MARIA CRISTINA DO AMARAL WESTIN

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EXPRESSION OF THE PROTEINS MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 AND VEGF-A IN THE CIN 3 AND CERVICAL CANCER

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Tocoginecologia da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do título de Doutora em Ciências da Saúde, na área de concentração em Oncologia Ginecológica e Mamária

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Dedico este trabalho...

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Sumário

Símbolos, Siglas e Abreviaturas

CBD – Domínio ligante do colágeno *(Collagen binding domain)*

CIN – *Cervical intraepithelial neoplasia*

CIN 3 – *Cervical intraepithelial neoplasia grade 3*

C-terminal – Carboxi-terminal

IARC – *International Agency for Research on Cancer*

INCA – Instituto Nacional do Câncer

MEC – Matriz extracelular

MMPs – Metaloproteinases de Matriz Extracelular (*Metalloproteinases)*

mRNA – Ácido ribonucleico mensageiro

MT-MMP – MMP tipo membrana *(Membrane type MMP)*

MT1-MMP – O mesmo que MMP-14 *(The same as MMP-14)*

NIC – Neoplasia intraepitelial cervical

NIC 3 – Neoplasia intraepitelial cervical de grau 3

N-terminal – Amino-terminal

PDGF – Fator de crescimento derivado de plaqueta

TIMPs – Inibidores teciduais das metaloproteinases de matriz extracelular *(Tissue inhibitors of matrix metalloproteinases)*

VEGF – Fator de crescimento endotelial vascular

VEGF-A – *Vascular endothelial growth fator A*

VEGFR – Receptor de VEGF

Resumo

Introdução: O carcinoma escamoso do colo uterino é precedido pela neoplasia intraepitelial cervical grau 3 (NIC 3). A invasão tumoral envolve a degradação da matriz extracelular e membrana basal do epitélio por enzimas proteolíticas denominadas metaloproteinases (MMPs). Os inibidores teciduais das metaloproteinases (TIMPs) também interferem no processo de invasão. Angiogênese é condição indispensável para a progressão tumoral. **Objetivo:** Analisar a expressão de MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 e VEGF-A na NIC 3 e carcinoma do colo uterino. **Sujeito e Métodos:** Estudo do tipo comparativo observacional constituído de três grupos:- Grupo 1: 55 casos com diagnóstico de NIC 3, Grupo 2: 30 casos com NIC 3 e carcinoma associados e Grupo 3: 46 casos com carcinoma. A expressão proteica foi pesquisada separadamente nas células tumorais e estromais por reação imunoistoquímica. Para estabelecer a porcentagem de células imunopositivas utilizou-se *software* morfométrico. **Análise Estatística:** Aplicou-se o Teste T-pareado ou de Mann-Whitney ou Wilcoxon Signed Rank. **Resultados:** Em todos os grupos, a expressão tumoral de MMP-14 foi maior que a estromal. Inversamente, a expressão de TIMP-2 foi maior nas células estromais que nas tumorais, em cada grupo diagnóstico. A expressão

de MMP-9 foi maior nas células estromais que nas tumorais, com exceção do componente invasor do Grupo 2. A expressão estromal de TIMP-1 foi maior que a tumoral no carcinoma e, ao contrário, sua expressão foi maior nas células tumorais da NIC 3. A expressão de VEGF-A foi maior apenas nas células tumorais da NIC 3. Comparando a expressão dos marcadores entre os grupos, foram encontradas as maiores diferenças entre grupos extremos, ou seja, entre NIC 3 e carcinoma. A expressão de MMP-2 nas células estromais foi maior no componente NIC 3 do Grupo 2 que no NIC 3 do Grupo 1. A expressão de VEGF-A nas células estromais do carcinoma foi maior que nas células estromais da NIC 3. **Conclusões:** Os resultados deste estudo sugerem que a expressão de TIMP-1 aumenta nas células do estroma e diminui nas células tumorais quando a NIC 3 progride para carcinoma invasor. MMP-9 e TIMP-2 tiveram expressão similar na NIC 3 e no carcinoma, o que limita inferências sobre seu papel na progressão neoplásica. O padrão imunoistoquímico da expressão das MMPs, TIMPs e VEGF-A na NIC 3 e no carcinoma invasivo, quando estas lesões estavam associadas, foi semelhante. A expressão do VEGF-A foi maior nas células tumorais do que nas estromais da NIC 3, porém quando esta lesão progride para carcinoma invasivo sua expressão aumenta nas células do estroma e não se altera nas tumorais. A expressão de MMP-14, MMP-2, TIMP-1 e VEGF-A aumentou com a gravidade da neoplasia.

Palavras-chave: Metaloproteinase de matrix extracelular, MMP, inibidor tecidual de metaloproteinase, TIMP, moduladores de angiogênese, VEGF-A, tumor, estroma, neoplasias do colo do útero.

Summary

Introduction: Squamous cell carcinoma of the cervix is preceded by cervical intraepithelial neoplasia grade 3 (CIN 3). Tumor invasion involves degradation of extracellular matrix and epithelium basement membrane by proteolytic enzymes called metalloproteinases (MMPs). Tissue inhibitors of metalloproteinases (TIMPs) are also involved in the invasion process. Angiogenesis is a prerequisite for tumor progression.. **Objective:** To analyze the expression of MMP-2, MMP-9 and MMP-14, TIMP-1, TIMP-2 and VEGF-A in CIN 3 and invasive carcinoma. **Subject and Methods:** This comparative observational study study was consists of three groups: Group 1: 55 cases diagnosed with CIN 3, Group 2: 30 cases with CIN 3 associated with invasive carcinoma and Group 3: 46 cases with invasive carcinoma. Protein expression was investigated separately in tumor and stromal cells by immunohistochemistry and evaluated by the percentage of cells positive for immunostaining using morphometric software. **Statistical Analysis:** were performed applying paired t-test or Mann-Whitney or Wilcoxon Signed Rank. **Results:** In each diagnostic group, expression markers were significantly higher: MMP-14 in tumor cells, and TIMP-2 in stromal cells; also MMP-9 expression was significantly higher in stromal cells, except in invasive

component of group 2, and TIMP-1 had significantly higher expression in stromal cells of invasive carcinoma and in tumor cells of CIN 3. VEGF-A expression was significantly higher only in tumor cells CIN 3. Comparing the expression of markers between groups, two by two, we find the greatest differences between the extreme groups, i.e. between invasive carcinoma and CIN 3. The expression of MMP-2 was significantly greater in the stromal component CIN 3 in group 2 than in CIN 3 only. The expression of VEGF-A was significantly higher in the group stromal cell carcinoma when compared to stromal cells CIN 3. **Conclusions:** The results of this study suggest that the expression of TIMP-1 increases in the stromal cells and decreases in tumor cells when CIN 3 progresses to invasive carcinoma. MMP-9 and TIMP-2 had similar expression in CIN 3 and invasive carcinoma, which limits inferences about its role in neoplastic progression. The immunohistochemical pattern of expression of MMPs, TIMPs and VEGF-A in CIN 3 and invasive carcinoma, as these lesions were associated, was similar. The expression of VEGF-A was higher in tumor cells than in stromal cells in CIN 3, but when the lesion progresses to invasive carcinoma its expression increases in the stromal cells and the tumor cells does not change. The expression of MMP-14, MMP-2, TIMP-1 and VEGF-A was increased with the severity of the neoplasia.

Keywords: Matrix metalloproteinase, MMP, tissue inhibitor of metalloproteinase, TIMP, Vascular Endothelial Growth Factor A, VEGF-A, tumor cells, stromal cells, cervical neoplasia.

1. Introdução

O carcinoma do colo uterino é um grave problema de saúde pública. Segundo dados da *International Agency for Research on Cancer* (IARC) – GLOBOCAN 2008 – é o terceiro câncer mais comum nas mulheres em todo o mundo, com estimativa de 530.000 novos casos em 2008. Mais de 85% do total global ocorrem em países em desenvolvimento, sendo responsáveis por 13% de todos os cânceres femininos. Em geral, a taxa de mortalidade é de 52%, representando 275.000 mortes em 2008, das quais 88% ocorrem em países em desenvolvimento, sendo 31.700 na América Latina e Caribe (1).

De acordo com as estimativas do Instituto Nacional do Câncer (INCA) para o ano de 2012, as previsões eram de 17.540 casos novos de câncer do colo do útero, com um risco estimado de 17 casos a cada 100 mil mulheres. Sem considerar os tumores da pele não melanoma, o câncer do colo uterino foi o mais incidente na região Norte (24/100 mil); nas regiões Centro-Oeste (28/100 mil) e Nordeste (18/100 mil) ocupa a segunda posição mais frequente; na região Sudeste (15/100 mil), a terceira, e na região Sul (14/100 mil), a quarta posição, ultrapassado apenas pelo câncer de mama (2).

Considerando os tipos histológicos de câncer do colo uterino, os carcinomas escamosos são os mais frequentes, representando de 75% a 85% do total (3, 4).

As neoplasias intraepiteliais cervicais (NIC) são as lesões precursoras do câncer cervical e o aumento no grau de severidade destas neoplasias correlaciona-se com o aumento de risco para carcinoma invasor (5). Estima-se que 12% a 22% das NIC 3 progridam a carcinoma invasor quando não tratadas (6). Esta progressão é feita à custa do rompimento da membrana basal subepitelial. As metaloproteinases da matriz extracelular (MMPs), sendo enzimas proteolíticas potentes, têm um papel chave nesse processo de degradação dos vários componentes da membrana basal, bem como da matriz extracelular (MEC) (7) e consequente invasão tumoral e metástase (8).

Os Inibidores Teciduais das Metaloproteinases (TIMPs) controlam as atividades das MMPs e, por isso, minimizam a degradação da matriz. Ambos, MMPs e TIMPs, estão envolvidos na remodelação tecidual e regulam a progressão de células tumorais, incluindo a angiogênese tumoral (9).

Os TIMPs funcionam como principais inibidores das MMPs através da ligação ao local ativo e, em seguida, formando complexos estáveis enzima-inibidor que se tornam inativos. Desequilíbrios nas atividades extracelulares de MMPs e TIMPs foram associados à destruição patológica de tecidos, como encontrada no câncer. Os TIMPs diferem na sua especificidade para a inibição de MMPs, com TIMP-2 que tem uma afinidade mais elevada para a MMP-2 (7).

Angiogênese é uma importante ocorrência durante o processo neoplásico. Este complexo procedimento, também conhecido como neovascularização, é essencial para o desenvolvimento tecidual, cicatrização de feridas e reprodução, e um requisito indispensável para a progressão tumoral, invasão e metástase (10).

O fator de crescimento endotelial vascular (VEGF) é um mitógeno potente que está envolvido na angiogênese, sobrevida endotelial e indução da hematopoiese (11).

1.1. Metaloproteinases da Matriz Extracelular (MMPs)

Em 1962, Jerome Gross e Charles Lapiere, de Havard, descreveram uma "atividade" que estava presente na cauda de girinos durante sua metamorfose e que tinha a capacidade de degradar feixes rígidos de colágeno. Esta "atividade" revelou-se ser uma colagenase intersticial, uma enzima com a habilidade quase singular de degradar a hélice tripla do colágeno, em pH neutro. Desta observação, nasceu um novo campo de pesquisas biomédicas que iria crescer para abarcar toda uma família de enzimas – as Metaloproteinases de Matriz Extracelular (MMPs).

Em 1994, Motoharu Seiki e colaboradores descreveram o clone de uma nova MMP ancorada à membrana celular – a MT1-MMP (ou MMP-14). Mostraram também que essa nova MMP podia ativar a MMP-2 (8). A MT1-MMP pode degradar vários componentes da membrana basal incluindo fibronectina, vitronectina, fibrina, laminina 1 a 5 e colágeno tipo I, II e III. Como ativadora específica da pró-MMP-2 (forma inativa), age na superfície celular. Para isto, a

MT1-MMP forma um complexo tri-molecular com TIMP-2 e a pró-MMP-2. Uma vez o complexo ternário formado, outra MT1-MMP cliva o pró-domínio N-terminal da pró-MMP-2, gerando assim um composto intermediário que resulta na enzima MMP-2 plenamente ativa. Por isso, MT1-MMP é considerada uma enzima-chave que contribui para invasão tumoral e metástase, através da degradação direta da matriz extracelular (MEC) e/ou ativação de outras MMPs. A atividade da MT1-MMP pode ser inibida pela interação com as TIMPs ou a enzima pode ser destruída através da degradação autoproteolítica ou lisossomal (6). Atualmente, a Base de Dados MEROPS[1] alberga as várias famílias das metaloproteinases que estão agrupadas em clãs. As metaloproteinases humanas são endopeptidases pertencentes ao clã MA, caracterizado por apresentar um só íon metálico bivalente no sítio catalítico, e à família M10 onde esse íon é o Zinco^{++} (12). São também conhecidas como Metaloproteinases de Matriz Extracelular (MMPs). Até agora, pelo menos 24 MMPs têm sido encontradas expressas no tecido humano. Estas MMPs compartilham alta homologia na sequência proteica e têm estrutura do domínio definida. De acordo com a especificidade do substrato e organização estrutural podem ser divididas em cinco subgrupos, sendo dois deles de interesse para este estudo:

1) Gelatinases, i.e., MMP-2 (gelatinase A) e MMP-9 (gelatinase B), caracterizadas pela presença de um "domínio ligante do colágeno" (*Collagen*

MEROPS: Base de Dados das Peptidases (proteases, proteinases ou enzimas proteolíticas) iniciada em 1993 (Rawlings & Barrett), estruturada em famílias e clãs. A inspiração para o nome adveio de um pássaro da família dos abelharucos (bee-eater), Merops apiaster, que vive às margens do rio Zambeze, em colônias divididas em famílias e clãs. Na mitologia grega, Merops é o nome do profeta troiano sogro de Príamo, rei de Tróia.

Binding Domain - CBD) e que estão aptas para processar vários substratos da MEC, tais como colágeno I e colágeno IV.

2) Tipo Membrana (MT-MMPs), em um total de 6 MT-MMP, apresentam em C-terminal um domínio adicional representado por uma região intramembrana completada por uma curta cauda citoplasmática. A MT1-MMP (também denominada MMP-14) será objeto desta pesquisa. Somente MMP-14 e MMP-16 têm se mostrado aptas a clivar o colágeno fibrilar tipo I (mais abundante e difundido no organismo, aparecendo no tecido conjuntivo frouxo e denso de ossos, dentina, tendões e derme).

As MMPs têm importante papel em uma variedade de processos biológicos como crescimento, remodelação esquelética e cicatrização de feridas. O desequilíbrio da sua atividade e expressão é geralmente a base das doenças como câncer, neurodegeneração, inflamação, artrite e doenças cardiovasculares. Por esta razão, são consideradas interessantes alvos para drogas, embora sua inibição terapêutica tenha levantado muitas perguntas, uma vez que: 1) a atividade dessas enzimas é pleiotrópica (podem atuar sobre muitos tipos celulares diferentes), de modo que sua inibição pode modular negativamente algumas funções celulares; 2) um grande número de inibidores não são seletivos para uma enzima única (13).

