

Vascular Endothelial Growth Factor Concentrations in Dogs with Spirocercosis

V. Mukorera, R.M. Kirberger, P. Mabeta, and E. Dvir

Background: Vascular endothelial growth factor (VEGF) is a potent proangiogenic factor associated with tumor development. *Spirocera lupi* is a nematode of canids that induces an esophageal nodule that progresses to a sarcoma in 25% of cases. Determination of neoplastic transformation is challenging and usually based on endoscopy-guided biopsies under general anesthesia, an expensive procedure that often yields nondiagnostic, necrotic samples.

Hypothesis: Circulatory VEGF concentrations are increased in dogs with neoplastic spirocercosis and can distinguish between dogs with neoplastic and nonneoplastic disease.

Animals: A total of 24 client-owned dogs, 9 nonneoplastic, 9 neoplastic, and 6 controls.

Methods: Case-control study. Plasma and serum VEGF concentrations at the time of diagnosis were compared with those of healthy controls. Measurement of VEGF was performed using a canine-specific ELISA. Kruskal-Wallis and Dunn's tests were used for statistical analysis with significance set at $P < .05$.

Results: The median plasma VEGF concentrations of dogs with neoplastic spirocercosis were 629 pg/mL (range, 282–2,366) higher than both the nonneoplastic (<39.5 pg/mL; range, <39.5 –716) and control dogs (<39.5 pg/mL; all values, <39.5 ; $P = .0003$). The median serum VEGF concentration of the neoplastic dogs was 69 pg/mL (range, <39.5 –212) higher than the nonneoplastic (<39.5 pg/mL; range, <39.5 –44.13) and control dogs (<39.5 pg/mL; all values, <39.5 ; $P = .001$).

Conclusions and Clinical Importance: Both plasma and serum VEGF concentrations can be used to differentiate nonneoplastic and neoplastic spirocercosis. The role of VEGF in neoplastic transformation of *S. lupi*-induced nodules and the potential utility of anti-VEGF drugs in spirocercosis-induced sarcoma warrant further investigation.

Key words: Canine esophagus; Neoplastic; Nonneoplastic.

Spirocera lupi is a nematode of canids that infests the esophagus, causing the formation of a nodule that may undergo neoplastic transformation into osteosarcoma, fibrosarcoma, or anaplastic sarcoma in approximately 25% of cases.¹ Nonneoplastic spirocercosis can be successfully treated with doramectin.^{[1].a} Once neoplastic transformation occurs, treatment options become limited to surgical excision of the neoplastic mass with or without chemotherapy and the prognosis is much poorer compared with dogs with nonneoplastic cases. Definitive antemortem diagnosis of neoplastic transformation is achieved by histopathology of endoscopically obtained biopsies, a technique that is expensive, requires general anesthesia, and is not available in most practices. In addition, nondiagnostic necrotic material from the periphery of the nodule is commonly obtained.² The lobulated, cauliflower-like endoscopic appearance of the neoplastic nodules is very characteristic, and the lesions are ulcerated and necrotic and very different from the smooth, rounded, and often operculated nonneoplastic nodule.¹ Endoscopic appearance, however, is operator dependent, subjective, and does not always correlate with the state of malignancy.^{1,2}

Abbreviations:

CRP	C-reactive protein
CV	coefficient of variation
OVAH	Onderstepoort Veterinary Academic Hospital
SPSS	Statistical Package for the Social Sciences
VEGF	vascular endothelial growth factor

Response to doramectin treatment excludes the possibility of neoplastic transformation.¹ This approach, however, requires time and may not be suited to compromised dogs. Anemia, hyperglobulinemia, leukocytosis, thrombocytosis, and eosinopenia have been associated with neoplastic transformation.¹ These findings are nonspecific and associated with a variety of other conditions. Increased serum C-reactive protein (CRP) concentrations have been reported in neoplastic and nonneoplastic spirocercosis. In contrast to dogs with neoplasia, serum CRP concentrations decreased in dogs with nonneoplastic disease shortly after treatment, implying that persistently high concentration levels despite treatment would be associated with neoplastic transformation.³ In light of the difficulties in identifying neoplastic transformation in dogs with spirocercosis, there is a need for identification of a reliable and readily available method of identifying neoplastic transformation.

Vascular endothelial growth factor (VEGF) is one of the most potent proangiogenic factors described to date and is increased in a variety of other tumors.^{4,5} Tissue expression of VEGF has been documented in both the peritumoral and tumoral regions in several canine soft-tissue sarcomas of dogs, including extraskelatal osteosarcoma, neoplastic peripheral nerve sheath tumor, hemangiosarcoma, and liposarcoma.⁶ Importantly,

From the Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa (Mukorera, Kirberger, Mabeta, Dvir). This study was presented as an abstract at the European College of Veterinary Internal Medicine—Companion Animals (ECVIM-CA) Annual Congress held in September 2011, Seville, Spain.

