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“JÚLIO DE MESQUITA FILHO”
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AVALIAÇÃO DA EXPRESSÃO GÊNICA E PROTÉICA DA
VIA mTOR/4EBP1/eIF4E NOS CARCINOMAS
PROSTÁTICOS CANINOS

Luis Gabriel Rivera Calderón
Médico Veterinário e Zootecnista

2018

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CANINOS**

Luis Gabriel Rivera Calderón

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Co-orientadora: Profa. Dra. Rosemeri de Oliveira Vasconcelos

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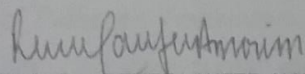
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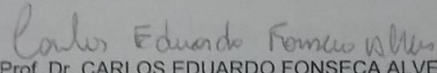
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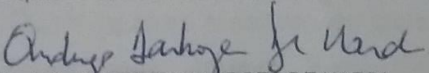
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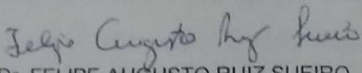
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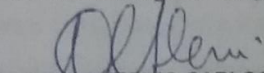
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Jaboticabal, 26 de março de 2018

DADOS CURRICULARES DO AUTOR

LUIS GABRIEL RIVERA CALDERÓN – nascido em venticinco de janeiro de 1990, na cidade de Florencia, Caquetá, Colômbia, filho de William Rivera Cortés e Analid Calderón Molina. Iniciou sua graduação no curso de Medicina Veterinária e Zootecnia em fevereiro de 2007 na Universidad de la Amazonia (Florência, Colômbia), tendo finalizado a mesma em dezembro de 2011. Durante a graduação, participou de vários cursos em Reprodução animal e Produção animal. Foi integrante de dois grupos de pesquisa: em Fauna Silvestre e em Produção bovina, com os quais conseguiu publicar dois artigos científicos em revistas nacionais, e participar como ouvinte ou organizador de vários semanários e congressos nacionais e internacionais. No terceiro ano do curso obteve menção honrosa por excelência acadêmica. Realizou seu estágio curricular no Departamento de Patologia Animal, FCAV/UNESP, câmpus Jaboticabal, sob orientação do Prof. Dr. Gervásio Bechara, colaborando no desenvolvimento do projeto intitulado: *“Inhibition of Pathogen Transmission by Vaccination with Bm86 Antigen”*. Em março de 2012 foi aprovado para cursar seu Mestrado no Programa de Pós-Graduação em Medicina Veterinária (Patologia Animal), na FCAV/UNESP, câmpus Jaboticabal, sob orientação da Profa. Dra. Renée Laufer Amorim e co-orientação da Profa. Dra. Rosemeri de Oliveira Vasconcelos. No mestrado participou no grupo de pesquisa: “Biomarcadores preditivos do câncer: estudo comparativo”, liderado pela Profa. Dra. Renée Laufer Amorim. Junto com esse grupo tem publicado alguns resumos e artigos enfocados na carcinogênese prostática canina. Em agosto de 2014 começa a executar seu Doutorado, na FCAV/UNESP, câmpus Jaboticabal, baixo a orientação da Profa. Dra. Renée e co-orientação da Profa. Dra. Rosemeri. Durante seu curso de Doutorado participou em alguns congressos e simpósios, nacionais e internacionais relacionados à Patologia Veterinária e Oncologia comparada. Atualmente, encontra-se como pesquisador colaborador do Projeto intitulado: Efeitos de fucoidam em *Fucus vesiculosus* sobre a morfologia, a ploidia e metabolismo do endotélio corneal de coelhos.

Epígrafe

PONTE

Não vamos dramatizar a dor! A prova, seja ela qual for, não vem para esmagar, mas para induzir a crescer. A pedra de tropeço pode ser transformada em degrau. Está no próprio homem escolher ser abismo ou ponte!

Chico Xavier

Dedico minha Tese à minha mãe, meu pai, meus irmãos, meus sobrinhos (Thomas e Sasa), minha vovó Luz (In memoriam), meu primo Jairito (In memoriam) e Ge, que foram meu guia e inspiração durante esta etapa acadêmica.

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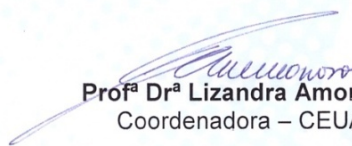
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CERTIFICADO

Certificamos que o projeto intitulado "**Avaliação da expressão gênica e proteica da via mTOR-4EBP1-eI4F nos carcinomas prostáticos caninos**", protocolo nº 10.162/16, sob a responsabilidade da Prof^a Dr^a Renée Laufer Amorim, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de junho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 06 de julho de 2016.

Vigência do Projeto	01/08/2016 a 01/05/2018
Espécie / Linhagem	<i>Canis familiaris</i>
Nº de animais	20
Peso / Idade	Entre 10 a 20 kg / maiores de 5 anos
Sexo	Machos
Origem	Amostras de próstatas coletadas no serviço de Patologia, FMVZ, Unesp.

Jaboticabal, 06 de julho de 2016.


Profª Drª Lizandra Amoroso
Coordenadora – CEUA

LISTA DE ABREVIATURAS

4E-BP1: Fator de iniciação eucariótico

CP: Carcinoma prostático

EG: Escore de Gleason

eIF4E: Fator de iniciação de tradução eucariótico

HPB: Hiperplasia Prostática Benigna

INCA: Instituto Nacional do Câncer

LOX: lisil-oxidase

MEC: Matriz Extracelular

MMP: Metaloproteinase

mTOR: Alvo da rapamicina do mamífero

PIA: Atrofia Inflamatória Prolifetativa

PIN: Neoplasia Intraepitelial Prostática

PSA: Antígeno prostático específico

PSR: Picrosirius

PTEN: Fosfatase e tensina homóloga deletada do cromossomo 10

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RESUMO

O câncer é uma doença complexa que precisa de um microambiente favorável para seu crescimento e progressão. Esse microambiente tumoral está constituído por células neoplásicas, vasos sanguíneos, células imunes, fibroblastos e a matriz extracelular (MEC). Geralmente, as células neoplásicas apresentam modificações nas suas vias de sinalização. No homem, a via mTOR/4E-BP1/eIF4E foi descrita como alterada em diferentes tumores, incluindo o câncer de próstata (CP). Além do homem, o cão é espécie doméstica que desenvolve com mais frequência o CP, sendo considerada um potencial modelo para estudos na área de Oncologia Comparada. Devido à limitada informação sobre a via mTOR/4E-BP1/eIF4E e os componentes da MEC nos CP caninos, o objetivo deste estudo foi avaliar a expressão gênica e protéica de mTOR, 4E-BP-1 e eIF4E neste tipo de tumor. Outrossim, avaliar a expressão dos colágenos (C-I e C-III) pela técnica Picrosirius (PSR) e imuno-histoquímica no tecido prostático normal e neoplásico. Foram utilizadas 35 amostras de tecido prostático caninos. Identificou-se alta expressão protéica de p-mTOR e eIF4E nos CP caninos com alto GS (≥ 8), assim como, correlação positiva entre essas proteínas. Nos colágenos não foi observada diferença de expressão quando comparadas amostras de próstata normal e CP canino. De forma similar com o CP humano, estes resultados sugerem que p-mTOR e eIF4E podem ser bons marcadores para o processo carcinogênico prostático canino e estão correlacionados com alto GS. Além disso, a distribuição de colágenos foi similar no tecido prostático normal e neoplásico.

Palavras-chave: cão, via de sinalização mTOR, Matriz extracelular, câncer de próstata, oncologia.

ABSTRACT

Cancer is a complex disease that needs a favorable microenvironment for its growth and progression. This tumor microenvironment consists of neoplastic cells, blood vessels, immune cells, fibroblasts and extracellular matrix (ECM). Generally, neoplastic cells show modification in their signaling pathways. In men, the mTOR/4E-BP1/eIF4E pathway has been described as altered in different tumors, including prostate cancer (PC). Apart from men, the dog is the only species that develops with high frequency the PC, being considered a potential model for comparative oncology initiatives. Due to limited information on this pathway and ECM components in canine tumors, this study aimed to investigate mTOR, 4E-BP1 and eIF4E gene and protein expression in canine PC. Additionally, to evaluate the expression of collagens (C-I and C-III) by Picrosirius Red and Immunohistochemistry in normal samples and canine PC. Were used a total of 35 formalin-fixed paraffin-embedded (FFPE) samples from canine prostatic tissue. We identified higher p-mTOR and eIF4E protein levels in the canine PC with higher GS (≥ 8), as well as, significant positive correlation between these proteins. No difference statistical was observed in the collagen expression between normal samples and canine PC. Similar to human PC; our data suggested that p-mTOR and eIF4E good markers for canine prostatic carcinogenic process and are correlated with higher GS. Also, the distribution and collagen levels are similar in normal and neoplastic canine prostate.

Keywords: dog, mTOR pathway, extracellular matrix, prostate cancer, oncology

CAPÍTULO 1 – Considerações gerais

INTRODUÇÃO

O câncer de próstata (CP) é uma doença com alta taxa de incidência no homem (FERLAY, 2010; SIEGEL et al., 2018). Segundo dados do Instituto Nacional do Câncer (INCA) são esperados 68.220 novos casos de CP para o ano 2018, sendo a segunda neoplasia com maior apresentação entre os homens (atrás apenas do câncer de pele não melanoma) (INCA, 2018). Os principais fatores que favoreceram o aumento da sua incidência incluem, o uso de testes diagnósticos como o PSA (Antígeno prostático específico) e o toque retal, o aumento da expectativa de vida da população e a melhoria na qualidade de registros (INCA, 2018). Embora, exista uma preocupação por esse aumento, só 10% desses casos irão apresentar progressão tumoral ou evolução clínica da doença (BANGMA et al., 2007).

Além do homem, o cão é a espécie que desenvolve de forma espontânea e com alta frequência, lesões prostáticas, tais como, a hiperplasia prostática benigna (HPB), a atrofia inflamatória proliferativa (PIA), a neoplasia intraepitelial prostática (PIN) e o CP (SMITH, 2008). O CP canino é menos comum que o CP humano, não obstante, tem um comportamento mais agressivo e um prognóstico desfavorável (CORNELL et al., 2000 ARGYLE, 2009). Em ambas as espécies, a neoplasia prostática possui algumas semelhanças clínico-patológicas, como o aumento de ocorrência com a idade, nos sinais clínicos identificados (disúria, estrangúria, tenesmo, hematúria, dor na região lombar), e na predileção por metástases óssea (CORNELL et al., 2000; LEROY; NORTHRUP, 2008). Por esse motivo, o CP canino seria um potencial modelo de estudo para o CP invasivo/metastático do homem (BELL et al., 1991; BOSTWICK et al., 2000).

O CP humano é comumente correlacionado com a superexpressão de via de sinalização de sobrevivência celular e perda de funções dos genes supressores tumorais (KARAN et al., 2003; LONG et al., 2014). Recentes estudos demonstraram que o CP canino também apresenta desregulação na expressão de alguns oncogenes (*c-MYC*, *MDM2*, *STAT3*) e genes supressores tumorais (*PTEN*, *NKX3.1*)

(FONSECA-ALVES et al., 2013; LIN; PALMIERI, 2016; RIVERA-CALDERÓN et al., 2016).

A PI3K/Akt/mTOR é uma das vias de sinalização que tem gerado maior interesse nos anos recentes na busca de potenciais alvos para a terapia no homem e no cão (KENT et al., 2009; PAOLONI et al., 2010; CHEN et al., 2012; MURRAI et al., 2012a; RODRIGUEZ et al., 2012; KARLSSON et al., 2013; DELGADO et al., 2015; LORUSSO, 2016). Entre os constituintes da PI3K/Akt/mTOR, o alvo da rapamicina do mamífero (mTOR) é muito importante no processo de transformação neoplásica e no crescimento tumoral (LAPLANTE; SABATINI, 2012; CARGNELLO et al., 2015). A proteína mTORC1 produz a fosforilação do seu efetor, fator de iniciação eucariótico (4E-BP1), que a sua vez, ativa o fator de iniciação de tradução eucariótica (eIF4E), essas duas proteínas, contribuem no crescimento e proliferação celular e foram identificadas com alterações no CP humano (CARGNELLO et al., 2015). No cão, a mTOR/4E-BP1/eIF4E foi observada com alterações nos hemangiossarcomas e nos carcinomas mamários (DELGADO et al., 2015; MURAI et al., 2012a; MURAI et al., 2012b).

