8^+$, no estágio tardio da doença. No estágio tardio da doença, a DHA-d levou à redução da expressão de marcadores do estroma reativo e da transição epitélio-mesenquimal. O consumo da DHA-d modulou diferencialmente a expressão de ANXA1 na próstata, pois aumentou sua expressão no epitélio acinar e reduziu nos fibroblastos associados ao câncer. A DHA-d reduziu os níveis séricos de TNF-α e da prostaglandina PGE_2, em ambos os estágios, e os níveis teciduais de TNF-R1 e PTGS2, no estágio tardio. Os dados com este modelo pré-clínico reforçam as propriedades anti-tumorais do DHA e mostram que elas ocorrem pela modulação do microambiente tumoral em direção a um espectro anti-tumoral, anti-inflamatório, e inibição da transição epitélio-mesenquimal indicando que intervenções dietéticas com esse ômega-3 podem ser eficazes para

mitigar a progressão da doença, e que podem ser associadas a outras abordagens terapêuticas como possível adjuvante.
Palavras–chave: Ácido docosahexaenoico. Linfócitos. Macrófagos associado a tumor. Morte Celular Regulada. TRAMP.

ABSTRACT

Prostate cancer (PCa) is one of the most frequent malignancies in men worldwide and the second cause of death by cancer-related cases in Brazil. Inflammation has a pivotal role in PCa development and progression. The omega-3 polyunsaturated fatty acids have several anti-tumor effects, and although recent reports attest that its intake can mitigate PCa aggressiveness, the effects upon the inflammatory microenvironment need to be better elucidated. This study aimed to assess the effect of omega-3 docosahexaenoic acid (DHA) intake upon tumor progression in an early- and late-stage disease in the transgenic adenocarcinoma of the mouse prostate (TRAMP), emphasizing tumor microenvironment response, lymphocyte and macrophage infiltration, epithelial-to-mesenchymal transition process, annexin A1 (ANXA1) expression, and programmed cell death pathways. Animals were fed with a DHA-enriched diet (DHA-d) for four (8-12th weeks of age) or ten weeks (8-18th weeks of age). The ventral prostate was processed for morphological, immunohistochemistry, and Western Blotting analyses, and blood samples were collected for serum measurement of inflammatory molecules. The DHA-d intake reduced pre-malignant and malignant lesions in mice prostate which was linked to reduction of cell proliferation and increased apoptosis. Mechanistically, DHA-d intake leads to activation of ERK1/2 in early-stage disease and inhibition of Akt in late-stage disease. For both disease stages, pyroptosis was down-regualated while necroptosis increased in late-stage disease. In addition, regardless of disease stage, DHA-d reduced CD4+ lymphocyte and M2 macrophage infiltration, resulting in increased CD8+ lymphocyte in the late-stage disease. In late-stage disease, DHA-d reduced the expression of reactive stroma markers and impaired epithelial-to-mesenchymal transition. The DHA-d intake differentially modulated anxA1 expression in the ventral prostate, up-regulating epithelial expression, and down-regulating in cancer-associated fibroblast in late-stage disease. Reduction of serum levels of PGE2 and TNF-α were observed for both disease stages, however, only late-stage disease exhibited down-regulation of TNF-R1 and PTGS2 tissue protein levels. Our data obtained with this pre-clinal model reinforces the anti-tumor effects of DHA and demonstrates that this occurs via modulation of the tumor microenvironment into an anti-tumoral and anti-inflammatory state with decreased epithelial-to-mesenchymal transition process. This indicates that dietary intervention with this omega-3 might be efficient in mitigating disease progression and even be used as a possible adjuvant in other therapeutical approaches.

Keywords: Docosahexanoic acid. Lymphocytes. Tumor-associated macrophages. Regulated cell death. TRAMP.

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ADT	terapia de ablação adrogênica
Akt	proteína quinase B
anxA1	anexina a1
AP	próstata anterior
AR	receptor de andrógeno
ASC	proteína adaptadora
ATM	serina/treonina quinase
BRCA	gene de câncer de mama
BUT	ultrassom trans retal
C	carbono
Ca^{2+}	cálcio
CAF	fibroblastos associados ao câncer
CaP	câncer de próstata
CARD	domínio de recrutamento de caspase
CCL	ligante de quimiocina
CD	cluster de diferenciação
CHEK	quinase de checkpoint
cIAP	inibidor celular de apoptose
CRPC	câncer de próstata resistente à castração
CXCL	quimiociona de ligação com motivo C-X-C
DAMP	danos moleculares associados a patógenos
DC	células dendríticas
DHA	ácido dosahexaenóico
DISC	complexo sinalizador de morte celular
DLP	próstata dorsolateral
ECM	matriz extracelular
EDR	exame dígito retal
EGF	fator de crescimento epidermal
ER	receptor de estrógeno
ETS	fator de transcrição com motivo hélice-volta-hélice conservado
FAP	proteína ativada de fibroblastos
Fib	fibroblasto
GEM	camundongo geneticamente modificado
GF	fato de crescimento
GPR	receptor acoplado à proteína G
GRO	ligante de quimiocina
GSDM	gasdermina
I	insaturação
IGF	fator de crescimento semelhante à insulina
IL	interleucina
INCA	instituto nacional do câncer
iNOS	óxido nítrico sintase induzida
IκB	quinase capa B
MCP	proteína quimioatrativas de monócitos
mCRPC	câncer de próstata resistente à castração metastático
MDSCs	células supressoras derivadas de miolóide
MHC	complexo de histocompatibilidade humano
MLH	proteína de reparo de incompatibilidade homóloga a MuTL
MSH	proteína de reparo de incompatibilidade homóloga a Msh

mTOR	alvo de mamíferos da rapamicina
MUFA	ácido graxo monoinsaturado
MYC	fator de transcrição bHLH
N-3	ômega-3
N-6	ômega-6
NF-κB	fator nuclear capa B
ºC	graus celsius
P53	proteína de tumor p53
PALB	parceiro e localizador de BRCA
PAMP	padrões moleculares associados a patógenos
PCD	morte celular programada
PD-L1	ligante de morte programada 1
PI3K	fosfatidilinositol-3 quinase
PIA	atrofia epitelial inflamatória
PIN	neoplasia intraepitelial prostática
PMS	endonuclease de reparo por incompatibilidade
PPAR	receptor ativado por proliferador de peroxissomos
PR	prostatectomia radical
PSA	antígeno prostático específico
PTEN	fosfatase homóloga à tensina
PTGS2	prostaglandina E2 sintase
PUFA	ácido graxo poliinsaturado
RB	retinoblastoma
RIPK	serina/treonina-proteína quinase
rPB	probascina de rato
SFA	ácido graxo saturado
SLUG	proteína dedo de zinco SNAI2
SMA	actina do músculo liso
SMC	célula muscular lisa
SNAIL	proteína dedo de zinco SNAI1
SPM	mediadores especializados de pró-resolução
STAT	transdutores de sinais e ativadores da transcrição
TAM	macrófago associado a tumor
TEM	transição epitélio-mesenquimal
TGF	fator de crescimento transformante
TME	microambiente tumoral
TNF	fator de necrose tumoral
TNF-R	receptor do fator de necrose tumoral
TRAD	proteína com domínio de morte associado ao TNFR
TRAMP	camundongo transgênico para adenocarcinoma de próstata
Treg	célula T reguladora
TWIST	proteína 1 relacionada à torção
VIM	vimentina
VP	próstata ventral
WHO	organização mundial da saúde
ZA	zona anterior
ZC	zona central
ZEB	fator de transcrição dedo de zinco com domínio homeobox 1 de ligação a E-box
ZP	zona periférica

ZT	zona de transição

LISTA DE SÍMBOLOS

%	porcentagem
Δ	delta
N	ômega
A	alfa
B	beta
γ	gama
κ	capa

SUMÁRIO

1- INTRODUÇÃO

1.1 Próstata

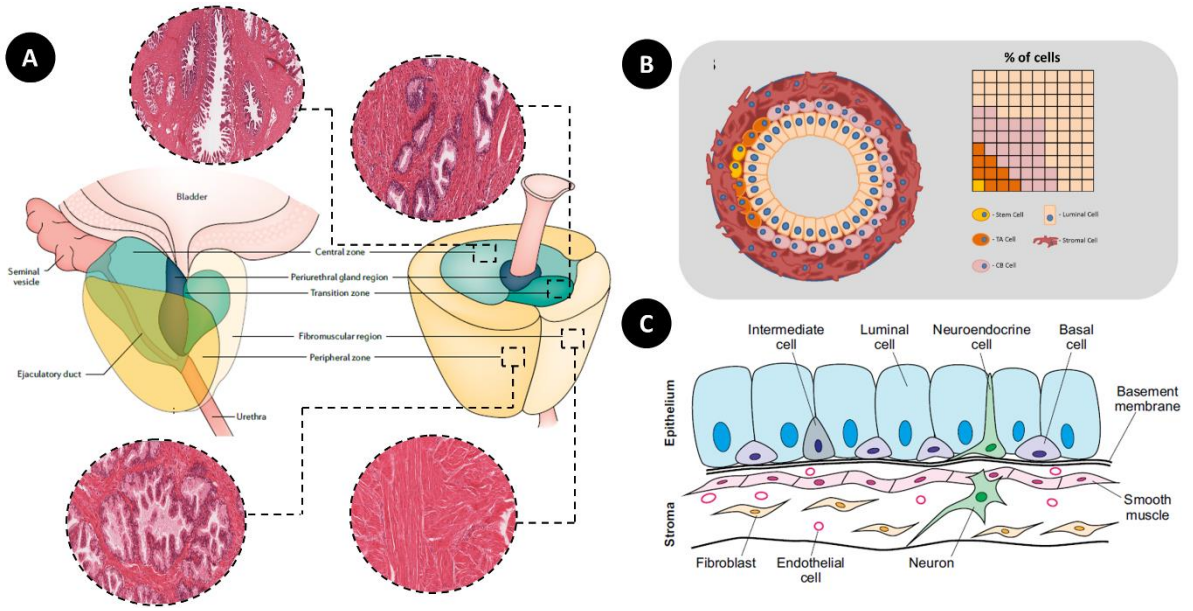
1.1.1. Aspectos morfofuncionais da próstata

A próstata é uma glândula acessória do sistema reprodutor masculino de mamíferos placentários, também encontrada em fêmeas, que se localiza na base da bexiga urinária, circundando a região da uretra denominada uretra prostática (LEE; AKIN-OLUGBADE; KIRSCHENBAUM, 2011). Essa glândula contribui ativamente para a formação do sêmen, sendo sua secreção rica em citrato e zinco, além de outros fatores, tendo como principal função estimular a motilidade e sobrevivência espermática, aumentando assim o sucesso reprodutivo do indivíduo (HUGGINS; SCOTT; HEINEN, 1942; PANG; CHOW; WONG, 1979; KAVANAGH, 1985). Morfologicamente, a próstata humana é um órgão compacto, de aproximadamente 20g, e que se diferencia em quatro regiões distintas: (1) zona periférica (ZP), zona central (ZC), zona de transição (ZT) e zona anterior ou região fibromuscular (ZA) (TIMMS, 2008) (Fig 1A), em condições normais, as regiões zonais da próstata ocupam, respectivamente, 75, 25 e 5% do volume total da glândula, quando desconsiderada a região fibromuscular (MCNEAL, 1981; ITTMANN, 2018).

A próstata é glândula tipo túbulo-alveolar com três componentes distintos – o epitélio secretor, o lúmen e o estroma glandular. A região epitelial secretora, apresenta uma constituição que varia de pseudoestratificada colunar à cúbica enquanto a região ductal apresenta um epitélio simples cúbico (CUNHA et al., 1987). Diferentes tipos celulares compõem o epitélio maduro, sendo que, aproximadamente 40% das células epiteliais correspondem a células basais e células transientes em amplificação as quais se organizam em uma camada continua na próstata humana, ambas derivadas de células-tronco, tendo como principal função a renovação celular do epitélio glandular (PACKER; MAITLAND, 2016a). Ainda, os quase 60% restantes são majoritariamente compostos por células luminais secretoras, as quais são responsáveis pela produção da secreção glandular, e, em menor quantidade, por células neuroendócrinas, as quais podem estabelecer contato tanto com as células epiteliais quanto com as estromais (CUNHA et al., 1987; PACKER; MAITLAND, 2016a; TOIVANEN; SHEN, 2017) (Fig 1B). Por sua vez, o compartimento estromal é caracterizado por uma alta diversidade de células além de exibir uma alta plasticidade frente a estímulos endógenos e exógenos. Nele, é possível observar a região da membrana basal, a qual estabelece o limite morfofuncional entre a região epitelial e

estromal, além de células musculares lisas (SMC) e fibroblastos (Fib), as quais se concentram preferencialmente em torno dos alvéolos prostáticos (ITTMANN, 2018). No microambiente glandular, as SMC, devido sua capacidade de contração, contribuem para a liberação do fluido prostático no ejaculado, ao passo que os Fib são responsáveis principalmente pela síntese de elementos da matriz extracelular (ECM) e secreção de fatores de crescimento (GF), os quais modulam a histofisiologia prostática, impactando diretamente diferentes processos essenciais à glândula, como a dinâmica de proliferação e morte celular (TUXHORN; AYALA; ROWLEY, 2001; TUXHORN et al., 2002). Ainda, encontram-se no estroma células endoteliais, telócitos, nervos e células do sistema imune (Fig 1C). Dessa forma, a interação recíproca entre os compartimentos epitelial e estromal é responsável por manter a homeostasia glandular, de forma que quaisquer fatores que interfiram negativamente nessa dinâmica são capazes de comprometer a atividade da próstata e mesmo induzir o estabelecimento de lesões pré-malignas e malignas (LI; MASON; MELNICK, 2015).

Figura 1. Aspectos morfológicos da próstata humana. (A) Esquema representando a morfologia da próstata humana, sua organização em zonas e a caracterização histológica dessas regiões. A zona periférica corresponde a maior parte da próstata e é a região mais suscetível à inflamação e neoplasia. A zona central ocupa a região do utrículo e ductos ejaculatórios. A zona de transição circunda a uretra e é mais suscetível a hiperplasia benigna prostática. A zona anterior apresenta uma constituição fibromuscular e com ausência de glândulas. **(B)** Esquema da organização celular do alvéolo prostático e do estroma sub-epitelial, aqui representado pelas células musculares. O diagrama representa a contribuição em densidade das células tronco, células transientes em amplificação, células basais e células luminais na composição o epitélio. **(C)** Esquema simplificado dos compartimentos epitelial e estromal.

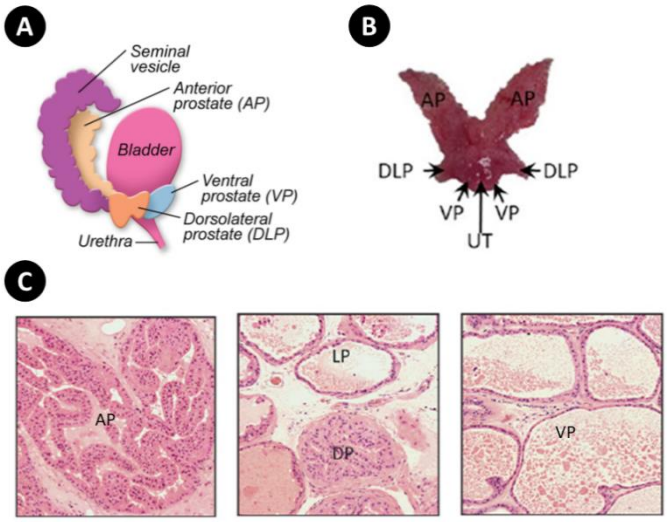


Fonte: Esquemas adaptados de PACKER; MAITLAND, 2016b; TOIVANEN; SHEN, 2017; SATHIANATHEN et al., 2018; REBELLO et al., 2021, fotomicrografias extraídas do site

https://histology.medicine.umich.edu/resources/male-reproductive-system#v-prostate-gland acesso em 08/01/2024 às 12:07.

Apesar da próstata ser encontrada em todos os grupos de mamíferos placentários, sua organização anatômica e histológica pode variar dependendo do grupo em questão. Nos roedores, o modelo não humano mais utilizado em pesquisa sobre a biologia prostática, a próstata se organiza em uma estrutura multilobulada e bilateral denominada complexo prostático, composta pelos lobos anteriores (AP), dorsolaterais (DLP) e ventrais (VP), (LEE; AKIN-OLUGBADE; KIRSCHENBAUM, 2011) (Fig 2A e B). O VP localiza-se ventralmente à uretra na região medial e é caracterizado pela presença de um estroma frouxo, sendo a região epitelial caracterizada por um epitélio simples colunar, com o núcleo celular localizado a região basal do citoplasma e nucléolo pouco evidente, além disso, nesse lobo não são observados dobramentos epiteliais frequentes como nos demais. O DLP dispõe-se parcialmente de forma lateral e dorsal à uretra, chegando à base das vesículas seminais, este lobo apresenta um estroma denso e unidades glandulares com epitélio simples colunar com o citoplasma frequentemente mais eosinofílico e núcleo disposto na região central da célula, além de apresentar uma maior frequência de dobramentos epiteliais quando comparado ao VP. Por fim, o AP, também denominado glândula coaguladora, localiza-se cranialmente aos demais lobos, associado à curvatura menor da vesícula seminal; o componente glandular apresenta um epitélio simples colunar e núcleo localizado centralmente em um citoplasma mais acidófilo, além de apresentar um intenso crescimento papilar, diferente dos demais lobos, e estroma subepitelial denso (MARKER et al., 2003; LEE; AKIN-OLUGBADE; KIRSCHENBAUM, 2011; ITTMANN, 2018) (Fig 2C).

Figura 2. Complexo prostático de camundongos (A) Representação esquemática do complexo prostático em visão lateral. **(B)** Morfologia do complexo prostático isolado. **(C)** Caracterização histológica dos lobos prostáticos. AP: lobo anterior, DLP: lobo dorsolateral, VP: lobo ventral, UT: uretra.



Fonte: Adaptado de BRZEZINSKA et al., 2015; WANG et al., 2018b

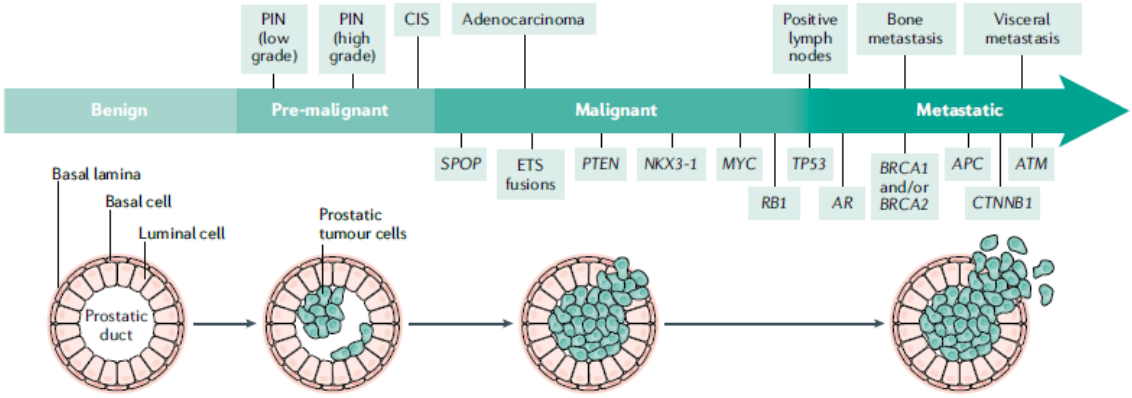
1.1.2 Incidência e etiologia do câncer de próstata

O câncer de próstata (CaP) é um dos cânceres mais incidente mundialmente em homens, apesentando uma alta taxa de mortalidade por casos relacionados a câncer (REBELLO et al., 2021). Em nível nacional, as estimativas do Instituto Nacional do Câncer (INCA) indicam que o CaP será o câncer mais incidente em homens no triênio 2023-2025, sendo a segunda causa de óbitos por câncer no Brasil (INCA, 2023), reforçando a importância dessa doença. Sua etiologia, além de complexa, não é totalmente conhecida, contudo sabe que o risco de desenvolvimento de CaP aumenta com a idade, sendo sua incidência positivamente correlacionada com o índice de desenvolvimento humano. Além disso, fatores genéticos e o estilo de vida, em particular o consumo de lipídeos, também são capazes de modular o risco do desenvolvimento da doença (KALRA et al., 2016; BRAY et al., 2018; KWAN et al., 2019; LABBÉ et al., 2019; REBELLO et al., 2021; COTTER; RUBIN, 2022).

Dentre os fatores genéticos, estima-se que, 4.6% dos casos de CaP localizado sejam referentes a mutações germinativas, sendo que essa porcentagem pode chegar a 16,2% em casos de CaP metastático (PRITCHARD et al., 2016; CASTRO et al., 2019). Os demais casos resultam de mutações somáticas adquiridas ao longo de envelhecimento, sendo a maior parte dessas alterações causadas por rearranjos gênicos (COTTER; RUBIN, 2022). Dentre as mutações germinativas destacam-se mutações em genes envolvidos com o processo de recombinação homóloga, como *BRCA1/2*, *ATM*, *CHEK2*, e *PALB2*, e no reparo por mal pareamento (*miss match repair*) como *MLH1*, *PMS2*, *MSH2*, e *MSH6* (CHENG et al., 2019). Por outro lado, mutações somáticas em oncogenes e genes supressores de tumor como *NKX3.1*,

PTEN, *MYC*, *TP53*, *RB1*, *AR* e fusão *ETS* são alguns exemplos de mutações frequentes no CaP (COTTER; RUBIN, 2022). Assim, coletivamente, diversas mutações herdadas e/ou adquiridas ao longo da vida estimulam o processo de iniciação e progressão do CaP (Fig 3).

Figura 3. Principais alterações genéticas encontradas no CaP ao longo de sua progressão.



Fonte: Extraído de REBELLO et al., 2021.

Além dos fatores genéticos, fatores relacionados ao estilo de vida do indivíduo, como por exemplo a obesidade, prática de exercícios físicos, tabagismo e dieta são reportados como capazes de modular o risco de desenvolvimento e progressão do CaP (HUNCHAREK et al., 2010; ADESUNLOYE, 2021; BRASSETTI et al., 2021; KENFIELD; CHAN, 2022). Interessantemente, nas últimas décadas, grande ênfase tem sido dada para o papel da dieta e em especial o consumo de lipídeos na gênese e progressão do CaP (MARSHALL, 2012; HUANG et al., 2016; HAYASHI et al., 2018). Nesse sentido, os ácidos graxos ganham grande destaque por apresentarem um grande espectro de implicações clínico-metabólicas; assim, dietas hiperlipídicas já foram associadas ao desenvolvimento de quadros de resistência à insulina, aumento da pressão sanguínea, dislipidemia e estabelecimento de estresse oxidativo (HANCOCK et al., 2008; MATSUZAWA-NAGATA et al., 2008; BHANDARKAR; BROWN; PANCHAL, 2019; FEILLET-COUDRAY et al., 2019).

No que diz respeito ao CaP, estudos demonstram que apesar da quantidade de lipídeos ser um fator importante para o desenvolvimento da doença (CHO et al., 2015; LABBÉ et al., 2019), a natureza de lipídeo também é igualmente importante na iniciação e progressão tumoral (BERQUIN et al., 2007). O consumo de ácidos graxos saturados (SFA) e poli-insaturados (PUFA) ômega-6 (n-6) sensibilizam a glândula ao estabelecimento e progressão do CaP por estimularem um estado hiperproliferativo, através da ativação da via PI3k/Akt/mTOR, estímulo da via do fator de crescimento semelhante à insulina (IGF-1), aumento na expressão do receptor

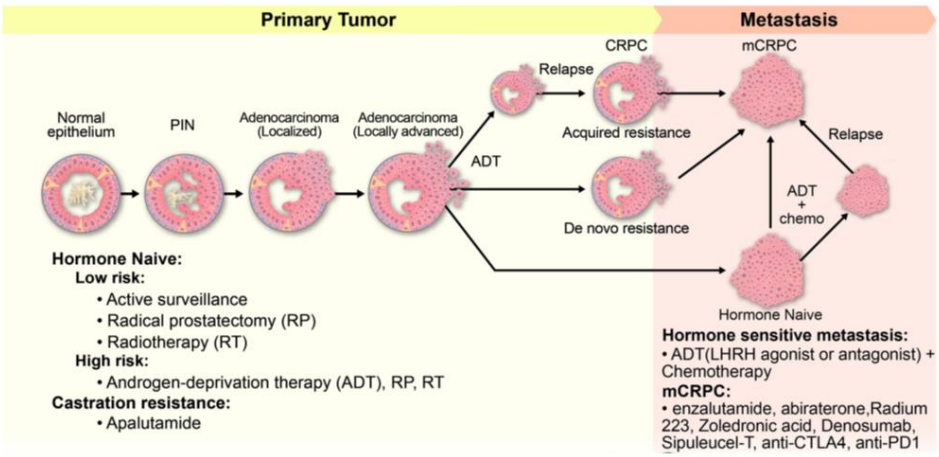
de andrógeno (AR), estabelecimento de um microambiente pró-inflamatório e amplificação da sinalização Myc (WANG et al., 2012; SILVA et al., 2015; XU et al., 2015; KWAN et al., 2019; LABBÉ et al., 2019). Interessantemente, os efeitos do consumo excessivo de SFA e PUFAs n-6 parecem ser tão prejudiciais à saúde prostática que apenas a exposição durante a fase gestacional já é o suficiente para desregular a histofisiologia prostática na fase adulta e/ou senil, estabelecendo um microambiente suscetível a lesões pré-malignas e malignas, devido ao aumento da ativação da via Akt e desativação de PTEN, e desregulação da vias dos receptores nucleares como AR, ERα, ERβ, PPARγ e LXRα (BENESH et al., 2013; PYTLOWANCIV et al., 2016; YANG et al., 2018). Por outro lado, o consumo de PUFAs ômega-3 (n-3) tem sido associado a efeitos positivos na saúde prostática, atrasando a progressão tumoral por redução da proliferação celular através da inativação da via Akt, redução do infiltrado de macrófagos associados a tumor (TAM), redução da expressão de IGF-1, aumento da apoptose e estímulo da resposta antitumoral do sistema imune (AKINSETE et al., 2012; GU et al., 2013; LLOYD et al., 2013; GEVARIYA et al., 2018; LIANG et al., 2020; AMARO et al., 2022). Dessa forma, modular a frequência e quantidade da ingestão de componentes alimentares, a fim de se estabelecer uma melhor qualidade nutricional, representa uma maneira interessante de se modular os aspectos envolvidos no microambiente tumoral.

