LAÍS CAPELASSO LUCAS PINHEIRO

ANÁLISE DE PROTEÍNAS ENVOLVIDAS NA CONSTITUIÇÃO E REMODELAÇÃO DA MATRIZ EXTRACELULAR COMO CANDIDATAS A MARCADORES PREDITIVOS DO PROCESSO METASTÁTICO NO CÂNCER DE PRÓSTATA

Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Genética e Biologia Molecular, da Universidade Estadual de Londrina, como requisito final para a obtenção do título de Mestre.

Orientador: Profa. Dra. Roberta Losi Guembarovski.
Coorientador: Prof. Dr. Carlos Alberto Miqueloto.

Londrina
2023

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RESUMO

A metástase é o maior problema no tratamento e a maior causa de mortes em pacientes com câncer de próstata (CaP). Este estudo objetivou quantificar a deposição de proteínas que constituem a matriz extracelular (MEC), sendo elas colágeno total, colágeno I (Col I) e colágeno III (Col III) em amostras de CaP metastáticas e não metastáticas, avaliar a integridade da membrana basal, e também a imunomarcação de Metaloproteinase (MMP)-2 e MMP-9, enzimas responsáveis pela remodelação da MEC, nessas mesmas amostras. Sessenta pacientes foram divididos em três grupos prognósticos: melhor prognóstico (n=20), pior prognóstico (n=23) e metastático (n=17). Para quantificação dos colágenos, utilizou-se a técnica Picrosirius, para a análise da integridade de membrana basal, a técnica de Ácido Periódico de Schiff, e para avaliar a imunomarcação das MMPs, foi aplicada a técnica de imunohistoquímica indireta. Observamos maior razão Col I/ Col III no grupo metastático em relação aos grupos de melhor e pior prognóstico. Acerca da integridade da membrana basal, constatamos que a piora de sua integridade correlacionou-se positivamente com parâmetros de agressividade. Quanto às MMPs, a imunomarcação de MMP-9 se correlacionou positivamente com os grupos prognósticos, grau ISUP, extensão extraprostática e recidiva bioquímica. Nosso estudo indicou que a ausência de Col III pode constituir um marcador preditivo para metástase, que a integridade da membrana basal no tecido tumoral encontra-se alterada, o que está correlacionado a fatores de pior prognóstico e que a MMP-9 parece constituir um marcador para fatores de invasão tecidual no CaP, essenciais para a metástase.

Palavras-chave: colágeno, metaloproteinases, parâmetros prognósticos, biomarcador.

PINHEIRO, L. C. L. **Analysis of proteins involved in the constitution and remodeling of extracellular matrix as candidates for predictive markers of prostate cancer metastatic process.** 2023. 121 p. Dissertation presented to Postgraduate Program in Genetics and Molecular Biology, at the State University of Londrina, as a final requirement for obtaining a master's degree, 2023.

ABSTRACT

Metastasis is the biggest problem in treatment and major cause of deaths in patients with prostate cancer (PCa). This study aimed to quantify the deposition of proteins that constitute the extracellular matrix (ECM), being total collagen, collagen I (Col I) and collagen III (Col III) in metastatic and non-metastatic PCa samples, to evaluate the integrity of the basement membrane, and also the immunostaining of Metalloproteinase (MMP)-2 and MMP-9, enzymes responsible for the ECM remodeling in these same samples. Sixty patients' were divided in three prognostic groups: better prognosis (n=20), worse prognosis (n=23) and metastatic (n=17). To quantify collagen, PicroSirius Red staining was used, the Periodic Acid-Schiff technique was used for basement membrane integrity analysis, and to evaluate the MMPs immunostaining, indirect immunohistochemistry technique was applied. We observed a higher ol I/Col III ratio in the metastatic group compared to the better and worse prognosis groups. Regarding the basement membrane integrity, it was observed that it's integrity worsening was positively correlated with aggressiveness parameters. As for MMPs, the MMP-9 immunostaining positively correlated with prognostic groups, ISUP grade, extraprostatic extension and biochemical recurrence. Our study indicated that the absence of Col III may constitute a predictive marker for metastasis, the basement membrane integrity in tumor tissue was altered, which is correlated with worse prognosis factors, and the MMP-9 seems to constitute a marker for tissue invasion factors, essential in the metastasis.

Key-words: collagen, metalloproteinases, prognostic parameters, biomarker.

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LISTA DE ABREVIATURAS E SIGLAS

AJCC	Comitê Conjunto Americano de Câncer, do inglês *American Joint Committee on Cancer*
AKT	Serina-Treonina Quinase, do inglês *Serine-Threonine Kinase*
AR	Receptor Androgênico, do inglês *Androgen Receptor*
CaP	Câncer de Próstata
Col I	Colágeno do tipo I
Col III	Colágeno do tipo III
DHT	Di-hidrotestosterona
DNA	Ácido Desoxirribonucleico, do inglês *Deoxyribonucleic Acid*
EMT	Transição Epitelial-mesenquimal, do inglês *Epithelial-mesenchymal Transition*
HCL	Hospital do Câncer de Londrina
IHQ	Imunohistoquímica
ISUP	Sociedade Internacional de Patologia Urológica, do inglês *International Society of Urological Pathology*
LOX	Enzima Lisil Oxidase, do inglês *Lysyl Oxidase*
MEC	Matriz Extracelular
MMP	Metaloproteinase
NCCN	Rede Nacional Abrangente do Câncer, do inglês *National Comprehensive Cancer Network*
NKX3.1	NK3 homeobox 1
p53	Proteína Tumoral 53
PAS	Ácido Periódico de Schiff, do inglês *Periodic Acid Schiff*
PD-1	Proteína de Morte Celular Programada 1, do inglês *Programmed Cell Death Protein 1*
PDGF	Fator de Crescimento Derivado de Plaquetas, do inglês *Platelet-derived growth Factor*
PD-L1	Ligante de Proteínas de Morte Celular Programada 1, do inglês *Programmed Death-ligand 1*
PET-scan	Tomografia por Emissão de Pósitrons
PSA	Antígeno Prostático Específico, do inglês *Prostate-specific Antigen*
PTEN	Fosfatase Homóloga à Tensina, do inglês *Phosphatase and Tensin*

	Homologue
TNM	Tumor-linfonodo-metástase, do inglês *Tumor-node-metastasis*
TP53	Gene da Proteína Tumoral 53
TRPM8	Receptor de Potencial Transitório, Membro 8 da Melastatina, do inglês *Transient Receptor Potential, Melastatin Member 8*
UICC	União Internacional Contra o Câncer, do inglês *International Union Against Cancer*
VEGF	Fator de Crescimento Endotelial Vascular, do inglês *Vascular Endothelial Growth Factor*

SUMÁRIO

1. INTRODUÇÃO

O câncer é um grave problema de saúde pública por todo o mundo sendo responsável por milhões de novos casos e mortes todos os anos. Dentre todos os tipos, destaca-se o câncer de próstata (CaP), o mais comum em homens no Brasil, atrás apenas do câncer de pele do tipo não melanoma (INCA, 2023). Para o triênio 2023-2025 estima-se 72 mil novos casos de CaP a cada ano no Brasil (INCA, 2022).

Apesar de existirem métodos de diagnóstico muito eficazes para o CaP, ainda enfrenta-se algumas dificuldades, como resultados falso-positivos, rebiópsias e tratamentos inadequados (LITWIN; TAN, 2017). Dessa forma, busca-se novos marcadores moleculares que sejam capazes de distinguir tumores prostáticos agressivos de tumores indolentes, que são aqueles de evolução lenta e relativamente benigna, para que a conduta terapêutica seja cada vez menos agressiva e mais adequada à particularidade de cada paciente.

No CaP, a metástase é a principal causa de morte e, para que ela ocorra, é necessário que as células cancerosas se desprendam do epitélio de origem, migrem pela matriz extracelular (MEC) circundante e adentrem os vasos do sistema circulatório. Durante esse processo ocorre a modificação de diversas proteínas responsáveis pela homeostase tecidual (GANESH; MASSAGUÉ, 2021). Dentro desse contexto, o estudo da integridade da membrana basal, e de proteínas que participam da constituição da MEC, como os colágenos I e III, e de seu remodelamento, como as metaloproteinases (MMPs), se mostra interessante na busca de indicadores prognósticos. Considerando o papel fundamental dessas proteínas no processo metastático, elas foram selecionadas para investigação no presente estudo.

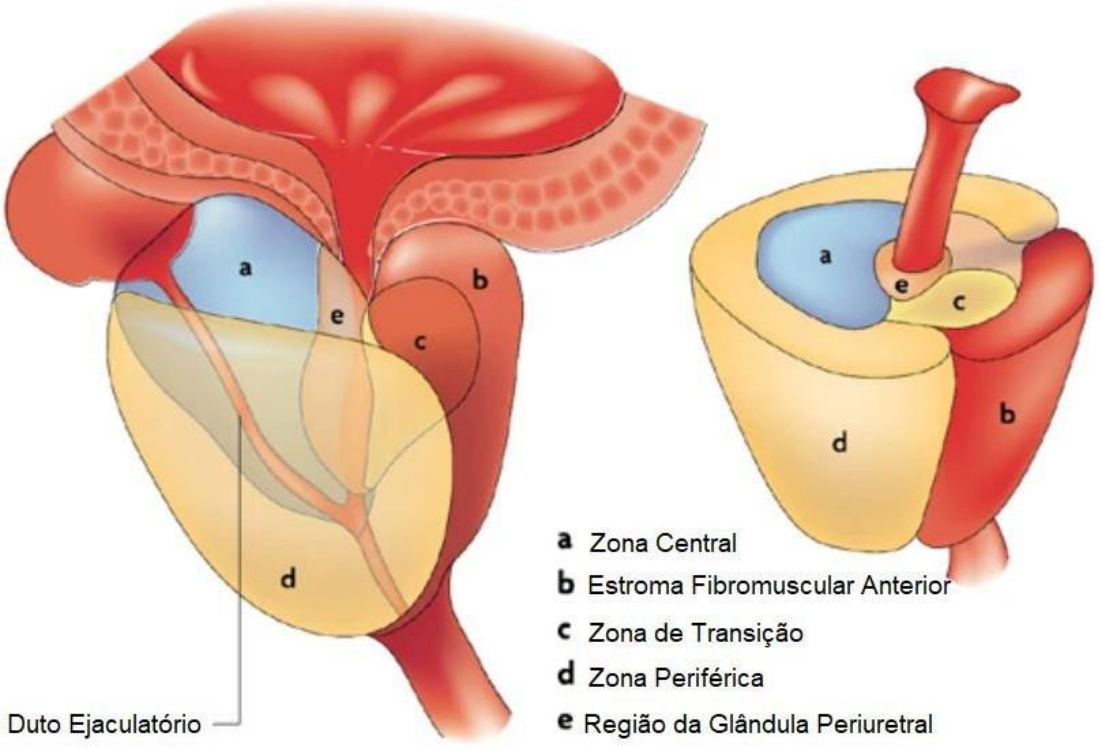
Além disso, para o estudo de tais proteínas e estruturas, foram utilizadas nesse trabalho metodologias que fazem uso de cortes histológicos de amostras tumorais. Dentre elas, a técnica de imunohistoquímica, que já é uma metodologia padrão ouro na rotina clínica da oncologia. Assim, analisamos biomarcadores candidatos para a carcinogênese prostática com potencial de serem implementados futuramente na clínica oncológica como preditores do prognóstico tumoral.

2. FUNDAMENTAÇÃO TEÓRICA

2.1. A Próstata

A próstata é uma glândula exócrina anexa do sistema reprodutor masculino, que se localiza na parte inferior do abdômen, ventralmente à bexiga e a anteriormente ao reto, envolvendo a porção inicial da uretra. Ela é anatomicamente dividida em quatro regiões, sendo elas: zona periférica, que representa cerca de 70% da glândula; zona central, que compreende 15% da próstata; zona de transição, que corresponde a apenas 5% do órgão; e estroma fibromuscular anterior, que forma a superfície anterior prostática completa (MCNEAL, 1988; MCNEAL, 1981). As regiões são envolvidas por uma cápsula de músculo liso recoberta por colágeno (LEE; AKIN-OLUGBADE; KIRSCHENBAUM, 2011) (Figura 1).

Figura 1. Visão sagital e coronal das regiões prostáticas evidenciando principalmente as zonas central, de transição e periférica, além do estroma fibromuscular anterior.

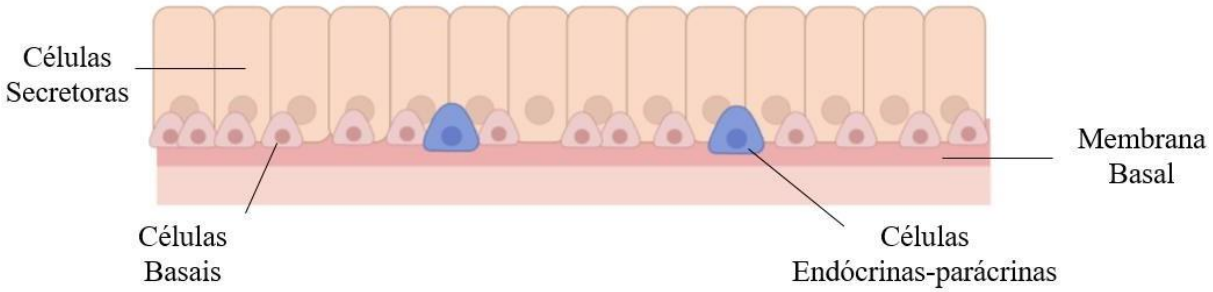


Fonte: DE MARZO et al., 2007 com modificações.

A principal função da próstata é produzir o líquido prostático, que possui um pH alcalino que facilita a locomoção dos espermatozoides, e que, ao se juntar com a secreção da vesícula seminal, formam o sêmen (MCNEAL, 1981).

Quanto aos tipos celulares que compõem a próstata, pode-se dizer que ela é composta majoritariamente pelas i) células basais, que separam a membrana basal das células secretoras. Elas se localizam paralelas à membrana basal, possuem um núcleo filiforme e normalmente apresentam pouco citoplasma. ii) células endócrinas-parácrinas que se organizam em pequenas e isoladas populações entre as células basais e as células secretoras, possuem função parácrina em resposta a estímulos neurais. iii) e células secretoras que contribuem na secreção de uma grande variedade de produtos no plasma seminal (MCNEAL, 1988).

Figura 2. Imagem representativa do epitélio prostático.



Fonte: próprio autor.

Um dos produtos liberados pelas células secretoras prostáticas é o antígeno prostático específico (PSA, do inglês *prostate specific antigen*), uma serina protease da família das calicreínas teciduais. Sua principal função é hidrolisar as proteínas de alto peso molecular produzidas pelas vesículas, permitindo assim a liberação de espermatozoides (DE LAMIRANDE, 2007).

Existem 3 processos patológicos que mais comumente acometem a próstata: as inflamações, as hiperplasias benignas e os tumores malignos (GROSSMAN, 2018).

2.2. Desenvolvimento do Câncer

O corpo humano é formado por conjuntos de células que se organizam harmonicamente em tecidos. De maneira geral, todas as células possuem um destino: elas amadurecem, se multiplicam e morrem. Porém, alterações, como mutações no DNA, que perturbam a harmonia dos tecidos, são danosas para o corpo humano. Tais mutações podem ocorrer em genes que controlam o destino das células, dando a elas uma vantagem sobre as demais e permitindo que elas cresçam e se dividam mais vigorosamente, tornando-se fundadoras de um clone mutante que se divide exacerbadamente e fora de contexto. Os tumores podem ser classificados em benignos, que são aqueles cujas células são bem diferenciadas e que ainda não adquiriram a

capacidade de invadir novos tecidos, ou malignos, que se caracterizam por elevado número de mutações, grande número de células indiferenciadas e pelo poder de invasão de tecidos adjacentes (KUMAR et al., 2013; ALBERTS et al., 2017).

Os genes que sofrem mutações e acarretam a formação do câncer podem ser classificados em três categorias: os genes supressores tumorais, os oncogenes e os genes responsáveis pelo reparo do DNA (PIERCE, 2016).

Os supressores tumorais são genes que codificam proteínas que funcionam como freios da divisão e têm a função de inibir o ciclo celular. Essa classe de genes pode perder sua função por deleção, mutação pontual, metilação (adição de um grupo metil no DNA, inibindo sua transcrição) ou inserção viral, fazendo com que o crescimento passe a ser descontrolado, levando a formação de tumores (PIERCE, 2016). Um exemplo de gene supressor tumoral é o *TP53* (Proteína Tumoral 53), que codifica o fator de transcrição p53, capaz de regular a entrada da célula em apoptose ou induzir o reparo do DNA danificado. Esse gene é frequentemente perdido ou mutado na maioria dos cânceres humanos (NIGRO et al., 1989). Os genes supressores tumorais possuem um padrão de herança recessiva ou dominante, ou seja, necessitam que haja o silenciamento dos dois alelos do gene ou de apenas um (haploinsuficiência), para um possível desenvolvimento tumoral (INOUE; FRY, 2017).

Os proto-oncogenes são genes que codificam proteínas responsáveis pela regulação da proliferação celular, e que controlam a diferenciação e o crescimento. Quando mutados, formam os oncogenes, que atuam como aceleradores do ciclo celular, contribuindo para o aumento exacerbado da divisão. Por se comportarem como genes com padrão de herança dominante, a mutação em apenas uma das cópias dos oncogenes pode contribuir para a progressão tumoral (PIERCE, 2016).

Existem 4 formas de ativar um proto-oncogene a oncogene, sendo elas: mutação pontual, amplificação gênica, translocação cromossômica e ativação retroviral. Na mutação pontual, um proto-oncogene pode se transformar em oncogene apenas por uma substituição de base. Já na amplificação gênica, o aumento do número de cópias pode acarretar uma superexpressão de seus produtos. Na translocação cromossômica, o rearranjo de cromossomos pode ativar uma proteína já existente ou ainda criar uma proteína nova. Por fim, na ativação retroviral, um vírus pode ativar um oncogene quando se insere no genoma do hospedeiro (KONTOMANOLIS et al., 2020).

A mutação de genes depende de dois fatores: a taxa de erros que surgem após a replicação ou exposição a fatores mutagênicos, e a eficiência com que esses erros são corrigidos. Defeitos nos genes que codificam componentes do sistema de reparo de DNA estão

relacionados a vários tipos de câncer, como o colorretal e o de mama (PIERCE, 2016). Essa classe inclui genes de reparo por mismatch (MMR), reparo por excisão de nucleotídeo (NER) e reparo por excisão de base (BER) (VOGELSTEIN; KINZLER, 2004). Esses defeitos nos genes de reparo podem também contribuir para gerar rearranjos cromossômicos e instabilidade genética (PIERCE, 2016).

Dessa forma, evidencia-se que o câncer é uma doença exclusivamente genética, que deriva de alterações em genes responsáveis pelo controle do reparo de DNA, crescimento e divisão celular. Sem esse controle, ocorre um desbalanço na homeostase dos tecidos, que pode ocasionar o surgimento de neoplasias malignas, como as que ocorrem no câncer de próstata (CaP) (PIERCE, 2016).

2.3. O Câncer de Próstata

O CaP pode se manifestar com alto grau de agressividade ou de maneira mais lenta e sem muitas manifestações clínicas. A maneira mais comum é a de progressão lenta, e é essa característica que permite diagnóstico do tumor ainda em sua forma inicial, quando os primeiros sintomas ainda não são detectados (VICKERS; ROOBOL; LILJA, 2012).

O eixo de sinalização androgênica desempenha um papel fundamental na patogênese do câncer de próstata, visto que a próstata possui uma dependência de testosterona para que ocorra seu amadurecimento. O desbalanço na função ou nos níveis de androgênios, ou dos fatores de crescimento dependentes da testosterona, podem causar a proliferação excessiva da glândula, levando assim ao desenvolvimento de doenças proliferativas prostáticas (DAI; HEEMERS; SHARIFI, 2017).

O receptor androgênico (AR) presente na próstata é responsável por mediar os efeitos fisiológicos androgênicos a partir de sua ligação com a DHT (di-hidrotestostenona), um precursor da testosterona. O AR ativo no núcleo celular é capaz de se ligar em regiões conhecidas como elemento responsivo aos androgênios (AREs) em seus genes alvos, podendo recrutar diferentes fatores de transcrição e também diferentes cofatores, que atuam inibindo ou ativando a transcrição gênica (DAI; HEEMERS; SHARIFI, 2017).

2.3.1. Epidemiologia e fatores de risco

O câncer é um importante problema de saúde pública, e foi responsável por 9.958.133 óbitos em 2020. O CaP é o segundo tipo de câncer mais frequente em homens e, segundo o levantamento de 2020, foram constatados cerca de 1,4 milhões de novos casos no mundo (GLOBOCAN, 2021). Esta doença é a segunda neoplasia maligna com maior incidência em

homens no Brasil, com 65.840 novos casos em 2020, o que representa 29% dos novos casos de câncer no país, só ficando atrás dos tumores de pele não melanocíticos. Quanto à taxa de mortalidade, o CaP é a segunda causa de morte relacionada ao câncer em homens adultos, com 15.983 mortes em 2019, e é superado apenas pelo câncer de pulmão (INCA, 2021).

O início da progressão do CaP raramente apresenta sintomas e, quando ocorrem, são inespecíficos, como dificuldade para urinar e jato urinário fraco e intermitente (MERRIEL; FUNSTON; HAMILTON, 2018; GROSSMAN et al., 2018). Nesse estágio, as células cancerosas se restringem à próstata. Com a progressão do tumor, essas células podem invadir outros tecidos, como vesícula seminal, bexiga e uretra, podendo gerar sintomas como dor ao urinar e sangue na urina (NICE, 2015). Em estágios mais avançados da doença, o CaP pode resultar em metástase e gerar sintomas associados aos órgãos afetados, que são normalmente ossos, linfonodos, fígado e pulmão (BUBENDORF et al., 2000).

O principal e mais bem estabelecido fator de risco para o desenvolvimento de CaP é a idade. Enquanto apenas 1 em 350 homens abaixo de 50 anos desenvolveria o CaP, essa proporção aumenta para 1 em 52 homens entre 50 e 59 anos. A incidência para homens com mais de 65 anos chega a 60% (PERDANA et al., 2017).

O segundo fator de risco considerado é a descendência. Homens afro-americanos possuem uma taxa de incidência e mortalidade maior do que caucasoides (JAYADEVAPPA et al., 2011). A incidência mais baixa de CaP é encontrada em asiáticos, o que pode estar associado não apenas a fatores genéticos de suscetibilidade, como também a fatores ambientais e dietéticos (AKAZA et al., 2011). Dentre os fatores ambientais, pode-se destacar a exposição ativa ou passiva à fumaça de cigarro, que pode contribuir para progressão do câncer (HUNCHAREK et al., 2010). Quanto aos fatores dietéticos, o consumo aumentado de gorduras animais saturadas, carne vermelha e laticiníos tem sido associados com elevado risco de desenvolvimento de CaP (ARONSON et al., 2010; GIBSON et al., 2010; ROHRMANN et al., 2007).

O histórico familiar e a predisposição genética também podem ser considerados fatores de risco. É estimado que cerca de 20% dos pacientes com CaP possuem familiares com essa condição. Isso pode estar associado não apenas aos genes compartilhados, mas também a estilos de vida e padrões de exposição a fatores carcinogênicos em comum (RAWLA, 2019).

2.3.2. Diagnóstico

Para fazer o diagnóstico do CaP, é necessário que o médico urologista reúna o máximo de informações possíveis, além de averiguar os sintomas relatados pelo paciente. Para isso,

utiliza-se: o toque retal, a dosagem sanguínea de PSA, a ultrassonografia transretal e a biópsia prostática (REDA et al., 2018).

No toque retal, o urologista avalia a próstata manualmente a partir do toque na região posterior do reto do paciente. Esse exame é importante para identificar alterações na consistência e no volume da glândula (MISTRY; CABLE, 2003).

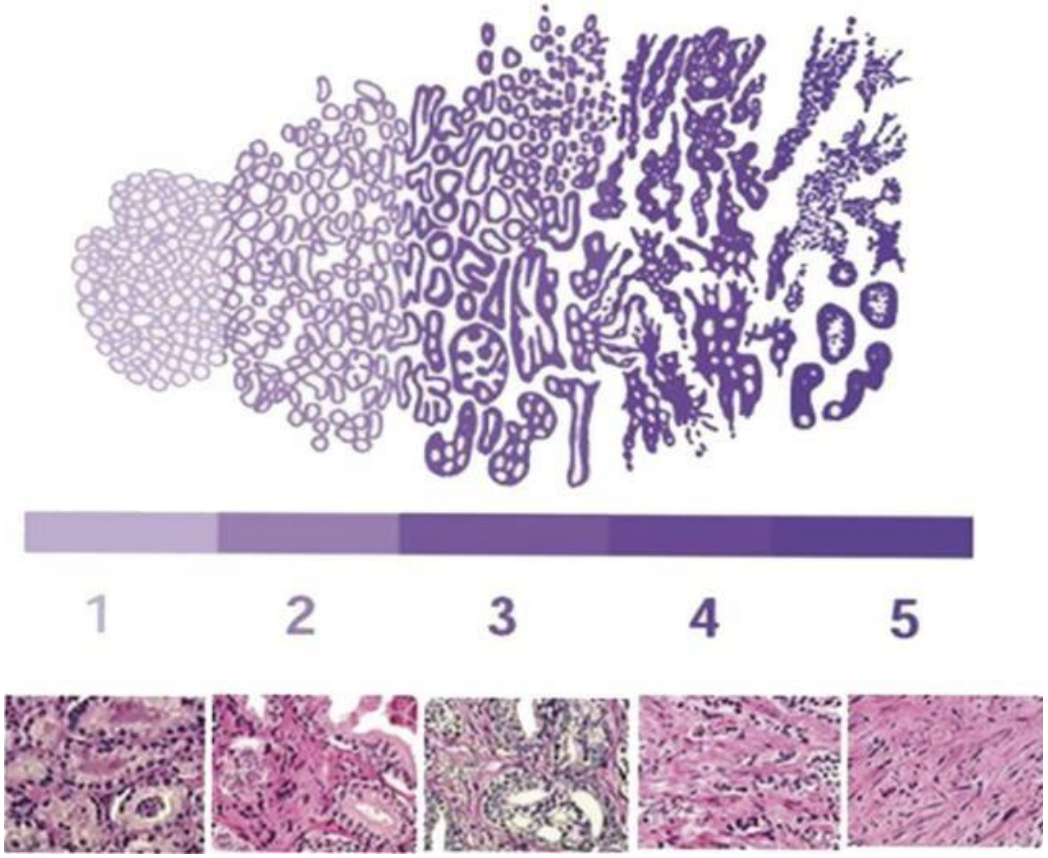
O PSA é uma glicoproteína produzida pelo epitélio acinar e ductal da próstata que está presente normalmente em baixas concentrações no sangue, com a função de liquefazer o fluido seminal (BALK; KO; BUBLEY, 2003). O aumento na dosagem do PSA sanguíneo pode ser um indício de alterações na próstata, como hiperplasias benignas, prostatites e o CaP (REDA et al., 2018).

