LUCIANA REGINA MOREIRA

AVALIAÇÃO DA ANGIOGÊNESE E LINFANGIOGÊNESE NOS CARCINOMAS COLORRETAIS: COMPARAÇÃO ENTRE MÉTODOS DE MENSURAÇÃO E DE DIFERENTES MARCADORES VASCULARES COM INDICADORES ANATOMOPATOLÓGICOS DE PROGNÓSTICO

Tese de Doutorado

ORIENTADOR: Prof. Dr. JOSÉ VASSALLO

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para obtenção do Título de Doutor em Ciências Médicas, área de concentração em Anatomia Patológica

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Dedico este trabalho...

Aos meus pais, Adilson e Maria do Carmo,
pela dedicação e ensinamentos,
presentes em todos os momentos da minha vida.

Ao Anderson,
pelo amor, carinho, paciência e apoio,
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O futuro não é um lugar para onde estamos indo,
mas um lugar que estamos criando.
O caminho para ele não é encontrado, mas sim construído,
e o ato de fazê-lo muda tanto o realizador quanto o destino.

F. Shaar

Estrutura da Tese

Esta tese está sendo apresentada no formato alternativo de disponibilização de dissertações e teses de mestrado e de doutorado na UNICAMP, de acordo com o disposto em *"Normas, procedimentos e orientações para publicação de dissertações e teses da Faculdade de Ciências Médicas"* (2009).

Trata-se de uma introdução sobre o tema, os objetivos da tese, dois artigos originais (um aceito e outro submetido às revistas cientifícas) - com a descrição dos métodos e resultados obtidos – e, por fim, uma discussão geral e os anexos. Nos anexos foram incluídas planilhas com informações dos casos estudados.

Sumário

Símbolos, Siglas e Abreviaturas

APC – Polipose adenomatosa colônica, do inglês *adenomatous polyposis coli*

CI – Intervalo de confiança, do inglês *confidence interval*

CRC – Carcinoma colorretal, do inglês *colorectal cancer*

DCC – Deletado no câncer do cólon, do inglês *deleted in colon cancer*

DNA – Ácido desoxirribonucleico, do inglês *deoxyribonucleic acid*

F – Feminino

H_2O_2 – Peróxido de hidrogênio

HR – Razão de risco, do inglês *hazard ratio*

IQ – Imunoistoquímica

INCA – Instituto Nacional do Câncer

K-ras – do inglês *Kirsten retrovirus-associated DNA sequences*

M – Masculino

mM – milimol

MSI – Instabilidade de microssatélites, do inglês *microsatellite instability*

MVD – Densidade microvascular, do inglês *microvessel density*

OMS – Organização Mundial da Saúde

OS – Sobrevida global, do inglês *overall survival*

PAF – Polipose adenomatosa familiar

pH – Potencial hidrogeniônico

SD – Desvio padrão, do inglês *standard deviation*

TVA – Área vascular total, do inglês *total vascular area*

VEGF – Fator de crescimento endotelial vascular, do inglês *Vascular endothelial growth factor*

UNICAMP – Universidade Estadual de Campinas

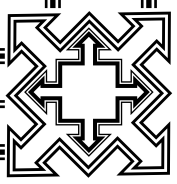
µm – Micrômetro

Resumo

Contexto: A angiogênese e linfangiogênese são processos em que as células endoteliais se dividem e migram para a formação de novos capilares, dando suporte para a progressão tumoral. A mensuração destes processos pode discriminar diferentes extratos prognósticos no câncer. O valor prognóstico da determinação da angiogênese e linfangiogênese no carcinoma colorretal (CRC) é assunto controverso na literatura. Isto pode ser devido a variações na forma de análise, como local de quantificação dentro da amostra tumoral, escolha do marcador imunoistoquímico e o método de quantificação. No presente trabalho, a angiogênese e linfangiogênese são estudadas através de programas de análise de imagem, comparando métodos de quantificação (densidade microvascular - MVD *versus* estimativa da área vascular total - TVA), diferentes marcadores imunoistoquímicos (pan-endoteliais *versus* vasos neoformados) e áreas de análise (campo central da lesão *versus* periferia *versus* campo de invasão mais profunda no carcinoma). **Objetivos:** Comparar esses parâmetros entre adenomas de pacientes sem e com carcinoma na mucosa adjacente; compará-los nos tecidos colorretais não neoplásicos, em adenomas e carcinomas, além de encontrar um meio de quantificação de angiogênese e linfangiogênese que fosse mais fidedigno como fator prognóstico no CRC. **Métodos:** 60 CRC esporádicos, 30 adenomas e 10 tecidos colorretais não neoplásicos foram submetidos a estudo imunoistoquímico para a detecção dos antígenos CD31, CD34, CD105, VEGF-A, VEGF-C e D2-40. Imagens dos preparados imunoistoquímicos foram capturadas para avaliar a MVD e a TVA em um programa de análise de imagem. Também foram

analisadas a porcentagem e a intensidade de expressão protéica de VEGF-A e VEGF-C nas células carcinomatosas. **Resultados:** A imunocoloração da maioria dos marcadores, bem como a expressão de VEGF-A e VEGF-C, mostraram aumento estatisticamente significante nos adenomas e carcinomas quando comparados com tecido não neoplásico para MVD e TVA nos diversos campos da lesão. Os adenomas de pacientes com carcinoma apresentaram aumento estatisticamente significante na TVA determinada pelo CD105 (p= 0,019) e na MVD determinada pelo D2-40 (p= 0,041), quando comparadas com os adenomas de pacientes sem carcinoma. Dentre os carcinomas, apenas a MVD determinada pelo marcador CD34 no campo central da lesão diferencia-se estatisticamente com a recorrência/metástase (p= 0,04) e a sobrevida (p= 0,02). **Conclusões:** Os achados apóiam o fato da angiogênese e o aumento da vascularização linfática ocorrer precocemente, ainda nos adenomas. Também corroboram o fato de que angiogênese e aumento da contagem de vasos linfáticos ocorram mais em adenomas de pacientes com carcinomas na mucosa adjacente, possivelmente influenciados por fatores produzidos pelo tumor. A MVD na área central do carcinoma determinada pelo marcador imunoistoquímico CD34 adiciona critério prognóstico, associando-se com recidiva/metástase e sobrevida, enquanto os outros meios de quantificação vascular e de expressão de fatores de crescimento não apresentaram resultados estatisticamente significantes. Este método é um fator prognóstico independente e adicional no CCR.

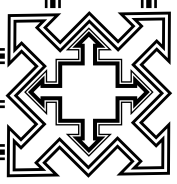
Summary



Background: Angiogenesis and lymphangiogenesis play an important role in the progression of solid tumors, and its quantification may be associated with prognostic stratification. However, the prognostic value of the assessment of angiogenesis and lymphangiogenesis in colorectal cancer (CRC) is still controversial. This may be due to variations in the methods of analysis, as the precise location of assessment within a tumor sample, the choice of immunohistochemical markers and the quantification system. In the present study, angiogenesis and lymphangiogenesis were assessed using image analysis software, comparing two methods of quantification (microvessel density - MVD *versus* estimation of the total vascular area - TVA), different immunohistochemical markers (pan-endothelial *versus* neovessel) and areas of analysis (periphery *versus* inner portions of the lesion *versus* the deepest invasion area of carcinoma). **Objectives:** To compare angiogenic and lymphangiogenic patterns in adenomatous polyps from patients without and with sporadic CRC; to compare angiogenesis and lymphangiogenesis in non neoplasic colorectal tissue, adenomas and cancer, and to search for a reliable approach to quantify angiogenesis and lymphangiogenesis, which could be of clinical value as prognostic factor in CRC. **Methods:** 60 sporadic CRC, 30 colorectal adenomas and 10 non neoplasic colorectal tissues were submitted to immunohistochemical analysis for CD31, CD34, CD105, VEGF-A, VEGF-C and D2-40. MVD and TVA were determined by digitalizing the immunohistochemical reactions and examining them by computer image analysis. Immunostaining for VEGF-A and VEGF-C was evaluated using a parameter based on the

percentage of tumor area stained and staining intensity. **Results:** Staining for most markers, as well as for VEGF-A and VEGF-C, exhibited significant increase in adenomas and carcinomas, when assessment of MVD and TVA in different tumor fields was compared with non neoplastic colorectal tissues. Adenomas from patients with carcinoma showed significantly higher values of TVA determined by immunostaining for CD105 (p = 0.019) and of lymphatic MVD determined by D2-40 (p = 0.041) when compared with adenomas from patients without cancer. Among patients with CRC, only MVD determined by immunostaining for CD34 in the central areas of the tumor was significantly correlated with recurrence/metastasis (p=0.04) and survival rates (p=0.02). **Conclusions:** Our results support that angiogenesis and lymphatic vascularization, plays a role in early tumor development at the stage of adenoma formation. The findings further support the notion that neoangiogenesis and elevated lymphatic vessel counts occur in colorectal adenomas from patients with CRC when compared to those without carcinoma, possible under the influence of factors produced by the carcinoma. Our results suggest that MVD determined with staining for CD34 in the inner part of the tumor is more closely related with relapse/metastasis and survival than other means of vascular quantification. The method is an additional prognostic factor in CCR.

1. Introdução



1.1. Epidemiologia

O carcinoma colorretal (CRC) representa 8,5% das neoplasias malignas no mundo. A Organização Mundial da Saúde (OMS) estima que 940 mil casos novos ocorram anualmente, com 492 mil mortes. Esta neoplasia tem uma distribuição mundial, com taxas de mortalidade mais altas na América, Europa Oriental, Austrália e parte da Ásia. Os fatores ambientais, sobretudo os hábitos alimentares, são implicados nos contrastes geográficos (1). No Brasil, o CRC está entre as cinco principais causas de morte por câncer. O Instituto Nacional do Câncer (INCA) estima que no ano de 2008, 26.990 casos novos dessa neoplasia incidiram em nosso meio. À exceção dos cânceres não melanocíticos da pele, entre os cinco tipos de tumor que mais acometeram os brasileiros em 2008, o CRC ficou com o terceiro lugar na população feminina e quarto lugar na população masculina (2).

1.2. Patogênese do carcinoma colorrretal

O CRC desenvolve-se esporadicamente, como parte de uma síndrome de câncer hereditário ou num contexto de doença inflamatória intestinal (3). A forma esporádica corresponde à maioria dos casos, com cerca de 80 a 85% dos casos (4). As formas hereditárias principais são: a polipose adenomatosa familiar (PAF) e o CRC hereditário não relacionado à polipose. Na PAF, os pacientes geralmente desenvolvem mais de cem adenomas colorretais mesmo

em idades precoces (50% ao redor dos 15 anos e 95% ao redor dos 35 anos). Nesta forma estão incluídas as variantes Síndrome de Gardner (com cistos epidérmicos, tumores desmóides ou anormalidades dentárias) e a Síndrome de Turcot (relacionada a tumores cerebrais, em especial meduloblastoma) (5,6). A PAF é uma doença autossômica dominante, com 80% dos casos apresentando mutação do gene supressor tumoral APC (do inglês *adenomatous poliposis coli*), quando usados métodos de rotina, e com mais de 95% dos casos com mutação, quando usada análise de mutação monoalélica. Este gene é localizado no cromossomo 5q21, sendo responsável pela inibição de transdução de sinais relativos à proliferação celular (3).

Já a forma do CRC hereditário não relacionado à polipose é uma doença autossômica dominante, apresenta tumores da porção proximal do cólon, bem circunscritos e ricos em linfócitos. É relacionada a mutações hereditárias em qualquer um dos genes de reparo do DNA: hMSH2, hMSH6 hMLH1, hPMS1 e hPMS2. As mutações destes genes de reparo de DNA são detectadas por alterações difusas nas repetições de seqüências de nucleotídeos do DNA, denominado instabilidade de microssatélites - MSI (3,5,6).

Na forma esporádica, dentre os fatores de risco incluem-se: dieta rica em carboidratos refinados, baixo teor de fibras vegetais, ingestão excessiva de carnes vermelhas, além do hábito do fumo, ingestão excessiva de álcool e idade avançada. É proposto que a lentificação do trânsito intestinal exponha o epitélio a maior quantidade de subprodutos oxidativos (ingestão de ácidos graxos saturados de carne vermelha e carboidratos refinados). Estes poderiam ser

convertidos em carcinógenos potenciais pelas bactérias intestinais (7). Além disso, seria necessário acúmulo de alterações genéticas ao longo do tempo, tais como: mutação do gene regulador da proliferação celular - APC (presente em 85% dos casos); mutação do gene transmissor de sinais promotores de mitose - K-ras (presente em 50% dos casos); mutação do gene supressor tumoral responsável pela interrupção do ciclo celular frente à lesão de DNA - gene p53 (70 - 85% dos casos); mutação de genes de reparo de erros do DNA levando à instabilidade de microssatélites (15% dos casos); deleção do gene codificador de proteína de adesão celular – gene DCC (em 70-75% dos casos); anormalidades de metilação do DNA, entre outros (5,7,8).

Três vias principais representam estas alterações genéticas: instabilidade cromossômica (anormalidades de cariótipo, perda e ganho de cromossomos); instabilidade de microssatélites (alterações de pequenas seqüências de nucleotídeos) e alterações epigenéticas (padrões de alterações de expressão de gene que não afetam diretamente a seqüência primária do DNA, por exemplo, alteração de metilação) (3,9).

1.3. Fatores de prognóstico do carcinoma colorretal

O estádio baseado na classificação do TNM continua sendo o fator de prognóstico predominante no CRC (5). Entretanto, sabe-se que estádios idênticos podem evoluir de maneiras diferentes (10,11). Aspectos morfológicos como: comprometimento de linfonodal, tipo e graduação tumoral, invasões linfática e venosa, além de extensão tumoral são, ainda, fatores morfológicos

importantes para o estabelecimento do prognóstico. Evidências sugerem que a configuração da borda tumoral, perda da coesão celular na margem invasora, e linfócitos intratumorais são aspectos morfológicos adicionais, mas ainda não essencias para o prognóstico (12,13).

Na tentativa de se encontrar fatores prognósticos adicionais no CRC, têm sido propostas classificações baseadas nos achados moleculares, refletindo os mecanismos de carcinogênese (14). Este sistema de classificação parece ser útil na correlação com outros fatores moleculares (por exemplo, o *status* do gene p53 parece ter pouco efeito em tumores com altas taxas de metilação e instabilidade de microssatélites) (3). Quanto à sobrevida dos pacientes com CRC, vários autores demonstraram associação entre a classificação molecular e o prognóstico, enquanto outros não confirmaram estes resultados (3,9,12).

Além disto, diversos trabalhos com marcadores biológicos relacionados à invasão tumoral, ciclo celular, apoptose, proliferação celular, reparo de DNA, fatores de crescimento, entre outros têm sido realizados. O uso de tais marcadores como indicadores de valor prognóstico é questionado, apresentando resultados divergentes na literatura (12).

1.4. Angiogênese, linfangiogênese e carcinoma colorretal

O fato de a progressão tumoral poder ser dependente da angiogênese e linfangiogênese tem estimulado pesquisas para novos fatores prognósticos e desenvolvimento de novas estratégias terapêuticas. Isto é bem vindo já que 20-

30% dos pacientes com CRC tratado com cirurgia potencialmente curativa irão recidivar, sugerindo que fatores prognósticos convencionais não são suficientes, havendo necessidade de fatores adicionais (10,11).