1.1.3 Progressão e diagnóstico do câncer de próstata

O processo de transformação maligna da próstata está associado ao estabelecimento de lesões pré-malignas como a atrofia inflamatória proliferativa (PIA) e neoplasias intraepiteliais (PIN), seguido pelo desenvolvimento do câncer localizado, culminando em processos de invasão local e eventos de metástases (TAICHMAN et al., 2007; WANG et al., 2018b) (Fig 4). As PINs são lesões bem aceitas como precursoras do CaP e são caracterizadas por uma proliferação celular excessiva contida no epitélio glandular, o que leva a perda da polaridade celular, além de ser possível observar claramente certas atipias nucleares como aumento da área nuclear e nucleolar (BOSTWICK; QIAN, 2004; BOSTWICK; CHENG, 2012). Por sua vez, as PIAs também são consideradas promotoras da carcinogênese prostática. Essas lesões são caracterizadas por um epitélio atrófico com alta taxa proliferativa associadas a processos inflamatórios (WOENCKHAUS; FENIC, 2008). Evidências morfológicas da transição de PIA para PIN de alto grau (HGPIN) e adenocarcinoma já foram reportadas em humanos, validando o papel das PIAs como precursoras do CaP (WANG; BERGH; DAMBER, 2009). Ainda, estudos demonstram que a enzima PTGS2 é hiperexpressa nas PIAs, contribuindo para o aumento do recrutamento de linfócitos T $CD4^+$ no microambiente glandular, o que reforça a

relação da inflamação com o processo de oncogênese prostática (ZHA et al., 2001; STAHL et al., 2010).

Figura 4. Progressão do câncer de próstata e abordagens terapêuticas.



Fonte: Extraído de WANG et al., 2018b

Durante o processo de transformação, a progressão das lesões pré-malignas para um estágio maligno é regulada por andrógenos e seu receptor, o AR, sendo essa uma característica marcante do CaP, e por isso a terapia de ablação androgênica (ADT) é um dos métodos padrões para o tratamento da doença (COTTER; RUBIN, 2022). Contudo, inevitavelmente a resistência à ADT é desenvolvida, levando ao estabelecimento do CRPC primário ou CRPC metastático (mCRPC), um estágio letal e sem cura, onde os tratamentos só visam atrasar a progressão da doença (WANG et al., 2018b). Diferentes mecanismos podem levar ao estabelecimento do CRPC e do mCRPC, dentre eles destacam-se as alterações da sinalização do AR e na esteroidogênese local, inflamação e no processo de transição epitélio-mesenquimal (TEM) (DEVLIN; MUDRYJ, 2009; JIN; DAYYANI; GALLICK, 2011). Assim, compreender os fenômenos que governam a progressão da doença é um processo crucial para se estabelecer intervenções terapêuticas mais eficientes.

Apesar de sua alta incidência em homens, o CaP é de difícil diagnóstico uma vez que, na maioria das vezes, é assintomático, e os sintomas quando presentes se assemelham aos da hiperplasia benigna prostática, como incontinência urinária, hematúria e noctúria (MALINOWSKI et al., 2019). Detectar a doença de forma precoce é essencial para um tratamento eficaz, uma vez que a agressividade está diretamente ligada ao estadiamento inicial no diagnóstico (TABAYOYONG; ABOUASSALY, 2015). As ferramentas de rotina no diagnóstico do CaP são o exame digital retal (EDR), dosagem dos níveis séricos do antígeno prostático específico (PSA) e biópsias guiadas por ultrassom trans retal (BUT)

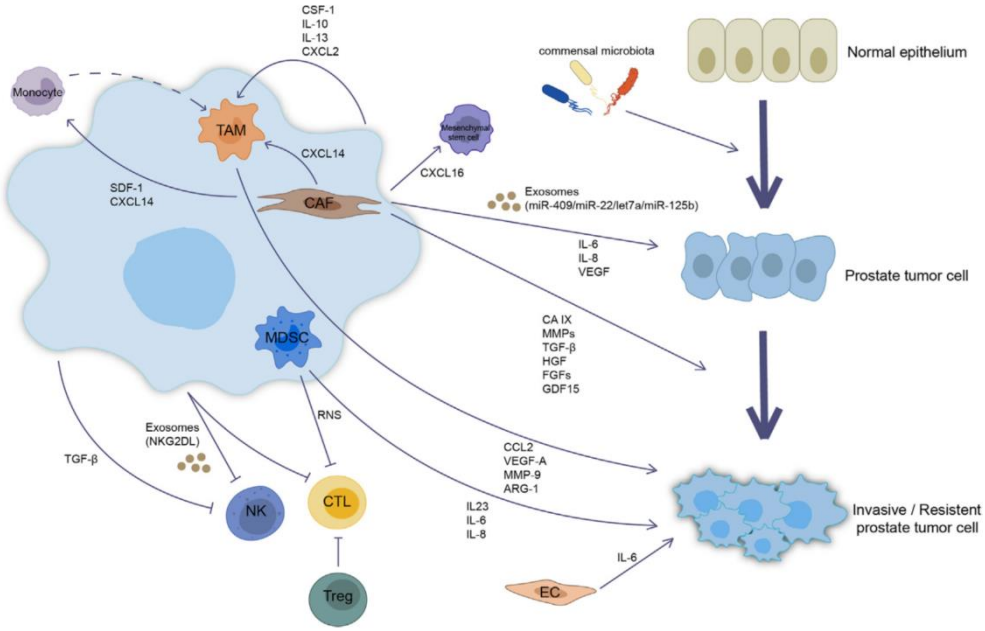
(HEIDENREICH et al., 2011). Uma vez detectada a doença, diferentes estratégias de tratamento podem ser traçadas de acordo com o seu estadiamento, entre elas: (i) vigilância ativa, para caso de CaP localizado com baixo risco de progressão, onde exames em intervalos de tempo regulares são feitos para acompanhar a progressão da doença; (ii) prostatectomia radical (PR) associada ou não à radioterapia, para CaP não metastático, com expectativa de vida do indivíduo de mais de 10 anos e, (iii) aqueles não elegíveis para a PR e que apresentam a doença em um estágio mais agressivo são indicados para a ADT, a qual tem como principal função reduzir ou eliminar a produção de testosterona (HEIDENREICH et al., 2011; TABAYOYONG; ABOUASSALY, 2015; MALINOWSKI et al., 2019). Contudo, estima-se que cerca de 20-40% dos indivíduos que realizam PR terão a reincidência da doença e ainda, indivíduos sobre ADT podem eventualmente adquirir resistência ao tratamento, levando ao desenvolvimento do CRPC (LU-YAO et al., 1996; BOCCON-GIBOD et al., 2004; CORNFORD et al., 2017; TOURINHO-BARBOSA et al., 2018).

1.2 Microambiente tumoral

Por ser um câncer muito comum em homens, há um grande esforço da comunidade cientifica em compreender os mecanismos envolvidos na transformação maligna, iniciação e progressão do CaP. Levando em consideração que essa doença é altamente heterogênea (TOLKACH; KRISTIANSEN, 2018; HAFFNER et al., 2021), nos últimos anos, devido a sua complexidade e plasticidade, grande atenção vem sendo dada ao microambiente tumoral (TME), uma vez que esse participa ativamente do processo oncogênico (BIANCHI-FRIAS et al., 2016). Entende-se por TME o conjunto de células tumorais, células estromais (i.e., fibroblastos, células do sistema imune e células endoteliais) e seus produtos, e os elementos da MEC (BAGHBAN et al., 2020). As interações complexas e recíprocas desses elementos são responsáveis por afetar estimular todos os estágios da progressão tumoral, induzindo a proliferação e sobrevivência celular, evasão de apoptose, invasão, migração celular, TEM, angiogênese e resistência à quimioterapia (ANDERSEN et al., 2018; BONOLLO et al., 2020; ZHU et al., 2021). Dessa forma, modular os aspectos envolvidos no TME vem se mostrando uma forma eficaz de atrasar a progressão tumoral e aumentar a eficiência terapêutica (MONTICO et al., 2015; MATEUS et al., 2019) (Fig 5).

Figura 5. *Crosstalk* entre as células tumorais e os componentes do TME. Interações complexas afetam o crescimento tumoral e levam a resistência a mecanismos antitumorais do sistema imune. Componentes da microbiota intensificam a inflamação no microambiente tumoral e promovem a tumorigênese. Vesículas extracelulares (exossomos), fatores de crescimento secretados por CAFs estimulam a transformação, progressão tumoral e metástase. O aumento em fatores quimioatrativos secretados por CAFs como CXCL14 e SDF-1, aumentam o influxo de monócitos e estimulam a polarização para o perfil M2. Células T reguladoras (Treg)

aumentam a supressão do sistema imune e secretam fatores que aumentam a polarização de macrófagos para o perfil M2. Macrófagos M2 e células supressoras derivadas de mieloides (MDSCs) secretam fatores que levam a imunossupressão e proliferação e metástases das células tumorais. Os níveis altos de TGF-β são responsáveis por inibir a ação de células NK e linfócitos $CD8^+$ além de estimular a evolução do estroma reativo e transição epitélio-mesenquimal. Ação simultânea de MDSCs e Tregs leva a exaustão de linfócitos $CD8^+$. TAM: macrófago associado a tumor, CAF: fibroblasto associado a câncer, CLT: linfócito T citotóxico ($CD8^+$), NK: natural killer, Treg: linfócito T regulador, MDSC: célula supressora derivada de mieloide.



Fonte: Extraído de LIN; SUN; LV, 2023

1.2.1 Estroma reativo

A plasticidade dos componentes do compartimento estromal permite que um conjunto de respostas sejam arquitetadas frentes a um estímulo, como o reparo tecidual e retorno à homeostase, contudo, essa mesma característica pode favorecer o estabelecimento de lesões e progressão do CaP (ELO et al., 2010). A ativação do estroma, conhecido como estroma reativo, envolve um processo que é caracterizado pela mudança do fenótipo de fibroblastos em miofibroblastos, aumento do influxo de leucócitos, maior biodisponibilidade de fatores de crescimento e citocinas inflamatórias bem como um intenso *turnover* dos componentes da ECM e produção de proteases que contribuem tanto para a renovação da ECM quanto para a invasão das células tumorais (TUXHORN; AYALA; ROWLEY, 2001; TUXHORN et al., 2002).

A mudança do fenótipo de fibroblastos em miofibroblastos é um processo importante na progressão tumoral e o principal indutor dessa diferenciação é o fator de crescimento transformante β (TGF-β) (SCHARENBERG et al., 2014). Essas células podem ser detectadas pela expressão de diferentes fatores como, vimentina (VIM), proteína ativada de fibroblastos (FAP) e α-actina do músculo liso (αSMA) e são responsáveis pela secreção de fatores de

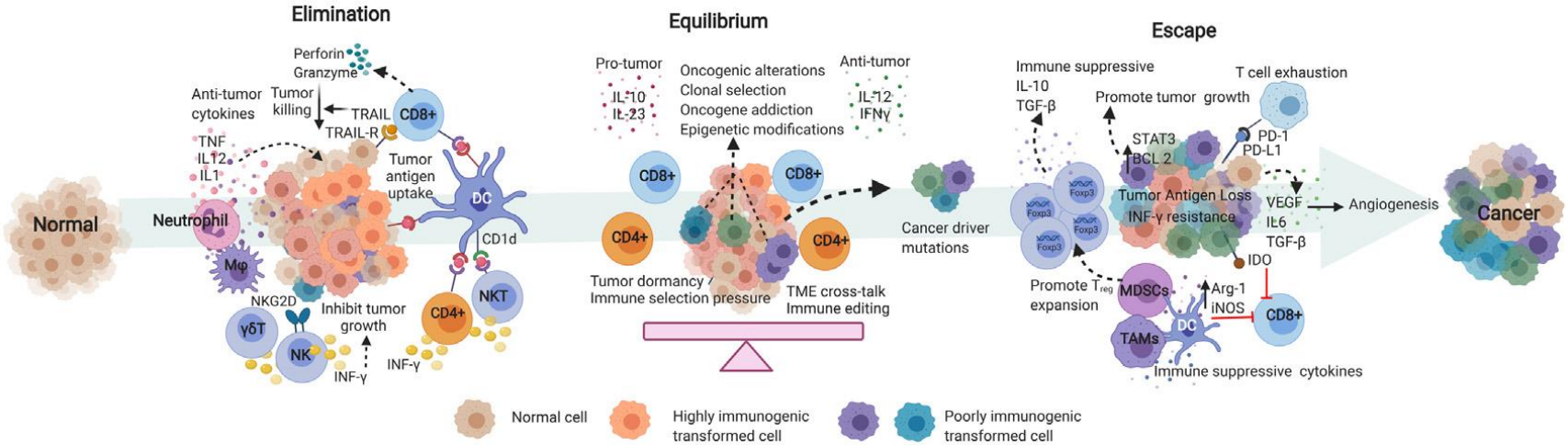
crescimento, citocinas pró-inflamatórias e fatores angiogênicos que desregulam a interação epitélio-estroma e favorecem a progressão tumoral (TUXHORN; AYALA; ROWLEY, 2001; COMITO et al., 2014; BONOLLO et al., 2020; HINTZ et al., 2020). Por impactarem diretamente diferentes processos do TME em um contexto tumoral a população de fibroblastos e miofibroblastos são denominadas de fibroblastos associados ao câncer (CAF) (JAESCHKE et al., 2020). Recentemente, foi demonstrado que as populações de CAF são altamente heterogêneas e que podem ser classificadas em seis *clusters* com base em seus perfis de assinatura gênica, contudo, a maioria deles se encontram em dois subgrupos caracterizados pela expressão das citocinas quimioatrativas CCL2 e CXCL12, demonstrando a importância dessas células na modulação do microambiente inflamatório (VICKMAN et al., 2020).

1.2.2 Microambiente inflamatório

Nos últimos anos um progresso substancial na área de imunologia dos tumores permitiu uma melhor compreensão de como o TME afeta o crescimento tumoral, levando a formulação da 'Teoria dos 3 Es" de imunoedição de tumores, onde cada "E" representa: eliminação, equilíbrio e escape (DUNN; OLD; SCHREIBER, 2004) (Fig 6). Assim, a primeira fase estabelecida durante o processo de transformação é a eliminação. Como consequência do processo de transformação e do estabelecimento de um estroma reativo, a produção de moléculas quimioatrativas é responsável por recrutar diferentes componentes do sistema imune inato e adaptativo (DUNN; OLD; SCHREIBER, 2004). No início da tumorigênese, células transformadas apresentam antígenos de superfície via complexo de histocompatibilidade principal I (MHC I), os quais podem ser reconhecidos por células dendríticas (DC) e macrófagos M1, levando a morte imunogênica das células tumorais, a qual resulta no aumento local de citocinas pro-inflamatórias (NAIR et al., 2020). Ainda, como parte do sistema imune adaptativo, células natural killer (NK) levam à eliminação das células tumorais pela secreção de moléculas como porfirina e granzima B, ou pela *up regulação* de ligantes de morte celular como FasL e TRAIL. Como consequência, isso resulta na liberação de padrões moleculares associados a danos (DAMPs), o que permite a opsonização de antígenos tumorais pelas DC, as quais migram para os linfonodos drenantes e os apresentam aos linfócitos T $CD4^+$ e $CD8^+$. Uma vez ativadas, as células $CD4^+$ passam a secretar IFγ, o qual estimula a ação citotóxica de células $CD8^+$ por mecanismos semelhantes às células NK (DUNN; OLD; SCHREIBER, 2004; NAIR et al., 2020). Contudo, muitas células tumorais escapam da fase de eliminação, entrando na fase de equilíbrio, a qual células NK e $CD8^+$ serão responsáveis por manter a dormência do tumor

e, inevitavelmente com o tempo e a pressão imposta pela resposta do sistema imune, leva a seleção de mutações adquiridas que permitem as células a adentrarem a fase de escape. Essa última fase é caracterizada pelo estabelecimento de um microambiente extremamente imunossuprimido, onde as células tumorais apresentam supressão do MHC, contribuindo para a evasão do sistema imune, bem como o alto infiltrado de células imunossupressoras, como células supressoras derivadas de mielóides (MDSCs), macrófagos M2, e células T reguladoras (Treg), sendo que esta última pode se originar de células de $CD4^+$ presentes no TME. Coletivamente essas células suprimem a função de linfócitos T e células NK, além de contribuir para processos de TEM, sobrevivência celular e angiogênese (DUNN; OLD; SCHREIBER, 2004; KARAVITIS et al., 2012; NAIR et al., 2020; THUMKEO et al., 2022). Assim, abordagens que visem anular essas ações imunossupressoras são maneiras de restaurar a ação citotóxica do sistema imune e conter a tumorigênese (EKMEKCIOGLU; GRIMM; ROSZIK, 2017; LIN; SUN; LV, 2023).

Figura 6. Microambiente inflamatório ao longo da progressão do tumoral, representando a "Toria dos 3 Es". Durante o processo de eliminação, elementos do sistema imune inato e adaptativo medeiam respostas citotóxicas que destroem as células tumorais, contudo, eventualmente a progressão para a fase de equilíbrio ocorre, onde o sistema imune adaptativo mantém o tumor dormente. Ao longo da progressão tumoral, o acúmulo de mutações e a pressão seletiva exercida pelo sistema imune favorece o escape das células tumorais e o estabelecimento de um microambiente imunossuprimido, sendo essa fase conhecida como escape. MΦ: macrófagos, NK: natural killer, CD4: linfócito T auxiliar, CD8: linfócito T citotóxico, NKT: linfócitos T natural killer, γδT: linfócitos T gama delta, DC: célula dendrítica, FOXP3: células T reguladoras (expressando *forkhead box P3*), TAM: macrófagos associado a tumor, MDSC: célula supressora derivada de mieloide.



Fonte: Extraído de NAIR et al., 2020.

1.2.3 Citocinas pro-inflamatórias no CaP

Conforme mencionado anteriormente, o processo inflamatório é uma característica muito marcante do câncer, já sendo relatado que inflamações crônicas podem acelerar o desenvolvimento de lesões pré-neoplásicas na próstata de camundongos (GAO et al., 2019). Ainda, dados experimentais indicam que o tratamento de camundongos transgênicos para adenocarcinoma de próstata (TRAMP) com agentes anti-inflamatórios é capaz de atrasar a progressão tumoral e que o controle do processo inflamatório mesmo em estágios iniciais do CaP é responsável por diminuir a proliferação celular em estágios avançados da doença (KIDO et al., 2016). Assim, compreender o estabelecimento e progressão do microambiente inflamatório no CaP é essencial para fazer correlações mais precisas com o prognóstico do indivíduo, bem como traçar estratégias terapêuticas efetivas.

A IL-6 é a citocina mais estudada no CaP, e evidências demonstram que a secreção de IL-6 no microambiente tumoral é responsável por aumentar a resistência ao tratamento do CaP por doxorrubicina (CHETEH et al., 2020), acelerar a progressão tumoral pela fosforilação de STAT3 (HAYASHI et al., 2018) e estimular o processo de TEM (ROJAS et al., 2011). Ainda, elevados níveis de IL-6 já foram correlacionados com o risco de recorrência do CaP (ALCOVER et al., 2010). O TNF-α, é outra molécula chave no CaP, seu mecanismo de ação ocorre pela via de sinalização do fator nuclear kappa B (NF-κB) (GARG et al., 2012), levando ao aumento da expressão de genes pró-inflamatórios importantes como PTGS2, MCP-1, GRO1, IL-6, entre outros (ZHOU et al., 2003). Por fim, a IL-1β é outra citocina de grande destaque no CaP, e também apresenta um mecanismo de ação mediado pelo NF-κB, afetando a expressão de genes relacionados a resposta inflamatória e imune que estimulam a progressão do CaP (THOMAS-JARDIN et al., 2018). Além disso, é relatado que a exposição crônica à IL-1β é responsável por estimular a aquisição de um fenótipo andrógeno-independente de células LNCaP (DAHL et al., 2020) e aumentar o infiltrado de células $CD4^+$ e a suscetibilidade ao desenvolvimento de lesões pré-malignas, em particular as PIAs (ASHOK et al., 2019).

1.2.4 Macrófagos e linfócitos no CaP

Apesar da importância das citocinas pró-inflamatórias na iniciação e progressão tumoral, o perfil do infiltrado leucocitário, em particular o perfil de macrófagos e linfócitos,

também vem se mostrando importantes alvos na terapêutica no câncer (GEVARIYA et al., 2018). Os macrófagos compõem um grupo de células com alta diversidade funcional e que apresentam um impacto direto na homeostase tecidual e em condições patológicas (LIU et al., 2018; SILVA et al., 2018; WANDERLEY et al., 2018). Esses fagócitos teciduais podem ser classificados, de maneira simplificada em: macrófagos M1 ($CD68^+$/$iNOS^+$), M2 ($CD163^+$) e associados a tumor (TAM). Essa subdivisão se dá com base no perfil de resposta imunológica estimulado por essas células, onde macrófagos M1 estimulam respostas tipicamente pró-inflamatórias e dirigem uma resposta antitumoral do sistema imune (ZHANG et al., 2023), ao passo que os macrófagos M2 estimulam uma resposta de natureza imunossupressora, pró-remodelação e favorecem a progressão tumoral (COMITO et al., 2014). Os TAM por sua vez consistem em uma população altamente plástica de macrófagos que é modulada pelo microambiente local e que podem favorecer ou conter a progressão tumoral (COMITO et al., 2014; NOY; POLLARD, 2014). Embora tipicamente não possam ser classificados em M1 ou M2 pelo fato de serem encontrados apenas em condições patológicas, estudos demonstram que os TAM podem apresentar assinaturas de polarização M1 ou M2 (LIU et al., 2018).

As variações nas populações de linfócitos no microambiente tumoral também vêm sendo amplamente investigadas no câncer (DAVIDSSON et al., 2013; WANG et al., 2017). Em relação à próstata, é relatado que o principal subtipo encontrado em amostras de CaP são linfócitos T $CD4^+$ (T auxiliares) (MERCADER et al., 2001), sendo relatado que células $CD4^+$ podem promover a progressão tumoral e levar a aquisição de resistência à terapia em casos de CaP resistentes à castração (CRPC) (ISCARO et al., 2018; WANG et al., 2018a). Por outro lado, os linfócitos T $CD8^+$ (T citotóxicos) estão relacionados com uma resposta anti-tumoral, sendo capazes de estimular diretamente a morte de células tumorais via granzima B ou ligantes de morte celular (IVANOVA et al., 2017; NAIR et al., 2020), sendo seus efeitos anti-tumorais já relatados no CaP e em câncer de mama (YI HUANG et al., 2015; HUSAINI et al., 2020). Ainda, recentemente foi reportado que o alto influxo de células $CD8^+$ está relacionado ao aumento da sobrevivência em indivíduos submetidos à prostatectomia radical (YANG et al., 2021b). Portanto, avaliar as populações de leucócitos no microambiente tumoral vem se mostrando uma ferramenta útil no prognóstico de pacientes com câncer (WANG et al., 2017).

1.2.5 Anexina A1

A anexina A1 (ANXA1) é membro da superfamília das proteínas ligantes de fosfolipídios de maneira dependente de Ca^{2+}, com ação anti-inflamatória e pró-resolução (SHEIKH; SOLITO, 2018). Sua expressão é induzida por glicocorticóides, sendo sua ação

desencadeada via receptores de formil peptídeos (FPRs) localizados na membrana plasmática (PERRETTI; D'ACQUISTO, 2008; SHEIKH; SOLITO, 2018; FOO et al., 2019). Assim, os clássicos efeitos anti-inflamatórios e de pró-resolução da ANXA1 se baseiam na inibição da fosfolipase A2 citosólica (cPLA2) e subsequente supressão do metabolismo do ácido araquidônico e produção de eicosanóides (KIM et al., 1994).

Recentemente, grande atenção tem se voltado para o possível papel da ANXA1 no câncer, sendo demonstrado que dependendo do tipo de tumor, uma expressão diferencial da ANXA1 pode ser observada. Níveis aumentados de ANXA1 foram detectados em câncer de fígado, pulmão, pâncreas, colorretal e pele (GANESAN et al., 2020). Por outro lado, o CaP localizado exibe baixa expressão de ANXA1 (PAWELETZ et al., 2000; PATTON et al., 2005). Nesse sentido, estudos demonstram que a ANXA1 é abundantemente expressa no epitélio normal da próstata, contudo, sua expressão é drasticamente reduzida em PINs de alto grau (HGPIN) e no CaP (PAWELETZ et al., 2000; PATTON et al., 2005; ALAN et al., 2023), sugerindo um papel protetor da anxA1 no desenvolvimento do CaP. De fato, a perda da expressão de ANXA1 leva ao aumento da secreção de IL-6 em linhagens prostáticas benignas e malignas e resulta na hiperativação da via do STAT3, promovendo a aquisição de características tumorigênicas (INOKUCHI et al., 2009). Por outro lado, no CaP metastático e em linhagens celulares de CaP metastático observa-se alta expressão de ANXA1, principalmente em contextos de hipóxia ou ablação androgênica (VARAMBALLY et al., 2005; SETLUR et al., 2008; BIZZARRO et al., 2017; MOTA et al., 2020; YANG et al., 2021a). Assim, a perda da expressão ANXA1 pode ser um fator chave no estabelecimento do CaP, porém, em estágios mais agressivos, a reexpressão de ANXA1 pode representar um evento importante de resistência à terapia e aumento da capacidade invasiva das células tumorais.