Além das alterações nas vias de sinalização celular, o CP humano pode apresentar um estroma reativo responsável pela sua progressão e metástase. O estroma reativo possui uma abundante formação de colágeno, principalmente, tipo 1 e 3 (TUXHORN et al., 2001; PALUMBO et al., 2012; ZHANG et al., 2013). Também é possível identificar degradação de colágeno IV que forma parte da membrana basal (LI et al., 2010; PENET et al., 2017). Na medicina veterinária, poucos estudos foram conduzidos para entender a relação entre o câncer e as fibras de colágeno, comparado com a medicina humana (CASE et al., 2017). Picrosirius (PSR) é uma técnica histoquímica utilizada para a visualização de fibras de colágeno e sua leitura com microscópios de luz polarizada permite a diferenciação das fibras de acordo com sua cor de birefringência (JUNQUEIRA et al., 1979).

Devido ao limitado conhecimento sobre a biologia dos CP em cães, a presente pesquisa realizou um estudo morfológico, histoquímico, imuno-histoquímico e molecular para a caracterização das neoplasias prostáticas caninas.

OBJETIVOS

- Avaliar a expressão gênica e protéica da via mTOR/4E-BP1/eIF4E nos CP caninos
- Identificar e caracterizar as fibras de colágenos e elastina na próstata normal e nos CP caninos

OBJETIVOS ESPECIFICOS

- Correlacionar a expressão gênica e protéica da via mTOR/4E-BP1/eIF4E com o Escore de Gleason (EG) dos CP caninos.
- Correlacionar os níveis de colágenos I e III utilizando a técnica de PSR e Imuno-histoquímica nas amostras de próstata normal e no CP canino
- Avaliar a expressão de elastina e colágeno IV no tecido prostático normal e nos carcinomas prostáticos caninos.

REVISÃO DE LITERATURA

Câncer de próstata no cão

Dentro das lesões proliferativas diagnosticadas na próstata canina, o CP é a menos frequente. Ocorre de forma espontânea em 5-7% dos cães com prostatopatias, geralmente, em indivíduos com idade avançada (média de 10 anos)(SMITH, 2008; LEROY; NORTHUTUP, 2009). Há alta prevalência do CP em cães machos castrados, os quais podem desenvolver a doença em um tempo variável após a castração, mas na mesma idade que em cães inteiros, o que sugere que a castração não é um fator inibitório do CP (TESKE et al., 2002).A idade avançada, é o único fator de predisposição conhecido para o surgimento do CP no cão. No homem, sabe-se de outros fatores endógenos e exógenos que aumentam o risco de CP, tais como, o histórico familiar, a raça e fatores nutricionais (LEROY; NORTHUTUP, 2009).

Os sinais clínicos dos animais com CP são similares com os que apresentam homens com CP em estágio avançado, elencam-se, anorexia, perda de peso, hematúria, estrangúria, tenesmo, dor na região lombar e debilidade nos membros pélvicos (BELL et al., 1990; SMITH, 2008). Esses sinais são identificados principalmente em animais com metástase nos linfonodos regionais e nos ossos (CORNELL et al., 2000; JACOBS).

O diagnóstico de CP canino é confirmado por exame histopatológico. Diferentes padrões histológicos podem ser observados no CP do cão, tal como acontece no CP do homem, e sua classificação tem sido relatada por vários autores (CORNELL et al., 2000; LAI et al., 2008; PALMIERI et al., 2014). Entre os diferentes padrões de crescimento do CP canino se encontram, cribriforme, sólido, de pequenos ácinos, túbulo-papilar, micropapilar e sarcomatoide (LAI et al., 2008; PALMIERI et al., 2014).

Depois de identificar-se o padrão de crescimento é possível definir o comportamento ou a agressividade do tumor utilizando o sistema de Gleason. O sistema de Gleason é um importante fator prognóstico, o qual é realizado, aplicando uma escala de 1 a 5, segundo o grau de diferenciação celular observado por cada padrão de crescimento (sendo a escala 5, a de menor diferenciação).Normalmente, em um CP são observados dois padrões histológicos, a soma da escala desses

padrões dará o Escore de Gleason (EG) Final (EPSTEIN et al., 2005). Em outras ocasiões, é encontrado só um padrão histológico, este deve ser somado duas vezes para obter o EG final, finalmente, podem identificar-se CP com três padrões de crescimento, nesse caso são selecionados o de menor e maior escala para gerar o EG final (EPSTEIN et al., 2005). O sistema tem sido modificado nos últimos anos na Medicina humana, procurando diminuir sua margem de erro (EPSTEIN et al., 2016). Na Medicina Veterinária um sistema de Gleason foi empregado recente (PALMIERI; GRIECO, 2015). No cão, o CP tem um comportamento mais agressivo que o CP do homem, apresentando, com maior frequência EG igual o superior a 8 (4+4) (PALMIERI; GRIECO, 2015).

Via PI3K/AKT/mTOR

A PI3K/Akt/mTOR é uma importante via de sinalização intracelular, envolvida em vários eventos como, apoptose, transformação neoplásica, progressão, metástase e radioresistência (LORUSSO et al., 2016), por esse motivo, atualmente, essa via é considerada um alvo promissor para o desenvolvimento de novos medicamentos antineoplásicos no homem. Na Figura 1, observa-se o esquema da via PI3K/Akt/mTOR e seus principais eventos.

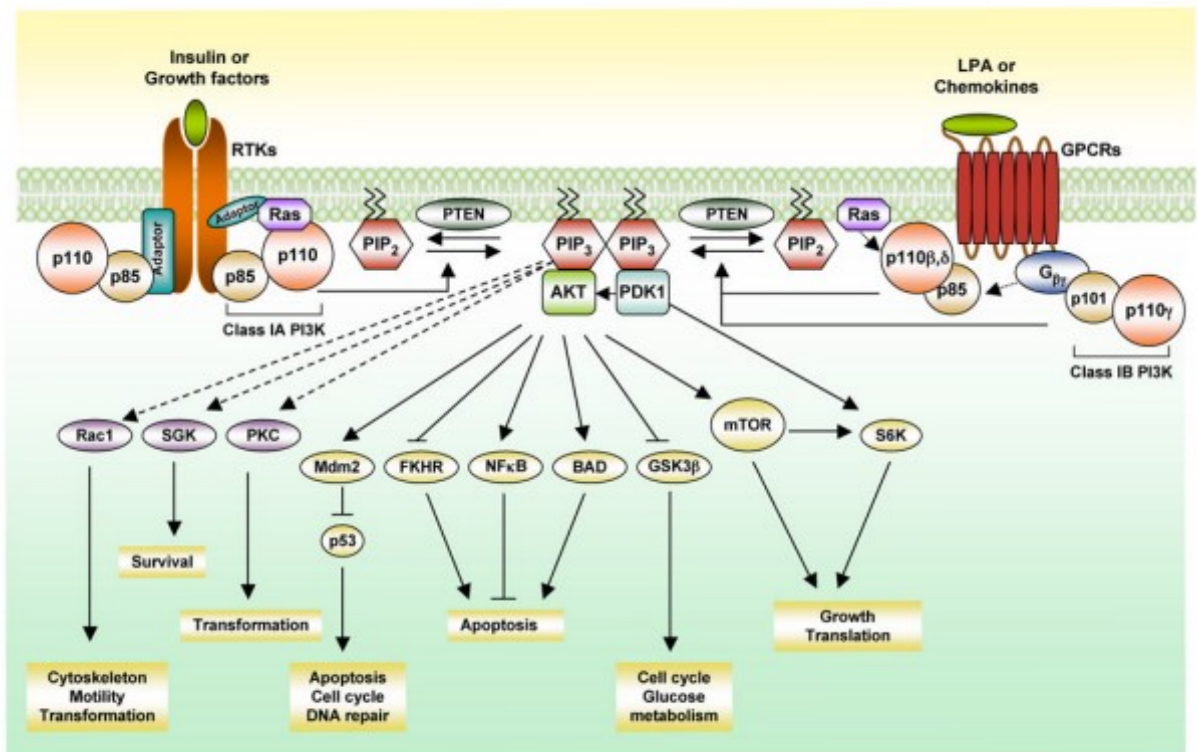


Figura 1. Esquema da via de sinalização das fosfatidilinositol-3 quinases (PI3Ks). A ativação das classes IA e IB é efetuada por meio da estimulação das RTKs e GPCRs. Na classe A1, depois da conversão por subunidades catalíticas p110 de PtdIns (4,5)P₂ (PIP₂) para PtdIns (3,4,5) P₃ (PIP₃) proporcionar locais de acoplamentos nas proteínas de sinalização que tem domínios de homologia plecstrina, incluindo a PDK1 e o AKT. De forma independente ao AKT, as PI3Ks também estão implicadas na regulação da atividade de outros alvos celulares, como a soro-e-quinase induzida por glicocorticóides (SGK) e as proteínas G pequenas RAC1 e a PKC. A atividade desses alvos leva à sobrevivência, à reorganização do citoesqueleto e a transformação celular. PTEN (Homologo de fosfatase e tensina) antagonista das PI3Ks por desfosforilação da PIP₃; BAD, BCL-2 agonistas associados com morte celular; FOXO1, (também conhecido como fator de transição forkhead (FKHR); GSK3β, glicogênio sintetase quinase; mTOR, alvo da rapamicina em mamíferos; NFκB, factor nuclear-kB; PKC, proteína quinase; S6K, proteína ribossomal quinase; LPA, ácido lisofosfático. Fonte: Liu et al., (2009).

A PI3K/Akt/mTOR está constituída pelas fosfatidilinositol-3 quinases (PI3Ks), uma família de enzimas divididas em três classes, consideradas proteínas quinases de serina/treonina. Depois de fosforiladas, as PI3Ks são responsáveis pela conversão de PIP2 (fosfatidilinositol-4, 5-bisfosfato) em PIP3 (fosfatidilinositol-3, 4, 5-trifosfato), recrutando, assim, a AKT e a PDK1 (LAPLANTE; SABATINI, 2012).

Na via das PI3Ks, existe um antagonista importante chamado PTEN (fosfatase e tensina homóloga deletada do cromossomo 10) que permite a desfosforilação de PIP3 em PIP2. Mutações e deleções do PTEN podem ser observadas nos CP humanos, associadas à progressão de andrógeno-independência e metástase (SONG et al., 2012; LORUSSO, 2016), enquanto nos CP caninos, a perda ou baixa expressão deste supressor tumoral está possivelmente associada com progressão tumoral (LIN ; PALMIERI, 2016; RIVERA-CALDERÓN et al., 2016). Outrossim, a perda de PTEN indiretamente estimula a atividade das PI3Ks, levando a ativação constitutiva do AKT e regulação positiva do mTOR (SONG et al., 2012).

A regulação de mTOR pela proteína AKT pode ocorrer de forma direta ou indireta (LAPLANTE ; SABATINI, 2009). No primeiro caso, a proteína AKT pode ativar mTOR pela fosforilação nos domínios Thr 2446 e Ser 2448 dessa proteína e, no segundo, ela pode inibir a atividade do complexo TSC1/TSC2 (hamartina/tuberina) (ZHANG et al., 2003).

A proteína mTOR é uma serina/treonina quinase, que se apresenta sob a forma de dois complexos. No complexo TORC1, mTOR fosforila seus efetores S6K1 (quinase ribossomal S6) e 4EBP1 (fator de iniciação eucariótico 4E ligante de proteína). A fosforilação do 4EBP1 culmina com a liberação de eIF4E (Fator de iniciação da tradução eucariótica 4E), que até então estavam ligados, induzindo assim a ativação de eIF4E. Desta forma, através da fosforilação de seus efetores, mTOR influencia o crescimento e proliferação celular (LAPLANTE ; SABATINI, 2009).

