BIOMARKERS OF NEOPLASTIC TRANSFORMATION IN CANINE SPIROCERCOSIS

by

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Biomarkers of neoplastic transformation in canine spirocercosis

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I, Eran Dvir, hereby declare that the work on which this thesis is based, is original and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree at this or any other University, Tertiary Education Institution, or Examining Body.

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SUMMARY

Spirocerca lupi is a nematode that infects the dog's oesophagus and promotes the formation of an inflammatory fibroblastic nodule that progresses to sarcoma in approximately 25% of cases. Differentiating neoplastic from non-neoplastic cases ante-mortally is challenging and has major therapeutic and prognostic implications. More importantly, spirocercosis-associated oesophageal sarcoma is an excellent and under-utilized spontaneous model of parasite-associated malignancy and the pathogenesis of the neoplastic transformation is poorly understood.

The current study objective was to investigate potential clinical, clinicopathological, radiological and tissue biomarkers for the malignant transformation and an attempt to use these biomarkers to gain a deeper understanding of the pathogenesis of the neoplastic transformation. Our central hypothesis was that the parasite produces excretory product(s) which diverts the immune response from a T helper 1 (Th1) to Th2 cell response, typical of many nematode infections, and further to an immunoregulatory (immunosuppressive), FoxP3+ regulatory T cell-predominated response which then facilitates neoplastic transformation.

The following parameters were studied and compared between cases with non-neoplastic and neoplastic spirocercosis: clinical presentation, haematology, serum albumin and globulin, thoracic radiology, haematoxylin-eosin (H&E) histology, Immunohistochemistry for expression of vascular endothelial growth factor (VEGF)-A, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), MAC387 (myeloid cells), CD3 (T cells), Pax5 (B cells) and FoxP3 (T regulatory cells) and plasma cytokine concentrations including IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, GM-CSF and MCP-1.

Hypertrophic osteopathy showed 100% specificity for neoplastic transformation but relatively poor sensitivity (40%). Female gender, anaemia, leukocytosis, thrombocytosis, spondylitis and bronchial displacement were significantly more common in neoplastic cases, but appeared in non-neoplastic cases as well. The H&E study revealed 2 stages in the non-neoplastic nodules: early inflammation, characterized by fibrocytes and abundant collagen, and a pre-neoplastic stage, characterized by activated fibroblasts and reduced collagen. The neoplastic cases were all sarcomas, primarily osteosarcoma with very aggressive features comparable to other appendicular osteosarcoma in the dog. The inflammation in spirocercosis is characterized by pockets of pus (MAC387+ cells) surrounded by organized lymphoid foci (CD3+ and to a lesser degree Pax5+ cells). There was no evidence of a local accumulation of FoxP3+ cells, unlike many previous studies which have reported an increase in Foxp3+ T cells in both malignancies and parasite infections. Interleukin-8 plasma concentration was higher in the neoplastic group compared to the non-neoplastic and the control groups. Interleukin-18 concentration was higher in the non-neoplastic group followed by the control group and finally the neoplastic group.

As with most similar studies, no ideal biomarker with high sensitivity and specificity was identified. However, if examined together, a panel of the biomarkers that were identified more commonly in the neoplastic cases should substantially increase the index of suspicion for neoplastic transformation in a diagnosed spirocercosis case. The inflammatory response showed features of increased myeloid (innate) response and lymphocytic response with pro-inflammatory cytokines. This was not our initial hypothesis and the question remains whether the response is secondary to the worm infection, or to a symbiotic bacterium that is carried by the worm. The role of such a reaction in neoplastic transformation remains to be elucidated.