Considerando a importância do TME no processo de iniciação e progressão tumoral, estudos demonstram que a ANXA1 estromal detém grande relevância no processo de carcinogênese (ALAN et al., 2023). Camundongos *knockout* para ANXA1, com o intuito de abolir a expressão estromal de ANXA1, apresentam o crescimento tumoral reduzido por comprometimento da angiogênese e capacidade metastática em modelo alográfico/xenográfico de melanona e carcinoma de pulmão (YI; SCHNITZER, 2009). Ainda, uma alta expressão estromal de ANXA1 foi reportada para hepatocarcinoma celular (HCC), em particular em TAMs M2, onde a hiperexpressão de ANXA1 resulta na proliferação e migração das células tumorais, além de comprometer a atividade citotóxica de linfócitos $CD8^+$ pela expressão de PD-L1 (SONG et al., 2023). A secreção deANXA1 por CAFs também foi demonstrada como um

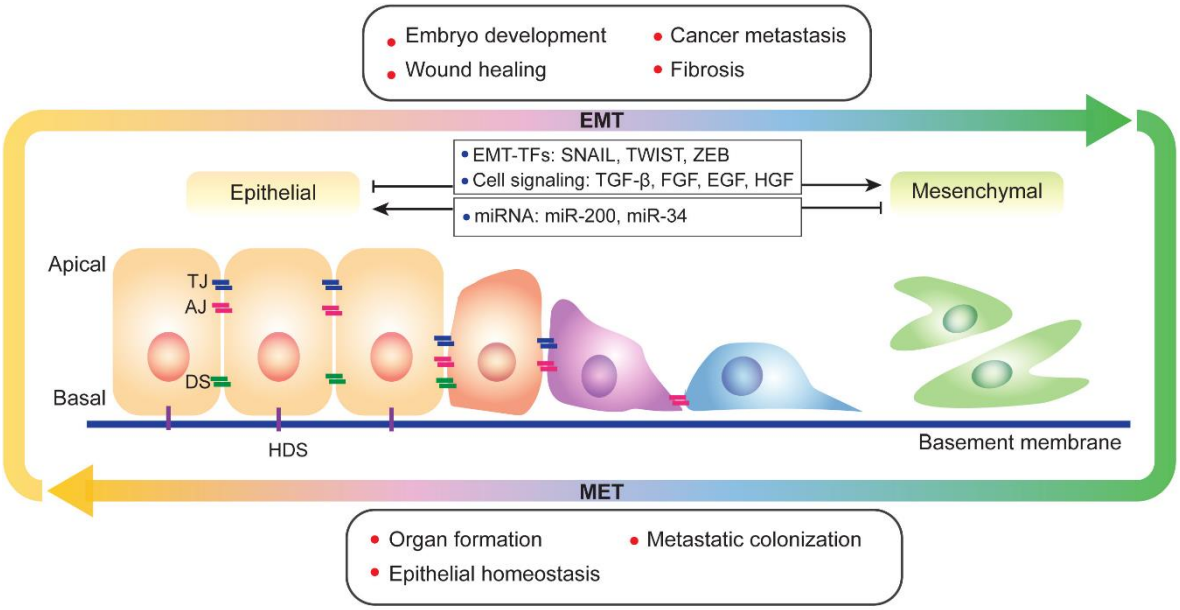
fator chave na desdiferenciação de células tumorais levando a geração de populações de células-tronco e indução de TEM no CaP (GEARY et al., 2014). Assim, esses achados sugerem um possível papel diferencial da ANXA1 no TME. Contudo, a compreensão do papel da ANXA1 no CaP ainda é um pouco desafiadora, principalmente no que diz respeito às suas variações no TME em diferentes estágios da progressão tumoral, bem como em contextos de modulação do microambiente inflamatório.

1.2.6 Transição Epitélio-Mesenquimal

A TEM é definida como um processo biológico no qual células epiteliais perdem suas características fenotípicas e adquirem características mesenquimais. Durante a TEM, as células epiteliais perdem as junções célula-célula, polaridade celular e marcadores epiteliais, e adquirem alta mobilidade, morfologia fusiforme e marcadores mesenquimais (GIANNONI et al., 2010; LAI et al., 2020).

O processo de TEM é essencial durante a embriogênese, no qual células epiteliais passam por mudanças morfológicas e moleculares que promovem sua conversão em células fenotipicamente mesenquimais (KHAN et al., 2015). Contudo, esse mesmo processo é responsável por aumentar a capacidade de migração e invasão celular, estimular processos metastáticos e promove quimioresistência aos fármacos utilizados no tratamento do câncer (KWON et al., 2016). Assim, o processo de TEM deve ser finamente regulado e, embora alguns marcadores mesenquimais possam mudar dependendo do contexto biológico, uma rede complexa de fatores de transcrição é necessária para a indução da TEM, incluindo SNAIL, SLUG, TWIST e ZEB (LI; MASON; MELNICK, 2015). Ainda, sabe-se que a TEM é estimulada por componentes do TME, em particular alguns fatores de crescimento como o TGF-β, IGF-1, EGF e citocinas pró-inflamatórias (GAO; MITTAL, 2012) (Fig 7). Portanto, modular múltiplos aspectos do TME representa uma abordagem importante para conter a progressão tumoral.

Figura 7. Processo de transição epitélio-mesenquimal. O processo de transição epitélio-mesenquimal é importante para diferentes processos biológicos, incluindo o processo de oncogênese e metástase. Diferentes fatores podem induzir o processo de transição epitélio-mesenquimal, bem como o processo contrário, denominado transição mesenquimal-epitelial. TJ: junção de oclusão, AJ: junção aderente, DS: desmossomos, HDS: hemidesmossomos, EMT-TFs: fatores de transcrição relacionados ao processo de transição epitélio-mesenquimal, miRNA: micro RNAs envolvidos na transição mesenquimal-epitelial.



Fonte: Extraído de LAI et al., 2020

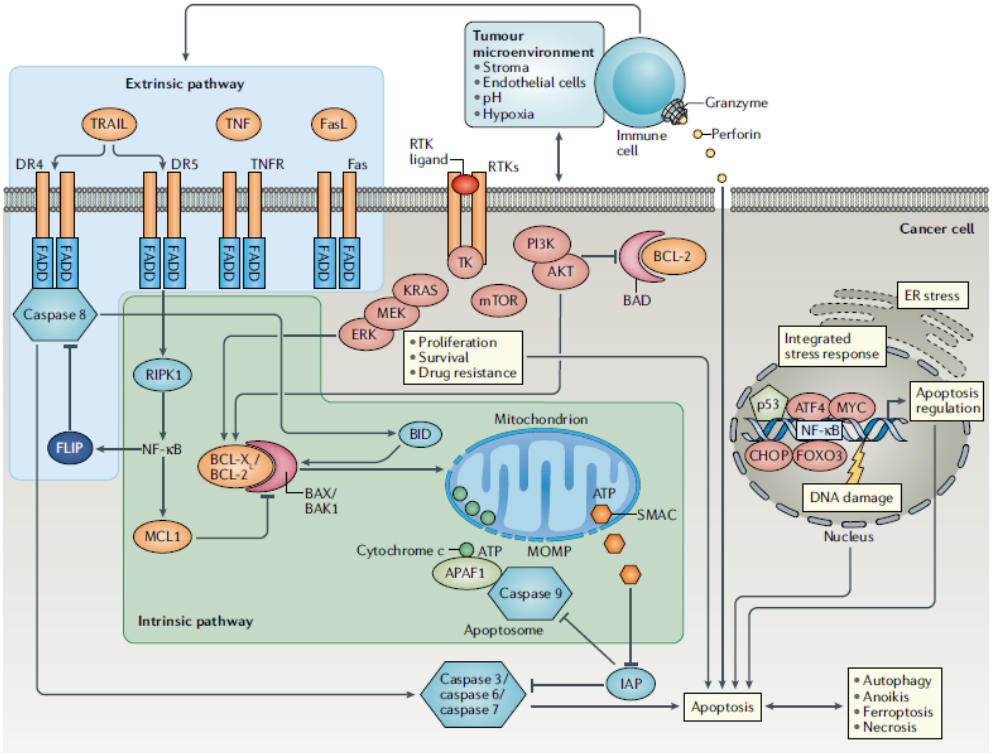
1.3 Morte celular regulada

A morte celular é um processo fundamental na manutenção da homeostase tecidual, sendo um processo natural envolvido no desenvolvimento dos tecidos e sua posterior manutenção e reparo frente a alguma injúria (PARK et al., 2023). O processo de morte pode ser dividido em (i) morte celular acidental e (ii) morte celular regulada/programada (PCD). Nesse sentido, a PCD ganha grande destaque, uma vez que sua indução e execução depende de vias controladas geneticamente e que apresentam grande importância no desenvolvimento, resposta imune e tumorigênese (PENG et al., 2022). Dentre as PCDs de maior destaque no câncer, tem-se a (i) apoptose, (ii) a necroptose e (iii) a piropotose.

A apoptose foi a primeira PCD a ser descoberta tendo sido reconhecida pela indução de um programa de morte envolvendo a ação de proteases baseadas em cisteínas, as caspases, com a degradação controlada de vários alvos celulares (ELMORE, 2007). Atualmente, sabe-se, que a apoptose pode ser induzida por três vias de ativação: (i) via extrínseca, (ii) via intrínseca e (iii) via mediada por porifirina/granzima B (Fig 8). A via extrínseca é ativada via ligantes extracelulares que ao se associarem aos receptores de morte celular leva formação de um complexo sinalizador de morte celular (DISC), o qual recruta caspases iniciadoras, entre elas a 8 e 10, que por sua vez ativam as caspases efetoras, como as 3, 6 e 7 e levam a degradação proteolítica de diversos substratos celulares, culminando na morte celular (ELMORE, 2007; PARK et al., 2023). Por sua vez, a via intrínseca é ativada mediante a diferentes tipos de estresses intracelulares que resultam na permeabilização da membrana mitocondrial externa e

liberação do citocromo c para o citoplasma, onde em associação com a proteína Apaf-1 e procaspase-9, formam um complexo supramolecular denominado apoptossomo, o qual leva a ativação de caspase-9 e subsequente ativação das caspases efetoras 3, 6 e 7 (PARK et al., 2023). Por fim, a via mediada por granzima B, derivada de linfócitos citotóxicos e células NK, caracteriza-se pela ativação da caspase iniciadora 10 ou mesmo diretamente da caspase 3 efetora, deflagrando a cadeia proteolítica que leva a morte celular (ELMORE, 2007; PARK et al., 2023).

Figura 8. Aspectos moleculares envolvidos na indução de apoptose. A via intrínseca da apoptose envolve o aumento da permeabilidade da membrana externa da mitocôndria e liberação do citocromo c no citoplasma. O complexo supramolecular denominado apoptossomo é formado a partir associação do citocromo c com a proteína Apaf1 e pro-caspase-9. A ativação da caspase-9, leva a ativação da caspase-3 e resulta no processo de apoptose. A via extrínseca é ativada por ligantes de morte celular e seus receptores, formando o complexo DISC. A ativação da caspase-8 resulta na ativação da caspase-3 e apoptose. Linfócitos $CD8^+$ e células NK podem induzir a apopotse por através da produção de porfirina e granzima B. Ainda, estímulos estressantes, como danos ao DNA, estresse de retículo endoplasmático e intensa autofagia são processos que podem resultar na ativação da via apoptótica. DISC: complexo sinalizador de morte celular, NK: natural killer, DNA: ácido desoxirribonucleico.

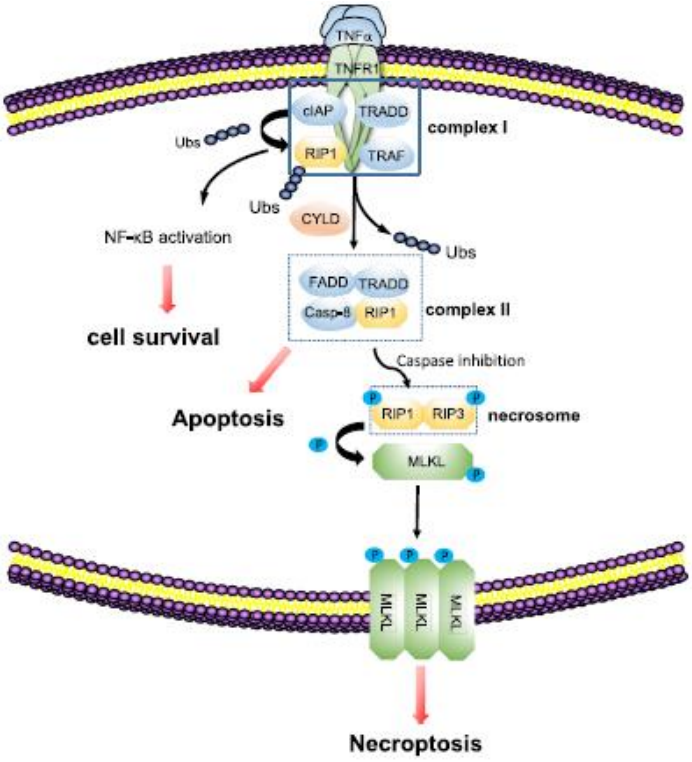


Fonte: Extraído de CARNEIRO; EL-DEIRY, 2020

Assim como a apoptose, a necroptose é um processo de necrose regulada que pode ser induzido a partir da sinalização de receptores de morte celular, contudo, diferente da apoptose, não há ativação de caspases (TANG et al., 2019) (Fig 9). A via de ativação mais estudada de necroptose é a mediada via TNF-α/TFN-R1. Uma vez ativado, a via do TNF-R1 leva a formação

de um complexo de sinalização (complexo I) pelo recrutamento de RIP31, proteína com domínio de morte associado ao TNFR1 (TRADD), inibidores celulares de apoptose (cIAPs) e fator 2 associado ao TNFR (TRAF2) (ZHU et al., 2019). Nesse complexo, diversas proteínas levam a ubiquitinação do RIPK1, o que permite a associação do NFκB e recrutamento e ativação das quinase IκB (IKK) α/β, ativando a via NFκB (ZHU et al., 2019). A desubiquitinação do RIPK1 é um evento chave para inativar a via do NFκB e deflagrar a via apoptótica ou necroptótica. Em contextos de não ativação da caspase-8, a desubiquitinização do RIPK1 leva a sua dissociação do complexo I, onde o mesmo é autofosforilado e uma vez ativo, interage com RIPK3, o qual também é fosforilado, formando um complexo denominado necrossomo, o qual tem como alvo a proteína MLKL (GONG et al., 2019). Uma vez fosforilado pelo RIPK3, a MLKL forma oligômeros que são transportados à membrana plasmática, esse processo resulta no comprometimento de sua integridade e rompimento celular (GONG et al., 2019; ZHU et al., 2019).

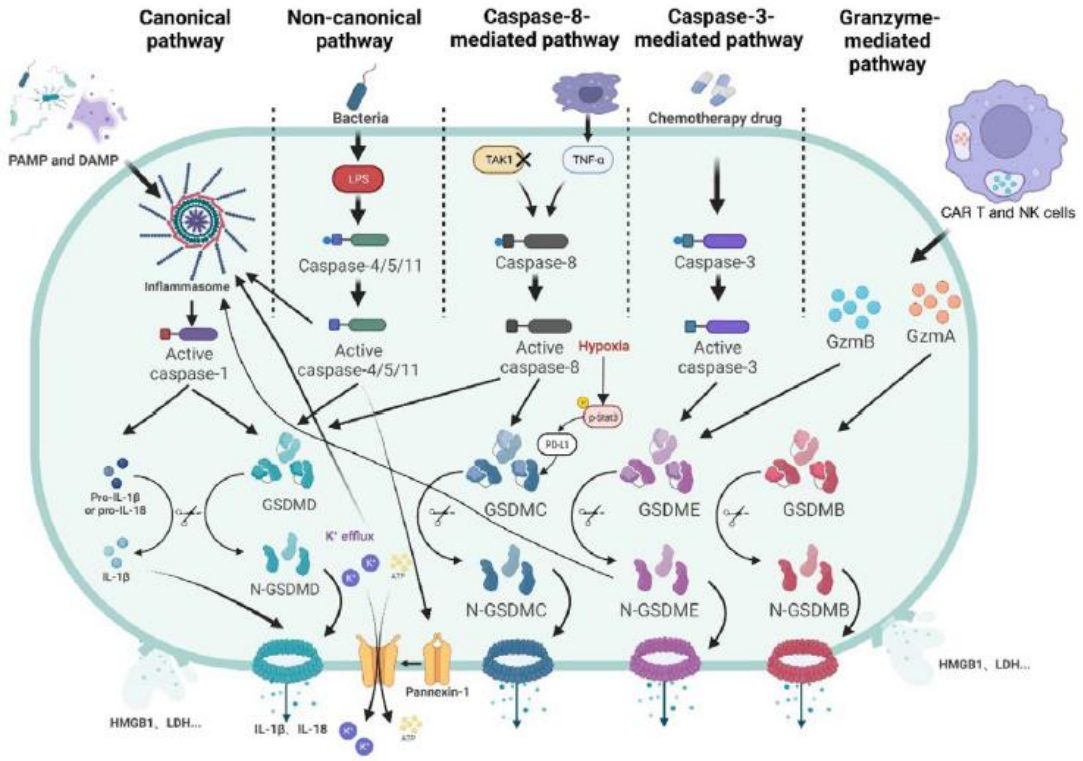
Figura 9. Esquema representativo da via de indução de necroptose. A associação de um fator de morte ao seu receptor leva a formação do complexo I de sinalização, o que permite a ativação inicial da via do NFκB. A desubiquitinação de RIP1K inativa a via do NFκB e o dissocia do complexo I levando a formação do complexo II na presença de caspase e indução de apoptose ou a formação do necrossomo na ausência de ativação de caspase. Ativado, o RIP3K fosforila o MLKL o qual forma oligômeros que são translocados a membrana e resultam na morte celular por necroptose.



Fonte: Extraído de GONG et al., 2019

Por fim, a piroptose também é um tipo de PCD com morfótipo necrótico executada pela família de proteínas denominada gasderminas (GSDM), composta por: GSDM A/B/C/D/E e DFNB59, onde quase todos os membros já foram relatados com função de formação de poros na membrana plasmática (YU et al., 2021) (Fig 10). Canonicamente, a piroptose é deflagrada a partir da detecção padrões moleculares associados a danos (DAMPs) ou padrões moleculares associados a patógenos (PAMPs) devido à formação dos inflamassomos. Esses consistem de um complexo supramolecular formado por: (i) proteínas detectoras, (ii) proteína adaptadora (ASC) contendo domínio de recrutamento de caspase (CARD) e (iii) pro-caspase-1(WEI et al., 2022). Uma vez que o inflamassomo é ativado, a caspase-1 é clivada, fazendo que com a mesma aja sobre alguns alvos específicos, entre eles a GSDMD e pro-IL-1β e pro-IL-18. Como consequência, a clivagem da GSDMD permite a sua oligomerização e translocação para a membrana, resultando na morte por rompimento da membrana plasmática e liberação de IL-1β e IL-18 madura para o meio extracelular (WEI et al., 2022; LIU; KUANG; CHEN, 2023). Apesar da via canônica ser a mais comum, vias alternativas de ativação da piroptose já foram relatadas, seja mediada por LPS, caspase-8, caspase-3 e granzima B(YU et al., 2021; LIU; KUANG; CHEN, 2023).

Figura 10. Vias de indução de piroptose. A via canônica se dá pela detecção de PAM/DAMPs pelo inflamassomo, resultando na ativação da caspase-1 e clivagem da GSDMD. A via não canônica ocorre pela ativação da caspase-4/5/11 por LPS extra ou intracelular, culminando na clivagem de GSDMD. Macrófagos M1 podem induzir a piroptose mediada pela clivagem da GSDMC/D via ativação da caspase-8 em situações de hipóxia e translocação nuclear do PD-L1. Alguns quimioterápicos já foram relatados como capazes de induzir a ativação da caspase-3 e clivagem de GSDME. Por fim, granzima B derivada de linfócitos e células NK podem induzir a clivagem de GSDMB/E e levar a piroptose.



Fonte: Extraído de LIU; KUANG; CHEN, 2023

1.4 Ácido docosahexaenoico

1.4.1 Ácidos graxos: Estrutura e nomenclatura

Os lipídeos são componentes essenciais à saúde, classicamente caracterizados por uma classe de moléculas heterogêneas que compartilham entre si a característica de serem predominantemente hidrofóbicos (BURDGE; CALDER, 2015). Contudo, essa classificação é bem vaga, uma vez que pode abranger compostos orgânicos não lipídicos. Assim, em uma nova definição, lipídeos são caracterizados como pequenas moléculas hidrofóbicas ou anfifílicas que podem ser formadas parcialmente ou totalmente a partir da condensação de unidades tioésteres e ou/ isoprenos. Dessa maneira, as moléculas lipídicas se organizam em oito classes gerais que podem apresentar subclasses (FAHY et al., 2005), todavia, no que diz respeito à alimentação, a classe de maior reconhecimento são os ácidos graxos (BURDGE; CALDER, 2015).

Os ácidos graxos são um grupo diverso de moléculas caracterizadas pela repetição de uma série de grupamentos metileno, característica que confere hidrofobicidade à molécula, podendo haver ao longo dela diferentes graus de insaturação, sejam elas *cis* ou *trans* (FAHY et al., 2005). Dessa maneira, essas moléculas podem ser classificas como saturadas (SFA), monossaturadas (MUFA) e poli-insaturadas (PUFA) e, consequentemente a contagem dos números e localização das insaturações pode se dar utilizando três métodos de nomenclatura: (i) sistemática (Δ), onde todas as insaturações são listadas a partir do grupamento carboxila; (ii)

sistema ômega (n), onde a apenas a primeira insaturação é listada a partir do grupamento metil; e (iii) sistema de classificação simplificada (C:I), onde o número de carbonos (C) é dado em função do número de insaturações (I) (RUSTAN; DREVON, 2005; RATNAYAKE; GALLI, 2009) (Tab 1).

Tabela 1. Exemplo de nomenclatura de ácidos graxos

Nomenclatura		Abreviações*	
Comum	**Sistemática**	**Δ**	**n**
Ácido α-linolênico	Ácido todas-cis-^{9,12,15}-octadecatrienóico	18:3 $\Delta^{9, 12, 15}$	18:3, n-3

*Referente ao grupo carboxila (COOH - nomenclatura Δ) ou metil (CH3 - nomenclatura n).

Fonte: Baseado em RATNAYAKE; GALLI, 2009.

1.4.2 Ácido Docosahexaenoico

De acordo com diretrizes da Organização Mundial da Saúde (WHO) e de agências nacionais de saúde, recomenda-se que o consumo diário de lipídeos não ultrapasse 15-30% do valor total de calorias ingeridas no dia, com o intuito de prevenir o desenvolvimento de doenças cardiovasculares (NISHIDA et al., 2004; SOUZA et al., 2015) (Tab 2). Nesse sentido, ainda se recomenda um baixo consumo de gorduras *trans* (<1%) e SFA (7-10%), e um balanço na ingestão de PUFAs n-6 e n-3 (4:1/5:1). Contudo, alterações no estilo de vida e o aumento crescente da ocidentalização da dieta têm sido os principais responsáveis por desequilibrar a proporção de lipídeos na alimentação e contribuir para o estabelecimento de diversos distúrbios metabólicos como dislipidemia, diabetes e doenças cardiovasculares (FAHY et al., 2005; SOUZA et al., 2015).

Tabela 2. Referência para o consumo diário de lipídeo. Dados extraídos de NISHIDA et al., 2004; SOUZA et al., 2015.

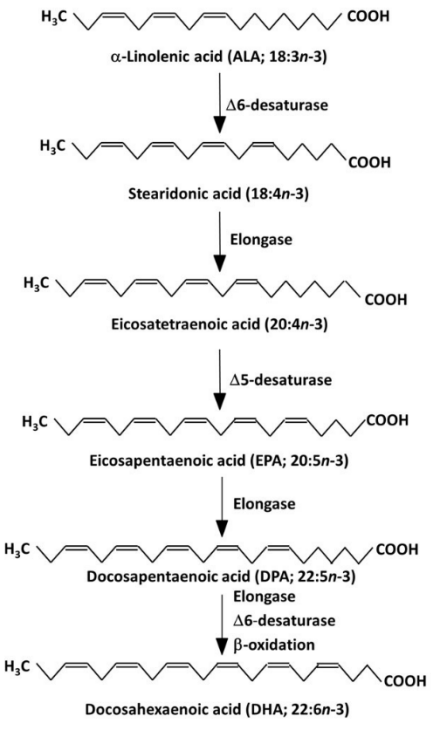
	WHO 2002	**Pesquisa de dieta nacional**
Total de lipídeos	15-30%	15-30%
SFAs	<10%	<7%
PUFAs	6-10%	10%
PUFAs n-6	5-8%	-
PUFAs n-3	1-2%	-
Ácidos graxos *trans*	<1%	<1%
MUFAs	Por diferença*	-
Razão n-6:n-3	4:1 – 5:1	4:1 – 5:1

*Calculado por: total de lipídeos – (SFA + PUFA + ácidos graxos *trans*). WHO: organização mundial da saúde, SFAs: ácidos graxos saturados, PUFAs: ácidos graxos poli-insaturados, n: ômega.

Assim, diversos estudos na literatura ressaltam a importância da manipulação dietética, em particular pelo aumento do consumo de PUFAs n-3, como o ácido eicosapentaenoico (EPA, C20:5) e docosahexaenóico (C22:6), na amenização e reversão de quadros de distúrbios metabólicos, atribuindo um efeito benéfico do consumo desses lipídeos à saúde e bem-estar (KALUPAHANA; CLAYCOMBE, 2010; ISHIDA et al., 2013; PAVLISOVA et al., 2016; NIAZI et al., 2017).

O ácido docosahexaenóico (DHA) é um dos PUFAs n-3 de maior relevância biológica. Devido a sua alta taxa de insaturação, o DHA apresenta um ponto de fusão extremamente baixo (-44ºC) e propriedades biológicas particulares (CALDER, 2016). Sua síntese ocorre a partir do ácido α-linolênico, um PUFA n-3 essencial, porém, essa rota biosintética é particularmente ineficiente em humanos e influenciada por hormônios sexuais (CALDER, 2015) (Fig 11). Assim, mulheres apresentam uma maior eficiência de bioconversão, uma vez que o estradiol é capaz de aumentar a expressão da enzima Δ-6 desaturase, o passo limitante da reação (GILTAY et al., 2004; PLOURDE; CUNNANE, 2007; KITSON et al., 2013). Consequentemente, a aquisição de DHA ocorre majoritariamente pela alimentação, seja pelo consumo de organismos de água fria, em particular peixes, ou alternativamente pela suplementação alimentar com fontes ricas em DHA que provém mais efetivamente a aquisição da dose recomendada de 0.2–1g/dia (KRIS-ETHERTON; GRIEGER; ETHERTON, 2009; CALDER, 2017; RACEY et al., 2021; PARK, 2022).