Matriz Extracelular no câncer

A matriz extracelular (MEC) é uma complexa rede não celular, constituída principalmente por proteoglicanos e proteínas fibrosas (colágenos, elastinas,

fibronectinas e lamininas) (THEOCHARIS et al., 2016). Sua função é gerar suporte às células e resistência ao tecido (FRANZ et al., 2010). Atualmente, sabe-se que a MEC, além de auxiliar na ligação das células para formação de tecidos, também serve como reservatório para muitos hormônios, controlando o crescimento e a diferenciação celular (THEOCHARIS et al., 2016).

A MEC sempre está em constante remodelação, e sua síntese e degradação acompanha a morfogênese, a regeneração, a cura de feridas, os processos fibróticos crônicos e os processos neoplásicos (FANG et al., 2014). No câncer, a MEC é a fonte de processos e estímulos positivos que permitem à célula evadir adesões e gerar metástase (FRANZ et al., 2010). Um componente importante na remodelação da MEC no microambiente tumoral é o colágeno. Anteriormente, acreditava-se que o colágeno era uma importante barreira passiva contra a proliferação das células tumorais, sem embargo, na atualidade sabe-se que a degradação e re-deposição desta proteína promovem a infiltração tumoral, angiogênese, invasão e migração (THEOCHARIS et al., 2016). Os processos de degradação e redeposição estão controlados basicamente por duas famílias de enzimas, as lisil-oxidase (LOX), essenciais para a estabilização das fibras de colágeno e para a integridade e elasticidade da elastina e as metaloproteinase (MMPs) as principais enzimas utilizadas para degradar colágeno (FANG et al., 2014).

Em alguns tumores, tais como, o câncer de mama, câncer colorretal e o CP foi identificada uma abundante formação de colágenos, responsável pela progressão e metástase (TUXHORN et al., 2001; PALUMBO et al., 2012; ZHANG et al., 2013). Além disso, alta densidade de Colágeno tipo I e degradação de Colágeno tipo IV foram observadas em tumores sólidos, associados à metástase (LI et al., 2010; PENET et al., 2017). Na medicina veterinária, poucos estudos foram conduzidos para o entendimento da relação entre o câncer e as fibras de colágeno, comparado com a medicina humana.

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1 **p-mTOR, p-4EBP-1 and eIF4E expression in canine prostatic carcinoma**

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22 ABSTRACT

23 The mTOR/4E-BP1/eIF4E pathway plays an important role in the neoplastic
24 transformation process and in tumor growth. In men, the mTOR/4E-BP1/eIF4E
25 pathway was described to be altered in different tumors, including prostate cancer
26 (PC). Apart from men, the dog is the only species that develops with high frequency
27 the PC, being considered a good model for comparative oncology initiatives. Due to
28 limited information on this pathway in canine tumors, this study aimed to investigate
29 mTOR, 4E-BP1 and eIF4E gene and protein expression in canine PC, its metastasis
30 and normal tissue and evaluate the correlation between their expression and
31 Gleason score (GS) in PC. A total of 35 formalin-fixed paraffin-embedded (FFPE)
32 samples, including 13 normal prostatic tissue, 17 PC and 5 metastasis, were
33 evaluated by immunohistochemistry and RT-qPCR. *mTOR* gene mutation in the
34 kinase region also was investigated. We identified higher p-mTOR and eIF4E
35 protein levels in the canine PC with higher GS (≥ 8), as well as, significant positive
36 correlation between these proteins. eIF4E overexpression also was observed in
37 metastasis when compared to normal samples. Similar to human, our data suggests
38 that p-mTOR and eIF4E are correlated with higher GS in canine PC. More studies
39 should be performed in the mTOR/4EBP1/eIF4E pathway to identify possible
40 correlations with clinical and pathologic findings in canine PC, as well as, the role of
41 these proteins as therapeutic targets.

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43 **Keywords:** dog, mTOR pathway, prostate cancer, oncology, Gleason Score.

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46 INTRODUCTION

47 Prostate cancer (PC) has an increasing significance worldwide (Siegel et al.,
48 2018). In men, PC is the second neoplasia most frequently diagnosed (after lung
49 cancer) (Ferlay, 2010). The increased incidence of human PC is possibly influenced
50 by the development of early detection tests, such as screening for prostate-specific
51 (PSA) in serum (Schröder et al., 2014). Apart from men, the dog is the only species
52 that develops, with high frequency, PC (Leroy and Northrup, 2009). In both species,
53 PC shares some clinical similarities, such as, PC is often found in elderly patients
54 and bone metastasis is a common site (Cornell et al., 2000; Leroy and Northrup,
55 2009). Besides, men and dogs share to the same environment factors that may
56 contribute for the carcinogenic process (LeBlanc et al., 2016; Vail and Macewen,
57 2000). Canine prostate cancer is a very invasive and aggressive disease. Similar to
58 late stage humans PC, canine PC is resistant to anti-androgen treatment (Lai et al.,
59 2008), usually, animals affected are euthanized due to poor quality of life (Argyle,
60 2009).

61 In humans, PC is commonly correlated with the overexpression of cell survival
62 signaling pathways and loss of tumor suppressor functions (Karan et al., 2003; Long
63 et al., 2014). Recent studies showed that canine PC also can present deregulation
64 expression of some oncogenes (*c-MYC*, *MDM2*, *STAT3*) and tumor suppressor
65 genes (*PTEN*, *NKX3.1*) (Fonseca-Alves et al., 2013; Lin and Palmieri, 2016; Rivera-
66 Calderón et al., 2016).

67 The PI3K/Akt/mTOR is one of the signaling pathways that have generated
68 major interest in recent years in the search of attractive targets for cancer therapy in
69 humans and dogs (Armstrong et al., 2013; Chen et al., 2012; Delgado et al., 2015;

70 Karlsson et al., 2013; Kent et al., 2009; LoRusso, 2016; Murai et al., 2012a; Paoloni
71 et al., 2010; Rodriguez et al., 2012). These proteins are also related to prognosis,
72 since some studies suggested that the activation of PI3K/Akt/mTOR pathway is
73 strongly involved in PC progression(Chang et al., 2013; Kremer et al., 2006).

74 Among the constituents of the PI3K/Akt/mTOR pathway, the mammalian
75 target of rapamycin (mTOR) is very important in the neoplastic transformation
76 process and tumor growth (Cargnello et al., 2015; Laplante and Sabatini, 2012).
77 Several mTOR inhibitors are currently undergoing evaluation in clinical trials to test
78 their efficacy in the treatment of different forms of cancer (Hurvitz et al., 2015;
79 Rathkopf et al., 2015; Templeton et al., 2013).In dogs, the mTOR/4E-BP1/eIF4E
80 pathway was observed altered in hemangiosarcoma and mammary carcinoma
81 (Delgado et al., 2015; Murai et al., 2012a; Murai et al., 2012b). The aim of this study
82 was to evaluate mTOR, 4E-BP1 and eIF4E gene and protein expression in canine
83 prostatic carcinomas, it's metastasis and normal tissue and investigate the correlation
84 between their expression and Gleason Score (GS) in canine PC.

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86 **MATERIALS AND METHODS**

87 *Subjects*

88 All procedures were performed under the approval of the Ethics Committee on
89 the Use of Animals of FCAV, UNESP, Jaboticabal, Sao Paulo, Brazil (protocol No.
90 10.162/16). This study cohort included 35 formalin-fixed paraffin-embedded (FFPE)
91 samples: 13 normal prostates, 17 PC and 5 metastasis (Supplementary Table 1),
92 retrieved from the archives of the Veterinary Pathology Service, FMVZ, UNESP,
93 Botucatu, SP, Brazil. The samples were from dogs between 3 and 16 years old, with

94 no breed predilection. The specimens were collected during necropsy (26 of 35),
95 prostatectomy (1 of 35) or biopsy (8 of 35). Necropsy was performed on animals that
96 had an interval between death and necropsy, less than 6 hours.

97 The clinical data from dogs with PC were obtained from 8 of the 17 PC cases
98 (Supplementary Table 2). The mean age was 9.4 (ranging from 10 to 16 years), none
99 of these animals were neutered (5/6), and one we did not have the information. The
100 most frequent clinical signs were hematuria (2/6) and anorexia (2/6), others clinical
101 signs are described in the Supplementary Table 2.

102

103 *Histologic evaluation, classification and grading*

104 Hematoxylin and eosin stained (HE) slides were used for histopathological
105 diagnosis, which were examined by three pathologists using a multi-head
106 microscope. The samples were classified as normal prostate or PC according to the
107 human WHO from Tumors of the Urinary System and Male Genital Organs (Eble et
108 al., 2004), which was recently adapted to canine PC (Palmieri et al., 2014). A
109 Gleason grading was performed according to Palmieri and Grieco (2015)
110 (Supplementary Table 3). The GS is one the most important prognostic factor in men
111 and patients showing $GS \geq 8$ present poor prognosis (Epstein et al., 2005)

112

113 *Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)*

114 In HE slides, the areas of interest were selected by the pathologists and
115 marked with a pen. The area on the paraffin block was used for macrodissection with
116 needles (16 gauge), according to Pires et al., (2006) and Rivera-Calderón et al.,
117 (2016). mRNA extraction was achieved using a commercial RecoverAll™ Total

118 Nucleic Acid Kit (Ambion, Life Technologies, MA, USA), according to manufacturer's
119 instructions. The RNA concentration was measured with a spectrophotometer
120 (NanoDrop™ND-8000, Thermo Scientific, Wilmington, DE, USA), and the RNA
121 quality was analyzed in Bioanalyzer (Agilent 2100 Bioanalyzer 6000 Nanochip,
122 Agilent Technologies, Waldbronn, Germany). cDNA was synthesized according to
123 the procedure described by Rivera-Calderón et al., (2016). RT-qPCR was done to
124 identify the expression of *mTOR*, *4E-BP1*, *eIF4E* and reference genes
125 (Supplementary Table 4) in a total volume of 10 µL, containing Power SYBR Green
126 PCR Master Mix, (Applied Biosystems; Foster City, CA, USA), 1 µL cDNA (1:10) and
127 0.3 µL of each primer. qRT-PCR was performed in duplicate in 384-well plates using
128 QuantStudio™ 12K Flex Thermal Cycler equipment (Applied Biosystems; Foster City,
129 CA, USA). To verify PCR product specificity, we performed a dissociation curve for all
130 samples. The most stable reference genes (*GAPDH*, *HPRT*, *RPL8* and *RPS5*) were
131 selected, according to Brinkhof et al., (2006) and identified using geNorm software
132 (Vandesompele et al., 2002). Relative gene quantification was performed by the 2⁻
133 $\Delta\Delta CT$ method (Livak and Schmittgen, 2001).

134

135 *Immunohistochemistry (IHC)*

136 Normal prostate and PC paraffin sections were placed on charged slides
137 (Startfrost® - Knitell, Bielefeld, Germany) and deparaffinized. Sections were stained
138 with primary antibody against p-mTOR (rabbit monoclonal), 4EBP-1 (rabbit
139 monoclonal) and eIF4E (rabbit polyclonal). The antibodies, dilutions and incubation
140 period are described in detail in Supplementary Table 5.

141 Antigen retrieval was performed with citrate buffer (pH 6.0) in a pressure
142 cooker (Pascal; Dako, Carpinteria, CA, USA). Endogenous peroxidase activity was
143 inhibited with 4% hydrogen peroxide in methanol for 10 min at room temperature
144 (RT). Then, the slides were treated with protein block serum-free for 15 min RT
145 (Dako, Carpinteria, CA, USA).