As células tumorais secretam MMPs e induzem as células do estroma, como fibroblastos, células inflamatórias e células endoteliais, também a produzi-las (14). MMP-2 e MMP-9 são potentes enzimas proteolíticas com um papel

importante na digestão do colágeno tipo IV da membrana basal, mecanismo importante para a invasão vascular e metástase. MMP-14 é uma enzima-chave na invasão tumoral e parece implicada tanto no rompimento das membranas basais pelas células tumorais como na invasão celular através dos tecidos intersticiais do colágeno tipo I (15).

1.2. Inibidores Teciduais das Metaloproteinases de Matriz Extracelular

Inibidores teciduais das metaloproteinases de matriz extracelular (*Tissue Inhibitors of Matrix Metalloproteinases* – TIMP-1 a 4) são inibidores naturais dessas enzimas. São proteínas multifuncionais que não só inibem as MMPs, mas também promovem o crescimento celular, induzem à apoptose e inibem a angiogênese (16, 14). TIMP-1 foi purificado em 1979, por um grupo de pesquisadores da Universidade de Washington. Recentes evidências sugerem que os TIMPs, especialmente os TIMP-1 e TIMP-2, podem ter propriedades biológicas ímpares, independente da sua habilidade de inibir MMPs. Isto é apoiado pela identificação de receptores de superfície celular para esses dois membros da família TIMP. TIMP-2 atinge o microambiente tumoral por, inicialmente, promover a diferenciação das células endoteliais e fibroblastos e, possivelmente, das células epiteliais. No entanto, a subsequente progressão e massiva produção e ativação das MMPs por uma variedade de tipos celulares que compreendem o microambiente tumoral, incluindo células tumorais, células endoteliais, células do sistema imunológico e fibroblastos associados às células tumorais, induz a alteração da função de TIMP-2 para inibidor das metaloproteinases. Finalmente,

a progressão continuada e a posterior diminuição dos níveis de TIMP-2 poderiam novamente mudar a função de TIMP-2, de inibidor para ativador das MMPs, facilitando a ativação da pró-MMP-2 pela MMP-14 (MT1-MMP) (17). Entre os TIMPs, a atividade de TIMP-2 é incomparável uma vez que sua função é tanto inibitória como ativadora das MMPs (18).

1.3. Fator de Crescimento do Endotélio Vascular (*Vascular Endothelial Growth Factor* - VEGF)

VEGF é uma glicoproteína; o mais estudado e conhecido membro da família de ligantes VEGF. Essas proteínas fazem parte da família do fator de crescimento derivado de plaqueta (PDGF), produzidas por vários tipos celulares em resposta a inúmeros estímulos. O VEGF age sobre as células endoteliais vasculares ligando-se a receptores específicos transmembrana; essa ligação ativa várias vias sinalizadoras (19). Foi primeiramente identificado por Senger et al. (20), em 1983, como um fator que aumentava a permeabilidade vascular dos vasos que revestiam a cavidade peritoneal de mamíferos com ascite tumoral; foi o primeiro artigo a sugerir um mecanismo através do qual a permeabilidade vascular aumentada foi estabelecida por ascite neoplásica. Anteriormente, em 1971, Folkman e colaboradores (21) isolaram um fator de angiogênese tumoral (sem denominação) de um tumor de mamífero como um alvo potencial para tratamento. VEGF tem sido desde então estabelecido como ferramenta de diagnóstico e terapêutica no tratamento dos cânceres humanos.

Até o momento, foram identificados seis membros da família VEGF, além do PlGF (Fator de crescimento placentário):

- VEGF-A – o mais importante para angiogênese, agindo nas células endoteliais vasculares, com função de manutenção vascular.
- VEGF-B – agindo nas células endoteliais vasculares.
- VEGF-C – agindo nas células endoteliais linfáticas.
- VEGF-D – agindo nas células endoteliais linfáticas.
- VEGF-E – codificado por vírus; agindo nas células endoteliais vasculares.
- VEGF-F – encontrado em veneno de certas cobras.

Existem 3 receptores para o VEGF:

- VEGFR-1: fraco estimulador da angiogênese.
- VEGFR-2: mais importante para a angiogênese.
- VEGFR-3: ligado à proliferação dos vasos linfáticos.

Os efeitos biológicos do VEGF-A parecem ser exercidos principalmente por meio da ligação ao receptor-2, que é expresso predominantemente nas células endoteliais vasculares (22).

O VEGF-A apresenta quatro principais funções biológicas:

- crescimento e proliferação de células endoteliais vasculares
- migração de células endoteliais vasculares
- sobrevida de células endoteliais imaturas por inibição da apoptose
- maior permeabilidade capilar

Recentemente, o VEGF tem sido relacionado também à linfangiogênese (23). Em presença do VEGF, as células endoteliais linfáticas proliferam para formar vasos linfáticos gigantes onde o fluxo de linfa é lento. Contudo, o VEGF não é necessário para a manutenção desses vasos, uma vez que eles se mantêm quando o VEGF já não está presente.

Além dessas ações nas células endoteliais, existem evidências de atuação sobre outros tipos de células como, p. ex., em células da medula óssea (19).

Vasos sanguíneos são os meios pelos quais o tumor é capaz de atender suas necessidades metabólicas, uma vez que o limite de difusão do oxigênio nos tecidos é de 100µm. Um tumor sólido em crescimento requer um suprimento de vasos sanguíneos para crescer além de poucos milímetros (24).

Em um tumor em desenvolvimento existe um equilíbrio dinâmico entre fatores pró e antiangiogênicos que regulam a habilidade de recrutar vasos sanguíneos e crescer a um tamanho considerável. O período conhecido como "chave angiogênica" muda este equilíbrio a favor da formação de vasos sanguíneos e permite ao tumor formar novos vasos sanguíneos a partir de capilares pré-existentes. Esta mudança ocorre em grande parte pelo efeito do tumor em seu meio ambiente. Certas condições no tecido humano promovem a indução da mudança angiogênica, como inflamação, hipóxia e transcrição de oncogênes, são alterações genéticas conhecidas por causar o câncer (24). A vascularização tumoral é um processo complexo com vários passos.

As fases chaves ocorrem na seguinte sequência: fatores de crescimento como o VEGF estimulam as células endoteliais; proteases degradam a MEC; células endoteliais proliferam e migram para dentro do tumor e novos vasos capilares são formados por um processo denominado de “brotamento”, pelo qual o tumor secreta fatores de crescimento como VEGF para induzir a proliferação das células endoteliais, as quais por sua vez levam a formação de novos capilares. Sob condições em que o Oxigênio é limitado, o gene VEGF será transcrito para criar o mRNA de modo que o tumor possa ultrapassar este obstáculo para o crescimento (24).

Dvorak verificou que VEGF era secretado pelas células tumorais, estava em alta concentração no líquido ascítico neoplásico e, portanto, era responsável pelo aumento da permeabilidade vascular tumoral, que é maior em relação aos vasos dos tecidos normais (25).

É clinicamente relevante destacar que estudo recente demonstrou o potencial do uso da terapêutica antiangiogênica no tratamento do carcinoma avançado do colo do útero. A adição de bevacizumabe (anticorpo monoclonal - VEGF) ao topotecan e cisplatino mostrou taxas mais altas de intervalo livre de progressão e sobrevida global (26).

1.4. A proposta do estudo

Com o objetivo de avaliar a variação da expressão destas proteínas entre a lesão precursora e invasora do colo do útero, foi construído um estudo que

incorporou três grupos, ordenados por progressão da lesão. Um grupo foi constituído de casos que apresentaram apenas NIC 3; outro grupo foi constituído de casos que apresentaram apenas carcinoma invasor. Foi incluído um terceiro grupo de presumida gravidade intermediária, constituído de casos que apresentaram NIC 3 e carcinoma invasor associados.

Optou-se também por avaliar as células do tumor e do estroma separadamente porque a avaliação da expressão das proteínas MMP e TIMP tem sido realizada principalmente em células epiteliais malignas e, portanto, há menos informação sobre os padrões de expressão proteica do estroma (6,7,10,11,13,18). Há indicações que o contato célula-célula entre as células do carcinoma cervical e os fibroblastos do estroma periférico aumentam a produção e ativação das MMPs e, portanto, o desequilíbrio subsequente entre MMPs e TIMPs pode resultar na progressão da invasão das células do carcinoma cervical. Observou-se que 15% a 20% de todas as neoplasias malignas são iniciadas ou exacerbadas pelo processo inflamatório (27).

Em resumo, o melhor entendimento dos mecanismos moleculares pelos quais as lesões pré-invasivas adquirem a habilidade para invadir o estroma cervical e metastatizar pode ter importância clínica. Neste sentido, este estudo avaliou a expressão de três das MMPs (MMP-2, MMP-9 e MMP-14), dois dos TIMPs (TIMP-1 e TIMP-2), bem como a expressão do VEGF-A na NIC 3 e no carcinoma invasor, nas células tumorais e estromais adjacentes.

2. Objetivos

2.1. Objetivo Geral

Analisar o papel das MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 e do marcador de angiogênese VEGF-A no processo de invasão tumoral da neoplasia do colo uterino com base na expressão imunoistoquímica.

2.2. Objetivos Específicos

- Artigo 1: Descrever e analisar a expressão das proteínas MMP-2, MMP-9, MMP-14, TIMP-1 e TIMP-2 em células tumorais e estromais na neoplasia intraepitelial cervical de grau 3 (NIC 3) e carcinoma escamoso invasivo do colo uterino.

- Artigo 2: Descrever e analisar a expressão da proteína VEGF-A em células tumorais e estromais na neoplasia intraepitelial cervical de grau 3 (NIC 3) e carcinoma escamoso invasivo do colo uterino.

3. Publicações

Artigo 1 – **Expression of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 in intraepithelial and invasive cervical neoplasia**

Artigo submetido à revista PLOS ONE.

Artigo 2 – **Expression of VEGF-A in intraepithelial and invasive cervical neoplasia**

Artigo submetido à revista Journal Clinical Pathology

3.1. Artigo 1

---------- Forwarded message ----------
From: PLOS ONE <plosone@plos.org>
Date: 2013/8/9
Subject: Submission Confirmation for Expression of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 in intraepithelial and invasive cervical neoplasia
To: Silvia Helena Rabelo-Santos <rabelo.silvia@gmail.com>

Dear Dr Rabelo-Santos,

Your submission entitled "Expression of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 in intraepithelial and invasive cervical neoplasia" has been received by PLOS ONE. You will be able to check on the progress of your paper by logging on to Editorial Manager as an author. The URL is http://pone.edmgr.com/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,

PLOS ONE

Expression of MMP-2, MMP-9, MMP-14, TIMP-1, TIMP-2 in intraepithelial and invasive cervical neoplasia

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Abstract

Background: Tumor invasion involves degradation of the extracellular matrix and epithelial basement membrane by metalloproteinases (MMPs). Tissue inhibitors of metalloproteinases (TIMPs) are involved in the invasion process. The aim of this study was to evaluate the expression of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 in Cervical intraepithelial neoplasia grade 3 (CIN 3) and invasive squamous cell carcinoma. *Methods:* This study comprised three groups: Group 1: 55 cases with CIN 3; Group 2: 30 cases with CIN 3 and invasive carcinoma components; Group 3: 46 cases with invasive carcinoma. Protein expression was investigated in tumor and stromal cells by immunohistochemistry and percentages of immunostained cells were determined by morphometric software. *Results:* The mean percentage of MMP-14 tumor cells was significantly higher in stromal cells in all groups. TIMP-2 and MMP-9 expression was significantly higher in stromal cells than in tumor cells, except for MMP-9 in carcinoma (Group 2). TIMP-1 had a significantly higher expression in stromal cells of carcinoma and tumor cells of CIN 3. The differences in protein expression were more evident between Group 1 and Group 3: Stromal cells expression of MMP-2, MMP-14, TIMP-1 was increased with the severity of cervical neoplasia. The pattern of immunohistochemical expression of MMPs and TIMPs in CIN 3 and invasive carcinoma when these lesions were associated, was similar, except for the expression of MMP-2 in stromal cells that was higher in CIN 3 component of Group 2 than in CIN 3 of Group 1. The proteins assessed were similarly expressed in CIN 3 and invasive cervical cancer when both lesions were associated. *Conclusions:* The gradual increase in MMP-2 expression from CIN

3 to invasive cervical cancer reinforces their relevant role in cervical cancer progression. From CIN 3 to invasive cervical carcinoma, TIMP-1 expression increased in stromal cells and decreased in tumor cells.

Keywords: MMP, TIMP, CIN 3, uterine cervical neoplasms, neoplasm invasiveness.

Introduction

Cervical intraepithelial neoplasia grade 3 (CIN 3) is the precancerous lesion that may progress to invasive carcinoma [2].Transformation from preinvasive neoplasia to invasive carcinoma starts by a focal disruption of the subepithelial basement membrane. Potent proteolytic enzymes termed matrix proteinases (MMPs) are known to play a key role in this process. These enzymes are able to degrade a variety of substrates that constitute the basement membrane and extracellular matrix [3].

Tumor cells secrete MMPs and induce stromal cells such as fibroblasts, inflammatory cells and endothelial cells to produce MMPs [4,5]. MMP-2 and MMP-9 are proteolytic enzymes with a major role in type IV collagen digestion, an important event in vascular invasion and metastasis. MMP-14 is a key enzyme in tumor cell invasion. This enzyme seems to be implicated in both breaching the basement membrane by tumor cells and cell invasion through interstitial type-I collagen tissues [6].

Tissue inhibitors of matrix metalloproteinases (TIMPs 1 to 4) are natural inhibitors of MMPs. TIMPs are multifunctional proteins that not only inhibit MMPs but also promote cell growth, induce apoptosis, and inhibit angiogenesis [5,7]. In fact, Stetler-Stevenson [8] reported that recent evidence suggests that TIMPs, in particular TIMP-1 and TIMP-2, may have unique biological properties, independent of their ability to inhibit MMPs. This evidence is supported by the identification of cell surface receptors for these two TIMP family members.

TIMP-2 impacts the tumor microenvironment by initially promoting cellular differentiation of endothelial cells and fibroblasts, and possibly cells in the

epithelial compartment. However, with the subsequent progression massive production and activation of MMPs by a variety of cell types present in the tumor microenvironment, TIMP-2 function shifts to metalloproteinase inhibitor. The continued progression and further depletion of TIMP-2 levels could again change TIMP-2 function from MMP inhibitor to activator by facilitating MMP-14 activation of pro-MMP-2 [8].

Expression of MMP and TIMP markers has been studied mostly in tumor cells. In stromal cells, expression of these markers has been less frequently evaluated. It has been shown that cell–cell contact between cervical carcinoma cells and peripheral stromal fibroblasts augments the production and activation of MMPs. Therefore, the subsequent imbalance between MMPs and TIMPs may result in the gradually increasing invasive potential of cervical cancer cells [9].