O Fator de crescimento endotelial vascular (VEGF, do inglês *vascular endothelial growth factor*) é uma importante glicoproteína estimuladora da angiogênese. A família VEGF inclui o VEGF-A, VEGF-B, ambos ligantes do receptor VEGF-R1, mediador da angiogênese, bem como o VEGF-C e VEGF-D, ambos importantes ligantes do receptor VEGF-R3, envolvido na linfangiogênese. Além do VEGF-R1, o VEGF-A é transmitido via receptor VEGF-R2, sendo um importante fator de sinalização para proliferação e migração endotelial vascular (15).

A expressão de VEGF-A no citoplasma das células colorretais pelo estudo imunoistoquímico (IQ) é maior em adenomas do que na mucosa colorretal normal e aumenta ainda mais no adenocarcinoma (16-19). O aumento de VEGF-A também é correlacionado com pior prognóstico na maior parte dos estudos (20-23). Enquanto outros não conseguiram confirmar esta correlação (24,25). A expressão da proteína VEGF-C apresenta resultados controversos na literatura também, com trabalhos associando seu aumento com envolvimento linfático ou metástases, enquanto outros não obtiveram estes resultados (26-32).

A quantificação vascular demonstra igualmente resultados divergentes nos CRC. Diversos trabalhos demonstraram pior prognóstico com o aumento de contagem microvascular - densidade de microvasos (MVD) (33,34,35). Também foi notado que a MVD está associada com metástases hematogênicas e com a

maior imunoexpressão da proteína p53 (35). Por outro lado, discutiu-se que a MVD não fornece nenhuma nova informação prognóstica, enquanto que a área total vascular (TVA) e o padrão de ramificação dos microvasos apresentam melhor poder discriminatório do prognóstico (36). E há, ainda, estudos onde o aumento da MVD correlacionou-se com melhor prognóstico (37,38).

O anticorpo para o antígeno CD34 representa um marcador vascular pan-endotelial utilizado e recomendado para quantificação vascular, segundo consenso internacional na especialidade (39). Ultimamente, o uso de anticorpo para CD34 tem sido preterido em favor do anticorpo para o CD105. Este tem sido considerado mais específico para microvasos neoformados. O CD105 ou Endoglin é uma proteína transmembrana expressa no endotélio vascular, sendo mais detectada na vascularização tumoral onde há proliferação endotelial, sugerindo possível distinção entre vasos vasculares pré-existentes de vasos neoformados (40). Recentemente, a expressão da proteína CD105 foi demonstrada em vasos tumorais linfáticos, sugerindo que a sua expressão não é confinada à vascularização sanguínea (41).

A MVD determinada pelo uso do anticorpo CD105 parece ser um fator independente de prognóstico no CRC, em contaste com o CD34 (42). A contagem de vasos positivos para CD105, também, foi associada à presença de metástases (43). Por outro lado, há relato recente da falta de associação da contagem de vasos expressos pelo CD105 com parâmetros clínicos e patológicos do CRC (44).

O anticorpo D2-40 identifica uma sialoglicoproteína que foi originalmente descrita em testículos fetais e tumores testiculares de células germinativas.

Este anticorpo parece ter uma expressão imunoistoquímica seletiva para vasos linfáticos, sendo útil na diferenciação destes com os vasos sanguíneos (45). A densidade de microvasos linfáticos foi associada a pior prognóstico e presença de metástases no CRC (46,47,48). Foi demonstrado que vasos linfáticos marcados com D2-40 são encontrados em mucosas colorretais, porém sua função ainda é incerta, uma vez que carcinomas intramucosos não metastatizam (49).

Do ponto de vista clínico, além de representar um potencial fator de valor prognóstico em CRC, a angiogênese pode servir ainda de alvo terapêutico. Neste particular, embora drogas antiangiogênicas estejam sendo experimentadas em vários tipos de neoplasias, seu exato mecanismo de ação é desconhecido. Portanto, é desejável que se disponha de métodos mais uniformes nos vários estudos relacionados à quantificação da angiogênese. Desta forma, os resultados de múltiplos centros podem ser comparáveis quando esse parâmetro for aplicado à compreensão do mecanismo de ação de fármacos antiangiogênicos, na avaliação da resposta da angiogênese após a administração dessas drogas, e, por fim, no estabelecimento de novos fatores de prognóstico.

O presente estudo abordará apenas o aspecto patogenético e o valor prognóstico da quantificação da angiogênese e linfangiogênese no CRC. A existência de estudos apontando conclusões diversas, o potencial valor prognóstico e preditivo da angiogênese e linfangiogênese nos CRC, além da carência de um único estudo que avalie os diversos métodos e marcadores imunoistoquímicos de quantificação no mesmo grupo de pacientes com CRC, estimularam-nos a apresentar o presente trabalho.

2. Objetivos

2.1. Objetivo Geral

Analisar o perfil imunoistoquímico de diferentes marcadores relacionados à angiogênese e linfangiogênese previamente descritos como tendo valor prognóstico controverso no CRC, utilizando métodos de mensuração: área vascular total (TVA) e densidade microvascular (MVD), além da porcentagem e a intensidade de células tumorais positivas para VEGF-A e VEGF-C. Comparar estes parâmetros nos tecidos colorretais não neoplásicos, adenomas e carcinomas; compará-los entre adenomas de pacientes sem e com carcinomas e correlacioná-los com fatores morfológicos e clínicos de utilidade bem estabelecida nos carcinomas colorretais.

2.2. Objetivos Específicos

- **Artigo 1 – Aceito para publicação no *Brazilian Journal of Medical and Biological Research***

  - Comparar a angiogênese e os vasos linfáticos entre os grupos de adenomas, um sendo de pacientes sem CRC e o outro de pacientes com CRC em local distinto na mucosa, usando densidade microvascular e área total vascular através de análise por programa de imagem em computador.

  - Determinar diferenças na angiogênese e os vasos linfáticos entre estes dois grupos de adenomas e, se é possível inferir que a presença de carcinoma possa influenciar o adenoma.

- **Artigo 2 – Submetido para publicação na revista *Modern Pathology***

  - Determinar o meio de quantificação de angiogênese e linfangiogênese mais fidedigno como fator prognóstico nos carcinomas colorretais dentre os diversos marcadores imunoistoquímicos e métodos de quantificação relacionados a estes processos.

  - Correlacionar os marcadores e métodos de quantificação relacionados à angiogênese e linfangiogênese com fatores morfológicos e clínicos de valor clínico bem estabelecido nos carcinomas colorretais.

  - Comparar angiogênese e linfangiogênese em tecidos colorretais não neoplásicos, adenomas e carcinomas.

3. Publicações

3.1. Artigo 1

COMPARISON OF BLOOD NEOANGIOGENESIS AND LYMPHATIC VASCULARIZATION IN COLORECTAL ADENOMAS FROM PATIENTS WITH AND WITHOUT CONCOMITANT COLORECTAL CANCER

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Blood and lymphatic vessel proliferation is essential for tumor growth and progression. Most colorectal carcinomas develop from adenomas (adenoma-carcinoma sequence) in a process due to accumulation of molecular genetic alterations. About 5% of adenomatous polyps are expected to become malignant, but data on the differential angiogenic patterns of these lesions in patients with and without concomitant cancer are missing. The aim of the present study is to compare the angiogenic and lymphatic patterns of adenomatous polyps from patients with and without sporadic cancer. Thirty adenomatous polyps (15 from patients with another principal malignant lesion, and 15 from patients without cancer) were submitted to immunohistochemical staining for CD105 (new formed blood vessels marker) and D2-40 (lymphatic endothelium marker). Microvessel density and total vascular area were determined by computer image analysis. Image was evaluated using this program to quantify the stained and total areas and to assess the number of microvessels. Adenomas from patients with carcinoma showed significantly higher values of total vascular area determined by immunostaining for CD105 (cut-off value = 4386 μm^2; p = 0.019) and of lymphatic microvessel density determined by immunostaining with D2-40 (cut-off value = 11.5; p = 0.041) when compared with those from patients without cancer. The present data indicate a significant increase in blood microvascular area and in lymphatic microvascular counts in adenomas removed from patients with cancer.

Introduction

Colorectal carcinoma (CRC) represents an important cause of cancer mortality in industrialized countries. Most cases (80%) correspond to sporadic carcinomas and arise from colorectal adenomas (1). The adenoma-carcinoma sequence involves accumulations of genetic alterations causing progressive disorders in the cell cycle (2). About 5% adenomatous polyps will probably become malignant (3).

Angiogenesis plays an important role in tumor progression and metastasis in most human solid tumors (4-7). This fact has led to new perspectives in the research of prognostic indicators and of new therapeutic strategies. The fact that 20-30% of patients with CRC treated with potentially curative surgery succumb from recurrent disease suggests that the conventional prognostic factors are not totally sufficient (8-11).

Most studies have evaluated angiogenesis as a potential prognostic or predictive factor in CRC both in early and advanced disease (12,13). Comparison of literature data has been frequently hindered by variations in patient management, such as indication of adjuvant therapy, and in the methods for analysis of angiogenesis (different immunohistochemical markers, quantification methods, parameters quantified, etc.) (12,14,15).

Endoglin (CD105) is a membrane glycoprotein, part of the TGF-beta receptor complex, involved in angiogenesis. Markers for this protein identify newly formed blood vessels, representing a helpful tool in the evaluation of neoangiogenesis (16). In CRC, CD105 has been correlated to prediction of metastasis (17).

D2-40 is a monoclonal antibody directed against the oncofetal antigen M2A, present in germ cells, lymphatic endothelium, and some neoplasms such as mesotheliomas (18,19). Using this antibody, it has been demonstrated that the colorectal mucosa indeed presents lymphatic vessels in normal, inflammatory and neoplastic conditions (20,21). However, in contrast to CD105, present in newly formed blood vessels, the presence of D2-40 does not indicate the degree of

neolymphangiogenesis. Lymphatic vessel density assessed by D2-40 has been correlated the prediction of metastasis and with a poor outcome of CRC (22-24).

In spite of the many reports on angiogenesis in cancer, data on the differential angiogenic patterns of adenomatous lesions in patients with and without concomitant CRC are not available. The purpose of the present study was to compare blood angiogenesis and lymphatic vessels between two groups of adenomatous polyps, one from patients with concomitant CRC at another site of the mucosa, and the other from patients without carcinoma, using microvessel counting and total vascular area determination with image analysis software. Our aim was to determine potential differences between adenomatous polyps from the two groups of patients, and weather the presence of carcinoma could influence the vascularization of colorectal adenomas.

Material and Methods

Tissue samples

A retrospective study was performed on 30 low-grade adenomatous polyps removed by endoscopy or surgery from 15 patients with sporadic CRC and 15 patients without carcinoma. The latter group did not show evidence of carcinoma from the time of the procedure throughout a 5-year follow-up. Hamartomatous and inflammatory polyps were excluded from the study.

The samples were selected from the files of the Department of Pathology, State University of Campinas, Campinas, SP, Brazil, and included patients diagnosed from 1987 to 2003. The group of patients with CRC consisted of 8 males and 7 females ranging from 33 to 82 years (median: 61 years); 5 cases were staged as I, 7 as II, 2 as III, and 1 as IV, according to the TNM pathological staging system (25). Only low-grade adenomatous polyps which had been removed from the colorectal mucosa concomitantly to or soon after the diagnosis of the main malignant lesion, without the effect of neoadjuvant therapy were included. There were 8 tubular, 6 tubulovillous, and 1 villous adenomas.

In the group of patients without a diagnosis of CRC 9 were males and 6 were females ranging from 20 to 82 years (median: 56 years). There were 11 tubular and 4 tubulovillous adenomas.

Immunohistochemistry

Tissue specimens had been fixed in 10% formalin and embedded in paraffin and 3-µm thick sections were placed on silanized slides. Endogenous peroxidase activity was quenched by incubating the slides with 3% H_2O_2 for 10 min. Antigen retrieval was achieved by microwaving tissue sections in 10 mM citrate buffer, pH 6.0, in four cycles of 5 min each. Sections were incubated at room temperature for 20 min with mouse monoclonal antibodies to CD105 (Endoglin, Clone SN6h, Dako, USA; diluted 1:15) and to D2-40 (Dako, diluted 1:400). Antigen-antibody binding was detected using the Advance system (Dako). Internal and external positive and negative controls were run concomitantly in each reaction batch.

Evaluation of immunohistochemistry

Digital images from two "hot spot" fields stained by each marker were captured. One area corresponded to the upper/inner portions of the lesions, and the other to the deeper area of the polyps. The upper/inner areas were grouped together because there were some small adenomas in which both areas appeared in the same image. Digitalization was done at 200X magnification, 120 dpi, using a digital camera (Leica DFC360 FX, Leica, Germany) connected to a bright field microscope (Leica DM5000 B).

The images were examined with image analysis software (Leica QWin Standard V3, Microsystem Imaging, Leica) set to detect color intensities in a fixed and constant range. Every image was evaluated using this standardized program to quantify the proportion between stained and total areas and to assess the number of microvessels. Immunostained blood and lymphatic vessels were marked with a circle by the pathologist who analyzed the image to perform automated quantification. An example of the resulting image prepared for analysis after selection of the immunostained vessels is shown in Figure 1.

This resulted in the evaluation of two parameters for each marker: microvessel density (MVD) and total vascular area (TVA).

Statistical analysis

Statistical analysis was performed using the SAS System for Windows software package (version 9.1.3). For the quantitative parameters, the minimum and maximum values, mean, standard deviation and median were analyzed. For the qualitative variables, the absolute and relative frequencies were analyzed. The non-parametric Mann-Whitney test was used to compare two groups and the Kruskal-Wallis test was used for three or more groups. The Dunn comparison test was used for multiple comparisons. The level of significance was set at 5% in all analyses.

Results

CD105 in adenomas from patients with CRC

MVD ranged from 0 to 15 (median 5; mean 5. 53) in "hot spots" of the upper/inner parts of adenomas and from 0 to 37 (median 1; mean 5.40) in the deeper areas. TVA ranged from 0 to 219905 μm^2 (median 7498; mean 25681.33 μm^2) in the "hot spots" of the upper/inner regions and from 0 to 27255 μm^2 (median 755; mean 6215 μm^2) in the deeper area.

CD105 in adenomas from patients without CRC

MVD ranged from 0 to 10 (median 2; mean 3.20) in "hot spots" of the upper/inner part of adenomas and from 0 to 10 (median 1; mean 2.53) in the deeper area. TVA ranged from 0 to 19660 μm^2 (median 183; mean 3810.20 μm^2), in the "hot spots" of the upper/inner parts and from 0 to 66522 μm^2 (median 143; mean 8114.40 μm^2) in the deeper area. The results for CD105 are summarized in Table 1.

D2-40 in adenomas in patients with CRC

MVD ranged from 4 to 177 (median 12; mean 29.20) in "hot spots" of the upper/inner parts of the adenoma and TVA ranged from 85 to 40691 μm^2 (median 3914; mean 9232 μm^2). MVD ranged from 1 to 37 (median 12; mean 14.67) in "hot spots" of the deeper area, and TVA ranged from 676 to 64650 μm^2 (median 9563; mean 18473.73 μm^2).

D2-40 in adenomas from patients without CRC

MVD ranged from 1 to 18 (median 8; mean 8.40) in "hot spots" of the upper/inner parts of the adenoma and TVA ranged from 1 to 53112 μm^2 (median 5836; mean 11592.47 μm^2). MVD ranged from 2 to 14 (median 8; mean 8.27) in "hot spots" of the deeper area and TVA ranged from 265 to 44491 μm^2 (median 8687; mean 14430.73 μm^2). The results for D2-40 are summarized in Table 2.

A plate with illustrations of cases with lower and higher vascularization using both markers is shown in Figure 2.