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ABBREVIATIONS

S. lupi – Spirocerca lupi
µg – microgram
µl – microlitre
BSA - bovine serum albumin
BVSc – Bachelor of Veterinary Science
C – Celsius
C – Collagen
CD - Cluster of differentiation
CRP- C-reactive protein
dl – decilitre
DL – detection limit
DN - degenerate neutrophils
DNA - deoxyribonucleic acid
DVM – Doctor of Veterinary Medicine
Dipl. ECVIM-CA - Diplomate of the European College of Veterinary Internal Medicine – Companion Animals
ED – Eran Dvir
EDTA - Ethylenediaminetetraacetic acid
F – fibroblast
FGF - fibroblast growth factor
Fig. – figure
FoxP3 - Forkhead box P3
GM-CSF - granulocyte-macrophages colony-stimulated factor
H& E / HE - Haematoxylin-eosin
HO - Hypertrophic osteopathy
Hons - Honours
Ht - Haematocrit
IHC – immunohistochemistry
IL - Interleukin
kg – kilogram
L1-5 – Larvae life cycle stage number
LP – lymphocytic-plasmacytic
LSAB - labelled steptavidin-biotin
MAC 387 - Macrophage marker
MCP - monocyte chemotactic protein
Martius, Scarlet and Blue – MSB
MCV - mean corpuscular volume
MCW – Mark C Williams
NF-κB - nuclear factor kappa-light-chain-enhancer of activated B cells
NGS – normal goat serum
Mi – mitoses

ml – millilitre
mm – milimeter
MMedVet – Master in Veterinary Medicine
MN – multinucleated
MVD- Microvessel density
O – osteoblasts
Osm – osteoid matrix
OVAH – Onderstepoort Veterinary Academic Hospital
p – probability
Pax5 - Paired box protein 5
PBS - phosphate buffer
PBST80 - phosphate-buffered saline (PBS) containing 0.5% Tween 80
PDGF - platelet-derived growth factor
pH - power of hydrogen
pg – picogram
RK – Robert Kirberger
RT – room temperature
S. lupi – Spirocerca lupi
SC – subcutaneous
SJC – Sarah J Clift
T – temperature
T1-12 – Thoracic vertebrae number
TGF - Transforming growth factor
Th1 - T helper type
Tregs - T regulatory cells
VEGF - vascular endothelial growth factor
UK – United Kingdom
vs – versus
WBC - White blood cell count
µg – microgram
µl – microlitre
χ^2 - Chi-square

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1 BACKGROUND

1.1 Canine spirocercosis

Spirocercosis is an emerging and highly prevalent disease in dogs in South Africa (van der Merwe et al., 2008). It is caused by *Spirocerca lupi (S. lupi)*, a spiurid nematode of carnivores, particularly Canidae, of worldwide distribution but most prevalent in the tropics and subtropics (Bailey, 1972). Dogs are the definitive hosts and become infested with the worm when they ingest either the coprophagous beetle intermediate hosts or a paratenic host (Bailey, 1972). Following ingestion of the intermediate or paratenic host *S. lupi* L3 larvae are liberated in the gastric lumen. Larvae penetrate the gastric mucosa and migrate through the wall of the gastric and coeliac arteries to the caudal thoracic aorta. Larvae spend up to three months in small nodules in the aortic wall, where they moult to L4 and finally to adults. Young adult worms then migrate from the aorta to the oesophagus below. Groups of three to six worms cluster together in the oesopageal submucosa and induce the formation of one or more nodules with a nipple-like protuberance (Bailey, 1963, 1972; van der Merwe et al., 2008). The nodules are often referred to as granulomatous (Bailey, 1963, 1972), but histologically this is inappropriate as the mature nodule is composed mostly of actively dividing immature fibroblasts with relatively pronounced vascularisation (van der Merwe et al., 2008). The host inflammatory reaction is commonly mild to moderate and is not characterized by a predominance of macrophages, as would be expected in granulomatous inflammation (Bailey, 1963; van der Merwe et al., 2008). Spirocercosis induces a few pathognomonic lesions; aortic scarring with aneurysm formation, thoracic vertebral spondylitis and caudal oesophageal nodule formation. The typical clinical signs associated with spirocercosis are related to the presence of

oesophageal nodules and include regurgitation, vomiting, dysphagia and weight loss, together with non-specific signs like pyrexia (Dvir et al., 2001; Mazaki-Tovi et al., 2002). The clinical diagnosis of spirocercosis is largely dependant upon thoracic radiography, which demonstrates the caudal oesophageal soft tissue masses, caudal thoracic vertebral spondylitis and aortic undulation due to aneurysm formation. Faecal flotation tests are also used to detect the typical embryonated eggs (Christie et al., 2011). Oesophagoscopy typically demonstrates one or more nodules with a nipple-like protuberance.