Corresponding author: V. Mukorera, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, 0110 Pretoria, South Africa. e-mail: varaidzo.mukorera@up.ac.za.

tissue concentrations of VEGF have been shown to be increased in the *S. lupi*-induced nodule. Tissue expression progressively increased from nonneoplastic to preneoplastic nodules, and was highest in the neoplastic nodules.⁷

Because neoplastic transformation of tissue is associated with angiogenesis and an increase in tissue expression of VEGF has been identified in *S. lupi*-induced neoplasms,⁷ it was expected that there would be an increase in serum and plasma VEGF concentrations in dogs with neoplastic spirocercosis.

Materials and Methods

This study was a prospective cohort study that was part of a larger study on the epidemiology of spirocercosis in dogs being conducted by the University of Pretoria. Dogs presented to the Onderstepoort Veterinary Academic Hospital and diagnosed with naturally acquired *S. lupi* infection between November 2008 and December 2010 were included in the study. The dogs were divided into nonneoplastic (9) and neoplastic (9) groups. The control group included healthy staff and client-owned dogs (6). The study was approved by the institutional animal use and care committee.

Routine physical examination, fecal analysis, urinalysis, hematology, serum biochemistry, thoracic radiographs, and abdominal ultrasound examination were performed on all dogs enrolled in the study to exclude concurrent disease. The diagnosis of *S. lupi* was achieved by a combination of identification of *S. lupi* worm eggs on fecal flotation, characteristic findings of *S. lupi* on thoracic radiographs (eg, caudodorsal mediastinal mass, spondylitis of the ventral bodies of the caudal thoracic vertebrae, undulation of the thoracic aorta, or some combination of these findings), and visual identification of typical caudal thoracic esophageal *S. lupi* nodules on esophageal endoscopy. Determination of nonneoplastic spirocercosis was confirmed by response to treatment as shown by resolution of the nodules at a 6-week posttreatment follow-up endoscopy. Additional endoscopies were scheduled at 6-week intervals until only a scar was seen at the original nodule sites. Determination of neoplastic transformation was made by histopathology of biopsy samples obtained either by endoscopy, esophagostomy, or necropsy. The presence of any concurrent disease resulted in exclusion from the trial. Healthy dogs used as controls were included in the trial if all the imaging and clinicopathologic results were within normal limits.

Blood samples were collected at presentation from each dog. Blood was collected by venipuncture from either the jugular or cephalic veins and immediately transferred into 4-mL ethylenediaminetetraacetic acid and plain plastic tubes. The tubes were centrifuged to separate red blood cells and plasma or serum. Samples were stored at -80°C until analysis for VEGF. Samples were analyzed as a batch when an adequate sample size had been obtained.

Measurement of VEGF was performed using a canine-specific VEGF Quantikine ELISA kit.^b VEGF in 100 μL of serum or plasma was determined using a quantitative sandwich enzyme immunoassay technique according to the manufacturer's guidelines. The system used a solid-phase monoclonal antibody and an enzyme-linked polyclonal antibody against canine VEGF. All analyses and calibrations were performed in triplicate. Optical densities were determined by using a microtiter plate reader at 450 nm.^c A standard curve was created using Gen 5 software.^c Intra-assay (within an experiment) precision was determined as the mean coefficient of variation (CV) based on 24 triplicates on the same plate. Interassay (between experiments conducted on

different days) precision was determined as the mean CV on the basis of triplicate analysis of 8 controls on 2 plates. The intra-assay CV was 5.53% and the interassay variation was 13.52%.

All VEGF concentrations below the detection limit of the test (39.5 pg/mL) were assigned a value of 20 pg/mL (a value between zero and the detection limit). Statistical analysis to compare VEGF concentrations in the different groups was performed using the Kruskal-Wallis test and Dunn's test as a posthoc test using a statistical software package.^d The chi-squared test was used to assess the sex distribution between the neoplastic and nonneoplastic groups.^e A *P* value of $<.05$ was considered significant for all tests. The data obtained did not have a normal distribution based on the Kolmogorov-Smirnov test run on a statistical package.^e

Results

At the time of the conclusion of this study, 115 dogs (56 neoplastic and 59 nonneoplastic) had fulfilled the criteria for enrollment into the main spirocercosis study. Of these dogs, 24 dogs were randomly selected for enrollment into the study. Of these, 18 client-owned dogs had naturally occurring canine spirocercosis, 9 with nonneoplastic spirocercosis, and 9 with neoplastic spirocercosis. The remaining 6 dogs were healthy, staff- and client-owned dogs. The neoplasms in the neoplastic group were osteosarcoma (8) and fibrosarcoma (1). Three of the dogs with neoplastic disease had evidence of pulmonary metastases on radiographs (2) or computed tomography (1). There was no significant difference in ages of the controls (4.1 ± 1.4 years), nonneoplastic (4.4 ± 1.8 years) cases, and neoplastic cases (5.3 ± 1.1 years).

