

**Research Article** 

# Microvessel Density Quantification in Gastric Cancer: Comparing Methods for Standard Measures

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#### Abstract

The quantification of angiogenic and linfangiogenic factors has been explored in an attempt to predict the prognosis of various malignancies. In gastric cancer (GC), a promising parameter is the microvessel density (MVD).

Objective: The aim of our study is to evaluate the different methods used for its quantification.

**Methods:** 52 cases of GC were labeled by immunohistochemistry for CD34, CD105 and D2-40. The quantification of the microvasculature was performed for each marker by counting microvessels (mv) in three "hot spots", using three different microscopic magnifications (100x, 200x and 400x). MVD was then calculated by dividing the number of vessels by the microscopic field area (measured in mm<sup>2</sup>) and compared between the three different evaluations.

**Results:** the MVD obtained for CD34 was 203 mv/mm<sup>2</sup> (100x), 311 mv/mm<sup>2</sup> (200x) and 490 mv/mm<sup>2</sup> (400x). The MVD score for CD105 was 127 mv/mm<sup>2</sup> (100x), 213 mv/mm<sup>2</sup> (200x) and 347 mv/mm<sup>2</sup> (400x). The MVD obtained for D2-40 was 35 mv/mm<sup>2</sup> (100x), 69 mv/mm<sup>2</sup> (200x) and 170 mv/mm<sup>2</sup> (400x). We found that MVD obtained in 100x magnification was lower than 200x, which was lower than in 400x. Those differences were statistically significant and occurred in a proportional way for all three markers. MVD obtained for CD34 was higher than for CD105. The MVD for lymphatics obtained by D2-40 was lower than the MVD for CD34 and CD105.

**Conclusion:** Our results show that the lack of standardized methods for assessing angiogenesis and lymphangiogenesis in GC can produce variations in the MDV value, impairing the reproducibility of the results and the comparison between different studies and populations. It is necessary to standardize the MVD determination methods to compare results and confirm its prognostic value in GC and in other types of tumors.

**Keywords:** Gastric cancer; Angiogenesis; Lymphangiogenesis; Microvessel count; Microvessel density; Prognosis; Immunohistochemistry; CD34; CD105; D2-40

#### Introduction

Current research on neoplastic diseases focus on revealing new indicators capable of predicting the biological behavior of tumors and the prognosis of patients in the early stages of the disease. The study of angiogenic and lymphangiogenic factors has been explored in an attempt to predict the prognosis of various types of cancers [1-5]. Angiogenesis and lymphangiogenesis can be assessed directly by counting vessels or indirectly by analysis of inducing factors [6-8].

In the first report on the correlation between tumor angiogenesis and metastasis, Weidner et al performed a quantitative study of blood vessels stained by immunohistochemistry (IHC) in areas of high vascular density in breast cancer [1]. From the pathological point of view, it is expected that the mechanisms responsible for the relationship between the tumor and local endothelial cells are particularly active in those highly vascularized areas, called *hot spots*. These areas presumably arise due to the existence of angiogenic tumor cell clone, which is more likely to enter the bloodstream and result in metastases [9]. Since then, subsequent studies have attempted to evaluate the association between tumor progression and angiogenic potential of different cancers [10,11]. A promising parameter in gastric cancer (GC) is the microvessel density (MVD), both lymphatic and blood vessel [12,13].

The method originally proposed by Weidner et al. in 1991 for determining MVD used the 200x microscopic magnification to perform the vessel count [1]. However, further studies involving MVD in other malignancies, including GC, used non-standard variations of the original method, such as 400x magnification vessel count, producing inconsistent results and problematic in terms of comparison [3,14-16]. In those reports, there is a tendency to describe MVD as number of vessels per microscopic field evaluated, rather than number of vessels per area, for example square millimeter (mm<sup>2</sup>). There are significant variations in the field area between microscope producers and models. Thus, the data reported in the literature are mixed and therefore inconsistent to allow a proper conclusion about which values of MVD would be considered at risk for metastasis or predictive of a bad prognosis.

To evaluate the methods for the study of angiogenesis and lymphangiogenesis in GC and compare the results between three different microscopic magnifications, we performed a vessel count and MVD determination using IHC with CD34, CD105 and D2-40 markers in a series of 52 cases of GC, analyzing three different microscopic magnifications for blood and lymph vessels.

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## Material and Methods

This is a cross-sectional study of a series of cases of primary GC with IHC study. We selected 52 cases of GC retrospectively from the database of the Gastrointestinal Pathology research laboratory from Hospital das Clinicas of Federal University of Minas Gerais. All patients underwent a gastrectomy and lymphadenectomy for GC in the same institution.