Valores de PSA de até 4,0 ng/mL são considerados o limite superior para pacientes saudáveis (MISTRY; CABLE, 2003). Segundo as diretrizes do *National Comprehensive Cancer Network* (NCCN) de 2016, valores entre 4,0 e 10,0 ng/mL indicam tumores prostáticos de risco muito baixo. Conforme o aumento gradativo dessa glicoproteína no sangue, há um aumento do risco da neoplasia maligna, sendo que valores acima de 20 ng/mL normalmente indicam cânceres de alto risco (MOHLER et al., 2016).

A biópsia prostática guiada por ultrassom transretal é indicada quando existe a suspeita da doença e, a partir dela, são obtidos cerca de 12 fragmentos da próstata. Após a biópsia, os fragmentos são submetidos a análises histopatológicas por três sistemas: o escore de Gleason, o sistema ISUP e o sistema tumor-linfonodo-metástase (TNM) (REDA et al., 2018; LITWIN; TAN, 2017). Ao final das análises, o médico terá uma visão da extensão da neoplasia para indicar o tratamento mais adequado a cada paciente (REDA et al., 2018).

O sistema de classificação tumoral escore de Gleason (Figura 3) se baseia no padrão de crescimento de adenocarcinoma de próstata e classifica o tumor de acordo com sua diferenciação histológica. Neste sistema, são atribuídos valores de 1 a 5 aos padrões de crescimento encontrados na biópsia ou na prostatectomia radical, onde os padrões de 1 a 3 abrangem estruturas glandulares bem delineadas com distâncias intraglandulares variáveis e circunscrição nodular; o padrão 4 compreende estruturas glandulares mal formadas, fundidas, glomeruloides e cribriformes; enquanto o padrão 5 compreende padrões de crescimento sem diferenciação glandular, como células únicas, cordões e campos sólidos, e a presença de comedonecrose. Dessa forma, o escore de Gleason é determinado através da soma entre os dois padrões mais abundantes na amostra analisada (VAN LEENDERS; VERHOEF; HOLLEMANS, 2020).

Figura 3. Padrões aplicados para análise histopatológica do CaP e definição do Escore de Gleason. Padrão 1: muito bem diferenciado; Padrão 2: bem diferenciado; Padrão 3: moderadamente diferenciado; Padrão 4: pouco diferenciado; Padrão 5: indiferenciado.



Fonte: HARNDEN et al., 2007.

Tumores com escores de Gleason com soma final de 6 são considerados de baixa malignidade, já os que têm soma de 7 são intermediários, enquanto os tumores com soma entre 8 e 10 possuem alta malignidade (MOHLER et al., 2016).

O sistema ISUP, proposto pela *International Society of Urological Pathology* em 2014, utiliza os mesmos padrões de diferenciação tecidual do escore de Gleason. Porém, nesse sistema, os tumores são divididos em 5 graus. Os tumores de Grau 1 são aqueles com escore de Gleason 3+3. Os de Grau 2 têm soma final 3+4. Os de Grau 3 possuem soma 4+3. Neoplasias de Grau 4 apresentam somas 4+4, 3+5 ou 5+3. E os de Grau 5 têm soma 4+5, 5+4 ou 5+5 (EPSTEIN et al., 2016).

O sistema TNM (Quadro 1) para CaP foi introduzido em 1992 a partir de um consenso entre a AJCC (*American Joint Committee on Cancer*) e a UICC (*International Union Against Cancer*) (SCHRÖDER et al., 1992). Nessa classificação é avaliada a extensão do tumor primário, o acometimento de linfonodos regionais e a presença ou ausência de metástases à

distância. Em tal sistema, tanto estadiamentos clínicos (via biópsia) quanto patológicos (via retirada do tumor) podem prover métodos para avaliar a extensão da disseminação do tumor e prever o prognóstico do paciente (CHENG et al., 2012).

Quadro 1. Classificação clínica TNM de tumores malignos de próstata.

TNM - Classificação Clínica	
T - Tumor Primário	
TX	O tumor primário não pode ser avaliado
T0	Não há evidência de tumor primário
T1	Tumor não diagnosticado clinicamente, não palpável ou visível por meio de exame de imagem
T1a	Achado histológico incidental em 5% ou menos do tecido ressecado
T1b	Achado histológico incidental em mais de 5% de tecido ressecado
T1c	Tumor identificado por biópsia por agulha (p. Ex., devido a PSA elevado)
T2	Tumor confinado à próstata
T2a	Tumor que envolve uma metade de um dos lobos ou menos
T2b	Tumor que envolve mais da metade de um dos lobos, mas não ambos os lobos
T2c	Tumor que envolve ambos os lobos
T3	Tumor que se estende através da cápsula prostática
T3a	Extensão extracapsular (uni- ou bilateral)
T3b	Tumor que invade vesícula(s) seminal(ais)
T4	Tumor está fixo ou invade outras estruturas adjacentes, que não as vesículas seminais: colo vesical, esfíncter externo, reto, músculos elevados do ânus, ou parede pélvica
N - Linfonodos Regionais	
NX	Os linfonodos regionais não podem ser avaliados
N0	Ausência de metástase em linfonodo regional
N1	Metástase em linfonodo regional
M - Metástase à Distância	
MX	A presença de metástase à distância não pode ser avaliada
M0	Ausência de metástase à distância
M1	Metástase à distância
M1a	Linfonodo(s) não regional(ais)
M1b	Osso(s)
M1c	Outra(s) localização(ões)

Fonte: *American Joint Committee on Cancer* (AJCC) *clinical TNM classification of prostatic tumors* (2010).

Para a identificação de metástases utilizam-se exames de imagem, como ressonâncias magnéticas e PET-scans (GROSSMAN et al., 2018). Após a identificação e classificação do tumor, o urologista deve associar todas as informações obtidas para direcionar o melhor tratamento para o paciente (LITWIN; TAN, 2017).

2.3.3. Tratamento

Homens diagnosticados com tumores localizados (sem acometimento de linfonodos ou metástases à distância) possuem, em geral, três opções: gestão expectante, cirurgia e radioterapia. A gestão expectante se resume em espera vigilante e vigilância ativa, onde os sintomas são tratados, é feito o acompanhamento dos valores sanguíneos de PSA e realização de outros exames clínicos (FILSON; MARKS; LITWIN, 2015). A cirurgia e a radioterapia continuam a ser tratamentos eficazes para homens com cânceres mais agressivos, como aqueles com nível de PSA superior a 10 ng/mL e aqueles com nódulos palpáveis no toque retal ao diagnóstico (LITWIN; TAN, 2017).

Já para os cânceres metastáticos, a privação androgênica continua sendo a primeira linha de tratamento, mesmo que cause efeitos colaterais, como diminuição da densidade mineral óssea, alterações metabólicas, disfunção sexual, ondas de calor, morbidade cardíaca e disfunção cognitiva (NGUYEN et al., 2015; NEAD et al., 2017). Huggins e Hodges demonstraram em 1941 que a privação androgênica pela redução dos níveis de testosterona mediada por castração poderia possibilitar o controle do potencial metastático do CaP. A privação androgênica pode ocorrer cirurgicamente, através da retirada dos testículos (chamada de orquiectomia), ou quimicamente, com o uso de medicamentos (DAI; HEEMERS; SHARIFI, 2017).

Em muitos casos, cânceres metastáticos são ou se tornam insensíveis a hormônios andrógenos (também chamados de CaP resistentes à castração) (LITWIN; TAN, 2017). Desde 2010 novas drogas foram desenvolvidas para melhorar a sobrevida desses pacientes. É o caso do acetato de abiraterona e do enzalutamide, que atuam no eixo androgênico e desaceleram a progressão da doença, aumentando o tempo de sobrevida (DE BONO et al., 2011; BEER et al., 2014).

2.4. Doença Metastática

O maior problema no tratamento do câncer e a maior causa de morte dos pacientes é a metástase (GEIGER; PEEPER, 2009). Esse processo consiste na migração e multiplicação de células cancerosas em novos locais do organismo. Após a disseminação do câncer pelo corpo, ele se torna quase impossível de erradicar, tanto por cirurgia quanto por tratamentos quimio e radioterápicos (ALBERTS et al., 2017).

Para que a célula cancerosa seja capaz de migrar, ela deve perder o controle dos mecanismos de adesão, que a deixam ligada às outras células dentro de um tecido. Essa mudança se assemelha à transição epitelial-mesenquimal (EMT, do inglês *epithelial-mesenchymal transition*), onde as células do epitélio mudam sua conformação e se tornam mais

parecidas com células mesenquimais, que vivem dispersas e não aderidas (ALBERTS et al., 2017). Nesse processo, as células perdem sua polaridade original e alteram a expressão de proteínas de ancoragem (GEIGER; PEEPER, 2009).

Após se desprenderem do seu epitélio de origem, as células cancerosas invadem a circulação sanguínea ou linfática, e podem atingir órgãos distantes. Para que essas células consigam atingir os vasos, elas precisam romper a membrana basal e remodelar a matriz extracelular (MEC) circundante ao tumor (WALKER; MOJARES; DEL RÍO HERNÁNDEZ, 2018).

Essas células, quando têm sucesso em alcançar os vasos linfáticos, podem ficar presas nos linfonodos, dando origem a metástases nos linfonodos, ou podem ganhar a via sanguínea atingindo diferentes órgãos, formando as metástases à distância. Porém, são poucas as células que conseguem se alojar em um novo local, sobreviver e proliferar, fundando um novo sítio metastático (ALBERTS et al., 2017).

A EMT se define como a transformação de células epiteliais em células mesenquimais. Esse processo pode ocorrer em três momentos do desenvolvimento humano: durante o desenvolvimento embrionário, na regeneração tecidual, e na progressão do câncer (KALLURI et al., 2009).

As células epiteliais possuem uma polaridade apical-basal e mantêm contato com células adjacentes através das chamadas junções aderentes, junções comunicantes e dos desmossomos. Por outro lado, células mesenquimais estão dispersas na MEC, não possuem membrana basal as separando do tecido adjacente e não possuem polaridade (RIBATTI; TAMMA; ANNESE, 2020). Para que a EMT ocorra, as células epiteliais devem perder as junções aderentes, além de haver uma diminuição na expressão de marcadores epiteliais, como citoqueratinas e E-caderinas. Em compensação, ocorre um aumento na expressão de marcadores mesenquimais, como fibronectina, N-caderina e vimentina, bem como o ganho de um fenótipo invasivo fibroblastóide e de resistência a apoptose (ZEISBERG et al., 2009).

Em cânceres de origem epitelial, como o adenocarcinoma de próstata, após a ativação da EMT, as células cancerosas perdem sua polaridade e suas ligações célula-célula para ganhar propriedades migratórias e invasivas, se tornando células mesenquimais (THIERY et al., 2009). E para que a metástase ocorra é necessário que além do ganho de mecanismos de invasão, a célula seja capaz de reorganizar seu citoesqueleto, alterar a expressão de moléculas de adesão celular e degradar a membrana basal através da ativação de Metaloproteinases (MMPs) (WICK; PLATTEN; WELLER, 2001).

Estudos em tumores de mama e pele em modelos de camundongo indicam que a ativação da EMT é necessária para a disseminação tumoral inicial, porém, após a chegada no novo local do organismo, a célula deve reverter este processo e voltar a apresentar características epiteliais (OCAÑA et al., 2012; TSAI et al., 2012), o que evidencia a importância deste processo na evolução tumoral e na metástase.

2.5. Matriz Extracelular

A MEC é comumente definida como um componente acelular que provê suporte estrutural e bioquímico para os tecidos (WALKER; MOJARES; DEL RÍO HERNÁNDEZ, 2018). A MEC é um componente fisiologicamente ativo do tecido, responsável pela comunicação célula-célula, pela adesão e proliferação celular (WALKER; MOJARES; DEL RÍO HERNÁNDEZ, 2018).

É composta pelo interligamento de água, minerais, proteoglicanos, glicosaminoglicanos, glicoproteínas adesivas e proteínas fibrosas secretadas por células residentes. Cada tecido possui uma composição particular desses elementos, determinada durante o desenvolvimento embrionário, e que depende da função exercida por cada órgão. A produção desses compostos é adaptada durante as fases do desenvolvimento e da progressão de doenças (BONNANS; CHOU; WERB, 2014).

No tecido epitelial, a MEC se organiza em uma estrutura muito delgada chamada membrana basal, que é composta principalmente por colágeno tipo IV, laminina, nidogênio e proteoglicanos de heparan-sulfato. Essa camada muito fina e flexível suporta todo o epitélio e é fundamental na manutenção da homeostase tecidual e arquitetura corporal (ALBERTS et al., 2017).

A constituição da MEC é muito dinâmica. Mesmo após o desenvolvimento embrionário, ela está constantemente sendo depositada, degradada e modificada para garantir a homeostase dos tecidos (LU et al., 2011; GATTAZZO; URCIUOLO; BONALDO, 2014). Pequenas mudanças nesse equilíbrio podem acarretar alterações significativas nos estímulos de proliferação de células cancerosas (FANG et al., 2014). Conforme as células tumorais proliferam, a MEC circundante passa por mudanças estruturais, como o aumento da secreção de fibronectina e colágenos I, III e IV. O aumento na deposição dos componentes de matriz promove a progressão tumoral através da interferência na adesão célula-célula e na polaridade celular, além de amplificar a sinalização de fatores de crescimento (MALIK; LELKES; CUKIERMAN, 2015).

2.5.1. Colágeno

Os colágenos são as mais abundantes proteínas do nosso corpo. A molécula de colágeno é uma tripla hélice helicoidal longa e rígida, onde três cadeias polipeptídicas, chamadas de cadeias l-, são enroladas entre si formando uma super-hélice semelhante a uma corda. Todos os colágenos são sintetizados pelas células às quais a MEC está interagindo (ALBERTS et al., 2017).

Existem 28 tipos de colágeno, dentre eles, 90% do colágeno que possuímos em nosso corpo é o do tipo I (Col I), formado por duas cadeias l-1 e uma cadeia l-2, com comprimento de aproximadamente 300 nm e largura de cerca de 1 a 5 nm (HENRIKSEN; KARSDAL, 2016). Essa proteína compõe o tecido conjuntivo dos nossos órgãos em forma de fibras espessas. O colágeno tipo III (Col III), composto por três cadeias l-1, forma uma estrutura fibrilar e ramificada, formando fibrilas mais finas e está presente na constituição da pele, vasos e órgãos. Outra classe muito abundante é o colágeno do tipo IV, que é o componente mais abundante e estrutural das membranas basais e forma uma estrutura semelhante a uma rede (SUN, 2021; ALBERTS et al., 2017).

Durante a progressão do câncer, o colágeno presente na MEC tem um papel muito importante. Foi observado que os fibroblastos associados às células cancerosas aumentam a deposição de colágeno, que passa a ser alinhado, formando ligações cruzadas entre as diferentes fibras. As enzimas lisil oxidases (LOX) são as responsáveis por catalisar as reações de ligação cruzada, o que aumenta a rigidez e o volume da MEC circundante (XIAO; GE, 2012). Esse aumento na rigidez ativa integrinas e promove a mobilidade celular (LU et al., 2011). Além disso, a organização das proteínas fibrosas da MEC agem como trilhas, onde as células proliferativas neoplásicas migram para fora do tumor (XIAO; GE, 2012).

Estudos *in vitro* realizados por Han e colaboradores (2016) avaliaram a migração de células provenientes de uma linhagem celular de câncer de mama metastático em um microambiente com fibras de Col I organizadas. A partir de seus resultados, esse grupo de pesquisa provou que as orientações das fibras de colágeno I têm um importante papel como guia das células cancerosas em seu caminho migratório até os vasos do sistema circulatório.

Resultados interessantes foram encontrados por Penet e colaboradores (2017) a partir da análise das fibras de Col I em amostras de tecido tumoral e não tumoral adjacente de pacientes com CaP. Foi constatado que em tecido neoplásico maligno as fibras de Col I se encontravam mais alinhadas e lineares do que aquelas observadas em tecido não tumoral, que se encontravam orientadas de maneira aleatória.

Ling e colaboradores (2017) analisaram a orientação das fibras de colágeno total em amostra de CaP com diferentes escores de Gleason. Seus resultados indicaram que essas fibras se encontram mais organizadas no tecido prostático de acordo com o aumento da agressividade dos tumores. Eles constataram que quanto menos agressivo o tumor, mais isotrópicas são as fibras, e com o aumento da agressividade ocorre um aumento da anisotropia. Dessa forma, observamos a relevância do estudo da orientação das fibras de colágeno no contexto da tumorigênese prostática.

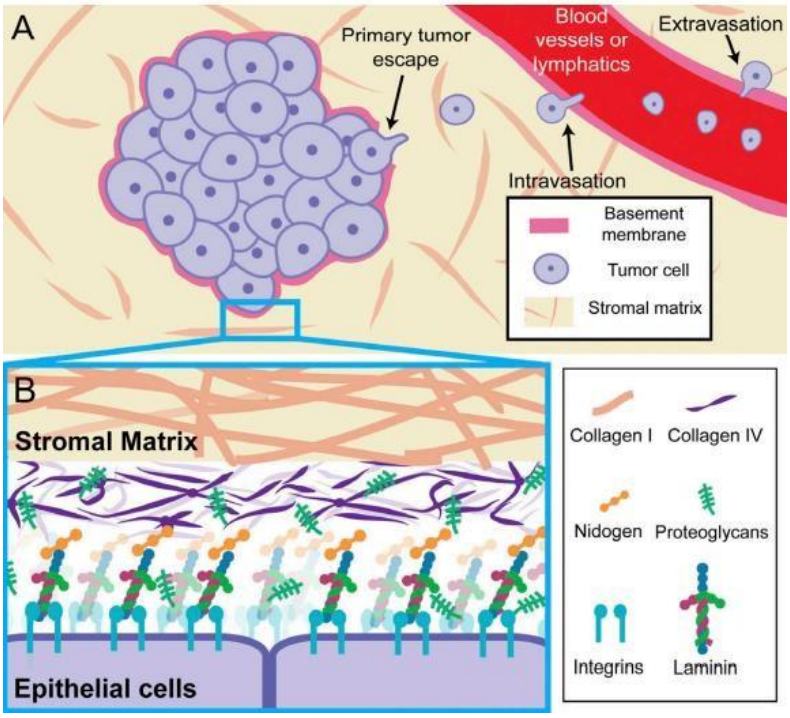
2.5.2. Membrana Basal

A membrana basal é uma lâmina fina e densa de MEC que desempenha um papel importante no desenvolvimento normal dos tecidos e de sua função (YURCHENCO, 2011). Essa estrutura é essencial na sinalização celular, na integridade estrutural e serve como barreira protetiva contra células e grandes moléculas (CHANG; CHAUDHURI, 2019). Ela separa o tecido epitelial, endotelial, adiposo, nervoso e cardíaco de seus respectivos tecidos conectivos (FIDLER et al., 2017).

Seus dois componentes mais abundantes são o colágeno tipo IV, que representa a sua principal proteína estrutural, e a laminina, que regulam a adesão, a migração, o crescimento e a diferenciação celular por conta de sua ligação com os receptores de membrana celular. Outros componentes incluem nidogênios, proteoglicanos e fatores de crescimento como VEGF (fator de crescimento endotelial vascular) e PDGF (fator de crescimento derivado de plaquetas) (CHANG; CHAUDHURI, 2019). O nidogênio tem a função de ligar de maneira não covalente a laminina e as redes de colágeno IV, proporcionando estabilização contra tensões mecânicas. Já os proteoglicanos desempenham funções diversas, como a regulação da atividade de fatores de crescimento e a promoção da angiogênese (IOZZO, 2005).

Anormalidades nas propriedades químicas e estruturais da membrana basal implicam em doenças, como por exemplo o câncer. Em tumores malignos do tipo carcinomas, a membrana basal epitelial serve como uma barreira física contra a invasão celular na MEC circundante, enquanto a membrana basal endotelial, presente nos vasos sanguíneos e linfáticos, impede a invasão de células para dentro e para fora dos vasos do sistema circulatório (LAMBERT; PATTABIRAMAN; WEINBERG, 2017) (Figura 4).

Figura 4. A- Esquema que representa o mecanismo de invasão de células tumorais através das membranas basais epiteliais e endoteliais. B- Representação das estruturas que compõem a membrana basal e como se comunicam com a MEC e com as células epiteliais.



Fonte: CHANG, CHAUDHURI, 2019.

Existem duas formas com que as células podem romper a membrana basal do tecido. A primeira e mais comum delas é a degradação dependente de proteases, onde as enzimas são usadas pelas células para degradar quimicamente a MEC, incluindo a membrana basal (CHANG; CHAUDHURI, 2019). Existem 6 classes de proteases que comumente estão envolvidas na invasão do câncer, porém, as MMPs são especialmente importantes durante a degradação de membrana basal (EATEMADI et al., 2017).

A outra forma de rompimento é a forçada, que sempre foi associada à células do sistema imune, porém também passou a ser vinculada às células cancerosas (WANG et al., 2006). Nesse sistema, as células tumorais aproveitam poros nanométricos (que medem entre 32 a 112 nm) presentes na estrutura da membrana basal para extravasar para a MEC. A partir da força de contato, as células aumentam o tamanho dos poros criando canais grandes o suficiente por onde elas podem passar (WISDOM et al., 2018).

No CaP, estudos indicam que alterações na integridade da membrana basal são peça chave em seu prognóstico (LIU et al., 2009).

2.5.3. Metaloproteinases

As MMPs são outra família de proteínas enzimáticas responsáveis pelo remodelamento da MEC. As MMPs são enzimas proteolíticas que atuam em diferentes substratos, mas que compartilham características estruturais similares. Elas são dependentes de zinco (Zn^{2+}) e são funcionais em pH neutro (CUI; HU; KHALIL, 2017; AMĔLINEI; CĔRUNTU; BĔLAN, 2007). Sua principal função é degradar as proteínas e glicoproteínas da MEC, receptores de membrana, citocinas e fatores de crescimento (KLEIN; BISCHOFF, 2011; MASKOS, 2005). Estão envolvidas em diversos processos biológicos, como o remodelamento e reparo dos tecidos, diferenciação e mobilidade celular (CUI; HU; KHALIL, 2017; AMĔLINEI; CĔRUNTU; BĔLAN, 2007).

Os tecidos humanos expressam 23 tipos de MMPs, que podem ser agrupadas segundo sua especificidade de substrato, similaridade sequencial e organização de domínio em: colagenases, gelatinases, estromelisinas, metrilisinas, MMPs de membrana e outras MMPs (CUI; HU; KHALIL, 2017). As colagenases (MMP-1, MMP-8, MMP-13 e MMP-18) têm como papel mais importante a clivagem dos colágenos fibrilares dos tipos I, II, III, IV e XI em dois fragmentos (CUI; HU; KHALIL, 2017; AMĔLINEI; CĔRUNTU; BĔLAN, 2007). Já as gelatinases (MMP-2 e MMP-9) são reconhecidas como as principais enzimas envolvidas na degradação da MEC e estão envolvidas na invasão tumoral e metástase (CUI; HU; KHALIL, 2017). As estromelisinas (MMP-3, MMP-10 e MMP-11) são muito parecidas com as colagenases, porém não possuem a capacidade de clivar o colágeno intersticial (CUI; HU; KHALIL, 2017). Além disso, elas são capazes de fragmentar outras proteínas que compõem a MEC, como proteoglicanos, glicoproteínas, fibronectina e laminina (ZÍTKA et al, 2010). As metrilisinas correspondem as MMP-7 e MMP-26, as MMPs de membrana são as MMP-14, -15, -16 -17, -24 e -25, e as outras MMPs correspondem a outros 8 tipos de MMPs (CUI; HU; KHALIL, 2017).

A desregulação na expressão de MMPs leva a progressão de diversas doenças, que podem ser agrupadas em: fibroses, que podem levar à cirrose hepática e aterosclerose; enfraquecimento de matriz, que podem causar aneurismas aórticos; e destruição de tecidos, que é um processo-chave no câncer e na metástase (CUI; HU; KHALIL, 2017; LARONHA; CALDEIRA, 2020).

Durante a proliferação tumoral e a metástase, as MMPs são responsáveis por diversos papéis. Elas auxiliam na degradação da MEC circundante das células cancerosas proliferativas, e também causam a liberação de fatores de crescimento ativos, promovendo a angiogênese tumoral. As MMPs conseguem liberar esses fatores de crescimento a partir do afrouxamento

do colágeno presente na MEC, fazendo com que eles sejam liberados para interagir com a membrana celular (DISCHER; MOONEY; ZANDSTRA, 2009; DERYUGINA; QUIGLEY, 2006).

A MMP-2, também chamada de gelatinase A, é codificada pelo gene presente no braço longo do cromossomo 16, e tem a capacidade de clivar os colágenos tipo I, III, IV, V, VII e X além de outros componentes da MEC. Ela degrada o colágeno em duas etapas, primeiro induzindo uma degradação semelhante à colagenase, e em seguida promovendo a lise da gelatina (LARONHA; CALDEIRA, 2020).

Em estudos *in vitro* que visavam avaliar a influência de proteínas na angiogênese tumoral, o grupo de pesquisa de Bergers (2000) constatou um aumento da expressão de MMP-2 em amostras de tumores pancreáticos e de isolados angiogênicos, mostrando assim, que essa proteína está associada com a progressão tumoral.

Em estudos em CaP, Escaff e seus colaboradores (2011) compararam a imunomarcação de MMP-2 em tecido tumoral e em hiperplasia benigna prostática a partir de análises imunohistoquímicas. Seus resultados mostraram uma diferença significativa na imunomarcação dessa enzima, que se apresentou de maneira mais abundante no tumor de próstata.

Também em CaP, Trudel e colaboradores (2003) estudaram o papel da MMP-2 na sobrevida livre de doença de pacientes com tumores prostáticos. Eles encontraram uma associação entre o aumento da expressão de MMP-2 e a diminuição da sobrevida livre de doença, sugerindo que essa proteína pode ser um preditor independente de prognóstico.