A better understanding of the mechanisms by which CIN 3 acquires the ability to invade the cervical stroma may have a significant clinical relevance. Marker expression in CIN 3 and cervical invasive carcinoma could be a good model for analyzing the role of the proteins involved in the tumor invasiveness. Therefore, the aim of this study was to describe and analyze the expression of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 in CIN 3 and invasive carcinoma of the cervix, in tumor cells and stromal cells.

Materials and Method

Ethics statement

All tissue samples were collected for histologic examination and diagnostic purposes and were thoroughly anonymized for the use in this study. Thus no

informed consent was needed. This study was approved by the State University of Campinas ethics committee (Approval No. 858/2007 - November 11th, 2007).

Tissue Samples

This comparative observational study of women with cervical neoplasia consisted of three groups. The first group comprised 55 cases of histologic diagnosis of unique CIN 3 (Group 1), the second group comprised 30 cases with both components: CIN 3 (Group 2) and invasive carcinoma (Group 2) and the third group was composed of 46 cases with a histologic diagnosis of invasive carcinoma (Group 3). The study design was constructed assuming that pure CIN 3 (Group 1), CIN 3 associated with invasive carcinoma (Group 2) and pure cervical invasive carcinoma (Group 3) represent progressive steps from CIN 3 to invasive carcinoma.

Case selection was made from records of pathology reports obtained from women consecutively managed in the Women's Hospital, State University of Campinas, Brazil, between 2002 and 2008. Hematoxilin-eosin paraffin-embedded sections were reviewed and the best representative sample was identified considering tumor tissue and the underlying stroma. Specimens were obtained by punch biopsies, conizations or hysterectomies. The number of cases varied in each analysis, as can be seen in the presentation of results. In some cases, lesions were not represented for all markers, due to serial section of the tissue block. Despite some limitations of immunohistochemistry analysis of the stroma cells, this tissue compartment was analyzed because there is

evidence that the stroma has a relevant role in cervical cancer progression [9,18].

Immunohistochemistry assay

Expressions of MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 were investigated in paraffin-embedded sections using the avidinbiotin-peroxidase complex method. The paraffin sections (5 µm thick) were deparaffinized and immersed in 3% hydrogen peroxidase in methanol to block endogenous peroxidase activity. Next, an gen retrieval procedure was performed by immersing the slides in 10 mM citrate buffer (pH 6.0) and autoclaving at 121˚C for 10 min. After washing in PBS, the tissue sections were preblocked using 10% normal goat serum for 15 min. The protocol for the Dako LSAB 2 peroxidase kit (Dako, Kyoto, Japan) was followed. The sections were incubated overnight with primary bodies in a humidity chamber at 4˚C.

For this study, the primary bodies used were polyclonal mouse human MMP-2, MMP-9, MMP-14, TIMP-1 and TIMP-2 (Nemarkers). The working dilutions for each primary body were: MMP-2 (1:100), MMP-9 (1:200), MMP-14 (1:100), TIMP-1 (1:100) and TIMP-2 (1:100). Sections were rinsed with PBS for 15 min and incubated for 1h with the secondary body (biotinylated goat -mouse and rabbit immunoglobulin G secondary body; Dako). The sections were then incubated with streptavidin-peroxidase complex using 3,3'- diaminobenzidine as a chromogen. The sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reactions was checked by omitting the primary body.

Immunohistochemistry assay and image acquisition

The methodology for interpretation of the immunostaining sections consisted in the following steps:

1. Identification of representative regions of the lesions selected, according to group (CIN 3, invasive carcinoma and underlying stromal lesion); selection criteria for these representative areas were based on: image sharpness, areas of higher intensity of cellular immunoreaction (but any intensity of immunostaining was considered positive), a similar proportion of stromal and tumor region in the same picture, excluding necrotic regions. Stromal regions with a high concentration of inflammatory cells were excluded.
2. Images of these regions were captured under a magnification of 400x. In groups of CIN 3 and invasive carcinoma, one to eight pictures were taken. In Group 2, which included cases of invasive cell carcinoma associated with CIN 3, pictures were taken separately.
3. Selection of one photograph per lesion and its underlying stroma was made for qutative and qualitative analysis of the immunohistochemical reaction.
4. Calculation of total cell number and number of immunostained cells (stromal and tumor cells separately) was made by two observers using morphometric software (Image Pro Plus®, version 6.3, Olympus). To determine the percentage of immunostained cells, at least 1,000 tumor cells and stromal cells per case were counted (Figure 1, Figure 2 and Figure 3).

Statistical Analysis

Statistical analysis was carried out, considering the mean percentage of immunopositive tumor cells and stromal cells within each group and between diagnostic groups. The CIN 3 and invasive carcinoma components of Group 2 were analyzed separately. The T-paired test was used to compare the mean percentage of tumor cells and stromal cells within each diagnostic group. The reason for this was because although the percentage distribution of immunopositive tumor cells and stromal cells was not normal, the differences between them were normally distributed. The percentages of tumor and stromal cells between two diagnostic groups were analyzed by the Mann-Whitney and Wilcoxon Signed Rank tests. Differences were considered significant when p value was less than 0.05.

Results

In all cases analyzed, an immunohistochemical reaction for MMP-14, MMP-2 and TIMP-1 was observed in tumor cells and stromal cells. These proteins were markedly expressed in most cases. In some cases, none of the cells expressed MMP-9 and TIMP-2 immunohistochemical reaction or expression of MMP-9 and TIMP-2 immunohistochemical reaction was an infrequent finding.

MMP-14

In all groups, the mean percentage of cells expressing MMP-14 was significantly higher in tumor than in stromal cells. For tumor cells, the mean percentage ranged from 80.2% (Group 2 - invasive carcinoma) to 89.1% (Group

2 – CIN 3); for stromal cells, the mean percentage ranged from 51.9% (Group 1 - CIN 3) to 66.1% (Group 3 – invasive carcinoma). Intergroup analysis of tumor cells showed that MMP-14 expression did not vary. Nevertheless, the percentage of stromal cells positive for MMP-14 was significantly higher (p=0.0212) in invasive carcinoma (Group 3) (median=69.8%) than in CIN 3 (Group 1) (median=57.2%) (Table 1 – 4 and Figure 4).

MMP-2

In all diagnostic groups, there was no statistically significant difference in the mean percentage of cells expressing MMP-2 in both tumor and stromal cells. The percentages of both tumor and stromal cells immunopositive for MMP-2 were significantly higher (p=0.0066 and p=0.0014, respectively) in invasive carcinoma (Group 3) compared to CIN 3 (Group 1). The percentage of stromal cells immunopositive for MMP-2 was significantly higher (p=0.0141) in the CIN 3 component (Group 2) than in CIN 3 (Group 1) (Table 1-4 and Figure 4).

MMP-9

The mean percentage of cells expressing MMP-9 was significantly higher in stromal than in tumor cells in the three groups, i.e.: CIN 3 (Group 1), CIN 3 component (Group 2) and invasive carcinoma (Group 3). For stromal cells, the mean percentage was 58.8% (p=0.0295) in CIN 3 (Group 1), 65.9% (p=0.0357) in the CIN 3 component (Group 2), and 53.2% (p=0.0009) in invasive carcinoma (Group 3). For tumor cells, the mean percentage was 49.9%, 52.3% and 42.0%,

respectively. When tumor and stromal cells were analyzed, MMP-9 expression did not vary between groups (Table 1-4 and Figure 4).

TIMP-1

In groups at opposite ends (of the range of severity), the mean percentage of cells expressing TIMP-1 was statistically higher in tumor cells (mean=83.8%; p=0.0002) from CIN 3 (Group 1). Inversely, it was statistically higher in stromal cells (mean=83.7%; p=0.0076) from invasive carcinoma (Group 3).. There was a significantly higher percentage of tumor cells expressing TIMP-1 (p=0.0119) than stromal cells, when CIN 3 (Group 1) was compared to invasive carcinoma (Group 3). Inversely, there was a significantly higher percentage of stromal cells expressing TIMP-1 (p=0.0082) than tumor cells, when invasive carcinoma (Group 3) was compared to CIN 3 (Group 1) (Table 1-4 and Figure 4).

TIMP-2

In all groups, the mean percentage of cells expressing TIMP-2 was significantly higher in stromal than in tumor cells. In stromal cells, the mean percentage ranged from 59.0% (p<0.0001) (CIN 3- Group 1) to 65.1% (p<0.0001) (invasive carcinoma - Group 3). In tumor cells, the mean percentage ranged from 37.7% (invasive carcinoma - Group 3) to 51.1% (CIN 3 component - Group 2).. However, TIMP-2 expression in tumor or stromal cells showed no statistical difference between the diagnostic groups examined () (Table 1-4 and Figure 4).

Discussion

The analyzed markers were similarly or more highly expressed in stromal cells than in tumor cells, except for MMP-14 expression. TIMP-2 was more highly expressed in stromal cells in all groups. MMP-9 also showed a higher expression in stromal cells, despite lack of statistical demonstration in invasive carcinoma of Group 2. In all groups, MMP-2 expression was similar in tumor and stromal cells. All diagnostic groups showed a higher MMP-14 expression in tumor cells. The marker TIMP-1 was the unique that changed the distribution between tumor and stromal cells, since TIMP-1 expression was higher in tumor cells of CIN 3, and higher in stromal cells of invasive carcinoma.

Similarly to this study, Nair et al. [10] observed that MMP-2, MMP-9 and TIMPs showed intense immunostaining in both stromal and tumor cells of invasive carcinoma in virtually all cases. Neoplastic changes occur in epithelial cells, but there is also a significant change in MMP and TIMP expression in stromal cells, reinforcing that stromal cells play an important role in the carcinogenic process. Under normal physiological conditions, the stroma acts as an important barrier to epithelial cell transformation. Nevertheless, the stromal compartment undergoes changes in response to emerging epithelial lesions. This compartment has a key role in cancer initiation and progression, including the recruitment of new stromal cells that provide factors involved in cell growth and matrix remodeling [11, 12].

Although this study showed that MMP-2, MMP-14 and TIMP-1 were highly expressed in all groups, major differences in marker expression were found between CIN 3 (Group 1) and invasive carcinoma (Group 3). MMP-2

expression was higher in tumor and stromal cells of invasive carcinoma than in these cells of CIN 3. This marker also showed higher expression in stromal cells of CIN 3 of Group 2 than in stromal cells of CIN 3 of Group 1. MMP-14 was more expressed in stromal cells of invasive carcinoma than in stromal cells of CIN 3. There was a different trend in TIMP-1 expression from CIN 3 to invasive carcinoma in tumor and stromal cells. TIMP-1 expression was higher in tumor cells of CIN 3 and, inversely, was higher in stromal cells of invasive carcinoma. There was a similar expression of MMP-9 and TIMP-2 in invasive carcinoma and CIN 3.

The immunohistochemical pattern of expression of MMPs and TIMPs in CIN 3 and invasive carcinoma, as these lesions were associated, was similar. Considering invasive carcinoma and invasive carcinoma associated with CIN 3, none of the markers showed any difference in their expression, in tumor cells and stromal cells.

Although MMP-2 and MMP-9 are almost identical proteinases, their contribution to biological or pathological processes can be very different. At present, it is still unclear which of these enzymes is more important in tumor progression and metastasis [13]. Evidence shows that MMP-2 is especially important in ECM degradation, cancer cell invasion and metastasis. Therefore, MMP-2 is a determinant of cancer cell behavior [10,13,14]. This study demonstrated a higher MMP-2 expression in tumor and stromal cells of invasive cervical carcinoma than in the corresponding cells of CIN 3. In addition, MMP-2 was statistically more expressed in CIN 3 stromal cells of Group 2. However, no difference was demonstrated when the expression of this protein in tumor and

stromal cells within the same group was analyzed. In contrast, no difference in MMP-9 expression was demonstrated between CIN 3 and invasive carcinoma, however, this marker showed a different pattern of expression, which was higher in stromal than in tumor cells in almost all groups. These findings suggest that MMP-2 could have a role in the neoplastic progression of the lesion and MMP-9 would act through an interaction between tumor and stromal cells.

In agreement with results of this study, Nair et al. [10] also found a progressive increase in MMP-2 positivity related to the severity of cervical neoplasia. Fernandes et al. [15] also showed that MMP-2 expression was higher in stromal cells of invasive carcinomas than of CIN 3 and indicated that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN 3 to advanced invasive tumor. Brummer et al. [16] suggested that MMP-2 expression, when focally observed in high-grade intraepithelial lesions of the cervix, may indicate tumor regions with an increased risk for invasive growth.

MMP-14 may have a very important role in tumor cell progression. Cell invasion is a multistep process, involving degradation of the extracellular matrix (ECM) and cell mobility. While ECM degradation can be orchestrated collectively by MMPs, cell migration is likely to be predominantly associated with MMP-14 [17]. This understanding is considered concordant with findings in this study which showed that MMP-14 expression was higher in stromal cells in invasive carcinoma than in stromal cells of CIN 3.

Recent studies have demonstrated that TIMP-1 possesses additional functions, including enhancement of malignant transformation, stimulation of cell

growth and inhibition of apoptosis, as well as promotion of migration, invasion and angiogenesis. This indicates a potential tumor-promoting role of TIMP-1 in the early stages of tumorigenesis [18]. Elevated TIMP-levels have been reported in association with cancer progression and were identified as poor prognostic indicators in several human types of tumor. This study showed a higher TIMP-1 expression in CIN 3 tumor cells, where malignant transformation, stimulation of cell growth and inhibition of apoptosis occur. In invasive carcinoma, this study showed a higher TIMP-1 expression in stromal cells, where the promotion of migration, invasion and angiogenesis take place. While MMPs are important in the late stage of tumor progression leading to metastasis, the -apoptotic effects of some TIMPs may favor tumor growth during tumor onset and early primary tumor growth [19].

The power of this study was the design that analyzed three steps of cervical cancer progression ranging from CIN 3 to invasive carcinoma. However, findings would be more conclusive, if normal cervical tissue had been included as control group. Limitations of the study were subjectivity and variability in immunostaining evaluation.

Conclusions

The progression of CIN 3 to invasive carcinoma seems be determined by complex interactions between tumor and stromal cells. These findings reinforce the evidence of the role of stromal cells in tumor invasiveness. Thus, the main findings from this study were:

– Immunohistochemistry features for all study MMPs and TIMPs were similar between CIN 3 and invasive cervical cancer when both lesions were associated.
– The gradual increase in MMP-2 expression from CIN 3 to invasive carcinoma in tumor cells and especially in stromal cells reinforces its major role in cervical cancer progression.
– MMP-14 was highly expressed in tumor cells in CIN 3 and invasive carcinoma; in stromal cells, its expression was higher in invasive cervical carcinoma than in CIN 3.
– TIMP-1 showed a higher expression in stromal cells of invasive carcinoma and CIN 3 tumor cells, suggesting that TIMP-1 expression increases in stromal cells and decreases in tumor cells from CIN 3 to cervical carcinoma.
– Although MMP-9 and TIMP-2 may have role in the progression of cervical neoplasia, their expression showed no variation between CIN 3 and invasive cervical carcinoma, neither in stromal nor in tumor cells. Nevertheless, MMP-9 and TIMP-2 were more highly expressed in stromal cells than in tumor cells, suggesting that stromal cells may play a relevant role in malignant transformation and/or tumor invasion.