Figura 11. Rota biosintética do DHA



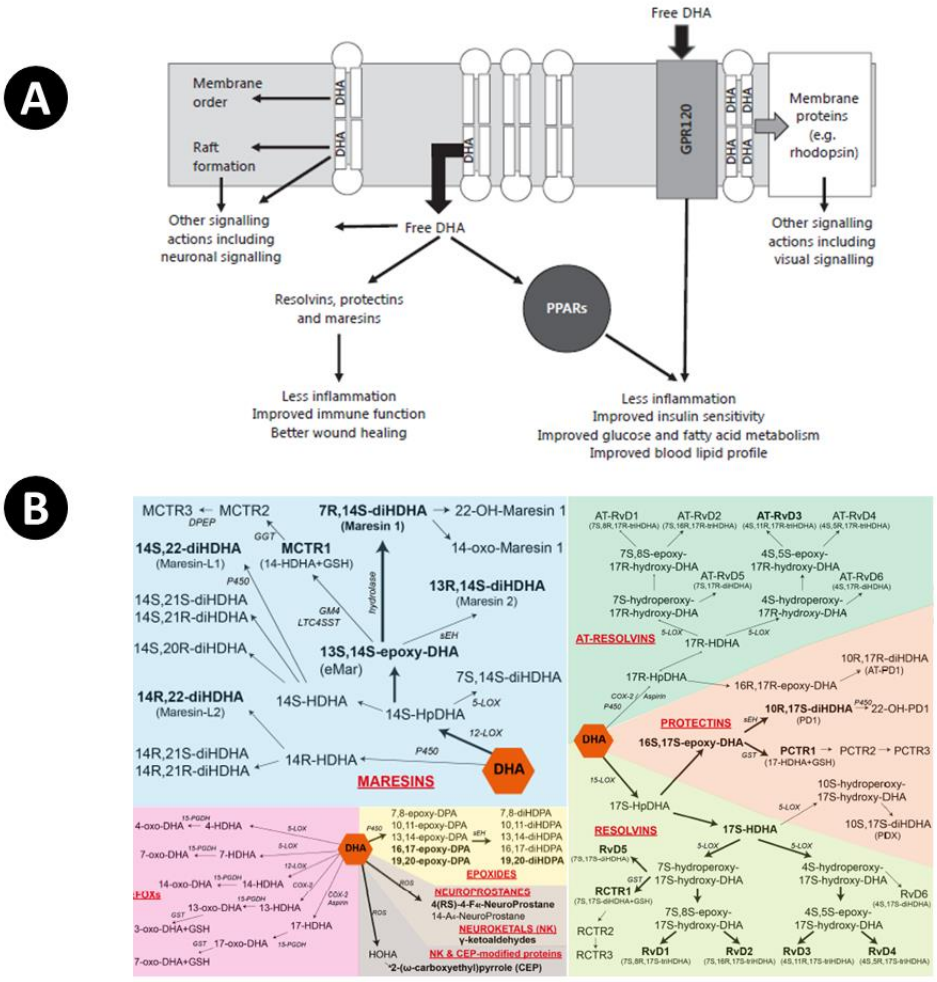
Fonte: Extraído de CALDER, 2017

Uma vez que o DHA foi adquirido pela alimentação, esse lipídeo pode tanto ser armazenado em gotículas lipídicas na forma de estéril de colesterol ou triacilglicerídeos, ou ainda ser incorporado aos fosfoglicerídeos de membrana, preferencialmente na fosfatidiletalonamina e fosfatidilcolina (STILLWELL; WASSALL, 2003). Devido ao seu alto grau de insaturação, a alta incorporação de DHA nas biomembranas resulta em aumento da fluidez da membrana e afeta diretamente a composição e organização dos domínios de membrana (CALDER, 2017) (Fig 11A). Estudos demonstram que membranas ricas em DHA podem levar a exclusão de colesterol e esfingomielina nos domínios de membrana e consequentemente reduzir o recrutamento de algumas proteínas essenciais para certos processos de sinalização (FAN et al., 2003, 2004; STILLWELL et al., 2005). Por outro lado, de forma paradoxal, a incorporação de DHA também pode ser responsável por aumentar o tamanho e estabilidade dos domínios de membrana, a partir da segregação de regiões de membrana ricas em DHA - regiões de "não-domínio" - e regiões ricas em colesterol - domínios de membrana (SONI et al., 2008; SHAIKH et al., 2009; LEVENTAL et al., 2016). De fato, apesar de contraditórios, os dois mecanismos não são totalmente excludentes, e evidências demonstram que os efeitos anti-tumorais do DHA pode se dar das duas formas, seja pela depleção de colesterol e indução de apoptose (STILLWELL et al., 2005) ou por maior estabilidade e concentração de proteínas essenciais envolvidas com o processo de necroptose (NEWELL et al., 2022).

Ainda, o DHA é reconhecido como ligante do receptor de membrana GPR-120 (FFAR) e dos receptores nucleares PPAR (PPARα e PPARγ) e RXRα (KREY et al., 1997; GANI; SYLTE, 2008; OH et al., 2010) (Fig 8A). O GPR-120 é um transportador de ácido graxo de cadeia longa, sendo demonstrado que os efeitos anti-inflamatórios e de sensibilização à insulina do DHA são mediados pelo mesmo, uma vez que esses efeitos não são observados camundongos *knockout* para GPR-120 (OH et al., 2010). Ainda, alguns efeitos antitumorais do DHA já foram demonstrados serem dependentes do GPR-120, como redução do infiltrado de TAM de perfil M2, redução da expressão de fatores angiogênicos e inibição da supressão de células T $CD8^+$ por TAMs M2 (LIANG et al., 2019, 2022, 2023). Por ser um ligante endógeno do PPARα/γ e do seu parceiro de heterodimerização RXRα, o DHA pode estimular a transcrição de genes alvos do PPAR, e consequentemente modular mecanismos envolvidos no metabolismo, inflamação e sobrevivência celular, o que pode impactar positivamente a

progressão tumoral (PATEL et al., 2001; DE VOGEL-VAN DEN BOSCH et al., 2008; YU et al., 2008; O'FLAHERTY et al., 2012; CHANG et al., 2015; SONG et al., 2017; NAEINI et al., 2020). Por fim, o DHA também é alvo de oxidação enzimática e não-enzimática, levando a formação de diversos lipídios bioativos, sendo que a maior parte deles constituem mediadores especializados de pró-resolução (SPM) (CALDER, 2020) (Fig 12A). Nesse sentido, a metabolização enzimática pela via da lipo-oxigenase (LOX), ciclo-oxigenase e citocromo P450 leva a formação de classes chamadas resolvinas, protectinas, maresinas, oxo-derivados eletrofílicos e epóxidos, sendo a oxidação não enzimática responsável pela formação de neuroprostanos (KUDA, 2017) (Fig 12B). Interessantemente, já foi observado que parte dos efeitos antitumorais do DHA é mediado por seus SPM, a partir da modulação da polarização de macrófagos, e redução da angiogênese e metástase (GUODONG et al., 2013; SHAN et al., 2020).

Figura 12. Mecanismos de ação do DHA. (A) Esquema resumindo as principais vias de ação do DHA. **(B)** Lipídios bioativos formados a partir da oxidação enzimática e não-enzimática do DHA.



Fonte: Adaptado de CALDER, 2016 e KUDA, 2017

1.5 Camundongos transgênicos para adenocarcinoma de próstata (TRAMP)

Inúmeros esforços da comunidade científica são voltados para compreender os aspectos envolvidos na biologia do CaP, e com isso, diferentes modelos de estudo foram estabelecidos aos longos dos anos com esse propósito. Atualmente, a maioria esmagadora dos trabalhos usam linhagens celulares com diferentes *backgrounds* genéticos e responsividade androgênica a fim de compreender mecanisticamente os processos envolvidos na biologia do CaP. Contudo, apesar das linhagens celulares representarem uma forma rápida para identificação de vias e alvos no tratamento do CaP, células isoladas em cultura não reproduzem todas as interações celulares e moleculares que são estabelecidas em um microambiente prostático intacto (LEE; AKIN-OLUGBADE; KIRSCHENBAUM, 2011). Ainda, vale ressaltar que as principais linhagens celulares de CaP usadas em pesquisa são derivadas de tumores metastáticos, e, portanto, apresentam conjuntos de mutações características dessa fase da doença, e consequentemente acabam não sendo tão representativas de todo o espectro da progressão tumoral (SOBEL; SADAR, 2005a, 2005b). Dessa maneira múltiplas abordagens são necessárias para se compreender melhor o processo de tumorigênese prostática.

O uso de camundongos geneticamente modificado (GEM) representou um avanço no estudo do CaP, e múltiplas linhagens foram estabelecidas ao longo do tempo com o intuito de desenvolver um modelo em que a doença progrida de forma mais semelhante ao observado em humanos. Assim, um bom modelo de GEM recapitula várias etapas da progressão tumoral, como o desenvolvimento de PINs, as quais progridem para um carcinoma invasivo, seguindo para um adenocarcinoma bem diferenciado e indiferenciado, e por fim, culminando em eventos de metástase (GRABOWSKA et al., 2014). Contudo, uma característica comum a vários GEM é que os sítios de metástase geralmente diferem do humano, o qual ocorre nos ossos em 90% dos casos, sendo em camundongos mais frequentemente nos pulmões e linfonodos (WU et al., 2013; KANG et al., 2022). Ainda, boa parte dos modelos de GEM apresentam diferenciação neuroendócrina, que corresponde a apenas 2% dos casos de CaP em humanos, embora, a incidência de CaP com diferenciação neuroendócrina venha aumentando nos últimos anos, principalmente em casos de recorrência após prostatectomia radical e ADT (YUAN; VEERAMANI; LIN, 2007; EPSTEIN et al., 2014; YAMADA; BELTRAN, 2021) (Fig 13). Assim, apesar de algumas diferenças pontuais serem observadas entre camundongos e humanos, a utilização de GEM vem se mostrando uma ótima abordagem para se avaliar os

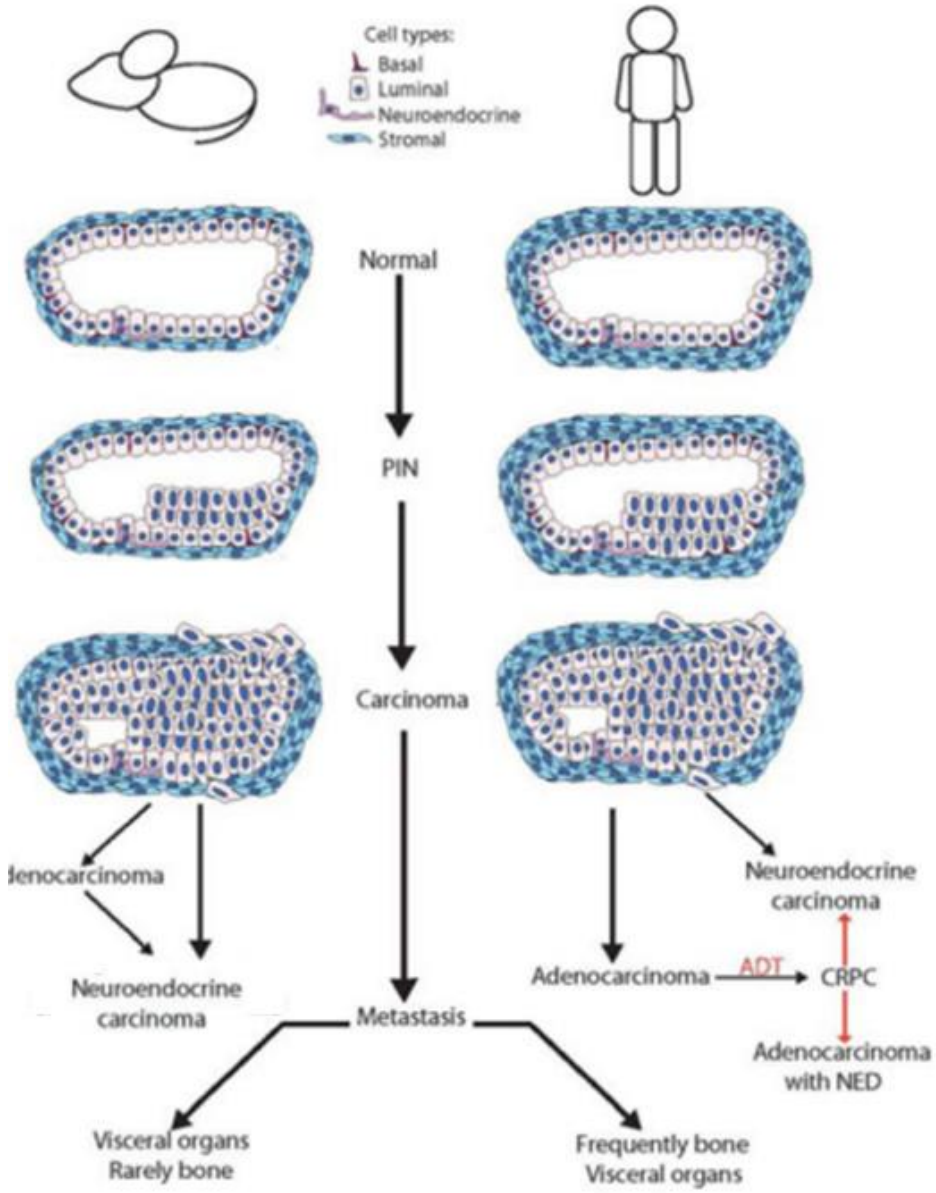
mecanismos envolvidos na progressão do CaP, bem como as novas abordagens terapêuticas no tratamento da doença (KIDO et al., 2019).

Entre os GEM, se destaca a linhagem TRAMP (do inglês, *Transgenic Adenocarcinoma of the Mouse Prostate*), a qual foi estabelecida por Greenberg e colaboradores (1995). Nesse modelo a carcinogênese é estimulada pela expressão heteróloga do antígeno T do vírus símio 40 (SV40), sob controle da probasina de rato (rPB), um elemento andrógeno-responsivo (GREENBERG et al., 1995). A expressão do rPB é restrita ao epitélio prostático (GREENBERG et al., 1994), de modo que a expressão do transgene ocorre com a maturação sexual do indivíduo devido ao aumento na produção de testosterona, resultando na inativação das proteínas p53 e Rb, importantes supressores de tumor, o que leva ao estabelecimento da doença (GREENBERG et al., 1995; AHUJA; SÁENZ-ROBLES; PIPAS, 2005). Incialmente, a linhagem foi estabelecida com o *background* C57BL/6, e, posteriormente, constatou-se que camundongos F1 gerados do cruzamento de fêmeas TRAMP C57BL/6 (B6) com camundongos da linhagem FVB apresentavam uma progressão da doença de forma mais rápida e agressiva (GREENBERG et al., 1995; GINGRICH et al., 1996, 1999).

Semelhante ao observado em humanos, camundongos TRAMP apresentam uma forma progressiva da doença ao longo do envelhecimento, que se manifesta pelo estabelecimento de lesões pré-malignas e malignas, e eventos de metástases (GREENBERG et al., 1995; GINGRICH et al., 1996, 1999; KAPLAN-LEFKO et al., 2003). Assim, os animais apresentam alterações nucleares compatíveis PINs a partir da 6ª semana de vida, sendo que a partir da 8ª semana já se pode observar diversos focos de lesões proliferativas estabelecidos na glândula. Com o decorrer da idade, a doença progride para um fenótipo mais agressivo, onde ocorre uma prevalência de PINs de alto grau (12-18 semanas) e lesões malignas como carcinoma in situ e adenocarcinoma bem diferenciado e indiferenciado, além de uma alta incidência de tumores primários (16-22 semanas) (KIDO et al., 2016; AMARO et al., 2022). Ainda, estudos demonstram a aquisição de fenótipo neuroendócrino e altamente metastático em estágios avançados da doença (MASUMORI et al., 2001). Interessantemente, a castração não detem totalmente a progressão da doença, de forma que os tumores primários proveniente de animais castrados apresentam morfologia pouco diferenciada, uma característica de agressividade tumoral, sugerindo a aquisição de fenótipos resistentes à castração frente a ADT como observado em humanos (GINGRICH et al., 1997). De fato, mutações espontâneas no gene do *AR* que conferem maior afinidade a testosterona já foram relatadas no modelo, um dos mecanismos de resistência observado no CRPC (HAN et al., 2001). Dessa forma, o fato de o

microambiente glandular ser preservado no modelo TRAMP permite a análise de mecanismos envolvidos em várias etapas da progressão tumoral, bem como a pesquisa de compostos e alvos terapêuticos no tratamento da doença (KIDO et al., 2019).

Figura 13. Comparação dos aspectos envolvidos na progressão do CaP em humanos e GEM.



Fonte: Extraído de GRABOWSKA et al., 2014

2- PROBLEMÁTICA DO ESTUDO

Estima-se que 1.2 milhões de indivíduos serão diagnosticados com CaP anualmente, dos quais aproximadamente 30% irão falecer por conta da doença, fazendo com que este seja uma das principais causas de óbito relacionado ao câncer (REBELLO et al., 2021). Apesar de apresentar uma etiologia complexa, nos últimos anos a dieta vem sendo demonstrada como um fator chave no risco de desenvolvimento da doença (MOKBEL; WAZIR; MOKBEL, 2019). Nesse sentido, os lipídeos da alimentação ganham muito destaque, em particular os PUFAs n-3. Contudo, majoritariamente, os estudos envolvendo esses lipídeos se baseiam em abordagens *in vitro*, e os poucos estudos clínicos que correlacionam os níveis desses PUFAs com o risco de desenvolvimento da doença são contraditórios e algumas vezes inconclusivos (NORRISH et al., 1999; LEITZMANN et al., 2004; CROWE et al., 2008; BRASKY et al., 2011; SORONGON-LEGASPI et al., 2013). Dessa maneira, mais estudos, em particular focando abordagens *in vivo* são necessárias para compreender as repercussões do consumo de DHA sobre a progressão do CaP.

Existe vasta literatura mostrando que o DHA tem uma ação anti-tumoral por inibir vários *hallmarks* do câncer, incluindo diferentes elementos do microambiente inflamatório (NARAYANAN; NARAYANAN; REDDY, 2005; DE FREITAS RODRIGUES et al., 2023). Estudo recente do nosso laboratório demonstrou que o consumo de uma dieta enriquecida com DHA é capaz de atrasar as fases iniciais da progressão tumoral em camundongos TRAMP, sendo esse efeito relacionado à redução da proliferação celular e da sinalização de AR (AMARO et al., 2022). Nesse estudo, também se constatou que o DHA é capaz de preservar o estroma prostático, impedindo o aumento de fibras colágenas, expressão de αSMA e o infiltrado de células T CD3+. Esses achados sugerem que o DHA pode modular múltiplos aspectos do TME. De fato, estudos *in vitro* demonstram que o DHA é capaz de reduzir a ativação de fibroblastos estimulados por TGF-β (BIANCHINI et al., 2012; ZENG et al., 2017), modular a polarização de macrófagos (SHAN et al., 2020) e inibir a angiogênese (ASLAN et al., 2020). Considerando o amplo espectro de ação do DHA e a múltiplas alterações observadas no TME, a utilização de um modelo animal em que este encontra-se preservado, bem como as influências das condições homeostáticas e metabólicas, é essencial para compreender como DHA é capaz de modular diferentes fenômenos no TME, tais como a transição-epitélio mesenquimal, a resposta inflamatória e a dinâmica de proliferação e morte celular. Nesse contexto, estudos experimentais que investiguem os mecanismos pelos quais o DHA modula o microambiente tumoral no CaP em modelos animais são extremamente relevantes para esclarecer as vias de

ação do DHA bem como ultrapassar sua utilização em modelos pré-clínicos, fomentando sua aplicação em estudos clínicos para que seja solidificado o seu uso como quimioprotetor.

3- OBJETIVOS

3.1 Objetivos gerais

O presente trabalho teve como objetivo avaliar o impacto de uma dieta enriquecida em ácido docosahexaenoico sobre a progressão do CaP em camundongos transgênicos para adenocarcinoma de próstata (TRAMP) com enfoque para as alterações no TME.

3.2 Objetivos específicos

(i) Avaliar a progressão tumoral com base em análises histopatológicas, perfil de proliferação e morte celular e vias de sinalização de sobrevivência celular;
(ii) Caracterizar o infiltrado de linfócitos no TME;
(iii) Caracterizar o perfil de TAM;
(iv) Quantificar a expressão sérica de TNF-α e PGE2, e os níveis intra-teciduais do TNF-R1 e PTGS2;
(v) Avaliar o processo de transição epitélio-mesenquimal;
(vi) Caracterizar o padrão de expressão de ANXA1 ao longo da progressão tumoral no epitélio glandular e a expressão de ANXA1 nos CAFs.

4- RESULTADOS

Os resultados a seguir serão apresentados na forma de dois manuscritos, intitulados:

Manuscrito I: *"Dietary docosahexaenoic acid impairs prostate cancer progression through modulation of inflammatory microenvironment"* – Submetido para a revista ***"Prostate Cancer and Prostatic Diseases"***

Manuscrito II: *"Docosahexaenoic acid improves prostate cancer microenvironment by modulation of Annexin A1 expression"* – A ser submetido para a revista ***"Inflammation"***

Dietary docosahexaenoic acid impairs prostate cancer progression through modulation of inflammatory microenvironment

Running title: Immunomodulatory effects of docosahexaenoic acid for prostate cancer management

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ABSTRACT

Background: Prostate cancer (PCa) is one of the leading causes of death worldwide and inflammation is critical for its initiation and prognosis. The polyunsaturated fatty acid docosahexaenoic acid (DHA) has impressive anti-inflammatory and pro-resolution properties but its therapeutic use in PCa requires pre-clinical evidence. Here, the transgenic adenocarcinoma of the mouse prostate (TRAMP) was used as a pre-clinical model to assess the effect of DHA intake on the inflammatory microenvironment and proliferative and survival pathways in early- and late-stage disease.

Methods: TRAMP mice were fed with standard rodent or DHA-enriched diet (DHA-d) for 4 (early stage) or 10 weeks (late stage). The ventral prostate was evaluated by using histopathological, immunohistochemical, and western blotting analysis. Serum samples were collected for TNF-α measurement.

Results: DHA-d preserved ventral prostate morphology resulting in a lower frequency of pre-malignant and malignant lesions, thereby reducing PCa progression and aggressiveness. Dietary DHA reduced cell proliferation and increased apoptosis by inhibiting the Akt pathway in late-stage disease and activating ERK1/2 signaling in early-stage disease. DHA-d down-regulated pyroptosis in both periods and up-regulated necroptosis in the late stage. Regardless of the period, the intake of DHA reduced $CD4^+$ T-cell and M2-like macrophage and increased $CD8^+$ T-cell infiltration only in the late stage. TNF-α systemic level was down-regulated by DHA-d in both periods but the TNF R1 protein level in the prostate diminished only in the late stage.

Conclusions: DHA-d has a protective effect on prostate carcinogenesis of TRAMP mice through negative modulation of proliferative and survival pathways, induction of apoptosis, and ERK 1/2 activation. DHA also changed the tumor microenvironment into

a low inflammatory and anti-tumor feature, reducing the $CD4^+/CD8^+$ ratio and downregulating the M2-like TAM profile. Such immunomodulatory effects suggest DHA might be a good adjuvant to PCa therapy.

Keywords: omega-3, lymphocytes, tumor-associated macrophages, necroptosis, pyroptosis, TRAMP

1. Introduction

Prostate cancer (PCa) is the most frequent malignancy in men and the second leading cause of death by cancer-related cases in the United States.[1] Moreover, approximately 1.2 million new cases of PCa are diagnosed globally per year resulting in a high incidence of death due to PCa-related cases.[2] Although PCa is a highly heterogeneous disease, inflammation has been linked to increasing PCa development risk and tumor progression.[3] Persistent inflammation in the prostate microenvironment results in an overproduction of reactive oxygen species (EROs), growth factors, and cytokines that collectively lead to genomic instability, up-regulation of proliferative and survival pathways, immune surveillance evasion, and androgen independence. [2,4]

Beyond these molecules, the tumor-infiltrated immune cells are recognized to have a significant role in PCa outcome, particularly T-cell infiltration and tumor-associated macrophages.[3] In the tumor microenvironment (TME), $CD4^+$ T-cells are recruited by tumor cells-secreted chemoattractant molecules such as PGE_2 and CXCL9[5,6] and trigger PCa progression and metastatic capacity through up-regulation of MMP9 and epithelial-mesenchymal transition (EMT)[5,7]. Contrary to $CD4^+$ T-cells, the cytotoxic $CD8^+$ T-cells are reduced in tumor microenvironments (TEM) and mediate anti-tumor responses[8]. The increase in $CD8^+$ T-cells infiltration, expansion, and function is reported to overcome the immunosuppressive PCa microenvironment and inhibit tumor growth.[9,10] Nevertheless, high $CD8^+$ T-cell infiltrate was related to improved survival of patients undergoing radical prostatectomy.[11] Therefore, screening the profile of tumor-infiltrate lymphocytes, particularly the $CD4^+/CD8^+$ ratio in TME has been proven a useful tool in cancer prognostic.[12]

Macrophages play diverse roles in the prostate microenvironment and under pathological conditions such as cancer. These immune cells compose a highly heterogeneous group of cells, namely tumor-associated macrophages (TAMs).[3] However, despite their plasticity, TAMs can be clustered into two general groups based on their

surface markers, protein expression pattern, and function: the antitumor M1-like and pro-tumor M2-like TAM. The M1-like profile displays a gene expression signature related to the Toll-like receptor 4 (*Tlr4*) pathway, pro-inflammatory cytokines such as IL-12 (*Il-12a and b*) and tumor necrosis factor (*Tnf*), and interferon-related gene response such as iNOS (*Nos2*), while the M2-like typically exhibits a metabolic change leading to up-regulation of gene-related to arginine and lipid metabolisms such as *Arg1* and *Cpt1a*.[13] In the TME, M1-like TAM is related to reduced prostate and ovarian tumor growth[14,15] while M2-like TAM correlates with poor PCa prognostic and induces tumor progression by IL-6 up-regulation.[16,17] Consequently, targeting TAM polarization in the TME has shown to be a good strategy for cancer management. [18]

The imbalanced inflammatory TME results in the overactivation of proliferative and survival pathways. To counter this, programmed cell death (PCD), an essential mechanism to keep tissue homeostasis, selectively removes transformed cells and avoids tumor onset and progression. Interestingly, as the tumor progresses, PCa cells develop therapy resistance by apoptosis evasion.[19] Recently, targeting other PCDs with necrotic morphotypes, such as necroptosis and pyroptosis, has emerged as an alternative to bypass apoptosis resistance. [20,21] Necroptosis is a regulated form of necrosis without caspase activation whose hallmark is the phosphorylation of activate mixed lineage kinase domain-like protein (MLKL) by receptor-interacting protein kinase 1 (RIP1) and 3 (RIP3). Necroptosis is induced downstream of death domain receptors (e.g., TNFR and Fas) and Toll-like receptor (TLR)-4 or TLR-3 activation following the formation of necrosome, a supramolecular complex formed by phosphorylated RIP1, RIP3, and MLKL. Then, oligomers of MLKL translocate to the cell surface and trigger cell death by membrane disruption.[21] Pyroptosis, another form of necrotic-like PCD, can be mediated by inflammasome assembly that results in the activation of caspase-1 or through alternative pathways such as activation of caspase-3/4/8/11 or via granzyme B, leading to cleavage of target substrates including the pore-forming proteins known as gasdermin (GSDM), like GSDM D (GSDMD).[20] Proteolytic cleavage of GSDMD (cGSDMD) leads to its oligomerization and membrane pore formation that culminate in cell death and inflammatory response by mature IL-18 and IL-1β release. [20] Thus, targeting multiple or specific PCD might help to overcome apoptosis resistance in cancer.