A metanálise realizada pelo grupo de pesquisa de Xie (2016) analisou 8 trabalhos que associaram a expressão de MMP-2 com o CaP. Seus resultados indicaram que a superexpressão dessa proteína está relacionada com o CaP, além de associar o aumento de sua expressão com o aumento do escore de Gleason dos pacientes. Mostrando assim que tal proteína está correlacionada com tumores mais avançados.

A MMP-9, codificada pelo gene localizado no braço longo do cromossomo 20, é produzida por uma variedade de células, como as epiteliais e os fibroblastos. Ela é sintetizada em sua forma inativa de pro-MMP-9, e depende de outras proteases para ser clivada e gerar sua forma ativa (CUI; HU; KHALIL, 2017; VANDOOREN; VAN DEN STEEN; OPDENAKKER, 2013).

Estudos da expressão de MMP-9 em carcinoma de próstata através da técnica de imunohistoquímica, realizados por Oguic e colaboradores (2014), correlacionaram a alta expressão da proteína com tumores que apresentaram recidiva bioquímica. Evidenciando, assim, a associação dessa proteinase com tumores de pior prognóstico. Resultados parecidos

foram encontrados por Baspinar et al. (2017) que constataram um aumento na imunomarcação de MMP-9 em amostras de CaP com escores de Gleason e estadiamentos TNM altos.

Já as análises comparativas executadas pelo grupo de pesquisa de Medina-González (2020) demonstraram um aumento na expressão de MMP-9 em amostras de CaP. Nessa análise eles compararam a expressão em tecido tumoral com amostras de biópsias prostáticas negativas dos mesmos pacientes, realizadas anteriormente.

Esses resultados mostram a relevância de estudos que avaliam as MMPs em carcinomas prostáticos, ressaltando que tais moléculas podem constituir bons marcadores candidatos de uso futuro para a predição de processo metastático.

2.6. Uso de Técnicas Histológicas no Câncer

Atualmente, a análise histológica de amostras coletadas em biópsia guiada por ultrassom transretal continua sendo o exame padrão ouro para confirmar o diagnóstico de CaP em todas as situações (DESCOTES, 2019). Ela começou a ser usada em 1989, quando se mostrou superior à amostragem por biópsia dirigida digitalmente em um estudo de referência (HODGE et al., 1989). No entanto, outras técnicas que fazem uso de análises histológicas também são importantes para o diagnóstico e estabelecimento do prognóstico em diferentes tipos de câncer.

É o caso da técnica de imunohistoquímica (IHQ), que se baseia na ligação específica entre um anticorpo e uma proteína (antígeno) de interesse. Nessa técnica, a amostra tecidual é preparada, os epítopos dos antígenos são recuperados e o anticorpo primário é ligado a eles, sendo sua coloração revelada em seguida (FERRO, 2014).

Ela é muito utilizada para a subclassificação de tumores de mama. Nessas neoplasias malignas, os cânceres luminais, triplo-negativos e HER2-enriched possuem tratamentos distintos, e sua classificação exata é essencial para o direcionamento correto da conduta terapêutica. Essa classificação por IHQ só é possível pois cada tipo tumoral expressa proteínas diferentes (BONACHO; RODRIGUES; LIBERAL, 2020).

Além de ser usada na clínica oncológica, a IHQ é utilizada na pesquisa básica e clínica para exploração de novos biomarcadores potenciais (KIM; ROH; PARK, 2016). Uma das vantagens de se usar tal metodologia é que ela é realizada sem que haja a destruição da arquitetura tecidual, e assim é possível avaliar a imunomarcação da molécula no contexto do microambiente celular (SCHACHT; KERN, 2015).

No CaP, a IHQ é largamente utilizada para avaliação de marcadores candidatos. Como exemplo, Pereira et al. (2022) avaliaram a imunomarcação das proteínas TRPM8 (*transient*

melastatin 8), NKX3.1 (*NK3 homeobox 1*), AKT (*serine-threonine kinase*) e PTEN (fosfatase homóloga à tensina) em amostras tumorais metastáticas e não metastáticas, e encontraram evidências de que tanto a proteína AKT quanto TRPM8 podem agir como marcadores de prognóstico para essa neoplasia. Sharma, Yang e Miyamoto (2019) avaliaram as proteínas PD-1 (proteína de morte celular programada 1) e PD-L1 (ligante de proteína de morte celular programada 1) em 220 amostras de tecido tumoral prostático para avaliar sua significância prognóstica, porém não encontraram resultados significativos.

A técnica histoquímica Picrosirius também se utiliza de cortes histológicos e é baseada no realce da birrefringência natural do colágeno, que pode ser observada através da polarização da luz (RITTIÉ, 2017; ZUNDER et al., 2020). Tal metodologia é barata, porém necessita de uma coloração adicional para visualização das outras estruturas teciduais (ZUNDER et al., 2020). No câncer, ela é utilizada, por exemplo, no estudo de carcinoma oral de células escamosas, onde a análise das alterações na MEC circundante ao tumor indicou uma relação entre a mudança nas fibras de colágeno e piora no prognóstico tumoral (SHARMA et al., 2015; KALELE et al., 2014).

A coloração histoquímica Ácido Periódico de Schiff (PAS) também se utiliza de cortes histológicos e tem a função de corar as ligações 1,2-glicol de carboidratos para produzir aldeídos (MCMANUS, 1948). Ela é muito utilizada para avaliação de infecções por microrganismos (DADACI et al., 2015; FANTRY et al., 2016; PAI; PAI; SHARMA, 2015). No câncer, o PAS foi utilizado como método para investigar a relação da membrana basal com carcinoma espinocelular de esôfago em vários estágios. Constatou-se uma relação direta entre essa estrutura de MEC e o desenvolvimento dessa neoplasia maligna (ZHANG et al., 2008).

Dessa forma, vê-se a importância de técnicas que façam uso de cortes histológicos no diagnóstico e prognóstico do câncer, incluindo aquele da próstata, e em especial, na busca por novos marcadores para uso futuro, conforme proposto neste trabalho.

3. OBJETIVOS

3.1. Objetivos gerais

O objetivo deste trabalho foi avaliar a presença e os perfis de imunomarcação de proteínas envolvidas na constituição e remodelação da MEC em amostras de tecido de pacientes com CaP metastático e não metastático como marcadores preditivos do processo de metástase desta neoplasia.

3.2. Objetivos específicos

Esse trabalho objetivou, de maneira específica:

- Selecionar amostras teciduais incluídas em parafina de pacientes com CaP pertencentes aos grupos de melhor prognóstico, pior prognóstico sem metástase e metastático do Biobanco do Laboratório de Mutagênese e Oncogenética da UEL;
- Quantificar a presença dos colágenos I e III em amostras de CaP de melhor prognóstico, pior prognóstico sem metástase e metastáticas, bem como em tecido não tumoral adjacente, para sua comparação;
- Avaliar semi-quantitativamente a imunomarcação das metaloproteinases 2 e 9 em amostras de CaP de melhor prognóstico, pior prognóstico sem metástase e metastáticas, bem como em tecido não tumoral adjacente, para sua comparação;
- Avaliar a integridade da membrana basal do epitélio prostático em amostras de CaP de melhor prognóstico, pior prognóstico sem metástase e metastáticas e correlacioná-la com parâmetros clínico-patológicos dos pacientes;
- Correlacionar os perfis de imunolocalização de tais proteínas com parâmetros clínico-patológicos dos pacientes;
- Colaborar na identificação de possíveis biomarcadores candidatos para a carcinogênese prostática e que possam ser utilizados na clínica futuramente.

4. CAPÍTULO 1

Collagen III deposition and basement membrane integrity alterations: possible predictive markers in metastatic prostate cancer

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Collagen III deposition and basement membrane integrity alterations: possible predictive markers in metastatic prostate cancer

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ABSTRACT

Metastasis represents the major cause of deaths in cancer patients, and the tumor surrounding extracellular matrix (ECM) passes through changes in its organization during the evolution of this process. Therefore, the aim of the present study was to quantify the deposition of proteins that constitute the ECM, namely total collagen, collagen I (Col I) and collagen III (Col III) in samples from patients with metastatic (mPCa) and non-metastatic prostate cancer (PCa), in addition of evaluating the basement membrane integrity. Tissue samples from 60 patients were divided into three groups according to parameters ISUP grade, TNM staging and PSA concentration: better prognosis (n=20), worse prognosis (n=23) and metastatic (n=17). To quantify collagen, the Picrosirius Red technique was used with further analysis under a polarization microscope, and to basement membrane analysis the Periodic Acid Schiff (PAS) technique was employed, where the staining was classified in G1, G2 and G3. It was observed that the Col I/ Col III ratio was higher in the metastatic group in relation to better prognosis (p=0.012) and worse prognosis (p=0.018) groups. About the basement integrity, it was observed that its constitution in the malignant tumor tissue differed from the adjacent non-tumor tissue (p=0.000). Also, the worsening in the tumor tissue integrity was positively correlated with worse prognosis parameters (advanced ISUP grade, extraprostatic extension and perineural invasion). Our study indicates that the absence of Col III can constitute a marker for potential metastatic tumors. The basement membrane integrity also seems to be an indicator of poor prognosis in malignant prostatic tumors.

Keywords: Extracellular Matrix; Collagen I; Col I/ Col III ratio; Picrosirius; PAS.

1 INTRODUCTION

Worldwide, prostate cancer (PCa) is the second most common malignant neoplasia in men, and represents the fifth leading cause of cancer-related deaths [1]. In 2020, in Brazil, was responsible for about 65,840 new cases, second only to non-melanocytic skin tumors [2].

According to the Urology Brazilian Society, PCa screening is performed through digital rectal examination and measurement of prostate-specific antigen (PSA) in the bloodstream [3]. The increase in PSA concentration may indicate, in addition to PCa, benign prostatic hyperplasia and prostatitis [4]. Screening based on this antigen can lead to the diagnosis of indolent tumors, the so-called overdiagnosis [4].

The onset of PCa progression rarely presents symptoms, and when they do occur, they are nonspecific, such as difficulty urinating and a weak and intermittent urinary stream [4, 5]. In advanced stages of the disease, it can cause metastases, which are the biggest cause of death for patients [6], and generate symptoms associated with affected organs, which are normally bones, lymph nodes, liver and lung [7]. So, the study of biomarkers capable of differentiating more or less aggressive tumors is of great relevance in the management of patients with this malignant neoplasm.

Metastasis consists in the migration and multiplication of cancerous cells in new organism places after the loss of control over their adhesion mechanisms [8]. After detaching from their epithelium of origin, these cells rupture the basement membrane and remodel the surrounding extracellular matrix (ECM) so that they can invade the blood or lymphatic circulation [9]. This way, the study of proteins that constitute the ECM seems to be a promising field in the context of cancer aggressiveness, including PCa.

Collagen, the most abundant protein in the human body, is the most significant ECM component. Each of its 28 subtypes is composed of three homologous or non-homologous polypeptide chains that coil with an asymmetric axis [9, 10]. The fibrillar collagens, like Col I and Col III, form fibrous structures [10], such as the fibromuscular stroma that surrounds the prostate [11]. Evidences indicate that the organization of Col I fibers plays an important role on the migration of neoplastic cells through tissue, both in breast cancer [12] as in PCa [13]. The spatial orientation of total collagen fibers also seems to interfere in the aggressiveness of prostate tumors [14].

Another ECM structure, the basement membrane, is a thin and dense sheet that acts in cell signaling and structural integrity, serving as a protective barrier against cells and large molecules [15]. Its two most abundant components are collagen IV and laminin, while other elements include nidogens, proteoglycans and growth factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) [15]. Abnormalities in chemical and structural properties of basement membrane implicate in the development of diseases, including cancer [16]. According to Liu et al. [17], its integrity is a key piece in the prognosis of PCa.

Since Col I and Col III are the main fibrous proteins that constitute the surrounding ECM of malignant tumors, and considering the lack of markers capable of predicting aggressiveness and metastatic capacity, together with the high incidence of prostate tumors, this study aimed to quantify the deposition of total collagen, Col I and Col III in metastatic and non-metastatic PCa samples, in addition to evaluating the integrity of the basement membrane, in the search for candidate markers of prognostic in PCa.

2 METHODS

2.1 Study Group and Sample Characterization

This retrospective and longitudinal study comprehended the same sample group analyzed by Pereira et al. [18], which corresponds to 60 samples of tissues embedded in paraffin from patients with PCa and their respective adjacent non-tumor tissues. The samples were selected from patients diagnosed with PCa in the Hospital do Cancer de Londrina (HCL) between the years 2006 and 2016. Of this amount, 50 samples are from radical prostatectomy, 5 from biopsy and 5 from transurethral resection. A Table containing all the clinical and pathological characteristics of the patients was included as Online Resource 1.

The study was approved by the Research Ethics Committee Involving Human Beings of the State University of Londrina - Brazil, under number 176/2013. Patients participated voluntarily and signed a free and informed consent form.

Histopathological data were obtained from medical records, which were used, together with the guidelines of the National Comprehensive Cancer Network [19], for the classification of patients into three experimental groups: 1) PCa with better prognosis (n=20); 2) PCa with worse prognosis (n=23); and 3) metastatic PCa (n=17). Patients with ISUP grade ≤ 2 (3+4), TNM staging $\leq$ T2a, and PSA $\leq$ 10ng/mL were considered to have a better prognosis. Patients with ISUP grade ≥ 3 (4+3), TNM staging $\geq$ T3a, and PSA $\geq$ 20 ng/mL were considered to have worse prognosis. Patients with metastasis at diagnosis were classified according to the presence of lymph node invasion and/or distant metastasis and/or positive bone scintigraphy.

2.2 Histopathological Analysis

Tissues obtained from biopsies were stained with hematoxylin and eosin to confirm the diagnosis of PCa and to verify the presence of tumor and adjacent non-tumor tissue for further comparison. The histopathological classification used was based on international standards established by the World Health Organization, such as ISUP grade [20] and clinical staging determined by the Tumor/Lymph Node/Metastasis (TNM) system, following the recommendations of the AJCC (American Joint Committee on Cancer).

2.3 Collagen Quantification

Based on Pupim et al. [21], one slide per sample was made with one histological cut of 6µm from the 60 tissue samples. The sections were stained with Picrosirius Red, which enhances the birefringent characteristic of collagen, and counterstained with hematoxylin. Thus, it was possible to quantify Col I, Col III and total collagen using a polarized light microscope. Five images were captured from each sample, most of them containing tumor and adjacent non-tumor tissue (3 samples showed only tumor tissue). The images were obtained, using regular and polarized light, using an AxioCam high resolution camera (Carl Zeiss, Jena, Germany) attached to an Axioscop Plus light microscope (Carl Zeiss, Jena, Germany; x10 objective) using the software AxioVisionRel 4.1 (Carl Zeiss, Jena, Germany). Additionally, the quantification of collagen deposition was performed in a normal prostate sample as a positive control.

The images using regular light were evaluated with the software Image-Pro Plus 4.5 (Media Cybernetics, Silver Spring, EUA) to obtain the number of pink pixels (which correspond to total collagen), and images in polarized light were quantified with the same software to count yellow and red pixels (which refer to Col I), and green pixels (which correspond to Col III). Results were summed and expressed in percentage (%) and area (μm^2).

2.4 Evaluation of Basement Membrane Integrity (PAS)

One slide per sample was made with a 6µm histological cut of the 60 samples and processed for histochemical staining of Periodic Acid Schiff (PAS), in order to reveal the epithelium basement membrane. Sections were counterstained with hematoxylin. Ten images were captured from each sample, 5 from malignant tumor e 5 from adjacent non-tumor tissue, using a AxioCam high resolution camera attached to an Axioscop Plus light microscope (x40 objective) using the software AxioVisionRel 4.1.

Images were analyzed for the basement membrane integrity, both in the malignant and non-malignant tissues. For this, the scores proposed by Colling et al. [22], ranging from G1 to G3 depending on the intensity, thickness, and continuity of PAS staining. The G1 was considered when the staining was barely visible and discontinuous. The G2 was designed when the labeling was relatively easy to see, but with varying thickness and discontinuity in some places. The G3, on the other hand, represented thick, well-visible and continuous stains. Additionally, the observation of the basement membrane profile was made in a normal prostate sample as a positive control.

2.5 Statistical Analysis

In collagen deposition analysis, all data initially passed through the Shapiro-Wilk normality test. Those with a normal distribution were compared between groups (PCa with better prognosis, PCa with worse prognosis,

and metastatic PCa) by one-way ANOVA, followed by Tukey's post-hoc test. Data that did not pass the normality test were submitted to the non-parametric Kruskal Wallis test, followed by paired analysis.

In the analysis of basement membrane integrity, the McNemer test was used to compare PAS staining between malignant tumor and adjacent non-tumor tissue. Kendall's Tau test was applied to the correlation between histochemical staining and clinical and pathological parameters of the patients. The parameters used in the correlation were: prognostic groups, age, ISUP grade, TNM staging, PSA quantification, seminal vesicle invasion, extraprostatic extension, perineural invasion and biochemical recurrence.

Some data were excluded from the statistical analysis due to the lack of information contained in medical records or because some samples did not have malignant tumor and adjacent tissue in the same slice for comparison.

All statistical analyses were carried out using IBM® SPSS® software Statistics for Windows, version 20.0 (IBM® Corp., Armonk, N.Y., USA), considering a significance level (ł) of 5%.

3 RESULTS

3.1 Collagen Deposition

In a general way, it was possible to quantify the total collagen deposition in all samples and in the positive control from images captured in common light (Fig 1a), and Col I and Col III (Fig 1b) in images captured in polarized light. Tables containing the quantification values were provided in the Online Resource 2-5.

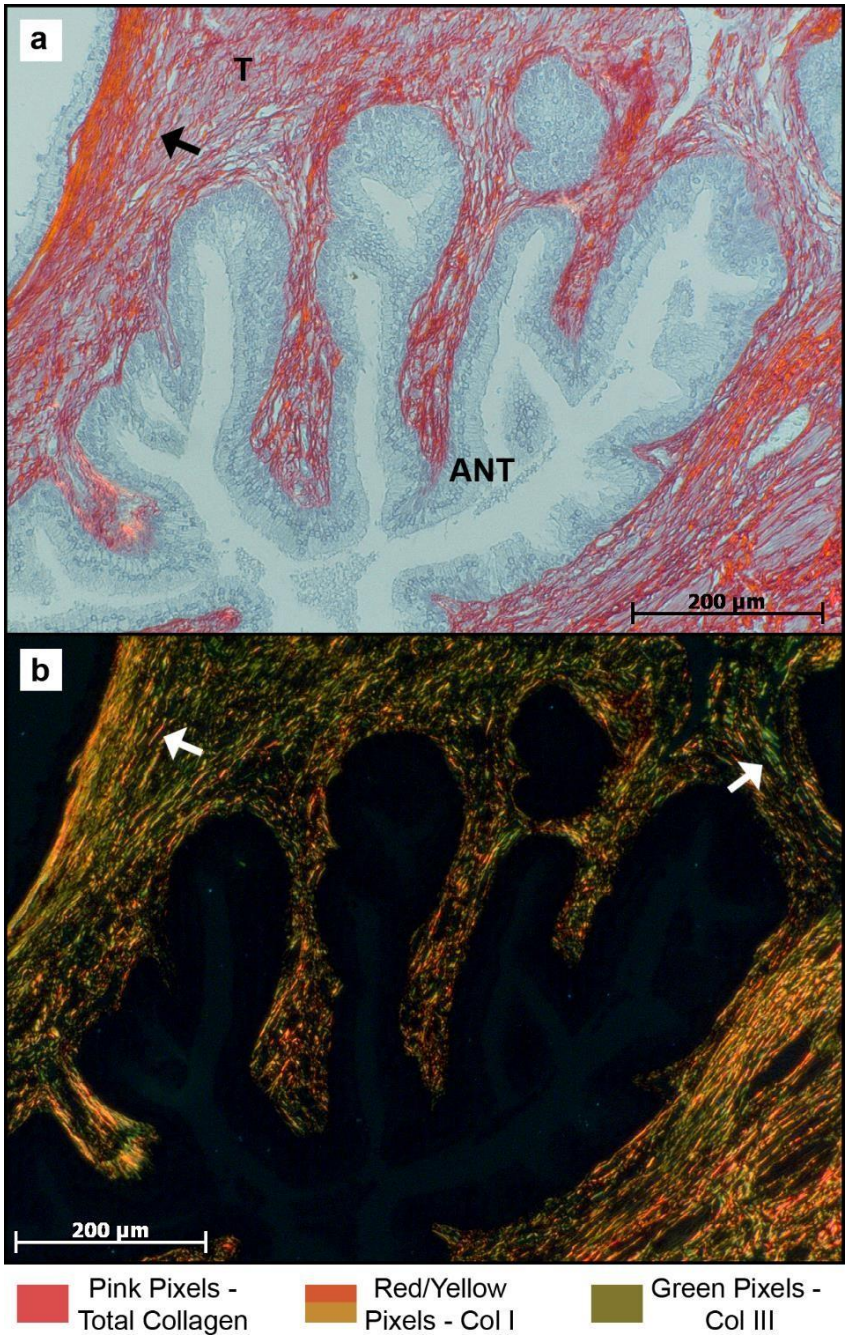

Fig 1 Example of total collagen, Col I and Col III deposition profile in tissue sample from a patient with better prognosis PCa. Photomicrograph of a PCa tissue sample after treatment with Picrosirius Red histochemical stain. Total collagen fibers under regular light (a), with arrow indicating a collagen fiber; and collagen fibers I and III under polarized light (b) with arrows indicating Col I (left arrow) and Col III (right arrow) fibers. Tumor (T) and adjacent non-tumor (ANT) tissues are present in most of the samples as represented in image a. 10x magnification.

Means of Col I deposition did not differ between the three prognostic groups (p=0.066) (Fig 2a). Regarding the deposition of Col III, a significant difference was found between the groups with better prognosis and metastatic PCa (p=0.001), where the first one had higher deposition of Col III (Fig 2b). Means of Col I/ Col III ratio presented significant differences between better prognosis and metastatic (p=0.012), and between worse prognosis and metastatic (p=0.018) (Fig 2c). In this analysis, the metastatic group presented a higher mean,

evidencing a decrease in Col III deposition in these samples. Regarding the means of total collagen deposition, a significant difference was found in the presence of collagen between better prognosis X metastatic (p=0.001) (Fig 2d), which the latter showed greater protein deposition.

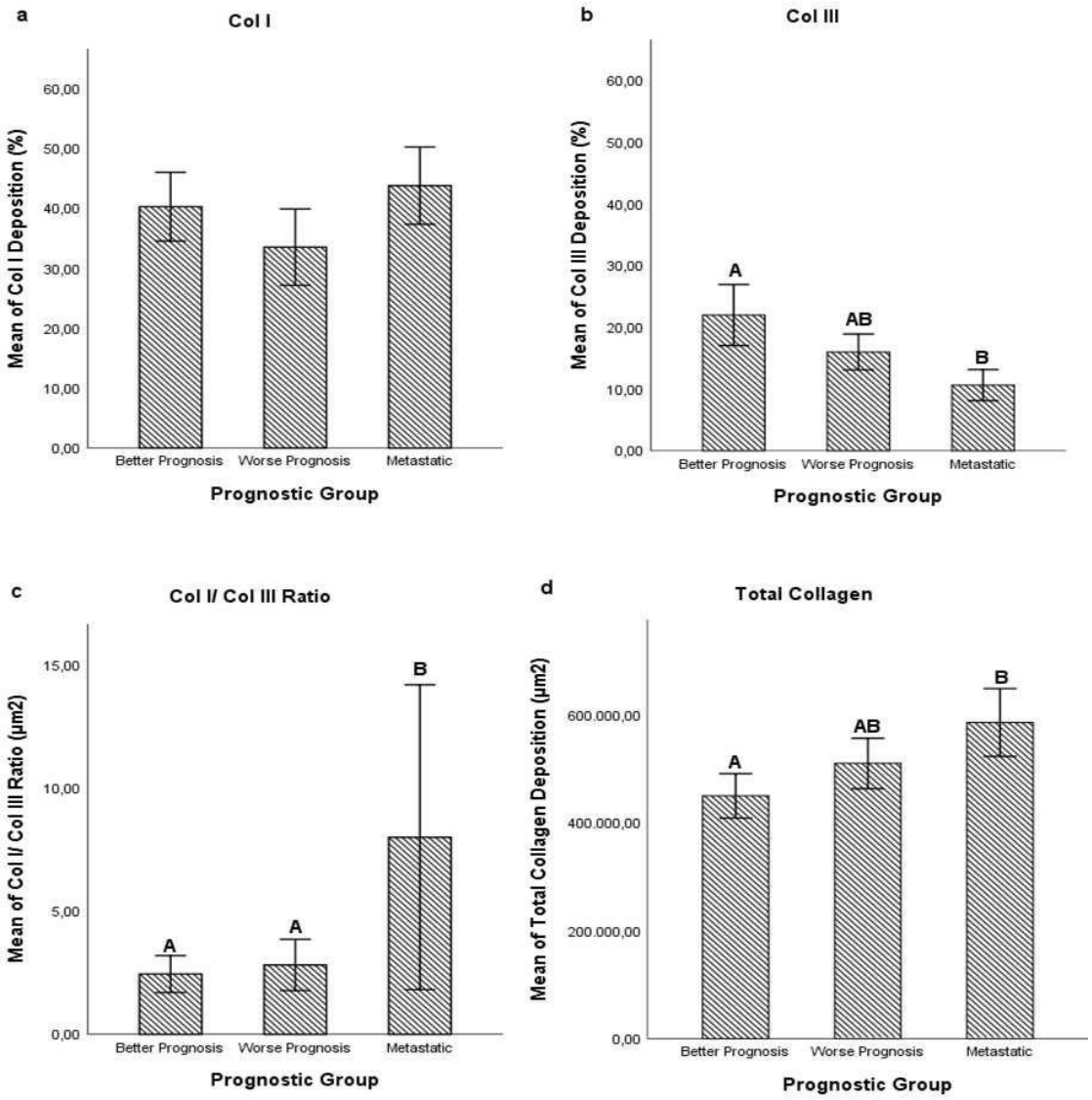

Fig 2 Graphics representing the collagen deposition by prognostic group. (a) Mean of Col I deposition (%) by prognostic group in patients with PCa. Normal distribution (p=0.413). One-way ANOVA test followed by Tukey's post-hoc. (b) Mean of Col III deposition (%) by prognostic group in patients with PCa. Abnormal distribution (p=0.002). Kruskal-Wallis test followed by pair comparison. (c) Mean of Col I/ Col III deposition (μm^2) by prognostic group in patients with PCa. Abnormal distribution (p=0.000). Kruskal-Wallis test followed by pair comparison. (d) Mean of total collagen deposition (μm^2) by prognostic group in patients with PCa. Normal distribution (p=0.222). One-way ANOVA test followed by Tukey's post-hoc. Significance level of p<0.05 with error bar representing the standard error. Different letters indicate means significantly different.