The IHC study was performed in tumor sections using streptavidinbiotin peroxidase (Dako LSAB<sup>\*</sup> + System) with CD34, CD105 and D2-40 markers. All slides were pre-treated in a buffer at 98°C in a steamer for 20 minutes for antigen retrieval. Blockage of endogenous peroxidase and avidin protein for 15 minutes each were carried out. The D2-40, CD34 and CD105 primary antibodies were incubated for 30 minutes at room temperature. The slides were revealed with diaminobenzidine. The background staining was performed with hematoxylin. Positive and negative controls were included in all reactions.

The quantification of the microvasculature was performed according to the method described by Weidner et al. [1]. The three *hot spots* were initially detected at lower magnification (40x) and selected in each marker. Those areas were captured and digitized for morphometric image analysis. The microvessel count was performed in the same hot spot area using the following microscopic magnifications: 100x, 200x

and 400x. Isolated endothelial cells or groups of cells highlighted by the endothelial markers, with or without lumen, were considered as individual vessels and counted manually using the software Image J (Figure 1). The mean values of microvessel count from the three *hot spots* in each magnification and each marker was calculated. The MVD was then determined dividing the number of vessels (mean microvessel count) by the microscopic field area of each magnification (in mm<sup>2</sup>) for each marker. The method used is illustrated in Figure 2.

## Results

The clinicopathologic characteristics of the sample are shown in Table 1. We observed the following distribution of cases according to Lauren's classification: 25 cases of intestinal type GC (48.1%), 12 of the diffuse type (23.1%) and 15 of mixed type (28.8%). Seven cases represented early CG (pT1) and 45 cases advanced CG, of which the majority (33 cases) shows tumor invasion up to the serosa (pT3). Lymph node metastasis was detected in 39 cases (75.0%).

The mean MVD obtained for CD34 was 203 microvessels/mm<sup>2</sup> (100x), 311 microvessels/mm<sup>2</sup> (200x) and 490 microvessels/mm<sup>2</sup> (400x). The mean MVD obtained for CD105 was 127 microvessels/mm<sup>2</sup> (100x), 213 microvessels/mm<sup>2</sup> (200x) and 347 microvessels/mm<sup>2</sup> (400x). The mean MVD obtained for D2-40 was 35 microvessels/mm<sup>2</sup> (100x), 69 microvessels/mm<sup>2</sup> (200x) and 170 microvessels/mm<sup>2</sup> (400x).



Figure 1: Case example of microvessels highlighted by CD34 and analyzed in three different magnifications on the same hot spot. Example shows 163 microvessels in 100x field; 67,6 microvessels in 200x field; 23 microvessels in 400x field.

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The MVD obtained for CD34 was higher than the MVD by CD105. Lymphatic MVD obtained by D2-40 was considerably lower than the MVD by CD105 and CD34. We observed that the mean MVD for 100x magnification was lower than that for the 200 x magnification, which was lower than the mean MVD for 400x magnification. Those differences occurred in a proportional and similar way form the three IHC markers studied. To assess whether those differences were statistically significant, we performed the ANOVA test, confirming the significance of our observation (Table 2). Figure 3 shows the comparison of mean MVD of each microscopic magnification and each IHC marker used.

## Discussion

In this study, the group of 52 patients reflected the profile usually described in the literature for GC on the following characteristics: the majority of patients were male (65.4%), tumor topography was predominantly in the distal third of the stomach (55.8%) and the most frequent histological type according to Laurén corresponded to the intestinal type (48.1%) [17]. Cases of GC in advanced stages were the vast majority, and 67.3% were at least on stage pT3 tumor depth. Our sample is in agreement with the literature that indicates that the majority of diagnoses of GC in our country are made in a late stage, already as an advanced disease [17]. Note also that three fourth of cases (75.0%) had already lymph node metastasis at diagnosis.

If we try to compare the MVD values of blood and lymph vessels found in our study with the results of other studies available in the literature, we find a lack of standardization on the MVD determining method. It is possible to observe microvessel count values expressed only by a microscopic field and not by mm<sup>2</sup>. Some authors do not even register the precise definition of the microscopic field area used for counting. Previous series of GC using CD34 found MVD values much lower as compared to our study, but those values were expressed only by number of microvessels per field of 200x or 400x, but not by mm<sup>2</sup> [11,14,18]. Only two other studies show similar values to our MVD results [13,19].

Regarding the lymph vessel MVD, the literature is still scarce and most authors demonstrate microvessel count only in the microscopic magnification of 200x. Perhaps this choice is justified by the fact that lymphatic vessels are larger and more distended than blood vessels, thus in need of a higher size field [20]. Our mean lymph vessels counted per 200x field was similar to the ones found in two other series of GC [13,21].