Regarding PCa prevention, it is well known that dietary habits can impact directly on inflammation and consequently the risk of PCa development. Particularly, the amount and nature of lipid intake correlate with disease onset and progression. [22,23] Pre-clinical

models of PCa highlighted that saturated (SFA) and omega-6 polyunsaturated fatty acids (n-6, PUFAs) intake accelerate tumor progression by exacerbating prostate inflammatory signaling through up-regulation of IL-6 and activation of STAT3 and NFκB pathway.[24,25] Contrary to these reports, the consumption of omega-3 (n-3) PUFAs, such as eicosapentaenoic (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), decreases tumor growth suppressing PGE_2 production and increasing pro-resolving lipids mediators and impairing IL-6 and IL-8 production and NFκB activity in PCa cells.[26–29] Therefore, increasing n-3 PUFAs intake by changes in lifestyle or dietary supplementation could positively impact prostate health, however, the majority of DHA studies comprise *in vitro* approaches, therefore, *in vivo* studies are needed to attest to the possible benefits of the immunomodulatory properties of DHA in PCa.

Thus, considering the effects of inflammation on PCa onset and progression, we used the transgenic adenocarcinoma of the mouse prostate (TRAMP) to assess the effect of DHA intake on tumor growth and inflammatory features. TRAMP mice display a progressive and well-established disease progression.[30] In addition, the TRAMP PCa microenvironment displays a disturbance in inflammatory pathways and apoptosis signaling similar to human PCa.[31] Here, we correlated the effects of DHA intake on disease progression with inflammatory molecules, T-cells infiltrate, and TAM profile, and for the first time, we reported the pattern of necroptosis and pyroptosis in TRAMP PCa and its modulation by DHA.

2. Materials and Methods

2.1 Experimental design

Forty-eight transgenic mice (C57BL/6-Tg [TRAMP] 8247 Ng/J X FVB/Unib) were provided by the Multidisciplinary Center for Biological Investigation of the State University of Campinas (CEMIB/UNICAMP) at 6 weeks of age. Animals were randomly distributed into four experimental groups according to their diet and age. Control groups (C) were fed an AIN-93G standard diet while DHA-fed groups (D) received an AIN-93G modified diet enriched with 10% of fish oil (DHA-d) as previously reported [27]. Diet was offered from 8 to 12 (C12 and D12) or 8 to 18 weeks of age, and at the end of the experiment, animals were euthanized with xylazine hydrochloride (5 mg/kg, intramuscular) and ketamine hydrochloride (60 mg/kg, intramuscular). The ventral prostate was harvested for morphological and molecular analyses. Animal care and

experiments were conducted according to Brazilian guidelines for the care and use of animals for scientific and educational purposes (BDCA) - CONCEA, and were approved by the Ethics Committee on Animal Use (CEUA) of UNICAMP (Protocol: 5884-1/2021).

2.2 Biometric analyses

Food intake was assessed every two days to calculate the weekly food (g) intake. Energy intake was obtained by multiplying the amount of food ingested in a week by the energy (kcal) provided by each diet[27]. Additionally, the relative weight gain was calculated through the following formula: RW = (FW-IW × 100)/IW, where FW means final weight and IW initial weight. The adiposity index was estimated by the following formula: [(sum of epididymal, retroperitoneal, and visceral fat/body weight) × 100[32].

2.3 Histopathological analyses

Ventral prostate samples were weighted and fixed in buffered paraformaldehyde 4% for 24h and processed for paraffin and plastic polymer infiltration (Paraplast, Merck). Samples were sectioned at 4µm thickness and stained with Hematoxylin-Eosin for histopathological analyses. The microscopic images were obtained on a Zeiss-Jenaval microscope, AxioCam HRc digital camera coupled, and AxioVision Image analysis system (Zeiss, Germany).

The histopathological analysis was carried out as described before[27], for each animal (n = 5/group) a total of 15 images at 400x magnification were analyzed. Each image was divided into four quadrants and in each quadrant, all morphological features were counted. Lesion grading was based on Berman-Booty and colleagues (2012) with modifications and classified as normal epithelium, low-grade intraepithelial neoplasia (LGPIN), high-grade intraepithelial neoplasia (HGPIN), carcinoma *in situ* (CIS). The incidence of well-differentiated adenocarcinoma (WDAC) and undifferentiated adenocarcinoma (UDAC) was calculated considering the prevalence in the number of 18-week-old mice analyzed. Additionally, WDAC e UADC incidence was calculated considering the number of affected animals. All morphological features are represented and characterized in Supplementary Figure 1.

2.4 Immunohistochemistry and immunofluorescence

Prostate sections were subject to immunohistochemistry using the antibodies anti-pHH3 (9701S, rabbit monoclonal, Cell Signaling, MA, USA), anti-CD163 (sc-58965,

mouse monoclonal, Santa Cruz Biotechnology, TX, USA), anti-CD4 (25229S, rabbit monoclonal, Cell Signaling, MA, USA) and anti-CD8α (85336S, rabbit monoclonal, Cell Signaling, MA, USA). Epitope retrieval was conducted in citrate buffer (10mM, pH 6.0) for 60min at 98ºC following peroxidase blockade in 0.3-3% H_2O_2 solution for 15-30min. The nonspecific binding blockade was achieved by incubating sections in BSA 3% for 60min at room temperature. Primary antibodies were diluted at 1:75-100 in BSA 1% and incubated overnight at 4ºC. Then, samples were incubated with mouse anti-rabbit HRP-conjugated (1:150, sc-2357, Santa Cruz Biotechnology, TX, USA) secondary antibody for 2h30min at 37ºC or with a HRP-based polymer system (Leica Biosystem, Germany). Peroxidase activity was detected using diaminobenzidine (Roche, Merck, NJ, USA) as the chromogen, and Harris hematoxylin was used for counter-staining.

Histological sections were scanned with a BX61VS camera (Olympus Corporation) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5). Then, quantitative analyses were performed in 10 random fields at 400x magnification per animal (n = 5/group), and the number of positive cells in each field was counted. The result was expressed in mean cells/field.

The immunofluorescence assay was carried out using the primary antibodies anti-CD68 (97778S, rabbit monoclonal, Cell Signaling, MA, USA), and anti-iNOS (MA5-17139, mouse monoclonal, ThermoFisher Scientific, MA, USA). Epitope retrieval and the nonspecific binding blockade were realized as described for immunohistochemistry. Primary antibodies were diluted at 1:100 in BSA 1% and incubated overnight at 4ºC. Then, samples were incubated with anti-rabbit IgG #FITC (1:150, F0382, Sigma Aldrich, MO, USA) and anti-mouse IgG #TRITC (1:150, T5393, Sigma Aldrich, MO, USA) for 2h30min at 37ºC. Next, samples were mounted in a DAPI medium (Fluoroshield™ with DAPI, F6057, Sigma Aldrich, MO, USA).

For the immunofluorescence analyses, 10 random fields were captured at 400x magnification per animal (n= 5/group) on the AX10 Fluorescence Microscope (Zeiss) coupled with AxioVision (Zeiss, Germany) software. The number of positive cells co-localizing each antigen in each field was counted and the result was expressed in mean cells/field.

2.5 TUNEL assay

Apoptosis was detected through the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using the ApopTag® Peroxidase In Situ Apoptosis

Detection Kit (S7100, Merck, NJ, USA) according to the manufacturer's instructions. Image acquisition and TUNEL-positive cells quantification followed the same method described for immunohistochemistry analyses. The result was expressed in mean cells/field.

2.6 Western Blotting

Prostate samples were powdered in liquid nitrogen and resuspended in RIPA extraction buffer with protease (P8340) and fosfatase (P5726, Sigma-Aldrich, MO, USA) inhibitor cocktail. Total protein content was determined with Bradford reagent (Bio-Rad Laboratories, CA, USA) and measured in an Epoch microplate reader (BioTek Instruments Inc., VT, USA) at 595 nm. A total of 50µg of protein was subject to electrophoresis in SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Life Science, MA, USA). The nonspecific binding blockade was achieved by incubating membranes in BSA 3% for 60min or non-fat milk 5% for 30min at room temperature, then the membranes were incubated with their respective primary antibody overnight at 4ºC in a dilution range of 1:350-1.200. The following antibodies were used: anti-phospho-AKT (rabbit polyclonal, Santa Cruz Biotechnology, TX, USA), anti-AKT (rabbit polyclonal, Immuny); anti-phospho-ERK1/2 (4370S, rabbit polyclonal, Cell Signaling, MA, USA), anti-ERK1/2 (9102, rabbit polyclonal, Cell Signaling, MA, USA), anti-TNF R1 (sc7895, rabbit polyclonal, Santa Cruz Biotechnology, TX, USA), anti-phospho-MLKL (37333S, rabbit monoclonal, Cell Signaling, MA, USA), anti-cleaved-GSDMD (10137S, rabbit monoclonal, Cell Signaling, MA, USA) and anti-β-actin (sc47778, mouse monoclonal, Santa Cruz Biotechnology, TX, USA). Next, the membranes were incubated with mouse anti-rabbit HRP-conjugated (sc-2357, Santa Cruz Biotechnology, TX, USA) or anti-mouse IgGκ HRP-conjugated (sc-516, Santa Cruz Biotechnology, TX, USA) antibodies in a dilution range of 1:3500-5000 for 2h at 4ºC. ECL system was applied for band development and the images were acquired in a G: BOX Chemi XRQ photodocumentator (Syngene Synoptics, TF, UK). Relative densitometry was assessed by Image J software (https://imagej.nih.gov/ij/; NIH) and β-actin was used as endogenous control. For pAKT and pERK1/2 expression, the total protein AKT and ERK1/2 levels were used for protein normalization.

2.7 ELISA

After euthanasia, blood samples were collected through cardiac puncture from the left ventricle and centrifuged at 1620g for 20 min, 4°C; the serum obtained was used to determine the levels of TNF-α (R&D System, MN, USA) using a specific commercial kit according to the manufacturer's instructions. Sample absorbance was read in an Epoch microplate reader (BioTek Instruments Inc, VT, USA).

2.7 Statical analyses

Statistical analyses were performed using GraphPad Prism software (version 8.0.1, MA, USA). The data were compared by *two-way* ANOVA followed by Tukey's test. Data was expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

3. Results

3.1 DHA intake decreases ventral prostate weight

To assess the effect of DHA intake on prostate cancer progression we fed TRAMP mice with a DHA-enriched diet from 8 to 12 and 8 to 18 weeks of age. First, we examined if DHA intake could affect some biometric parameters. Overall, animals under DHA-d did not exhibit significant changes in food intake or relative weight gain (Supplementary Fig. 2A, 2C), however, as expected, due to its hypercaloric composition both D12 and D18 animals displayed an increase of 1.48- and 1.34-fold in energy intake compared to same age control groups (Supplementary Fig. 2B).

For both ages, animals under DHA-d had a reduction of approximately 1.53-fold of prostate wet weight (Supplementary. Fig 2D) and prostate relative weight (data not shown). Interestingly, even consuming more lipids in their diet, animals of DHA-d groups did not display any alterations in fat pad weights or adiposity index (Supplementary Fig. 2D, 2E), suggesting that DHA has no obesogenic effects.

3.2 DHA intake impairs PCa progression through modulation of proliferative and survival pathways and programmed cell death pathways

As expected, at 12 weeks of age the ventral prostate of TRAMP mice was mostly characterized by a higher incidence of pre-malignant lesions such as LGPIN and HGPIN, comprising up to 80% of the gland (Fig. 1). Epithelial areas without proliferative disorders

were 3-fold more frequent in the D12 group and HGPIN and CIS were respectively 2.8- and 2.3-fold less frequent (Fig. 1). The ventral prostates from mice in the C18 group were marked by the high frequency of LGPIN (46%), HGPIN (38%), and CIS (7%) (Fig. 1). Normal epithelial areas in the ventral prostate of D18 animals were 4.3-fold more frequent than in C18 whereas the frequencies of HGPIN and CIS decreased by 2.2- and 1.7-folds respectively (Fig. 1). Aggressive lesions such as WDAC and UDAC were registered only in 18 weeks of age mice whereas the D18 group displayed a 40% reduction in the incidence of WDAC, however, the UDAC incidence was not altered by DHA intake (Fig 2).

To investigate the mechanism by which DHA impairs PCa progression, we evaluated different proliferative and survival pathways as well as some PCD mechanisms. First, we observed that DHA-d downregulated the proliferative activity by approximately 2-fold in both DHA-d-fed groups (Fig. 2A-D, M). It was observed higher activation of the ERK1/2 pathway in the D12 group and the inhibition of Akt signaling in the D18 group (Fig. 2R and S). Additionally, it was also observed an increase in Akt activation during tumor progression, highlighting the constitutive activation of the Akt pathway in TRAMP mice carcinogenesis. The apoptosis rate did not change over tumor progression in control groups and increased 1.5- and 1.9-fold in the D12 and D18 groups, respectively (Fig. 2E-H, N). Next, we evaluated the expression of cleaved Gasdermin D (cGSDMD) and phosphorylated MLKL (pMLKL), specific markers for pyroptosis and necroptosis, respectively. The number of cGSDMD-positive cells was reduced by 1.9-fold in animals of the D18 group (Fig. 2I-L, O) and and both DHA-d-fed groups displayed a 3.4-fold reduction of 3.4-fold in cGSDMD protein levels (Fig. 2P). Interestingly, the pMLKL protein level was reduced by 3-fold in C18 when compared to C12, suggesting a possible suppression of necroptosis during tumor progression. The level of pMLKL protein increased 2.5-fold in the D18 group in comparison to C18 animals (Fig. 2Q).

3.3 T-cell infiltrate is affected by DHA intake

To characterize the T-lymphocyte infiltration in the ventral prostate of TRAMP mice, we selectively identified CD4+ and CD8+ T-cells (Fig. 3). T-cells were most abundant in the prostate stroma for all groups, however, a predominance of CD4+ was observed in control groups (Fig. 3A-D, J). CD4+ cells reduced 43% in D12 and 59% in D18 groups (Fig. 3J). The CD8+ T-cell infiltration did not vary among experimental groups, except for the D18 group which displayed approximately a 1.8-fold increase in

C8+ T-cell infiltration (Fig. 3E-H, K). Additionally, the overall modulation in T-cell infiltration by DHA-d intake decreased the CD4+/CD8+ ratio for both D12 and D18 groups (Fig. 3L).

3.4 DHA intake downregulates M2-like TAM in TRAMP mice ventral prostate

Next, we investigated the TAM profile in the TRAMP mice prostate after DHA-d intake. Both M1- and M2-like TAMs, were observed preferentially in the prostate stroma (Fig. 4A-P), although M2-like TAMs were also frequently observed in subepithelial and intraepithelial areas. The DHA-d intake downregulated M2-like TAMs in TRAMP mice ventral prostate while no effect on M1-like TAMs infiltration was observed.

3.5 DHA impairs TNF-α signaling

To assess the possible effects of DHA-d intake on TNF-α signaling we first measured its systemic levels. Regardless of the time, DHA intake reduced the systemic levels of TNF-α (Fig. 5A), however, TNF R1 protein levels in the ventral prostate were only affected in animals of the D18 group, which were downregulated by 2.4-fold (Fig. 5B).

4 Discussion

The impact of inflammation, particularly the chronic one, on PCa development risk and progression, has been widely investigated over the past years in several pre-clinical models.[3,25] The capacity of dietary compounds to modulate tumor inflammatory microenvironment has been previously reported to slow PCa progression [33], highlighting that targeting inflammatory processes might be a useful strategy in PCa therapy. Interestingly, DHA has a broad-spectrum action, including anti-infammatory and pro-resolution, effects that have been associated with decreasing PCa burden and aggressiveness.[27,34] Thus, dietary supplementation with DHA might be a beneficial adjuvant in PCa management. Here, we used TRAMP mice as a pre-clinical PCa model to assess the effect of DHA-d on the progression of disease and inflammatory response. The present data indicate that DHA dietary supplementation delays PCa progression in the TRAMP model and decreases its aggressiveness. These effects were linked to DHA immunomodulatory properties by deregulating TNF-α signaling, decreasing the

$CD4^+/CD8^+$ ratio, and M2-like TAMs infiltration and downregulating pyroptosis in the TRAMP prostate.

Similar to human PCa, the prostate of TRAMP mice exhibits constitutive activation of Akt and NF-κB that leads to higher cell proliferation and apoptosis evasion [31]. The present results show the antiproliferative and pro-apoptotic effects of dietary DHA, corroborating previous *in vitro* and *in vivo* studies. [27,35] Consistent with our findings, the anti-tumor effects of DHA and other n-3 PUFAs in PCa are usually linked to their capacity to downregulate PI3K/Akt pathway[36] resulting in lower proliferating rates[27,34] or cell cycle arrest.[35] Akinsete and colleagues (2012) have also reported that dietary supplementation with fish oil increases apoptosis in C3(1)Tag mice prostate through downregulation of the anti-apoptotic protein Bcl-2 and suppression of NFκB signaling.[34] Our results show that dietary DHA leads to ERK1/2 phosphorylation and apoptosis in the TRAMP prostate. Although activation of the ERK1/2 pathway in many cancers usually promotes cell proliferation, several evidence reveal that different stimuli including ROS result in ERK 1/2 activation and apoptosis [35]. Increased oxidative damage, a well-established DHA action mechanism[37], can lead to ERK1/2 phosphorylation, triggering apoptosis in mouse fibroblast L929 cells[38]. We had previously observed that DHA increases superoxide production in PNT1A cells triggering ERK 1/2 activation and apoptosis[35]. The activation of the apoptotic pathway by DHA has also been reported in PCa cells through upregulation of reactive oxygen species (EROs)-mediated autophagy and downregulation of the Akt/mTOR pathway. [37] Therefore, our data reinforces that one of the anti-tumor effects of DHA is based on targeting proliferative and survival pathways. The suppression of cell proliferation and increasing apoptosis by direct modulation of EKR 1/2 and Akt pathways in TRAMP mice highlights the therapeutic benefits of this fatty acid for PCa management.

Unfortunately, in advanced stages of cancer progression, tumor cells frequently exhibit mechanisms of apoptosis evasion, therefore other PCDs have been a target for cancer management such as pyroptosis and necroptosis [20,21]. Here we observed different actions of DHA dietary on these two types of immunogenic cell deaths, inhibiting the former in both intake periods and favoring the latter in the longer period. Pyroptosis is a programmed necrotic-like cell death characterized by proteolytic cleavage of members of the gasdermin family, such as GSDMD, resulting in cell membrane pore formation.[20] One of the hallmarks of pyroptosis is IL-1β release, a pro-inflammatory cytokine strongly related to PCa onset and progression[39], therefore constant induction of pyroptosis might

unleash tumorigenesis. However, the actual role of pyroptosis in PCa is yet unclear. Recently, Wu and colleagues reported that all members of GSDM are highly expressed in PCa samples except GSDMDE whose expression is reduced in PCa, suggesting different roles of pyroptosis effectors in PCa [40]. The authors demonstrated that inducing pyroptosis through GSDME significantly reduces TRAMP-C2 tumor growth. On the other hand, the NLR3P inflammasome has been reported to promote PCa progression via the caspase-1 pathway.[41] Also, the NLRP12 inflammasome is up-regulated in PCa samples, and their downstream targets IL-1β and IL-18 are up-regulated in the PC3 and DU145 cells line, suggesting that the canonical pathway of pyroptosis might contribute to tumor onset and progression.[39] Interestingly, DHA was shown to inhibit inflammasome assembly in macrophages through NK-κB inactivation and induction of autophagy[42], which could explain the downregulation herein observed in cGSDMD levels. However, for the MDA-MB-231 triple-negative breast cancer cells, DHA triggers pyroptosis through the upregulation of GSDMD and reduces cell viability.[43] To the best of our knowledge this is the first report of cGSDMD in PCa pre-clinal model and the effects of DHA in modulating pyroptosis in this disease. Thus, it seems that the effect of DHA on pyroptosis might differ according to tumor type and could be time- and dose-dependent. Also, it seems that pyroptosis might have a dual role in PCa progression according to its effector and disease stage. Comparing our results in the face of the mechanism known to induce pyroptosis through GSDMD cleavage we suggest that DHA might reduce this cell death by its negative effect on inflammasome assembly and NK-κB signaling.

Necroptosis is another necrotic-like cell death modulated by cell receptors such as the TNF receptor that upon ligand binding induces necrosome assembly leading to cell death and exposure to damage-associated molecular patterns (DAMPs). [21] Here, for the first time we report the pattern of necroptosis in TRAMP mice tumor progression. Our data indicated that necroptosis is reduced in advanced stages of the disease (i.e., C18 animals). Consistent with our findings, RIP3, a key kinase involved in MLKL phosphorylation is downregulated in PCa samples and castration-resistant prostate cell lines, and the overexpression of RIP3 suppresses cell proliferation and impairs epithelial-mesenchymal transition (EMT)[44]. Necroptosis has been linked to inducing an anti-tumor immunity response through cross-priming and expansion of $CD8^+$ T cells[45] and overcoming apoptosis resistance to docetaxel in PCa cells.[46] Additionally, a positive correlation between $CD8^+$ T-cell infiltration and necroptosis-related genes was observed in hepatocellular carcinoma, highlighting that the immunogenic characteristics of

necroptosis are important for $CD8^+$ cell recruitment into the TME. [47] Nevertheless, DHA intake reduces tumor burden by increasing necroptosis in mice xenograft triple-negative breast cancer model, through downregulation of TNF-R1 expression.[48] Therefore, induction of necroptosis might be an effective mechanism to overcome apoptosis resistance and trigger a cytotoxic immune system response against tumor cells.

Among the components of the TME, T-cell infiltration has been reported to have a prognostic value for PCa.[12] PCa microenvironment is composed of a high infiltration of $CD4^+$ T-cells and a low density of $CD8^+$ T-cells.[49] In the TEM, some immunosuppressor factors such as PGE2 and TFG-β induce $CD4^+$ T-cells to acquire a regulatory T-cell (Treg) phenotype[6,50] that is strongly related to PCa therapy resistance and suppression of $CD8^+$ T-cells function. On the other side, $CD8^+$ T-cells suppress tumor growth by induction of cell death via death ligands or cytotoxic granzyme B action.[20,21] We previously demonstrated that DHA reduces $CD3^+$ lymphocytes in TRAMP ventral prostate [27]. Here it was demonstrated that DHA ingestion modulates T-cell infiltrates in the prostate of TRAMP into an anti-tumoral pattern, reducing $CD4^+$ T-cells regardless of the time of intake and increasing the $CD8^+$ T ones in advanced stages of disease and longer intake time. As previously reported, DHA reduces the migratory capacity of $CD4^+$ and Treg lymphocytes ($CD4^+/CD25^+$) migration through downregulation of CCR-4 and CXCR-4 mRNA levels and impairment of cytoskeleton function. [51,52] Additionally, DHA can increase $CD8^+$ T-cell recruitment into the TME and enhance their cytotoxic functions by reversing PD-L1 immune suppression. [53] The high $CD8^+$ T cell infiltration was reported to improve survival in patients undergoing radical prostatectomy[11] and to inhibit PCa progression in murine models by enhancing anti-tumor immunity.[54] Therefore, the reduction in $CD4^+/CD8^+$ T-cell ratio might elicit a stronger anti-tumoral immune system response through the prevalence of $CD8^+$ T-cell response, leading to increased levels of apoptosis and necroptosis as herein reported.