3.2 Basement Membrane Integrity

From the PAS histochemical staining, it was possible to identify the basement membrane of malignant tumor and adjacent non-tumor prostatic epithelium (Fig 3). In the adjacent epithelium, the pattern G1 basement membrane was found in 8 of 54 samples (14.8%), G2 pattern in 31 of 54 (57.4%), and G3 in 15 of 54 (27.8%). Regarding the tumor epithelium, the G1 basement membrane was observed in 34 of 59 samples (57.6%), G2 in 24 of 59 (40.7%), and G3 in 1 of 59 samples (1.7%) (Online Resource 6-8). The normal prostate tissue used as control presented a G3 pattern in the epithelium.

A significant difference was found in the basement membrane integrity between the malignant tumor tissue and adjacent non-tumor tissue (p=0.000). Furthermore, its integrity in tumor tissue was significantly correlated with ISUP grade (p=0.015; Tau=0.067), with extraprostatic extension (p=0.049; Tau=0.273) and with perineural invasion (p=0.044; Tau= 0.288) (Table 1).

Table 1 Comparison analysis between the integrity of the tumor basement membrane and the clinical and pathological parameters of patients with PCa.

Clinical-patological Data	Basement Membrane Integrity Score			p value of χ^2	p value of Kendall (value of Tau)
	G1 (%)	G2 (%)	G3 (%)		
Experimental Groups					
Better Prognosis	7 (20,6%)	11 (45,8%)	1 (100,0%)		
Worse Prognosis	16 (47,1%)	7 (29,2%)	0 (0,0%)	0,175	0,066 (0,226)
Metastatic	11 (32,4%)	6 (25,0%)	0 (0,0%)		
Age					
≤64 years	12 (35,3%)	8 (33,3%)	0 (0,0%)	0,761	0,738 (-0,044)
≥65 years	22 (64,7%)	16 (66,7%)	1 (100,0%)		
ISUP					
1	5 (15,2%)	11 (45,8%)	1 (100,0%)		
2 e 3	19 (57,6%)	9 (37,5%)	0 (0,00%)	0,067	**0,015*** (0,304)
4 e 5	9 (27,3%)	4 (16,7%)	0 (0,00%)		
TNM					
≤T1a	5 (17,2%)	7 (30,4%)	1 (100,0%)		
T2b a T2c	5 (17,2%)	6 (26,1%)	0 (0,00%)	0,225	0,059 (0,247)
≥T3a	19 (65,5%)	10 (43,5%)	0 (0,00%)		
PSA (ng/mL)					
<10	11 (33,3%)	12 (50,0%)	1 (100,0%)		
10 à 20	11 (33,3%)	5 (20,8%)	0 (0,00%)	0,519	0,231 (0,149)
>20	11 (33,3%)	7 (29,2%)	0 (0,00%)		
Seminal Vesicle Invasion					
Não	18 (66,7%)	18 (81,8%)	1 (100,0%)	0,406	0,191 (0,185)
Sim	9 (33,3%)	4 (18,2%)	0 (0,00%)		
Extraprostatic Extension					
No	9 (32,1%)	13 (56,5%)	1 (100,0%)	0,115	**0,049*** (0,273)

Yes	19 (67,9%)	10 (43,5%)	0 (0,00%)		
Perineural Invasion					
No	18 (66,7%)	19 (90,5%)	1 (10,00%)	0,126	**0,044*** (0,288)
Yes	9 (33,3%)	2 (9,5%)	0 (0,00%)		
Biochemical recurrence					
No	12 (50,0%)	7 (41,2%)	0 (0,00%)	0,577	0,581 (-0,087)
Yes	12 (50,0%)	10 (58,8%)	0 (0,00%)		

Kendall's Tau Correlation and Chi-square test. * Significance level of $p<0.05$. Due to lack of data and wear of paraffin blocks, some variables do not include the total number of patients.

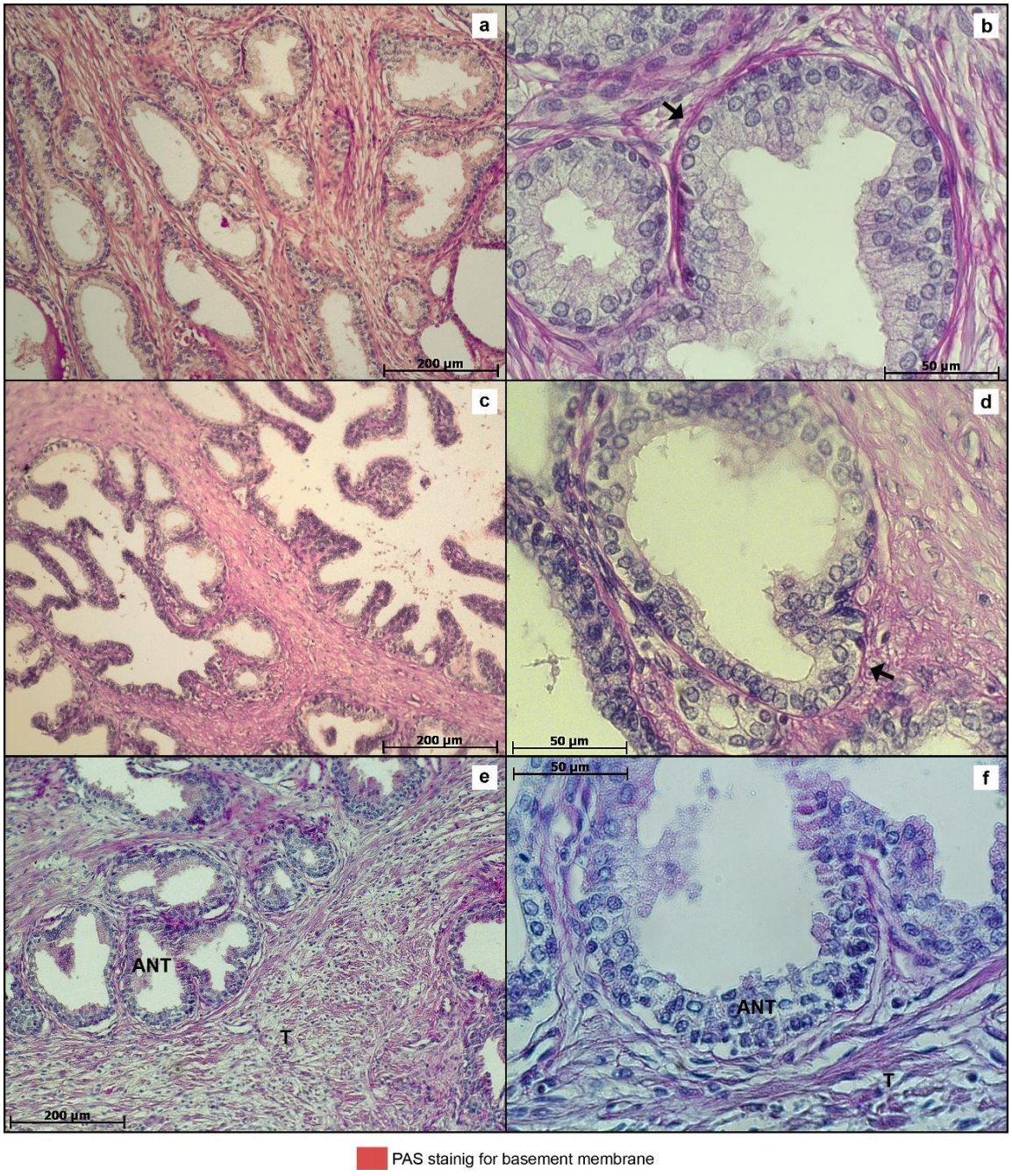

Fig 3 PAS staining profile in basement membrane with different integrity patterns in tissue samples from patients with PCa. Photomicrographs of PCa tissue samples after treatment with PAS histochemical stain with G3 (a and b), G2 (c and d) and G1 (e and f) patterns and arrows indicating the basement membrane. Tumor (T) and adjacent non-tumor (ANT) tissues are present in most of the samples analyzed, as represented in images e and f. 10x (first collum) and 40x (second collum) magnification.

4 DISCUSSION

In the present study, the quantities of total collagen, Col I and Col III were evaluated, as well as the basement membrane integrity in malignant prostate tumor samples. This analysis indicated that there was differential collagen deposition between the sample groups (patients with better prognosis PCa, worse prognosis PCa and with metastasis at diagnosis). The main results were the difference between the Col I/Col III ratio in the

metastatic group in relation to the groups with better and worse prognosis, evidencing a low deposition of Col III in this group. In addition, a decrease in basement membrane integrity was observed in relation to worse prognostic factors (advanced ISUP grade, presence of extraprostatic extension and perineural invasion).

According to Hanahan and Weinberg [23], the tumor microenvironment, composed by the ECM, is one of the factors that influence carcinogenesis, because to invade and migrate, cancer cells need to change the tissue microenvironment. Regarding the analysis of collagen deposition, our work is pioneer in the quantification of this protein in tissue samples from patients with PCa. We observed that Col I remained stable between the prognostic groups (better prognosis, worse prognosis and metastatic), while Col III was present in less quantity in the metastatic group when compared to the best prognosis group (p=0.001). These analyses were performed through the study of images captured in polarized light, capable of differentiating Col I and Col III fibrils, treated with Picrosirius Red staining, according to Rittié [24]. This technique is widely used to analyze interstitial collagen [25-27], and in cancer has already been used to analyze tumor staging of patients with oral squamous cell carcinoma (OSCC) [28, 29] and gastric cancer [30].

In the Col I/Col III ratio analysis, a higher mean value was found in the metastatic group, which differed from the mean values of the better (p=0.012) and worse prognosis (p=0.18) groups, indicating a decrease in Col III in these samples, since it is the denominator of the division. The *in vivo* and *in vitro* studies by Brisson et al. [31] evaluated the role of Col III in the development and metastasis of breast cancer. The authors found that Col III suppresses the metastatic behavior of triple negative breast cancer cells in vitro, in addition to slowing the growth and metastasis of tumors in vivo of mice models. Our results agree, as we found a higher concentration of this protein in tumors with a better prognosis when compared to metastatic tumors.

Studies based on the analysis of collagen fiber alignment in cancer are common in the literature [14, 32, 33] and aim to associate them with tumor prognosis. The study by Garcia et al. [32] analyzed the alignment of collagen fibers in tissue samples of PCa and found that there was an increase in alignment in tumors with higher Gleason score, therefore more aggressive. Thus, in healthy tissues, interstitial collagen is isotropically oriented, whereas collagen in tumors is often aligned and anisotropic. In breast, pancreatic, and other cancers, alignment of collagen fibers correlates with a worsening tumor prognosis [12, 34, 35].

Analysis carried out by the Second Harmonic Generation (SHG) found that for Col I to organize itself in a linear way, Col III must be present in low concentration [36]. This occurs because Col III associates between Col I fibrils, causing them to be misaligned [31, 36]. In addition, a study indicates that Col III is essential for the normal fibrogenesis of Col I in the ECM of several organs [37]. An ECM whose Col I becomes linear favors the migration of cancer cells through the matrix, as they form structures similar to pathways that direct this process [12].

However, analyses that assess the amount of collagen present in the tissue, such as the one performed in our work, are scarce in the literature. In the total collagen quantification, which was analyzed from images captured in common light, it was observed that the metastatic group had a higher mean value when compared to the group with the better prognosis (p=0.001), indicating that in this group there is an abundance of this protein. However, this analysis considers all types of collagens present in the tissue, not just the types I and III. In the organs of our body, such as the prostate, there are found the collagen types: I, III, IV, VII, among others [10], which could explain the higher average of total collagen in the metastatic group, even though this group had shown less Col III.

It is known that in solid tumors the surrounding ECM becomes fibrous, in other words, rich in fibrillar proteins [38]. Tumor fibrosis is characterized by chronic inflammation and high numbers of contractile

myofibroblasts, which abundantly secrete ECM proteins and remodel enzymes that reorganize and harden the matrix. In addition, there is the release of cytokines and growth factors that stimulate the proliferation and invasion of tumor cells, producing a differentiated stroma [39, 40]. Our results, which indicate a more fibrous ECM in the metastatic group, corroborate these studies, showing a worsening in the prognosis and aggressiveness of the tumor according to the increase in interstitial fibrosis.

The basement membrane is mainly formed by type IV collagen (which is network-shaped) and laminin, and in the epithelium it acts as a physical barrier against the invasion of cancer cells into the surrounding stromal tissue. In the endothelium, this structure prevents tumor cells from entering the blood and lymph vessels during the metastasis process [15, 16]. Liu et al. [17] observed that disruption of basement membrane continuity is a key factor in PCa invasion and progression, and Oka et al. [41] state that changes in its integrity can favor the metastatic process. In the samples of the present study, it was found that the basement membrane of the adjacent non-tumor tissue differs from that of the tumor tissue (p=0.000), indicating a worsening in its integrity in the altered malignant cells.

A significant correlation was also observed between the worsening of the basement membrane integrity and a higher ISUP grade, presence of extraprostatic and perineural extensions, factors that indicate aggressiveness and worse prognosis in PCa. According to Berman and Epstein [42], for epithelial cancers, such as prostate adenocarcinoma, basement membrane invasion is a very important parameter for defining malignancy. Prostatic tumors with ISUP grade 1 and higher are defined by the infiltrative pattern of tumor cells, which may irregularly extend into the surrounding ECM and extraprostatic tissues. This feature agrees with our results, which positively correlate invasive parameters with worsening of basement membrane integrity.

5 CONCLUSION

This study, using simple techniques, demonstrated for the first time in PCa malignant tissue samples that Col III and basement membrane integrity can be good predictive markers of worse prognosis. Col III absence seems to indicate a predictive candidate marker of metastatic tumors, and basement membrane integrity was correlated with worse prognosis invasive parameters. All in all, the use of simple methodologies to evaluate cellular structures seems interesting and easy to apply in oncology clinical routines.

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DECLARATIONS

Ethics Approval - This study was approved by the Institutional Ethics Committee Involving Humans at State University of Londrina, Londrina—Paraná (PR), Brazil (CEP/UEL 176/2013; CAAE 19769913.0.0000.5231, in accordance with Resolution 466/12 of the National Research Ethics Commission).

Consent to Participate - The study purpose and procedures were explained to all the patients and written informed consent was obtained from all the individual participants included in the study.

Consent for Publication - Not applicable.

Competing Interests - The authors have no competing interests to declare that are relevant to the content of this article.

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Online Resource 1 Patients clinical and histopathological characteristics.

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT144	Better Prognosis	RP	2008	61	6	pT2a	04.62	No	No	No	No
PT149	Better Prognosis	RP	2006	65	6	pT2a	08.61	No	No	No	No
PT178	Better Prognosis	RP	2008	61	6	pT2a	04.24	No	No	No	Yes
PT180	Better Prognosis	RP	2008	66	6	pT2a	11.07	No	No	No	Yes
PT188	Better Prognosis	RP	2006	66	6	pT2a	12.33	No	No	No	No
PT235	Better Prognosis	RP	2011	62	6	pT2a	03.62	No	No	No	Yes
PT240	Better Prognosis	RP	2011	61	6	pT2a	03.68	No	No	No	No
PT246	Better Prognosis	RP	2011	63	7 (3+4)	pT2a	-	No	No	No	-
PT266	Better Prognosis	RP	2012	69	6	pT2a	04.04	No	No	No	No
PT271	Better Prognosis	RP	2014	65	7 (3+4)	pT2b	07.74	No	No	No	-
PT308	Better Prognosis	RP	2014	67	6	pT2b	07.14	No	No	No	-
PT318	Better Prognosis	RP	2014	72	7 (3+4)	pT2b	05.65	No	No	No	No
PT320	Better Prognosis	RP	2014	60	6	pT2a	07.88	No	No	No	No
PT332	Better Prognosis	RP	2015	66	7 (3+4)	pT2c	06.34	No	No	No	No
PT345	Better Prognosis	RP	2015	56	6	pT2b	05.78	No	No	No	No
PT347	Better Prognosis	RP	2015	59	6	pT2a	06.69	No	No	No	Yes
PT359	Better Prognosis	RP	2015	74	7 (3+4)	pT2b	08.90	No	No	No	-
PT374	Better Prognosis	RP	2015	59	7 (3+4)	pT2b	05.87	No	No	No	-
PT383	Better Prognosis	RP	2016	70	6	pT1a	06.00	No	No	No	-
PT384	Better Prognosis	RP	2015	68	6	pT2a	06.79	No	No	No	-
PT012	Worse Prognosis	RP	2006	74	8 (4+4)	pT3a	09.63	No	Yes	Yes	Yes
PT074	Worse Prognosis	RP	2007	76	7 (4+3)	pT3a	23.69	No	Yes	No	Yes
PT089	Worse Prognosis	RP	2007	70	6	pT3a	23.15	No	Yes	No	Yes

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT116	Worse Prognosis	RP	2007	65	8 (3+5)	pT3c	09.26	Yes	Yes	Yes	Yes
PT126	Worse Prognosis	RP	2008	72	8 (3+5)	pT3a	09.38	No	Yes	No	No
PT148	Worse Prognosis	RP	2006	61	8 (5+3)	pT3b	31.00	Yes	Yes	No	Yes
PT175	Worse Prognosis	RP	2007	65	7 (4+3)	pT3b	9.36	Yes	Yes	No	No
PT199	Worse Prognosis	RP	2008	70	8 (4+4)	pT3a	19.29	No	Yes	No	Yes
PT212	Worse Prognosis	TUR	2011	58	-	-	28.31	-	-	-	-
PT247	Worse Prognosis	RP	2011	67	7 (3+4)	pT3b	31.51	Yes	Yes	Yes	Yes
PT248	Worse Prognosis	RP	2011	67	8 (3+5)	pT3b	14.30	Yes	Yes	No	Yes
PT259	Worse Prognosis	RP	2011	61	8 (3+5)	pT3a	26.43	No	Yes	Yes	No
PT263	Worse Prognosis	RP	2012	71	7 (3+4)	pT3b	22.93	Yes	Yes	Yes	No
PT281	Worse Prognosis	RP	2014	72	7 (4+3)	pT3b	15.00	Yes	Yes	No	-
PT282	Worse Prognosis	RP	2014	66	7 (3+4)	pT3a	06.15	No	Yes	No	-
PT283	Worse Prognosis	RP	2014	55	7 (3+4)	pT2c	18.82	No	No	No	No
PT288	Worse Prognosis	RP	2014	72	7 (4+3)	pT3a	09.13	No	Yes	No	No
PT291	Worse Prognosis	RP	2014	73	7 (3+4)	pT3a	19.36	No	Yes	No	Yes
PT324	Worse Prognosis	RP	2014	71	7 (3+4)	pT3a	21.69	No	Yes	-	-
PT339	Worse Prognosis	RP	2015	64	7 (3+4)	pT3a	13.37	No	Yes	No	No
PT357	Worse Prognosis	RP	2015	76	7 (3+4)	pT3a	16.74	No	Yes	No	-
PT371	Worse Prognosis	RP	2015	57	7 (3+4)	pT3a	14.77	No	Yes	Yes	Yes
PT379	Worse Prognosis	RP	2015	63	7 (3+4)	pT3a	21.32	No	Yes	No	-
PT024	Metastatic	RP	2006	59	6	pT2c	30.69	No	No	Yes	No
PT129	Metastatic	RP	2008	84	8 (4+4)	-	30.85	-	-	-	Yes
PT140	Metastatic	TUR	2008	83	7 (3+4)	pT2a	618.7	-	-	-	No
PT163	Metastatic	TUR	2008	62	9 (4+5)	-	25.15	-	-	-	Yes

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT202	Metastatic	RP	2008	59	6	pT2b	04.48	No	No	No	Yes
PT249	Metastatic	RP	2011	56	8 (3+5)	pT3bN1	18.09	Yes	Yes	No	Yes
PT269	Metastatic	RP	2012	71	7 (3+4)	pT3aN1	18.05	No	Yes	Yes	-
PT280	Metastatic	BIOP	2014	82	7 (3+4)	pT3a	520.7	-	Yes	Yes	-
PT284	Metastatic	RP	2014	74	8 (4+4)	pT3bN1	16.00	Yes	Yes	No	No
PT287	Metastatic	RP	2014	73	7 (4+3)	pT3b	141.7	Yes	Yes	-	Yes
PT295	Metastatic	BIOP	2014	84	8 (4+4)	-	15.70	-	-	Yes	-
PT326	Metastatic	BIOP	2013	71	6	pT2cNxM1a	23.27	-	No	-	Yes
PT333	Metastatic	BIOP	2014	83	7 (3+4)	pT4	11.81	Yes	Yes	-	-
PT338	Metastatic	BIOP	2014	80	7 (4+3)	-	16.00	-	-	-	-
PT361	Metastatic	RP	2015	58	7 (4+3)	pT3b	56.03	Yes	Yes	Yes	Yes
PT377	Metastatic	TUR	2015	66	7 (3+4)	-	35.00	-	-	-	No
PT385	Metastatic	RP	2015	68	8 (4+4)	pT3a+bN1	03.28	Yes	Yes	No	Yes

The exam type are presented as follow: (RP) Radical Prostatectomy; (TUR) Transurethral Resection; (BIOP) Biopsy; (-) no data. Due to lack of data, some variables did not include the total of patients

Online Resource 2 Picrosirius analysis of better prognosis samples of PCa.

Patient	Photo	Collagen I	Collagen III	Total Collagen
PT144	1	80754.81	36225.28	179863.80
	2	83517.75	55722.26	272201.03
	3	117333.39	73716.18	198127.84
	4	127413.80	54657.52	241560.13
	5	59555.66	16370.16	229800.55
	Sum (pixels)	468575.42	236691.40	1121553.35
	Percentage	41.78%	21.10%	100.00%
	Area (μm²)	197577.76	99802.41	472910.00
PT149	1	152906.27	25716.24	329482.59
	2	139728.55	25062.51	298640.56
	3	120623.19	31657.09	217427.89
	4	214705.94	21793.41	216892.81
	5	173399.69	50540.86	268990.97
	Sum (pixels)	801363.64	154770.10	1331434.82
	Percentage	60.19%	11.62%	100.00%
	Area (μm²)	337900.00	65259.78	561407.83
PT178	1	46835.65	15130.10	123179.71
	2	63035.33	12055.71	173241.55
	3	64365.26	17096.58	161811.38
	4	78121.38	33440.75	233295.44
	5	52510.87	18623.87	117244.41
	Sum (pixels)	304868.49	96347.01	808772.49
	Percentage	37.70%	11.91%	100.00%
	Area (μm²)	128549.71	40625.32	341023.99
PT180	1	215478.59	34799.32	261180.80
	2	161743.97	25205.23	230955.39
	3	169722.23	60472.82	242502.52
	4	135442.73	78094.51	263613.34
	5	192451.92	72529.86	246304.88
	Sum (pixels)	874839.44	271101.75	1244556.93
	Percentage	70.29%	21.78%	100.00%
	Area (μm²)	368881.53	114311.75	524775.23
PT188	1	93942.06	23462.64	317541.81
	2	93169.16	22221.71	240774.14
	3	81485.91	18811.77	237524.88
	4	140385.81	8145.13	197419.03
	5	60269.44	12174.48	202347.59
	Sum (pixels)	469252.39	84815.73	1195607.45
	Percentage	39.25%	7.09%	100.00%
	Area (μm²)	197863.21	35763.08	504135.37
PT235	1	40969.39	29249.45	155131.77
	2	42095.21	35259.32	225662.98
	3	19010.37	47696.49	195539.78
	4	19229.21	56194.55	161248.39

Patient	Photo	Collagen I	Collagen III	Total Collagen
	5	32249.11	39118.74	175219.92
	Sum (pixels)	153553.29	207518.54	912802.84
	Percentage	16.82%	22.73%	100.00%
	Area (μm²)	64746.71	87501.49	384889.04
PT240	1	104833.86	48835.81	270188.88
	2	63077.67	51960.70	371774.31
	3	56545.79	19577.50	261961.11
	4	80535.92	37438.02	249587.19
	5	93221.03	39701.89	255324.67
	Sum (pixels)	398214.27	197513.91	1408836.16
	Percentage	28.27%	14.02%	100.00%
	Area (μm²)	167909.54	83282.98	594044.59
PT246	1	121522.59	24625.99	279125.91
	2	108802.07	15581.04	193274.98
	3	198717.31	22079.19	329025.97
	4	78557.51	8447.46	274078.25
	5	54370.47	15398.47	270954.22
	Sum (pixels)	561969.94	86132.15	1346459.33
	Percentage	41.74%	6.40%	100.00%
	Area (μm²)	236958.15	36318.16	567743.01
PT266	1	60854.70	45576.40	173048.58
	2	46296.59	42246.58	91652.05
	3	49862.96	54047.48	153665.45
	4	57935.15	46895.34	165993.42
	5	104564.00	58873.75	218537.27
	Sum (pixels)	319513.39	247639.56	802896.77
	Percentage	39.80%	30.84%	100.00%
	Area (μm²)	134724.82	104418.77	338546.45
PT271	1	49954.46	50367.26	165990.47
	2	44149.52	48046.89	126125.82
	3	50707.96	70398.04	177173.63
	4	67571.68	77341.88	174870.97
	5	59824.17	40705.85	105685.19
	Sum (pixels)	272207.78	286859.92	749846.08
	Percentage	36.30%	38.26%	100.00%
	Area (μm²)	114778.12	120956.28	316177.30
PT308	1	57010.03	31382.61	114475.03
	2	43133.75	66960.70	243512.81
	3	55074.63	74930.00	173171.27
	4	53807.13	23761.17	105110.89
	5	43309.58	37702.82	136619.16
	Sum (pixels)	252335.12	234737.29	772889.16
	Percentage	32.65%	30.37%	100.00%
	Area (μm²)	106398.68	98978.45	325893.56
PT318	1	61671.02	47636.19	206474.95

Patient	Photo	Collagen I	Collagen III	Total Collagen
	2	80271.13	29639.48	156662.16
	3	59164.27	20897.71	145877.89
	4	89033.56	38558.78	204857.05
	5	30774.16	25610.56	95240.34
	Sum (pixels)	320914.14	162342.71	809112.39
	Percentage	39.66%	20.06%	100.00%
	Area (μm²)	135315.46	68452.82	341167.31
PT320	1	74049.59	88311.27	272753.69
	2	68961.46	45633.32	187741.27
	3	58309.58	43633.83	167280.88
	4	65505.98	21265.39	139156.31
	5	110409.42	20367.26	193000.50
	Sum (pixels)	377236.03	219211.08	959932.65
	Percentage	39.30%	22.84%	100.00%
	Area (μm²)	159063.94	92431.72	404761.62
PT332	1	68493.42	72631.56	184119.98
	2	51826.61	75477.73	166597.64
	3	54466.18	53557.09	163834.97
	4	45106.68	141000.58	221760.41
	5	88328.56	177348.63	301808.91
	Sum (pixels)	308221.45	520015.59	1038121.91
	Percentage	29.69%	50.09%	100.00%
	Area (μm²)	129963.50	219267.83	437730.61
PT345	1	67449.40	34592.68	228446.19
	2	84241.86	36131.72	180623.77
	3	92580.53	17890.03	162851.00
	4	159888.25	33157.36	205644.44
	5	140470.98	59993.67	299858.03
	Sum (pixels)	544631.02	181765.47	1077423.43
	Percentage	50.55%	16.87%	100.00%
	Area (μm²)	229647.08	76642.55	454302.34
PT347	1	92762.06	58653.48	221029.67
	2	60360.49	24240.94	190472.92
	3	35583.89	29781.36	201966.53
	4	31694.09	32272.06	176667.91
	5	66033.51	67418.06	237077.30
	Sum (pixels)	286434.04	212365.89	1027214.33
	Percentage	27.88%	20.67%	100.00%
	Area (μm²)	120776.71	89545.41	433131.36
PT359	1	162385.38	117680.52	285008.53
	2	70081.45	29603.83	228227.69
	3	220568.38	100250.46	305308.56
	4	66397.82	47329.91	245550.70
	5	118605.61	58495.77	285370.19
	Sum (pixels)	638038.64	353360.49	1349465.67

Patient	Photo	Collagen I	Collagen III	Total Collagen
	Percentage	47.28%	26.19%	100.00%
	Area (µm²)	269032.99	148996.66	569010.66
PT374	1	181022.02	32514.78	221632.67
	2	119376.96	105613.80	285717.75
	3	221270.13	35815.60	339412.72
	4	140623.69	13179.48	248121.14
	5	174854.73	38830.08	251516.22
	Sum (pixels)	837147.53	225953.74	1346400.50
	Percentage	62.18%	16.78%	100.00%
	Area (µm²)	352988.50	95274.81	567718.21
PT383	1	95857.16	67526.88	184307.22
	2	100647.37	93903.89	236199.19
	3	77458.40	67345.38	226346.77
	4	28451.87	64304.03	194662.67
	5	76069.88	99122.53	219101.88
	Sum (pixels)	378484.67	392202.70	1060617.73
	Percentage	35.69%	36.98%	100.00%
	Area (µm²)	159590.43	165374.73	447216.11
PT384	1	42326.28	23681.06	175146.73
	2	92943.58	27222.55	216477.48
	3	60796.93	20743.80	182018.89
	4	45946.20	24146.57	168641.42
	5	44526.48	36703.07	254764.72
	Sum (pixels)	286539.46	132497.04	997049.24
	Percentage	28.74%	13.29%	100.00%
	Area (µm²)	120821.16	55868.21	420412.06

Quantification of collagen fibbers by Picrosirius analysis

Online Resource 3 Picrosirius analysis of worse prognosis samples of PCa.