146 The sections were washed with Tris-buffered saline (pH 7.4) and incubated
147 with LSAB system (Dako, Carpinteria, CA, USA) for 1 hour at RT, as indicated by the
148 manufacturer. Peroxidase activity was visualized by 3',3'-Diaminobenzidine
149 chromogen (DAB, Substrate System, Dako) for 5 min. Slides were then
150 counterstained with Harris's hematoxylin, dehydrated and mounted. As a negative
151 control, primary antibodies were replaced with by Tris-buffered saline. The following
152 positive control tissues and samples were used: p-mTOR, normal canine prostate; p-
153 4E-BP1, normal canine stomach; and eIF4E, normal canine lymph node, according
154 to the Human Protein Atlas (<https://www.proteinatlas.org/>).

155

156 *Interpretation of IHC staining*

157 Five High Power Field (HPF) were selected in each HE slide. The same areas
158 were captured for each antibody, with a digital camera (Axioncam MRc, Zeiss®
159 Vision, Germany) coupled in a microscopy (Axio Imager A1, Zeiss®, Germany) and
160 analyzed with ImageJ 1.49v software (National Institutes of Health, USA) as per Da
161 Silva et al., (2017). Staining was assessed by setting a threshold using the Image J
162 threshold tool. In the present study, cytoplasmic, with and without nuclear
163 immunostaining, was considered positive for all antibodies used (Supplementary
164 Table 6).

165 *DNA extraction and sequencing*

166 DNA extraction of FFPE prostate tissues was performed using RecoverAll™
167 Total Nucleic Acid Kit (Ambion, Life Technologies, MA, USA), according to
168 manufacturer's instructions. We selected five samples of canine PC and one pool of
169 normal prostate(Supplementary Table 1).The amplification of the kinase region of
170 *mTOR* gene was performed in the Veriti 96-Well Thermal Cycler (Thermo Fisher
171 Scientific, MA, USA). In each reaction was used 40,5 µL of nuclease free water, 5 µL
172 of 10x *PfuUltra* II, 0,5 µL of 100 µM dNTP, 1 µL of each oligonucleotide in 10 µM
173 (Forward 5'CCTGGGGTTAAGCTGCTAGG3' and Reverse
174 5'ACAAACGCCCGTGAACAAAC3'), 1 µL genomic DNA (100ng/µl) e 1 µL *PfuUltra* II
175 fusion HS DNA polymerase (Agilent, CA, USA).

176 After of the amplification, the region of the *mTOR* gene was analyzed in 2%
177 agarose gel stained with Neotaq Brilliant Plus DNA Stain (Neobio, Brazil). The PCR
178 products contained in the agarose gel were cut and centrifuged. The supernatant
179 was treated with ammonium acetate and ethanol, finally, the DNA precipitate was
180 centrifuged with ethanol 70% at RT and resuspended with nuclease free water.

181 The DNA sequencing was performed with BigDye™ Terminator v 3.1 Cycle
182 Sequencing Kit version 3.1, according to the manufacturer's instructions (Applied
183 Biosystems, Thermo Fisher Scientific, MA, USA). The RT-amplicons were directly
184 sequenced at ABI 3500 (Applied Biosystems, Thermo Fisher Scientific, MA, USA).
185 The resulting nucleotide sequences were compared to data of the NCB I (National
186 Center for Biotechnology Information, USA).

187

188 *Data analysis*

189 Statistical analysis was performed using GraphPad Prism 6 (GraphPad
190 Software Inc. La Jolla, CA). Descriptive statistics was used to define the median and
191 percentile of gene and protein expression for each group. After establishing the
192 median expression in the normal group, the PC was considered as having under or
193 over expression, compared to normal median. Kruskal-Wallis or Mann Whitney *U* test
194 was applied to compare mTOR, 4E-BP1 and eIF4E gene and protein expression
195 among the diagnosis. Differences among the 3 groups were assessed by ANOVA
196 test, and t-tests were used to make comparisons between normal and canine PC and
197 to evaluate their differential DNA sequencing. Spearman test was used to evaluate
198 the correlation between gene and protein expression, as well as the correlation
199 between mTOR, 4E-BP1 and eIF4E expression and Gleason Score in the canine PC.
200 Statistical significance was set at $p < 0.05$.

201

202 **RESULTS**

203 This is the first time that the protein and gene expression of mTOR/4EBP-
204 1/eIF4E pathway is studied in the canine PC (Fig. 1). We observed higher protein
205 levels of eIF4E in PC and metastasis, compared to normal tissue (Table 1 and Fig.
206 2). p-mTOR had higher levels in PC compared to normal tissue (Fig. 2), as well as, a
207 positive correlated with higher GS in canine PC (Supplementary Table 6).

208 There was no difference in *4E-BP1* expression among the groups. Higher *4E-*
209 *BP1* and p-4E-BP1 levels were detected in 3 PC when compared to normal (case
210 No. 16, 20 and 22). Interestingly, in these cases, GS was ≥ 9 . No statistic differences
211 in *mTOR*, *4E-BP1* and *EIF4E* transcript levels were observed between PC,

212 metastasis and normal samples ($p>0.05$) (Table 1; Supplementary Table 7) and no
213 correlation was observed between *mTOR*, *4E-BP1* and *EIF4E* levels and GS
214 ($p>0.05$; Supplementary Table 8). However, positive correlation was found between
215 *mTOR* and *4E-BP1* transcript and protein levels in the PC ($R= 0.6727$, $p=0.0233$ and
216 $R=0.5130$, $p=0.0220$, respectively) (Supplementary Table 9). Compared with normal
217 prostate glands, higher p-mTOR protein levels were detected in PC ($p=0.0471$) (Fig.
218 1). This difference increased when compared p-mTOR levels with PC higher GS (≥ 8)
219 ($p=0.0006$) (Fig. 2). Paired mTOR protein and transcript expression was assessed in
220 8 PC and 2 metastasis. No correlation was observed between gene and protein
221 expression ($p>0.05$). Nevertheless, there was overexpression of *mTOR* transcript
222 levels and p-mTOR levels in 5 cases compared to normal (case No. 16, 17, 18, 20
223 and 22). These five samples had GS ≥ 9 .

224 eIF4E expression was significantly different among all groups ($p=0.0181$; Fig.
225 1 and Fig. 2). Compared with normal prostate, eIF4E overexpression was observed
226 in PC with GS ≥ 8 ($p= 0.0004$) and metastasis ($p=0.0046$) (Fig. 2). Additionally, we
227 found positive correlation between eIF4E and p-mTOR ($R= 0.8231$, $p<0.0001$), as
228 well as positive correlation between eIF4E and p-4E-BP1 protein levels ($R= 0.5812$,
229 $p= 0.0037$). Paired eIF4E protein and gene expression was assessed in 6 PC,
230 without correlation ($p> 0.05$), however, in three cases with GS 10 (case No. 18, 19
231 and 20) we identified gene and protein overexpression compared to normal prostate.

232 No significantly difference was identified in the DNA sequencing analysis
233 between normal and PC samples (Fig. 3 and Fig. 4).

234

235 **DISCUSSION**

236 In this study, we observed higher protein levels of p-mTOR and eIF4E in PC
237 compared to normal tissue, as well as, a correlated with higher GS in canine PC.
238 Kremer et al., (2006) reported that mTOR protein levels and cytoplasmic p-mTOR
239 were greater in prostate intraepithelial neoplasia (PIN) and human PC when
240 compared to normal prostate, suggesting that alterations in p-mTOR expression may
241 occur in the early stages of prostate carcinogenesis and maintained in PC.
242 Interesting, p-mTOR protein overexpression was found in canine PC with higher GS;
243 probably mTOR is involved not only in canine PC development but also in it's more
244 aggressive forms. Once the Gleason system for veterinary medicine is recent
245 (Palmieri and Grieco, 2015) a bigger group of tumors should be used to confirm this
246 result and correlated to clinical data, such as survival time and metastasis.

247 In two previous studies evaluating canine hemangiosarcoma and mammary
248 carcinoma, p-mTOR was overexpressed when compared to benign lesion and
249 normal tissue (Delgado et al., 2015; Murai et al., 2012b), showing similar results to
250 us.

251 We analyzed by qRT-PCR, mTOR/4E-BP1/eIF4E pathway in the canine PC,
252 but no statistical significance was found when compared to normal prostate.
253 However, we identified correlation between mTOR and 4E-BP1 gene and protein
254 levels in the canine PC. More than 75 % of canine PC with higher mTOR and 4E-
255 BP1 had GS \geq 8 (Supplementary Table 4). Similar to our results, significant
256 correlation was observed between p-mTOR and p-4E-BP1 in humans PC (Dai et al.,
257 2009). On the other hand, some studies identified overexpression in 4E-BP1
258 transcripts and protein levels in the human PC (Balakumaran et al., 2009;

259 Jendrossek et al., 2008; Kremer et al., 2006). Balakumaran et al., (2009)
260 demonstrated that *4E-BP1* gene overexpression may be promoted by amplification of
261 *MYC* oncogene in human PC.

262 In a previous work from our group, MYC protein overexpression was detected
263 in prostatic proliferative inflammatory atrophy (PIA) and canine PC compared to
264 normal prostate (Fonseca-Alves et al., 2013), so we cannot assume that there is a
265 correlation between MYC and 4E-BP1 expressions in canine PC, as in humans.

266 In the mTOR1/4E-BP1/eIF4E pathway, it is thought that eIF4E affects cell
267 proliferation and malignant transformation by promoting the translation of specific
268 mRNAs coding for pro-oncogenic proteins regulation cell cycle progression, survival,
269 energy metabolism, angiogenesis and metastasis (Laplante and Sabatini, 2012). We
270 observed increased eIF4E protein expression in canine PC in higher Gleason scores
271 and in metastasis, when compared to normal tissue. Also, Graff et al., (2009)
272 detected eIF4E cytoplasmic significantly increased in high-grade PC compared to
273 adjacent Benign Prostatic Hyperplasia (BPH).

274 The activation of mTOR pathway has been studied in some canine cell lines
275 and neoplasias of patients routinely treated in veterinary hospitals (Delgado et al.,
276 2015; Murai et al., 2012a; Murai et al., 2012b; Chen et al., 2012; Paolini et al., 2010).
277 These authors mentioned the importance of the pathway in these tumors and
278 highlight the potential therapeutic targets within this pathway. Based on our results,
279 we believe that some components this pathway also may be potential therapeutic
280 targets in the canine PC.

281 Our data suggested that p-mTOR and eIF4E are correlated with higher GS in
282 canine PC. More studies should be performed in the mTOR/4EBP1/eIF4E pathway to

283 identify possible clinical and pathological correlations in canine PC, as well as,
284 therapeutic targets for it's treatment.

285

286 **DECLARATION OF CONFLICTING INTERESTS**

287 The authors declared no potential conflicts of interest with respect to the
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289

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296

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478 **Table 1.** Median, 25% percentile and 75% percentile values of mTOR, 4E-BP1 and
 479 eIF4E gene and protein according to the diagnosis in canine prostatic tissue.

	Group	Gene expression				<i>p</i>	Protein expression				
		n	25%	Median	75%		n	25%	Median	75%	<i>p</i>
	Normal	7	0.28	0.74	4.25		11	26.78	31.08	32.92	
mTOR	PC	11	0.33	1.39	3.00	0.7134	13	26.85	46.84	53.15	0.1112
	MTS	2	1.13	2.03	2.93		5	20.93	37.52	49.31	
	Normal	6	1.22	1.71	2.21		11	10.08	27.36	36.25	
4E-BP1	PC	13	0.47	0.96	1.91	0.2729	13	7.42	35.86	43.57	0.7689
	MTS	LAT	LAT	LAT	LAT		5	1.29	18.85	49.71	
	Normal	3	1.27	1.35	5.56		11	30.04	32.26	35.93	
eIF4E	PC	8	0.29	0.62	3.86	0.2788	13	29.18	40.62	53.05	0.0181
	MTS	LAT	LAT	LAT	LAT		5	37.58	43.21	58.18	

480 mTOR, mammalian target of rapamycin; 4E-BP1, Eukaryotic translation initiation
481 factor 4E-binding protein 1; eIF4E, Eukaryotic translation initiation factor 4E; PC,
482 Prostate cancer; MTS, Metastasis; LAT, not used to limited amount of tissue.