Breed distribution for the nonneoplastic cases was as follows: 2 Staffordshire Bull Terriers, 2 Bull Terriers, and 1 each of Scottish Terrier, Chow Chow, Miniature Pinscher, Rottweiler, and Labrador Retriever. The breed distribution for the neoplastic cases was as follows: 2 Jack Russell Terriers, 1 each of Labrador Retriever, Doberman Pinscher, Boerboel, Border Collie, Boxer, German Shepherd, and a medium-sized mixed-breed dog. There were 4/9 (44%) males and 5/9 (56%) females in the nonneoplastic group, and 5/9 (56%) males and 4/9 (44%) females in the neoplastic group. There was no difference in the sex distribution between the nonneoplastic and neoplastic cases (*P* = .5).

Plasma VEGF Concentrations

Median plasma VEGF concentrations in the control, nonneoplastic, and neoplastic groups were <39.5 pg/mL (all values, <39.5), <39.5 pg/mL (range, <39.5 –716), and 629 pg/mL (range, 282–2,366), respectively. There was an overall difference in the plasma VEGF concentrations of the control, nonneoplastic, and neoplastic groups (*P* = .0003; Fig 1). The plasma VEGF concentration of the neoplastic group was significantly higher than that of both the control (*P* $<.001$) and nonneoplastic groups (*P* $<.01$). There was no difference in the plasma VEGF concentrations of the control and nonneoplastic groups (*P* $>.05$; Fig 1).

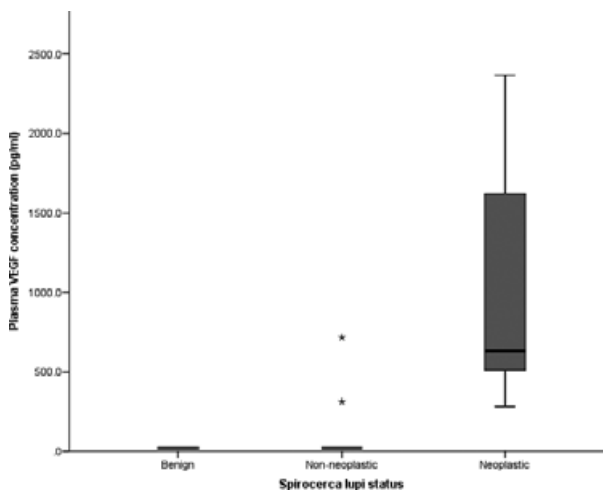


Fig 1. Box and whisker plot representing plasma vascular endothelial growth factor (VEGF) concentrations in control, nonneoplastic, and neoplastic dogs. The box represents the 25–75th percentile range, the horizontal line through the box represents the median, and the outliers are represented by an asterisk.

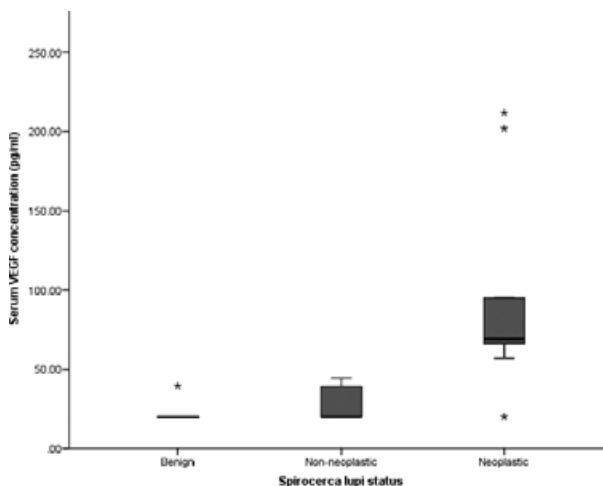


Fig 2. Box and whisker plot representing serum vascular endothelial growth factor (VEGF) concentrations in control, nonneoplastic, and neoplastic dogs. The box represents the 25–75th percentile range, the horizontal line through the box represents the median, and the outliers are represented by an asterisk.

Serum VEGF Concentrations

Median serum VEGF concentrations in the control, nonneoplastic, and neoplastic groups were <39.5 pg/mL (all values, <39.5), <39.5 pg/mL (range, <39.5–44.13), and 69 pg/mL (range, <39.5–212), respectively. There was an overall difference in the serum VEGF concentrations of the control, nonneoplastic, and neoplastic groups ($P = .001$; Fig 2). The serum VEGF concentration of the neoplastic group was significantly higher than that of both the control ($P < .01$) and nonneoplastic groups ($P < .01$). There was no difference in the serum VEGF concentration of the control and nonneoplastic groups ($P > .05$; Fig 2).