Competing interests: The authors declare no competing interests.

Author contributions

MCAW participated in the construction of study design, case selection, was responsible for the evaluation of immunohistochemistry assay, calculated the

percentage of immunostained cells, was data manager and wrote the manuscript. SHRS participated in the construction of study design, helped in study coordination, was responsible for the calculation of total cell number and number of immunostained cells and helped in manuscript drafting and revising. LALAL was responsible for histopathological reports and revising the manuscript. SFMD participated in the construction of study design and revised the manuscript. GAP oversaw all laboratory aspects of the study and revised the manuscript. SSM was responsible for statistical analysis and helped draft the manuscript. PLF was responsible for immunohistochemistry assay and participated in manuscript drafting. LCZ is the project manager and principal investigator of the study, conceptualized the study and was involved in study design, study coordination and revising the manuscript. All authors read and approved this manuscript.

References

1. The International Agency for Research on Cancer (IARC). Globocan 2008. Estimated cancer Incidence, Mortality, Prevalence and Disability-adjusted life years (DALYs) Worldwide in 2008 – [http://globocan.iarc.fr/]
2. Snijders PJF, Steenbergen RDM, Heideman DAM, Meijer CJLM. (2006) HPV-Mediated Cervical Carcinogenesis: Concepts And Clinical Implications. J Pathol 208:152-164.
3. Wu YC, Wang PH, Tsai A, Yang SF, Chen SC. (2011) Semi-Qutative Expression of Tissue Inhibitor of Matrix Metalloproteinase-2 in Cancer of Uterine Cervix. J Surg Oncol 104:210–215.
4. Chung AS, Yoon SO, Park SJ, Yun CH. (2003) Roles of matrix metalloproteinases in tumor metastasis and angiogenesis. J Biochem Mol Biol 36(1):128-137.
5. Boyd S, Tolvanen K, Virolainen S, Kuivanen T, Kyllönen L, et al. (2008) Differential expression of stromal MMP-1, MMP-9 and TIMP-1 in basal cell carcinomas of immunosuppressed patients and controls. Virchows Arch 452:83–90.
6. Poincloux R, Lizarraga F, Chavrier P. (2009) Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. J Cell Sci 122:3015–3024.
7. Brew K, Dinakarpandian D, Nagase H. (2000) Tissue inhibitors of metalloproteinases: Evolution, structure and function. Biochim Biophys Acta 1477:267–283.

8. Stetler-Stevenson WG. (2008) The tumor microenvironment: regulation by MMP - independent effects of tissue inhibitor of metalloproteinases-2. Cancer Metastasis Rev 27(1):57–66.
9. Sato T, Sakai T, Noguchi Y, Takita M, Hirakawa S, Ito A. (2004) Tumor–stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. Gynecol Oncol 92:47–56.
10. Nair AS, Karunagaran D, Nair MB, Sudhakaran PR. (2003) Changes in matrix metalloproteinases and their endogenous inhibitors during tumor progression in the uterine cervix. J Cancer Res Clin Oncol 129:123–131.
11. Tlsty TD, Coussens LM. (2006) Tumor Stroma and Regulation of Cancer Development. Annu Rev Pathol Mech Dis 1:119–150.
12. Del Casar JM, González LO, Alvarez E, Junquera S, Marín L, González L, et al. (2009) Comparative analysis and clinical value of the expression of metalloproteinases and their inhibitors by intratumor stromal fibroblasts and those at the invasive front of breast carcinomas. Breast Cancer Res Treat 116:39–52.
13. Zhang X, Wang Y, Yamamoto G, Tachikawa T. (2009) Expression of matrix metalloproteinases MMP-2, MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 in the epithelium and stroma of salivary gland pleomorphic adenomas. Histopathology 55:250–260.
14. Xu X, Wang Y, Chen Z, Sternlicht MD, Hidalgo M, Steffensen B. (2005) Matrix metalloproteinase-2 contributes to cancer cell migration on collagen. Cancer Res 65(1):130-136.

15. Fernandes T, de Angelo-Andrade LA, Morais SS, Pinto GA, Chagas CA, et al. (2008) Stromal cells play a role in cervical cancer progression mediated by MMP-2 protein. Eur J Gynaecol Oncol 29(4):341-344.
16. Brummer O, Böhmer G, Hollwitz B, Flemming P, Petry KU, et al. (2002) MMP-1 and MMP-2 in the cervix uteri in different steps of malignant transformation – an immunohistochemical study. Gynecol Oncol 84(2):222-227.
17. Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, et al. (2012) Human matrix metalloproteinases: An ubiquitarian class of enzymes involved in several pathological processes. Mol Aspects Med 33(2):119-208.
18. Jensen SA, Vainer B, Bartels A, Brünner N, Sorensen JB. (2010)Expression of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1) by colorectal cancer cells and adjacent stroma cells – Associations with histopathology and patients outcome. European J Cancer 46:3233–3242.
19. Jiang Y, Goldberg ID, Shi YE. (2002) Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene 21:2245-2252.

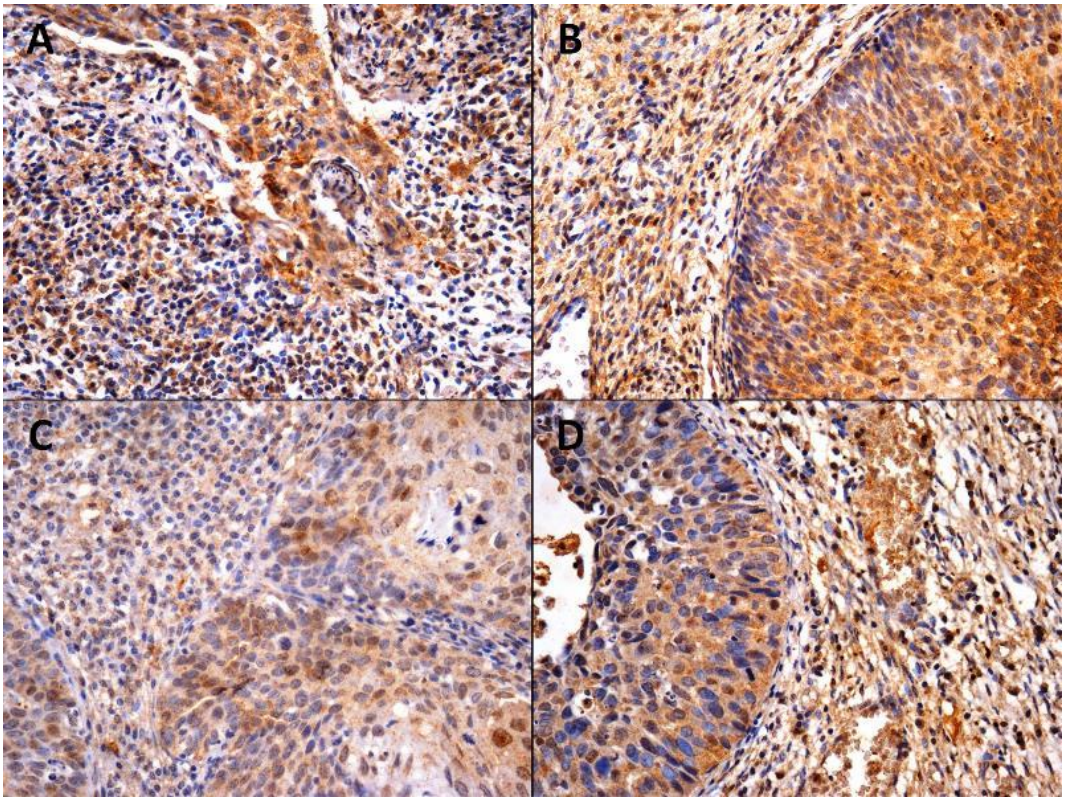

Figure 1 Percentage of tumor and stromal cells expressing MMP-2. A: Invasive carcinoma (Group 3): Stromal cells 97.5%, Tumor Cells 97.8%; **B:** CIN 3 (Group 1): Stromal cells 89.6%, Tumor Cells100%; **C:** Invasive carcinoma compartment (Group 2): Stromal cells-88.2%, Tumor Cells-82.4%; **D:** CIN 3 compartment (Group 2): Stromal cells-83.8%, Tumor Cells-84.5%.
MMP-Metalloproteinases, Cervical Intraepithelial Neoplasia.

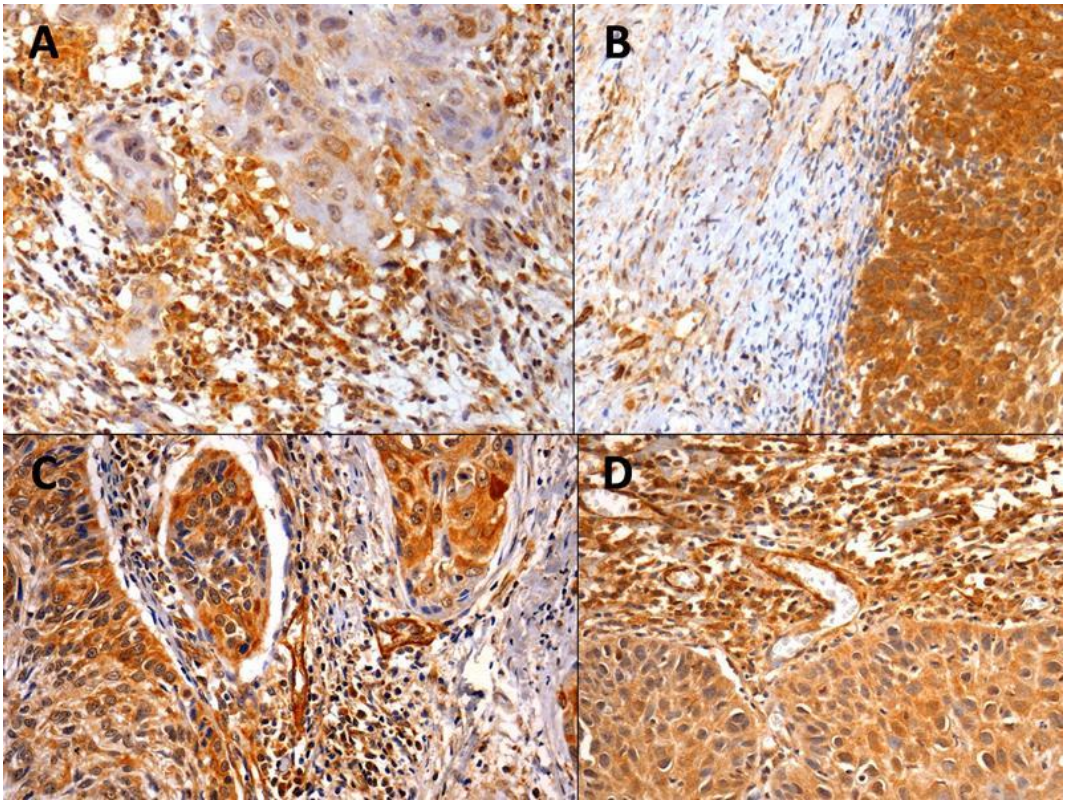

Figure 2 Percentage of tumor and stromal cells expressing TIMP- 1. A: Invasive carcinoma (Group 3): Stromal cells 89.6%, Tumor Cells 56,2%; **B:** CIN 3 (Group 1): Stromal cells 98.7%, Tumor Cells-19.8%; **C:** Invasive carcinoma compartment (Group 2): Stromal cells-87.2%, Tumor Cells-98.2%; **D:** CIN 3 compartment (Group 2): Stromal cells-88.8%, Tumor Cells-82.0%.

TIMP-Tissue inhibitors of metalloproteinases, Cervical Intraepithelial Neoplasia.

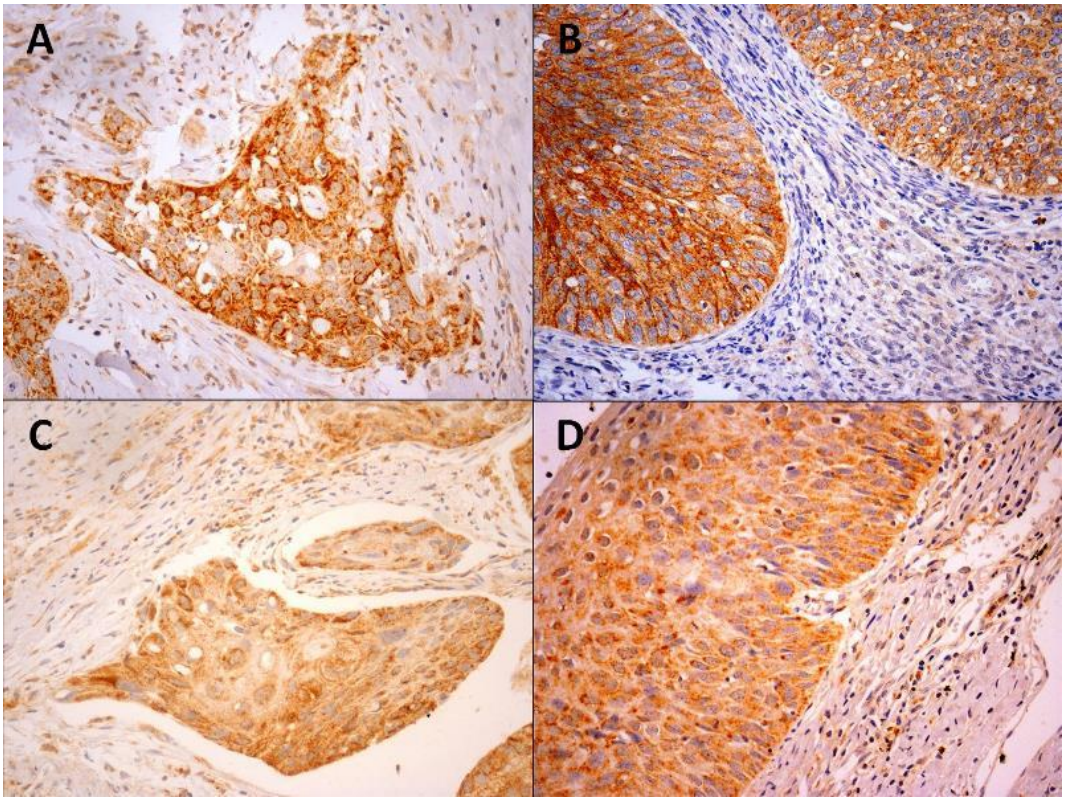

Figure 3 Percentage of tumor and stromal cells expressing MMP-14. A: Invasive carcinoma (Group 3): Stromal cells 67%, Tumor Cells 100%; B: CIN 3 (Group 1): Stromal cells 21.3%, Tumor Cells-100%; C: Invasive carcinoma compartment (Group 2): Stromal cells-62.2%, Tumor Cells-100%; D: CIN 3 compartment (Group 2): Stromal cells-49.9%, Tumor Cells-100%.