Statistical analysis of these data showed significantly higher values of TVA determined by Immunostaining for CD105 (p = 0.019) and of MVD determined by Immunostaining for D2-40 (p = 0.041) when compared with those from patients without CRC.

For both markers, there was no significant difference among histological types of adenoma (tubular, tubulovillous and villous) and MVD or TVA counts, in the groups of patients with and without CRC.

The cut-off value for TVA determined by CD105 in the upper/inner parts of the adenomas was 4386, or approximately 4400 μm^2 (sensitivity and specificity: 66.7%; predictive positive and predictive negative values: 66.7%; accuracy: 66.7%). The cut-off value of MVD determined by D2-40 in the upper/inner parts of the adenomas was 11.5 (60.0% sensitivity, 66.7% specificity, 64.3% predictive positive value, 62.5% predictive negative value, and 63.3% accuracy).

Discussion

The present data indicate a significant increase in blood microvascular area and in lymphatic microvascular counts in the upper and inner portions of adenomas removed from patients with CRC compared to those without carcinoma.

Cancer cells might have an influence on the vascularization of adenomas, evidence supported by data showing increased levels of angiogenic factors in colorectal tissues distant from the primary tumor. Hanrahan et al. (26) showed that vascular endothelial growth factor (VEGF) plays a role early in tumor development at the stage of adenoma formation. Moreover, increased levels of VEGF in normal tissue collected from sites distant from the primary tumor have indicated environmental changes that could help explain our findings, although this was not directly assessed in our material.

The more significant increase in lymphatic MVD in the upper/inner areas of the adenomas is in keeping with a previous study reporting a more superficial location of lymphatic vessels in adenomas. This finding supports the hypothesis sustained by Fogt et al. (20) that superficial lymphatic vessels may be immature in normal colonic mucosa and may not communicate with deeper vessels, changing and maturing through the adenoma-carcinoma process. An equivalent assumption could be made about the increase in newly formed blood vessels detected by CD105, which suggests that they may originate superficially on adenomas, developing and meeting deeper vessels during the progression of malignancy.

The assessment of TVA using immunostaining for CD105 showed significantly higher values in adenomas from patients with CRC, while assessment of MVD did not. The opposite was seen in the assessment of lymphatic vessels using the D2-40 antibody: in contrast to MVD, TVA did not differ significantly between the two groups of lesions.

These differences might reflect variations in the mechanisms of proliferation of blood and lymphatic vessels, the former affecting predominantly architectural scores, and the latter numerical scores. Unlike normal blood vessels, newly formed blood vessels incorporated during tumor angiogenesis are tortuous and

dilated, a fact that could explain the higher value of TVA using CD105 in patients with CRC, an aspect supported by experimental studies (27). The higher MVD evaluated by D2-40 in patients with CRC could be explained by recent evidence showing elevated lymphatic vessel counts as an event preceding the increased number of blood vessels in early gastrointestinal tumors (28). It should be noted that computer image analysis seems to be more objective and reproducible, reducing to minimum intraobserver variability from case to case, and increasing the reliability of information in the study of angiogenesis (29).

The findings reported in the present study support the notion that neoangiogenesis and elevated lymphatic vessel counts occur in colorectal adenomas from patients with CRC, allowing us to assume that either angiogenic factors produced by the carcinoma or constitutional defects of the colorectal epithelial cells might account for these observations.

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Table 1. Microvessel density (MVD; number of microvessels counted) and total vascular area (TVA; μm^2) determined by immunostaining for CD105 in the 2 areas (upper/inner and deeper) of adenomas in 15 patients with CRC and 15 patients with no CRC.

Variables	Area	Patient groups	Range	Mean ± SD	Median
MVD	Upper/inner	CRC	0-15	5.53 ± 5.22	5
		No CRC	0-10	3.20 ± 3.41	2
	Deeper	CRC	0-37	5.40 ± 9.56	1
		No CRC	0-10	2.53 ± 3.27	1
TVA	Upper/inner	CRC	0-219905	25681.33 ± 55338.71*	7498
		No CRC	0-19660	3810.20 ± 5910.57	183
	Deeper	CRC	0-27255	6215 ± 9110.95	755
		No CRC	0-66522	8114.40 ± 17675.36	143

*p<0.05 compared to No CRC (non-parametric Mann-Whitney test)

CRC = colorectal cancer; No CRC = no colorectal cancer; SD = standard deviation

Table 2. Lymphatic microvessel density (MVD; number of lymphatic microvessels counted) and total vascular area (TVA; μm^2) determined by immunostaining for D2-40 in the 2 areas (upper/inner and deeper) of adenomas in 15 patients with CRC and 15 patients with no CRC.

Variables	Area	Patient groups	Range	Mean ± SD	Median
MVD	Upper/inner	CRC	4-177	29.20 ± 44.74*	12
		No CRC	1-18	8.40 ± 5.58	8
	Deeper	CRC	1-37	14.67 ± 10.31	12
		No CRC	2-14	8.27 ± 3.28	8
TVA	Upper/inner	CRC	85-40691	9232.00 ± 11023.34	3914
		No CRC	1-53112	11592.47 ± 15384.84	5836
	Deeper	CRC	676-64650	18473.73 ± 20906.02	9563
		No CRC	265-44491	14430.73 ± 14044.96	8687

*p<0.05 compared to No CRC (non-parametric Mann-Whitney test)

CRC = colorectal cancer; No CRC = no colorectal cancer; SD = standard deviation.

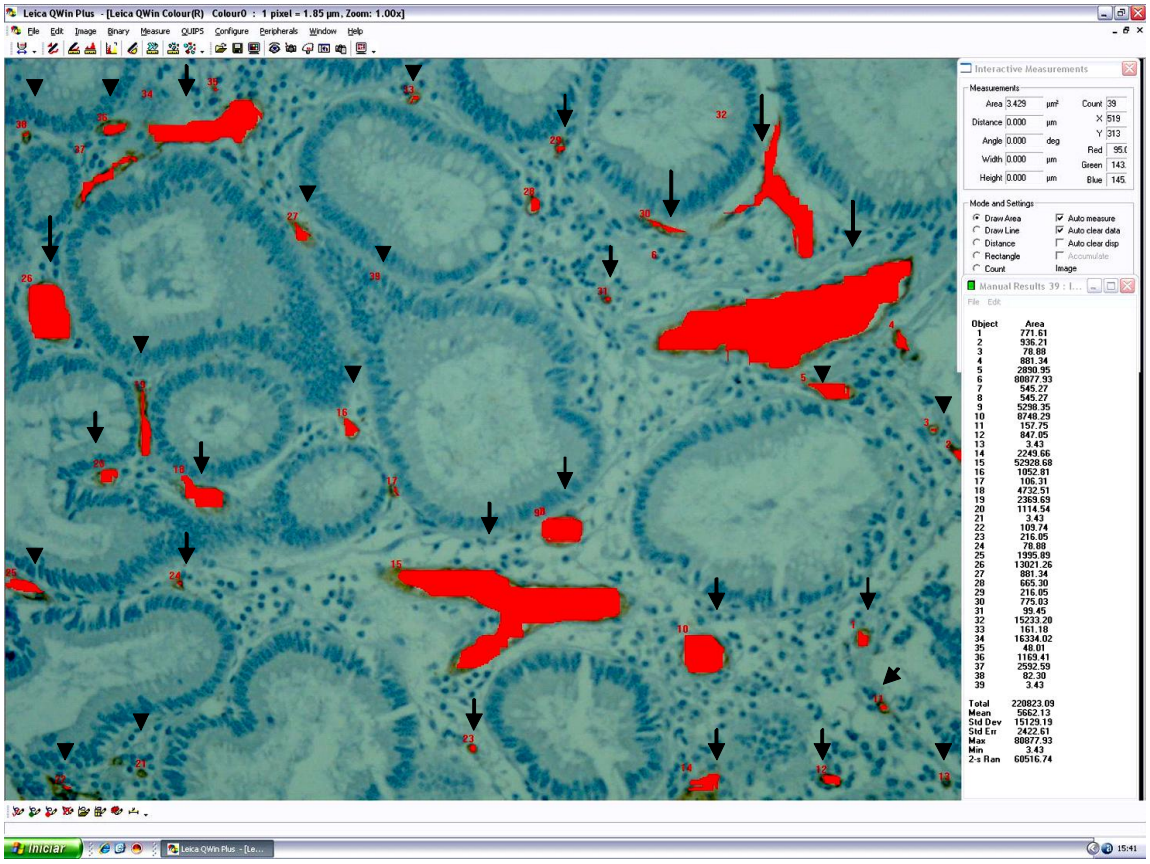

Figure 1. Evaluation of microvessels in immunostained sections by computer image analysis: the positive vessels are automatically delimited and their area was calculated by the software.

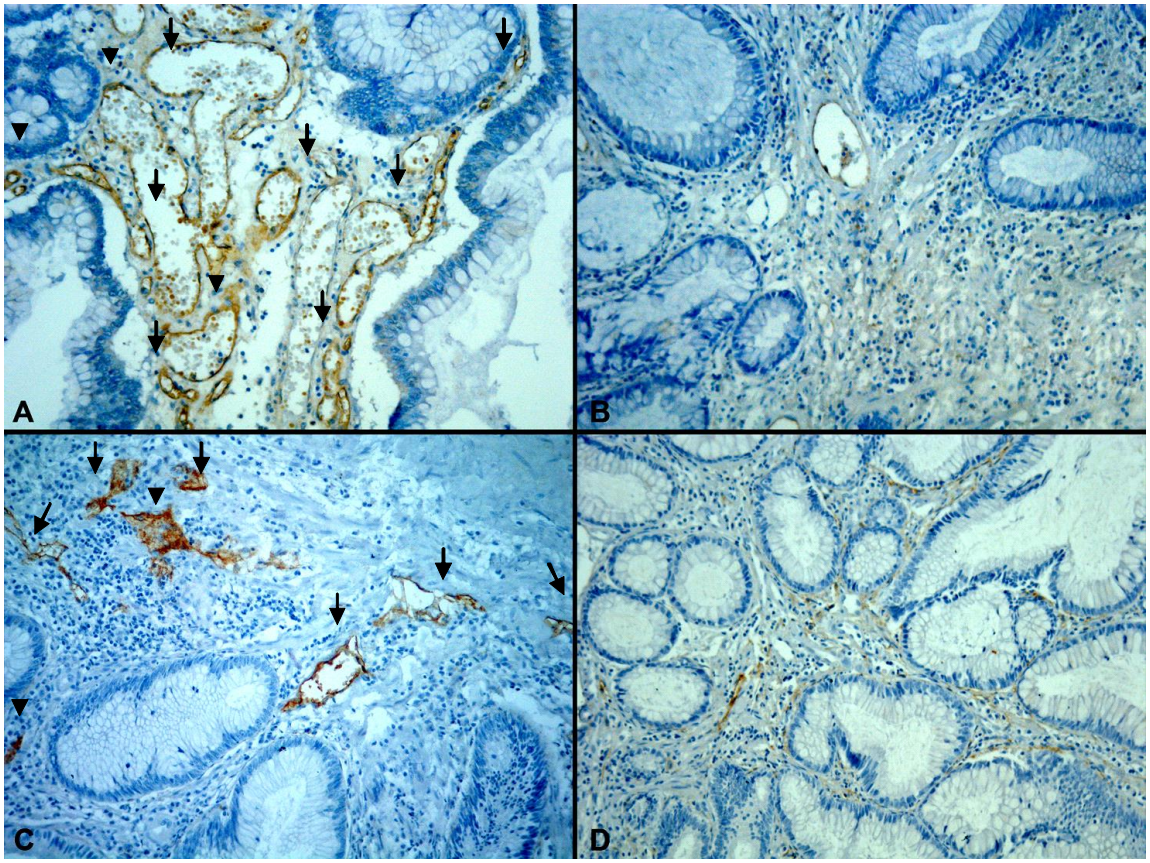

Figure 2. Various degrees of vascularization using CD105 and D2-40: low and high values are shown (original magnification 200X). *A*, CD105, High vascularization (arrows); *B*, CD105, low vascularization; *C*, D2-40, high vascularization (arrows); *D*, D2-40, low vascularization.

3.2. Artigo 2

ANGIOGENESIS AND LYMPHANGIOGENESIS IN COLORECTAL CARCINOMA: COMPARISON BETWEEN QUANTIFICATION METHODS AND DIFFERENT IMMUNOHISTOCHEMICAL MARKERS WITH ANATOMOPATHOLOGIC PROGNOSTIC FACTORS

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Conflict of interests: The authors declare no conflict of interests.

Authors' contributions:

LRM and JV participated in all phases of the study, from design to final manuscript; AAS and PLF participated mainly in the preparation and analysis of

immunohistochemical reactions; CSPL was responsible for clinical follow-up and data management; MAST reviewed all histopathological diagnoses. All authors read and are in accordance with the final manuscript.

Ethical aspects:

The present study was approved by the Committee for Ethics in Medical Research of our Institution (State University of Campinas Medical School)

Abstract

Background: Blood and lymphatic vessels play an important role in the progression of solid tumors, and have been considered as potential targets for therapy. Thus, reliable evaluation of these parameters may have an impact on patients' management. Analysis of angiogenesis and lymphangiogenesis in colorectal cancer (CRC) is controversial in the literature, which may be due to variations in the methods of analysis: the precise location within the tumor sample, the choice of immunohistochemical markers and the method of quantification have been differently evaluated in the literature. **Objectives:** Therefore, in the present study it was aimed to search for a reliable approach in the quantification of angiogenesis and lymphangiogenesis as prognostic factor in CRC. It was also intended to compare these parameters between non neoplastic tissue, adenomas and cancer, in order to contribute to the understanding of angio- and lymphangiogenesis in the progression of these lesions. **Methods:** 60 sporadic CRC, 30 colorectal adenomas and 10 colorectal non neoplastic tissues were submitted to immunohistochemical evaluation of CD31, CD34, CD105, VEGF-A, VEGF-C and D2-40. Microvessel density (MVD) and total vascular area (TVA) were determined by computer image analysis for all markers. **Results:** The majority of markers showed progressive vessel counts from non neoplastic tissue to carcinoma, both for MVD and TVA. Only MVD determined by immunostaining for CD34 in the central areas of the lesion was significantly correlated with recurrence or metastasis (p=0.04) and survival

rates (p=0.02) in patients with CRC. **Conclusions:** Our results corroborate the increasing in vascularization of carcinoma and suggest that MVD determined with staining for CD34 in the inner part of the tumor might represent a valuable parameter to be considered in the management of patients with CRC, as it is more closely related with relapse/metastasis and survival.

Keywords: angiogenesis; lymphangiogenesis; colorectal cancer; immunohistochemistry; CD31; CD34; CD105; VEGF-A; VEGF-C; D2-40.

Introduction

Colorectal carcinoma (CRC) is an important cause of mortality worldwide.[1] The fact that tumor growth is dependent on angiogenesis has supported recent researches for new prognostic parameters and in the development of novel therapeutic strategies. This is welcome, in view that 20%-30% of patients with CRC treated with potentially curative surgery, will succumb from recurrent disease, suggesting that the conventional prognostic factors may not be sufficient, and that additional parameters, either morphological or molecular, are needed for clinical management.[2, 3] In spite of its importance, data concerning the prognostic value of parameters related to angiogenesis in CRC remain controversial.