Analyzing the differences found on the mean MVD between IHC markers studied, the lymph vessel MVD was considerably lower than the blood vessel MVD by CD34 and CD105. The lymphatic system is known to be scarcer than the blood network on the gastric wall [20]. This difference was consistent with the results of two other studies regarding the MVD obtained by markers of the lymphatic and blood endothelium in CG [13,18].

The MVD evaluation using markers CD34 and CD105 showed distinct patterns of expression. As previously reported by Ding and colleagues in 2006, the microvessel count with CD105 is lower than the one assessed by CD34 [15]. It is expected that CD105 marker highlights

Gender	34 (65 4)		
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Male	34 (65.4)		
Female	18 (34.6)		
Tumor topography			
Proximal third	8 (15.4)		
Medium third	3 (5.8)		
Distal third	29 (55.8)		
Proximal+medium	1 (1.9)		
Medium +distal	3 (5.8)		
Proximal+medium+distal	7 (13.5)		
Esophagogastric junction	1 (1.9)		
Curvature			
Small curvature	27 (51.9)		
Large curvature	4 (7.7)		
Small and large	13 (25.0)		
Not evaluated	8 (15.4)		
Tumor depth (pT)			
Mucosa (pT1a)	2 (3.8)		
Submucosa (pT1b)	5 (9.6)		
Muscular propria (pT2a)	6 (11.5)		
Subserosa (pT2b)	4 (7.7)		
Serosa (pT3)	33 (63.5)		
Structure invasion (pT4)	2 (3.8)		
Lymph node metastasis			
Negative	13 (25.0)		
Positive	39 (75.0)		
Organ invasion			
Negative	27 (51.9)		
Duodenum	13 (25.0)		
Esophagus	6 (11.5)		
Esophagus + duodenum	3 (5.8)		
Other	3 (5.8)		
Laurén classification			
Intestinal	25 (48.1)		
Diffuse	12 (23.1)		
Mixed	15 (28.8)		

Table 1: Clinico-pathological caracteristics of 52 cases of GC.

just newly formed blood vessels, while CD34 is a pan-endotelial marker [22,23].

Our most intriguing result was the uniform and almost proportional difference in the mean MVD between the three microscopic magnifications studied. This is perhaps the most interesting result of our work, since it is yet unpublished. We observed that the values of MVD for 100x, expressed as number of vessels per mm<sup>2</sup>, was consistently lower than MVD for 200x, which was lower than MVD for 400x. This variation could be identified not only in the mean values of MVD, but also for each individual case. Initially the study, we expected to obtain values of MVD similar or identical between the different microscopic magnifications, since the field captured vessel count was always the same. However, the difference in MVD values was statistically significant and occurred in all three IHC markers. This variation can be interpreted as reflecting the uneven distribution of blood and lymph vessels in the gastric wall and also in the tumor tissue. Even the most vascularized tumor areas, called hot spots, show a higher concentration of vessels in its central region. When examining the periphery of the *hot spot*, we notice a lower concentration of vessels in contrast to the central region. Maybe that is why we obtained higher densities using higher microscopic magnifications, as 400x. Also, those fields are more detailed and can increase the number of microvessels identified.

Once established the significant difference of results depending on the method chosen for microvessel count, it is necessary to carry on new prognostic studies to determine which microscopic magnification would be most adequate for MVD count in GC. However, the literature still lacks comparative studies using different microscopic magnifications and field areas to study angiogenesis and lymphangiogenesis in GC. Our study evaluated the MVD using three IHC markers for blood and lymph vessels endothelium, in three different microscopic magnifications, applying the same method described by Weidner [1]. In our opinion, studies using the *hot spot* method for vessel count should always express MVD values by microvessels/mm<sup>2</sup> and the magnification field area used should always be given.

## Conclusion

In summary, our results show that the lack of standardized methods

IHC Marker	Microscopic magnification	Mean MVD	Standart -deviation	P value
CD34	100x	203.06	66.14	< 0,001*
	200x	311.44	105.09	
	400x	490.01	166.05	
D2-40	100x	35.64	10.64	< 0,001*
	200x	69.59	20.34	
	400x	170.12	59.48	
CD105	100x	127.26	58.58	< 0,001*
	200x	213.97	95.36	
	400x	347.27	133.56	

\*difference statistically significant (ANOVA test)

Table 2: Analysis of variance between the mean MVD values found in each microscopic magnification. (N = 52).

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for assessing angiogenesis and lymphangiogenesis in GC can produce variations in the MVD value, impairing the reproducibility of the results and the comparison between different studies and populations. The standardization of MVD quantification is required to help confirm its prognostic value in CG and in other types of malignancies.

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