1.2 *Spirocerca lupi*-induced sarcoma

The oesophageal nodule may undergo neoplastic neoplastic transformation (Bailey, 1963; Seibold et al., 1955). Over time up to 25% of these nodules undergo neoplastic transformation (Dvir et al., 2001), making spirocercosis a highly attractive model to study the association between cancer, helminth infection and inflammation.

The association between *S. lupi* infection and oesophageal sarcoma was first described in 1955 (Seibold et al., 1955). This association was based on the finding of *S. lupi* worms and related oesophageal nodules close to the neoplastic neoplasm or the pathognomonic findings of spondylitis or aortic aneurysm in conjunction with the malignant neoplasm. Macroscopically, an increased size, and progression to cauliflower-like shape and area of necrosis in the neoplastic nodule compared to the smooth appearance of the benign nodule may be indicative of neoplastic transformation (Ranen et al., 2004). Histologically the malignant neoplasms are classified as fibrosarcoma, osteosarcoma or anaplastic sarcoma (Ranen et al., 2007; Ranen et al., 2004). The histopathological characteristics of the *S. lupi*-induced fibrosarcoma include interwoven bundles of tapered to plump spindle shaped cells, variable amounts of intercellular collagenous matrix, and a high mitotic index (Bailey

et al., 1963). Histological characteristics of the *S. lupi*-induced osteosarcoma include foci of polygonal osteoblasts, and variable numbers of osteoclasts and/or interwoven bundles of spindle cells, variable amount of osteoid matrix with or without foci of chondroid differentiation. Sometimes obvious spicules or trabeculae of bone are identified amidst solid foci of neoplastic spindle or polygonal cells (Bailey, 1963). Anaplastic sarcomas are characterized histologically by the presence of obviously neoplastic, plump, roughly spindle-shaped cells, usually in an interwoven or interlacing pattern, without the presence of clearly identifiable intercellular matrix, and numerous mitoses. In areas where spirocercosis does not exist, malignant neoplasms of the oesophagus are extremely rare (Ridgway and Suter, 1979), making spirocercosis the major cause of malignant oesophageal neoplasms in the dog. Spirocercosis-induced sarcoma metastasizes commonly to the lung but also to a variety of abdominal organs (Bailey, 1963; Dvir et al., 2001).

Benign spirocercosis is treated successfully with avermectins [doramectin (Dectomax, Pfizer, France) 400 µg/kg SC at 2-week intervals] (Lavy et al., 2002). However, malignant neoplasms can only be treated by surgical excision with or without chemotherapy and the success rate is lower (Ranen et al., 2004). This difference in prognosis emphasizes the need to improve diagnostic and prognostic markers for the antemortem diagnosis of the oesophageal nodule and the need for a better understanding of the neoplastic transformation that may improve treatment for neoplastic cases.

1.3 Inflammation / Infection-induced cancer

Epidemiologic evidence suggests that approximately 25% of all human cancers worldwide are associated with chronic inflammation, chronic infection or both (Morrison, 2012). The association between chronic infection-induced inflammation

and cancer is now well-described and is thought to be the mechanism responsible for up to 18% of global cancers (Vennervald and Polman, 2009). Reports suggest that the same proportions exist in domestic animals (Morrison, 2012) and judging by the fundamental similarities between animal and human cancer, there is no reason to assume that the figures should be fundamentally different.

Several other organisms have been implicated as causing neoplasia in humans by virtue of the chronic inflammatory reaction associated with them. These include Epstein-Barr virus, human papillomaviruses, hepatitis B and hepatitis C viruses, human immunodeficiency virus type 1, *Helicobacter pylori*, *Clonorchis sinensis*, *Opisthorchis viverrini* and *Schistosoma hematobium* (Herrera et al., 2005; Schottenfeld and Beebe-Dimmer, 2006).