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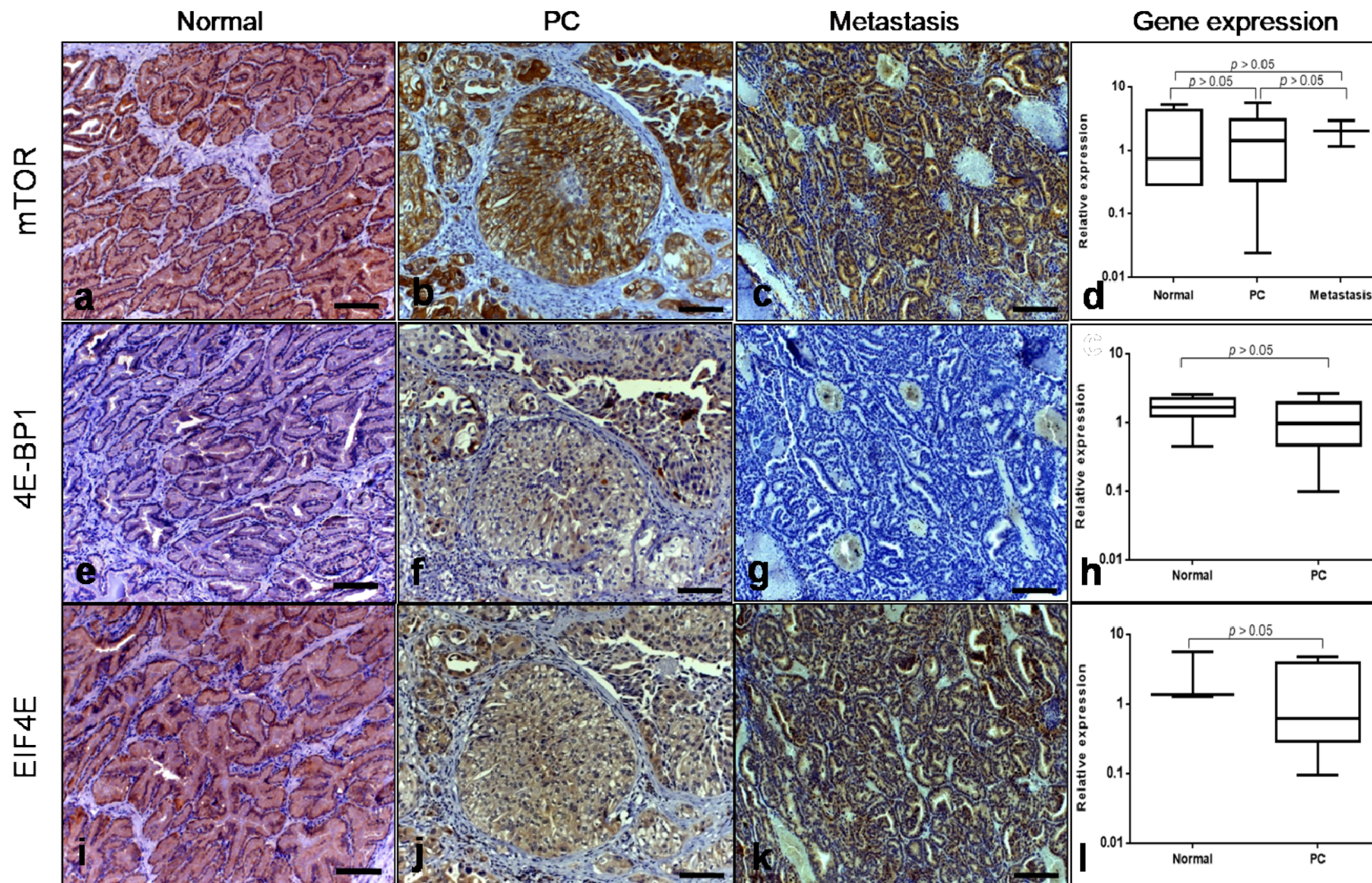


Fig. 1 Immunohistochemistry and gene expression of canine prostatic tissue. a: diffuse staining of p-mTOR in the cytoplasm of epithelial cells (case No. 10). b: overexpression of p-mTOR in PC with Gleason score 10 (case No. 16). c: moderate cytoplasmic staining of p-mTOR in the metastatic foci (lung) (case No. 34). d: gene expression of mTOR. No difference statistic was observed. e: weak staining of p-4E-BP1 in the cytoplasm of canine normal prostate (case No. 10). f: weak and diffuse staining of p-4E-BP1 staining in the cytoplasm of neoplastic epithelial cells (case No. 16). g: metastatic foci negative for p-4E-BP1(case No. 34). h: gene expression of p-4E-BP1 without statistic difference between the groups. i: moderate staining of eIF4E in canine normal prostatic tissue (case No. 10). j: weak or moderate expression of eIF4E in PC with cribriform pattern (case No. 16). k: moderate cytoplasmic staining of eIF4E in the pulmonary metastasis (case No. 34). l: gene expression of eIF4E demonstrate no difference statistic between normal prostate and PC.

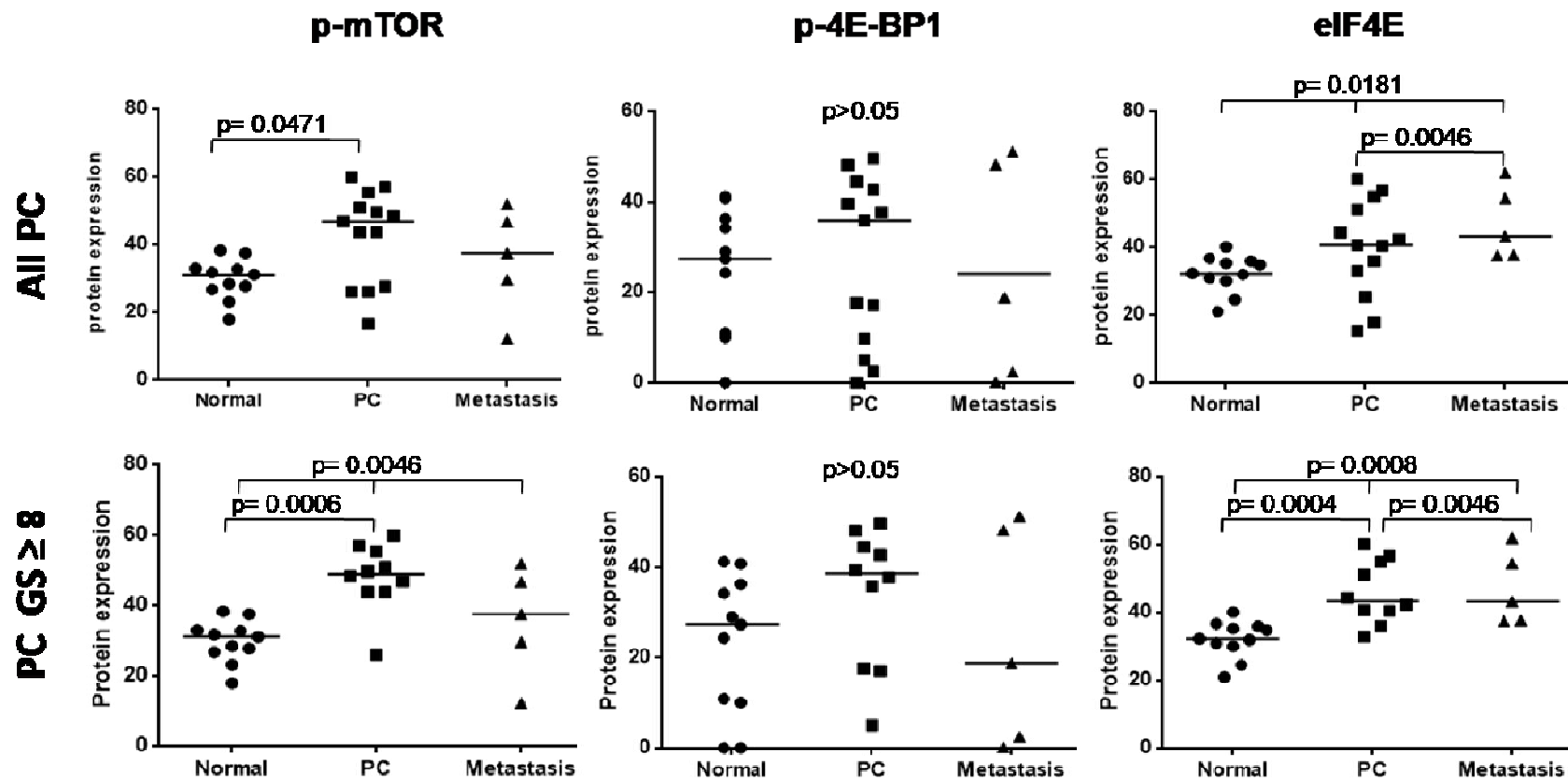


Fig. 2 Median and statistics difference observed in all canine PC and PC with $GS \geq 8$ compared to normal samples and metastases for p-mTOR, p-4E-BP1 and eIF4 protein expression.

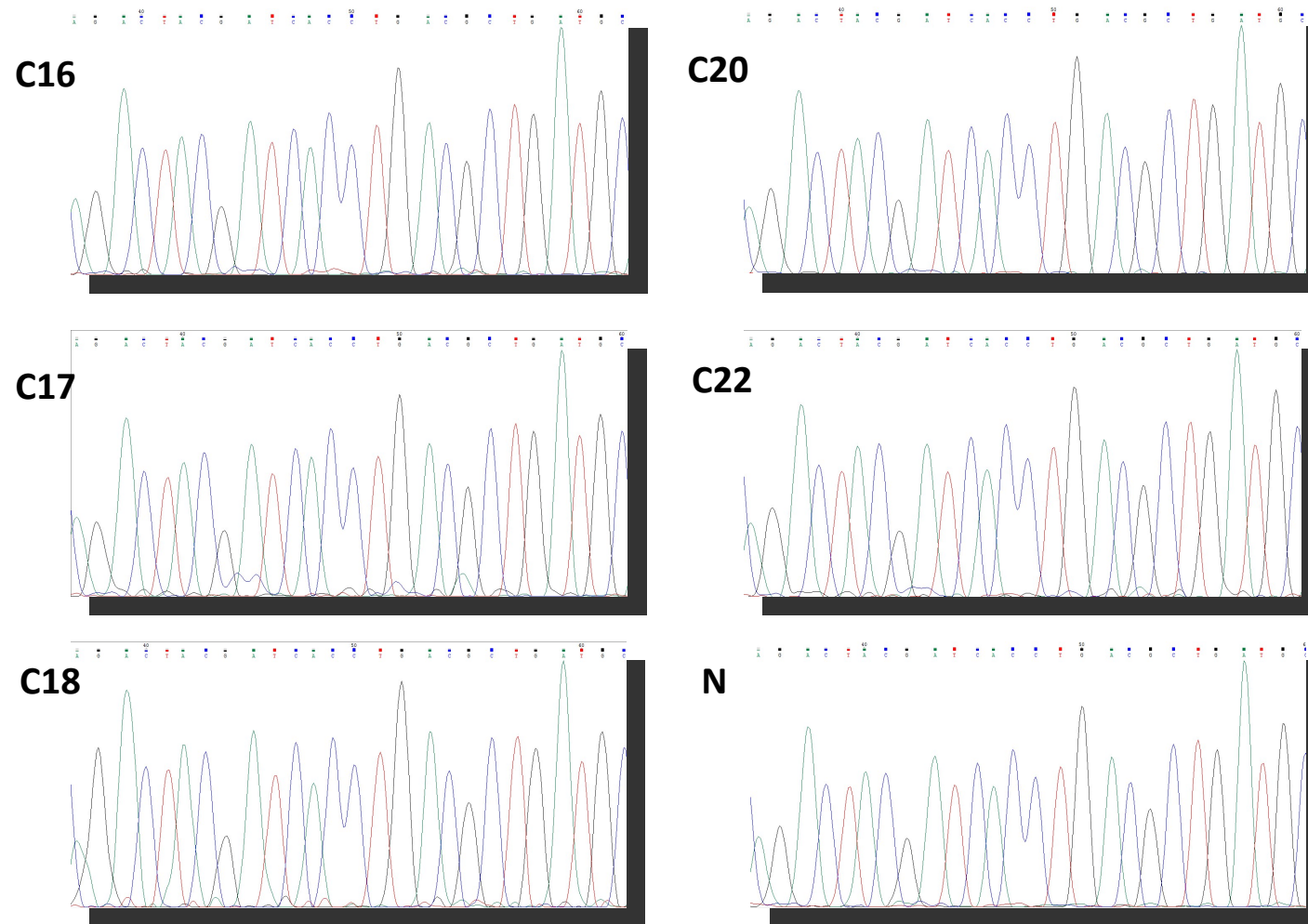


Fig. 3 Electropherogram of the kinase region of *mTOR* gene in five samples of canine PC and one pool of normal prostate; C, case number; N, normal.