TAMs are also important players in PCa onset and progression, in particular, the M2-like TAM that is highly abundant in TME.[17] Both M1 and M2 macrophages have a high degree of plasticity and can be converted into each other upon tumor microenvironment changes or therapeutic interventions. [15,55] Our results demonstrated the capacity of dietary DHA to modulate TAM cell populations by blocking the M2-like profile, which is related to the mitigation of PCa progression in this model. The PCa cells secrete several factors such as CCN3 that reprogramme M1-like TAM to an M2-like profile, leading to increased proliferation of tumor cells, metastatic capacity, and

angiogenesis.[55,56] Also, high M1-like TAM density has been reported for organ-confined tumors while high M2-like TAM infiltration is associated with extracapsular extension, i.e., aggressive prostate tumors, suggesting a protective role of M1-like TAM in PCa.[57] Although M1-like TAM was not affected by DHA intake, a previous report indicated that DHA could indirectly increase M1-like cytotoxic effects by downregulating the CD47 "don't eat me signal" in tumor cells. [58] Comparative approaches between n-3 and n-6 PUFA diets have also demonstrated that n-3 PUFAs can impair PCa mice model growth by decreasing infiltration and gene-related expression of M2-like TAM[59,60]. In addition, DHA can act through GPR120 in M2-like TAM reducing its pro-tumor effects by downregulating angiogenesis, and inhibiting T-cell suppression by decreasing PD-L1 expression, an immunosuppressive factor involved in T-cell exhaustion.[61] Therefore, the reduction in M2-like TAM infiltrate promoted by DHA could relieve the immunosuppressive TME promoted by these cells, leading to the anti-tumor effects reported herein.

TNF-α is a Th1 cytokine that positively correlates with Gleason grade[62] and induces PCa progression through up-regulation of NFκB and PD-L1.[63] TNF signaling is mediated by its receptor TNF R1 and can result in both pro-inflammatory or pro-apoptotic/necroptotic response.[64] Interestingly, the pro-inflammatory effects of TNF R1 rely on its translocation to lipids raft upon ligand binding that results in NFκB activity, however, impairing raft stability by cholesterol depletion enhances the pro-apoptotic effects of TNF R1.[64] Due to its highly unsaturated nature, DHA incorporation into cell membrane disrupts lipids rafts by down-regulating cholesterol and sphingomyelin content.[65,66] Thus, the possible remodeling of the cell membrane and the downregulation of the TNF-α/TNF R1 pathway by DHA intake could elicit a switch from pro-inflammatory to pro-apoptotic/necroptotic signaling as observed herein.

Our data confirms that DHA intake effectively impairs PCa progression in the TRAMP model. This protective effect, as reflected by the histopathological gland profile, was related to the immunomodulation of tumor microenvironment into low inflammatory features, due to impairing TNF signaling and decreasing $CD4^+$ T-cell and M2-like TAM in the gland, and increased $CD8^+$ T-cell infiltrate at later disease stage. Additionally, DHA differentially modulated proliferative and survival pathways and PCD in TRAMP prostate, downregulating pyroptosis and promoting apoptosis and necroptosis. The present pre-clinical data point to DHA as a promising adjuvant in PCa treatment due to

its immunomodulatory actions and support further clinical studies to clarify its potential benefits in PCa management and immunotherapy.

Conflict of Interest: The authors declare no conflict of interest.

Author Contributions: GMA and RMG were involved with project conception and design. GMA, LPM, and ADTS were involved with development of methodologies. GMA, LPM, ADTS, VHAC and RMG were involved with analyses and interpretation of data. GMA, VHAC and RMG were involved with writing and review of the manuscript. RMG was involved with study supervision.

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5. Figures

Figure 1. DHA-d reduces PCa progression and aggressiveness in TRAMP mice. (A) Normal epithelium frequency. **(B)** Low-grande PIN **(C)** High-grade PIN **(D)** Carcinoma *in situ* **(E)** Well-differentiated adenocarcinoma **(F)** Undifferentiated adenocarcinoma. Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, $n = 5$.

Figure 2. Proliferative and survival pathways and programmed cell death are differentially modulated by DHA-d. (A-L) Representatives photomicrographies of pHH3 and cGSDMD immunohistochemistry, and TUNEL assay. **(M-O)** Quantification of pHH3-, TUNEL-, and cGSDMD-positive cells. **(P)** Relative protein level of cGSDMD, pMLKL, pAKT and pERK-1/2. Arrows indicate positive cells. Scale bars: 20µm. Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, immunohistochemistry and TUNEL n = 5, Western Blotting n = 3-4/group.

Figure 3. DHA alters $CD4^+/CD8^+$ T-cells ratio in TRAMP ventral prostate. (A-D) Immunohistochemistry for $CD4^+$ T-cells. **(E-H)** Immunohistochemistry for $CD8^+$ T-cells **(J)** Quantification of $CD4^+$ T-cells. **(K)** Quantification of $CD8^+$ T-cells (**L**) $CD4^+/CD8^+$

ratio. Arrows indicate positive cells. Scale bars: 20µm Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, n = 5/group.

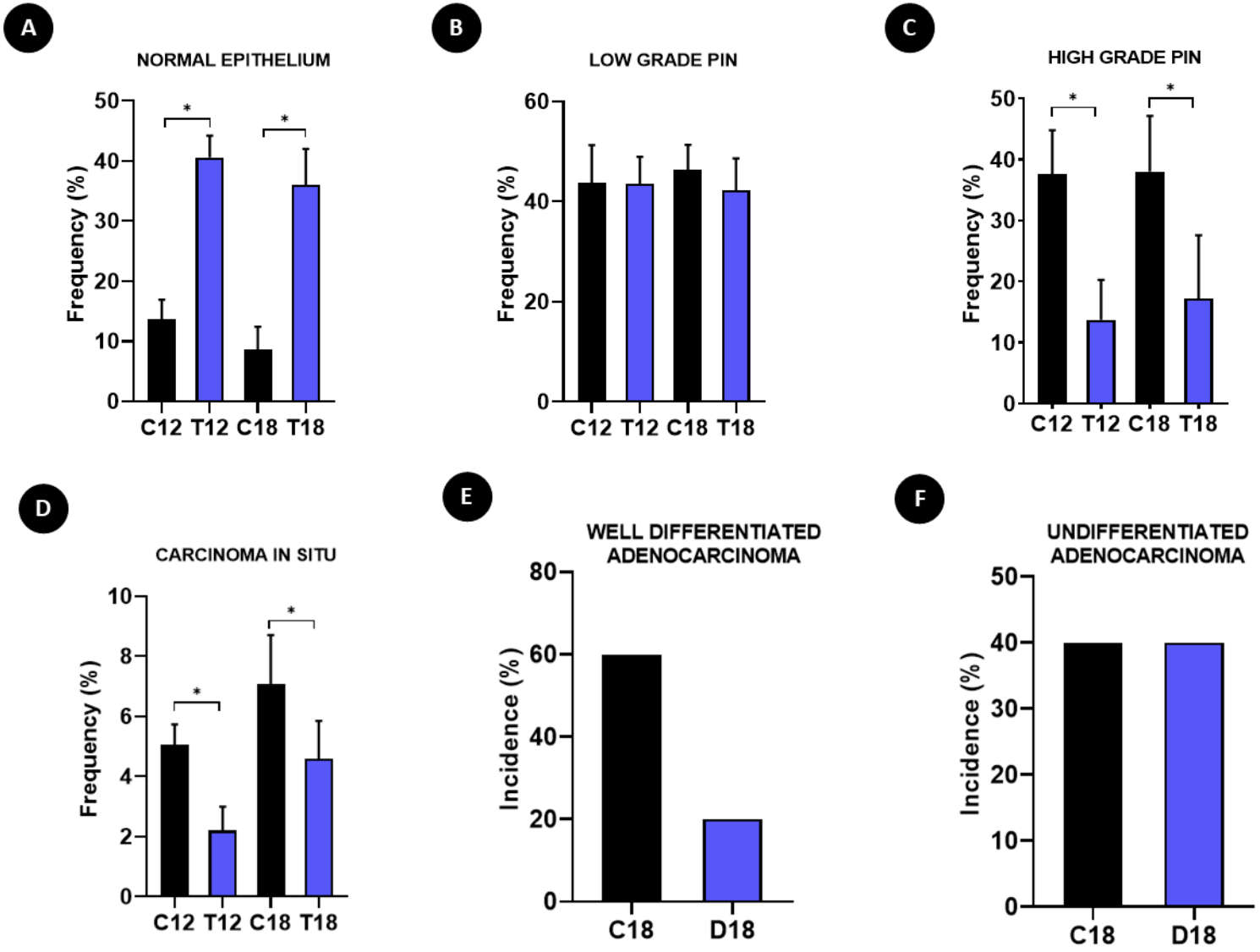
Figure 4. DHA reduces M2-like TAM infiltrate in TRAMP ventral prostate. (A-L) Double immunofluorescence for M1-like TAM detection ($CD68^+/iNOS^+$) **(M-P)** Immunohistochemistry for M2-like TAM detection ($CD163^+$) **(Q)** Quantification of M1-like TAM. **(R)** Quantification of M2-like TAM. Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, n = 5/group.

Figure 5. DHA impairs TNF-α signaling. (A-D) (A) Sistemic levels of TNF-α **(B)** Relative protein level of TNF R1**.** Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, ELISA n = 8-10/group, Western Blotting n = 3-4/group.

SUPPLEMENTARY INFORMATION

Supplementary Figure 1. Lesion grading in TRAMP mice ventral prostate. (A) Normal epithelium. **(B)** LGPIN is characterized by epithelial cell stratification. **(C)** HGPIN is marked by cell proliferation foci with papillary and cribriform pattern growth toward the glandular lumen. **(D)** CIS, an invasion of epithelial cells into the surrounding stroma, arrowheads indicated basal membrane rupture site. **(E)** WDAC, the epithelial growth leads to the formation of micro acini-like structures. The nearby stroma frequently shows a reactive phenotype marked by the replacement of smooth muscle cells phenotype by reactive fibroblasts. **(F)** UDAC, loss of glandular architecture, and cells displaying marked nuclear atypia. Ep: epithelium, L: lumen, St: stroma. Scale bars: A,B,D,F - 10µm, C,E - 20µm.

Supplementary Figure 2. DHA-d intake reduced ventral prostate weight in TRAMP mice. (A) Mean weekly food intake. **(B)** Mean weekly energy intake. **(C)** Relative weight gain. **(D)** Ventral prostate weight. **(E)** Epidimal (eWAT), visceral (vWAT), and retroperitoneal (rWAT) white adipose tissue weight. **(F)** Adiposity index. Statistic: *two-way* ANOVA followed by Tukey post-test, * $p < 0.05$, n = 8-10/group.



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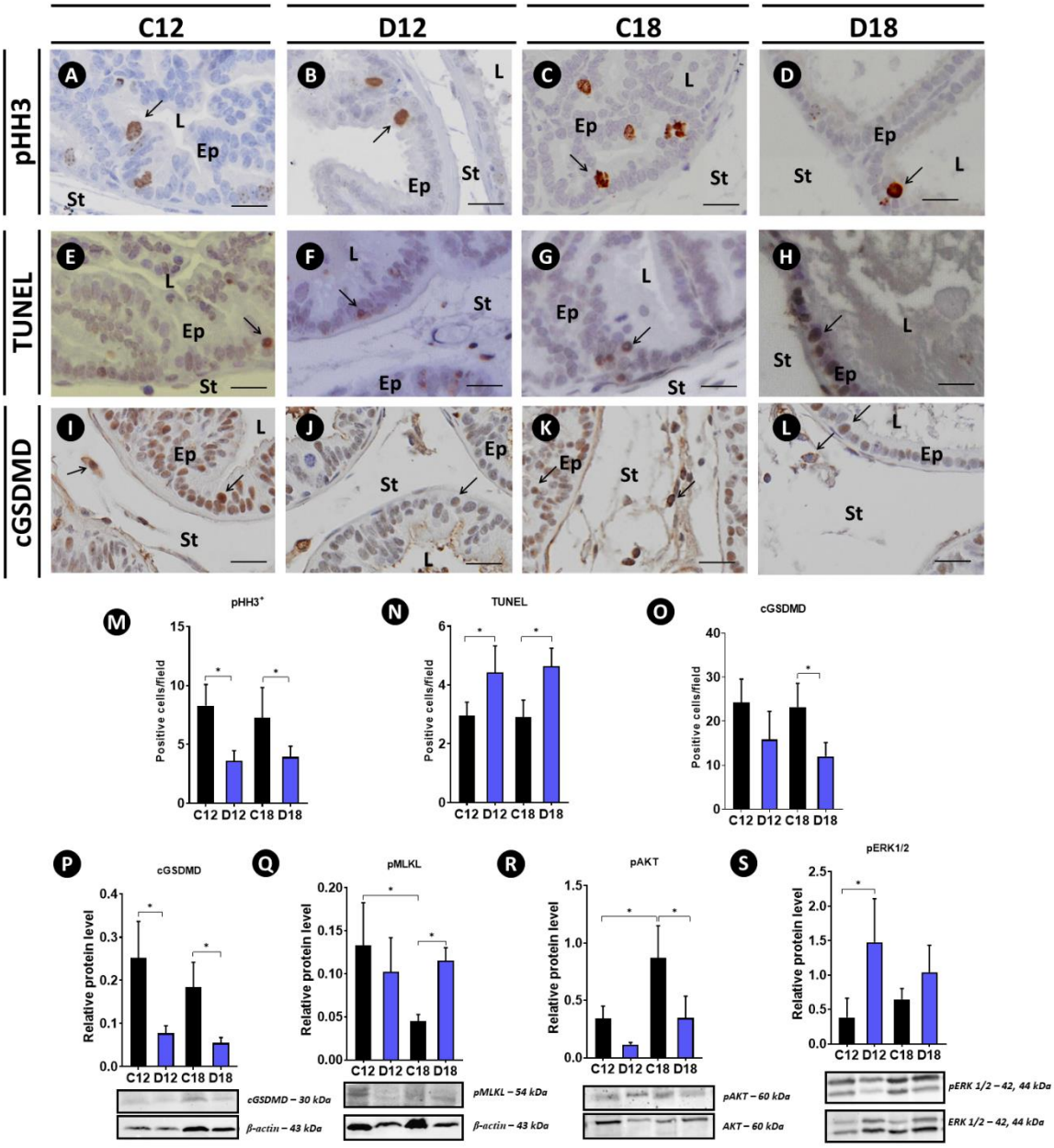

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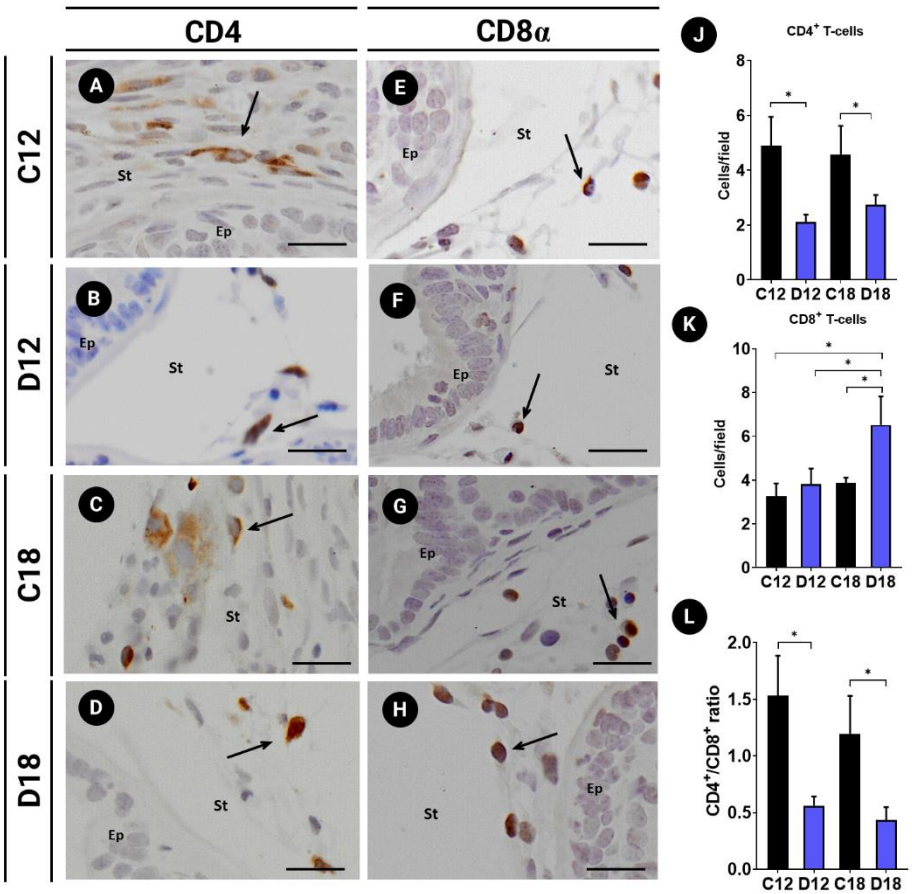

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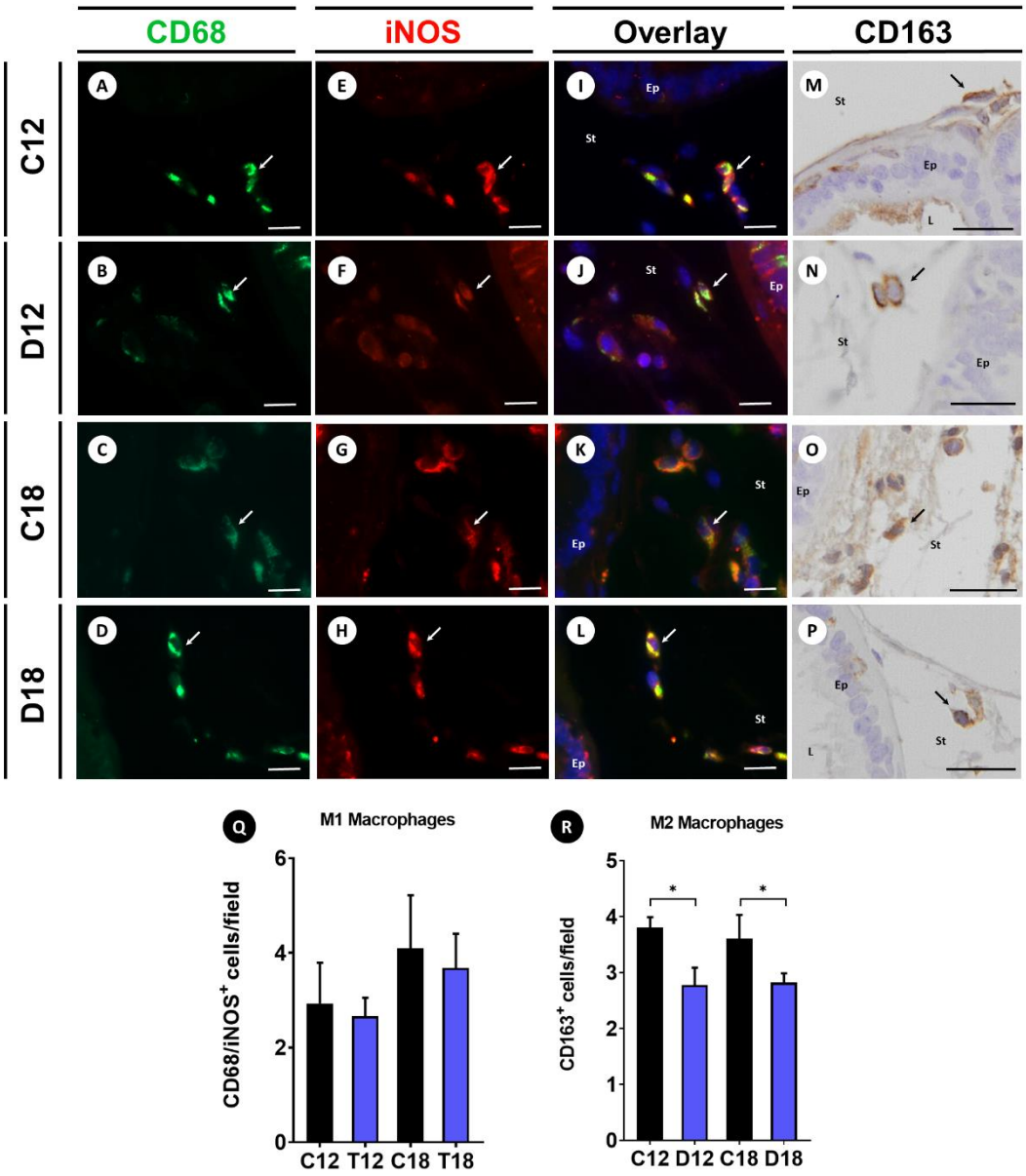

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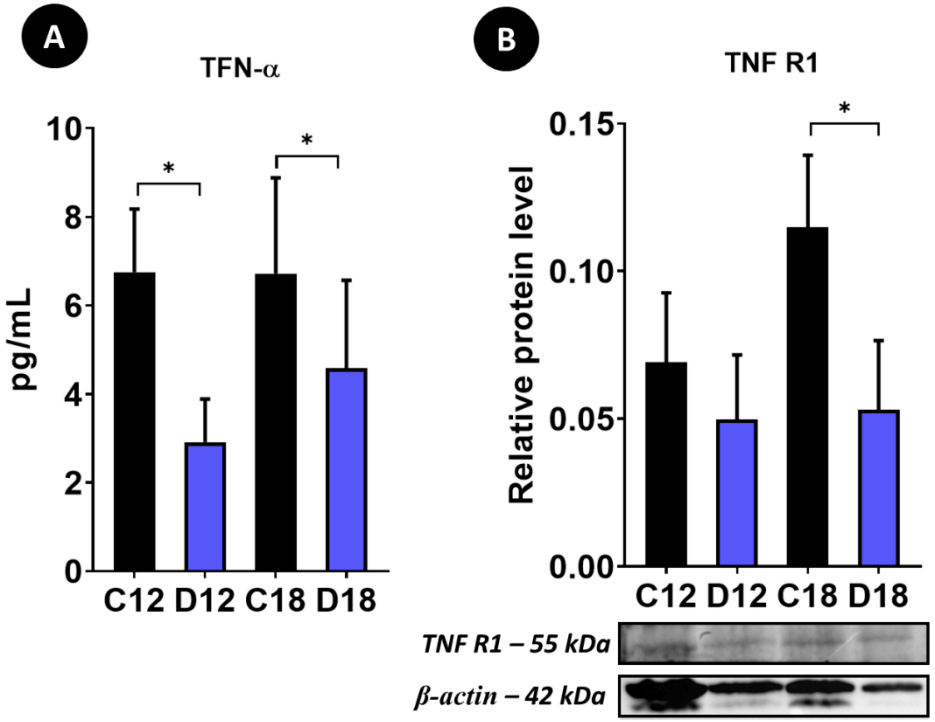

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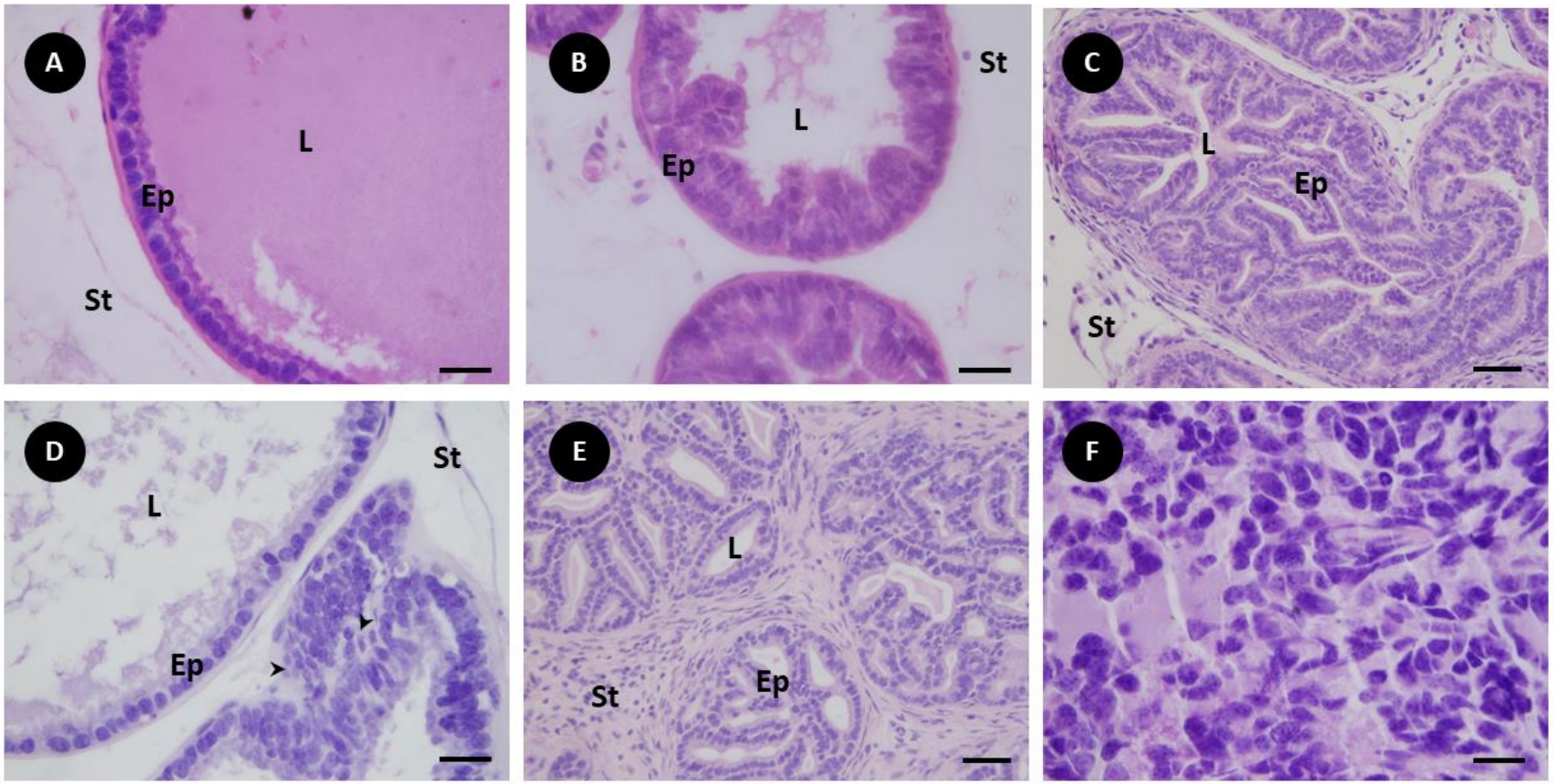