The most commonly proposed mechanism for inflammation-induced cancer is attributing genetic instability to reactive oxygen and nitrogen species, cytokines, chemokines and growth factors (Morrison, 2012). Chronic inflammation assures that DNA damage is not repaired and that mutations persist which lead to cancer formation. This approach is oversimplistic and there is clear evidence that the immune system plays a major role in the induction of inflammation/infection-induced cancer. For example, in *Helicobacter*-induced gastritis, an ineffectual helper lymphocyte type 1 (Th1) response and the associated cytokines are thought to play a significant role in carcinogenesis (Wilson and Crabtree, 2007).

T regulatory cells (Tregs) are often suggested as having a major role in the association between certain infections and cancer formation, as they are often highly prevalent and very active in chronic infections that lead to cancer (Erdman and Poutahidis, 2010). Tregs can inhibit the anti-tumour immune response (Beyer and Schultze, 2006)

and an increase in their number may facilitate tumour development. Tregs secrete interleukin (IL)-10 and transforming growth factor (TGF)β which are also known to have tumourogenic activity (Coussens and Werb, 2002). Numerous clinical studies on human patients with various types of cancer have shown increased Tregs proportions in the peripheral blood, draining lymph nodes and within the tumours (Curiel et al., 2004; Heimberger et al., 2008; Liyanage et al., 2002; Wolf et al., 2003; Woo et al., 2001). The same phenomena was observed in murine models of cancer (Imai et al., 2007), including models of fibrosarcoma (Betts et al., 2007) and canine patients with various cancer types (Biller et al., 2007).

1.4 Helminth-induced inflammation and cancer

It is widely accepted that helminths and their antigens induce a Th2 response, which is characterized by IL-4 and IL-5 secretion and stimulation (Maizels et al., 2009), and although a Th2 response to the parasite is essential for the host to clear the infection, it is imperative that the immune response is well controlled. The Th2 response can be tightly controlled by Tregs, which are characterized by the expression of CD4, CD25 and the intracellular forkhead box P3 (FoxP3) transcription factor and the secretion of IL-10 and TGFβ (Maizels et al., 2009). While Tregs are essential in the prevention of autoimmune and allergic diseases via their inhibition of an autopathogenic immune responses, induction of Tregs by helminths can facilitate long-lasting infection (Maizels et al., 2009). This immune response is well-described across species. It is associated with fibroblastic proliferation (as seen in spirocercosis) and has been classified as a delayed hypersensitivity type 3 (Meeusen, 1999). The potential link between switching from Th1 to Th2 response and cancer formation was demonstrated in *Schistosoma mansoni*-infected mice that were injected with fibrosarcoma cells. The

infected mice had up-regulation of their Th2 responses and consequently had a significantly weaker rejection of the cancer cells compared to the non-infected mice that showed a Th1 response and stronger rejection (Yoshida et al., 2002).

Three helminth infections have been classified as carcinogenic in humans, namely *Schistosoma haematobium, Clonorchis sinensis* and *Opisthorchis viverrini* and the presence of chronic inflammation induced by parasites or their deposition is considered a key element in their carcinogenesis (Vennervald and Polman, 2009). *Schistosoma haematobium* is associated with transitional cell carcinoma of the bladder and it was proposed that the egg antigen-induced inflammation and nitrogen species play a role in the neoplastic transformation (Mostafa et al., 1999). *Opistochris viverrini* is associated with the emerging epidemic of cholangiocarcinoma in East Asia and the mechanism proposed is through an excretory / secretory product that mimics TGF-β and stimulates Treg response (Thuwajit et al., 2006). *Schistosoma mansoni* is another helminth that is suspected to be carcinogenic (Yoshida et al., 2002). In dogs, oesophageal sarcoma (excluding leiomyosarcoma) is almost invariably associated with *S. lupi* infections, whereas in human oncogenic helminth-associated neoplasia the association is limited to only a portion of the specific cancer cases (Herrera et al., 2005), making spirocercosis a highly attractive model to study the association between cancer, helminth infection and inflammation. One of the major objectives of this study was to characterize the cellular immune response [with the aid of haematoxylin-eosin (H&E) histology, immunohistochemistry for expression of vascular endothelial MAC387 (myeloid cells), CD3 (T cells), Pax5 (B cells) and FoxP3 (T regulatory cells)] and the cytokine milieu (measuring plasma concentrations of GM-CSF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18 and MCP-1) in neoplastic and non-neoplastic spirocercosis cases and to compare them against the