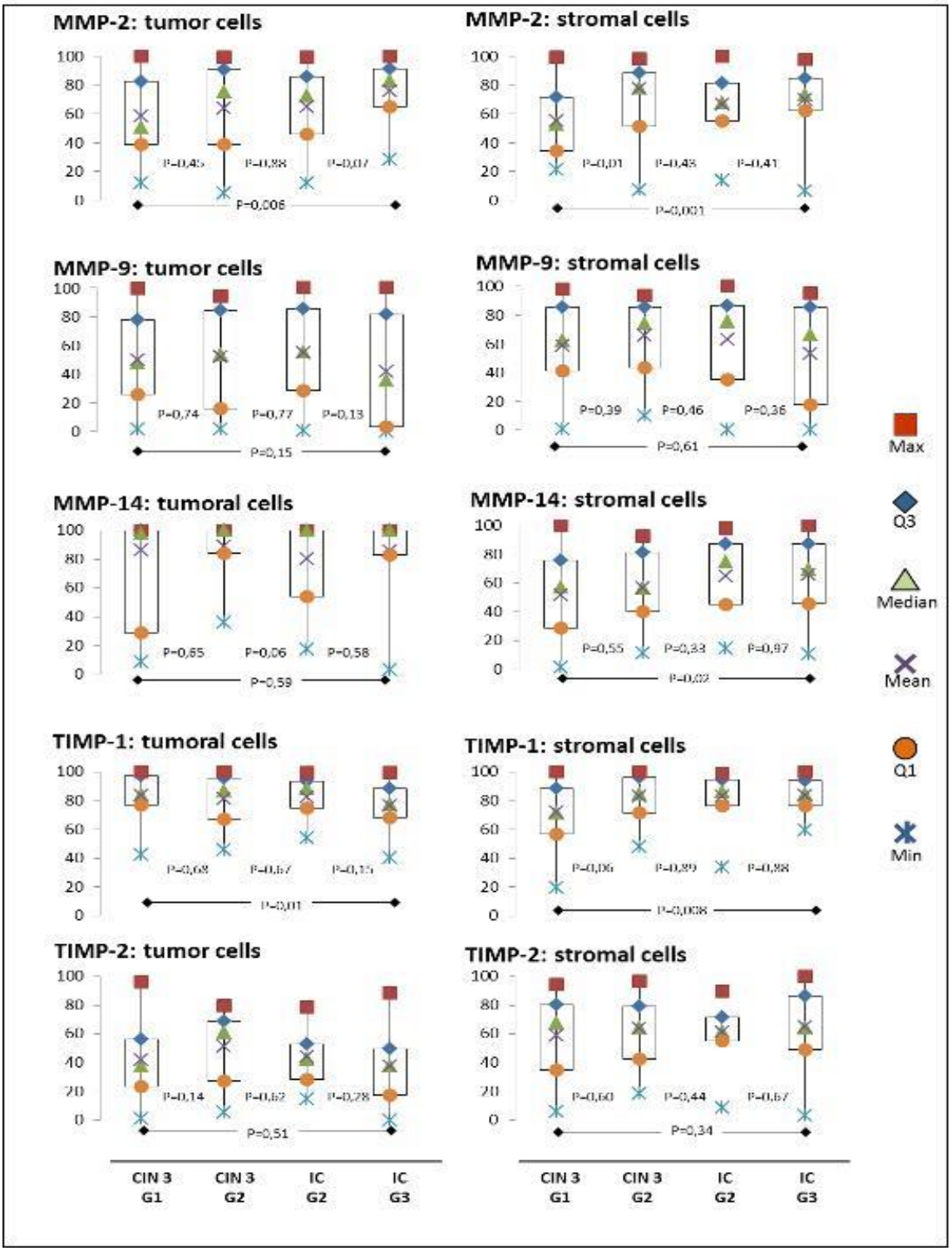

Figure 4 MMP-2, MMP 9, MMP 14, TIM-1 and TIMP 2 expressions in Cervical Neoplasia

MMP- Metalloproteinases, TIMP- Tissue inhibitors of metalloproteinases, CIN – Cervical Intraepithelial Neoplasia, IC – Invasive carcinoma, Group 1-G1, Group-G2, Group 3-G3, Min-Minimum, Max-Maximum, Q1- 25th percentile, Q3-75th percentile.

Higher percentages of stromal and tumoral cells expressing MMP 2 were observed in invasive carcinoma (Group 3). Higher percentages of stromal cells expressing MMP-2 were detected in CIN 3 of Group 2 when compared with CIN 3 of Group 1. There was a significantly higher percentage of tumor cells expressing TIMP-1 than stromal cells, when CIN 3 of Group 1 was compared to invasive carcinoma of Group 3. There was a significantly higher percentage of stromal cells expressing TIMP-1 than tumor cells, when invasive carcinoma of Group 3 was compared to CIN 3 of Group 1.

Table 1. Comparison of mean percentages of tumor and stromal cells immunopositive in CIN 3 (Group 1)

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	51	58.42	26.42	11.66	38.82	50.64	82.33	100.0	
	Stromal	51	55.32	21.48	21.44	34.31	52.78	71.31	99.15	0.3245
MMP9	Tumor	55	49.87	29.85	1.62	26.14	47.71	77.65	99.65	
	Stromal	55	58.78	29.58	0.62	41.0	62.49	85.15	97.98	0.0295
MMP14	Tumor	51	86.25	20.41	8.43	78.91	97.84	100.0	100.0	
	Stromal	51	51.89	28.53	1.25	28.8	57.17	75.68	99.79	< 0.0001
TIMP1	Tumor	53	83.75	14.81	42.49	77.1	84.29	96.98	100.0	
	Stromal	53	72.31	20.3	19.77	56.64	71.67	88.46	100.0	0.0002
TIMP2	Tumor	52	42.08	25.73	0.77	23.29	37.53	56.22	96.39	
	Stromal	52	59.04	25.58	5.72	35.0	67.77	80.17	94.34	< 0.0001

Comparisons between means were performed using the paired t-test

Table 2. Comparison of mean percentages of tumor and stromal cells immunopositive in CIN 3 (Group 2)

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	30	63.81	30.13	4.9	38.5	75.46	90.48	99.36	
	Stromal	30	67.8	26.15	6.9	51.54	77.86	88.3	98.2	0.5244
MMP9	Tumor	26	52.28	32.32	1.73	15.87	54.03	84.57	94.29	
	Stromal	26	65.94	24.13	9.77	43.35	74.17	85.23	93.56	0.0357
MMP14	Tumor	27	89.13	17.48	35.79	84.17	100.0	100.0	100.0	
	Stromal	27	56.46	24.95	11.39	40.29	56.93	81.46	92.67	< 0.0001
TIMP1	Tumor	18	81.58	16.73	45.74	67.3	86.73	95.78	99.79	
	Stromal	18	82.81	15.62	48.1	71.84	84.82	96.37	100.0	0.7674
TIMP2	Tumor	19	51.11	23.96	5.19	27.27	60.97	68.59	79.48	
	Stromal	19	63.91	22.04	18.39	42.73	64.24	79.18	96.46	0.0123

Comparisons between means were performed using the paired t-test

Table 3. Comparison of mean percentages of tumor and stromal cells immunopositive in Invasive Carcinoma (Group 2)

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	30	64.85	26.87	12.1	45.63	72.71	85.99	99.27	
	Stromal	30	66.54	20.39	13.57	55.08	67.81	81.35	100.0	0.7785
MMP9	Tumor	26	54.68	31.59	0.71	28.61	55.49	85.62	100.0	
	Stromal	26	63.19	28.87	0.17	35.37	75.36	86.33	100.0	0.2659
MMP14	Tumor	27	80.18	26.26	17.21	54.27	100.0	100.0	100.0	
	Stromal	27	64.96	27.93	14.58	45.3	74.57	87.32	98.14	0.0224
TIMP1	Tumor	18	82.3	15.12	54.07	74.85	88.41	93.32	99.51	
	Stromal	18	82.76	16.03	33.94	76.74	86.15	94.26	98.96	0.9072
TIMP2	Tumor	19	44.3	19.65	14.69	28.59	42.47	53.01	78.76	
	Stromal	19	61.15	16.9	8.43	55.24	61.9	71.33	89.4	0.0174

Comparisons between means were performed using the paired t-test

Table 4. Comparison of mean percentages of tumor and stromal cells immunopositive in Invasive Carcinoma (Group 3)

Markers	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
MMP2	Tumor	37	76.05	20.14	28.58	64.81	82.99	91.39	99.96	
	Stromal	37	69.99	18.87	6.51	62.21	72.99	84.9	97.81	0.1953
MMP9	Tumor	46	42.01	38.56	0.07	3.45	35.74	81.42	100.0	
	Stromal	46	53.2	35.51	0.07	17.89	66.23	85.74	95.41	0.0009
MMP14	Tumor	35	85.75	25.15	3.39	83.23	100.0	100.0	100.0	
	Stromal	35	66.06	23.79	10.9	45.64	69.83	87.49	100.0	< 0.0001
TIMP1	Tumor	46	76.53	15.08	40.26	68.03	77.66	88.45	99.65	
	Stromal	46	83.72	11.64	59.55	76.37	84.52	93.81	100.0	0.0076
TIMP2	Tumor	38	37.69	24.58	0.0	17.42	37.44	49.46	88.57	
	Stromal	38	65.11	21.73	3.13	49.08	64.21	86.38	100.0	< 0.0001

Comparisons between means were performed using the paired t-test

3.2. Artigo 2

27-Aug-2013

Dear Prof. Rabelo-Santos:

Your manuscript entitled "Expression of VEGF-A in intraepithelial and invasive cervical neoplasia" has been successfully submitted online and is presently being given full consideration for publication in the Journal of Clinical Pathology.

Your manuscript ID is jclinpath-2013-201937.

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Thank you for submitting your manuscript to the Journal of Clinical Pathology.

Respectfully,

Editor, Journal of Clinical Pathology

Profª Drª Silvia Helena Rabelo dos Santos
Faculdade de Farmácia-UFG

Expression of VEGF-A in intraepithelial and invasive cervical neoplasia

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ABSTRACT

Background and Aims: In cervical carcinogenesis, angiogenesis seems to be an early event that is associated with disease progression in cervical intraepithelial neoplasia (CIN). The aim of this study was to describe and analyze VEGF-A expression in CIN 3 and invasive cervical carcinoma, in tumor cells and stromal cells. **Methods:** This comparative observational study comprised three groups: Group 1: 55 cases with CIN 3; Group 2: 30 cases with both CIN 3 and invasive carcinoma components; Group 3: 46 cases with invasive carcinoma. The study design was based on the assumption that Group 1, Group 2 and Group 3 represent progressive steps from CIN 3 to invasive carcinoma. Protein expression was investigated separately in tumor and stromal cells by immunohistochemistry and percentages of immunostained cells were determined by morphometric software. Statistical analysis was carried out, considering the mean percentage of immunopositive cells. **Results**: VEGF-A expression was higher in stromal cells in cases of invasive carcinoma (Group 3) than in CIN 3 cases (Group 1) and this difference was statistically significant. VEGF-A expression showed a borderline association between stromal cells in invasive carcinoma (Group 3) and invasive carcinoma associated with CIN 3 (Group 2). The mean percentage of cells expressing VEGF-A was significantly higher in tumor cells in CIN 3 than in stromal cells in CIN 3 (Group 1). **Conclusions:** This study corroborates findings that stromal cells play an important role in tumor invasion and progression, mediated by a gradual increase in VEGF-A expression from CIN 3 to advanced invasive tumor.

INTRODUCTION

Cervical cancer is the third most common cancer in women worldwide and more than 85% of the global burden occurs in developing countries [1]. Cervical cancer develops from a precancerous lesion referred to as cervical intraepithelial neoplasia (CIN). [2] Invasion of cervical squamous cell carcinoma begins at the basal cell layer in a field of high-grade cervical intraepithelial neoplasia (CIN 3) [3].

Angiogenesis is the formation of neovasculature from preexisting blood vessels. It is crucial for normal body development and growth. Tumor angiogenesis has been extensively investigated in many human neoplastic conditions. Vascular endothelial growth factor (VEGF) is a potent mitogen responsible for the induction of angiogenesis [4, 5].

The vascular endothelial growth factor (VEGF) family is comprised of highly conserved secreted glycoproteins that regulate vasculogenesis, hematopoiesis, angiogenesis, lymphangiogenesis and vascular permeability. These glycoproteins are implicated in many physiologic and pathologic processes [6, 7]. Six VEGF members have been currently identified and the most important member is VEGF-A (termed VEGF). Other family members are VEGF-B, VEGF-C and VEGF-D. A number of VEGF-related proteins encoded by viruses (VEGF-E) and in the venom of some snakes (VEGF-F) have also been discovered. While VEGF-A and VEGF-B are mainly involved in hemangiogenesis, VEGF-C and its close homolog VEGF-D primarily take part in lymphatic vessel growth.

Histologic analysis of premalignant, noninvasive lesions, arising in a variety of organs, has revealed the early tripping of the “angiogenic switch”[4, 7]. In several types of carcinoma, including cervical cancer, a high VEGF expression has been associated with disease progression, leading to poor patient outcome [8-12]. The term “angiogenic switch” refers to a time-restricted event during tumor progression where the balance between proangiogenic and angiogenic factors tilts towards a proangiogenic outcome, resulting in the transition from dormant avascularized hyperplasia to outgrowing vascularized tumor and eventually to malignant tumor progression [8]. It has been indicated that a switch from a lymphangiogenic phenotype towards a hemangiogenic phenotype occurs with invasion, and disease progression in cervical neoplasia. The key molecule involved in this process is VEGF-A, which acts mainly as a potent mitogen on blood endothelial cells via the receptor tyrosine kinase VEGFR-2 [9].

Some studies have reported VEGF expression in premalignant lesions of the uterine cervix [10, 11, 12]. In fact, based on clinical and experimental evidence, it was demonstrated that angiogenesis precedes overt tumor formation during chemically induced carcinogenesis [4, 13].

In summary, angiogenesis in cervical carcinogenesis seems to be an early event that occurs in premalignant changes of the cervix. Furthermore, it is associated with disease progression and may serve as a prognostic indicator of cervical squamous cell carcinoma. A better understanding of the angiogenic mechanism by which CIN 3 acquires the ability to invade the cervical stroma provides opportunities, e.g. interference with initial tumor development by blocking the “angiogenic switch” which precedes the progression to invasive

cancer. Therefore, the aim of this study was to describe and analyze the expression of VEGF-A in CIN 3 and invasive carcinoma of the cervix, in tumor cells and stromal cells.

MATERIALS AND METHODS

Patients and samples: Case selection

This comparative observational study of women with cervical neoplasia consisted of three groups. The first group comprised 55 cases of histologic diagnosis of unique CIN 3 (Group 1), the second group comprised 30 cases with both components: CIN 3 (Group 2) and invasive carcinoma (Group 2) and the third group was composed of 46 cases with a histologic diagnosis of invasive carcinoma (Group 3). The study design was constructed assuming that pure CIN 3 (Group 1), CIN 3 associated with invasive carcinoma (Group 2) and pure cervical invasive carcinoma (Group 3) represent progressive steps from CIN 3 to invasive carcinoma.