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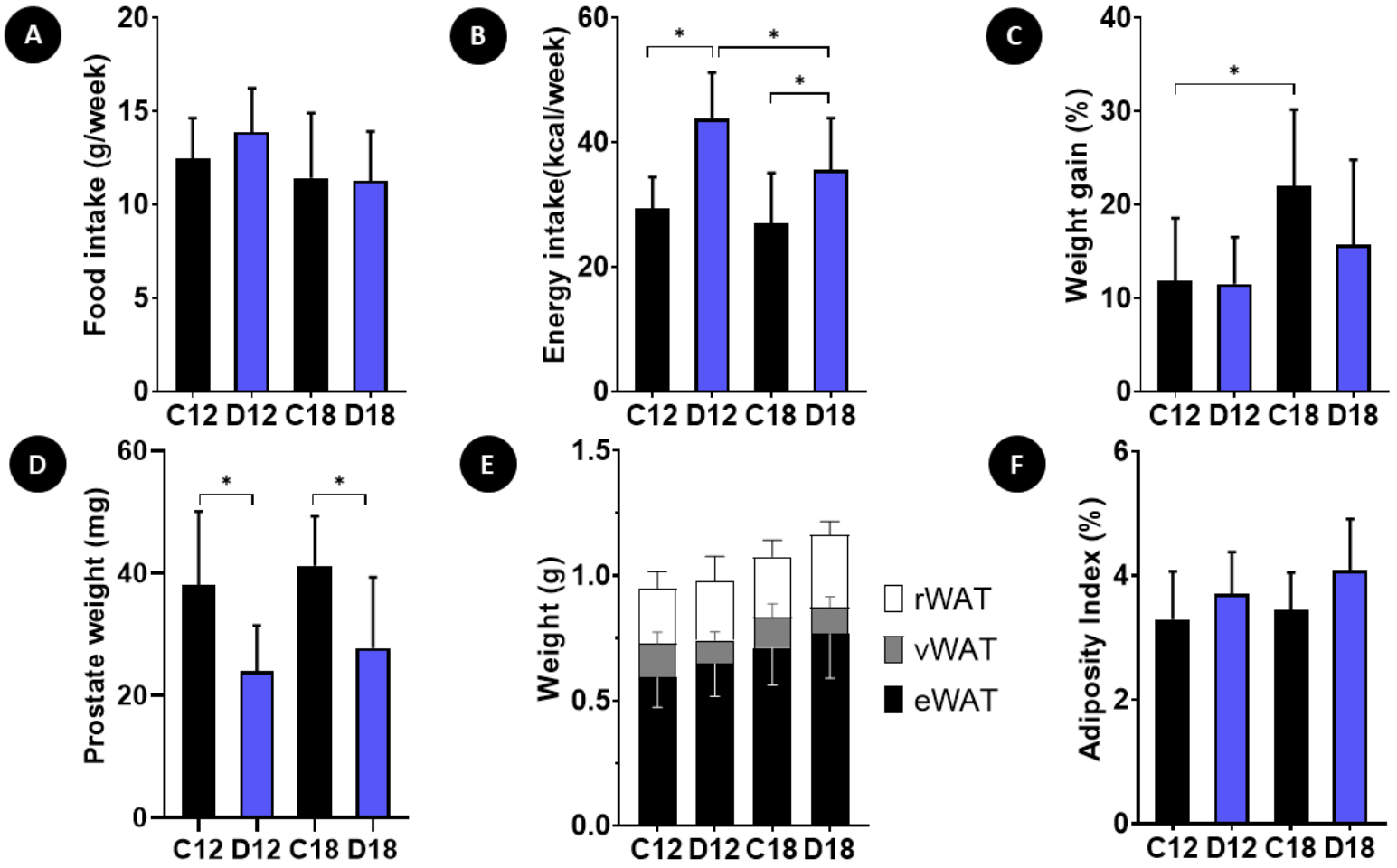

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Docosahexaenoic acid improves prostate cancer microenvironment by modulation of Annexin A1 expression

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Abstract

The omega-3 docosahexaenoic acid (DHA) has antitumor action through different mechanisms, including the modulation of inflammatory response. However, the consequences of dietary DHA on the prostate tumor microenvironment (TME) are unknown. Annexin A1 (ANXA1) has been proposed as a target for prostate cancer therapy however this remains controversial. Here it was investigated how DHA enriched diet (d-DHA) modulates TME in transgenic adenocarcinoma of the mouse prostate (TRAMP). DHA-d was provided for 4 (early-stage PCa) or 10 weeks (late-stage PCa), and the ventral prostate and blood samples were subjected to morphological, immunohistochemistry, molecular, and biochemical analyses. The DHA-d intake increased epithelial ANXA1 in both periods, however, ANXA1 expression in cancer-associated fibroblasts was decreased in the late-stage disease. In early-stage disease, DHA-d intake downregulated TGFB-RII expression and in late-stage disease decreased αSMA expression and epithelial-mesenchymal transition-related factors. In addition, DHA-d reduced the systemic levels of PGE2 and the protein level of PGTGS2 in late-stage disease. Thus, our data on this pre-clinical model reveal that anti-tumor effects of DHA are due to the modulation of TME, reducing anxA1 expression by CAFs, impairment of reactive stroma progression, and epithelium-to-mesenchymal transition, highlighting the benefits of DHA intake for prostate cancer management.

Introduction

Prostate cancer (PCa) is the second most diagnosed cancer in men worldwide, representing the fifth cause of death by cancer-related cases [1]. Although PCa etiology is not fully understood it is known that lifestyle and inflammatory processes can impact

directly the onset and progression of this disease [2]. Dietary habits, particularly regarding lipid intake, have emerged as a relevant topic for the management of PCa risk, and in this scenario, high lipid intake may favor inflammatory response and trigger disease onset and progression [3–5].

The polyunsaturated omega-3 fatty acid (n-3 PUFA) docosahexaenoic acid (DHA, C22:6) is commonly found in marine fishes, and their byproducts such as fish oil, which consumption has been linked to several improvements in metabolic disturbances [3, 6]. Interestingly, a great amount of evidence attributes to DHA multiple anti-tumor effects. The treatment of androgen-responsive and -nonresponsive cell lines with DHA impairs cell proliferation and survival through down-regulating of androgen signaling, inhibition of NF-κB nuclear translocation, and cell cycle arrest [7–9]. We have also attested some of the anti-tumor effects in the transgenic adenocarcinoma of the mouse prostate (TRAMP), where the intake of a DHA-enriched diet delayed the disease progression by modulating some inflammatory and stromal features [3]. Thus, the overall reports indicate that DHA can act in multiple targets of the tumor microenvironment (TME) suggesting that dietary supplementation with DHA might be a useful tool for PCa management.

Annexin A1 (ANXA1) is a Ca^{2+}-dependent phospholipid-binding protein with anti-inflammatory and pro-resolution properties [10]. There is some evidence that ANXA1 may play a role in tumorigenesis, however, its expression is highly variable between different types of cancer [11]. For PCa, a tumor suppressor role has been proposed for ANXA1, since the loss of this protein is observed in both localized and recurrent disease [12, 13]. Accordingly, the increase of ANXA1 in LNCaP cells induces apoptosis by activating p38 and JNK signaling [14]. However, although this tumor suppressor effect might be attributed to ANXA1, several cells of TME overexpress and secret ANXA1, which have been implicated in the tumor progression [15, 16]. Therefore, until now is difficult to properly attribute a causal effect of ANXA1 in prostate tumorigenesis due to its heterogeneous expression and function in the TME.

During cancer progression, the imbalance of inflammatory signaling and proliferative activity alters the crosstalk between the epithelial and stromal compartments and leads to the establishment of the so-called reactive stroma. This event is characterized by increased cell proliferation, extracellular matrix turnover, high growth factors biodisponibility, and high inflammatory influx [17, 18]. In PCa, the evolution of the reactive stroma is marked by an increase of cancer-associated fibroblasts (CAFs), which increases the inflammatory disturbance, cancer cell proliferation, and invasion,

particularly by up-regulating epithelial-to-mesenchymal transition (EMT) via TGF-β signaling [17, 19]. In the EMT process, tumor cells lose epithelial markers and express mesenchymal markers, leading to increased motility and promoting local invasion and metastasis, which is often a result of the development of a chemoresistance mechanism [20, 21].

Based on the above, we used TRAMP mice to assess the effects of DHA intake on TME. TRAMP mice display a preserved TME that resembles human PCa with an establishment of a reactive stroma marked by increased CAF and high expression of inflammatory molecules such as PTGS2 and NF-κB that support tumor progression by increasing cell proliferation and evading apoptosis [22–24]. Here, we demonstrated that the anti-tumor effects of DHA are related to differential modulation of ANXA1 expression in epithelial and stromal compartments, impairment of CAF onset and EMT process as well as downregulation of PGE2/PTGS2 signaling.

Materials and Methods

Experimental design

Forty-eight transgenic mice (C57BL/6-Tg [TRAMP] 8247 Ng/J X FVB/Unib) were provided by the Multidisciplinary Center for Biological Investigation of the State University of Campinas (CEMIB/UNICAMP) at 6 weeks of age. Animals were randomly distributed into four experimental groups according to their diet and age. Control groups (C) were fed an AIN-93G standard diet while DHA-fed groups (D) received an AIN-93G modified diet enriched with 10% of fish oil (DHA-d) as previously reported [3]. Diet was offered from 8 to 12 (C12 and D12) or 8 to 18 weeks of age (C18 and D18), and at the end of the experiment, animals were euthanized with xylazine hydrochloride (5 mg/kg, intramuscular) and ketamine hydrochloride (60 mg/kg, intramuscular). The ventral prostate was harvested for morphological and molecular analyses. Animal care and experiments were conducted according to Brazilian guidelines for the care and use of animals for scientific and educational purposes (BDCA) - CONCEA, and were approved by the Ethics Committee on Animal Use (CEUA) of UNICAMP (Protocol: 5884-1/2021).

Immunohistochemistry and immunofluorescence

Prostate sections were subject to immunohistochemistry using the antibodies anti-annexin A1(ANXA1, 71-3400, rabbit polyclonal, Invitrogen) and anti-SNAIL (#3879, rabbit monoclonal, Cell Signaling). Epitope retrieval was conducted in citrate buffer

(10mM, pH 6.0) for 60min at 98ºC following peroxidase blockade in 0.3 H_2O_2 solution for 15min. The nonspecific binding blockade was achieved by incubating sections in BSA 3% for 60min at room temperature. Primary antibodies were diluted at 1:75 in BSA 1% and incubated overnight at 4ºC. Then, samples were incubated with mouse anti-rabbit HRP-conjugated (1:150, sc-2357, Santa Cruz Biotechnology) secondary antibody for 2h30min at 37ºC or with an AP-based polymer system (#18653, Cell Signaling). Peroxidase activity was detected using diaminobenzidine (Roche, Merck) as the chromogen, while alkaline phosphatase activity was detected using the chromogen Signal Stain® Vibrant Red (#76713, Cell Signaling). Harris hematoxylin was used for counter-staining.

Histological sections were scanned with a BX61VS camera (Olympus Corporation) coupled to an Olympus VS120® Virtual Microscopy Slide Scanning System (VS120-S5). Then, quantitative analyses were performed in 10 random fields at 400x magnification per animal (n = 5/group), and the immunoreactivity was determined using Image J software (https://imagej.nih.gov/ij/; NIH).

The immunofluorescence assay was carried out using the primary antibodies anti-ANXA1 (71-3400, rabbit polyclonal, Invitrogen), anti-FAP (sc-71094, mouse monoclonal, Santa Cruz Biotechnology), and anti-αSMA (sc-32251, mouse monoclonal, Santa Cruz Biotechnology). Epitope retrieval and the nonspecific binding blockade were realized as described for immunohistochemistry. Primary antibodies were diluted at 1:75-100 in BSA 1% and incubated overnight at 4ºC. Then, samples were incubated with anti-mouse IgG #FITC (1:150, F0382, Sigma Aldrich) and mouse anti-rabbit IgG-TR (1:150, sc-3917, Santa Cruz Biotechnology) for 2h30min at 37ºC. Next, samples were mounted in a DAPI medium (Fluoroshield™ with DAPI, F6057, Sigma Aldrich).

For the immunofluorescence analyses, 10 random fields were captured at 400x magnification per animal (n= 5/group) on the AX10 Fluorescence Microscope (Zeiss) coupled with AxioVision (Zeiss) software. The number of positive cells co-localizing AnxA1 and FAP in each field was counted and the result was expressed in mean cells/field. For αSMA, quantitative analyses were performed using Image J software, and data was expressed in relative frequency.

Western Blotting

Prostate samples were powdered in liquid nitrogen and resuspended in RIPA extraction buffer with protease (P8340) and fosfatase (P5726, Sigma-Aldrich) inhibitor

cocktail. Total protein content was determined with Bradford reagent (Bio-Rad Laboratories) and measured in an Epoch microplate reader (BioTek Instruments Inc.) at 595 nm. A total of 50µg of protein was subject to electrophoresis in SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Life Science). The nonspecific binding blockade was achieved by incubating membranes in BSA 3% for 60min or non-fat milk 5% for 30min at room temperature, then the membranes were incubated with their respective primary antibody overnight at 4ºC in a dilution range of 1:350-1.200. The following antibodies were used: anti-TGFβ-RII (#37135, rabbit monoclonal, Cell Signaling) anti-E-cadherin (CDH1, 3195, rabbit monoclonal, Cell Signaling), anti-N-cadherin (CDH2,13116, rabbit monoclonal, Cell Signaling), anti-calponin (CALP, sc28545, rabbit polyclonal, Santa Cruz Biotechnology), anti-prostaglandin E2 synthase (PTGS2, #12282, rabbit monoclonal, Cell Signaling), and anti-β-actin (sc47778, mouse monoclonal, Santa Cruz Biotechnology). Next, the membranes were incubated with mouse anti-rabbit HRP-conjugated (sc-2357, Santa Cruz Biotechnology) or anti-mouse IgGκ HRP-conjugated (sc-516, Santa Cruz Biotechnology) antibodies in a dilution range of 1:3500-5000 for 2h at 4ºC. ECL system was applied for band development and the images were acquired in a G: BOX Chemi XRQ photodocumentator (Syngene Synoptics). Relative densitometry was assessed by Image J software and β-actin was used as endogenous control.

ELISA

After euthanasia, blood samples were collected through cardiac puncture from the left ventricle and centrifuged at 1620*g* for 20 min, 4°C; the serum obtained was used to determine the levels of PGE2 using a specific commercial kit according to the manufacturer's instructions (Cayman). Sample absorbance was read in an Epoch microplate reader (BioTek Instruments Inc).

Statical analyses

Statistical analyses were performed using GraphPad Prism software (version 8.0.1). The data were compared by *two-way* ANOVA followed by Tukey's test. Data regarding ANXA1 expression on tumor progression was compared by *one-way* ANOVA or Kruskal-Wallis followed by Tukey or Dunn's test, respectively. Data was expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

Results

AnxA1 is differentially modulated by DHA-d in epithelial and stromal compartments

To assess the effect of DHA-d on anxA1 expression during tumor progression, it was first examined its expression pattern along progressive pathological lesions in the TRAMP ventral prostate. Densitometric analysis of ANXA1 immunolabelling in acinar epithelium revealed a similar level of tissue expression in NE, LGPIN, and MGPIN, around 40% expression of total epithelial area, with an up-regulation of 14% in HGPIN, and down-regulation in aggressive lesions such as WDAC (17%) and UDAC (35%) (Fig 1A, B). Regardless of disease stage, DHA-d led to an up-regulation of ANXA1 in NE, LGPIN, MGPIN, WDAC, and UADC (Fig 1A, C). However, DHA-d intake did not affected ANXA1 in HGPIN in early-stage disease and down-regulated it in late-stage (Fig 1A, B). Following it was evaluated the expression of ANXA1 by CAFs using double immunofluorescence for such protein and FAP, a specific marker for CAF. ANXA1 was colocalized with all FAP-positive cells, and $ANXA1^{+}/FAP^{+}$ cells increased in the prostate during tumor progression, as noticed when compared C12 with the C18 group, while DHA-d avoided this increase (Fig 2A, B).

DHA-d intake improves stromal features in TRAMP mice and impairs EMT

Next, it was investigated the effects of DHA-d intake on some aspects of reactive stroma and the EMT process. The immunoreactivity of αSMA increased by 40% in the prostate during tumor progression from 12 to 18 wk of age and CALP levels were unchanged (Fig. 3A-E). DHA-d intake preserved the proportion of αSMA-positive cells of D18 animals similar to early-stage disease (Fig. 3A, D) Interestingly, animals from the C18 group exhibited lower levels of TGFβ-RII in comparison to C12 suggesting that the expression of this receptor might be suppressed as the tumor progresses in the TRAMP model (Fig 3D, E). DHA-d reduced TGFβ-RII expression in early-stage disease (Fig 3D, E). Additionally, DHA-d impaired EMT in late-stage disease by down-regulating 40% of CDH2 protein levels and reducing 43% of SNAIL immunoreactivity (Fig 4A-E).

DHA-d down-regulates PTGS2/PGE2 pathway in TRAMP mice

Finally, to assess the effect of DHA intake on the PTGS2/PGE2 pathway it was measured the systemic levels of PGE2. The dietary supplementation with DHA reduced

approximately 57% of PGE2 levels for both periods (Fig 5A) and 51% of the prostatic content of PTGS2 at late-stage disease (Fig 5B).

Discussion

The concern on how diet, and particularly lipid intake, may impact PCa onset and progression is a growing topic of attention. In this scenario, due to its anti-tumoral effects, DHA has been constantly investigated although most of the reports consist of *in vitro* approaches. We had previously constated the anti-tumor effects of DHA in TRAMP mice by modulation of cell proliferation, T-cell infiltration, and nuclear receptor expression [3]. Here, we sought to investigate how DHA intake would affect some aspects of TME, focusing on inflammatory molecules and stromal features. Our data states the anti-tumor effects of DHA by up-regulating ANXA1 expression, maintenance of stromal features, and impairment of EMT and PGE2/PTGS2 signaling.

ANXA1 expression has been explored for several types of cancer, however, it is still hard to properly attribute a role of ANXA1 carcinogenesis since its expression may vary according to tumor type and in the components of TME [11, 25]. For the prostate, loss of ANXA1 is reported in HGPIN and localized PCa [12, 25, 26], which might implicated in a possible protective role of ANXA1 in prostate tumorigenesis. Interestingly, the knockout of anxA1 in benign prostate cells increases IL-6 secretion and overactivates the STAT-3 pathway which promotes the gain of a malignant phenotype [10]. However, metastatic PCa and metastatic PCa cell lines overexpress ANXA1, especially under androgen ablation therapy and hypoxia [27–31]. Contrary to these reports, ANXA1-dependent inhibition of cell proliferation and induction of apoptosis have been reported for the prostate DU145 cell line by sodium butyrate treatment [32]. Thus, the causal relationship of ANXA1 in PCa is still unclear, however, it seems that loss of ANXA1 in early-stage disease might unleash tumorigenesis, however, in the aggressive disease stage, the restatement of ANXA1 expression might be a key factor for therapy resistance and increased metastatic capacity. Here we examined the variation of ANXA1 expression using lesion-specific-type quantification in a well-established PCa pre-clinical model to acquire more accurate information of ANXA1 in PCa progression. Our data slightly differs from human reports, since loss of ANXA1 expression occurs only in malignant lesions. Interestingly, DHA up-regulates epithelial ANXA1 expression in almost all lesion-grade which contributed to a less aggressive disease phenotype, possibly due to impairment of IL-6/STAT3 signaling, which is a known targetable

pathway of DHA [33]. Also, it is possible that the up-regulation of anxA1 herein observed could resulted from the negative effect of DHA on AR signaling as previously reported [3, 27].

Although the expression of ANXA1 by tumor cells may impact disease prognosis, recent reports also attest that secreted ANXA1 from TME cells can also affect directly tumor progression [25]. ANXA1-deficient mice display decreased tumor growth through impairment of angiogenesis and metastasis, suggesting that ANXA1 provided from different cells of the TME can modulate the disease progression [34]. Accordingly, high stromal ANXA1 is reported for hepatocellular carcinoma, particularly in M2-like tumor-associated macrophages (TAM), in which its overexpression leads to high proliferation of tumor cells and inhibition of $CD8^+$ T-cell function by PD-L1 expression [16]. In addition, CAF-secreted ANXA1 induces prostate cancer cells to gain stem cell-like characteristics that lead to increased expression of EMT factors [15]. These reports suggest that TME ANXA1 is a suitable target for cancer therapy since the expression of ANXA1 by tumor cells might differ according to the disease stage. Here, we demonstrated that DHA intake downregulates ANXA1 in CAFs in late-stage disease which was linked to the reduction of EMT-related factors, suggesting that one of the mechanisms by which DHA impairs tumor invasiveness is through TME ANXA1 modulation.

The onset and coevolution of a reactive stroma is a hallmark of PCa [17, 18] and have been targeted in several therapeutic approaches to slow the disease progression [24, 35]. In TRAMP carcinogenesis, αSMA-positive cells accumulate particularly in areas of HGPIN and WDAC [3] resulting in a displacement of a smooth muscle cell phenotype to a CAF phenotype [24]. Here, we observed that the increase in α-SMA-positive cells was not accomplished by any alteration in CALP expression, suggesting the increase of CAF onset in the reactive stroma in late-stage disease as attested by the up-regulation of FAP-positive cells. According to these findings, DHA has been shown to impair reactive stroma evolution and tumorigenesis by downregulating α-SMA and blocking TGF-β-stimulated CAF onset in PCa [3, 35]. Additionally, we previously observed that DHA intake reduces M2-like TAM [data not shown], which alongside CAF, secrete TGF-β into the TME and enhances PCa cell migration and invasion by up-regulating EMT [36]. Therefore, the inhibitory effect of DHA on TME is an effective mechanism in suppressing carcinogenesis.

The effects of TGF-β are mediated by its receptors, namely TGFβ-RI and TGFβ-RII which may trigger anti- or pro-tumor effects depending on the disease stage [37]. Interestingly, as PCa progresses, the loss of TGFβ-RI and TGFβ-RII is accomplished by overexpression of TGF-β, leading to high sensitivity of cancer cells to TGF-β signaling that results in a pro-tumor effect of TGF-β/TGFβ-R signaling [38, 39] Similar to human PCa we also detected a reduction in TGFβ-RII protein levels in late-stage disease which was associated with increased SNAIL expression. Accordingly, dysfunctional TGFβ-RII signaling leads to early disease onset through up-regulation of EMT in TRAMP mice [38]. Intriguingly, we also detected a down-regulation in TGFβ-RII in early-stage disease as a result of DHA intake. Although TGFβ-RII usually confers anti-tumoral effects in early-stage disease it is reported that DHA can down-regulate TGF-β secretion by tumor cells [40]. Thus, we hypothesize that DHA intake might modulate the TGF-β/TGFβ-R pathway to pro-apoptotic signaling since DHA intake greatly increases apoptosis in mice prostate cancer models [41].

As the tumor progresses to an aggressive disease stage, neoplastic cells often display increased motility and invasive capacity that leads to localized invasion and subsequential metastatic spread [3, 42], due to increased stimulation of EMT Overexpression of SNAIL or SLUG increases stem cell features of prostate cells e promotes the nuclear translocation of syndecan-1 intracellular domain, resulting in the up-regulation of EMT [43, 44]. Our data demonstrated that DHA was capable of impairing EMT in late-stage disease in TRAMP mice by downregulating the SNAIL transcriptional factor and CDH2, suggesting that this effect might be time-dependent. In agreement with our findings, DHA was capable of reversing EMT in docetaxel-resistant PC3 cells by inhibition of the PI3K/Akt pathway through downregulation of GTPπ, leading to increased apoptosis and ferroptosis [21]. In addition, up-regulation of syndecan-1 by DHA via PPARγ activation has been reported as a mechanism to antagonize EMT and increase apoptosis through the PDK1/Akt/Bad signaling [45, 46]. Therefore, targeting EMT by DHA is a promising mechanism to delay disease progression and reduce tumor burden.

PGE_2 is a bioactive lipid derived from prostaglandin E2 synthase (PTGS) arachidonic acid (AA) metabolism that exhibits pro-tumorigenic effects such as up-regulation of the PI3K/Akt pathway. [47] PTGS2, the inducible form of PTGS is overexpressed in pre-neoplastic and neoplastic prostate tissue [48, 49]. In the TME, PCa cells can preferably metabolize AA into PGE2 resulting in increased cell proliferation

and invasion by up-regulation of SNAIL via EP4 receptor [47, 50, 51]. Inhibition of PTGS2 by DHA or pharmacological agents such as celecoxib effectively delays PCa progression by impairing cell proliferation, angiogenesis, and CAF onset, and downregulates inflammatory mediators such as IL-6, IL-8, pSTAT-3, and NFκB.[22, 23, 52, 53]. Here we confirmed the inhibitory effects of DHA on PTGS2/PGE2 signaling, resulting in a less aggressive disease phenotype. Thus, targeting the PGE2/PTGS2 axis by DHA can improve PCa progression as we reported herein.

Conclusion

Our data reinforces the anti-tumor effects in the TRAMP mice pre-clinical model of PCa via modulation of TME. These effects rely on the up-regulation of epithelial ANXA1 during disease progression and the down-regulation of ANXA1 in CAFs in late-stage disease. Additionally, DHA attenuates reactive stromal and EMT markers in late-stage disease and impaired PGE2/PTGS2 signaling. These pre-clinical data highlight the anti-tumor effects of DHA and support its application for clinical trials which will help to clarify the possible benefits of DHA in PCa therapy.

Conflict of Interest: The authors declare no conflict of interest.

Author Contributions: GMA and RMG were involved with project conception and design. GMA, LPM, and ADTS were involved with development of methodologies. GMA, LPM, ADTS, SRT, SMO, VHAC and RMG were involved with analyses and interpretation of data. GMA, VHAC, SMO and RMG were involved with writing and review of the manuscript. RMG was involved with study supervision.