Patient	Photo	Collagen I	Collagen III	Total Collagen
PT12	1	33688.65	14281.92	276884.78
	2	48031.29	14778.63	270039.63
	3	70415.33	16929.50	327891.28
	4	87789.25	20928.91	303361.44
	5	58764.97	16345.08	267693.13
	Sum (pixels)	298689.48	83264.04	1445870.26
	Percentage	20.66%	5.76%	100.00%
	Area (μm²)	125944.29	35108.80	609660.25
PT74	1	28119.83	15041.32	245779.20
	2	27891.72	21616.63	157900.98
	3	17287.06	13860.69	124613.34
	4	20652.30	45287.15	168418.78
	5	16017.88	26466.52	172199.78
	Sum (pixels)	109968.79	122272.30	868912.08
	Percentage	12.66%	14.07%	100.00%
	Area (μm²)	46369.03	51556.88	366382.22
PT89	1	70994.69	31499.41	213524.20
	2	96817.34	27218.33	277555.66
	3	54542.50	29904.71	210488.27
	4	51286.05	63321.38	225245.19
	5	46431.94	24447.63	237664.02
	Sum (pixels)	320072.52	176391.46	1164477.34
	Percentage	27.49%	15.15%	100.00%
	Area (μm²)	134960.58	74376.56	491009.17
PT116	1	40562.07	7306.88	248779.28
	2	29235.54	9250.29	139655.86
	3	27125.57	27072.86	203592.94
	4	25469.73	41362.37	149686.06
	5	27685.95	25713.86	176655.14
	Sum (pixels)	150078.85	110706.27	918369.28
	Percentage	16.34%	12.05%	100.00%
	Area (μm²)	63281.69	46679.99	387236.16
PT126	1	47491.99	32537.53	180196.03
	2	42217.49	6265.81	110701.36
	3	50815.90	8218.50	128031.41
	4	118064.17	34386.07	253310.47
	5	61114.44	17802.33	220729.17
	Sum (pixels)	319703.99	99210.23	892968.44
	Percentage	35.80%	11.11%	100.00%
	Area (μm²)	134805.19	41832.62	376525.74
PT148	1	57114.61	12122.20	164400.31
	2	82173.63	19563.16	187620.56
	3	112527.83	23895.68	182858.55
	4	109541.23	16896.61	213512.13

Patient	Photo	Collagen I	Collagen III	Total Collagen
	5	116333.27	22468.80	301911.28
	Sum (pixels)	477690.57	94946.45	1050302.83
	Percentage	45.48%	9.04%	100.00%
	Area (μm²)	201421.22	40034.76	442866.77
PT175	1	107454.29	31961.93	200396.97
	2	92076.17	43546.73	233722.30
	3	128902.33	26392.44	246244.09
	4	145080.86	78216.53	255238.63
	5	85632.69	40046.79	241769.72
	Sum (pixels)	559146.34	220164.42	1177371.71
	Percentage	47.49%	18.70%	100.00%
	Area (μm²)	235767.56	92833.71	496446.16
PT199	1	135355.06	39812.88	224878.97
	2	77264.13	65864.79	174801.42
	3	108585.98	84234.48	215129.27
	4	105968.41	97881.79	247422.48
	5	52907.78	27819.28	281025.78
	Sum (pixels)	480081.35	315613.21	1143257.92
	Percentage	41.99%	27.61%	100.00%
	Area (μm²)	202429.31	133080.29	482061.87
PT212	1	87118.82	17301.82	245149.69
	2	101062.57	41209.73	246220.27
	3	57046.72	40880.42	181722.47
	4	93835.81	42936.41	245843.72
	5	79000.25	33305.36	223005.56
	Sum (pixels)	418064.16	175633.75	1141941.71
	Percentage	36.61%	15.38%	100.00%
	Area (μm²)	176279.37	74057.07	481506.88
PT247	1	110304.45	35350.41	15991.17
	2	56777.74	31902.46	169194.05
	3	77650.46	23507.03	129361.79
	4	88955.97	23133.47	163265.97
	5	71466.45	17539.31	185755.84
	Sum (pixels)	405155.07	131432.67	663568.82
	Percentage	61.06%	19.81%	100.00%
	Area (μm²)	170836.17	55419.41	279797.95
PT248	1	116232.97	22987.66	234629.34
	2	81949.06	79267.17	246946.77
	3	56001.55	25332.55	221386.39
	4	55553.98	10591.42	315071.66
	5	129662.66	14940.24	303433.13
	Sum (pixels)	439400.21	153119.03	1321467.29
	Percentage	33.25%	11.59%	100.00%
	Area (μm²)	185275.85	64563.60	557204.96
PT259	1	231051.88	23762.09	334781.16

Patient	Photo	Collagen I	Collagen III	Total Collagen
	2	154992.13	11972.01	242319.53
	3	154624.30	16596.59	278016.53
	4	44480.63	60307.19	217259.23
	5	97949.63	99839.45	248479.08
	Sum (pixels)	683098.56	212477.34	1320855.53
	Percentage	51.72%	16.09%	100.00%
	Area (μm²)	288032.79	89592.40	556947.01
PT263	1	197931.38	48549.71	395296.81
	2	218478.55	14457.87	387578.00
	3	196031.41	11813.43	320825.59
	4	197324.33	12820.01	383816.81
	5	235228.88	85425.65	360816.75
	Sum (pixels)	1044994.55	173066.66	1848333.96
	Percentage	56.54%	9.36%	100.00%
	Area (μm²)	440628.50	72974.64	779361.60
PT281	1	51670.60	42012.98	178216.39
	2	75947.46	14983.98	283456.31
	3	39509.19	50866.50	367117.13
	4	95718.08	19775.26	298021.16
	5	52298.02	11421.40	170415.33
	Sum (pixels)	315143.36	139060.12	1297226.32
	Percentage	24.29%	10.72%	100.00%
	Area (μm²)	132882.17	58635.57	546983.61
PT282	1	45874.93	38849.72	268146.81
	2	46240.93	113955.13	263340.78
	3	55734.95	83430.59	278045.19
	4	57086.35	75853.85	290859.75
	5	41177.26	82629.45	264951.50
	Sum (pixels)	246114.43	394718.74	1365344.03
	Percentage	18.03%	28.91%	100.00%
	Area (μm²)	103775.69	166435.63	575705.87
PT283	1	28533.06	77046.72	315947.03
	2	37559.03	74876.88	247656.84
	3	24881.51	70537.61	173660.39
	4	24032.30	46580.37	209235.53
	5	42627.76	29983.13	274346.84
	Sum (pixels)	157633.66	299024.70	1220846.63
	Percentage	12.91%	24.49%	100.00%
	Area (μm²)	66467.22	126085.64	514777.63
PT288	1	49651.29	31202.98	228624.97
	2	20620.68	7806.12	126983.89
	3	10275.76	47787.57	191415.06
	4	71078.59	68803.34	362878.66
	5	14381.43	52316.58	171603.13
	Sum (pixels)	166007.75	207916.59	1081505.71

Patient	Photo	Collagen I	Collagen III	Total Collagen
	Percentage	15.35%	19.22%	100.00%
	Area (μm²)	69998.21	87669.33	456023.66
PT291	1	148869.11	633.75	285335.66
	2	188166.63	2664.87	293688.22
	3	154647.48	38910.02	316748.59
	4	108727.44	8944.17	244774.41
	5	138937.42	11891.13	296486.75
	Sum (pixels)	739348.08	63043.93	1437033.63
	Percentage	51.45%	4.39%	100.00%
	Area (μm²)	311750.75	26582.87	605934.23
PT324	1	98197.64	10034.17	309932.09
	2	110589.47	35867.58	307750.88
	3	140459.81	17408.03	239039.55
	4	105619.96	16149.90	238940.78
	5	228365.14	38604.09	356445.84
	Sum (pixels)	683232.02	118063.77	1452109.14
	Percentage	47.05%	8.13%	100.00%
	Area (μm²)	288089.06	49782.33	612290.92
PT339	1	29166.39	37769.75	151400.31
	2	22327.76	25201.27	184210.23
	3	42101.38	11393.61	135205.34
	4	98676.05	84278.09	241411.70
	5	120136.41	61312.90	309406.72
	Sum (pixels)	312407.99	219955.61	1021634.30
	Percentage	30.58%	21.53%	100.00%
	Area (μm²)	131728.78	92745.66	430778.50
PT357	1	45241.19	37634.93	195479.42
	2	30800.73	66363.63	300379.91
	3	23719.51	49801.40	238891.03
	4	19877.30	59636.11	263094.09
	5	30829.40	78919.29	224611.66
	Sum (pixels)	150468.12	292355.36	1222456.11
	Percentage	12.31%	23.92%	100.00%
	Area (μm²)	63445.82	123273.47	515456.28
PT371	1	82408.96	56560.13	183344.16
	2	109079.37	63477.17	283145.97
	3	99435.94	52375.63	274830.06
	4	85870.13	35526.18	164365.41
	5	101712.11	70759.41	206833.34
	Sum (pixels)	478506.50	278698.51	1112518.94
	Percentage	43.01%	25.05%	100.00%
	Area (μm²)	201765.27	117514.97	469100.58
PT379	1	83530.10	15952.10	341016.59
	2	136681.56	45135.77	392634.91
	3	130248.35	83010.63	320881.25

Patient	Photo	Collagen I	Collagen III	Total Collagen
	4	24675.75	60305.28	287895.50
	5	114873.92	65992.58	340734.94
	Sum (pixels)	490009.68	270396.35	1683163.19
	Percentage	29.11%	16.06%	100.00%
	Area (μm²)	206615.65	114014.32	709716.31

Quantification of collagen fibbers by Picrosirius analysis

Online Resource 4 Picrosirius analysis of metastatic samples of PCa.

Patient	Photo	Collagen I	Collagen III	Total Collagen
PT24	1	159372.56	15997.22	241628.31
	2	131561.39	13574.38	257105.11
	3	114063.50	12186.29	188140.81
	4	185735.78	19542.92	244036.20
	5	100125.23	26472.84	257631.95
	Sum (pixels)	690858.46	87773.65	1188542.38
	Percentage	58.13%	7.38%	100.00%
	Area (μm²)	291304.80	37010.31	501156.34
PT129	1	39638.64	25577.25	210375.69
	2	98681.48	13603.90	235528.33
	3	42993.34	13824.42	106594.70
	4	162322.89	28606.00	307954.13
	5	69959.94	18406.56	166677.34
	Sum (pixels)	413596.28	100018.13	1027130.19
	Percentage	40.27%	9.74%	100.00%
	Area (μm²)	174395.46	42173.27	433095.88
PT140	1	58168.32	25259.74	286959.00
	2	99278.12	14028.50	262958.75
	3	113108.45	37516.86	279868.84
	4	89868.44	15790.60	199211.08
	5	110045.12	21905.46	269212.34
	Sum (pixels)	470468.45	114501.17	1298210.01
	Percentage	36.24%	8.82%	100.00%
	Area (μm²)	198375.97	48280.14	547398.39
PT163 (4 fotos boas)	1	121824.92	19210.24	324407.16
	2	186966.59	31740.17	358425.53
	3	173967.36	55784.70	386119.06
	4	210269.02	26970.40	372462.47
	5	128523.35	21805.53	317809.47
	Sum (pixels)	821551.24	155511.04	1759223.69
	Percentage	46.70%	8.84%	100.00%
	Area (μm²)	346412.23	65572.20	741787.69
PT202	1	110834.45	35594.96	332289.53
	2	76081.97	17468.38	253661.11
	3	83726.59	26422.67	222132.66
	4	101909.68	24510.88	282725.31
	5	162328.80	28407.40	313659.06
	Sum (pixels)	534881.49	132404.28	1404467.67
	Percentage	38.08%	9.43%	100.00%
	Area (μm²)	225536.13	55829.09	592202.59
PT249	1	49824.17	29918.20	298164.66
	2	39978.91	20877.89	296391.13
	3	43634.25	26803.42	314865.19

Patient	Photo	Collagen I	Collagen III	Total Collagen
	4	37587.70	21681.14	195834.50
	5	68320.12	43828.64	259250.44
	Sum (pixels)	239345.16	143109.29	1364505.92
	Percentage	17.54%	10.49%	100.00%
	Area (µm²)	100921.38	60342.93	575352.47
PT269	1	132247.00	53952.18	386140.16
	2	80043.01	43413.73	300634.56
	3	78772.13	27458.26	216727.52
	4	114883.20	40282.93	279074.88
	5	83571.84	39381.43	199903.02
	Sum (pixels)	489517.19	204488.53	1382480.14
	Percentage	35.41%	14.79%	100.00%
	Area (µm²)	206407.99	86223.87	582931.41
PT280 (3 fotos boas)	1	59093.12	30395.88	167870.73
	2	70795.98	32290.12	223475.36
	3	28747.02	50219.28	203464.73
	4	55762.34	21122.94	155348.06
	5	97122.77	72756.28	174040.22
	Sum (pixels)	311521.23	206784.50	924199.10
	Percentage	33.71%	22.37%	100.00%
	Area (µm²)	131354.88	87191.98	389694.34
PT284	1	217714.69	45370.92	291816.75
	2	152979.39	14586.06	280142.09
	3	295741.78	26803.00	413850.94
	4	192291.83	22121.15	321127.66
	5	261564.92	75362.84	450259.47
	Sum (pixels)	1120292.61	184243.97	1757196.91
	Percentage	63.75%	10.49%	100.00%
	Area (µm²)	472378.40	77687.63	740933.09
PT287	1	76269.18	31168.83	217212.84
	2	73758.64	33162.00	265322.97
	3	52554.39	24662.67	276627.16
	4	82149.60	40224.74	279891.19
	5	63431.44	35742.11	234500.33
	Sum (pixels)	348163.25	164960.36	1273554.49
	Percentage	27.34%	12.95%	100.00%
	Area (µm²)	146805.22	69556.57	537002.23
PT295	1	132454.45	4506.66	323549.47
	2	90022.77	2232.25	280190.16
	3	106233.34	3662.08	320611.81
	4	168733.33	1552.96	345093.19
	5	197003.70	623.63	278902.84
	Sum (pixels)	694447.59	12577.58	1548347.47
	Percentage	44.85%	0.81%	100.00%

Patient	Photo	Collagen I	Collagen III	Total Collagen
	Area (μm²)	292818.18	5303.42	652870.41
PT326	1	140306.11	10144.21	317881.59
	2	144288.23	15068.31	308288.50
	3	192402.17	8779.30	327305.59
	4	175751.39	8245.49	350643.44
	5	234679.11	7980.69	418800.78
	Sum (pixels)	887427.01	50218.00	1722919.90
	Percentage	51.51%	2.91%	100.00%
	Area (μm²)	374189.16	21174.73	726479.97
PT333	1	218386.48	12099.32	267471.41
	2	98771.64	33824.01	157969.61
	3	39615.52	23061.22	104738.48
	4	92112.73	12138.09	112044.07
	5	108254.27	46906.57	237542.05
	Sum (pixels)	557140.65	128029.21	879765.62
	Percentage	63.33%	14.55%	100.00%
	Area (μm²)	234921.85	53984.32	370958.69
PT338	1	108685.27	35530.02	183127.42
	2	186011.55	63381.26	358540.22
	3	128991.39	99160.48	393583.63
	4	166826.61	27391.63	306033.03
	5	145392.98	46146.90	368344.16
	Sum (pixels)	735907.80	271610.29	1609628.46
	Percentage	45.72%	16.87%	100.00%
	Area (μm²)	310300.13	114526.18	678709.93
PT361	1	139934.70	16571.04	251103.02
	2	177405.34	25625.93	322216.16
	3	181223.77	20715.01	382278.00
	4	156462.14	58294.01	351696.63
	5	235794.50	10873.36	376986.00
	Sum (pixels)	890820.45	132079.35	1684279.81
	Percentage	52.89%	7.84%	100.00%
	Area (μm²)	375620.02	55692.09	710187.14
PT377	1	161554.55	61725.66	243627.39
	2	38573.25	23589.85	186587.55
	3	40619.47	38237.14	255773.42
	4	32578.66	10039.45	152362.66
	5	31156.22	24131.69	187279.16
	Sum (pixels)	304482.15	157723.79	1025630.18
	Percentage	29.69%	15.38%	100.00%
	Area (μm²)	128386.81	66505.22	432463.39
PT385	1	203896.94	12202.73	341404.09
	2	263515.75	9614.61	360797.34
	3	211206.36	40697.42	401349.72
	4	199841.88	33035.92	358504.78

Patient	Photo	Collagen I	Collagen III	Total Collagen
	5	192242.36	34434.14	333445.34
	Sum (pixels)	1070703.29	129984.81	1795501.27
	Percentage	59.63%	7.24%	100.00%
	Area (μm²)	451468.75	54808.91	757084.36

Quantification of collagen fibbers by Picrosirius analysis

Online Resource 5 Picrosirius analysis of normal prostate sample.

Patient	Photo	Colagen I	Colagen III	Total Colagen
PTN	1	16229,97	21684,52	129654,66
	2	22557,34	12398,38	101567,30
	3	17877,80	18888,09	72701,55
	4	28479,51	20040,90	93430,59
	5	21002,70	8470,23	96347,61
	Sum (pixels)	106147,32	81482,12	493701,71
	Percentage	21,50%	16,50%	100,00%
	Area (µm²)	44757,68	34357,45	208172,42

Quantification of collagen fibers by Picrosirius analysis.

Online Resource 6 PAS analysis of better prognosis samples of PCa,

Better Prognosis		
Pacient	**Basement Membrane**	
	Adjacent non-tumor	**Tumor**
PT149	G2	G2
PT178	G3	G2
PT180	G2	G2
PT188	G2	G2
PT235	G2	G1
PT240	G2	G1
PT246	G2	G1
PT266	G2	G1
PT271	G3	G2
PT308	G2	G2
PT318	G3	G2
PT320	G3	G2
PT332	G2	G1
PT345	G3	G1
PT347	G2	G2
PT359	G2	G1
PT374	G3	G2
PT383	G3	G3
PT384	G2	G2

Analysis of PAS staining patterns for visualization of the basement membrane of tumor and adjacent non-tumor from PCa patients' samples. The G1 pattern represented barely visible and discontinuous colorations. The G2 assigned to markings seen relatively easily, but with varying thickness and discontinuity in some places. And G3, on the other hand, represented thick, well-visible and continuous stains.

Online Resource 7 PAS analysis of worse prognosis samples of PCa.

Worse Prognosis		
Pacient	**Basement Membrane**	
	Adjacent non-tumor	**Tumor**
PT12	G2	G1
PT74	G1	G1
PT89	G2	G2
PT116	G3	G1
PT126	G3	G2
PT148	G3	G2
PT175	G3	G1
PT199	G1	G1
PT212	G2	G1
PT247	G1	G1
PT248	G2	G1
PT259	G2	G1
PT263	G2	G2
PT281	G1	G1
PT282	G2	G1
PT283	G1	G1
PT288	G2	G1
PT291	G2	G1
PT324	G2	G1
PT339	G3	G2
PT357	G3	G2
PT371	G2	G1
PT379	G3	G2

Analysis of PAS staining patterns for visualization of the basement membrane of tumor and adjacent non-tumor from PCa patients' samples. The G1 pattern represented barely visible and discontinuous colorations. The G2 assigned to markings seen relatively easily, but with varying thickness and discontinuity in some places. And G3, on the other hand, represented thick, well-visible and continuous stains.

Online Resource 8 PAS analysis of metastatic samples of PCa.

Metastatic		
Pacient	**Basement Membrane**	
	Adjacent non-tumor	**Tumor**
PT24	G1	G1
PT129	-	G2
PT140	-	G1
PT163	-	G1
PT202	G2	G2
PT249	G1	G1
PT269	G2	G2
PT280	G2	G1
PT284	G2	G1
PT287	G2	G2
PT295	-	G1
PT326	G3	G2
PT333	G2	G1
PT338	-	G1
PT361	G2	G1
PT377	G1	G1
PT385	G2	G2

Analysis of PAS staining patterns for visualization of the basement membrane of tumor and adjacent non-tumor from PCa patients' samples. The G1 pattern represented barely visible and discontinuous colorations. The G2 assigned to markings seen relatively easily, but with varying thickness and discontinuity in some places. And G3, on the other hand, represented thick, well-visible and continuous stains. Due to paraffin blocks wear, some data did not include the total of patients.

5. CAPÍTULO 2

Metalloproteinase 9 immunostaining profile: an interesting prognostic marker in prostate cancer

Laís Capelasso Lucas Pinheiro, Érica Romão Pereira, Amanda Letícia Francelino, Alda Fiorina Maria Losi Guembarovski, Paulo Emílio Fuganti, Karen Brajão de Oliveira, Carlos Alberto Miqueloto, Juliana Mara Serpeloni and Roberta Losi Guembarovski.

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Metalloproteinase 9 immunostaining profile: an interesting prognostic marker in prostate cancer

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ABSTRACT

Metastasis is the main problem in the treatment of prostate cancer (PCa), and for it to occur, proteolytic enzymes must remodel the extracellular matrix (ECM) surrounding the tumor. The most important group of enzymes with this action include the matrix metalloproteinases (MMPs), which act on various substrates cleaving ECM components. The aim of the present study was to evaluate the immunostaining of MMP-2 and MMP-9 in PCa patients using the indirect immunohistochemical technique in 60 tissue samples divided into groups: better prognosis (n=20), worse prognosis (n=23) and metastatic (n=17). Immunostaining profile was evaluated by a pathologist using a crosses' system: (0) absence of staining, (+) weak and (++ and +++) strong staining. The malignant tumor cytoplasmic MMP-2 immunostaining was statistically more intense than in ECM (p=0.001), but it did not correlate with any clinical-pathological parameter. The MMP-9 staining was similar in all cell compartments and showed significant correlation with prognostic groups (p=0.038; Tau=0.253), ISUP grade (p=0.044; Tau=0.249), extraprostatic extension (p=0.025; Tau=0.309) and biochemical recurrence (p=0.048; Tau=0.306). Our results suggest that MMP-9 protein plays a role in prognostic aspects and seems to constitute a marker for tissue invasion factors, essential in the metastatic process of PCa.

INTRODUCTION

Prostate cancer (PCa) represents one of the most common cancers in the world, ranked first in 12 regions of the planet [1]. In the United States of America, more than 3 million new cases occurred in 2022 [2] and in Brazil there were about 70,000 new cases with a mortality rate of 13.5% [3].

The exams used in the urological clinic for its detection consist of a digital rectal examination and quantification of prostate-specific antigen (PSA) in the blood, a biomarker that may indicate changes in the prostate but is not specific for PCa [4]. To confirm the diagnosis, histopathological analysis of fragments collected at biopsy are the gold standard technique [5]. In this context, studies searching for new markers that are more specific to the tumor and may indicate the prognosis of PCa are of great importance.

The biggest problem in treating PCa is metastasis [6], which involves the cancer cells migration to new locations in the body [7]. For this process to occur, the components of the extracellular matrix (ECM) must be remodeled in a way that allows the passage of cells to the circulatory system vessels [8].