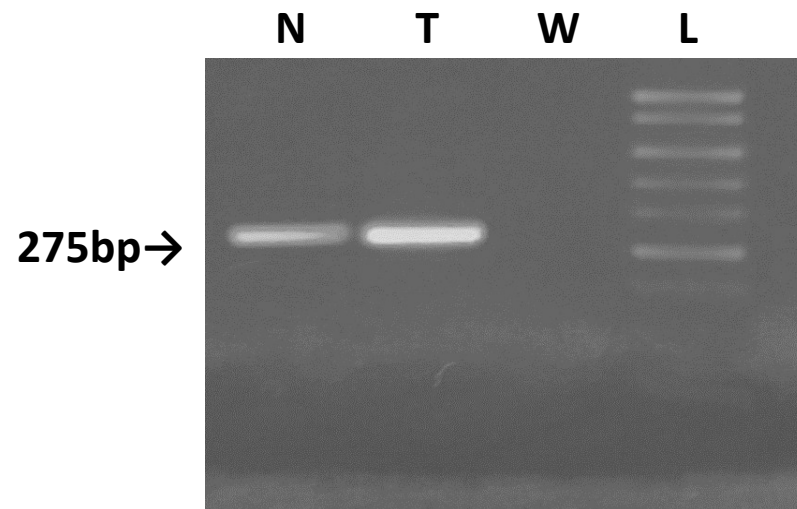


Fig. 4 Gel electrophoresis with DNA samples and ladder showing the size of the kinase region (275 bp); N, normal; T, tumor; W, white; L, ladder.

Supplementary Table 1. Samples used for Immunohistochemistry (IHC), RT-qPCR and DNA sequencing

Case No.	Histological diagnosis	IHC	RT-qPCR	DNA sequencing
1	Normal	X	X	X
2	Normal	X	X	X
3	Normal	X	X	X
4	Normal	X	X	X
5	Normal	X	X	X
6	Normal	X	X	X
7	Normal	X	LAT	N/U
8	Normal	X	LAT	N/U
9	Normal	X	LAT	N/U
10	Normal	X	LAT	N/U
11	Normal	X	LAT	N/U
12	Normal	LAT	X	N/U
13	Normal	LAT	X	N/U
14	PC	X	X	N/U
15	PC	X	X	N/U
16	PC	X	X	X
17	PC	X	X	X
18	PC	X	X	X
19	PC	X	X	N/U
20	PC	X	X	X
21	PC	X	X	N/U
22	PC	X	X	X
23	PC	X	LAT	N/U
24	PC	X	LAT	N/U
25	PC	X	LAT	N/U
26	PC	X	LAT	N/U
27	PC	LAT	X	N/U
28	PC	LAT	X	N/U
29	PC	LAT	X	N/U
30	PC	LAT	X	N/U
31	Metastasis (regional lymph node)from case no. 19	X	X	N/U
32	Metastasis (pelvis)from case no. 19	X	X	N/U
33	Metastasis (lumbar vertebra)from case no. 19	X	LAT	N/U
34	Metastasis (lung)	X	LAT	N/U
35	Metastasis (Intestine)	X	LAT	N/U

PC, Prostate cancer; LAT, not used to limited amount of tissue; N/U, not used.

Supplementary Table 2. Clinical data of dogs with prostate cancer.

No. Case	Neutered	Breed	Age	Clinical Signs	Surgical Treatment	Survival Time
17	No	Mixedbreed	10 years	Anorexia, tenesmus	Prostatectomy	2years
18	No	Dachshund	10 years	No data	No data	No data
19	No	Mixedbreed	10 years	Lameness, urinary incontinence	None	Euthanasia
20	No	German Shepherd	13 years	Dyschezia, hematuria	No data	No data
21	No	Mixedbreed	16 years	Anorexia, vomit	None	Euthanasia
23	No data	Boxer	12 years	No data	No data	No data
25	No	ScottishTerrier	13 years	Hematuria	None	No data
26	No	Cocker Spaniel	10 years	Cachexia	None	Euthanasia

* Survival time after first consultation.

Supplementary Table 3. Histological type and Gleason score of 17 canine prostatic carcinomas.

Histologic Type	Case Number	Gleason score
Single histological pattern		
Cribriform with comedonecrosis	19, 21	(5+5)=10
Solid	17, 22	(5+5)=10
Small acinar/ductual	14, 15, 26, 28	(3+3)=6
Mixed histological patterns		
Solid, cribriform with comedonecrosis	16, 24, 25	(5+5)=10
Cribriform with comedonecrosis, solid	18	(5+5)=10
Cribriform with comedonecrosis, cribriform without comedonecrosis	20	(5+4)=9
Cribriform without comedonecrosis, solid	23	(4+5)=9
Cribriform without comedonecrosis, cribriform with comedonecrosis	27	(4+5)=9
Tubulo-papillary, solid	29, 30	(3+5)=8

Supplementary Table 4. Primers sequences used for gene expression (endogenous and targets genes).

Gene	Foward	Reverse
<i>mTOR</i>	5'-CTGGCCGGATGTAAACGAA-3'	5'-GCGTATCGATTCTCGCAATGA-3'
<i>4EPB-1</i>	5'-CACCCCGGGAGGTACCA-3'	5'-GTGAGTTCCGACACTCCATCCA-3'
<i>EIF4E</i>	5'-CGCAGCACACCCTTGTGA-3'	5'-CACAGTCGCCATCTTAGGATCGA-3'
<i>GAPDH</i>	5'-CATCAACGGGAAGTCCATCT-3'	5'-TACTCACCACCAGCATCACC-3'
<i>HPRT</i>	5'-AGCTTGCTGGTGAAAAGGAC-3'	5'-TTATAGTCAAGGGCATATCC-3'
<i>RPL8</i>	5'-CCATGAATCCTGTGGAGC-3'	5'-GTAGAGGGTTTGCCGATG-3'
<i>RPS5</i>	5'-TCACTGGTGAGAACCCCT-3'	5'CCTGATTCACACGGCGTAG-3'

Supplementary Table 5. Primary antibodies used in the IHC analysis

Primary antibody	Clone	Specificity	Dilution	Incubation Period/ Temperature
p-mTOR	Rabbit, 49F9	Ser2448	1 in 100	18 hours/4°C
p-4E-BP1	Rabbit, 236B4	Thr37/46	1 in 500	2 hours/RT
eIF4E	Rabbit, n/a	Ser209	1 in 100	18 hours/4°C

p-mTOR, phosphorylated mammalian target of rapamycin; p-4E-BP1, phosphorylated Eukaryotic translation initiation factor 4E-binding protein 1; eIF4E, Eukaryotic translation initiation factor 4E; n/a, not applicable; RT, room temperature.

Supplementary Table 6. Immunolocalization of p-mTOR, p-4E-BP1 and eIF4E in the normal, PC and metastasis samples.

Group	p-mTOR				p-4E-BP1				eIF4E			
	C/N	C	N	n	C/N	C	N	n	C/N	C	N	n
Normal	4	7	0	0	3	7	0	1	3	8	0	0
PC	2	11	0	0	2	10	0	1	2	11	0	0
Metastasis	4	1	0	0	4	0	0	1	4	1	0	0

C/N, cytoplasm and nucleus; C, cytoplasm; N, nucleus; n, negative.

Supplementary Table 7. Correlation between mTOR, 4E-BP1 and eIF4E expression level and Gleason score in the canine PC.

Spearman's correlation coefficient		
	R	<i>P</i> *
p-mTOR	0.6637	0.0176
p-4E-BP1	0.4109	0.1631
eIF4E	0.5847	0.0358
<i>mTOR</i>	0.1794	0.5976
<i>4E-BP1</i>	0.5250	0.0655
<i>eIF4E</i>	-0.3546	0.3888

*Spearman's rank correlation test

Supplementary Table 8. Protein and gene expression of mTOR/4E-BP1/EIF4E pathway and Gleason score of the canine PC used in this study.

No. Case	Histological Diagnosis	<i>mTOR</i>	p-mTOR	<i>4E-BP1</i>	p-4E-BP1	<i>EIF4E</i>	eIF4E	Gleason Score
14	PC	LAT	27.64	0.15	2.48	LAT	25.39	6
15	PC	0.02	26.04	0.1	9.76	LAT	17.75	6
16	PC	1.62	46.86	1.65	37.72	0.32	35.9	10
17	PC	5.7	55.43	2.63	17.63	LAT	40.62	10
18	PC	1.07	56.91	1.03	35.86	4.63	56.76	10
19	PC	0.33	50.87	0.96	44.48	1.56	54.92	10
20	PC	2.88	43.71	2.07	49.59	0.82	42.26	9
21	PC	0.1	43.7	0.56	5.09	0.09	40.41	10
22	PC	3	49.62	1.74	48.1	0.28	44.38	10
23	PC	LAT	48.3	LAT	39.49	LAT	51.18	9
24	PC	LAT	26.05	LAT	17.1	LAT	32.97	10
25	PC	LAT	59.94	LAT	42.66	LAT	60.12	10
26	PC	LAT	16.53	LAT	0	LAT	15.39	6
27	PC	4.42	LAT	0.93	LAT	LAT	LAT	9
28	PC	1.28	LAT	0.37	LAT	0.41	LAT	6
29	PC	1.39	LAT	2.55	LAT	4.82	LAT	8
30	PC	LAT	LAT	0.15	LAT	LAT	LAT	8

PC, Prostate cancer; mTOR, mammalian target of rapamycin; 4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1; eIF4E, Eukaryotic translation initiation factor 4E; LAT, not used to limited amount of tissue.

Supplementary Table 9. Correlation between mTOR, 4E-BP1 and eIF4E expression level in the canine PC.

	Spearman's correlation coefficient			
	Protein		Gene	
	R	<i>P</i> *	R	<i>P</i> *
mTOR				
4E-BP1	0.5130	0.0220	0.6727	0.0233
eIF4E	0.9121	<0.0001	-0.0711	0.8665
4E-BP1				
eIF4E	0.7418	0.0037	0.3810	0.3518

Characterization of collagens fibers (I, III, IV) and elastin in the extracellular matrix of normal and neoplastic canine prostate.

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Abstract

Collagen (Coll) is the most common protein in the ECM, responsible for providing structure and support of the tissues. In some types of cancer, including prostate cancer (PC) abundant collagenous was identified and related to tumor progression and metastasis. In the veterinary medicine, few studies were performed to understand the relationship between cancer and collagens fibers. Apart from men, dog is the only species that develops with high frequency PC, being considered a potential model for comparative oncology initiatives. This study aimed to investigate Coll-I, III, IV and elastin in normal canine prostatic tissue and PC, using the Picrosirius red (PSR) and Immunohistochemical (IHC) analysis. Eight normal prostates and 10 PC from formalin-fixed paraffin-embedded samples were used. Canine PC was classified according to the human WHO from Tumors of the Urinary System and Male Genital Organs. Collagen fibers were analyzed with ImageJ software. Five fields of PRS and IHC staining were acquired for each slide with a 20x objective lens on the digital camera. Staining was assessed by setting a threshold using the image J threshold tool being obtained the quantification of area percentage for each collagen and elastin fibers. Mann-Whitney U test was applied to compare the area percentage among normal and canine PC. The distribution and percentage area for collagen and elastic fibers were similar in normal and neoplastic canine prostate when analyzed with PSR and IHC test.