leading theory of Th2 and Tregs dominated response that may lead to cancer development.

1.5 Cancer biomarkers

A biomarker is a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention (Mishra and Verma, 2010). Most of the research on cancer biomarkers is focused on proteins and recently on molecular research, including gene expression (Tainsky, 2009). However, the broader approach to biomarkers includes other clinical diagnostic fields such as imaging (Mishra and Verma, 2010). This study adopted this broader approach for biomarkers, using clinical and imaging parameters as the preliminary study that was later followed by a search of various proteins (growth factors and cytokines) as well as tissue biomarkers using immunohistochemistry.

Cancer biomarkers are in the forefront of biomedical research, but there has been very limited success in finding an optimal biomarker that has high sensitivity and specificity (Chatterjee and Zetter, 2005). Over 1000 candidates were studied over the last years, 5% of those were studied extensively, including various cytokines and growth factors (Polanski and Anderson, 2007). Biomarkers have a huge potential to improve early diagnosis of cancer and consequently save cost and improve prognosis. The current study aims to study various biomarkers to improve the differentiation between neoplastic and non-neoplastic spirocercosis. Spirocercosis is an ideal model for such an investigation because the lesion is readily accessible by endoscopy and has distinctive neoplastic and non-neoplastic stages. In the future a successful biomarker may replace the need of endoscopy, which is relatively expensive and requires anaesthesia. As we propose spirocercosis as a model for cancer formation,

any knowledge gain in the validity of certain biomarkers can be applied in other cancers across species. Moreover, biomarkers that are found useful in differentiating neoplastic from non-neoplastic disease may provide insights into the pathomechanistic processes during neoplastic transformation. One of the most important conclusion out of the failure to find a single ideal biomarker is the need for wide screening of a panel of biomarkers (Chatterjee and Zetter, 2005), which is one of the aims of the current study.

1.6 Diagnosis of neoplastic vs. non-neoplastic spirocercosis

While spirocercosis has a few pathognomonic lesions, the *ante-mortem* differentiation of neoplastic cases and non-neoplastic cases has not been studied. The objectives of the present study were to identify clinical ante-mortem differences between neoplastic and benign spirocercosis cases to assist in diagnosis, treatment and prognostication.

Ante-mortem differentiation between non-neoplastic and neoplastic cases is challenging, yet clinically, therapeutically and prognostically very important. A few studies have attemted to investigate criteria that might characterize dogs with *S. lupi*-induced oesophageal neoplasia (Bailey, 1963; Ranen et al., 2004; Seibold et al., 1955). Currently, antemortem diagnosis of neoplasia is based on surgical biopsies obtained by endoscopy (Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004). Macroscopically, the surface of oesophageal neoplasms tend to be cauliflower-like, ulcerated and necrotic (van der Merwe et al., 2008). Based on this characteristic appearance, Ranen and others (2004) reported that they were able to make a tentative diagnosis of *S. lupi*–induced sarcoma, using endoscopy, in all 15 cases examined. Benign nodules are typically small, smooth and rounded with a nipple-like protuberance (Dvir et al., 2001). Endoscopy-guided biopsy has limitations and

although highly specific, the procedure has very low sensitivity (Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004), because biopsies frequently include only the necrotic superficial layers of the tumour, rendering a definite diagnosis impossible. Thoracotomy and surgical resection of the mass with histology of the entire mass has the highest sensitivity and specificity, but is invasive with increased risk of complications.