The vascular endothelial growth factor (VEGF) has been identified as an important family of glycoproteins stimulating vascularity. The VEGF family includes VEGF-A (or VEGF) and VEGF-B, both ligands for receptor VEGF-R1, which mediates angiogenesis; VEGF-C and VEGF-D, both important members binding to the receptor VEGF-R3, which is mainly involved in lymphangiogenesis. VEGF-A signaling promoting endothelial proliferation, migration and survival is predominantly transmitted via VEGF-R2.[4] A variable proportion of cancer cells present a cytoplasmic immunostaining for VEGF, which is progressively expressed in adenomas and in CRC, when compared to colorectal normal tissue.[5, 6] Protein expression of VEGF has been associated with worse prognosis in most studies[7-9], but this has not always been the case, probably due to the different thresholds of positivity used in the studies.[10-12] VEGF-C protein expression has also involved some controversial data in the literature, with some reports correlating its expression with lymphatic involvement or the presence of metastasis, and others not.[13-19]

Vascular quantification has been the matter of diverging results, some manuscripts showing correlation of microvessel counting with poorer outcome or lymph nodes metastases.[20 -23] It has been also stated that outcome of patients with CRC was not correlated with vascular counting, but with vessel ramification and the total vascular area (TVA).[24] In contrast, others have shown that higher

microvascular counting was correlated with favorable outcome.[25, 26]

CD105 or Endoglin, a transmembrane protein highly expressed on human vascular endothelium, is up-regulated in tumor vasculature and proliferating cells, suggesting the possibility to distinguish newly formed tumor associated endothelial cells from pre-existing vessels.[27, 28] Recently, CD105 was showed in tumor lymphatics, suggesting that is not confined to the blood vasculature.[29] CD105+ microvessels have been preferentially observed in the surface area, while CD34+ microvessels were evenly distributed in adenomas. In carcinomas, expression of CD105, but not of CD34, presented significantly higher values in the adenoma-carcinoma sequence.[27] The microvessel counting assessed by anti-CD105 was shown as independent prognostic parameter for survival in CRC, in contrast to CD34.[28] CD105+ vessel counts have been equally strongly correlated with the occurrence of metastatic disease.[30] On the contrary, others have shown no significant correlation between CD105+ vessel counts and clinicopathologic characteristics.[31]

D2-40 is a monoclonal antibody directed to the M2A antigen, a surface sialoglycoprotein originally detected in germ cell neoplasia and fetal testicular gonocytes.[32] It has been also demonstrated to selectively immunoreact with the lymphatic endothelium, but not with blood vascular endothelium. Lymphatic vessel density was correlated with poor outcome and metastatic disease in colorectal cancer, [33-35] but the relationship between this parameter and VEGF-C is unclear. [22] It has been reported that lymphatic vessels labeled with D2-40 are more superficially located in adenomas and carcinomas than previously suspected, since intramucosal carcinomas do not metastasize.[36]

In view of the importance of the evaluation of angiogenesis as a potential prognostic and predictive parameter, and taking into account the controversial reports summarized above, it was the purpose of the present study to appraise the clinical value of angiogenesis in CRC comparing methods of assessment (microvascular density and total vascular area), and different immunohistochemical markers to detect angio- and lymphangiogenesis. It was also intended to compare

angio- and lymphangiogenesis counts between non neoplastic colorectal tissue, adenomas and carcinomas.

Materials and methods

Patient selection and tissue samples: A retrospective study was performed on 60 surgically resected sporadic colorectal carcinomas (**group 1**); 30 adenomatous polyps (**group 2**, obtained by polypectomy or surgical resection) and 10 non neoplastic colorectal tissues (**group 3**, obtained from surgery for benign conditions). All samples were selected from the files of the Department of Anatomical Pathology, State University of Campinas Hospital (Unicamp), São Paulo, Brazil, diagnosed from 1987 to 2003. The study was approved by the institutional Ethics Committee for Medical Research. Patients from **group 1** included 27 (45%) males and 33 (55%) females, with mean age of 60 years (range 24 to 81 y). Tumors consisted of adenocarcinomas primarily categorized according to the classification of the World Health Organization; pathological staging was based on the TNM classification.[37] No patient had received chemo- or radiation therapy before surgery. Eleven cases were staged as I (18.3%), 24 as II (40%), 20 as III (33.3%), and 5 as IV (8.3%). Follow up of patients ranged from 3 months to 13 years (median 5.34 y); 31 patients deceased. Routinely stained slides were revised, and, from each case, one tissue sample was selected, which included the deepest invasive tumor area, avoiding regions with prominent inflammation and necrosis. The whole section was submitted to vascular analysis, as recommended elsewhere.[38] **Group 2** consisted of 17 male (56.66%) and 13 female (43.33%) patients with mean age of 59 y (range 20 to 82 y). Nineteen cases were classified as tubular adenoma (63.33%), 10 as tubulovillous (33.33%) and one as villous adenoma (3.33%). Colorectal non neoplastic tissue (**group 3**) was obtained from five male and five female patients, with mean age of 55.5 y (range 32 to 71 y).

Immunohistochemistry: Tissue specimens were fixed in 10% formalin and

embedded in paraffin. Sections of three µm thick were placed on silanized slides. Endogenous peroxidase activity was quenched by incubating the slides in 3% H_2O_2 for 10 minutes. Antigen retrieval was achieved by microwaving tissue sections in 10 mM citrate buffer (pH= 6.0), four cycles of five minutes each. Sections were incubated at room temperature for 30 minutes and then overnight at 8 – 10^0 C with mouse monoclonal antibodies to CD31 (clone JC70A, Dako, Carpenteria, CA, USA, diluted at 1:20); CD34 (clone QBEnd 10, Dako, diluted at 1:100); CD105 (Endoglin, Clone SN6h, Dako, diluted at 1:15); D2-40 (clone D2-40, Dako, diluted at 1:400); VEGF-A (clone VG1, Dako, diluted at 1:25) and VEGF-C (clone Z-CVC7, Invitrogen, Carlsbad, CA, USA, diluted at 1:100). Antigen-antibody binding was detected using the Advance system (Dako). Internal and external positive controls included endothelial vascular cells, endothelial lymphatic cells and cases previously positive for VEGF-A and C. Negative controls were represented by the same tissue sample used for positive control, in which primary antibody was omitted.

Evaluation of Immunohistochemistry: Digital images from "hot spot" fields in different cancer areas (inner, periphery and area of deeper invasion) were captured at magnification x200, using a digital camera (Leica DFC360 FX, Solms, Germany) connected to a bright field microscope (Leica DM5000 B). Digital images from adenomas were captured at inner portions and peripheral areas. Images from the non neoplastic tissue samples were captured from the mucosa and the submucosa/muscular. The images were examined by image analysis software (Leica QWin Standard V3, Microsystem Imaging) setup to detect color intensities in a fixed and constant range. Every image was evaluated using this program to quantify the total vascular area (TVA) stained and to assess microvessel density (MVD). Blood and lymphatic vessel cross-sections were counted by a semi-automated procedure in the program. An example is shown in Figure 1.

Staining for VEGF-A and VEGF-C was evaluated using a visual score in four grades for percentage of positive cells: grade 1, no staining or less than

25% of the tumor area positive; grade 2, 25-50% of the tumor area stained; grade 3, 50-75% of the tumor area stained; grade 4, more than 75% of the tumor area stained. The four grades were used for intensity: grade 1, no staining; grade 2, weak staining; grade 3, moderate staining; grade 4, strong staining. Finally, a total score was obtained by adding the two scores. This visual system was validated by correlating the above scores in 20 cases with values obtained using the ACIS® Automated Cellular Imaging System (Dako; Figure 2).

***Statistical methods*:** Statistical analysis was performed using the SAS System for Windows software package (version 9.1.3). For the quantitative parameters, the minimum and maximum values, mean, standard-deviation (SD) and median were analyzed. For the qualitative variables, the absolute and relative frequencies were analyzed. The Pearson coefficient was used to evaluate the correlation between the visual and computer assessment of immunostaining for VEGF (see Figure 2). For the other parameters the nonparametric Mann-Whitney test to compare two groups and the Kruskal-Wallis test for three or more groups were used. The Dunn's comparison test was used for multiple comparisons. The Kaplan-Meier method was used to calculate survival curves and log-tests were performed on the data. The Cox adjusted regression was used for multivariate analysis. Significance level was set at a minimum of 5%.

Results

Pathologic colorectal tissue (groups 1+2) *vs.* colorectal non neoplastic tissues (group 3):

Evaluation of TVA and MVD stained with anti-CD105 in all fields, as well as the mean value of the three fields, showed significant higher values in pathologic colorectal tissues than in normal tissues ($p \leq 0.001$). The same results were obtained for MVD in immunostainings with D2-40 ($0.001 \leq p \leq 0.005$), TVA in immunostainings with anti-CD31 ($0.001 \leq p \leq 0.02$), TVA with anti-CD34 in the inner

field (p= 0.034) and the VEGF-A, VEGF-C grades ($p \leq 0.008$). (Tables 1 and 2).

Cancer (group 1) *vs.* adenomas (group 2) *vs.* colorectal non neoplastic tissues (group 3):

There was significant increase in values between CD105+ TVA and MVD in all fields ($p< 0.001$), CD31+ TVA and MVD in all fields ($0.001 \leq p \leq 0.003$), except in peripheral TVA ($p = 0.051$), CD34+ TVA and MVD in all fields ($0.006 \leq p \leq 0.045$), except in peripheral MVD ($p = 0.115$). D2-40+ MVD in all fields ($0.001 \leq p \leq 0.004$) and the VEGF-A, VEGF-C grades ($p \leq 0.001$) increase significantly in the sequence from non neoplastic tissue to adenoma and cancer. The other parameters did not show significant correlation. (Tables 1 and 2).

Cancer stages I+II *vs* III+IV:

For practical statistical analysis, we grouped the stages in ***low*** (I+II) and ***high*** (III+IV). Tumor staging (I+II *vs* III+IV) showed significant correlation with overall survival (OS) ($p = 0.0003$). There was no significant correlation between the angiogenic parameters for both stage groups. CD34+ MVD in the inner part of the tumor presented higher values in more advanced stages of cancer, although this difference did not reach significance (p= 0.098). (Table 3).

Tumor type:

CD105+ MVD at the periphery of the tumor showed higher values in poorly differentiated carcinoma than in the mucinous type (p= 0.036). The same was found for CD31+ TVA at the periphery of the tumor (p= 0.032), CD31+ MVD in all fields ($0.010 \leq p \leq 0.043$). CD34+ MVD in the deeper area of invasion showed also greater values in poorly differentiated carcinoma than in the mucinous type (p= 0.029). (Table 3).

Cancer relapse and metastasis:

CD34+ MVD in the inner part of the tumor showed significantly higher values in patients with relapse of the tumor or development of metastasis.

Patients without relapses or metastasis showed median 32.50 μm^2, mean 35.75μm^2 and SD= 15.63. Patients with relapse or metastasis presented median 42.50 μm^2, mean 43.89 μm^2 and SD= 16.52 (p= 0.04). Likewise, the MVD mean values of all three fields stained for CD34 were higher in patients who developed metastasis or relapse (p= 0.050). The other parameters did not show significant correlations. (Table 3)

Cancer overall survival:

Analysis preformed by Kaplan-Meier survival rate showed significant association only with high CD34+ MVD in the inner part of the tumor (p= 0.024). The median rate of MVD and TVA assessed by CD31, CD34, CD105 and D2-40 in all cancer fields (inner, periphery, deeper invasion and the mean of the three fields) were chosen as the cutoff point. The median rate of tumor cells expressing VEGF-A and VEGF-C percentage, intensity and the total score were also chosen as cutoff.

The median rate of MVD assessed by CD34 in inner field was 37 microvessels (range 12-85) and was the only significant association with overall survival (p= 0.024).

Adjusted Cox regression analysis showed that CD34+ MVD (hazard ratio (HR) = 3.36; 95% confidence interval (CI) =1.01-11,20; p= 0.048) and cancer stage (HR= 6.89; 95% CI = 1.91-24,90; p= 0.003) were significant prognostic factors for overall survival. (Table 4 and Figure 3)

VEGF-A and VEGF-C did not show significant correlations with clinicopathological factors in cancer. (Table 5)

Discussion

The data presented herein indicate that, concerning CRC, evaluation of MVD assessed by immunostaining for CD34 in the inner part of the tumor may represent an independent prognostic factor. This result is important, since it may

correspond to an additional parameter for clinicopathological risk evaluation, especially for those patients which, in spite of the rather favorable indicators at diagnosis and first therapeutic approach, will present adverse outcome. In addition, the present study contribute to unravel the controversial data of the related literature, since the same group of patients was studied with a variety of quantification methods and immunohistochemical markers for blood and lymphatic vessels.

While both blood and lymphatic vessels showed increased values in the progression from non neoplastic tissue to adenoma and cancer, only MVD using anti-CD34 proved to significantly correlate with the presence of relapse or metastasis and with overall survival in the group of patients with CRC. Despite previous studies have shown significant correlation between clinicopathological features and CD105 or CD31, [23,30] our data are more in accordance with recent studies in this aspect.[31,39] The present results further corroborate the recommendation to use CD34 as the eligible marker for the evaluation of tumor angiogenesis.[40]

That the use of the different vessel markers is not necessarily interchangeable may reflect variations in the mechanisms of proliferation of blood vessels in tumors, and in the spectrum of immunoreactivity of each marker. Unlike normal blood vessels, newly formed blood vessels incorporated during tumor progression are reported as tortuous and dilated.[40] This notion is corroborated by the significant increase in TVA values assessed with CD105 in the sequence from normal mucosa to cancer, found herein. Furthermore, it has already been shown that CD105 was expressed only in CD34-positive vessels, being unrelated to the expression of CD31 by endothelial cells.[41] Thus, although also considered a pan-endothelial marker, CD31 may not have its use superimposed to CD34. The latter seems to present a broader spectrum of reactivity in endothelial cells, supporting once more its eligibility in the evaluation of tumor angiogenesis.

While some studies emphasize the significance of evaluating angiogenesis at the front of the lesion, our results clearly demonstrate that the inner portion

may account for the most relevant changes in vascularization during tumor progression.[8] It was suggested elsewhere that the inner areas of carcinoma maintain the vasculature through a continuous “remodeling” of existing vessels, and migration of endothelial cells.[40]

CD34 is expressed by endothelial cells, including those positive for CD105, could represent the mechanism of both enhancements of vascular network (remodeling vessels and new endothelium proliferation). Also MVD method proved superior over TVA, especially in the inner areas, reflecting evidences about partitioning of the vessel lumen by insertion of interstitial tissue during carcinoma progression.[40, 42]

The ability of tumors to sustain a high vascular net in their inner portions, in relation to the invading fronts has emerged as an independent prognostic factor in tumors of the lung, colon and breast.[38, 43, 44, 45] A possible explanation for this finding might be a differential production of vascular survival factors (inhibitors of endothelial apoptosis) in the central tumor areas. VEGF, for example, shows properties which are related both to vascular maturation and to inhibition of endothelial degradation through apoptosis.[44]

Although there was no statistically significant correlation between VEGF-A immunostaining and clinicopathological parameters in CRC, microvessel counts were higher in cases positive for VEGF-A. This is in keeping with the stimulating role of VEGF-A in angiogenesis. The lack of significance between expression of VEGF-A and clinicopathological parameters may reflect the fact that in the present study we have examined only its expression by tumor cells. It is known that VEGF-A may be also expressed by platelets, granulocytes, monocytes, mast cells and lymphocytes.[40] Besides, previous studies have shown that VEGF is not the only factor promoting vascular proliferation, and pointed to the participation of a platelet-derived growth factor in colonic neovascularization.[46, 47] To further address the clinical value of the present results as predictive factor, evaluation of MVD assessed by CD34 immunostaining in central tumor areas should be used in future studies in which patients were submitted to additional therapy with angiogenic modulators.