Cases were selected from records of pathology reports obtained from women consecutively managed in the Women's Hospital, State University of Campinas, Brazil, between 2002 and 2008. Hematoxilin-eosin paraffin-embedded sections were reviewed and the best representative sample was identified considering tumor tissue and underlying stroma. Specimens were obtained by cervical punch biopsies, conizations or hysterectomies. Despite some limitations of immunohistochemistry analysis of stromal cells, this tissue compartment was analyzed because evidence shows that the stroma plays a relevant role in cervical cancer progression.

Imunohistochemistry assay

VEGF-A expression was investigated in paraffin-embedded sections using the avidin-biotinperoxidase complex method. Paraffin sections (5 µm thick) were deparaffinized and immersed in 3% hydrogen peroxidase in methanol to block endogenous peroxidase activity. Next, an gen retrieval procedure was performed by immersing the slides in 10 mM citrate buffer (pH 6.0) and autoclaving these slides at 121˚C for 10 min. After washing in PBS, the tissue sections were preblocked using 10% normal goat serum for 15 min. The protocol for the Dako LSAB 2 peroxidase kit (Dako, Kyoto, Japan) was followed. The sections were incubated overnight with primary bodies in a humidity chamber at 4˚C.

Primary bodies used for this study were polyclonal mouse human VEGF (DaKo). The working dilutions of primary bodies against VEGF-A were 1:100. Sections were rinsed with PBS for 15 min and incubated for 1 h with the secondary body (biotinylated goat -mouse and rabbit immunoglobulin G secondary body; Dako). The sections were then incubated with streptavidin-peroxidase complex, using 3,3'-diaminobenzidine as a chromogen. Sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reactions was checked by omitting the primary body.

The methodology for interpretation of immunostaining sections consisted in the following steps:

1. Identification of representative regions of lesions selected according to group (CIN 3, invasive carcinoma and underlying stromal lesion). Selection criteria for these representative regions were: image sharpness,

areas of higher intensity of cellular immunoreaction, a similar proportion of stromal and tumor regions in the same picture, with the exclusion of necrotic regions. Stromal regions with a high concentration of inflammatory cells were excluded.

2. Images were captured in these regions under a magnification of 400 x. In groups of CIN 3 and invasive carcinoma, from one to eight pictures were taken. In Group 2 which included cases of invasive cell carcinoma associated with CIN 3, pictures were taken separately.
3. Selection of one photograph per lesion and its underlying stroma was made for qutative and qualitative analysis of the immunohistochemical reaction.
4. Calculation of total cell number and number of immunostained cells (stromal cells and tumor cells, separately) was made by two observers using morphometric software (Image Pro Plus®, version 6.3, Olympus) (Figure 1). To assess the percentage of immunostained cells, at least 1,000 tumor cells and stromal cells per case were counted.

Statistical Analysis

Statistical analysis was carried out, considering the percentages of immunopositive tumor cells and stromal cells within each group and between diagnostic groups. The CIN 3 and invasive carcinoma components from group 2 were analyzed separately. Analyses of the percentages of tumor cells and stromal cells between two diagnostic groups were carried out using the Mann-Whitney and Wilcoxon Signed Rank tests. A comparison of the mean

percentage of tumor cells and stromal cells within each diagnostic group was made by using the paired t-test. The reason for this was because although the percentages of immunopositive tumor cells and stromal cells did not show a normal distribution, the differences between them were normally distributed. Differences were considered significant when *p* value was less than 0.05.

RESULTS

VEGF-A expression was observed in the cytoplasm of tumor cells and surrounding stroma in all analyzed cases. The mean percentage of cells expressing VEGF-A was significantly higher (p=0.0030) in tumor cells (mean=54.1%; SD=25.9) than in stromal cells (mean=43.7%; SD=26.1) in cases diagnosed as CIN 3 (Group 1) (Table 1). In invasive carcinoma (Group 3), the mean percentage of cells expressing VEGF-A was higher in stromal cells than in tumor cells (mean=48.1%; SD=28.8), but the difference was not statistically significant (p=0.0840) (Table 1). There was no statistically significant difference between the mean percentage of cells expressing VEGF-A in both tumor cells and stromal cells in CIN 3 and invasive carcinoma components from Group 2 (Table 1).

The percentage of stromal cells expressing VEGF-A was significantly higher (p=0.0154) in invasive carcinoma (Group 3) than in CIN 3 (Group 1). The percentage of stromal cells expressing VEGF-A in invasive carcinoma (Group 3) was higher than in the invasive carcinoma component from Group 2, but this difference was statistically borderline (p=0.0573). There was no statistically significant difference in the percentage of cells expressing VEGF-A in both tumor cells and stromal cells among: a) CIN 3 and invasive carcinoma components in

Group 2, b) between CIN 3 (Group 1) and CIN 3 (Group 2) and c) invasive carcinoma (Group 3) and invasive carcinoma component (Group 2) (Table 2).

DISCUSSION

This study showed that VEGF-A expression is higher in tumor cells than in stromal cells in CIN 3. However, in cervical cancer cases, stromal cells show a higher VEGF-A expression than tumor cells. Similarly, VEGF-A expression is higher in stromal cells in invasive carcinoma than in CIN 3.

There is increasing evidence to support the understanding that tumor cells and stromal cells contribute to tumor mass formation and characteristics [7, 14]. Based on clinical and experimental research, it has been demonstrated that tumor angiogenesis is dependent on stromal cell action and precedes overt tumor formation during chemically induced carcinogenesis [15, 13, 4).

High VEGF levels in the tumor microenvironment and consequent activation of a signalling pathway stimulate the growth and migration of lymphatic and blood endothelial cells, thereby promoting vascular invasion by tumor cells [15]. Tumor vasculature is dependent not only on endothelial cells but also other stromal cells, e.g. pericytes. Pericytes are affected by factors secreted by tumor cells. There is a loose association between tumor pericytes and endothelial cells which could affect their survival, as well as contribute to the presence of intercellular gap junctions or openings. These gaps could provide tumor cells with relatively easy access to the intravascular space [16, 17].

Inflammatory cells also regulate endothelial cell functions related to tumor angiogenesis. Tumor cells are surrounded by an infiltrate of inflammatory cells,

lymphocytes, neutrophils, macrophages and mast cells. These cells communicate through a complex network of intercellular signaling pathways, mediated by surface adhesion molecules, cytokines and VEGF expression. Therefore, inflammatory cells cooperate and synergize not only with stromal cells, but also malignant cells to stimulate endothelial cell proliferation and blood vessel formation [4].

angiogenic therapy acts by inhibiting the VEGF signalling pathways to reduce tumor growth and metastasis formation. However, resistance to -VEGF therapy has been observed [18]. Failure of this treatment suggests the existence of a connection between stromal and tumor cells. A modified microenvironment could contribute to resistance to angiogenic therapy in comparison with cytotoxic therapy, where resistance is due to tumor cells [19].

In cervical carcinogenesis, angiogenesis follows the current understanding of this issue. Angiogenesis occurs in premalignant changes of the cervix and is associated with disease progression [20, 4.] Some studies have reported VEGF-A expression in the stage of premalignant lesions of the uterine cervix [10, 11, 12]. Hammes et al.[12] showed that stromal VEGF expression was observed in 53.3 % and 75.0% cases of CIN 3 and invasive carcinoma, respectively. According to these authors, VEGF-A could be a factor in the development and progression of CIN lesions and a potential molecular target for studies of cervical cancer prevention and treatment. Stepan et al.[21] observed that VEGF-A is characteristic of cervical tumor angiogenesis in both early and advanced tumor stages.

Limitations of this study were failure to use normal tissue as control and the subjective nature of immunostaining in the evaluation of histologic samples.

However, despite these limitations, the data obtained reinforces the hypothesis that angiogenesis is an early event during tumorigenesis. Furthermore, the participation of stromal cells in tumor invasiveness is of critical importance.

In summary, the findings of this study are consistent with the role of VEGF-A in carcinogenesis. In CIN 3, VEGF-A expression was higher in tumor cells than in stromal cells. In a comparison between invasive carcinoma and CIN 3, VEGF-A expression showed no change in tumor cells, but increased significantly in stromal cells.

References

1. The International Agency for Research on Cancer (IARC). Globocan 2008. Estimated cancer Incidence, Mortality, Prevalence and Disability-adjusted life years (DALYs) Worldwide in 2008 – [http://globocan.iarc.fr/].
2. Tiltman AJ. **The pathology of cervical tumours.** *Best Pract Res Clin Obstet Gynaecol*. 2005 Aug,19(4):485-500.
3. Reich O, Pickel H, Tamussino K, Winter R. **Microinvasive Carcinoma of the Cervix: Site of First Focus of Invasion.** *Obstet Gynecol* 2001, 97:890-892.
4. Raica M, Cimpean AM, Ribatti D. **Angiogenesis in pre-malignant conditions.** *Eur J Cancer*. 2009 Jul, 45(11):1924-1934.
5. Delli Carpini J, Karam AK, Montgomery L. **Vascular endothelial growth factor and its relationship to the prognosis and treatment of breast, ovarian, and cervical cancer.** *Angiogenesis*. 2010 Mar, 13(1):43-58.

6. Van Trappen PO, Steele D, Lowe DG, Baithun S, Beasley N, Thiele W, et al. **Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR-3, during different stages of cervical carcinogenesis.** *Pathol.* 2003 Dec, 201(4):544-554.
7. Hanahan D, Weinberg RA. **Hallmarks of cancer: the next generation.** *Cell* 2011, 144:646–674.
8. Baeriswyl V, Christofori G. **The angiogenic switch in carcinogenesis.** *Semin Cancer Biol.* 2009 Oct, 19(5):329-337.
9. Kuemmel S, Thomas A, Landt S, Fuger A, Schmid P, Kriner M, Blohmer JU, Sehouli J, Schaller G, Lichtenegger W, Köninger A, Fuchs I. **Circulating vascular endothelial growth factors and their soluble receptors in pre-invasive, invasive and recurrent cervical cancer.** *cancer Res.* 2009 Feb, 29(2):641-645.
10. Smith-McCune KK, Weidner N. **Demonstration and characterization of the angiogenic properties of cervical dysplasia.** *Cancer Res.* 1994, 54(3):800–804.
11. Baritaki S, Sifakis S, Huerta-Yepez S, Neonakis IK, Soufla G, Bonavida B, Spandidos DA. **Overexpression of VEGF and TGF-beta1 mRNA in Pap smears correlates with progression of cervical intraepithelial neoplasia to cancer: implication of YY1 in cervical tumorigenesis and HPV infection.** *Int J Oncol.* 2007 Jul, 31(1):69-79.
12. Hammes LS, Tekmal RR, Naud P, Edelweiss MI, Kirma N, Valente PT, Syrjänen KJ, Cunha-Filho JS. **Up-regulation of VEGF, c-fms and COX-2**

expression correlates with severity of cervical cancer precursor (CIN) lesions and invasive disease. *Gynecol Oncol.* 2008 Sep, 110(3):445-451.

13. Hanahan D, Folkman J. **Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis.** *Cell.* 1996 Aug 9, 86(3):353-364.
14. Hanahan D, Weinberg RA. **The hallmarks of cancer.** *Cell.* 2000, 100:57–70.
15. Moserle L, Casanovas O. **-angiogenesis and metastasis: a tumor and stromal cell alliance.** *J Intern Med.* 2013, 273:128–137.
16. Gerhardt H, Semb H. Pericytes: **Gatekeepers in tumour cell metastasis?** *J Mol Med (Berl)*. 2008 Feb, 86(2):135-144.
17. Xian X, Hakansson J, Stahlberg A et al. **Pericytes limit tumor cell metastasis.** *J Clin Invest.* 2006, 116:642–651.
18. Bergers G, Hanahan D. **Modes of resistance to -angiogenic therapy.** *Nat Rev Cancer*. 2008, 8:592–603.
19. Ebos JM, Kerbel RS. **angiogenic therapy: impact on invasion, disease progression, and metastasis.** *Nat Rev Clin Onco.* 2011, 8:210–221.
20. Dai Y, Zhang X, Peng Y, Wang Z. **The expression of cyclooxygenase-2, VEGF and PGs in CIN and cervical carcinoma.** *Gynecol Oncol.* 2005 Apr, 97(1):96–103.
21. Stepan D, Simionescu C, Stepan A, Muntean M, Voinea B. **VEGF and CD105 immunoexpression in squamous cervical carcinomas and associated precancerous lesions.** *Rom J Morphol Embryol.* 2012, 53(3):585-589.

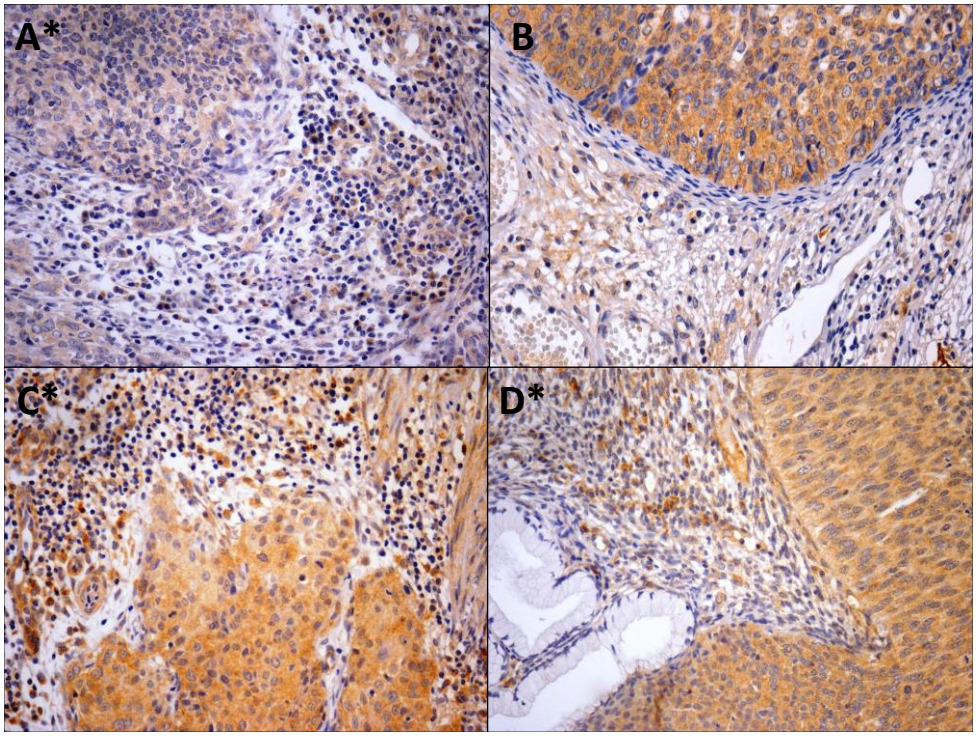

Figure 1. VEGF-A expression. A: Invasive carcinoma (Group 3) – low expression in tumor cells and low expression in stromal cells. **B**: NIC 3 (Group 1) – high expression in tumor cells and low expression in stromal cells. **C**: Invasive carcinoma compartment (Group 2) - high expression in tumor cells and high expression in stromal cells. **D**: NIC 3 compartment (Group 2) - high expression in tumor cells and high expression in stromal cells.