In summary, to date all the diagnostic procedures to dfferentiate neoplastic from non-neoplastic cases are invasive and expensive procedures that require general anaesthesia. This study aims to look for appropriate biomarkers that are cheaper and more readily available to diagnose neoplastic transformation in spirocercosis.

2 RESEARCH HYPOTHESES

- There are clinical, clinicopathological and radiological differences between neoplastic and non-neoplastic cases.[1]
- There are distinctive stages in the progression of the *S. lupi* oesophageal nodule from early infection to sarcoma, similarly to what is described in *Helicobacter*-associated gastric adenocarcinoma.[2]
- VEGF, FGF and PDGF would be expressed in *S. lupi*–induced nodules and their level of expression would increase with progression to malignancy.[3]
- Numerous Tregs will be present in spirocercosis-induced oesophageal nodules, and the number of Tregs will increase in neoplastic nodules.[4]
- The cytokine milieu in canine spirocercosis will show depressed pro-inflammatory / Th1 and increased Th2 responses in the non-neoplastic cases that will later will be diverted into immunoregulatory (immunosuppressive), FoxP3+ regulatory T cell- predominated response in the neoplastic cases.[5]
- Our central hypothesis, while investigating the neoplastic transformation and the inflammatory response in canine spirocercosis, was that the parasite produces excretory product(s) which diverts the immune response from a T helper 1 (Th1) to Th2 cell response, typical of many nematode infections, and further to an immunoregulatory (immunosuppressive), FoxP3+ regulatory T cell- predominated response which then facilitates neoplastic transformation.[6]

[1] This hypothesis is discussed in Chapter 4 of this thesis.
[2] This hypothesis is discussed in Chapter 5 of this thesis.
[3] This hypothesis is discussed in Chapter 6 of this thesis.
[4] This hypothesis is discussed in Chapter 7 of this thesis.
[5] This hypothesis is discussed in Chapter 8 of this thesis.
[6] This hypothesis is discussed in Chapter 7 and 8 of this thesis.

3 GENERAL METHODOLOGY

3.1 Cases

3.1.1 Retrospective cases

Medical records of 297 dogs diagnosed with spirocercosis at the Onderstepoort Veterinary Academic Hospital, University of Pretoria, South Africa, during 1995-2006, were retrospectively reviewed. From these records two groups of cases were selected: confirmed neoplastic cases and confirmed non-neoplastic oesophageal nodule cases.

The inclusion criteria for the non-neoplastic group were: an endoscopic diagnosis of spirocercosis with an obvious response to treatment within at least 6 weeks or a histological diagnosis of a non-neoplastic nodule after surgical resection of the whole nodule or diagnosis of non-neoplastic spirocercosis at necropsy, including histological appraisal of the entire nodule. A diagnosis of non-neoplastic nodule based only on endoscopic guided biopsy was judged to be unsatisfactory as it has been shown to be highly insensitive in a few studies (Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004). The inclusion criteria for the neoplastic group were: histological diagnosis of malignancy of an oesophageal nodule obtained either by endoscopic-guided biopsy, surgical resection or necropsy. Cases with radiographic diagnosis of caudal oesophageal nodules, spondylitis and thoracic metastases with no other obvious primary neoplasm elsewhere in the body without histological analysis of the oesophageal nodule or the metastasis were also included in the neoplastic group.

Sixty-two out of 297 dogs had adequate data to fulfil the inclusion criteria. Thirty one were included in the non-neoplastic group; 19 were based on endoscopic diagnosis and response to treatment, 12 were based on histology of the entire oesophageal

nodule, ten of which were necropsy cases, and two were surgical resection cases. Thirty one dogs were included in the neoplastic group; 27 were diagnosed histologically and four cases were selected according to the combination of a caudal oesophageal mass, spondylitis and thoracic metastasis.