There was some overlap in the concentration of the nonneoplastic and neoplastic cases in both the plasma and serum samples. Two of the nonneoplastic cases had plasma VEGF concentrations within the low end of the range of the neoplastic cases, and 5 neoplastic cases were within the high end of the range of the nonneoplastic cases. Three of the nonneoplastic cases had serum VEGF concentrations within the low end of the range of the neoplastic cases, and 1 neoplastic case was within the high end of the range of the nonneoplastic cases. Two of the nonneoplastic cases consistently overlapped with the neoplastic VEGF concentrations in both plasma and serum samples. The dogs were a 2-year-old intact female Rottweiler and a 4-year-old intact female Labrador Retriever. The sizes of their nodules were 4 and 10 cm in length, respectively, and were not larger than the average sizes of nodules in the nonneoplastic cases, which ranged from 1 to 12 cm.

There was a moderate positive linear relationship between plasma and serum VEGF concentrations in individual dogs with a correlation coefficient (r) of 0.45.

Discussion

This study serves as a proof of concept for the potential use of VEGF as a marker for neoplastic transformation in *S. lupi* nodules. Both plasma and serum VEGF concentrations were significantly higher in dogs with neoplastic spirocercosis compared with nonneoplastic and control groups. Vascular endothelial growth factor, therefore, potentially can be used to differentiate between neoplastic and nonneoplastic spirocercosis, with higher concentrations being associated with malignancy. VEGF is produced in other neoplasms,⁴ and hence a thorough evaluation for concurrent neoplastic disease is warranted.

It was not possible to determine if there was an association between neoplastic transformation and breed or age because the overall number of cases in this study was low and most of the breeds were represented by 1 dog each. A larger sample population with more numbers per breed will be needed to ascertain if there is any association between malignancy and breed.

Contrary to other published reports in which serum samples had higher VEGF concentrations,⁵ plasma samples in this study yielded consistently higher VEGF concentrations compared with serum. Results of both plasma and serum samples significantly differentiated between neoplastic and nonneoplastic cases. This finding implies that either plasma or serum VEGF concentrations can be used to differentiate between the 2 groups as long as sample consistency is maintained. Recently, serum VEGF has been shown to be unstable when subjected to repeated freeze and thaw cycles, leading to a rapid decrease in VEGF concentrations.⁸ Our serum (and plasma) samples underwent a single-freeze and -thaw cycle before analysis. This may explain the lower serum VEGF concentrations observed in this

study. No similar studies on plasma VEGF concentration could be identified. It remains to be determined whether plasma VEGF is more stable under storage and repeated freeze and thaw cycles compared with serum.

Antiangiogenic treatment has become an additional component for treatment of various human malignancies. Examples include the VEGF monoclonal antibody, bevacizumab, and the kinase inhibitor, sorafenib.⁹ Future studies evaluating the therapeutic benefit of antagonists of VEGF and its receptors in canine spirocercosis in combination with more traditional treatment modalities such as surgical excision and chemotherapy are warranted. The current cost of bevacizumab is prohibitive for use in most veterinary patients.

A previous study indicated that there is increased tissue expression of VEGF as well as other growth factors in *S. lupi*-induced sarcomas.⁷ This study has showed that there are increased concentrations of circulating VEGF. Vascular endothelial growth factor can be produced by the majority of cells in the body. It is now necessary to determine the source of the VEGF, the trigger for its secretion, and if it has any other function in the neoplastic transformation in canine spirocercosis apart from its known angiogenic properties. The possible sources of VEGF are first, the tumor itself after neoplastic transformation, second, inflammatory cells induced by the presence of the worm, and last, the worm itself. Hypoxia-induced production of VEGF is mediated by hypoxia-inducible factor 1.⁶ The stimulation for VEGF secretion can be the well-described hypoxia within the developing tumor, but it cannot be excluded that the worm itself may secrete a product that stimulates growth factor synthesis and secretion.

In conclusion, VEGF is a promising biomarker for neoplastic transformation in dogs with spirocercosis. This study serves as a basis for the evaluation of VEGF in a larger population. The study also justifies evaluating anti-VEGF treatment in conjunction with surgical excision and chemotherapy in the treatment of dogs with neoplastic spirocercosis.

Footnotes

^a Pfizer, Sandton, Johannesburg, South Africa

^b CAVE00; R&D Systems Inc, Minneapolis, MN

^c BioTek 800-BioTek, Bad Friedrichshall, Germany

^d GraphPad Prism 5 for windows, version 5.04; GraphPad Software, Inc, La Jolla, CA, www.graphpad.com

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Conflict of Interest Declaration: Authors disclose no conflict of interest.

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