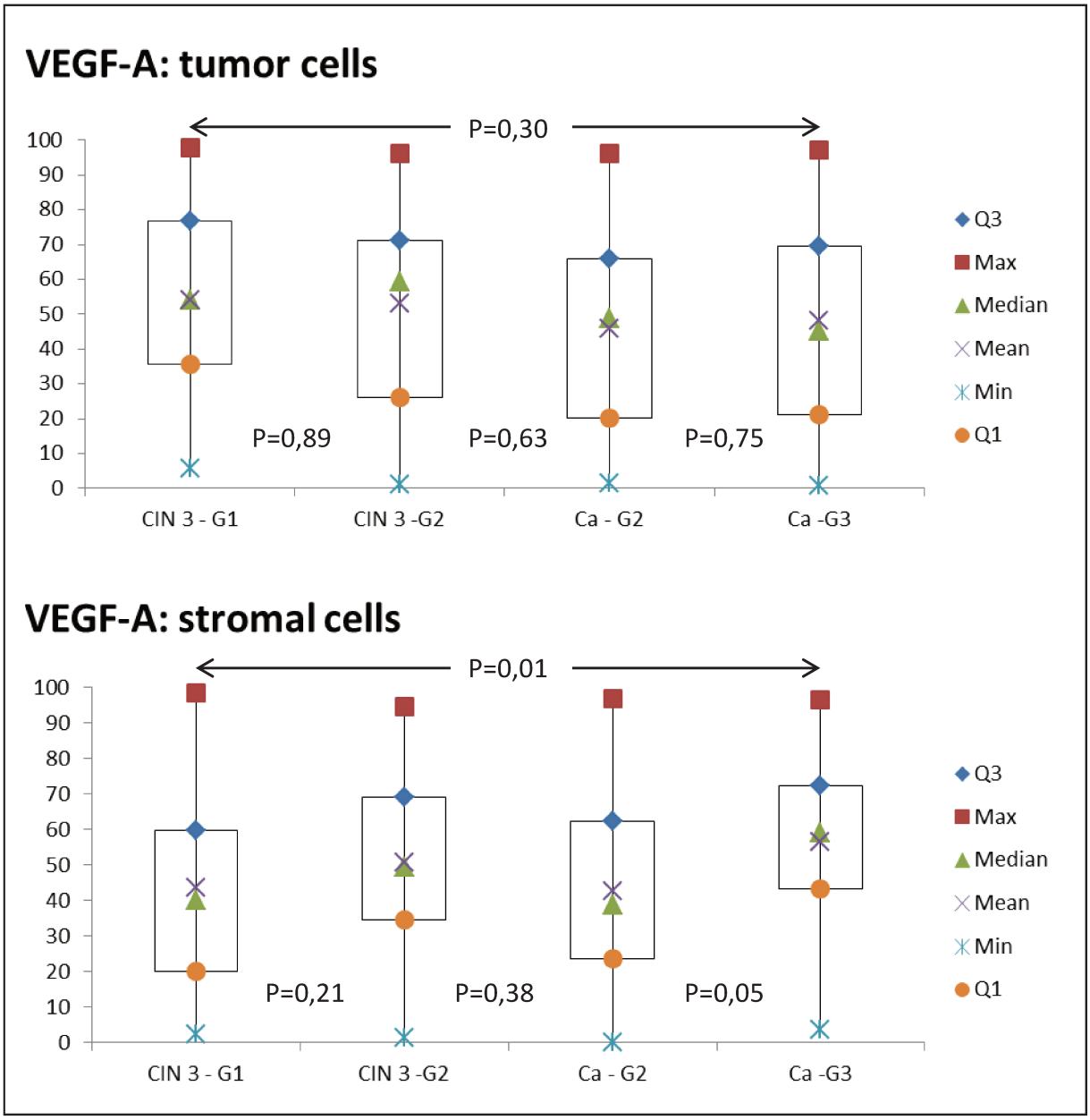

Figure 2. VEGF-A expressions in Cervical Neoplasia.

Tabela 1. Comparison of mean percentages of tumor and stromal cells VEGF-A immunopositive in each diagnostic group

	Cells	n	Mean	SD	Min	Q1	Median	Q3	Max	p-value
CIN 3 (Group 1)	Tumor	53	54.1	25.9	5.6	35.6	54.2	76.7	97.9	
	Stromal	53	43.7	26.1	2.5	20.2	40.1	59.8	98.5	0.0030
CIN 3 (Group 2)	Tumor	24	53.0	29.5	1.0	26.1	59.4	71.3	96.2	
	Stromal	24	50.8	25.6	1.4	34.6	49.5	69.2	94.6	0.7218
Invasive Carcinoma (Group 2)	Tumor	24	45.9	28.8	1.3	20.3	48.8	65.9	96.1	
	Stromal	24	42.8	27.4	0.2	23.6	38.7	62.3	96.7	0.6080
Invasive Carcinoma (Group 3)	Tumor	36	48.1	28.8	0.7	21.1	45.1	69.4	97.1	
	Stromal	36	56.5	23.5	3.6	43.3	59.1	72.2	96.4	0.0840

Comparisons between means were performed using the paired t-test.

CIN 3: cervical intraepithelial neoplasia grade 3; SD: standard deviation; Min: minimum;

Max.: maximum; Q1: 1º quartile (25%); Q3: 3º quartile (75%)

4. Discussão

Com base nos resultados deste estudo, o aumento progressivo da expressão de MMP-2, a partir de NIC 3, para carcinoma de células escamosas do colo uterino, em células tumorais e do estroma, reforça seu importante papel na progressão da neoplasia cervical. Outros estudos mostraram resultados semelhantes ou concordantes. Nair et al. (28) encontraram um aumento progressivo na positividade da MMP-2 relacionado com a gravidade da neoplasia cervical. Fernandes et al. (29) também mostraram que a expressão de MMP-2 foi maior nas células estromais de carcinomas invasivos do que de NIC 3 e indicaram que as células estromais desempenham um papel importante na invasão e progressão tumoral, mediada pelo aumento progressivo da expressão de MMP-2 a de NIC 3 para carcinoma invasivo avançado.

A MMP-14 foi altamente expressa em células tumorais de NIC 3 e carcinoma invasivo; contudo, sua expressão em células do estroma foi mais elevada no carcinoma invasivo do que em NIC 3. A invasão celular é um processo de várias etapas envolvendo a degradação de MEC e a mobilidade

celular. Embora a degradação de ECM possa ser orquestrada coletivamente pelas MMPs, é provável que a migração celular esteja associada predominantemente com a MMP-14, o que seria uma explicação para os achados deste estudo ao verificar a variação da expressão nas células estromais, entre NIC 3 e carcinoma invasivo (13).

Houve maior expressão de TIMP-1 nas células estromais do carcinoma e nas células tumorais de NIC 3, sugerindo que, na progressão de NIC 3 para carcinoma invasor, a expressão de TIMP-1 aumenta em células do estroma e diminui em células tumorais. Existem evidências que indicam um potencial papel de TIMP 1 como indutor tumoral nos estágios iniciais da tumorigênese, estimulando o crescimento das células e inibindo a apoptose, assim como promovendo a migração, angiogênese e invasão (30). Elevados níveis de TIMP são relatados em associação com a progressão do carcinoma e identificados como indicadores de mau prognóstico em vários tipos de tumores humanos (31).

De acordo com os resultados deste estudo, nenhum papel poderia ser inferido para a MMP-9 e TIMP-2 na progressão da neoplasia cervical porque estas proteínas foram expressas de forma similar nas células tumorais de NIC 3 e do carcinoma invasivo. No entanto, MMP-9 e TIMP-2 foram mais expressas nas células estromais do que nas células tumorais. Apesar da MMP-9 ser quase idêntica à MMP-2, a sua contribuição para os processos biológico ou patológico pode ser muito diferente (32). Estes achados sugerem que a MMP-2 teria papel mais centrado na progressão da neoplasia e a MMP-9 atuaria através do estroma.

Foram observadas diferenças significativas na expressão entre os grupos NIC 3 e o grupo carcinoma invasivo para várias MMPs e TIMP, porém nenhuma diferença foi demonstrada entre os componentes NIC 3 e carcinoma invasivo presentes no mesmo caso. A expressão das MMPs e TIMPs estudadas para ambas as lesões foi semelhante e parece seguir o padrão da lesão mais grave, isto é, do componente carcinoma invasivo, tanto para as células tumorais como estromais.

Este estudo também demonstrou que a expressão de VEGF-A foi maior nas células tumorais do que células estromais da NIC 3. Nos carcinomas invasivos do colo uterino, a expressão foi maior nas células estromais quando comparada às células tumorais. Em conformidade, a expressão de VEGF-A foi maior nas células estromais de carcinoma invasivo quando comparada à expressão em células estromais da NIC 3. Altos níveis de VEGF no microambiente tumoral e consequente ativação de vias de sinalização estimulam o crescimento e migração de células endoteliais linfáticas e sanguíneas, proporcionando assim invasão vascular das células tumorais (33). A vascularização do tumor não depende apenas das células endoteliais, mas também de outras células do estroma, como periquitos, que são afetados por fatores secretados pelas células tumorais (34, 35). Há cada vez mais evidências que sustentam o entendimento de que as células tumorais e estromais contribuem para a formação e as características da massa tumoral (36, 37). Com base em evidências clínicas e experimentais, foi demonstrado que a angiogênese tumoral é dependente da ação das células do estroma e que isto precede, de modo evidente, a formação do tumor durante a carcinogênese induzida quimicamente (38, 39).

A terapia angiogênica atua por inibição das vias de sinalização do VEGF, de maneira a reduzir o crescimento do tumor e formação de metástases. No entanto, resistência à terapia-VEGF tem sido observada (40). A falha no tratamento sugere que existam outras ligações que envolvem células estromais e tumorais. O microambiente modificado poderia contribuir para a resistência da terapia angiogênica, em comparação com a terapia citotóxica, em que a resistência acontece através de células tumorais (41). A angiogênese na carcinogênese cervical segue o entendimento atual e mais geral sobre o assunto, pois ocorre em alterações pré-malignas do colo do útero e está associada à progressão da doença (38,42). A expressão de VEGF-A na fase de lesão pré-maligna foi relatada para o colo do útero, em alguns estudos (43, 44, 45). Hammes et al. (45) mostraram que a expressão de VEGF estromal foi observada, respectivamente, em 53,3% e 75,0% em casos de NIC 3 e de carcinoma invasivo. De acordo com estes autores, VEGF-A pode ser um dos factores no desenvolvimento e progressão da NIC e pode ser um potencial alvo molecular para estudos de prevenção e tratamento do carcinoma do colo do útero. Stepan et al (46) observaram que o VEGF-A é característico para a angiogênese tumoral do colo do útero, em ambas as fases, precoce e avançada.

O poder deste estudo foi o seu desenho que analisou três etapas da progressão de neoplasia cervical: de NIC 3 ao carcinoma invasivo, mas os resultados poderiam ser mais conclusivos se um grupo representado por tecido cervical normal fosse incluído como controle. A subjetividade e a variabilidade da avaliação da imunoreatividade são limitações ao se comparar diferentes

sistemáticas para as análises qutativa e qualitativa. Este estudo avaliou a percentagem de células imunocoradas entre mais de 1000 células tumorais e estromais. Outros estudos avaliaram a expressão com base em características qualitativas e qutativas ou analisaram as células estromais separando fibroblastos e células inflamatórias.

Em resumo, a progressão da NIC 3 para carcinoma invasivo parece ser determinada por interações entre as células epiteliais e células do estroma e estes resultados reforçam as evidências do papel das células do estroma na invasão tumoral. Assim, os principais achados deste estudo foram:

- MMP-2 foi altamente expressa no carcinoma do colo do útero, nas células tumorais e estromais, o que indica que poderia ter um papel relevante na progressão da lesão neoplásica.

- MMP-14 foi altamente expressa em células tumorais e teria um papel importante na progressão do tumor, aumentando a sua expressão nas células do estroma à medida da progressão da neoplasia.

- MMP-9 foi altamente expressa nas células estromais e poderia exercer um papel na interação das células tumorais e estromais.

- TIMP-1 foi altamente expressa nas células tumorais da NIC 3 e nas células estromais do carcinoma invasivo, o que estaria em concordância com seu papel na transformação maligna e promoção da invasão.

– TIMP-2 foi altamente expressa em células do estroma e poderia ter papel na interação das células tumorais e estromais.

– Houve diferenças significativas na expressão de MMP-2, MMP-14 e TIMP-1 entre NIC 3 e carcinoma invasivo, e, portanto, essas proteínas poderiam ser testadas como marcadores de prognóstico para a NIC 3.

– VEGF-A foi mais expresso em células tumorais do que células estromais na NIC 3. Nos carcinomas invasivos do colo uterino, sua expressão foi maior nas células estromais.

5. Conclusões

- MMP-2

  – A expressão de MMP-2 nas células tumorais e estromais foi mais alta no carcinoma invasor do que na NIC 3.

  – Não houve diferença na expressão de MMP-2 entre as células tumorais e estromais em todos os grupos diagnósticos.

- MMP-14

  – A expressão de MMP-14 foi maior nas células tumorais do que nas estromais para NIC 3 e carcinoma invasivo.

  – A expressão de MMP-14 foi mais alta nas células estromais do carcinoma invasor na NIC 3.

- MMP-9

  – A expressão de MMP-9 foi maior nas células estromais do que nas células tumorais para NIC 3 e carcinoma invasivo. Esta diferença não foi observada no carcinoma invasivo quando associado à NIC 3.

- TIMP-1

  – A expressão de TIMP-1 foi maior nas células tumorais do que nas células estromais na NIC 3 e, ao contrário, a expressão de TIMP-1 foi significativamente maior nas células estromais do que nas células tumorais no carcinoma invasivo.

  – A expressão de TIMP-1 no carcinoma invasivo foi maior nas células estromais e menor nas células tumorais, quando comparado com a NIC 3.

- TIMP-2

  – A expressão de TIMP-2 nas células estromais foi maior que nas células tumorais para NIC 3 e carcinoma invasivo.

- VEGF-A

  – A expressão de VEGF-A foi mais alta nas células tumorais do que nas células estromais da NIC 3.

  – A expressão de VEGF-A nas células estromais do carcinoma invasor foi maior quando comparada às células estromais do NIC 3.

A expressão de MMP-2, MMP-14, TIMP-1 e VEGF variou entre os casos com diagnóstico de NIC 3 e aqueles com diagnóstico de carcinoma invasivo. Todavia, não houve diferença na expressão das MMPs, das TIMPs e do VEGF entre NIC 3 e carcinoma invasivo quando estas lesões estavam associadas no mesmo caso.

6. Referências Bibliográficas

1. The International Agency for Research on Cancer (IARC). Globocan 2008. Estimated cancer Incidence, Mortality, Prevalence and Disability-adjusted life years (DALYs) Worldwide in 2008 – Disponível em: http://globocan.iarc.fr/.