Keywords: dog, cancer, ECM, picrosirius, immunohistochemistry.

INTRODUCTION

The cancer is of second leading cause of mortality worldwide. In the men, prostate cancer (PC) is the third most common malignant neoplasia (after non-melanoma skin cancer and lung cancer) (Ferlay, 2014). In advance stage, human PC often shows metastasis to bones and resistance to anti-androgen treatment (Mundy, et al., 2002). Similar to the men, dogs are only species that spontaneously develops PC (Leroy and Northrup, 2009; Palmieri et al., 2014). In dogs, PC is very aggressive and highly metastatic (Argyle, 2009). Also, this canine neoplasia can present bone metastasis and usually is diagnosed at a late stage (Leroy and Northrup, 2009).

Due to similarities in the clinical and pathologic aspects of PC in both species, some authors suggest that the dog may be considered a good model for human PC (Argyle, 2009; Palmieri, et al., 2014; Palmieri and Grieco, 2015). Recently, has been demonstrated in the human PC that an interaction between the tumor cells and the proteins of extracellular matrix (ECM) plays an important role for their development and progression (Palumbo et al., 2012; Penet et al., 2016). The ECM is a complex network of macromolecules (Franz et al., 2010). The major constituents of ECMs are proteoglycans and fibrous proteins (collagens, elastins, fibronectins and laminins). (Theocharis et al., 2016). Collagen is the most common protein in the ECM (Franz et al., 2010). The main collagen function is to provide structure, support and tensile strength, as well as, regulation of cell adhesion, chemotaxis, migration and direct tissue development (Rozario and DeSimone, 2010).

In some human cancers such as, breast, colon cancer and PC exists a formation of abundant collagenous stroma (reactive stroma) in their tumor microenvironment (TME), responsible for the tumor progression and metastasis (Tuxhorn et al., 2001; Palumbo et al., 2012; Zhang et al., 2013). High density of type I collagen and degradation of type IV collagen are frequently observed in the solids cancers, associated with metastasis (Zeng et al., 1999; Tanjore et al., 2006; Provenzano et al., 2008; Li et al., 2010; Penet et al., 2017). In the veterinary medicine, few studies were conducted to understand the relationship between cancer and the collagens fibers, compared to human medicine (Case et al., 2017).

Picrosirius red (PSR) is a staining method useful to visualize collagen fibers in different connective tissues (Sweat et al., 1964; Junqueira et al., 1979). The

combination of PSR and polarized light microscopy allows distinguish and analyze the type I and III collagens fibers, in according with their birefringence color (Junqueira et al., 1979). The objective of this study was to characterize and compare the collagen fibers and elastin in the normal prostate and canine PC, using PSR and immunohistochemical test.

MATERIALS AND METHODS

The Subjects

Eight canine normal prostates and 10 PC were retrieved from the archives of the Veterinary Pathology Service, FMVZ, UNESP, Botucatu, SP, Brazil (Supplementary Table 1). The prostates were collected from animals that had an interval between death and necropsy less than 6 hours. Formalin-fixed paraffin-embedded (FFPE) samples from canine prostatic tissue were sectioned for histological diagnosis, which was performed by three pathologist (LGRC, CEFA, PEK), at the same time, in a multi-head microscopy. The histopathological classification was performed according to the human WHO from Tumors of the Urinary System and Male Genital Organs (Eble et al., 2004), which was recently adapted to canine PC (Palmieri et al., 2014). (Supplementary Table 2).

Picrosirius (PSR)

The slides were deparaffinized in xylene and rehydrated in alcohol. After, PSR staining was performed using a commercial kit (Histokit™, Easypath, SP, Brazil) in according to manufacturer's instructions. The slides were examined in an optical microscopy with polarized light (Axio Imager A1, Zeiss®, Germany). The collagen fibers that presented red-orange birefringence were considered type I, while the collagen fibers with green birefringence were interpreted as type III (Coleman et al., 2011).

Immunohistochemistry (IHC)

The slides were subject to immunohistochemical test using the peroxidase and DAB method. The antibodies, antigen retrieval, dilutions and incubation period are described in the Table 1. Endogenous peroxidase activity was inhibited with 4% hydrogen peroxide in methanol for 10 min at room temperature (RT). Then, the slides were treated with protein block serum-free for 15 min RT (Dako, Carpinteria, CA, USA). In each step of the immunohistochemical process, the slides were washed with Tris-buffered saline (pH 7.4). A LSAB system was used as secondary antibody; this was applied for 1 hour at RT, according to manufacturer's instructions (Dako, Carpinteria, CA, USA). Peroxidase activity was revealed with 3',3'-Diaminobenzidinechromogen (DAB, Substrate System, CA, Dako). For the counterstained, Harris's hematoxylin was used. As negative control, primary antibodies were replaced with Tris-buffered saline.

Table 1. Primary antibodies, retrieval antigen, dilution and incubation period used in the IHC test

Interpretation of PSR and IHC staining

Five fields (20x magnification) were selected in each HE slide. These same areas in each slide, were captured with a digital camera (Axioncam MRc, Zeiss® Vision, Germany) for each antibody and PSR. The areas stained were analyzed with Image J 1.49v software (National Institutes of Health, USA) and were assessed by setting a threshold using the Image J threshold tool in according to the procedure described by Bauman et al., 2014. Canine skin was used as positive control tissue from collagen and elastic fibers, according to the Human Protein Atlas (<https://www.proteinatlas.org/>). Also, was described of the distribution and intensity of staining in collagens and elastic fibers inside of the normal and neoplastic canine prostatic tissue.

Data analysis

Descriptive statistics was used to define the median and percentile of Coll-I, Coll-III and elastin of staining for normal and canine PC. After establishing the median in the normal group, the PC samples were considered as under or over expressed compared to normal median. Mann-Whitney U test was applied to

compare the area percentage among normal and canine PC. Statistical significance was set at $p < 0.05$. All statistical analysis was done using GraphPad Prism 6 (GraphPad Software Inc. La Jolla, CA).

RESULTS

In the normal prostates and PC stained with PSR, the distribution of Coll-I and Col-III was approximately 80% around and between prostatic ducts and acini, 15% amongst smooth muscle and 5% in blood vessels. A similar proportion of collagen distribution was observed for IHC test in both groups.

No statistic differences in the area percentage of PSR and IHC staining for type I and type III collagens fibers were observed between canine PC and normal samples ($p > 0.05$) (Figure 1). Table 2 shows the median, 25% percentile and 75% percentile values of Coll-I and Coll-III according to the diagnosis group and test applied.

Table 2. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-I and Coll-I in the normal and canine PC, according to the method used.

No statistic differences in the area percentage of immunohistochemical for Coll-IV and elastin was observed in normal prostates and canine PC ($p > 0.05$, Table 3).

Table 3. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-IV and elastin in the normal prostate and canine PC samples.

Immunostaining for collagen type IV (Coll-IV) was observed in the basal membrane (BM) of prostate acini, smooth muscle, blood vessels, and never fibers of normal and PC samples. Acinar BM, showed weak immunostaining for Coll-IV in more than of 70% normal samples and PC (Figure 2). Only the blood vessels were observed with strong immunostaining in the two groups. The distribution of Coll-IV was approximately 70% in acinar BM, 15% in smooth muscle, 10% in blood vessels BM and 5% in nerve fibers in both groups. Absence of Coll-IV immunostaining was

observed in the tumors with solid pattern due to loss of acinar BM (Figure 2). Immunostaining for elastin was observed with similar intensity and distribution than Coll-IV around of blood vessels to normal prostate and canine PC. Elastic fibers were found in the septa dividing the lobules and around the prostatic acini of normal samples. A high amount of elastic fibers was observed around the ducts and the urethra in normal and canine PC (Figure 2).

DISCUSSION

In cancer, the EMC is a network of macromolecules that allow the cellular evasion towards the defense of the organism, besides helping in their metastasis process (Franz et al., 2010). The collagens fibers are important components of remodeling of ECM in the TME, actually is know that their degradation and redeposition promote tumor infiltration, angiogenesis, invasion and migration (Theocharis et al., 2016). In this study, we identified and characterized the collagen and elastin fibers in the normal prostate and canine PC using PSR and IHC test.

The PSR birefringent color proportions (red-orange and green) were not statistically different when compared to normal prostates and canine PC samples. In humans, Bauman et al., (2014) evaluated of collagen content in normal prostates and benign prostatic hyperplasia (BPH) by PSR staining. The total birefringence was not statistically significant between normal and human BHP. Also, the normalized proportion of orange, red and green birefringent collagen bundles were not statistically different to normal and BHP samples. We do not find studies performed with human and canine PC evaluating the fibers collagens with PSR and IHC test.

In one study was evaluated of gene and protein expression of Col-I and Col-III in relation to Gleason score, using RT-qPCR and IHC (Duarte et al., 2012). No correlation was found between protein and gene expression in both collagens. However, the IHC analysis showed that Col-I and Col-III was significantly reduced in PC, in all Gleason scores, when compared to benign areas. These authors suggest that collagen reduction in PC could result from high metalloproteinase activity. In canine PC, the MMP-2 and MMP-9 were observed with overexpression when compared to normal prostate, but the activity of these metalloproteinase not was

correlated with the immunoexpression of collagen fibers (Faleiro et al., 2013).

Wegner et al., (2017) performed a study with fluorescence of PSR in prostate of C57BL/6J mice. They found that fluorescent PSR imaging is more sensitive than polarized light imaging for identify the collagen fibers. In addition, Fluorescent PSR imaging was compatible with the collagen expression by IHC test. The fluorescent PSR imaging method seems to be promising but it must still be studied in the comparative oncology.

Fewer studies with formalin-fixed paraffin-embedded (FFPE) samples of canine tumors were conducted to analyze the collagens fibers (Benazi et al., 1993; Murakami et al., 2009; Bedoya, 2016; Case, 2017). Bedoya et al., (2016) used PSR staining in canine squamous cell carcinomas (SCC), classified in well and poorly differentiated. The percentage of Col-I was approximately 30% for both differentiations of SCC, this value was higher than observed in our study to normal and canine PC. However, the percentage of Col-III in these SCC was similar when compared to canine PC.

The Col-IV is another important component of the ECM, localized mainly in the BM (Murakami et al., 2009). In the human prostatic tissue Coll-IV was found in the BM of epithelial and stromal elements (vessels, smooth muscle and nerves) (Sinha et al., 1991). In this study also was possible identify Coll-IV in BM of prostate acini, smooth muscle, blood vessels, and never fibers of normal and PC samples. Sinha et al., (1991) found that Coll-IV immunostaining was less uniform or absent in the acinar BM of poorly-differentiated PC when compared to acinar BM of well-differentiated PC, HBP and normal prostate. Similar results also were observed in feline and canine mammary tumors as well as in canine hemangiosarcomas (Benazzi et al., 1993; Murakami et al., 2009). In this study, the canine PC with solid pattern also shows absent of Coll-IV immunostaining when compared to others patterns of PC and normal samples.

We also found elastic fibers in the septa dividing the lobules, around the alveoli, ducts and the urethra. Marettová et al., (2017) performed an immunohistochemical localization of elastic system in the canine prostate. Just like our study, these authors observed elastic fibers around blood vessels, in the septa

supporting the lobules and between the secretory alveoli, as well as, a concentration of fibers around the ducts and in the area of the urethra.