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FIGURES

Figure 1. DHA-d increases epithelial ANXA1 in the ventral prostate of TRAMP mice. (A) Representative photomicrographs of ANXA1 immunohistochemistry. **(B)** Comparison of ANXA1 expression in normal epithelium (NE) and specific pathological lesions (LGPIN: low-grade intraepithelial neoplasia, MGPIN: moderate-grade intraepithelial neoplasia, HGPIN: high-grade intraepithelial neoplasia, WDAC: well-differentiated adenocarcinoma, UACD: undifferentiated adenocarcinoma). Scale bar: 20 µm. Statistic: *two-way* ANOVA, followed by Tukey test, * $p < 0.05$. C: control, D: docosahexaenoic acid-fed groups.

Figure 2. DHA-d decreases CAF expressing ANXA1 in the ventral prostate of TRAMP mice. (A) Double immunofluorescence of FAP and ANXA1. **(B)** Quantification of $FAP^+/ANXA1^+$ cells. Scale bar: 20 µm. Legend: Ep- Acinar epithelium; L-Acinar lumen; St- Stroma. Statistic: *two-way* ANOVA, followed by Tukey test. * $p < 0.05$. C: control, D: docosahexaenoic acid-fed groups.

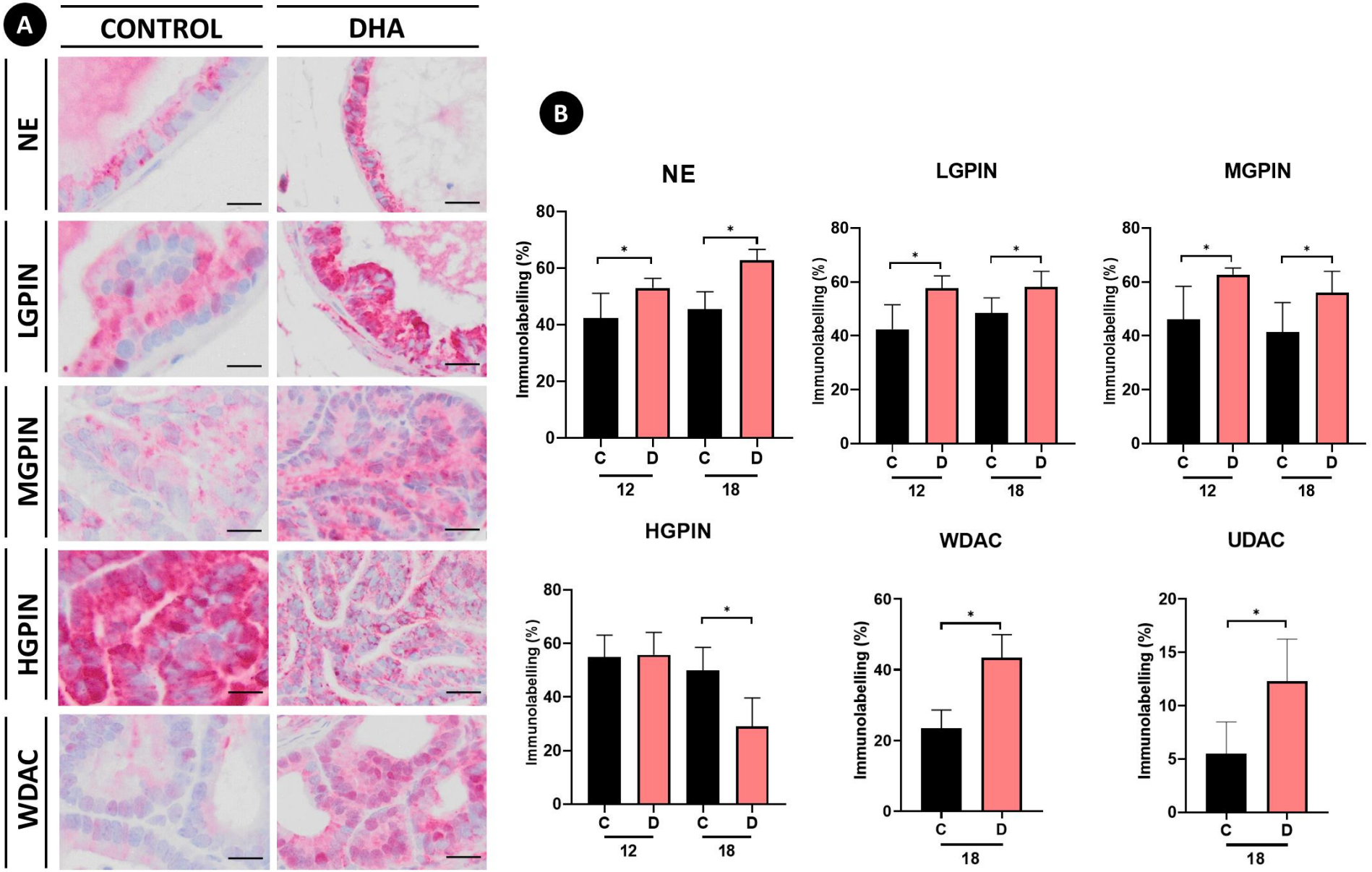
Figure 3. DHA-d improves stromal features in the ventral prostate of TRAMP. (A) Immunofluorescence of αSMA **(B)** Quantification of αSMA **(C-E)** Immunoblotting and quantification of CALP and TGF-β RII protein levels. Scale bar: 20 µm. Legend: Ep- Acinar epithelium; L-Acinar lumen; St- Stroma. Statistic: *two-way* ANOVA, followed by Tukey test, * $p < 0.05$. C: control, D: docosahexaenoic acid-fed groups.

Figure 4. DHA-d impairs EMT in the ventral prostate of TRAMP. (A) Immunohistochemistry of SNAIL **(B)** Quantification of SNAIL immunohistochemistry **(C-E)** Immunoblotting and quantification of CDH1 and CDH2 protein levels. Scale bar: 20 µm. Statistic: *two-way* ANOVA, followed by Tukey test, * $p < 0.05$. C: control, D: docosahexaenoic acid-fed groups.

Figure 5. DHA-d downregulates PGE2/PTGS2 signaling. (A) Serum levels of PGE2 **(B)** Immunoblotting and quantification of PTGS2 protein level in ventral prostate. Statistic: *two-way* ANOVA, followed by Tukey test, * $p < 0.05$. C: control, D: docosahexaenoic acid-fed groups.

SUPPLEMENTARY INFORMATION

Supplementary Figure 1. Lesion grading in TRAMP mice ventral prostate. (A) Normal epithelium (arrow) and LGPIN (arrowhead). (B) MGPIN. (C) HGPIN. (D) WDAC. (E) UDAC. Scale bar: 20 µm.



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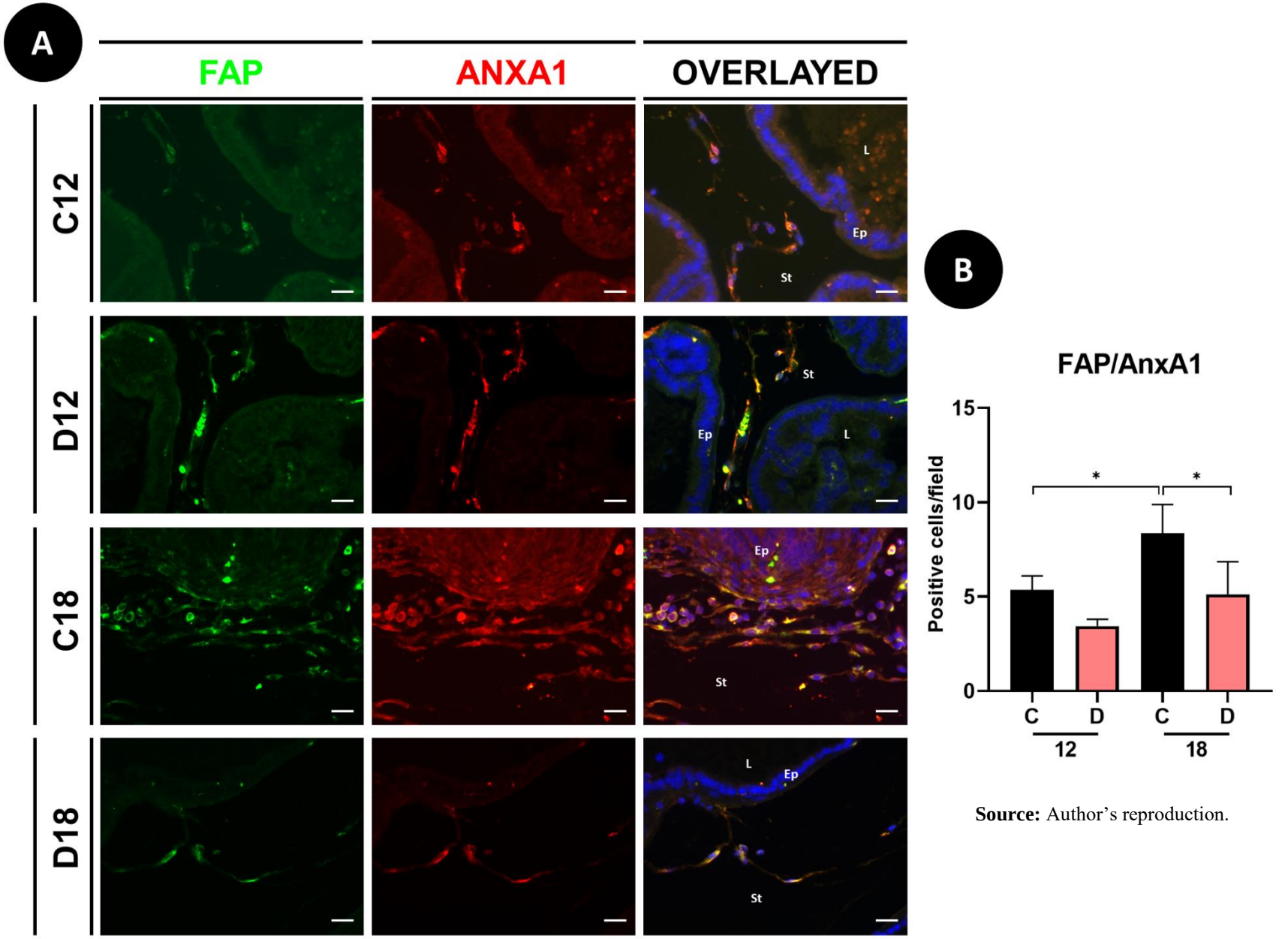

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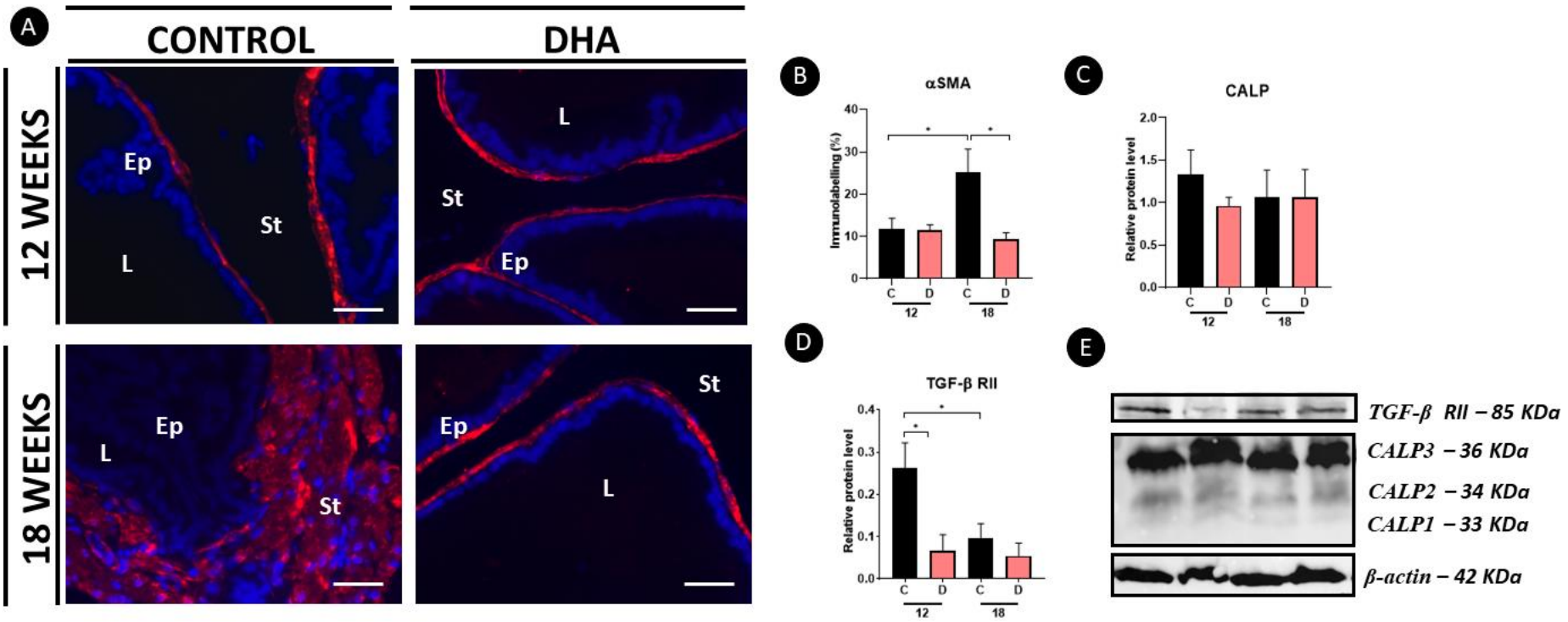

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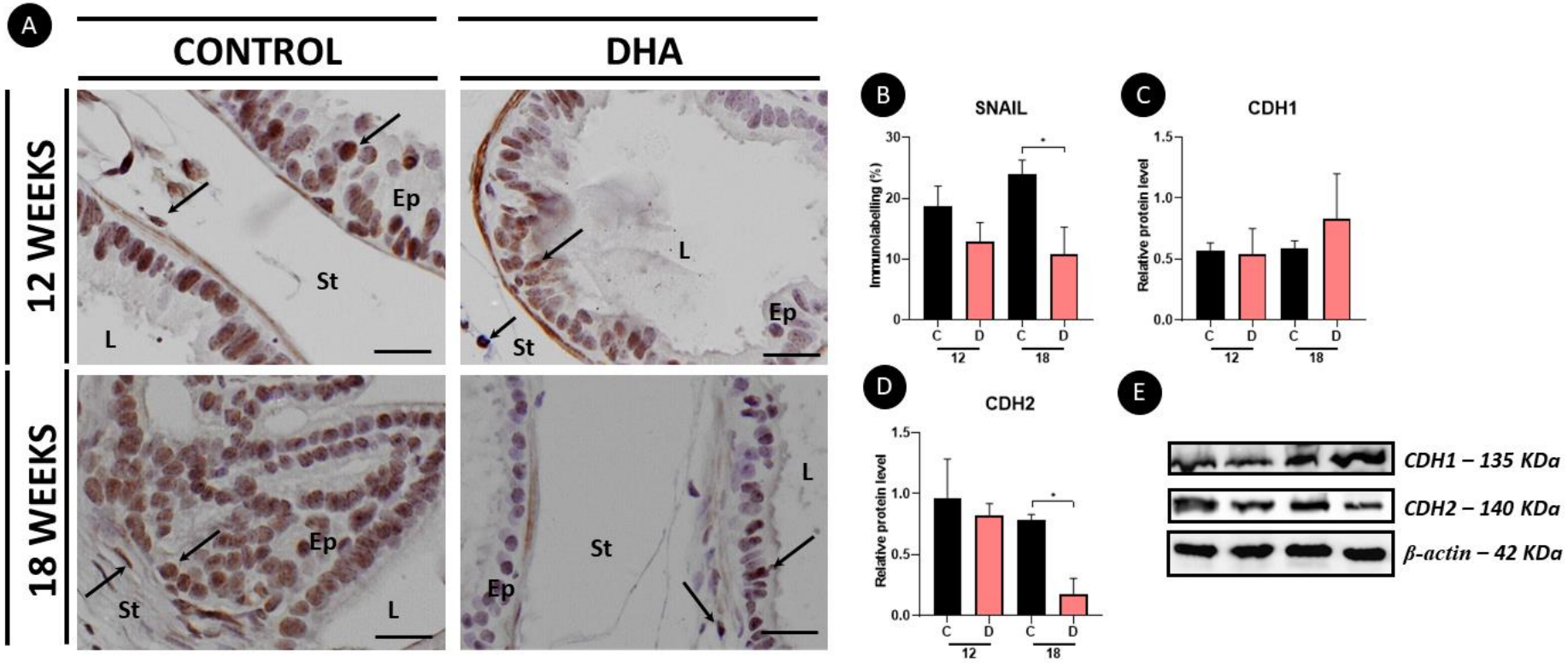

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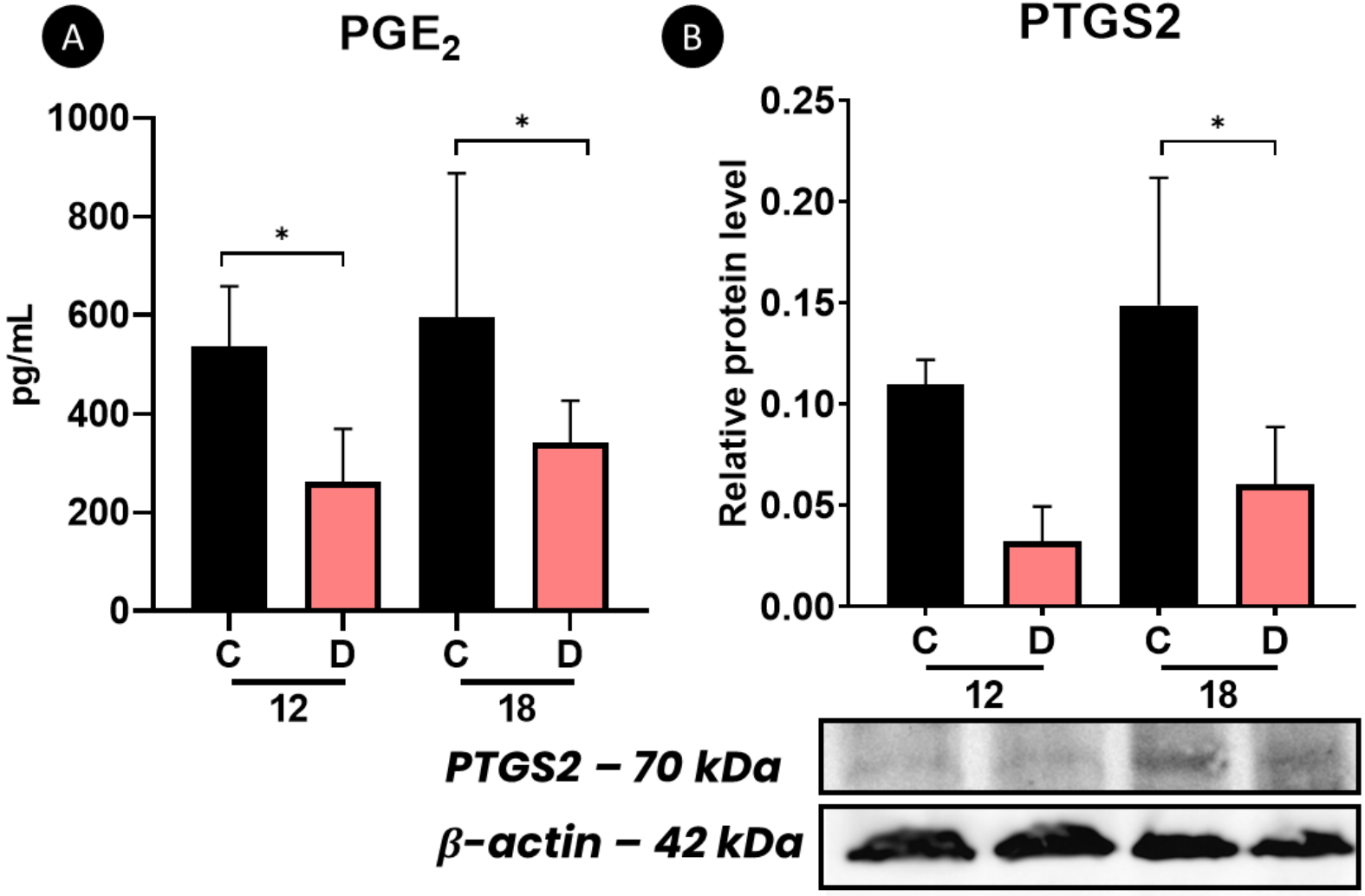

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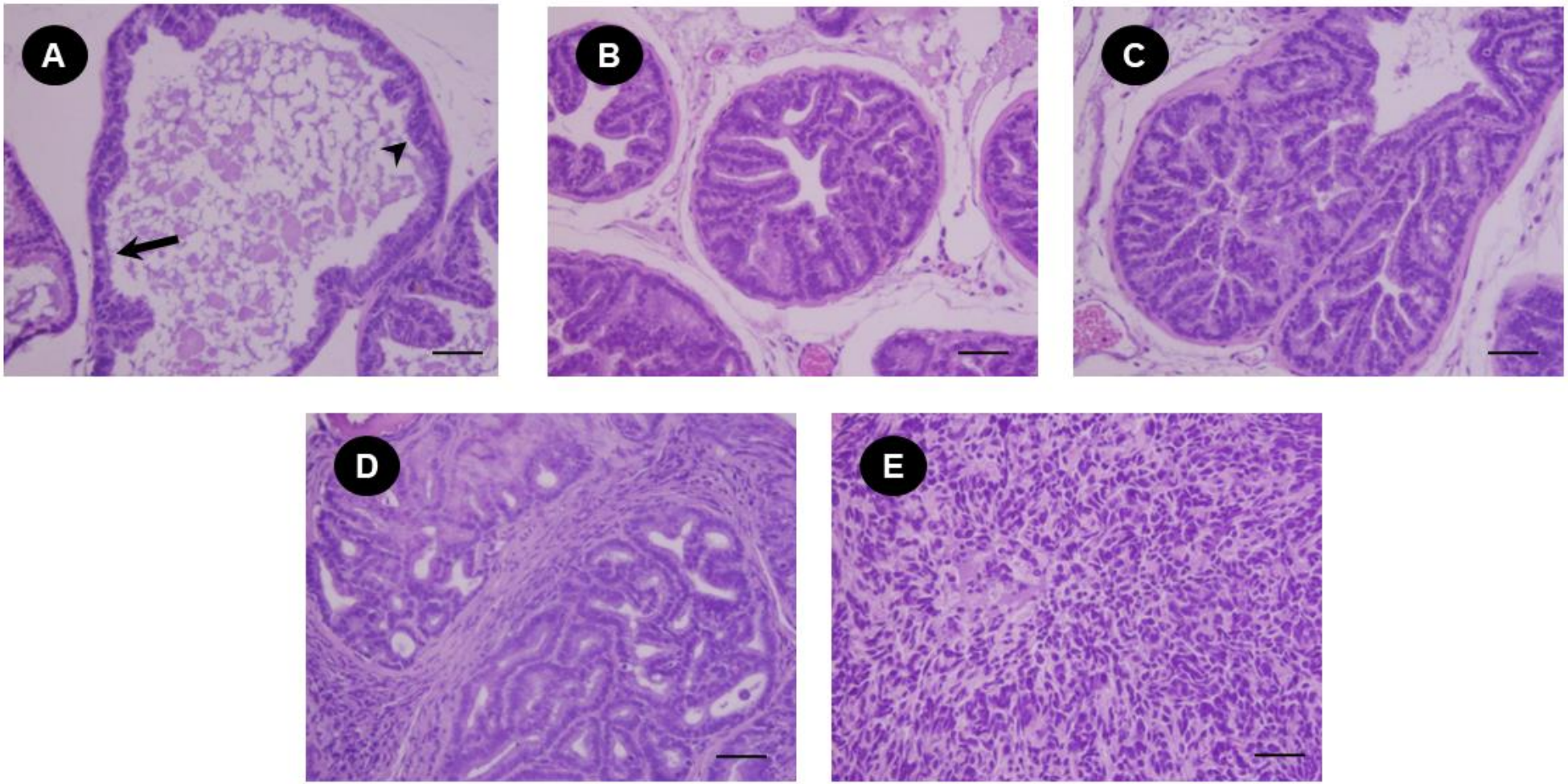

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5- CONCLUSÕES GERAIS

Nossos dados confirmam as ações anti-tumorais do DHA e suportam o seu uso em futuros testes clínicos para avaliar sua eficácia do tratamento do CaP, seja individualmente ou em associação a outros quimioterápicos. De forma geral, podemos concluir:

(I) O DHA exerce efeitos anti-proliferativos e pró-apoptóticos em ambos os estágios da doença;

(II) As vias de sobrevivência e proliferação celular são diferencialmente moduladas pelo DHA. No estágio inicial isso se dá pela ativiação da via ERK1/2 e no estágio tardio pela inativação da via Akt;

(III) O DHA inibe a piroptose em ambos os estágios da doença. No estágio tardio o consumo de DHA aumenta a necroptose, possivelmente devido a modulação da sinalização TNF-α/TNF-R1;

(IV) O DHA modulação o microambiente inflamatório, reduzindo o infiltrados de células $CD4^+$ e macrófagos M2 em ambos os estágios da doença. O aumento a densidade de células $CD8^+$ ocorre apenas no estágio tardio da doença;

(V) O DHA modula diferencialmente a expressão de anxA1, aumentando sua expressão nas células epiteliais em ambos os estágios da doença, e diminuindo nos CAFs no estágio avançado;

(VI) O DHA tem efeito negativo sobre a evolução do perfil de estroma reativo e sobre o processo de TEM no estágio tardio da doença e

(VII) O DHA exerce efeitos anti-inflamatórios, reduzindo os níveis séricos de TNF-α e PGE2 em ambos os estágios da doença, porém, os níveis proteicos intra-teciduais do TNF-R1 e da PTGS2 só foram reduzidos no estágio avançado da doença.

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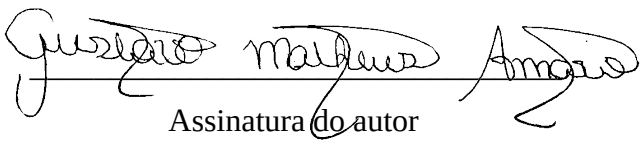

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