2. Brasil. Ministério da Saúde. Instituto Nacional de Câncer. Estimativa 2012: incidência de câncer no Brasil. Rio de Janeiro: INCA; 2011. Disponível em: http://www.inca.gov.br/estimativa/2012/tbregioes_consolidado.asp.

3. Smith HO, Tiffany MF, Qualls CR, Key CR. The rising incidence of adenocarcinoma relative to squamous cell carcinoma of the uterine cervix in the United States - a 24-year population-based study. Gynecol Oncol. 2000 Aug;78(2):97-105.

4. International Federation of Gynecology and Obstetrics (FIGO) –Staging Classifications and Clinical Practice Guidelines for Gynaecological Cancers. [acesso em 2007]. Disponível em: http://www.figo.org/publications.

5. Snijders PJF, Steenbergen RDM, Heideman DAM, Meijer CJLM. HPV-Mediated Cervical Carcinogenesis: Concepts and Clinical Implications. J Pathol. 2006;208:152-64.

6. Zhai Y, Hotary KB, Nan B, Bosch FX, Muñoz N, Weiss SJ, Cho KR. Expression of membrane type 1 matrix metalloproteinase is associated with cervical carcinoma progression and invasion. Cancer Res. 2005 Aug 1;65(15):6543-50.

7. Wu YC, Wang PH, Tsai A, Yang SF, Chen SC. Semi-Qutative Expression of Tissue Inhibitor of Matrix Metalloproteinase-2 in Cancer of Uterine Cervix. J Surg Oncol. 2011;104:210–5.

8. Brinckerhoff CE, Matrisian LM. Matrix metalloproteinases: a tail of a frog that became a prince. Nat Rev Mol Cell Biol. 2002 Mar;3(3):207-14.

9. Bourboulia D, Stetler-Stevenson WG. Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): Positive and negative regulators in tumor cell adhesion. Semin Cancer Biol. 2010 Jun;20(3):161-8.

10. Soufla G, Sifakis S, Baritaki S, Zafiropoulos A, Koumantakis E, Spandidos DA. VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix. Cancer Lett. 2005 Apr. 18;221(1):105-18.

11. Delli Carpini J, Karam AK, Montgomery L. Vascular endothelial growth factor and its relationship to the prognosis and treatment of breast, ovarian, and cervical cancer. Angiogenesis. 2010 Mar;13(1):43-58.

12. MEROPS. The Peptidase Database. Disponível em http://www.merops. sanger.ac.uk/cgi-bin/famsum?family=M10. [Acesso em 10 de junho. 2013].

13. Sbardella D, Fasciglione GF, Gioia M, Ciaccio C, Tundo GR, Marini S et al. Human matrix metalloproteinases: An ubiquitarian class of enzymes involved in several pathological processes. Mol Aspects Med. 2012 Apr;33(2):119-208.

14. Boyd S, Tolvanen K, Virolainen S, Kuivanen T, Kyllönen L, Saarialho-Kere U. Differential expression of stromal MMP-1, MMP-9 and TIMP-1 in basal cell carcinomas of immunosuppressed patients and controls. Virchows Arch. 2008;452:83–90.

15. Poincloux R, Lizarraga F, Chavrier P. Matrix invasion by tumour cells: a focus on MT1-MMP trafficking to invadopodia. J Cell Sci. 2009;122:3015–24.

16. Brew K, Dinakarpandian D, Nagase H. Tissue inhibitors of metalloproteinases: Evolution, structure and function. Biochim Biophys Acta. 2000;1477:267–83.

17. Stetler-Stevenson WG. The tumor microenvironment: regulation by MMP - independent effects of tissue inhibitor of metalloproteinases-2. Cancer Metastasis Rev. 2008;27(1):57–66.

18. Cardeal LB, Boccardo E, Termini L, Rabachini T, Andreoli MA, di Loreto C, et al. HPV16 oncoproteins induce MMPs/RECK-TIMP-2 imbalance in primary keratinocytes: possible implications in cervical carcinogenesis PLoS One. 2012;7(3):e33585.

19. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003 Jun;9(6):669-76.

20. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983;219(4587):983–5.

21. Folkman J, Merler E, Abernathy C, Williams G. Isolation of a tumor factor responsible for angiogenesis. J Exp Med. 1971;133(2):275–88.

22. Shibuya M, Luo JC, Toyoda M, Yamaguchi S. Involvement of VEGF and its receptors in ascites tumor formation. Cancer Chemother Pharmacol. 1999;43 Suppl:S72-7.

23. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ et al. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. J Exp Med. 2002 Dec 2;196(11):1497-506.

24. Kasper DL, Braunwald E, Fauci AS, Hauser SL, Longo DL, Jameson JL, Isselbacher KJ. Cancer cell biology and angiogenesis: introduction. In: Harrison T. (ed.). Harrison's Principles of Internal Medicine. 16th ed. New York: McGraw-Hill; 2005, n. 5.

25. Dvorak HF. Tumors: wounds that do not heal. N Engl J Med. 1986;315(26):1650–59.

26. Zighelboim I, Wright JD, Gao F, Case AS, Massad LS, Mutch DG et al.. Multicenter phase II trial of topotecan, cisplatin and bevacizumab for recurrent or persistent cervical cancer. Gynecol Oncol. 2013 Jul;130(1):64-8.

27. Sato T, Sakai T, Noguchi Y, Takita M, Hirakawa S, Ito A. Tumor–stromal cell contact promotes invasion of human uterine cervical carcinoma cells by augmenting the expression and activation of stromal matrix metalloproteinases. Gynecol Oncol. 2004;92:47–56.

28. Nair AS, Karunagaran D, Nair MB, Sudhakaran PR. Changes in matrix metalloproteinases and their endogenous inhibitors during tumor progression in the uterine cervix. J Cancer Res Clin Oncol. 2003;129:123–31.

29. Fernandes T, de Angelo-Andrade LA, Morais SS, Pinto GA, Chagas CA, Maria-Engler SS et al. Stromal cells play a role in cervical cancer progression mediated by MMP-2 protein. Eur J Gynaecol Oncol. 2008;29(4):341-4.

30. Jensen SA, Vainer B, Bartels A, Brünner N, Sorensen JB. Expression of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of metalloproteinases 1 (TIMP-1) by colorectal cancer cells and adjacent stroma cells – Associations with histopathology and patients outcome. European J Cancer. 2010;46:3233–42.

31. Jiang Y, Goldberg ID, Shi YE. Complex roles of tissue inhibitors of metalloproteinases in cancer. Oncogene. 2002;21:2245-52.

32. Zhang X, Wang Y, Yamamoto G, Tachikawa T. Expression of matrix metalloproteinases MMP-2, MMP-9 and their tissue inhibitors TIMP-1 and TIMP-2 in the epithelium and stroma of salivary gland pleomorphic adenomas. Histopathology. 2009;55:250–60.

33. Moserle L, Casanovas O. -angiogenesis and metastasis: a tumor and stromal cell alliance. J Intern Med. 2013, 273:128–37.

34. Gerhardt H, Semb H. Pericytes: Gatekeepers in tumour cell metastasis? J Mol Med (Berl). 2008 Feb, 86(2):135-44.

35. Xian X, Hakansson J, Stahlberg A et al. Pericytes limit tumor cell metastasis. J Clin Invest. 2006, 116:642–51.

36. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144:646–74.

37. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000, 100:57–70.

38. Raica M, Cimpean AM, Ribatti D. Angiogenesis in pre-malignant conditions. Eur J Cancer. 2009 Jul; 45(11):1924-34.

39. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996 Aug; 86(3):353-64.

40. Bergers G, Hanahan D. Modes of resistance to -angiogenic therapy. Nat Rev Cancer. 2008;8:592–603.

41. Ebos JM, Kerbel RS. angiogenic therapy: impact on invasion, disease progression, and metastasis. Nat Rev Clin Onco. 2011, 8:210–21.

42. Dai Y, Zhang X, Peng Y, Wang Z. The expression of cyclooxygenase-2, VEGF and PGs in CIN and cervical carcinoma. Gynecol Oncol. 2005;97(1):96–103.

43. Smith-McCune KK, Weidner N. Demonstration and characterization of the angiogenic properties of cervical dysplasia. Cancer Res. 1994, 54(3):800–4.

44. Baritaki S, Sifakis S, Huerta-Yepez S, Neonakis IK, Soufla G, Bonavida B et al. Overexpression of VEGF and TGF-beta1 mRNA in Pap smears correlates with progression of cervical intraepithelial neoplasia to cancer: implication of YY1 in cervical tumorigenesis and HPV infection. Int J Oncol. 2007 Jul, 31(1):69-79.

45. Hammes LS, Tekmal RR, Naud P, Edelweiss MI, Kirma N, Valente PT et al. Up-regulation of VEGF, c-fms and COX-2 expression correlates with severity of cervical cancer precursor (CIN) lesions and invasive disease. Gynecol Oncol. 2008 Sep, 110(3):445-51.

46. Stepan D, Simionescu C, Stepan A, Muntean M, Voinea B. VEGF and CD105 immunoexpression in squamous cervical carcinomas and associated precancerous lesions. Rom J Morphol Embryol. 2012, 53(3):585-89.

7. Anexos

7.1. Anexo 1 – Aprovação da Comissão de Pesquisa

Comissão de Pesquisa do DTG / CAISM

Campinas, 13 de agosto de 2007

Protocolo n°: 043/2007

O protocolo de pesquisa *"Papel das metaloproteinases da matriz extracelular (MMP-2, MMP-9 E MT-MMP) no processo de invasão tumoral da neoplasia do colo uterino"* das pesquisadoras, Maria Cristina do Amaral Westin, sob a orientação do Prof. Dr. Luiz Carlos Zeferino, foi aprovado pela Comissão de Pesquisa do DTG/CAISM.

Atenciosamente,

(original assinado)
Profa. Dra. Ellen Hardy
Presidente

7.2. Anexo 2 – Parecer do Comitê de Ética em Pesquisa

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA

www.fcm.unicamp.br/pesquisa/etica/index.html

CEP, 27/11/07.
(Grupo III)

PARECER CEP: N° 858/2007 (Este n° deve ser citado nas correspondências referente a este projeto)
CAAE: 0614.0.146.000-07

I - IDENTIFICAÇÃO:

PROJETO: **"PAPEL DAS METALOPROTEINASES DA MATRIZ EXTRACELULAR (MMP-2, MMP-9 E MT-MMP) NO PROCESSO DE INVASÃO TUMORAL DA NEOPLASIA DO COLO UTERINO".**
PESQUISADOR RESPONSÁVEL: Maria Cristina do Amaral Westin
INSTITUIÇÃO: Laboratório de Patologia Experimental / CAISM / UNICAMP
APRESENTAÇÃO AO CEP: 09/11/2007
APRESENTAR RELATÓRIO EM: 27/11/08 (O formulário encontra-se no *site* acima)

II - OBJETIVOS

Avaliar a expressão das proteínas MMP-2, MMP-9, MT1-MMP na carcinogênese do colo uterino e analisar a sua interação com TIMP-1, TIMP-2 e com os marcadores de angiogênese VEGF-A e D2-40

III - SUMÁRIO

Estudo do tipo corte transversal constituído de três grupos. O primeiro grupo corresponde a 45 mulheres com diagnóstico histológico de NIC 3, o segundo grupo composto por 45 mulheres portadoras de neoplasias de colo uterino com componentes intra-epiteliais e invasores e o terceiro grupo composto por 45 mulheres com apenas carcinoma invasor. A expressão protéica será pesquisada através de exame imunoistoquímico. Análise Estatística: Será calculado o odds ratio (OR), com seu respectivo intervalo de confiança de 95% (IC 95%), para estimar a magnitude da associação entre duas variáveis categóricas. A correlação entre a expressão das proteínas será testada pelo Coeficiente de correlação de Pearson.

IV - COMENTÁRIOS DOS RELATORES

O projeto apresenta-se bem redigido, com metodologia adequada. Os critérios de inclusão, exclusão e descontinuação dos sujeitos estão bem definidos; cálculo do tamanho amostral e análise estatistica muito bem embasados por cálculos estatísticos. Os aspectos éticos estão bem discutidos no corpo do projeto e o Termo de Consentimento Livre e Esclarecido é claro e adequado às recomendações. O orçamento é detalhado e prevê ressarcimento de custos com alimentação para as voluntárias. Os resultados permitiriam que as mulheres com lesões escamosas mostrando alto potencial para tornerem-se invasoras, deveriam ser tratadas rápida e eficintemente.

V - PARECER DO CEP

O Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas da UNICAMP, após acatar os pareceres dos membros-relatores previamente designados para o presente caso e atendendo todos os dispositivos das Resoluções 196/96 e complementares, resolve aprovar sem restrições o Protocolo de Pesquisa, bem como ter aprovado o Termo do Consentimento Livre e Esclarecido, assim como todos os anexos incluídos na Pesquisa supracitada.

O conteúdo e as conclusões aqui apresentados são de responsabilidade exclusiva do CEP/FCM/UNICAMP e não representam a opinião da Universidade Estadual de Campinas nem a comprometem.

VI - INFORMAÇÕES COMPLEMENTARES

O sujeito da pesquisa tem a liberdade de recusar-se a participar ou de retirar seu consentimento em qualquer fase da pesquisa, sem penalização alguma e sem prejuízo ao seu cuidado (Res. CNS 196/96 – Item IV.1.f) e deve receber uma cópia do Termo de Consentimento Livre e Esclarecido, na íntegra, por ele assinado (Item IV.2.d).

Pesquisador deve desenvolver a pesquisa conforme delineada no protocolo aprovado e descontinuar o estudo somente após análise das razões da descontinuidade pelo CEP que o aprovou (Res. CNS Item III.1.z), exceto quando perceber risco ou dano não previsto ao sujeito participante ou quando constatar a superioridade do regime oferecido a um dos grupos de pesquisa (Item V.3.).

O CEP deve ser informado de todos os efeitos adversos ou fatos relevantes que alterem o curso normal do estudo (Res. CNS Item V.4.). É papel do pesquisador assegurar medidas imediatas adequadas frente a evento adverso grave ocorrido (mesmo que tenha sido em outro centro) e enviar notificação ao CEP e à Agência Nacional de Vigilância Sanitária – ANVISA – junto com seu posicionamento.

Eventuais modificações ou emendas ao protocolo devem ser apresentadas ao CEP de forma clara e sucinta, identificando a parte do protocolo a ser modificada e suas justificativas. Em caso de projeto do Grupo I ou II apresentados anteriormente à ANVISA, o pesquisador ou patrocinador deve enviá-las também à mesma junto com o parecer aprovatório do CEP, para serem juntadas ao protocolo inicial (Res. 251/97, Item III.2.e)

Relatórios parciais e final devem ser apresentados ao CEP, de acordo com os prazos estabelecidos na Resolução CNS-MS 196/96.

VII - DATA DA REUNIÃO

Homologado na XI Reunião Ordinária do CEP/FCM, em 27 de novembro de 2.007.

Profa. Dra. Carmen Sílvia Bertuzzo
PRESIDENTE DO COMITÊ DE ÉTICA EM PESQUISA
FCM / UNICAMP