In accordance with previous studies, our data showed no correlation between intratumoral lymphatic vessel counts and clinicopathological features.[15, 33] This may be due to the fact that, while angiogenesis in intratumoral areas is essential for tumor development, lymphatic vessels are not, and are even compressed by proliferating cancer cells. On the other hand, lymph vessels seem more numerous in peritumoral areas, where they account for drainage of tissue fluid, and, eventually, metastatic cells.[15, 33, 34]

In summary, our findings corroborate the clinical value of the assessment of intratumoral MVD using anti-CD34 as an additional prognostic parameter in patients with CRC. The data presented herein also substantiate the concept that the inner areas of the carcinoma maintain the vasculature through a continuous "remodeling" of the existing vessels and migration of endothelial cells, which are better evaluated by a "broad spectrum" endothelial marker, as CD34. The ability of tumors to maintain a high vascular blood density in their inner portions may represent a promising parameter to evaluate tumor angiogenesis.

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Table 1: **Relationship between immunohistochemistry, quantification method and colorectal tissue (non neoplastic, adenomas and carcinomas)**

Marker	Method	Non neoplastic tissue X Pathologic tissue Mann-Whitney test (*p*)	Non neoplastic X Adenomas X Cancer Kruskal-Wallis test (*p*)
CD31	TVA	0.001 ≤ *p*≤ 0.020	0.001< *p*≤ 0.051**
	MVD	0.202 ≤ *p*≤ 0.968	*p*≤ 0.001
CD34	TVA	0.034 ≤ *p*≤ 0.573*	0.015≤ *p*≤ 0.045
	MVD	0.152 ≤ *p*≤ 0.495	0.006≤ *p*≤ 0.115***
CD105	TVA	*p*< 0.001	*p*<0.001
	MVD	*p*≤ 0.001	*p*<0.001
D2-40	TVA	0.053 ≤ *p*≤ 0.198	0.061≤ *p*≤ 0.437
	MVD	0.001 ≤ *p*≤ 0.005	0.001≤ *p*≤ 0.004

Legends:

TVA- Total Vascular Area (μm^2)

MVD- Microvessel Density (number of microvessels counted)

Pathologic tissue- adenomas and carcinomas

[*] Only TVA stained by CD34 in the inner field was significantly different (*p*= 0.034).

[**] Only TVA stained by CD31 in the peripheral field was not significantly different (*p*= 0.051).

[***] Only MVD stained by CD34 in the peripheral field was not significantly different (*p*= 0.115).

Table 2: **Relationship between immunohistochemical quantification parameters and colorectal tissue (non neoplastic, adenomas and carcinomas).**

Marker	Method	Non neoplastic tissue X Pathologic tissue Mann-Whitney test (*p*)	Non neoplastic tissue X Adenomas X Cancer Kruskal-Wallis test (*p*)
VEGF-A	**%**	*p*<0.001	*p*<0.001
	Intensity	*p*<0.001	*p*<0.001
	Total score	*p*<0.001	*p*<0.001
VEGF-C	**%**	*p*=0.008	*p*<0.001
	Intensity	*p*=0.001	*p*=0.001
	Total score	*p*=0.001	*p*<0.001

Table 3: **Relationship between immunohistochemistry, quantification method and clinicopathologic factors in cancer.**

Marker	Method	Field	Cancer **Tumor type [*]** Kruskal-Wallis test (*p*)	Cancer Stage **I+II versus III+IV** Mann-Whitney test (*p*)	Cancer **Relapse/Metastasis** Mann-Whitney test (*p*)	Cancer **Overall survival** Kaplan-Meier survival rate (*p*)
CD31	**TVA**	1	0.249	0.202	0.317	0.926
		2	0.032	0.300	0.899	0.368
		3	0.235	0.248	0.630	0.966
		M	0.233	0.129	0.619	0.771
	MVD	1	0.029	0.880	0.533	0.599
		2	0.043	0.922	0.899	0.602
		3	0.010	0.283	0.338	0.664
		M	0.013	0.584	0.578	0.476
CD34	**TVA**	1	0.764	0.787	0.738	0.149
		2	0.264	0.444	0.317	0.973
		3	0.612	0.833	0.490	0.896
		M	0.242	0.558	0.509	0.545
	MVD	1	0.748	0.098	0.044	0.024
		2	0.740	0.821	0.410	0.478
		3	0.029	0.471	0.624	0.316
		M	0.124	0.663	0.050	0.309
CD105	**TVA**	1	0.615	0.528	0.362	0.719
		2	0.224	0.908	0.399	0.848
		3	0.377	0.563	0.645	0.874
		M	0.318	0.216	0.382	0.367
	MVD	1	0.105	0.329	0.101	0.302
		2	0.036	0.776	0.855	0.631
		3	0.590	0.910	0.127	0.257
		M	0.184	0.416	0.987	0.490
D2-40	**TVA**	1	0.601	0.822	0.609	0.124
		2	0.834	0.845	0.662	0.276
		3	0.273	0.589	0.864	0.096
		M	0.671	0.685	0.588	0.752
	MVD	1	0.208	0.604	0.699	0.118
		2	0.265	0.868	0.858	0.939
		3	0.297	0.503	0.870	0.587
		M	0.155	0.910	0.410	0.138

Legends:

TVA- Total Vascular Area (μm^2)

MVD- Microvessel Density (number of microvessels counted)

Cancer field: 1- central field; 2- periphery; 3- deep invasion; M- mean value of the three fields

[*] Tumor types: well, moderately, poorly differentiated; mucinous

Table 4 - Adjusted Cox regression analysis - CD34 MVD in inner field cancer and stage.

Variable	Coefficients (β)	Hazard ratio	95% CI	*p* value
CD34				
≥37	1,21	3,36	(1,01 ; 11,20)	0,0486
<37	0	1,00	-	-
Stage				
III-IV	1,93	6,89	(1,91 ; 24,90)	0,0032
I-II	0	1,00	-	-
CD34 x Stage	-1,24	0,29	(0,06 ; 1,37)	0,1169

Legend: MVD- Microvessel Density

Adjusted CD34, Adjusted Stage and interaction between the variables.

Table 5 - Relationship between immunohistochemistry, quantification method and clinicopathologic factors in cancer.

Marker	Method	Cancer	Cancer Stage	Cancer	Cancer
				Relapse/	
		Tumor type	I+II versus III+IV	Metastasis	Overall survival
		Kruskal-Wallis test (*p*)	Mann-Whitney test (*p*)	Mann-Whitney test (*p*)	Kaplan-Meier survival rate (*p*)
VEGF-A	%	0.699	0.247	0.313	0.468
	Intensity	0.139	0.797	0.232	0.601
	Total score	0.434	0.438	0.237	0.544
VEGF-C	%	0.162	0.404	0.614	0.963
	Intensity	0.903	0.600	0.940	0.481
	Total score	0.685	0.566	0.969	0.604

Legends for the Figures

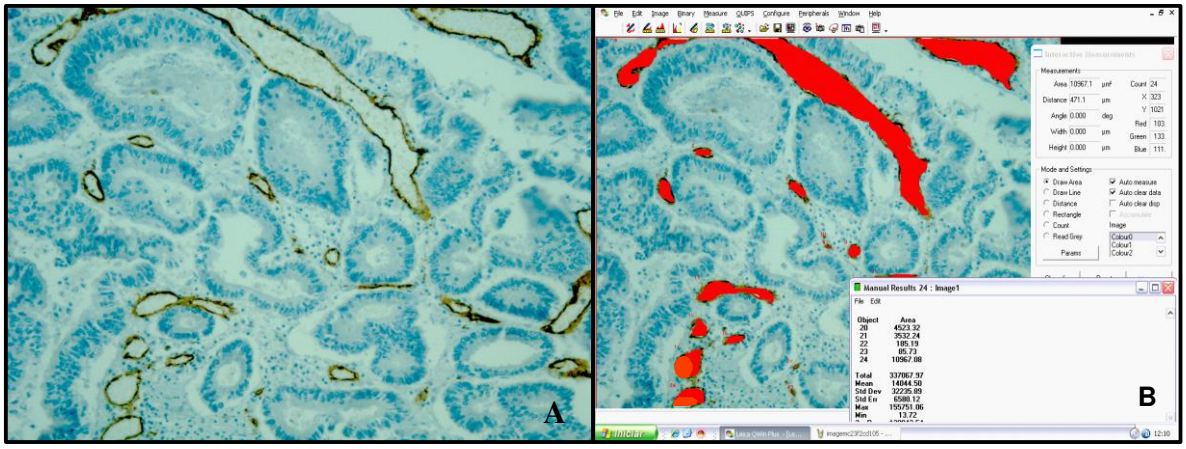


Figure 1: A- Immunostaining for CD34 showing positive vessels (200x). B- Image analysis software to assess the total vascular area (TVA) and microvessel density (MVD).

Figure 2: Significant correlation between visual evaluation and computer assessment of immunostainings for VEGF-A and C.

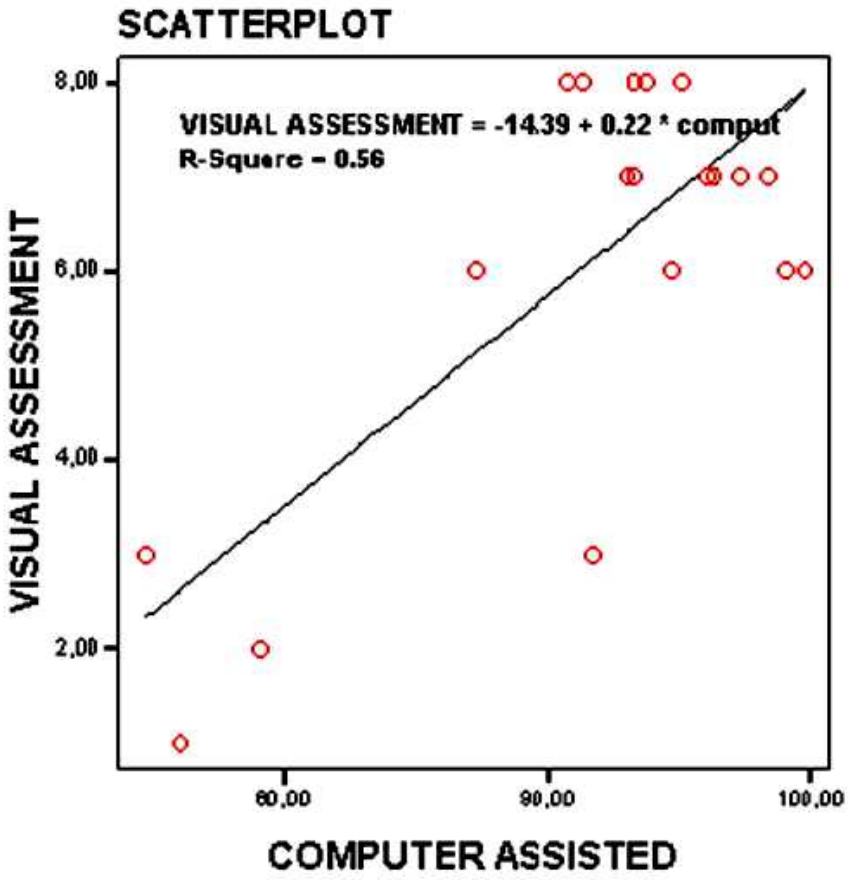
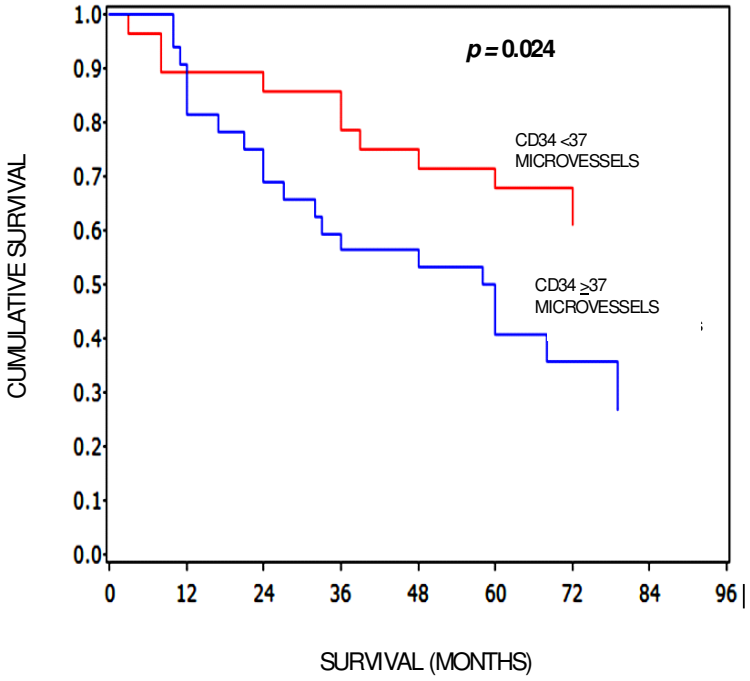


Figure 3: Survival of patients with microvessel density assessed by CD34 in the inner part of the tumour (Kaplan-Meyer)



Time (months)	Cumulative survival (%)	
	<37	≥37
0	100.0	100.0
12	89.3	81.2
24	85.7	68.7
36	78.6	56.2
48	71.4	53.1
60	67.9	50.0
72	61.1	35.5

4. Discussão

O CRC é uma causa importante de mortalidade e incidência entre as neoplasias (2). Aproximadamente 20-30% dos pacientes com esta neoplasia, tratados com cirurgia potencialmente curativa, apresentam recidiva, sugerindo que os fatores prognósticos convencionais não são suficientes e que há necessidade de fatores adicionais (10,11). O fato de a progressão tumoral poder ser dependente da angiogênese e linfangiogênese estimulou pesquisas nesta área, entretanto diversos resultados controversos são encontrados na literatura (50). Os critérios necessários para quantificação destes processos, tais como: escolha do local no tumor a ser quantificada a angiogênse, o marcador imunoistoquímico e o método de quantificação a serem empregados são questões não resolvidas (51).

Nesse estudo analisamos o perfil imunoistoquímico de diferentes marcadores relacionados à angiogênese e linfangiogênese previamente descritos como tendo valor prognóstico controverso no carcinoma colorretal, utilizando métodos de mensuração: área vascular total (TVA) e densidade microvascular (MVD), além da porcentagem e da intensidade de células tumorais positivas para VEGF-A e VEGF-C. Ressaltamos que para a determinação da MVD consideramos qualquer célula ou agrupamento de células endoteliais marcadas e que eram claramente separadas de outros microvasos adjacentes, por ser o método mais comumente utilizado na literatura e com menor possibilidade de viés (30,46,52). Esse viés poderia ser dado pela forte subjetividade embutida na ação de se restringir a contagem apenas aos vasos sem uma camada muscular, pois, como é sabido, esta pode ser

descontínua em arteríolas. Também comparamos estes parâmetros nos tecidos colorretais não neoplásicos, adenomas e carcinomas; nos adenomas de pacientes sem e com carcinomas, além de correlacioná-los com fatores morfológicos e clínicos de utilidade bem estabelecida nos CRC.

Os nossos resultados mostraram que a maioria dos marcadores e métodos exibiu valores progressivamente maiores de angiogênese e linfangiogênese entre tecido não neoplásico, adenoma e CRC. Estes achados sugerem que o aumento na angiogênese e linfangiogênese sejam eventos precoces, ocorrendo nas lesões precursoras e aumentando ainda mais nos carcinomas, corroborando dados prévios da literatura (53).