The ECM is highly hydrated and mainly composed of minerals, proteoglycans, glycosaminoglycans, glycoproteins, and fibrous proteins [9] and has the role of providing structural and biochemical support to tissues [8]. These components are in dynamic balance to maintain tissue homeostasis, and small changes in this harmony can lead to significant changes in cellular stimuli [10].

Essential to the migration and invasion process are the matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidases that are involved in ECM degradation and remodeling [11, 12]. Under healthy conditions, these enzymes act in inflammation, tissue repair after injury, and organogenesis. In cancer progression, MMPs are active during invasion of local and distant tissues [12]. Human tissues express 23 types of MMPs, which can be divided into 6 groups: collagenases, gelatinases, stromelysins, metrilysins, membrane-type MMPs, and other MMPs [13]. Among these groups, gelatinases stand out, composed of MMP-2 and MMP-9, which are recognized as the most important enzymes for ECM degradation and are involved in the processes of tumor invasion and metastasis [13].

In this context, the present study aimed to evaluate the immunostaining profile of ECM remodeling proteins MMP-2 and MMP-9 in different cellular regions of tissue samples from patients with metastatic and non-metastatic PCa (of better and worse prognosis) and relate them to clinical-pathological parameters, in the search for candidate markers of prognosis and prediction of metastasis.

METHODS

Sample selection

In this retrospective and longitudinal study, 60 paraffin-embedded tissue samples from patients with PCa were analyzed, corresponding to the same sample group used by Pereira et al. [14]. These samples were selected from patients from Cancer Hospital of Londrina diagnosed between the years 2006 and 2016. Out of the 60 samples, 50 were from radical prostatectomy, 5 from biopsy, and 5 from transurethral resections.

The study was approved by the Ethics Committee for Research on Human Subjects at the State University of Londrina - Brazil, under number 176/2013. All patients signed an informed consent form and voluntarily participated in this research.

All clinical-pathological information was obtained from medical record analysis used with National Comprehensive Cancer Network guidelines [15] to classify patients into three sample groups: PCa with better prognosis (n=20); PCa with worse prognosis (n=23); and metastatic PCa (n=17). Patients with an ISUP grade ≤ 2 (3+4), a TNM staging $\leq$ T2a, and a PSA level ≤ 10 ng/mL were classified as having a better prognosis. Patients with an ISUP grade ≥ 3 (4+3), a TNM staging $\geq$ T3a, and a PSA level ≥ 20 ng/mL were considered with worse prognosis. Patients with metastasis at diagnosis were classified according to the presence of lymph node invasion and/or distant metastasis and/or positive bone scintigraphy. A table with all clinical-pathological parameters is included as Supplementary Material 1.

Histopathological Analysis

Tissue samples obtained from the biopsies were stained with hematoxylin and eosin to confirm the diagnosis of PCa and to verify the presence of malignant tumor and adjacent non-tumor tissue in all samples for later comparison. The histopathological classification used was the ISUP grade, established by the International Society of Urological Pathology in 2014, and clinical staging was determined according to the tumor-lymph node-metastasis (TNM) system, as recommended by the AJCC (American Joint Committee on Cancer) [16].

Immunohistochemistry

Experiments were performed according to Pereira et al. [14]. Samples of tumor and adjacent non-tumor tissue embedded in paraffin and fixed in formalin were collected and 6 μm thick histological sections were fixed on silanized StarFrost® slides (Knittel glass, ALE).

Deparaffinization, rehydration, and antigenic retrieval were performed as described in the article cited above. Incubation of the primary antibody was performed overnight and with specific dilutions: (1) Rabbit Anti-MMP-2 [LF -183] (polyclonal, National Institute of Dental and Craniofacial Research) 1:1000 dilution; (2) Rabbit Anti-MMP-9 [LF -183] (polyclonal, National Institute of Dental and Craniofacial Research) 1:500 dilution. Dilutions were tested on positive control tissue according to the manufacturer's instructions: breast malignant tumor (MMP-2 and MMP-9).

Negative controls were performed in all batteries to ensure the specificity of the primary antibody, which was replaced with phosphate-buffered saline (PBS). The secondary antibody kit (mouse/rabbit detection kit HRP/DAB ABC, Abcam, Cambridge, MA, USA) was used according to the manufacturer's instructions.

Immunostaining profile was analyzed by a pathologist in both malignant tumor and adjacent non-tumor tissue, and different cell regions were evaluated: cytoplasm, nucleus, and ECM. When analyzing the adjacent non-tumor tissue, only normal glands and areas of benign hyperplasia were considered, and areas of atrophy were excluded. The classification was based on the intensity of staining: (0) absence of staining, (+) weak staining, and (++ and +++) strong staining.

Statistical analysis

To compare the immunostaining between malignant tumor X adjacent non-tumor tissue for each protein, the McNemer test for related samples was used. Kendall's Tau test was used to analyze the correlations between protein staining and clinical-pathological parameters, and logistic regression was performed on the variables that showed significance. Interaction analysis between proteins was also performed using the Kendall's Tau test. In addition, the Receiver Operating Characteristic (ROC) curve was performed to analyze the sensitivity and specificity of each protein as a prognostic biomarker for PCa.

Some data were not included in the statistical analysis due to the lack of information in the medical records and due to the wear of some of the paraffin blocks, making it impossible to obtain tissue for analysis. Furthermore, some patients did not have malignant tumor and adjacent non-tumor tissue on the same slide for comparison.

All statistical analyzes were performed using IBM® SPSS® Statistics for Windows software, version 26.0 (IBM® Corp., Armonk, N.Y., USA), considering a significance level (ł) of 5%.

RESULTS

In general, MMP-2 and MMP-9 showed positive immunostaining in both cytoplasmic regions of the malignant tumor and in the non-tumor adjacent tissues. No labeling was found in the nuclear region of either tissue. Therefore, the described markings always refer to the cytoplasm, either of the malignant tumor or of the adjacent non-tumor tissue. In addition, positive staining for both proteins was observed in the ECM.

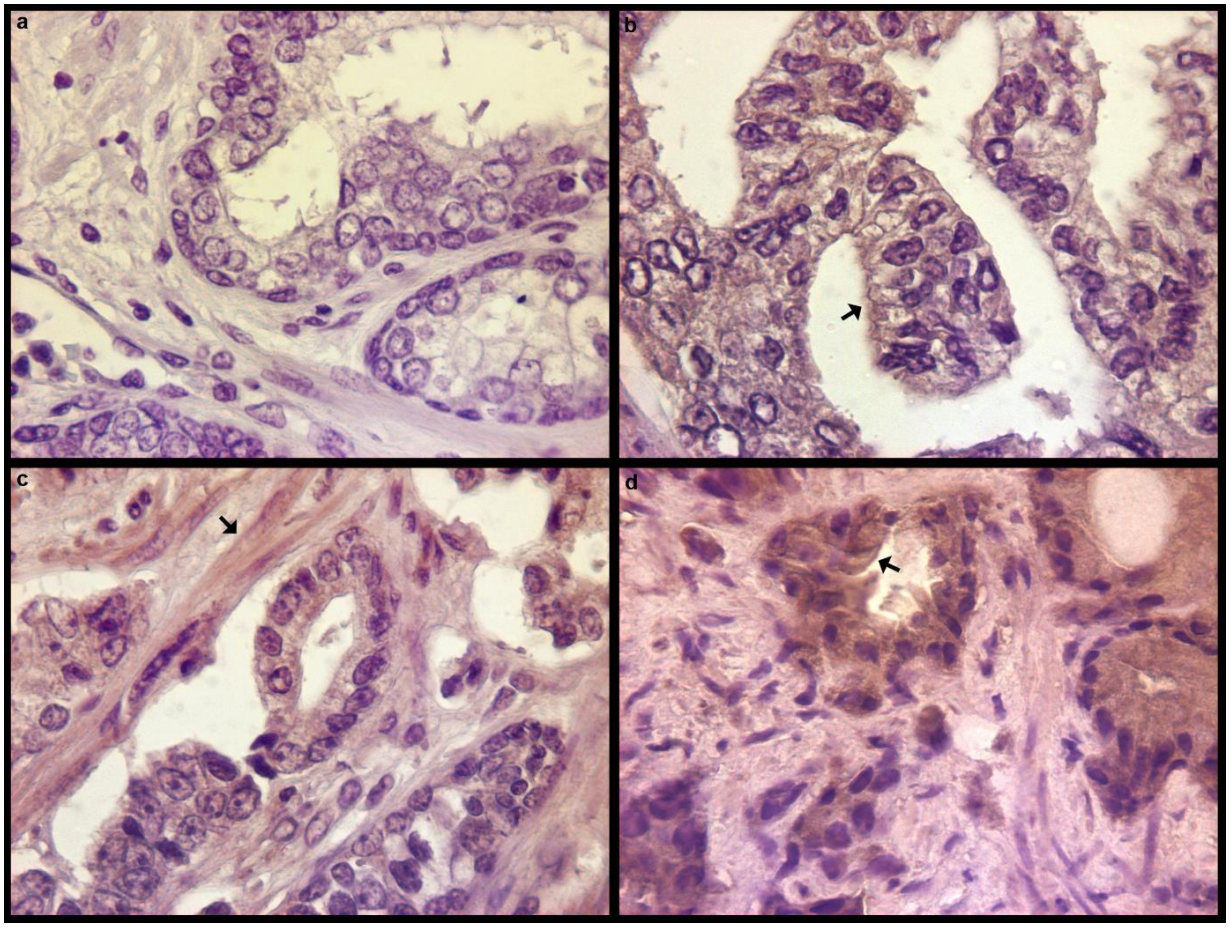

Figure 1. MMP-2 immunostaining profile in the malignant tumor cytoplasm (b and d) and ECM (c) using immunohistochemistry technique with arrows presenting the staining. (a) showing absence of staining (0), (b) weak staining (+), (c) and (d) strong staining (++ and +++, respectively). 40x magnification.

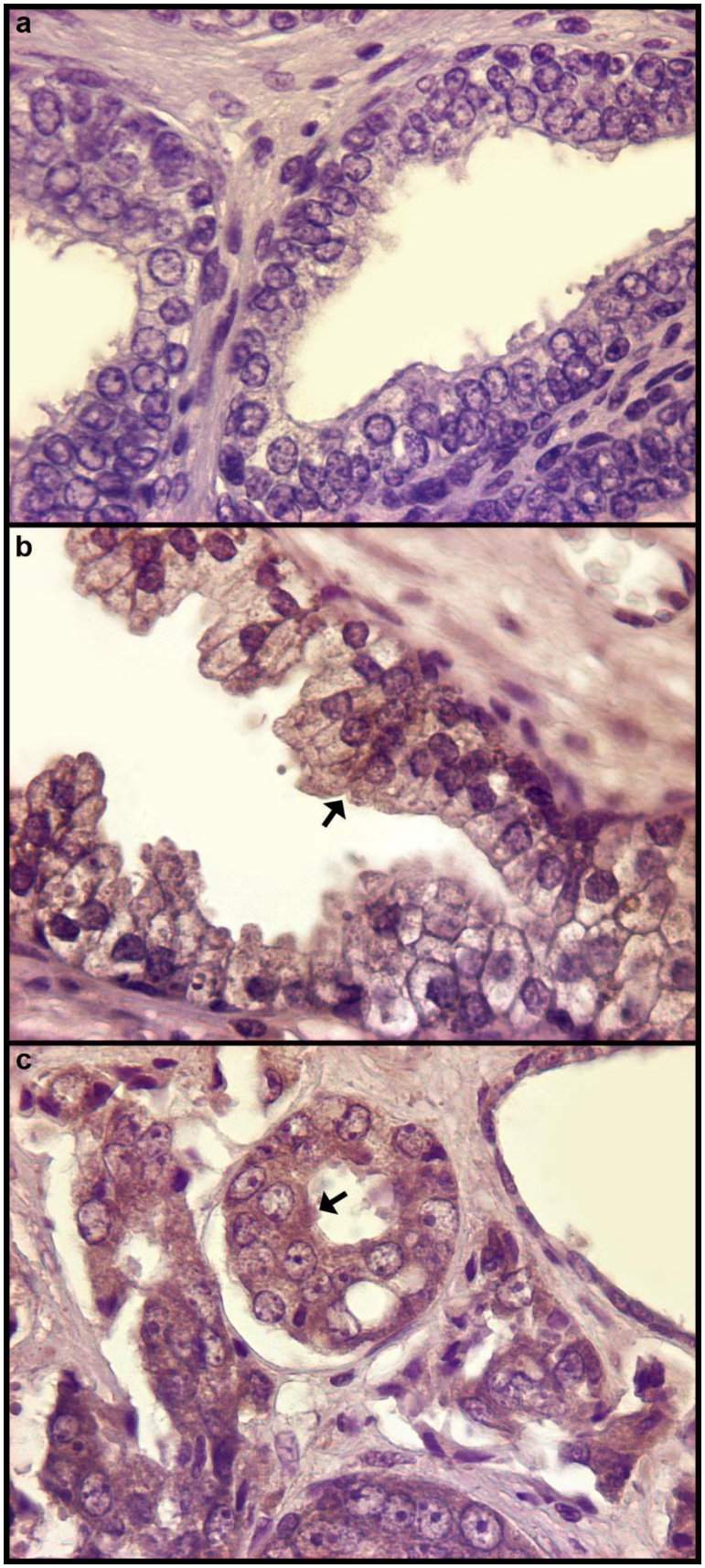

Figure 2. MMP-9 immunostaining profile in the malignant tumor cytoplasm (b and c) using immunohistochemistry technique with arrows presenting the staining. (a) showing absence of staining (0), (b) weak staining (+), and (c) strong staining (++). 40x magnification.

MMP-2

A predominance of weak (+) immunostaining was observed in the adjacent non-tumor tissue (34/58 - 58.62%) and in the ECM (38/60 - 63.33%). Although weak staining (+) was predominant in the malignant tumor (30/60 - 50.0%), a large number of strong markings (++ and +++) were observed (19/60 - 31.67%) (Supplementary Materials 2-4).

MMP-2 showed no significant differences in immunostaining profile between malignant tumor X adjacent non-tumor tissue (p=0.064). However, differences were observed between malignant tumor X ECM labeling (p=0.001) where the tumor tissue showed stronger labeling. MMP-2 staining did not show significant associations or correlations with clinical-pathological parameters (Supplementary Material 5).

MMP-9

MMP-9 showed mostly weak labeling in the three regions evaluated: adjacent non-tumor (38/58 - 65.52%), malignant tumor (42/60 - 70.0%), and ECM (37/60 - 61.67%) (Supplementary Materials 6-8). No significant differences were observed between immunostaining in malignant tumor X adjacent non-tumor tissue (p=0.132) or X ECM (p=0.061).

A significant association was found between weak (+) staining in malignant tumor and increased PSA levels (p=0.041), but no significant correlation with clinical-pathological parameters. Weak (+) immunostaining in the ECM showed a significant association with increased TNM (p=0.024) and a significant correlation with: Worsening prognosis group (p=0.038; Tau=0.253), advanced ISUP grade (p=0.044; Tau=0.249), presence of extraprostatic extension (p=0.025; Tau=0.309) and presence of biochemical recurrence (p=0.048; Tau=0.306) (Table 1).

Multinomial Logistic Regression

To assess whether the markers that showed significant associations with clinical-pathological parameters were independently associated, multinomial logistic regression analyzes were performed. None of the variables showed significant results, as shown in Supplementary Materials 9 and 10.

Protein Interaction

To determine whether there was a relationship between proteins immunostaining, an interaction analysis was performed considering only the malignant tumor and the ECM. A significant correlation was found between the staining of MMP-2 and MMP-9 in the tumor ($\chi^2 \leq 0.001$; $p \leq 0.001$; Tau=0.461) and between the staining of both proteins in the ECM ($\chi^2 \leq 0.001$; $p \leq 0.001$; Tau=0.443).

Table 1. Comparison analysis between clinical-pathological parameters and MMP-9 immunostaining in malignant tumor tissue and ECM of patients with PCa.

Clinical-patological Data	Absence of Staining		Weak Staining		Strong Staining		*p* value of χ^2		*p* value of Kendall (value of Tau)	
	Cytoplasm	**ECM**	**Cytoplasm**	**ECM**	**Cytoplasm**	**ECM**	**Cytoplasm**	**ECM**	**Cytoplasm**	**ECM**
Experimental Groups										
Better Prognosis	6 (46,2%)	11 (50,0%)	13 (31,0%)	8 (21,6%)	1 (20,0%)	1 (100,0%)				
Worse Prognosis	6 (46,2%)	8 (36,4%)	14 (33,3%)	15 (40,5%)	3 (60,0%)	0 (0,0%)	0,292	0,082	0,148 (0,173)	**0,038*** (0,253)
Metastatic	1 (7,7%)	3 (13,6%)	15 (35,7%)	14 (37,8%)	1 (20,0%)	0 (0,0%)				
Age										
≤64 years	4 (30,8%)	11 (50,0%)	16 (38,1%)	9 (24,3%)	1 (20,0%)	1 (100,0%)	0,679	0,053	0,962 (0,006)	0,120 (0,201)
≥65 years	9 (69,2%)	11 (50,0%)	26 (61,9%)	28 (75,7%)	4 (80,0%)	0 (0,0%)				
ISUP										
1	6 (46,2%)	9 (42,9%)	12 (29,3%)	8 (21,6%)	0 (0,0%)	1 (100,0%)				
2 and 3	5 (38,5%)	11 (52,4%)	19 (46,3%)	17 (45,9%)	4 (80,0%)	0 (0,0%)	0,366	0,059	0,140 (0,179)	**0,044*** (0,249)
4 and 5	2 (15,4%)	1 (4,8%)	10 (24,4%)	12 (32,4%)	1 (20,0%)	0 (0,0%)				
TNM										
≤T2a	4 (30,8%)	6 (28,6%)	9 (24,3%)	7 (21,9%)	1 (25,0%)	1 (100,0%)				
T2b to T2c	3 (23,1%)	8 (38,1%)	8 (21,6%)	3 (9,4%)	0 (0,0%)	0 (0,0%)	0,823	**0,024***	0,428 (0,101)	0,122 (0,200)
≥T3a	6 (46,2%)	7 (33,3%)	20 (54,1%)	22 (68,8%)	3 (75,0%)	0 (0,0%)				
PSA (ng/mL)										
<10	9 (69,2%)	13 (61,9%)	15 (35,7%)	11 (29,7%)	1 (25,0%)	1 (100,0%)				
10 to 20	1 (7,7%)	3 (14,3%)	12 (28,6%)	13 (35,1%)	3 (75,0%)	0 (0,0%)	**0,041***	0,118	0,187 (0,160)	0,113 (0,195)
>20	3 (23,1%)	5 (23,8%)	15 (35,7%)	13 (35,1%)	0 (0,0%)	0 (0,0%)				
Seminal Vesicle Invasion										
No	9 (69,2%)	17 (81,0%)	25 (75,8%)	18 (66,7%)	2 (66,7%)	1 (100,0%)	0,869	0,448	0,804 (-0,035)	0,375 (0,127)
Yes	4 (30,8%)	4 (19,0%)	8 (24,2%)	9 (33,3%)	1 (33,3%)	0 (0,0%)				

Extraprostatic Extension										
No	7 (53,8%)	14 (66,7%)	16 (44,4%)	9 (29,0%)	1 (25,0%)	1 (100,0%)	0,589	**0,015***	0,343 (0,128)	**0,025*** (0,309)
Yes	6 (46,2%)	7 (33,3%)	20 (55,6%)	22 (71,0%)	3 (75,0%)	0 (0,0%)				
Perineural Invasion										
No	9 (69,2%)	17 (85,0%)	25 (78,1%)	19 (70,4%)	3 (100%)	1 (100,0%)	0,505	0,428	0,299 (-0,148)	0,336 (0,139)
Yes	4 (30,8%)	3 (15,0%)	7 (21,9%)	8 (29,6%)	0 (0,0%)	0 (0,0%)				
Biochemical recurrence										
No	5 (55,6%)	9 (69,2%)	14 (43,8%)	11 (39,3%)	1 (100%)	0 (0,0%)	0,468	0,127	0,838 (0,032)	**0,048*** (0,306)
Yes	4 (44,4%)	4 (30,8%)	18 (56,3%)	17 (60,7%)	0 (0,0%)	1 (100,0%)				

Chi-square test and Kendall's Tau correlation. *Significance level of $p<0.05$. Due to lack of data and wear of paraffin blocks, some variables do not include the total number of patients.

ROC curve

To evaluate the potential of the proteins as predictors of metastasis, ROC curves were performed for malignant tumor and ECM immunostainings of the two proteins. None of the curves showed significant values, as shown in Supplementary Materials 11-13.

DISCUSSION

In the present study, the immunostaining profiles of MMP-2 and MMP-9 proteins in metastatic and non-metastatic prostate tumors were investigated. Cytoplasmic and ECM staining were observed, corroborating previous results in the literature [17, 18, 19 20]. Our main results indicated a significant difference between MMP-2 immunostaining between malignant tumor cells and ECM. ECM staining of MMP-9 also correlated with worse prognostic parameters. None of the proteins were able to effectively predict metastasis.

Tumor metastasis, the final step in cancer progression and the main cause of mortality and morbidity in this disease, depends on the spread of cancer cells from the primary tumor to nearby or distant tissues [21, 6]. The changes required for this process include epithelial-mesenchymal transition (EMT), degradation of cell-cell and cell-matrix adhesion, remodeling of the ECM, and cleavage of the basement membrane (BM) by enzymes such as MMPs [22]. The role of MMPs in tumorigenesis is mainly due to the digestion of ECM components, which facilitates the release of tumor cells [23].

Gelatinases, one of the six MMP families, have a distinct collagen-binding domain composed of three fibronectin repeats of the type II in the N-terminal catalytic domain which is required for gelatin binding and denaturation of type IV and V collagens [24]. Both gelatinases play a role in tumorigenesis of various cancers, e.g., breast [25, 26], prostate [27], and lung [28]. The two proteins evaluated in the present study, MMP-2 and MMP-9, belong to this family.

In the study by Ross et al. [17], the MMP-2 protein, also known as Gelatinase A, showed differential immunostaining between malignant tumor cells and the benign tissue elements. In tumor tissue, this protein showed moderate and intense immunostaining, while in non-tumor tissue it was weakly stained. This result differs from the results of the present study, in which no difference was observed in MMP-2 staining between malignant tumor and adjacent non-tumor tissue ($p=0.064$). One of the reasons that could explain this difference between the literature and our results is that the study by Ross et al. [17] compared tumor tissue with benign tissue, whereas our study compared malignant tissue with non-tumor tissue adjacent to the tumor.

Our results show stronger labeling of MMP-2 in malignant tumor tissue compared to ECM ($p=0.001$). This can be explained by the mechanism of MMP-2 production and activation, which occurs first in the cell and then in the tissue stroma. The pro-MMP-2, produced by the cells, is exported to the ECM and, in order to be effectively activated into MMP-2, it needs to bind to TIMP-2 (Tissue inhibitor of metalloproteinases 2), which is associated with MMP-14, one of the MMPs located on the cell surface [29, 18]. This suggests that although tumor cells have high protein production, their activation does not follow the same pattern.

Although no significant association and/or correlation between MMP-2 immunostaining and clinical-pathological parameters was found in our study, such results are common in the literature. High MMP-2 expression has been correlated with increased TNM grade and Gleason score [19], increase in tumor grade and stage [30], factors indicative of poorer prognosis. No significant results were found when evaluating the sensitivity and specificity of MMP-2 as a predictor of PCa metastasis, which is consistent with the findings of Salminen et al.

[31], who found no significant association on this protein as a predictor of bone metastasis in patients with PCa. Our analyzes were unable to distinguish the active form of MMP-2 from the immature form of pro-MMP-2. This may be one of the explanations for the lack of associations and correlations found between MMP-2 immunostaining and clinical-pathological parameters. Perhaps analyzes that can assess only the active form of this protein will be more successful in assessing its role as a biomarker for predicting metastasis.

In contrast to MMP-2, MMP-9 protein (also called Gelatinase B) showed no significant differences in immunostaining between the tissue localizations examined (tumor cytoplasm, adjacent non-tumor cytoplasm, and ECM), suggesting that this protein is produced and expressed in the same manner at these sites. This differs from the findings of Baspinar et al. [32], who found differences in MMP-9 immunostaining between malignant tumor tissue and benign prostatic hyperplasia. Cardillo et al. [33] also found higher expression of MMP-9 in tumor cells compared with stromal tissue. Our results indicate that MMP-9 is synthesized and secreted equally by malignant tumor, adjacent non-tumor, and stromal cells, where it may play a role in migration and growth factor release [22].

Regarding the association and correlation with clinical-pathological parameters, Cardillo et al. [33] found that high-grade prostate tumors had high expression of MMP-9 by immunohistochemistry and in situ hybridization. Miyake et al. [34] found a significant association between MMP-9 expression and several clinical and prognostic parameters, such as PSA, pathological stage, Gleason score, and seminal vesicle invasion. Incorvaia and colleagues [35] found an association between MMP-9 and PSA levels. Our results corroborate those mentioned above, as we also observed significant associations between MMP-9 staining and TNM (p=0.024), PSA (p=0.041), and extraprostatic extension (p=0.015). However, given the lack of significance of the multinomial logistic regression, it can be concluded that these variables are not independently associated with protein immunostaining.

In addition, we found a significant correlation between MMP-9 labeling and worsening of the experimental groups (p=0.038; Tau=0.253), advanced ISUP grade (p=0.044; Tau=0.249), presence of extraprostatic extension (p=0.025; Tau=0.309), and presence of biochemical recurrence (p=0.048; Tau=0.306). Tumor expansion outside the prostate is a common pathway for uncontrolled growth in PCa, which is also a major cause of morbidity, mortality, and poor prognosis [32]. All these significant correlations suggest that MMP-9 expression appears to play a role in invasive processes, which are essential for PCa evolution [36], and support the hypothesis that in prostate carcinomas large amounts of MMP-9 promote invasion and metastasis [37]. However, regarding the sensitivity and specificity as a biomarker for predicting metastasis, we found no significant results, as well as in previous studies [35], in which the protein was unable to distinguish patients with bone metastasis.

Protein interaction analysis, performed between immunostaining in the same cell compartments, showed that there is an interaction between MMP-2 and MMP-9 in both the malignant tumor tissue and the surrounding ECM. This can be explained by the fact that both proteins belong to the same subgroup of MMPs and have a very similar action in cleaving ECM elements [13]. Both are particularly involved in various types of cancer through the degradation of elastin, fibronectin, and vitronectin, as well as the activation of growth factors that activate the cell cycle [23].