3.1.2 Prospective cases

The study population comprised of 103 client-owned dogs admitted to the Onderstepoort Veterinary Academic Hospital, at the Faculty of Veterinary Science, University of Pretoria between 2008 and 2011. The dogs were divided into 3 groups, One hundred and three dogs were enrolled in the study and were divided into 3 groups, non-neoplastic (n=49), neoplastic (n=29) and healthy control (n=25). The same inclusion criteria were used as for the retrospective case series. This population was used for collecting plasma to investigate the cytokine concentrations, as described in chapter 8.

3.2 Samples

3.2.1 Histopathology

Sixty two paraffin blocks containing *S. lupi*-induced non-neoplastic or neoplastic oesophageal nodules, collected between 1998-2008, were retrieved from the archives of the Section of Pathology, Faculty of Veterinary Science, University of Pretoria. Per block, one 5-µm-thick hematoxylin and eosin–stained section was examined under a light microscope. In addition, 10 sections of normal distal third of oesophagus were evaluated and compared with the *Spirocerca*-induced nodules.

Only one nodule was selected per dog and if a dog had more than one nodule, the nodule that was most mature or advanced in relation to the progression of the nodule

toward neoplasia was selected for evaluation. If a nodule was sectioned more than once, the section with the most advanced fibroplasia was selected. Once the stages within the nodule progression were established (chapter 5), additional sections per block was used to study growth factors expression (chapter 6) and to label the different inflammatory cells (chapter 7) using immunohistochemistry. For the labelling of the different inflammatory cells, an additional 9 *S. lupi*-induced oesophageal nodule cases (5 neoplastic and 4 non-neoplastic) were collected prospectively together with the draining lymph nodes of the distal oesophagus (bronchial) and remote lymph nodes (popliteal) that served as control.

3.2.2 Plasma

Blood samples were collected from the 103 dogs enrolled in the prospective study at admission by jugular venipuncture with a 21g needle and a 5 mL potassium EDTA vacutainer syringe. The samples were then immediately centrifuged, separated, aliquoted and frozen at −80°C. The samples were batched and analyzed together.

4 CLINICAL DIFFERENTIATION BETWEEN DOGS WITH BENIGN AND MALIGNANT SPIROCERCOSIS

This chapter was published as a research paper:

Clinical differentiation between dogs with benign and malignant spirocercosis

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4.1 Abstract

Spirocerca lupi is a nematode infesting the canine oesophagus, where it induces the formation of a nodule that may transform into a malignant sarcoma. The current, retrospective study compared the clinical presentation, haematology, serum albumin and globulin and radiology of benign cases (n=31) and malignant cases (n=31) of spirocercosis.

Dogs with spirocercosis-induced sarcoma were significantly older (6.4±1.91 years) than benign cases (4.93±2.87). In the malignant cases there were significantly (p=0.03) more sterilized females (10/31) and fewer intact males (4/31) compared to 2/31 and 13/31, respectively, in the benign cases. Hypertrophic osteopathy was observed in 38.7% of malignant cases and in none of the benign cases (p=0.0002). Common clinical signs included weight loss, regurgitation, anorexia, pyrexia

(T≥39.5°), respiratory complications and salivation but did not differ in prevalence between groups. On haematology, the malignant group had significantly ($p<0.05$) lower haematocrit (0.34±0.08 vs. 0.41±0.07) and higher white cell count (31.6±27.83 vs. 17.71±13.18 $x10^3$/µl), mature neutrophil count (26.06±26.08 vs. 12.23±9.96 $x10^3$/µl) and thrombocyte count (493.15±151.61 vs. 313.27±128.54$x10^9$/µl). There were no differences in the mean corpuscular volume and immature neutrophil count. On radiology, the mass length was not significantly different, but the height and the width of the malignant masses were significantly larger (62.59 ± 15.15 and 73.93 ± 20.94 mm) compared to the benign group (46.43 ± 23.62 and 49.29 ± 25.56, respectively). Spondylitis was more prevalent in the malignant group (67.86% vs. 38.46%, $p = 0.03$). Examining secondary pulmonary changes revealed significantly higher prevalence of bronchial displacement in the malignant group (52% vs. 17%, $p = 0.008$).