Também foi observado que os adenomas provenientes de pacientes portadores de carcinomas em outro local da mucosa colorretal apresentaram valores aumentados de TVA determinada pela imunocoloração para CD105 e de MVD determinada com o uso do D2-40, quando comparados aos adenomas de pacientes sem carcinomas. Tais achados favorecem proposições existentes na literatura, de que uma área distinta da mucosa colorretal possa ser influenciada por fatores produzidos pelo tumor em outro sítio da mucosa (53). Apóiam ainda, dados de literatura sobre os vasos sanguíneos neoformados (CD105+) serem tortuosos, justificando a significante superioridade da TVA (39). Por sua vez, os vasos linfáticos apresentam MVD maior D2-40+, sendo um evento que precede o aumento de número de vasos sanguíneos em lesão gastrintestinal iniciais, segundo anteriormente proposto (54).

Entretanto, quando analisados os carcinomas, apenas a MVD determinada pelo marcador CD34 no campo central da lesão diferencia-se estatisticamente segundo a recorrência/ metástase (p= 0,04) e curva de sobrevida (p= 0,02). Estes dados não apóiam estudos prévios que demonstraram o valor prognóstico do uso dos demais marcadores imunoistoquímicos (35,43), mas corroboram estudos mais recentes que recomendam o uso do marcador CD34 (44,52).

O CD31 corresponde ao marcador PECAM-1, sendo também considerado um marcador pan-endotelial. Participa da adesão leucócito-endotélio na diapedese e, além disso, é expresso em monócito e células *Natural-Killer,* tendo relação com a imunidade tumoral (52). Este fato pode ter influenciado o resultado no CRC, uma vez que seu espectro de distribuição e função difere do CD34.

Há trabalhos sugerindo que, com o crescimento tumoral, as áreas centrais dos carcinomas mantêm sua vascularização através de remodelagem contínua de vasos existentes e de migração de células endoteliais. A habilidade do tumor em manter esta vascularização central tem sido relatada como um fator de prognóstico independente em vários tipos de tumor, incluindo pulmão, cólon e mama (51,55,56,57).

Sugere-se ainda que, com o crescimento tumoral, haja aumento da pressão intersticial e os vasos sanguíneos sofram remodelagem com rupturas de seu lúmen e ramificações (58,39).

Estas constatações explicam o fato de a MVD determinada pelo marcador CD34 diferenciar-se estatisticamente e ser um fator de prognóstico, sobretudo

na área central dos carcinomas, enquanto que o CD105 (marcador de vasos neoformados) não conduziu a este resultado.

O fato de não termos obtido resultados estatisticamente significantes entre o estudo imunoistoquímico dos fatores de crescimento vascular e parâmetros morfológicos e clínicos no CRC deve refletir a complexidade do controle da angiogênese, uma vez que foi relatado que o VEGF não é o único fator promotor de crescimento vascular. Outros componentes, como o Fator de crescimento derivado de plaquetas, participam desse controle (59,60). Além disto, neste estudo foi analisada a expressão do VEGF nas células tumorais, sem considerar outras células que poderiam expressar VEGF, como granulócitos, o que pode ser responsável pela falta de correlação citada (39).

Nossos dados não mostraram correlação estatisticamente significante dos vasos linfáticos intratumorais com fatores prognósticos estabelecidos para o CRC, em acordo com trabalhos anteriores (30,46). Há estudos postulando que enquanto os vasos sanguíneos são essenciais para o crescimento do tumor, os vasos linfáticos não o são. Com a progressão tumoral e o aumento da pressão intersticial, eles seriam mais numerosos nas áreas peritumorais, onde há maior drenagem de fluidos teciduais e, eventualmente, de células metastáticas (30,46,47).

Os presentes achados apóiam o fato de a angiogênese e o aumento da vascularização linfática ocorrer precocemente, ainda nos adenomas. Também corroboram o fato de a angiogênese e o aumento da contagem de vasos linfáticos ocorrerem mais pronunciadamente em adenomas de pacientes com carcinomas na

mucosa adjacente, possivelmente influenciados por fatores produzidos pelo tumor. A MVD na área central do carcinoma determinada pelo marcador imunoistoquímico CD34 adiciona um critério prognóstico, associando-se com recidiva/ metástase e sobrevida, enquanto os outros meios de quantificação vascular e de expressão de fatores de crescimento não apresentaram resultados estatisticamente significantes. Este representa um fator prognóstico adicional independente no CRC.

Além do valor prognóstico determinado com o uso do marcador CD34 no CRC, o presente trabalho possibilita um melhor entendimento de eventos fisiopatológicos relacionados à angiogênese e linfagiogênese e pode contribuir com linhas de pesquisa futuras no campo da terapêutica anti-angiogênica.

5. Conclusões

- A maioria dos marcadores vasculares e dos métodos de quantificação da angiogênese e linfangiogênese exibiu valores progressivamente maiores desde o tecido não neoplásico, ao adenoma e ao CRC, sugerindo que o incremento da vascularização é um evento precoce na carcinogênese colorretal.

- Adenomas de pacientes com carcinoma colorretal mostraram valores significantemente maiores da área vascular determinados com um marcador de CD105 e da densidade linfática determinada com o marcador D2-40, quando comparados com adenomas de pacientes sem carcinomas, sugerindo a influência de fatores tumorais nos adenomas distantes das lesões carcinomatosas.

- Quando analisados os carcinomas, apenas a MVD determinada pelo marcador de CD34 no campo central da lesão diferencia-se estatisticamente segundo a recorrência/ metástase e curva de sobrevida. Este método adiciona um fator de valor prognóstico independente no CRC.

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7. Anexos

ANEXO 1: Dados clínicos de pacientes com CCR

Biópsia	Sexo	Idade ao diagnóstico	Local do tumor	Estadio	Estadio final	Tipo	Tempo seguido	Recidiva/ metástase	Sobrevida
G23469/98	M	59a	reto	T4N0M0	II	adenoca.tub. mod. dif.	6a6m	não	vivo
B40497/90	F	53a	sigmóide	T2N0M0	I	adenoca.tub. mod. dif.	13a	não	vivo
B5780/95	M	33a	cólon D	T3N2M0	III	adenoca.tub. mod. dif.	5a11m	não	vivo
G26205/99	F	40a	reto	T3N1M0	III	adenoca.tub. mod. dif.	6a11m	sim	vivo
G644/92	F	68a	cólon D	T3N0M0	II	adenoca.tub. mod. dif.	13a	não	vivo
G21458/98	F	70a	sigmóide	T4N0M0	II	adenoca.tub. bem dif.	6a11m	não	vivo
G29617/00	M	70a	sigmóide	T3N0M0	II	adenoca.tub. bem dif.	5a2m	não	vivo
G29562/00	M	46a	cólon D	T4N0M0	II	mucinoso	5a	sim	óbito
G28418/00	M	57a	sigmóide	T3N2M0	III	adenoca.tub. mod. dif.	5a	sim	óbito
G28748/00	M	41a	cólon transverso	T2N0M0	I	adenoca.tub. mod. dif.	5a6m	sim	vivo
G37067/03	F	54a	reto	T1N0M0	I	adenoca.tub. mod. dif.	5a	não	vivo
G38926/03	M	63a	reto	T3N2M1	III	adenoca.tub. mod. dif.	2a3m	sim	óbito
G28956/00	F	77a	cólon	T2N0M0	I	adenoca.tub. mod. dif.	5a	não	vivo
G21359/98	F	66a	cólon D	T2N0M0	I	adenoca.tub.pouco dif.	7a4m	não	vivo
G37857/03	M	76a	sigmóide	T3N2M0	III	adenoca.tub. bem dif.	5a	não	vivo
G38185/03	M	55a	cólon E	T3N2M0	III	mucinoso	2a8m	sim	óbito
B14528/87	F	71a	reto	T1N0M0	I	adenoca.tub. bem dif.	16a	não	vivo
G26030/99	M	71a	reto	T3N1Mx	III	adenoca.tub. bem dif.	3a3m	sim	óbito
G27502/00	M	81a	cólon D	T3N2Mx	III	adenoca.tub. mod. dif.	5a	sim	óbito
G27695/00	F	51a	reto	T4N0Mx	II	adenoca.tub. mod. dif.	3a	sim	óbito
G27166/99	F	52a	reto	T3N0Mx	II	adenoca.tub. mod. dif.	5a	não	óbito
G25180/99	F	60a	sigmóide	T2N0Mx	I	adenoca.tub. mod. dif.	2a	sim	óbito
B03329/98	F	24a	reto	T4N2Mx	III	mucinoso	2a9m	sim	óbito
G27278/00	F	54a	cólon transverso	T3N1M1	IV	adenoca.tub. bem dif.	10m	sim	óbito
G22284/98	F	51a	sigmóide	T4NxMx	II	adenoca.tub. mod. dif.	7a5m	não	vivo
G27764/00	M	82a	reto	T3N0Mx	II	adenoca.tub. mod. dif.	5a1m	não	vivo
G30094/00	F	54a	reto	T2N0M0	I	adenoca. tub. bem dif.	5a7m	não	vivo
G31015/01	F	57a	cólon D	T3N1Mx	III	adenoca.tub. mod. dif.	5a1m	não	vivo
B05619/94	M	58a	sigmóide	T3N0Mx	II	adenoca.tub. mod. dif.	10a10m	sim	vivo
G31753/01	F	65a	reto	T4N2Mx	III	adenoca.tub. mod. dif.	1a5m	sim	óbito
G30608/01	F	41a	cólon D	T3N2Mx	III	adenoca.tub. mod. dif.	3a	sim	óbito
G31379/01	M	50a	cólon D	T3N0M0	II	adenoca.tub. mod. dif.	4a	sim	óbito
B0508/01	M	69a	sigmóide	T3N0Mx	II	adenoca.tub. mod. dif.	1a	não	óbito
G30237/00	M	37a	retossigmóide	T3N2MX	III	adenoca.tub. mod. dif.	2a	sim	óbito
G27962/00	F	70a	cólon D	T3N0MX	II	adenoca.tub. mod. dif.	1a	sim	óbito
G16494/96	M	74a	reto	T2N0MX	I	adenoca.tub. bem dif.	6a	sim	óbito
G20487/97	F	76a	sigmóide	T4NoMx	II	adenoca.tub. mod. dif.	4a	sim	óbito
IPC 01/388040	M	48a	cólon E	T4N0M0	II	mucinoso	5a	não	vivo
IPC 98/342823	M	64a	cólon transverso	T3N2Mx	III	adenoca.tub. mod. dif.	3a	sim	óbito
Americana (Gi) 2135/01	F	42a	cólon D	T3N2M0	III	adenoca.tub. pouco dif.	5a	não	vivo
Sta.Casa Rio Claro 199720	F	66a	cólon D	T3N2M1	IV	adenoca.tub. mod. dif.	1a9m	sim	óbito
B655/98	F	68a	cólon D	T4N2M1	IV	adenoca.tub. mod. dif.	2a	sim	óbito
B5582/00	F	80a	cólon transverso	T4N2M1	IV	adenoca.tub. mod. dif.	8m	sim	óbito
B8774/97	M	74a	cólon D	T1N0M0	I	adenoca.tub. mod. dif.	8a2m	não	vivo
B10508/00	F	63a	cólon transverso	T3N2M1	III	adenoca.tub. pouco.dif.	4a10m	sim	óbito
B1574/01	F	54a	sigmóide	T3N1MX	III	adenoca.tub. mod. dif.	6a2m	não	vivo
B11928/01	F	76a	cólon D	T3N0Mx	II	adenoca.tub. mod. dif.	5a	não	vivo
B3969/01	M	60a	cólon E	T3N2M0	III	adenoca.tub. mod. dif.	8m	não	óbito
B1914/98	M	54a	sigmóide	T3N2MX	III	adenoca.tub. pouco.dif.	10m	sim	óbito
B10576/00	F	46a	sigmóide	T3N0M0	II	adenoca.tub. mod. dif.	6a	não	vivo
B252/94	F	74a	cólon D	T3N0Mx	II	adenoca.tub. mod. dif.	11m	não	óbito
B10145/94	M	68a	cólon E	T3N1MX	III	adenoca.tub. mod. dif.	3m	não	óbito
B602/99	M	81a	cólon D	T3N0Mx	II	adenoca.tub. mod. dif.	1a	não	óbito
B1295/01	F	68a	reto	T3NOMX	II	mucinoso	6a7m	não	vivo
B3619/00	F	42a	cólon D	T2NOMX	I	adenoca.tub. mod. dif.	6a1m	não	vivo
B6358/01	M	48a	reto	T3N0Mx	II	adenoca.tub. mod. dif.	5a8m	sim	óbito
B5842/00	M	48a	sigmóide	T3N0Mx	II	adenoca.tub. bem dif.	6a	não	vivo
B8142/95	F	71a	cólon transverso	T3 N2 Mx	IV	adenoca.tub. mod. dif.	6a7m	sim	óbito
B9805/99	F	35a	sigmóide	T3N0Mx	II	adenoca.tub. mod. dif.	5a10m	não	vivo
G30907/01	M	68a	cólon transverso	T3N0M0	II	adenoca.tub. mod. dif.	5a9m	não	vivo

ANEXO 2: Casos CCR e valores de MVD e TVA determinados pelos marcadores CD31 e CD34