However, the present study has its positive and negative aspects. Among the negative ones, the sample size of the metastatic group can be highlighted, with a smaller number of patients compared with the better and worse prognosis groups. This fact is due to the low incidence of metastatic PCa in the population, which made it difficult to obtain samples. Meanwhile the positive aspects, the following ones stands out: 1) composition of sample groups based on prognostic parameters; 2) robust statistical analysis with comprehensive data exploration;

3) standardization of the methodology using ideal positive controls for each antibody; and 4) analysis of immunostaining profiles by an experienced pathologist, considering the usual staining in malignant tumor, adjacent non-tumor, and ECM tissues in virtually all samples, in addition to looking for different cell locations.

CONCLUSION

Although MMP-2 immunostaining was more intense in malignant tumor tissue, it does not seem to be able to clearly indicate prognosis in PCa patients or predict the metastatic process. Studies evaluating only its active form should be performed to measure its true prognostic role. MMP-9, while also unable to effectively predict metastasis, appears to constitute a marker for tissue invasion, essential to this process. Further studies on immunostaining of this protein should be performed to provide further clarification and possible future clinical application in prostate tumorigenesis.

ABBREVIATIONS

PCa: prostate cancer; ECM: extracellular matrix; MMPs: matrix metalloproteinases; MMP-2: metalloproteinase 2; MMP-9: metalloproteinase 9; PSA: prostate-specific antigen; ISUP: International Society of Urological Pathology; TNM: tumor-lymph node-metastasis; AJCC: American Joint Committee on Cancer; ROC: Receiver Operating Characteristic; EMT: epithelial-mesenchymal transition; BM: basement membrane; TIMP-2: tissue inhibitor of metalloproteinases 2; MMP-14: metalloproteinase 14.

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AUTHOR CONTRIBUTIONS

Laís Capelasso Lucas Pinheiro: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Visualization. Érica Romão Pereira: Methodology, Visualization. Amanda Letícia Francelino: Methodology, Investigation. Alda Fiorina Maria Losi Guembarovski: Investigation. Paulo Emílio Fuganti: Resources. Karen Brajão de Oliveira: Formal Analysis, Writing - Review & Editing. Carlos Alberto Miqueloto: Conceptualization, Resources, Writing - Review & Editing, Supervision. Juliana Mara Serpeloni: Conceptualization, Writing - Review & Editing. Roberta Losi Guembarovski: Conceptualization, Formal Analysis, Resources, Writing - Review & Editing, Supervision, Project Administration, Funding Acquisition.

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DATA AVAILABILITY

All data generated or analyzed during this study are included in this manuscript and its supplementary files.

DECLARATIONS

Ethics Approval - This study was approved by the Institutional Ethics Committee Involving Humans at State University of Londrina, Londrina—Paraná (PR), Brazil (CEP/UEL 176/2013; CAAE 19769913.0.0000.5231, in accordance with Resolution 466/12 of the National Research Ethics Commission).

Consent to Participate - The study purpose and procedures were explained to all the patients and written informed consent was obtained from all the individual participants included in the study.

Consent for Publication - Not applicable.

Competing Interests - The authors have no competing interests to declare that are relevant to the content of this article.

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HIGHLIGHTS

- Study group divided into better, worse and metastatic prognostic groups.
- MMP-2 strongly immunostained in tumor tissue compared with ECM.
- MMP-9 protein significantly correlated with important invasive parameters.
- MMP-2 and MMP-9 do not act as predictors of metastasis in PCa patients.
- MMP-9 as a candidate prognostic marker for invasive processes in PCa samples.

Supplementary Material 1. Clinical-pathological characteristics of the patients.

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	ISUP	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT144	Better Prognosis	RP	2008	61	6	1	pT2a	04.62	No	No	No	No
PT149	Better Prognosis	RP	2006	65	6	1	pT2a	08.61	No	No	No	No
PT178	Better Prognosis	RP	2008	61	6	1	pT2a	04.24	No	No	No	Yes
PT180	Better Prognosis	RP	2008	66	6	1	pT2a	11.07	No	No	No	Yes
PT188	Better Prognosis	RP	2006	66	6	1	pT2a	12.33	No	No	No	No
PT235	Better Prognosis	RP	2011	62	6	1	pT2a	03.62	No	No	No	Yes
PT240	Better Prognosis	RP	2011	61	6	1	pT2a	03.68	No	No	No	No
PT246	Better Prognosis	RP	2011	63	7 (3+4)	2	pT2a	-	No	No	No	-
PT266	Better Prognosis	RP	2012	69	6	1	pT2a	04.04	No	No	No	No
PT271	Better Prognosis	RP	2014	65	7 (3+4)	2	pT2b	07.74	No	No	No	-
PT308	Better Prognosis	RP	2014	67	6	1	pT2b	07.14	No	No	No	-
PT318	Better Prognosis	RP	2014	72	7 (3+4)	2	pT2b	05.65	No	No	No	No
PT320	Better Prognosis	RP	2014	60	6	1	pT2a	07.88	No	No	No	No
PT332	Better Prognosis	RP	2015	66	7 (3+4)	2	pT2c	06.34	No	No	No	No
PT345	Better Prognosis	RP	2015	56	6	1	pT2b	05.78	No	No	No	No
PT347	Better Prognosis	RP	2015	59	6	1	pT2a	06.69	No	No	No	Yes
PT359	Better Prognosis	RP	2015	74	7 (3+4)	2	pT2b	08.90	No	No	No	-
PT374	Better Prognosis	RP	2015	59	7 (3+4)	2	pT2b	05.87	No	No	No	-
PT383	Better Prognosis	RP	2016	70	6	1	pT1a	06.00	No	No	No	-
PT384	Better Prognosis	RP	2015	68	6	1	pT2a	06.79	No	No	No	-
PT012	Worse Prognosis	RP	2006	74	8 (4+4)	4	pT3a	09.63	No	Yes	Yes	Yes
PT074	Worse Prognosis	RP	2007	76	7 (4+3)	3	pT3a	23.69	No	Yes	No	Yes
PT089	Worse Prognosis	RP	2007	70	6	1	pT3a	23.15	No	Yes	No	Yes

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	ISUP	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT116	Worse Prognosis	RP	2007	65	8 (3+5)	4	pT3c	09.26	Yes	Yes	Yes	Yes
PT126	Worse Prognosis	RP	2008	72	8 (3+5)	4	pT3a	09.38	No	Yes	No	No
PT148	Worse Prognosis	RP	2006	61	8 (5+3)	4	pT3b	31.00	Yes	Yes	No	Yes
PT175	Worse Prognosis	RP	2007	65	7 (4+3)	3	pT3b	9.36	Yes	Yes	No	No
PT199	Worse Prognosis	RP	2008	70	8 (4+4)	4	pT3a	19.29	No	Yes	No	Yes
PT212	Worse Prognosis	TUR	2011	58	-	-	-	28.31	-	-	-	-
PT247	Worse Prognosis	RP	2011	67	7 (3+4)	2	pT3b	31.51	Yes	Yes	Yes	Yes
PT248	Worse Prognosis	RP	2011	67	8 (3+5)	4	pT3b	14.30	Yes	Yes	No	Yes
PT259	Worse Prognosis	RP	2011	61	8 (3+5)	4	pT3a	26.43	No	Yes	Yes	No
PT263	Worse Prognosis	RP	2012	71	7 (3+4)	2	pT3b	22.93	Yes	Yes	Yes	No
PT281	Worse Prognosis	RP	2014	72	7 (4+3)	3	pT3b	15.00	Yes	Yes	No	-
PT282	Worse Prognosis	RP	2014	66	7 (3+4)	2	pT3a	06.15	No	Yes	No	-
PT283	Worse Prognosis	RP	2014	55	7 (3+4)	2	pT2c	18.82	No	No	No	No
PT288	Worse Prognosis	RP	2014	72	7 (4+3)	3	pT3a	09.13	No	Yes	No	No
PT291	Worse Prognosis	RP	2014	73	7 (3+4)	2	pT3a	19.36	No	Yes	No	Yes
PT324	Worse Prognosis	RP	2014	71	7 (3+4)	2	pT3a	21.69	No	Yes	-	-
PT339	Worse Prognosis	RP	2015	64	7 (3+4)	2	pT3a	13.37	No	Yes	No	No
PT357	Worse Prognosis	RP	2015	76	7 (3+4)	2	pT3a	16.74	No	Yes	No	-
PT371	Worse Prognosis	RP	2015	57	7 (3+4)	2	pT3a	14.77	No	Yes	Yes	Yes
PT379	Worse Prognosis	RP	2015	63	7 (3+4)	2	pT3a	21.32	No	Yes	No	-
PT024	Metastatic	RP	2006	59	6	1	pT2c	30.69	No	No	Yes	No
PT129	Metastatic	RP	2008	84	8 (4+4)	4	-	30.85	-	-	-	Yes
PT140	Metastatic	TUR	2008	83	7 (3+4)	2	pT2a	618.7	-	-	-	No
PT163	Metastatic	TUR	2008	62	9 (4+5)	5	-	25.15	-	-	-	Yes

Patient	Experimental Group	Exam Type	Year	Age	Gleason Score	ISUP	TNM Staging	PSA (ng/mL)	Seminal Vesicle Invasion	Extraprostatic Extension	Perineural Invasion	Biochemical Recurrence
PT202	Metastatic	RP	2008	59	6	1	pT2b	04.48	No	No	No	Yes
PT249	Metastatic	RP	2011	56	8 (3+5)	4	pT3bN1	18.09	Yes	Yes	No	Yes
PT269	Metastatic	RP	2012	71	7 (3+4)	2	pT3aN1	18.05	No	Yes	Yes	-
PT280	Metastatic	BIOP	2014	82	7 (3+4)	2	pT3a	520.7	-	Yes	Yes	-
PT284	Metastatic	RP	2014	74	8 (4+4)	4	pT3bN1	16.00	Yes	Yes	No	No
PT287	Metastatic	RP	2014	73	7 (4+3)	3	pT3b	141.7	Yes	Yes	-	Yes
PT295	Metastatic	BIOP	2014	84	8 (4+4)	4	-	15.70	-	-	Yes	-
PT326	Metastatic	BIOP	2013	71	6	1	pT2cNxM1a	23.27	-	No	-	Yes
PT333	Metastatic	BIOP	2014	83	7 (3+4)	2	pT4	11.81	Yes	Yes	-	-
PT338	Metastatic	BIOP	2014	80	7 (4+3)	3	-	16.00	-	-	-	-
PT361	Metastatic	RP	2015	58	7 (4+3)	3	pT3b	56.03	Yes	Yes	Yes	Yes
PT377	Metastatic	TUR	2015	66	7 (3+4)	2	-	35.00	-	-	-	No
PT385	Metastatic	RP	2015	68	8 (4+4)	4	pT3a+bN1	03.28	Yes	Yes	No	Yes

The Exam type are presented as follow: (RP) Radical Prostatectomy; (TUR) Transurethral Resection; (BIOP) Biopsy; (-) no data. Due to lack of data, some variables did not include the total of patients.

Supplementary Material 2. Analysis of MMP-2 immunostaining in tissue samples from patients with better prognosis PCa.

Patients Better prognosis	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
PT144	+	+	+
PT149	+	0	+
PT178	++	+	+
PT180	++	++	+
PT188	+	++	+
PT235	++	+	0
PT240	+	++	+
PT246	++	+	0
PT266	++	+	0
PT271	+	++	0
PT308	0	+	+
PT318	0	+	0
PT320	+	++	++
PT332	+	+	+
PT345	+	+	0
PT347	+	+	+
PT359	+	+	+
PT374	0	0	0
PT383	+	+	+
PT384	+	+	+

0 = absence of staining; + = weak staining, ++ = strong steining.

Supplementary Material 3. Analysis of MMP-2 immunostaining in tissue samples from patients with worse prognosis PCa.

Patients Worse prognosis	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
PT12	+	+	+
PT74	+	++	+
PT89	+	++	+
PT116	+	+	+
PT126	0	++	+
PT148	+	++	+
PT175	0	0	0
PT199	+	+	++
PT212	+	++	++
PT247	0	0	0
PT248	0	0	0
PT259	+	++	++
PT263	0	0	0
PT281	0	+	+
PT282	0	0	0
PT283	0	0	0
PT288	+	+	0
PT291	0	0	0
PT324	+	+	+
PT339	+	+	+
PT357	++	+	+
PT371	++	+	+
PT379	0	0	0

0 = absence of staining; + = weak staining, ++ = strong staining.

Supplementary Material 4. Analysis of MMP-2 immunostaining in tissue samples from patients with metastatic CaP.

Patients	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
Metastatic			
PT24	+	0	0
PT129		+	+
PT140	+	+	+
PT163	0	++	+
PT202	0	++	0
PT249	+	+	+
PT269	+	++	+
PT280	+	+++	+
PT284	0	+	+
PT287	+	+	+
PT295	+	+	+
PT326	+	++	+
PT333	+	+	+
PT338		++	+
PT361	0	++	+
PT377	+	+	+
PT385	+	++	+

0 = absence of staining; + = weak staining, ++ and +++ = strong staining.

Supplementary Material 5. Comparison analysis between clinicopathological parameters and MMP-2 immunostaining in malignant tumor tissue and ECM of patients with PCa.

Clinical-patological Data	Absence of Staining		Weak Staining		Strong Staining		*p* value of $\chi 2$		*p* value of kendall (value of Tau)	
	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM
Experimental Groups										
Better Prognosis	2 (18,2%)	7 (38,9%)	13 (43,3%)	12 (31,6%)	5 (26,3%)	1 (25,0%)	0,065	0,096	0,319 (0,117)	0,289 (0,127)
Worse Prognosis	8 (72,7%)	9 (50,0%)	9 (30,0%)	11 (28,9%)	6 (31,6%)	3 (75,0%)				
Metastatic	1 (9,1%)	2 (11,1%)	8 (26,7%)	15 (39,5%)	8 (42,1%)	0 (0,0%)				
Age										
≤64 years	4 (36,4%)	8 (44,4%)	9 (30,0%)	10 (26,3%)	8 (42,1%)	3 (75,0%)	0,684	0,092	0,611 (-0,063)	0,798 (0,032)
≥65 years	7 (63,6%)	10 (55,6%)	21 (70,0%)	28 (73,7%)	11 (57,9%)	1 (25,0%)				
ISUP										
1	2 (18,2%)	5 (27,8%)	9 (30,0%)	12 (31,6%)	7 (38,9%)	1 (33,3%)	0,363	0,079	0,852 (-0,022)	0,275 (0,133)
2 and 3	8 (72,7%)	12 (66,7%)	14 (46,7%)	16 (42,1%)	6 (33,3%)	0 (0,0%)				
4 and 5	1 (9,1%)	1 (5,6%)	7 (23,3%)	10 (26,3%)	5 (27,8%)	2 (66,7%)				
TNM										
≤T2a	1 (9,1%)	3 (16,7%)	9 (33,3%)	10 (30,3%)	4 (25,0%)	1 (33,3%)	0,648	0,19	0,665 (-0,054)	0,772 (0,037)
T2b to T2c	3 (27,3%)	7 (38,9%)	5 (18,5%)	4 (12,1%)	3 (18,8%)	0 (0,0%)				
≥T3a	7 (63,6%)	8 (44,4%)	13 (48,1%)	19 (57,6%)	9 (56,3%)	2 (66,7%)				
PSA (ng/mL)										
<10	4 (36,4%)	10 (58,8%)	15 (51,7%)	14 (36,8%)	6 (31,6%)	1 (25,0%)	0,267	0,513	0,347 (0,111)	0,123 (0,186)
10 to 20	3 (27,3%)	3 (17,6%)	9 (31,0%)	12 (31,6%)	4 (21,1%)	1 (25,0%)				
>20	4 (36,4%)	4 (23,5%)	5 (17,2%)	12 (31,6%)	9 (47,4%)	2 (50,0%)				

Clinical-patological Data	Absence of Staining		Weak Staining		Strong Staining		p value of χ2		p value of kendall (value of Tau)	
	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM	Cytoplasm	ECM
Seminal Vesicle Invasion										
No	7 (63,6%)	14 (77,8%)	20 (76,9%)	19 (67,9%)	9 (75,0%)	3 (100,0%)	0,698	0,426	0,558 (-0,080)	0,887 (0,020)
Yes	4 (36,6%)	4 (22,2%)	6 (23,1%)	9 (32,1%)	3 (25,0%)	0 (0,0%)				
Extraprostatic Extension										
No	4 (36,6%)	10 (55,6%)	13 (50,0%)	13 (40,6%)	7 (43,8%)	1 (33,3%)	0,74	0,543	0,801 (-0,033)	0,276 (0,148)
Yes	7 (63,6%)	8 (44,4%)	13 (50,0%)	19 (59,4%)	9 (56,3%)	2 (66,7%)				
Perineural Invasion										
No	8 (72,7%)	15 (83,3%)	20 (83,3%)	20 (74,1%)	9 (69,2%)	2 (66,7%)	0,576	0,697	0,779 (0,039)	0,401 (0,119)
Yes	3 (27,3%)	3 (16,7%)	4 (16,7%)	7 (25,9%)	4 (30,8%)	1 (33,3%)				
Biochemical recurrence										
No	5 (62,5%)	8 (61,5%)	10 (50,0%)	10 (38,5%)	5 (35,7%)	2 (66,7%)	0,46	0,313	0,219 (0,183)	0,427 (0,121)
Yes	3 (37,5%)	5 (38,5%)	10 (50,0%)	16 (61,5%)	9 (64,3%)	33,3%)				

Chi-square test and Kendall's Tau correlation. *Significance level of $p<0.05$. Due to lack of data and wear of paraffin blocks, some variables do not include the total number of patients.

Supplementary Material 6. Analysis of MMP-9 immunostaining in tissue samples from patients with better prognosis PCa.

Patients Better prognosis	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
PT144	0	0	0
PT149	+	0	+
PT178	++	+	++
PT180	+	+	+
PT188	+	+	+
PT235	+	+	+
PT240	+	+	+
PT246	+	++	0
PT266	+	+	0
PT271	+	+	+
PT308	0	0	0
PT318	0	+	0
PT320	+	+	0
PT332	+	+	+
PT345	+	+	0
PT347	0	0	0
PT359	0	+	0
PT374	+	0	0
PT383	+	0	0
PT384	+	+	+

0 = absence of staining; + = weak staining, ++ = strong staining.

Supplementary Material 7. Analysis of MMP-9 immunostaining in tissue samples from patients with worse prognosis PCa.

Patients	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
Worse prognosis			
PT12	+	0	+
PT74	+	+	+
PT89	+	+	+
PT116	0	+	+
PT126	+	++	+
PT148	0	+	+
PT175	+	0	+
PT199	+	+	+
PT212	0	+	0
PT247	0	0	0
PT248	0	0	0
PT259	+	+	+
PT263	0	0	0
PT281	+	++	+
PT282	0	0	0
PT283	0	+	0
PT288	+	+	0
PT291	+	+	+
PT324	+	+	+
PT339	+	+	+
PT357	+	++	+
PT371	+	+	+
PT379	0	+	0

0 = absence of staining; + = weak staining, ++ = strong staining

Supplementary Material 8. Analysis of MMP-9 immunostaining in tissue samples from patients with metastatic CaP.

Patients / Metastasis	Adjacent Non-tumor Cytoplasm	Tumor Cytoplasm	ECM
PT24	0	0	0
PT129		+	+
PT140	0	+	+
PT163	0	+	+
PT202	+	+	0
PT249	+	+	+
PT269	+	+	+
PT280	+	+	+
PT284	+	+	+
PT287	+	+	+
PT295	+	+	+
PT326	+	+	+
PT333	+	+	0
PT338		++	+
PT361	0	+	+
PT377	0	+	+
PT385	+	+	+

0 = absence of staining; + = weak staining, ++ = strong staining.

Supplementary Material 9. Association between MMP-9 protein staining in tumor tissue with clinical–pathological parameters by multinomial logistic regression.

		MMP-9 cytoplasmic tumor staining		
		Absence of staining		
		χ^2 Wald	OR (95% CI)	*p* value
Weak Steaning	**Age**	0.302	0.665 (0.156 - 2.845)	0.583
	ISUP	0.747	1.961 (0.426 - 9.023)	0.387
	TNM	0.433	0.629 (0.158 - 2.503)	0.511
	PSA	1.715	1.935 (0.721 - 5.197)	0.19
Strong Steaning	**Age**	-	-	-
	ISUP	0.016	1.200 (0.070 - 20.473)	0.900
	TNM	-	-	-
	PSA	0.067	0.770 (0.107 - 5.554)	0.796

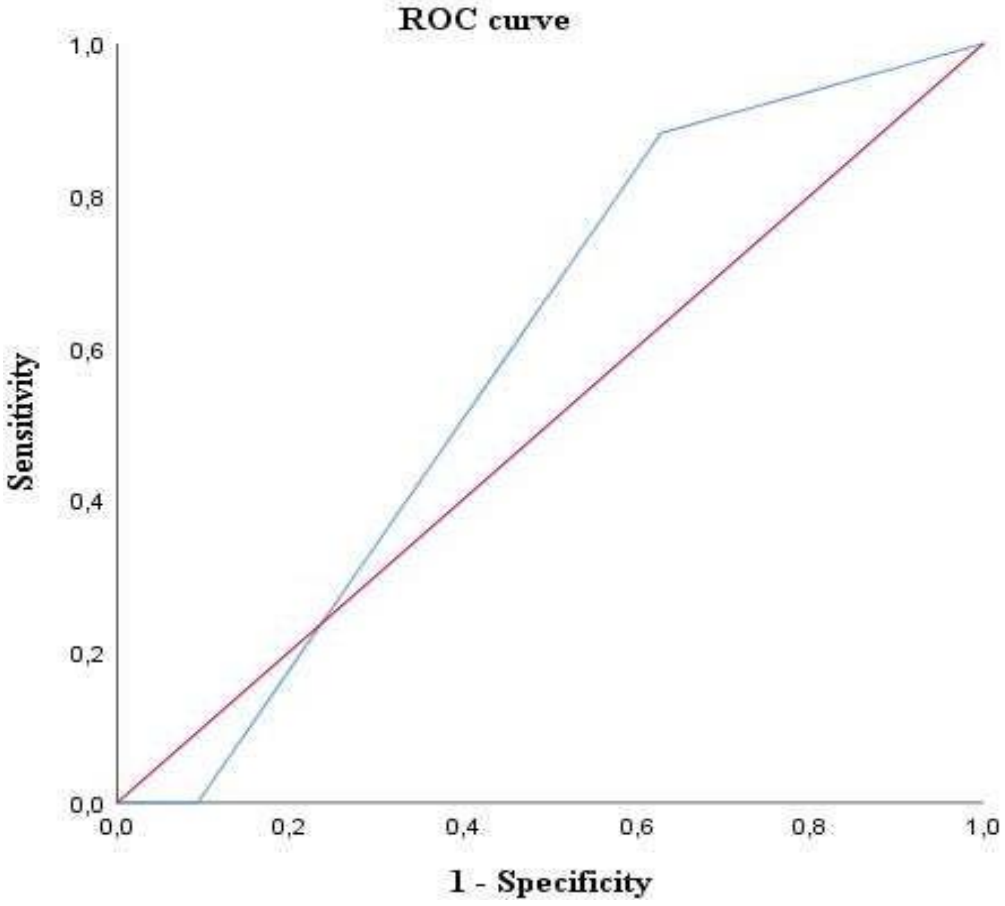
Multinomial logistic regression analysis, considering the staining of MMP-9 protein in tumor tissue. The reference category used was absence of staining. *Significance level of $p<0.05$

Supplementary Material 10. Association between MMP-9 protein staining in ECM tissue with clinical–pathological parameters by multinomial logistic regression.

		MMP-9 ECM staining		
		Absence of staining		
		χ^2 Wald	OR (95% CI)	*p* value
Weak Steaning	**Age**	1.336	2.159 (0.586 - 7.961)	0.248
	ISUP	0.587	1.699 (0.438 - 6.591)	0.444
	TNM	1.775	0.267 (0.038 - 1.864)	0.183
	Extraprostatic extension	3.595	19.701 (0.904 - 429.120)	0.058
Strong Steaning	**Age**	-	-	-
	ISUP	-	-	-
	TNM	-	-	-
	Extraprostatic extension	-	-	-

Multinomial logistic regression analysis, considering the staining of MMP-9 protein in tumor tissue. The reference category used was absence of staining. *Significance level of $p<0.05$.

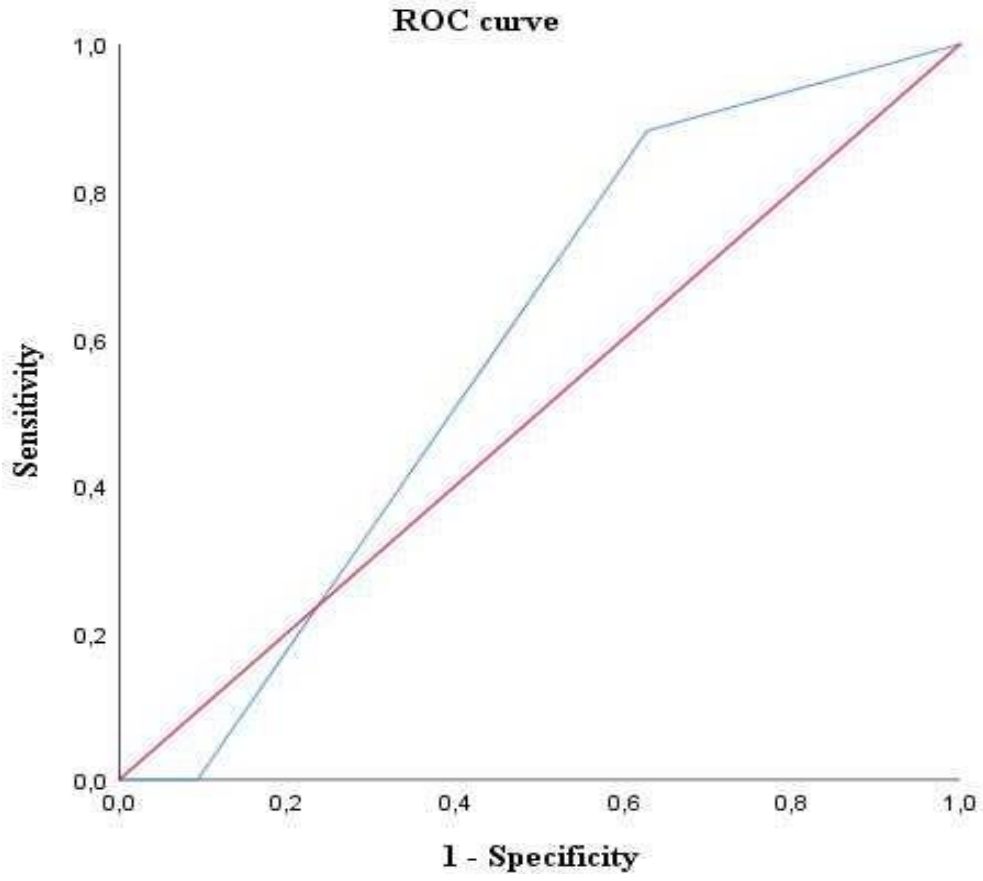
Supplementary Material 11. Analysis of sensitivity and specificity of tumor cytoplasmic staining of the MMP-2 protein as a biomarker for metastatic PCa.



p=0.078

ROC curve for MMP-2 tumor cytoplasmic immunostaining. *Significance level of $p<0.05$.