CONCLUSIONS

The distribution and area percentage of staining for collagen are similar in normal and neoplastic canine prostate when analyzed with PSR and IHC test. Also, the immunohistochemical localization of elastic system fibers is similar in both groups. Only, in canine PC with solid pattern was identified loss of Col-IV compared to others tumor patterns and normal prostate samples.

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Table 1. Primary antibodies, retrieval antigen, dilution and incubation period used in the IHC test

Primary antibody	Retrieval antigen	Dilution	Incubation Period
Collagen I, rabbit, Novotec	Citrate buffer pH 6,0, microwave, twice for 5 min	1:1000	Overnight at 4°C
Collagen III, rabbit, Novotec	Pepsin 2%, pH 1,4 in oven for 10 min at 60°C after for 30 min at 37°C.	1:1000	Overnight at 4°C
Collagen IV, rabbit, Biorbyt	Pepsin 2%, pH 1,4, in oven for 10 min at 60°C after for 30 min at 37°C.	1:1000	Overnight at 4°C
Elastin (BA-4), mouse, Santa Cruz.	Citrate buffer pH 6,0, pressure cooker (Pascal [®] , Dako, Carpinteria, CA, USA)	1:100	Overnight at 4°C

Table 2. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-I and Coll-III in the normal and canine PC, according to the method used.

		PSR test				IHC test			
		25%	Median	75%	<i>p</i>	25%	Median	75%	<i>p</i>
	N	1.25	1.89	2.27		2.85	4.73	8.03	
Coll-I	PC	2.09	2.24	2.43	0.099	3.31	6.18	8.56	0.447
	N	1.33	1.64	2.06		1.81	3.22	5.03	
Coll-III	PC	1.68	2.25	3.11	0.120	3.72	5.07	6.44	0.082

Coll-I: Collagen I, Coll-III: Collagen III, N: Normal, PC: Prostatic carcinoma

Table 3. Median, 25% percentile and 75% percentile values of area percentage staining for Coll-IV and elastin in the normal prostate and canine PC samples.

Group		IHC test			
		25%	Median	75%	<i>p</i>
	Normal	1.11	1.41	1.72	0.2722
Coll-IV	PC	0.58	1.14	1.61	
	Normal	0.25	0.26	0.42	0.0671
Elastin	PC	0.28	0.43	0.51	

Supplementary TableS1. Samples used for PSR and IHC test

Case number	Diagnosis	PSR	IHC
1	Normal	X	X
2	Normal	X	X
3	Normal	X	X
4	Normal	X	X
5	Normal	X	X
6	Normal	X	X
7	Normal	X	X
8	Normal	X	X
9	PC	X	X
10	PC	X	X
11	PC	X	X
12	PC	X	X
13	PC	X	X
14	PC	X	X
15	PC	X	X
16	PC	X	X
17	PC	X	X
18	PC	X	X

Supplementary Table S2. Histological type of 10 canine prostatic carcinomas, according to Eble et al., 2004 and Palmieri et al., 2014.

Histologic Type	Case Number
Single histological pattern	
Cribriform with comedonecrosis	14, 16
Solid	12, 18
Small acinar/ductual	9, 10
Mixed histological patterns	
Solid, cribriform with comedonecrosis	11, 17
Cribriform with comedonecrosis, solid	13
Cribriform with comedonecrosis, cribriform without comedonecrosis	15

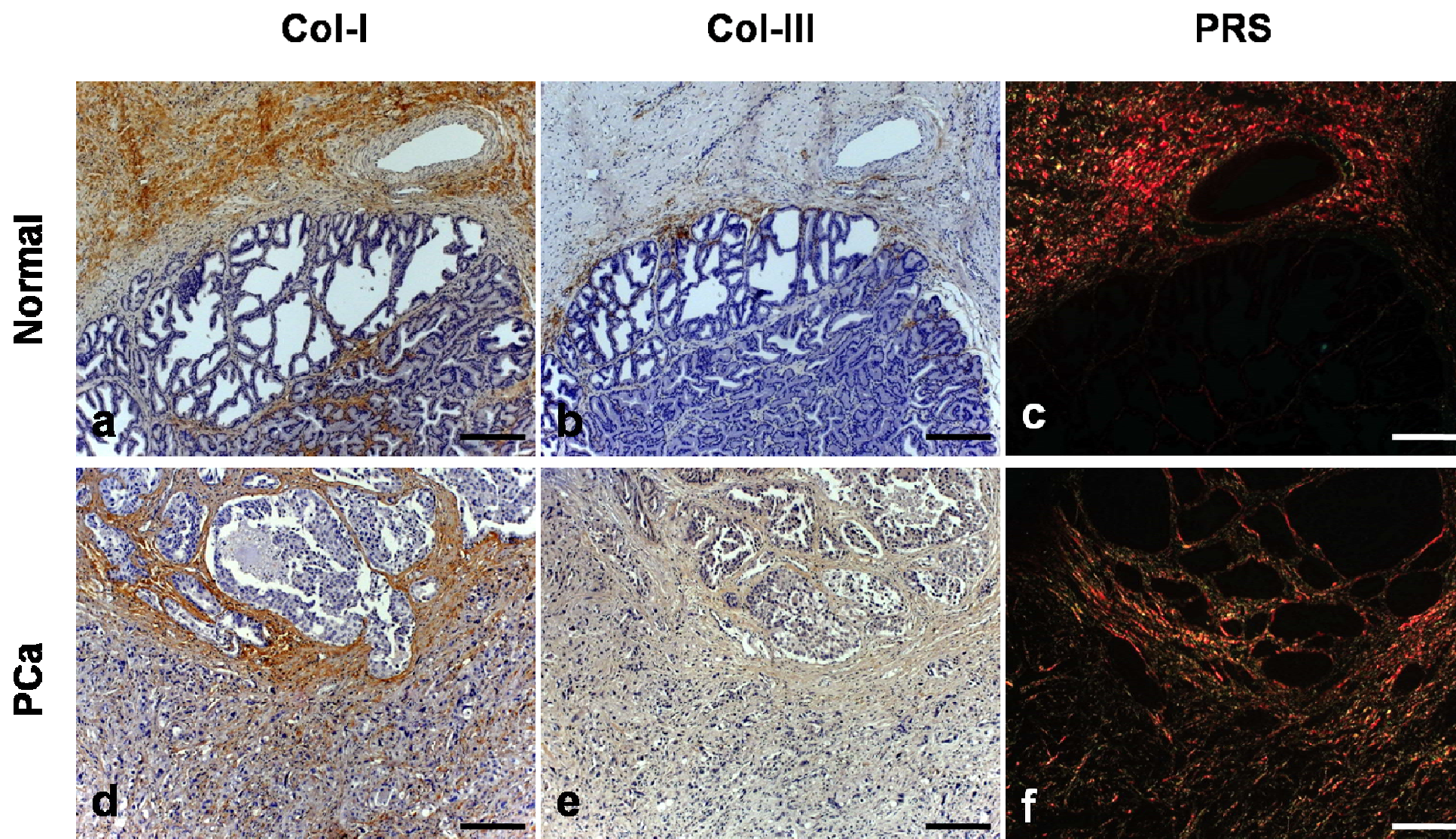


Figure 1. The immunohistochemistry and PSR stain in normal tissue and canine PC. **a:** immunostaining of Coll-I in the stroma of normal prostate (case No. 3). **b:** immunostaining of Coll-III in the stroma of the normal prostatic tissue (case No. 3). **c:** PSR staining observed in an optical microscopy with polarized light, the collagens fibers present red-orange birefringence (Coll-I) and green birefringence (Coll-III) in a smaller amount(case No. 3). **d:** immunostaining of Coll-I in the stroma of prostatic neoplastic tissue (case No. 11). **e:** immunostaining of Coll-III in the stroma of canine PC (case No. 11). **f:** PSR staining in the canine PC with similar amounts of Coll-I and Coll-III (case No. 11).

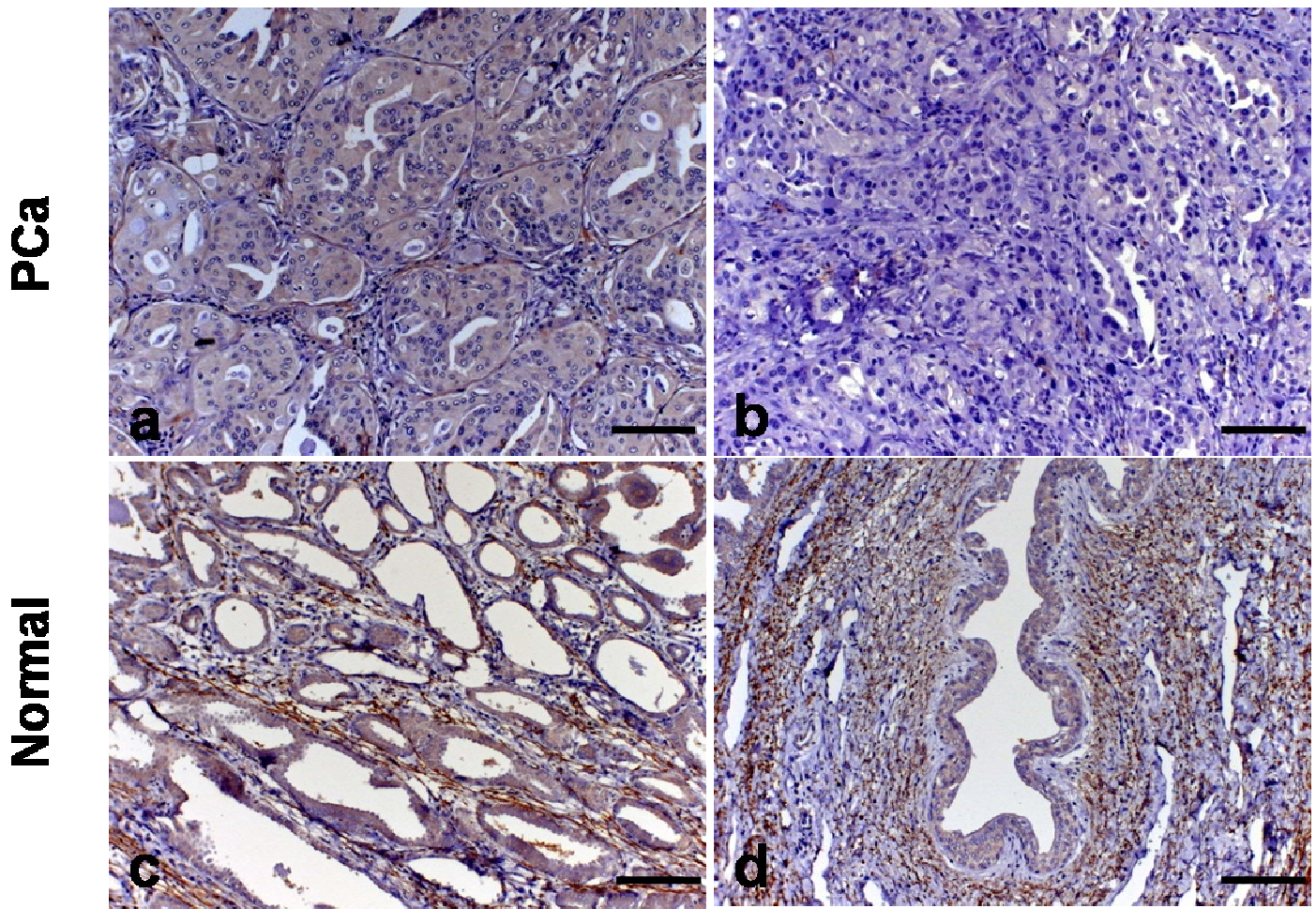


Figure 2. Immunostaining for Coll-IV and elastin in canine prostatic tissue. **a:** Coll-IV immunostaining in the basal membrane of canine PC with cribriform pattern (case No. 15). **b:** Absence of Coll-IV immunostaining in canine PC with solid pattern (case No. 12). **c:** Elastin fibers around the prostatic acini of normal samples (case No. 5). **d.** High amount of elastin fibers around the urethra (case No. 5).