Biópsia	CD31 TVA campo 1	CD31 TVA campo 2	CD31 TVAcampo 3	CD31 TVA média	CD31 MVD campo 1	CD31 MVD campo 2	CD31 MVD campo 3	CD31 MVD média	CD34 TVA campo 1	CD34 TVA campo 2	CD34 TVA campo 3	CD34 TVA média	CD34 MVD campo 1	CD34 MVD campo 2	CD34 MVD campo 3	CD34 MVD média
G23469/98	73015	38795	35134	48981,33	66	78	66	70,00	43906	8724	17862	23497,33	28	27	24	26,33
B40497/90	13642	3983	30922	16182,33	16	15	19	16,67	39879	34971	25815	33555,00	32	56	33	40,33
B5780/95	20499	13661	167914	67358,00	16	9	10	11,67	371463	351531	631137	451377,00	34	44	29	35,67
G26205/99	12270	7267	17328	12288,33	8	15	4	9,00	98974	42623	26341	55979,33	82	33	29	48,00
G644/92	20268	15162	8192	14540,67	28	35	23	28,67	42426	32872	14907	30068,33	48	54	52	51,33
G21458/98	14267	5765	26982	15671,33	26	13	21	20,00	18231	34971	21927	25043,00	25	56	40	40,33
G29617/00	15697	4350	13099	11048,67	21	6	14	13,67	79948	117280	30535	75921,00	50	78	59	62,33
G29562/00	25300	34	68	8467,33	9	1	1	3,67	15150	64649	48397	42732,00	15	19	15	16,33
G28418/00	60200	14667	20459,54	31775,51	19	19	25	21,00	59789	19719	13076	30861,33	50	86	35	57,00
G28748/00	51303,17	47369,69	42870,38	47181,08	13	26	14	17,67	47899	35190	30332	37807,00	38	46	30	38,00
G37067/03	6936	16608	22725	15423,00	10	9	24	14,33	54272	64671	64215	61052,67	49	37	56	47,33
G36926/03	11890	7556	6830	8759,33	19	4	13	12,00	37620	13549	39592	30253,67	70	43	36	49,67
G28956/00	1422	1656	437	1171,67	5	4	2	3,67	69060	20296	27104	38820,00	30	32	18	26,67
G21359/98	19705	12467	7906	13359,33	88	44	39	57,00	42411	81692	7530	43877,67	12	20	22	18,00
G37857/03	59581	3011	2191	21594,33	29	7	12	16,00	19821	21217	32433	24490,33	25	24	53	34,00
G38185/03	49578	10038	21749	27121,67	12	23	5	13,33	18373	29930	24936	24413,00	43	53	22	39,33
B14528/87	0	50	12	20,67	0	1	1	0,67	23536	56693	39971	40066,67	33	56	59	49,33
G26030/99	10393	11057	25498	15649,33	14	3	11	9,33	7960	24493	19803	17418,67	25	28	93	48,67
G27502/00	14478	4065	2376	6973,00	23	24	12	19,67	26159	22417	98264	48946,67	76	62	58	65,33
G27695/00	8783	10194	19765	12914,00	27	22	43	30,67	108635	30446	58178	65753,00	23	51	56	43,33
G27166/99	38147	10384	6998	18509,67	31	17	18	22,00	41499	22039	32078	31872,00	45	43	43	43,67
G25180/99	4916	10865	6884	7555,00	15	19	11	15,00	59842	23932	41598	41790,67	37	37	57	43,67
B03329/98	9956	5092	6716	7254,67	7	4	6	5,67	71232	34644	7045	37640,33	51	29	14	31,33
G27278/00	2432	5030	1950	3137,33	7	13	4	8,00	94776	18229	8666	40557,00	66	27	24	39,00
G22284/98	5421	15464	2151	7678,67	25	13	11	16,33	33255	13456	49227	31979,33	38	36	28	34,00
G27764/00	9718	1025	3219	4654,00	16	7	11	11,33	18846	3971	15043	12620,00	46	23	49	39,33
G30094/00	61271	32252	11291	34938,00	19	34	27	26,67	79923	38983	12250	43718,67	28	47	31	35,33
G31015/01	11778	7633	25843	15084,67	10	4	18	10,67	14955	9952	9361	11422,67	20	24	15	19,67
B05619/94	10968	22613	57566	30382,33	23	27	26	25,33	36429	27956	38996	34460,33	31	26	94	50,33
G31753/01	52332	32802	32795	39309,67	22	15	9	15,33	51418	35528	16160	34368,00	42	22	47	37,00
G30608/01	7972	2987	10028	6995,67	12	10	11	11,00	102270	38608	8335	49737,67	38	25	27	30,00
G31379/01	31915	4516	7396	14609,00	19	14	24	19,00	53026	13401	21256	29227,67	41	26	31	32,67
B0508/01	970,51	4722,22	17,15	1903,29	1	3	1	1,67	10540	11789	71757	31362,00	39	50	25	38,00
G30237/00	68971,21	93930,06	24406,73	62436,00	24	23	9	18,67	53886	27852	21712	34483,33	25	46	19	30,00
G27962/00	125490,43	27866,95	150637,89	101331,76	21	15	14	16,67	21496	56507	67798	48600,33	43	91	148	94,00
G16494/96	183624,87	11482	19471	71525,96	10	16	19	15,00	29431	14131	25300	22954,00	34	49	35	39,33
G20487/97	21124	3970	8910	11334,67	63	28	27	39,33	20101	23897	9669	17889,00	32	37	42	37,00
IPC 01/388040	16014	1891	2299	6734,67	4	13	9	8,67	81124	14833	16462	37473,00	31	37	39	35,67
IPC 98/342823	32667	17975	9687	20109,67	32	49	19	33,33	31393	7122	26785	21766,67	32	28	33	31,00
Americana (Gi) 2135/01	54217	12579	15410	27402,00	26	22	15	21,00	74990	19700	54861	49850,33	37	36	19	30,67
Santa Casa Rio Claro199720	18588	20910	12655	17384,33	30	26	25	27,00	75223	75854	49356	66811,00	54	38	66	52,67
B655/98	7141	6941	6462	6848,00	20	15	12	15,67	248831	53233	7261	103108,33	58	82	25	55,00
B5582/00	7061	8555	1876	5830,67	15	16	16	15,67	29801	35911	25994	30568,67	25	53	72	50,00
B8774/97	0	4093	1647	1913,33	0	5	3	2,67	61525	15569	35446	37513,33	36	25	47	36,00
B10508/00	33466	14499	7249	18404,67	49	33	27	36,33	62673	37091	27899	42554,33	60	42	36	46,00
B1574/01	34886,84	33882,04	19612,49	29460,46	30	18	32	26,67	81946	40775	24612	49111,00	48	37	30	38,33
B11928/01	57006,19	63672,85	59756,53	60145,19	35	53	37	41,67	87193	31217	17222	45210,67	26	49	37	37,33
B3969/01	64537,05	144708,54	52705,77	87317,12	52	53	39	48,00	29922	49850	80473	53415,00	29	64	68	53,67
B1914/98	135572,73	258621,46	51858,72	148684,30	28	51	18	32,33	33433	157466	243183	144694,00	51	60	39	50,00
B10576/00	21293	14606	9182	15027,00	35	34	25	31,33	115029	25176	37667	59290,67	32	20	44	32,00
B252/94	14678	7819	19470	13989,00	21	18	24	21,00	9316	40927	8656	19633,00	66	47	27	46,67
B10145/94	14525	58621,41	125514,43	66220,28	16	60	31	35,67	8012	25672	22550	18744,67	16	21	45	27,33
B602/99	1620	5702	9990	5770,67	14	14	19	15,67	35180	9781	10277	18412,67	61	25	36	40,67
B1295/01	4584	6002	0	3528,67	28	32	0	20,00	182374	16927	13262	70854,33	16	37	26	26,33
B3619/00	12284	9906	8533	10241,00	25	17	19	20,33	25526	19186	13746	19486,00	37	54	33	41,33
B6358/01	18442	11837	13295	14524,67	44	11	27	27,33	86446	68228	47246	67306,67	43	81	39	54,33
B5842/00	17758	11298	63006	30687,33	43	25	44	37,33	39046	51778	36951	42591,67	85	42	64	63,67
B8142/95	9247	2042	8950	6746,33	4	7	9	6,67	15756	11928	28069	18584,33	44	15	21	26,67
B9805/99	11112	25558	5177	13949,00	14	10	17	13,67	14059	12348	6470	10959,00	28	18	15	20,33
G30907/01	7173	10924	3953	7350,00	12	21	8	13,67	60280	11574	3712	25188,67	14	26	14	18,00

TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR
CAMPO 1 = PARTE CENTRAL DO TUMOR
CAMPO 2 = PARTE PERIFÉRICA DO TUMOR
CAMPO 3 = PARTE MAIS PROFUNDA TUMOR

ANEXO 3: Casos CCR e valores de MVD e TVA determinados pelos marcadores CD105 e D2.40

Biópsia	CD105 TVA campo 1	CD105 TVA campo 2	CD105 TVA campo 3	CD105 TVA média	CD105 MVD campo 1	CD105 MVD campo 2	CD105 MVD campo 3	CD105 MVD média	D2.40 TVA campo 1	D2.40 TVA campo 2	D2.40 TVA campo 3	D2.40 TVA média	D2.40 MVD campo 1	D2.40 MVD campo 2	D2.40 MVD campo 3	D2.40 MVD média
G23469/98	41867	11350	18657	23958,00	9	14	10	11,00	5210	3274	2	2828,67	16	4	2	7,33
B40497/90	68239	0	0	22746,33	8	0	0	2,67	14598	2229	35065	17297,33	24	6	10	13,33
B5780/95	29418	4078	16269	16588,33	5	6	3	4,67	54905	609	58579	38031,00	6	12	6	8,00
G26205/99	48679	5154	14255	22696,00	11	9	6	8,67	1906	27334	63786,02	31008,67	14	7	7	9,33
G644/92	11205	48686,57	38748,29	32879,95	16	8	7	10,33	2051	20092,6	12712,62	11618,64	10	15	8	11,00
G21458/98	111999,34	10860,77	73656	65505,37	32	8	32	24,00	58323,06	39499,32	25061,73	40961,37	40	11	5	18,67
G29617/00	1732	1139	0	957,00	21	4	0	8,33	1543,21	4705,08	63569,97	23272,75	11	7	15	11,00
G29562/00	0	0	0	0,00	0	0	0	0,00	10997,94	411,52	3223,59	4877,68	8	5	10	7,67
G28418/00	5277	3260	2134	3557,00	17	7	10	11,33	9032,92	5059	12196	8762,64	13	9	27	16,33
G28748/00	7373	13361	91683	37472,33	12	26	20	19,33	27742	14036	12439	18072,33	18	26	11	18,33
G37067/03	39494	6844	18648	21662,00	30	17	12	19,67	10361	637	2486	4494,67	13	6	7	8,67
G38926/03	34911	20784	2806	19500,33	17	4	4	8,33	15296	3594	7981	8957,00	31	23	31	28,33
G28956/00	4202	13799	45847	21282,67	13	4	10	9,00	9627	13477	6112	9738,67	9	2	5	5,33
G21359/98	7398	14751	21419	14522,67	7	20	10	12,33	3837	601	1	1479,67	8	2	1	3,67
G37857/03	14106	7672	0	7259,33	17	4	0	7,00	10011	40354	36870	29078,33	5	18	14	12,33
G38185/03	4516	8344	9692	7517,33	8	8	14	10,00	12763	7053	12250	10688,67	32	17	13	20,67
B14528/87	62940	0	0	20980,00	22	0	0	7,33	6764	6015	8486	7088,33	21	11	26	19,33
G26030/99	0	0	0	0,00	0	0	0	0,00	13902	370	4918	6396,67	21	3	6	10,00
G27502/00	182	418	0	200,00	1	1	0	0,67	6021	442	2461	2974,67	8	6	13	9,00
G27695/00	58348	45268	14804	39473,33	16	12	11	13,00	160334	272	160	53588,67	1	6	4	3,67
G27166/99	8712	19806	2200	10239,33	20	8	5	11,00	13886	10683	10145	11571,33	15	7	14	12,00
G25180/99	35632	25605	27964	29733,67	11	25	37	24,33	19088	7744	24476	17102,67	19	34	18	23,67
B03329/98	19268	4796	1478	8514,00	16	2	3	7,00	13719	10094	956	8256,33	39	10	3	17,33
G27278/00	30797	10767	4900	15488,00	35	12	13	20,00	5861	832	5857	4183,33	23	6	7	12,00
G22284/98	0	0	0	0,00	0	0	0	0,00	2260	1184	4178	2540,67	14	4	19	12,33
G27764/00	110624	1284	5369	39092,33	37	6	4	15,67	3480	1327	3489	2765,33	22	13	8	14,33
G30094/00	31810	7899	6194	15301,00	35	14	11	20,00	4082	2516	11618	6072,00	16	23	17	18,67
G31015/01	4129	6417	1209	3918,33	12	11	5	9,33	4673	1946	571	2396,67	7	14	7	9,33
B05619/94	17215	3414	12199	10942,67	55	12	15	27,33	963	13524	653	5046,67	8	4	7	6,33
G31753/01	41691	14268	17798	24585,67	13	28	15	18,67	18449	488	2520	7152,33	59	4	18	27,00
G30608/01	88089	14200	26086	42791,67	25	8	13	15,33	1676	14439	1545	5886,67	8	2	5	5,00
G31379/01	33309	2246	4234	13263,00	23	8	7	12,67	115	120	23840	8025,00	4	1	1	2,00
B0508/01	20261	195	8339	9598,33	6	4	3	4,33	14940	14940	4783	11554,33	39	20	7	22,00
G30237/00	12107	2597	27904	14202,67	7	4	3	4,67	785	808	3377	1656,67	3	1	6	3,33
G27962/00	1291	4799	3698	3262,67	2	4	9	5,00	25876	83939	23806	44540,33	47	19	28	31,33
G16494/96	29339	16713	53785	33279,00	13	12	22	15,67	2641	11355	9722	7906,00	22	12	35	23,00
G20487/97	3734	10814	8064	7537,33	6	17	15	12,67	1797	2196	1797	1930,00	4	11	4	6,33
IPC 01/388040	22834	22076	7879	17596,33	7	6	3	5,33	8347	1834	3177	4452,67	25	8	20	17,67
IPC 98/342823	7500	6902	10716	8372,67	6	9	11	8,67	5229	1078	10778	5695,00	17	7	6	10,00
Americana (Gi) 2135/01	24529	40145	4591	23088,33	14	15	9	12,67	21228	2590	27235	17017,67	18	4	4	8,67
Santa Casa Rio Claro199720	19734	9682	37901	22439,00	3	16	12	10,33	11687	17152	31	9623,33	44	12	1	19,00
B655/98	8455	4419	10585	7819,67	9	10	13	10,67	11161	4164	4328	6551,00	6	5	28	13,00
B5582/00	5789	30357	14097	16747,67	11	11	18	13,33	20910	164609	6410	63976,33	40	7	5	17,33
B8774/97	19681	23691	10171	17847,67	23	20	11	18,00	5079	6435	33142	14885,33	9	3	11	7,67
B10508/00	13180	8010	4724	8638,00	24	10	6	13,33	768	90466	19633	36955,67	5	7	33	15,00
B1574/01	7530	4113	8279	6640,67	10	10	3	7,67	3405	2377	31464	12415,33	11	10	11	10,67
B11928/01	50594	16570	16075	27746,33	19	23	18	20,00	7825	1484	7974	5761,00	16	6	41	21,00
B3969/01	18914	24510	12455	18626,33	31	31	18	26,67	1575	17213	15708	11498,67	8	4	8	6,67
B1914/98	46627	30462	75166	50751,67	21	17	10	16,00	16210	47453	145312	69658,33	34	8	6	16,00
B10576/00	40246	13038	2860	18714,67	18	1	7	8,67	9153	641	86	3293,33	9	5	2	5,33
B252/94	15867	9641	33817	19775,00	10	15	5	10,00	4770	28124	5931	12941,67	29	6	8	14,33
B10145/94	12181	31448	7313	16980,67	16	13	24	17,67	10511	1771	1334	4538,67	7	6	1	4,67
B602/99	10151	9682	6923	8918,67	10	3	8	7,00	7315	10327	1163	6268,33	19	6	2	9,00
B1295/01	9910	11701	9371	10327,33	12	9	7	9,33	21176	45639	24389	30401,33	38	45	37	40,00
B3619/00	6321	6698	12479	8499,33	16	8	6	10,00	7092	2548	41344	16994,67	15	13	35	21,00
B6358/01	7994	1493	2982	4156,33	10	2	4	5,33	14270	25731	40027,44	26676,15	24	9	11	14,67
B5842/00	9148	14384	18536	14022,67	13	1	9	7,67	11025	6536	7519	8360,00	9	4	7	6,67
B8142/95	13992	3775	2756	6841,00	2	7	2	3,67	8914	6747	6550	7403,67	7	6	2	5,00
B9805/99	14474	20290	1307	12023,67	21	23	4	16,00	20321	8046	509	9625,33	9	6	4	6,33
G30907/01	27810	9472	4527	13936,33	23	6	12	13,67	2295	114	0	803,00	8	3	0	3,67

TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR
CAMPO 1 = PARTE CENTRAL DO TUMOR
CAMPO 2 = PARTE PERIFÉRICA DO TUMOR
CAMPO 3 = PARTE MAIS PROFUNDA TUMOR