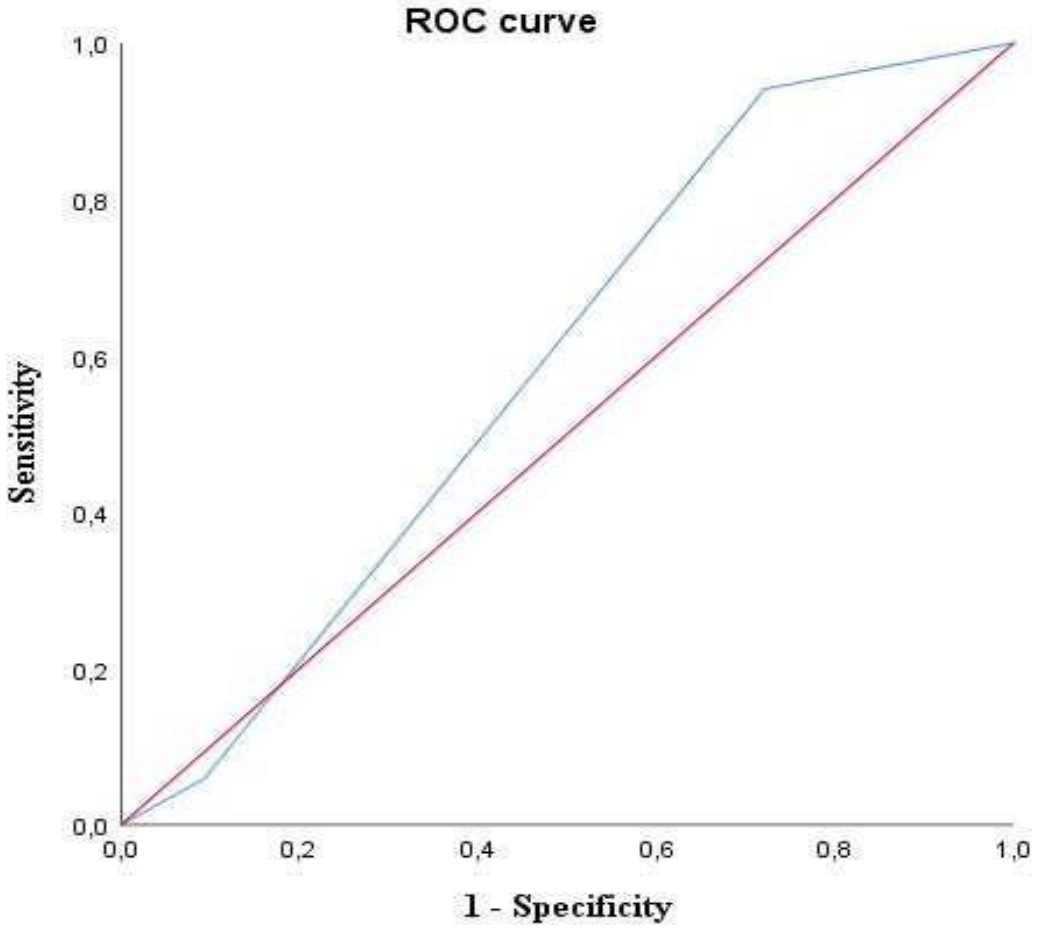
Supplementary Material 12. Analysis of sensitivity and specificity of ECM staining of the MMP-2 protein as a biomarker for metastatic PCa.



p=0.301

ROC curve for MMP-2 ECM immunostaining. *Significance level of p<0.05

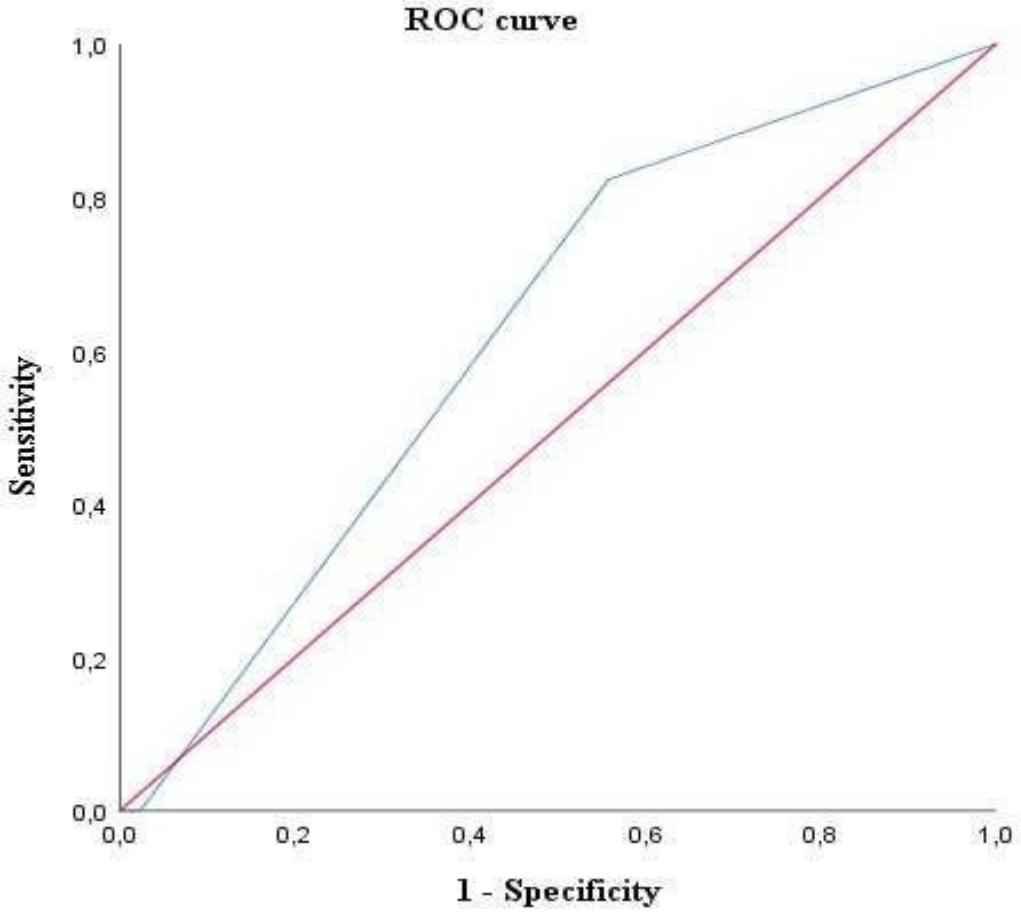
Supplementary Material 13. Analysis of sensitivity and specificity of cytoplasmic tumor staining of the MMP-9 protein as a biomarker for metastatic PCa.



p=0.294

ROC curve for MMP-9 tumor cytoplasmic immunostaining. *Significance level of $p<0.05$.

Supplementary Material 14. Analysis of sensitivity and specificity of ECM staining of the MMP-9 protein as a biomarker for metastatic PCa.



p=0.140

ROC curve for MMP-2 ECM immunostaining. *Significance level of $p<0.05$.

6. CONCLUSÃO GERAL

A MEC é uma estrutura essencial para a homeostase tecidual, e seu desbalanço pode causar severos danos na harmonia do corpo humano. O desenvolvimento de câncer, e muitas vezes de metástases, é um desses males. A MEC é constituída por muitas biomoléculas, e dentre elas destacam-se as proteínas fibrosas, como o colágeno, e a membrana basal, que apoia o tecido epitelial. Para que ocorra a progressão do câncer, é necessário que haja o remodelamento da MEC através de ação enzimática, e a principal família de proteínas com essa ação são as MMPs.

O presente estudo observou que a deposição de colágeno é extremamente importante na constituição da MEC, e que principalmente o Col III é essencial para que a matriz se estabeleça de forma correta. Nossos estudos indicam que a falta de Col III na MEC circundante ao tumor está relacionada com o desenvolvimento de metástases em tumores prostáticos.

Além disso, as análises da integridade da membrana basal indicaram que no tecido tumoral ocorre uma alteração em sua continuidade, tornando-a mais fina e falha. A perda de continuidade da membrana basal no epitélio da próstata maligna também está correlacionada com fatores de pior prognóstico tumoral, como grau ISUP avançado, presença de extensão extraprostática e de invasão perineural.

Acerca das análises de imunomarcação das MMP-2 e MMP-9, observamos que ambas possuem marcação citoplasmática e na MEC circundante ao tumor. A MMP-2 possui uma marcação mais intensa no tecido tumoral quando comparada a que ocorre na MEC tecidual, porém não se correlaciona com nenhum parâmetro clínico-patológico. Já a MMP-9 se expressa da mesma maneira em todos os tecidos, mas sua imunomarcação se correlaciona com fatores de pior prognóstico, como a piora no grupo amostral, o grau ISUP avançado, a presença de invasão perineural e a presença de recidiva bioquímica.

Dessa forma, podemos concluir que o Col III parece ser um bom biomarcador candidato preditivo no CaP metastático, e a análise de integridade da membrana basal também indica que ela pode ser usada para a predição antecipada de tumores prostáticos agressivos, o que deverá ser reavaliado para validação. As MMPs avaliadas não foram capazes de prever metástases, porém a MMP-9 parece indicar fatores invasivos que são essenciais para o processo metastático.

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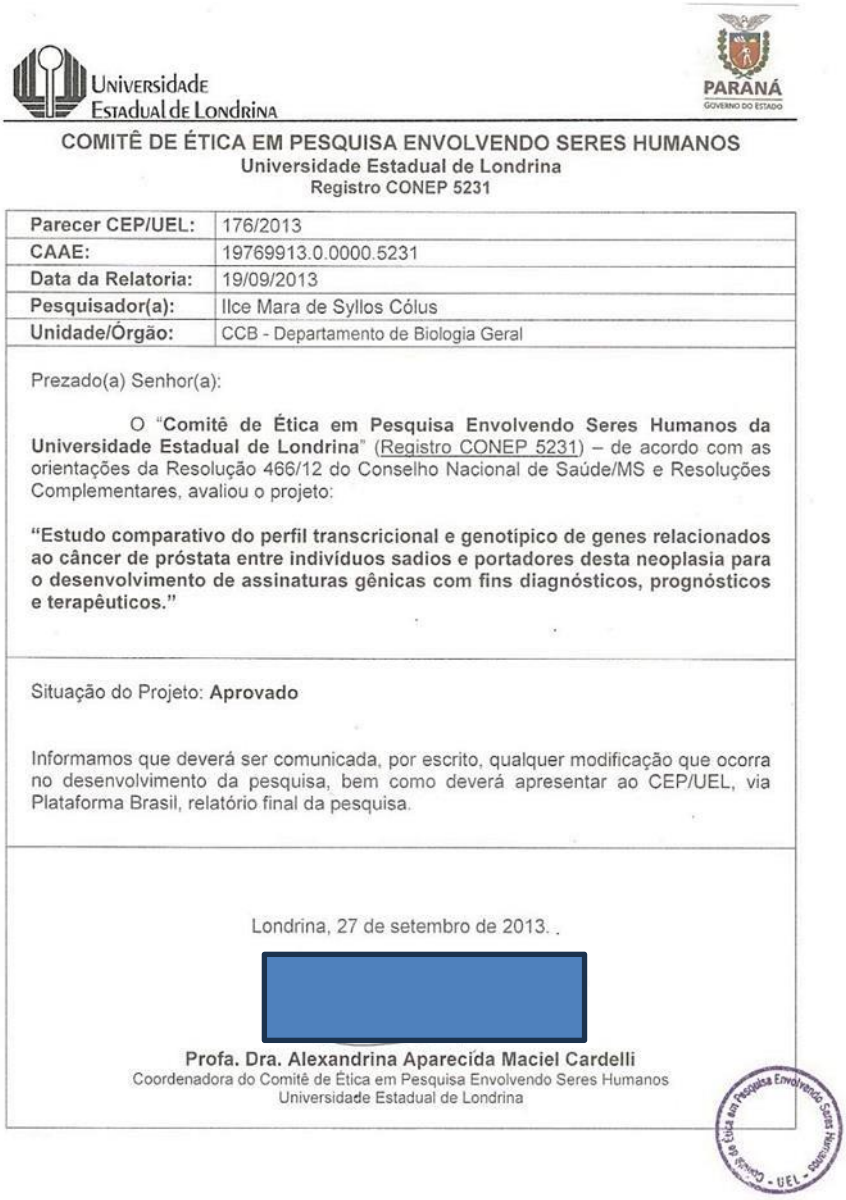
ANEXOS

Anexo A – Aprovação pelo Comitê de Ética em Pesquisa Envolvendo Seres Humanos da UEL.



Universidade Estadual de Londrina

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	176/2013
CAAE:	19769913.0.0000.5231
Data da Relatoria:	19/09/2013
Pesquisador(a):	Ilce Mara de Syllos Cólus
Unidade/Órgão:	CCB - Departamento de Biologia Geral

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo comparativo do perfil transcricional e genotipico de genes relacionados ao câncer de próstata entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 27 de setembro de 2013. .

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL

Campus Universitário: Rodovia Celso Garcia Cid (PR 445), Km 380 - Fone (43) 3371-4000 - PABX - Fax 3328-4440 - Caixa Postal 6001 - CEP 86051-990 - Internet http://www.uel.br
LONDRINA - PARANÁ - BRASIL

Form. Código 11.764 - Formato A4 (210x297)

Anexo B – Termo de Consentimento Livre e Esclarecido

Nós, Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza da Universidade Estadual de Londrina o convidamos para nossa pesquisa e solicitamos sua colaboração e o seu consentimento para incluí-lo em nosso projeto de pesquisa "Estudo comparativo do perfil transcricional e genotípico de genes relacionados ao câncer de próstata entre indivíduos sadios e portadores desta neoplasia para o desenvolvimento de assinaturas gênicas com fins diagnósticos, prognósticos e terapêuticos". O objetivo deste estudo é avaliar alguns fatores genéticos que possam auxiliar no diagnóstico, prognóstico e na terapia de pacientes portadores de câncer de próstata. Assim, solicitamos a sua colaboração como voluntário neste projeto onde vamos avaliar e comparar as semelhanças e diferenças entre dois grupos de pessoas: sadias e com câncer. Portanto, solicitamos a sua autorização para que uma pequena quantidade de seu sangue (10 ml) seja coletada via punção venosa (picada na veia) com seringa e agulha descartáveis. Esclarecemos que não haverá desconforto físico adicional para a sua pessoa, além da picada da agulha.

Caso o senhor tenha que realizar prostatectomia (operação de retirada parcial ou total da próstata), solicitamos também sua permissão para que, depois de realizada a cirurgia e da amostra da sua próstata ter sido utilizada pelo laboratório do Hospital para diagnóstico, possamos coletar uma pequena amostra deste tecido que não foi utilizado pelo Hospital, mas que fica armazenado. Desta forma, a coleta do material para análise genética ocorrerá somente após a finalização do seu diagnóstico e não trará riscos adicionais ao seu tratamento.

Pedimos sua autorização para que moléculas (DNA, RNA ou proteínas) obtidas a partir da amostra de sangue e/ou tecido possam ser armazenadas para estudos futuros no Laboratório de Mutagênese e Oncogenética da UEL, quando será solicitada nova autorização do Comitê de Ética em Pesquisa com Seres Humanos da UEL para a realização das pesquisas posteriores. O material obtido ficará armazenado no Laboratório de Mutagênese e Oncogenética da UEL, sob responsabilidade dos pesquisadores responsáveis por esta pesquisa. Esclarecemos ainda que a autorização para manutenção destas amostras é por prazo indeterminado, podendo ser cancelada por aviso escrito à responsável pelo Laboratório de Mutagênese e Oncogenética da Universidade Estadual de Londrina.

Solicitamos também sua autorização para que possamos consultar seu prontuário médico (que fica no Hospital do Câncer de Londrina) e obter alguns dados clínicos. Solicitamos-lhe o preenchimento de um questionário sobre seu estilo de vida, histórico de exposição ocupacional, onde o senhor será identificado apenas por um código, preservando sua identidade. Este questionário ficará armazenado no laboratório de Mutagênese e Oncogenética da Universidade Estadual de Londrina e somente poderão ter acesso a ele os pesquisadores responsáveis por esta pesquisa.

Sua identidade não será revelada e será mantido o caráter confidencial de todas as informações obtidas. Esclarecemos que o senhor a qualquer momento tem a liberdade de se recusar a contribuir com o estudo, sem ser prejudicado no seu tratamento e acompanhamento médico. Os resultados do estudo serão divulgados em congressos científicos e publicados em revistas especializadas, preservando sua identidade.

Esclarecemos que sua participação é voluntaria, não lhe trará nenhum gasto e que o senhor não terá quaisquer benefícios ou direitos financeiros sobre eventuais resultados desta pesquisa. Provavelmente os resultados desta pesquisa não trarão benefícios para a sua pessoa, mas poderão contribuir, no futuro, para uma melhora nos testes diagnósticos e prognósticos, assim como na conduta terapêutica para pacientes com câncer de próstata, melhorando assim, a qualidade de vida destes pacientes.

No caso de autorizado, o senhor deverá assinar este Termo de Consentimento.

Os pesquisadores responsáveis por este estudo Ilce Mara de Syllos Cólus e Marilesia Ferreira de Souza, poderão ser contatados pelos telefones 3371-4608, 3371-4191, [redacted] ou no endereço rodovia Celso Garcia Cid, Pr 445, Km 380, Campus Universitário, Centro de Ciências Biológicas, Bloco 11, Laboratório de Mutagenese e Oncogenética. Sempre que solicitados, estarão à sua disposição para esclarecimento de quaisquer questões relacionadas a esta pesquisa. O senhor também poderá entrar em contato com o Comitê de Ética em Pesquisa pelo telefone 3371-2490 ou pelo endereço Rua Robert Koch, 60 – Vila Operária ou pelo e-mail: cep268@uel.br.

Agradecemos-lhe a valiosa colaboração.

_____	_____
Profª. Drª. Ilce Mara de Syllos Cólus	Marilesia Ferreira de Souza
Assinatura do pesquisador responsável	Coletor / Entrevistador

Anexo C – Carta de Aceite de Transferência de Materiais

M20201341

Simple Letter Agreement for the Transfer of Materials

In response to RECIPIENT's request for the MATERIAL,

LF-41, LF-69, LF-99, LF-106, LF-113, LF-150, LF-178, LF-182, LF-183 and LF-184

the PROVIDER asks that the RECIPIENT and the RECIPIENT SCIENTIST agree to the following before the RECIPIENT receives the MATERIAL:

1. The above MATERIAL is the property of the PROVIDER and is made available as a service to the research community.
2. **THIS MATERIAL IS NOT FOR USE IN HUMAN SUBJECTS.**
3. The MATERIAL will be used for teaching or not-for-profit research purposes only.
4. The MATERIAL will not be further distributed to others without the PROVIDER's written consent. The RECIPIENT shall refer any request for the MATERIAL to the PROVIDER. To the extent supplies are available, the PROVIDER or the PROVIDER SCIENTIST agree to make the MATERIAL available, under a separate Simple Letter Agreement to other scientists for teaching or not-for-profit research purposes only.
5. The RECIPIENT agrees to acknowledge the source of the MATERIAL in any publications reporting use of it.
6. Any MATERIAL delivered pursuant to this Agreement is understood to be experimental in nature and may have hazardous properties. THE PROVIDER MAKES NO REPRESENTATIONS AND EXTENDS NO WARRANTIES OF ANY KIND, EITHER EXPRESSED OR IMPLIED. THERE ARE NO EXPRESS OR IMPLIED WARRANTIES OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, OR THAT THE USE OF THE MATERIAL WILL NOT INFRINGE ANY PATENT, COPYRIGHT, TRADEMARK, OR OTHER PROPRIETARY RIGHTS. Unless prohibited by law, RECIPIENT assumes all liability for claims for damages against it by third parties which may arise from the use, storage or disposal of the MATERIAL except that, to the extent permitted by law, the PROVIDER shall be liable to the RECIPIENT when the damage is caused by the gross negligence or willful misconduct of the PROVIDER.
7. The RECIPIENT agrees to use the MATERIAL in compliance with all applicable statutes and regulations.
8. The MATERIAL is provided at no cost, or with an optional transmittal fee solely to reimburse the PROVIDER for its preparation and distribution costs. If a fee is requested, the amount will be indicated here: none.

The PROVIDER, RECIPIENT and RECIPIENT SCIENTIST must sign both copies of this letter and return one signed copy to the PROVIDER. The PROVIDER will then send the MATERIAL.

PROVIDER INFORMATION and AUTHORIZED SIGNATURE

PROVIDER SCIENTIST:

Marian	*Young*	*myoung@dir.nidcr.nih.gov*
[first name]	[last name]	[email address]

PROVIDER Organization:

National Institute of Dental and Craniofacial Research ("NIDCR")

Address:

office: Building 30 RM 5A507
street: 30 Convent Dr MSC 4320
city: BETHESDA

state: MD
zip: 20892-4320
country: USA

Name of Authorized Official: [redacted] David W. Bradley, Ph.D.
Title of Authorized Official: [redacted] Technology Development Coordinator

Certification of Authorized Official: This Simple Letter Agreement has ☒ / has not ☐ [check one] been modified. If modified, the modifications are attached.

[redacted] Digitally signed by David W. Bradley -S
Date: 2020.04.17 10:38:58 -04'00'

Signature of Authorized Official — Date

RECIPIENT INFORMATION and AUTHORIZED SIGNATURE

RECIPIENT SCIENTIST:

Carlos Alberto	*Miqueloto*	*carlos.miqueloto@uel.br*
[first name]	[last name]	[email address]

RECIPIENT Organization:

Universidade Estadual de Londrina – State University of Londrina

Address:

office:	*Centro de Ciências Biológicas – Departamento de Biologia Geral Centre for Biological Science - General Biology Department*
street:	*Rodovia Celso Garcia Cid | PR 445 Km 380 | Campus Universitário – Caixa Postal 10.011*
city:	*Londrina*
state:	*Paraná*
zip:	*86.057-970*
country:	*Brazil*

Name of Authorized Official:

Paulo Cesar	*Meletti*	*pmeletti@uel.br*
[first name]	[last name]	[email address]

Title of Authorized Official:

Director of Centre for Biological Science

[redacted] Prof. Dr. Paulo César Meletti
Diretor do CCB/UEL
28/02/2020

Signature of Authorized Official — Date

Certification of RECIPIENT SCIENTIST: I have read and understood the conditions outlined in this Agreement and I agree to abide by them in the receipt and use of the MATERIAL.

[redacted] 28/02/2020

RECIPIENT SCIENTIST — Date

Prof. Dr. Carlos Alberto Miqueloto
Dept° Biologia Geral
CCB/UEL
Chapa Funcional: 0313940

Addendum

If checked the following Modification(s) apply:

☒ **Modification 1**

This Agreement may be executed in counterparts, each of which shall be deemed an original and all of which together shall be considered one and the same agreement.

☒ **Modification 2**

This MATERIAL will be used by RECIPIENT SCIENTIST solely in connection with the following research project described with specificity as follows: [insert plan below or as attachment]

Currently, I am part of a research group headed by professor Eduardo José de Almeida Araújo (Enteric Neuroscience Lab, Histology Department, State University of Londrina), which is interested on mechanisms involved in structural and functional changes in the enteric nervous system during infection and inflammation. In this context, the role of extracellular molecules is one of our targets.

In fact, the literature is sparse regarding the interaction between extracellular molecules and enteric neurons. We have some data showing the association of some extracellular molecules (tenascin X, aggrecan and phosphacan) with specific groups of submucosal (nitrergic/VIPergic) and myenteric neurons (cholinergic) of C57bl/6 mice. We usually perform immunofluorescence technique in frozen sections from fixed tissue in paraformaldehyde.

Our technical routine also includes histochemical stain (Azan and Picro Sirius) in paraffin sections, which have been showing ECM changes in the intestinal wall in our experimental models of inflammation (DSS induced-ulcerative colitis) and infection (Toxoplasmosis and Chagas disease). It would be remarkable to identify which ECM molecules are impaired in these clinical manifestations in order to elucidate mechanisms involved in the pathogenesis of these disease.

For that, we would like to check the possibility to send us some antibodies against ECM molecules from NIDCR to investigate the presence of collagens type I and III, proteoglycans (Versican, Decorin, Biglycan, Lumican and Fibromodulin) and metalloproteinases (MMP-2, MMP-3, MMP-9 e MMP-14). These antibodies will be applied in paraffin and frozen section from normal and colitis/parasite tissues fixed in paraformaldehyde aiming perform immunofluorescence and immunoperoxidase.

☒ **Modification 3**

Upon acceptance of materials RECIPIENT agrees to comply with human subjects regulations at 45 CFR Part 46, if applicable.

PROVIDER's ADDITIONAL SIGNATORY

_____ _____

Dr. Matthew Hoffman, Scientific Director [date]

☐ **Modification 4**

If email delivery is not acceptable, RECIPIENT requests the Agreement be printed, signed, and sent by mail.

☒ **Modification 5**

RECIPIENT requests that communication regarding this agreement be directed towards RECIPIENT CONTACT: [listed below]

Carlos Alberto	*Miqueloto*	*carlos.miqueloto@uel.br*
[first name]	[last name]	[email address]

☒ **Modification 6**

RECIPIENT requests MATERIALS be delivered to:

office:	*Centro de Ciências Biológicas – Departamento de Biologia Geral Centre for Biological Science - General Biology Department*
street:	*Rodovia Celso Garcia Cid | PR 445 Km 380 | Campus Universitário – Caixa Postal 10.011*
city:	*Londrina*
state:	*Paraná*
zip:	*86.057-970*
country:	*Brazil*
tel:	[redacted]
courier:	*No preference*
acct no.	

[redacted]