ANEXO 4: Casos CCR e quantificação dos marcadores VEGFA e VEGFC

Biópsia	VEGFA %	VEGFA intensidade	VEGFA Total	VEGFC %	VEGFC intensidade	VEGFC total
G23469/98	1	2	3	4	4	8
B40497/90	4	3	7	4	3	7
B5780/95	4	4	8	4	4	8
G26205/99	3	4	7	4	4	8
G644/92	4	4	8	4	4	8
G21458/98	4	4	8	4	2	6
G29617/00	3	4	7	4	4	8
G29562/00	3	3	6	3	3	6
G28418/00	2	2	4	4	3	7
G28748/00	1	2	3	3	2	5
G37067/03	1	1	2	2	4	6
G38926/03	4	3	7	4	3	7
G28956/00	4	4	8	4	3	7
G21359/98	1	1	2	3	4	7
G37857/03	4	3	7	4	3	7
G38185/03	1	3	4	1	2	3
B14528/87	4	3	7	4	4	8
G26030/99	4	3	7	4	4	8
G27502/00	4	3	7	4	3	7
G27695/00	4	3	7	4	4	8
G27166/99	4	3	7	4	3	7
G25180/99	3	3	6	4	3	7
B03329/98	4	4	8	4	4	8
G27278/00	4	3	7	3	3	6
G22284/98	4	3	7	4	3	7
G27764/00	4	4	8	4	3	7
G30094/00	1	2	3	2	3	5
G31015/01	1	2	3	3	3	6
B05619/94	4	4	8	4	4	8
G31753/01	4	4	8	2	3	5
G30608/01	4	4	8	4	4	8
G31379/01	4	4	8	4	3	7
B0508/01	1	2	3	1	3	4
G30237/00	4	4	8	4	4	8
G27962/00	1	1	2	2	2	4
G16494/96	3	4	7	1	3	4
G20487/97	4	4	8	4	3	7
IPC 01/388040	4	4	8	4	3	7
IPC 98/342823	3	3	6	4	4	8
Americana (Gi) 2135/01	4	3	7	3	3	6
Santa Casa Rio Claro 199720	3	4	7	4	3	7
B655/98	4	4	8	4	4	8
B5582/00	1	2	3	4	3	7
B8774/97	1	2	3	3	3	6
B10508/00	1	1	2	2	3	5
B1574/01	3	3	6	4	4	8
B11928/01	3	3	6	4	4	8
B3969/01	1	2	3	4	4	8
B1914/98	4	3	7	4	4	8
B10576/00	2	2	4	4	3	7
B252/94	4	4	8	4	4	8
B10145/94	4	3	7	4	3	7
B602/99	3	2	5	2	2	4
B1295/01	4	3	7	4	4	8
B3619/00	1	3	4	4	3	7
B6358/01	4	3	7	4	4	8
B5842/00	2	4	6	3	3	6
B8142/95	3	2	5	2	3	5
B9805/99	1	2	3	4	3	7
G30907/01	1	2	3	4	3	7

PORCENTAGEM: grau 1- menos de 25% do tumor positivo; grau 2- 25-50% ; grau 3- 50-75% ; grau 4- mais de 75% do tumor positivo
INTENSIDADE: grau 1- ausente ; grau 2- fraco ; grau 3- moderado ; grau 4- forte
TOTAL= SOMA DOS ANTERIORES

ANEXO 5: Dados clínicos de pacientes com Adenoma

Biópsia	Sexo	Idade ao diagnóstico	Câncer
G26147	M	33a	sim
G37067	F	54a	sim
G38143	M	55a	sim
G38419	F	71a	sim
G27166	F	52a	sim
G25180	F	60a	sim
G27224	F	54a	sim
G27764	M	82a	sim
G35590	F	54a	sim
G31379	M	50a	sim
G2917	M	74a	sim
B8774/97	M	74a	sim
B10576/00	F	46a	sim
B602/99	M	81a	sim
G30610	M	68a	sim
G36714	F	62a	não
G36710	M	56a	não
G37326	M	82a	não
G37410	F	50a	não
G37416	M	71a	não
G37449	M	72a	não
G37482	M	22a	não
G37468	M	20a	não
G37605	M	59a	não
G67624	M	55a	não
G37949	F	54a	não
G37973	F	71a	não
G37974	F	60a	não
G36965	F	29a	não
G37325	M	79a	não

ANEXO 6: Casos de Adenomas e quantificação dos marcadores VEGFA e VEGFC

Biópsia	VEGFA %	VEGFA intensidade	Total	VEGFC %	VEGFC intensidade	Total
G26147	2	1	3	1	3	4
G37067	1	1	2	2	2	4
G38143	1	1	2	4	4	8
G38419	1	2	3	2	3	5
G27166	1	2	3	2	3	5
G25180	1	2	3	4	4	8
G27224	2	2	4	3	3	6
G27764	2	3	5	2	2	4
G35590	1	2	3	2	3	6
G31379	3	3	6	3	3	6
G2917	4	4	8	2	3	5
B8774/97	1	1	2	1	3	4
B10576/00	1	2	3	2	3	5
B602/99	1	2	3	3	3	6
G30610	1	2	3	2	2	4
G36714	2	2	4	3	3	6
G36710	4	3	7	4	4	8
G37326	3	4	7	4	3	7
G37410	4	4	8	3	4	7
G37416	1	3	4	2	3	5
G37449	3	4	7	2	3	5
G37482	1	2	3	1	3	4
G37468	2	3	5	3	3	6
G37605	3	3	6	3	4	7
G67624	1	2	3	2	3	5
G37949	3	3	6	3	3	6
G37973	1	2	3	3	3	6
G37974	1	1	2	3	3	6
G36965	1	2	3	2	3	5
G37325	1	1	2	2	3	5

PORCENTAGEM: grau 1- menos de 25% do tumor positivo; grau 2- 25-50% ; grau 3- 50-75% ; grau 4- mais de 75% do tumor positivo
INTENSIDADE: grau 1- ausente ; grau 2- fraco ; grau 3- moderado ; grau 4- forte
TOTAL= SOMA DOS ANTERIORES

ANEXO 7: Casos de Adenomas e valores de MVD e TVA determinados pelos marcadores CD31 e CD34

Biópsia	CD31 TVA campo 1	CD31 TVA campo 2	CD31 TVA média	CD31 MVD campo 1	CD31 MVD campo 2	CD31 MVD média	CD34 TVA campo 1	CD34 TVA campo 2	CD34 TVA média	CD34 MVD campo 1	CD34 MVD campo 2	CD34 MVD média
G26147	24875	9195	17035	19	18	18,5	24989	34100	29544,5	26	16	21
G37067	40232	11	20121,5	9	1	5	34531	52547	43539	11	53	32
G38143	3859	20169	12014	12	22	17	66368	15315	40841,5	27	45	36
G38419	16554	26847	21700,5	6	7	6,5	51012	9944	30478	23	25	24
G27166	10	3	6,5	1	1	1	10817	7754	9285,5	34	58	46
G25180	8231	6859	7545	5	6	5,5	101632	60481	81056,5	37	29	33
G27224	5404	10436	7920	2	5	3,5	14575	12077	13326	56	28	42
G27764	20243	1722	10982,5	10	2	6	16155	35256	25705,5	23	48	35,5
G35590	9464	17138	13301	12	8	10	14615	13260	13937,5	26	27	26,5
G31379	747	2828	1787,5	1	2	1,5	43502	54583	49042,5	30	62	46
G2917	37827	21669	29748	18	21	19,5	845281,4	80984,24	463132,82	11	46	28,5
B8774/97	29780	13889	21834,5	3	10	6,5	102606,33	100154,35	101380,34	27	30	28,5
B10576/00	5363	17060	11211,5	3	12	7,5	244132,43	175353,26	209742,845	26	20	23
B602/99	4455	21543	12999	15	12	13,5	95456,12	85442,41	90449,265	54	36	45
G30610	12976	24611	18793,5	7	22	14,5	45243,49	281238,06	163240,775	31	17	24
G36714	4753	2865	3809	5	2	3,5	62534,31	118371,08	90452,695	17	24	20,5
G36710	8117	15523	11820	12	6	9	131220,88	103237,33	117229,105	17	43	30
G37326	7878	81140	44509	2	1	1,5	221831,32	26430,05	124130,685	24	41	32,5
G37410	4558	15833	10195,5	9	14	11,5	465572,8	281210,62	373391,71	41	34	37,5
G37416	9700	46811	28255,5	3	11	7	34893,7	111508,94	73201,32	24	32	28
G37449	3737	9543	6640	5	10	7,5	70727,04	140140,63	105433,835	62	63	62,5
G37482	1013	42701	21857	3	5	4	336680,47	201618,7	269149,585	21	15	18
G37468	4600	13201	8900,5	4	7	5,5	46779,85	48515,1	47647,475	17	21	19
G37605	98022	9856	53939	22	51	36,5	240140,66	32866,95	136503,805	53	55	54
G67624	7584	16115	11849,5	10	13	11,5	76296,31	47767	62031,655	17	30	23,5
G37949	5351	4857	5104	9	9	9	10981	54314	32647,5	31	19	25
G37973	33892	2155	18023,5	16	7	11,5	76952	11217	44084,5	22	11	16,5
G37974	2021	27905	14963	3	4	3,5	37944	24301	31122,5	32	36	34
G36965	14180	6100	10140	6	4	5	22333	14247	18290	44	30	37
G37325	15724	30124	22924	9	6	7,5	23221	74065	48643	28	38	33

CAMPO 1 = CENTRO/SUPERFÍCIE DO ADENOMA
CAMPO 2 = BASE/PERIFERIA DO ADENOMA
TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR

ANEXO 8: Casos de Adenomas e valores de MVD e TVA determinados pelos marcadores CD105 e D2.40

Biópsia	CD105 TVA campo 1	CD105 TVA campo 2	CD105 TVA média	CD105 MVD campo 1	CD105 MVD campo 2	CD105 MVD média	D2.40 TVA campo 1	D2.40 TVA campo 2	D2.40 TVA média	D2.40 MVD campo 1	D2.40 MVD campo 2	D2.40 MVD média
G26147	27528	17431	22479,5	7	1	4	19436	7062	13249	4	5	4,5
G37067	627	737	682	2	1	1,5	16496	9563	13029,5	6	8	7
G38143	0	0	0	0	0	0	21216	64650	42933	9	18	13,5
G38419	31463	15475	23469	8	5	6,5	2732	35073	18902,5	9	9	9
G27166	5719	282	3000,5	1	1	1	3433	5690	4561,5	12	21	16,5
G25180	219905	27255	123580	15	11	13	3914	3786	3850	27	22	24,5
G27224	2091	0	1045,5	5	0	2,5	2521	64099	33310	27	13	20
G27764	11021	0	5510,5	1	0	0,5	7178	676	3927	12	1	6,5
G35590	0	0	0	0	0	0	3045	26289	14667	29	34	31,5
G31379	12261	5000	8630,5	1	3	2	1170	19920	10545	21	6	13,5
G2917	44876	20151	32513,5	9	37	23	40691	15735	28213	177	37	107
B8774/97	15477	4542	10009,5	12	11	11,5	1429	5994	3711,5	15	12	13,5
B10576/00	329	755	542	1	1	1	9022	10959	9990,5	5	10	7,5
B602/99	6425	19	3222	7	2	4,5	6112	6112	6112	77	16	46,5
G30610	7498	1578	4538	14	8	11	85	1498	791,5	8	8	8
G36714	0	0	0	0	0	0	32250	4152	18201	12	6	9
G36710	0	5496	2748	0	1	0,5	13690	680	7185	6	6	6
G37326	19660	21638	20649	1	10	5,5	8934	8687	8810,5	4	14	9
G37410	157	143	150	2	2	2	53112	37643	45377,5	16	9	12,5
G37416	5468	20362	12915	6	3	4,5	401	31044	15722,5	8	8	8
G37449	2671	66522	34596,5	7	5	6	141	16058	8099,5	2	5	3,5
G37482	183	43	113	4	1	2,5	2	12341	6171,5	2	8	5
G37468	0	0	0	0	0	0	181	265	223	1	2	1,5
G37605	14577	4141	9359	10	9	9,5	1655	5884	3769,5	13	10	11,5
G67624	4078	2166	3122	6	3	4,5	5836	3099	4467,5	8	6	7
G37949	5665	0	2832,5	7	0	3,5	1	14509	7255	1	7	4
G37973	0	0	0	0	0	0	20642	26542	23592	13	12	12,5
G37974	0	0	0	0	0	0	9902	4717	7309,5	11	8	9,5
G36965	4694	1205	2949,5	5	4	4,5	1911	6349	4130	18	9	13,5
G37325	0	0	0	0	0	0	25229	44491	34860	11	14	12,5

CAMPO 1 = CENTRO/SUPERFÍCIE DO ADENOMA
CAMPO 2 = BASE/PERIFERIA DO ADENOMA
TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR

ANEXO 11: Casos de tecidos colorretais não neoplásicos e valores de MVD e TVA determinados pelos marcadores CD31 e CD34

Biópsia	CD31 TVA campo 1	CD31 TVA campo 2	CD31 TVA média	CD31 MVD campo 1	CD31 MVD campo 2	CD31 MVD média	CD34 TVA campo 1	CD34 TVA campo 2	CD34 TVA média	CD34 MVD campo 1	CD34 MVD campo 2	CD34 MVD média
63799	40974	7706	24340	17	5	11	18055	20638	19346,5	37	85	61
60425	34297	40957	37627	11	17	14	21331	23686	22508,5	19	68	43,5
66768	42181	5188	23684,5	27	7	17	42419	97602	70010,5	8	10	9
5398	24732	30521	27626,5	17	9	13	177870	48662	113266	19	19	19
75771	85229	15921	50575	17	10	13,5	14917	35363	25140	26	28	27
36878	26351	53413	39882	33	12	22,5	30180	38265	34222,5	36	61	48,5
60003	24829	15591	20210	19	1	10	24823	78357	51590	36	74	55
62477	40926	52110	46518	13	6	9,5	13469	27722	20595,5	32	29	30,5
59669	38345	17143	27744	13	30	21,5	45645	12861	29253	60	70	65
44146	61630	58504	60067	7	9	8	8589	61338	34963,5	29	62	45,5

CAMPO 1 = MUCOSA
CAMPO 2 = SUBMUCOSA/ MUSCULAR
TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR

ANEXO 12: Casos de tecidos colorretais não neoplásicos e valores de MVD e TVA determinados pelos marcadores CD105 e D2.40

Biópsia	CD105 TVA campo 1	CD105 TVA campo 2	CD105 TVA média	CD105 MVD campo 1	CD105 MVD campo 2	CD105 MVD média	D2.40 TVA campo 1	D2.40 TVA campo 2	D2.40 TVA média	D2.40 MVD campo 1	D2.40 MVD campo 2	D2.40 MVD média
63799	0	0	0	0	0	0	1194	7554	4374	1	2	1,5
60425	0	0	0	0	0	0	19825	9265	14545	5	2	3,5
66768	0	0	0	0	0	0	876	1825	1350,5	4	6	5
5398	1133	0	566,5	4	0	2	22630	3827	13228,5	10	3	6,5
75771	408	0	204	7	0	3,5	29957	13378	21667,5	9	4	6,5
36878	1238	0	619	5	0	2,5	21291	65276	43283,5	7	9	8
60003	0	0	0	0	0	0	37814	8845	23329,5	12	2	7
62477	221	0	110,5	4	0	2	4178	5769	4973,5	6	2	4
59669	131	0	65,5	4	0	2	19246	39677	29461,5	1	4	2,5
44146	0	0	0	0	0	0	57684	46066	51875	9	5	7

CAMPO 1 = MUCOSA
CAMPO 2 = SUBMUCOSA/ MUSCULAR
TVA = ÁREA VASCULAR TOTAL
MVD = MICRODENSIDADE VASCULAR

ANEXO 13: Permissão do Brazilian Journal of Medical and Biological Research

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