Hypertrophic osteopathy appeared to be a very specific but relatively rare (poor sensitivity) marker of malignancy. Female gender, anaemia, leukocytosis, thrombocytosis, spondylitis and bronchial displacement are significantly more common in malignant cases, but appear in benign cases as well. However, if found together in a specific case, they should increase the index of suspicion for malignancy in a diagnosed spirocercosis case.

Keywords: Spirocercosis; dog; sarcoma; oesophageal nodule; hypertrophic osteopathy.

4.2 Introduction

Spirocerca lupi (S. lupi) is a nematode of worldwide distribution, but it is most commonly found in tropical and subtropical areas (Bailey, 1972). Dogs are the definitive hosts and become infested by ingesting the coprophagous beetle intermediate hosts (Bailey, 1972). After ingestion the larvae are liberated in the gastric lumen, and migrate via the gastric mucosa, gastric arteries, and aorta and eventually through the thoracic aortic wall to the caudal oesophagus. Typically, the worms settle within the oesophageal wall, mature to adults and promote formation of a nodule (Bailey, 1963, 1972; van der Merwe et al., 2008). The nodules are often referred to as granulomatous (Bailey, 1963, 1972), but histologically this is inappropriate as the mature nodule is composed mostly of actively dividing immature fibroblasts with relatively pronounced vascularisation (van der Merwe et al., 2008). The host inflammatory reaction is commonly mild to moderate and is not characterized by a predominance of macrophages, as would be expected in granulomatous inflammation (Bailey, 1963; van der Merwe et al., 2008). Spirocercosis induces a few pathognomonic lesions; aortic scarring with aneurysm formation, thoracic vertebral spondylitis and caudal oesophageal nodule formation. The typical clinical signs associated with spirocercosis are related to the presence of oesophageal nodules and include regurgitation, vomiting, dysphagia and weight loss, together with non-specific signs like pyrexia (Dvir et al., 2001; Mazaki-Tovi et al., 2002). The clinical diagnosis of spirocercosis is largely dependant upon thoracic radiography, which demonstrates the caudal oesophageal soft tissue masses, caudal thoracic vertebral spondylitis and aortic undulation due to aneurysm formation. Faecal flotation tests are also used to detect the typical embryonated eggs. Oesophagoscopy typically demonstrates one or more nodules with a nipple-like protuberance.

The oesophageal nodule may undergo malignant neoplastic transformation (Bailey, 1963; Seibold et al., 1955). The association between *S. lupi* infection and oesophageal sarcoma was first described in 1955 (Seibold et al., 1955). This association was based on the finding of *S. lupi* worms and related oesophageal nodules close to the malignant neoplasm or the pathognomonic findings of spondylitis or aortic aneurysm in conjunction with the malignant neoplasm. Macroscopically, an increased size, and progression to cauliflower-like shape and area of necrosis in the malignant nodule compared to the smooth appearance of the benign nodule may be indicative of neoplastic transformation (Ranen et al., 2004). Histologically the malignant neoplasms are classified as fibrosarcoma, osteosarcoma or anaplastic sarcoma (Ranen et al., 2007; Ranen et al., 2004). The histopathological characteristics of the *S. lupi*-induced fibrosarcoma include interwoven bundles of tapered to plump spindle shaped cells, variable amounts intercellular collagenous matrix, and a high mitotic index (Bailey et al., 1963). Histological characteristics of the *S. lupi*-induced osteosarcoma include foci of polygonal osteoblasts, and variable numbers of osteoclasts and/or interwoven bundles of spindle cells, variable amount of osteoid matrix with or without foci of chondroid differentiation. Sometimes obvious spicules or trabeculae of bone are identified amidst solid foci of neoplastic spindle or polygonal cells (Bailey, 1963). In areas where spirocercosis does not exist, malignant neoplasms of the oesophagus are extremely rare (Ridgway and Suter, 1979), making spirocercosis the major cause of malignant oesophageal neoplasms in the dog. Spirocercosis-induced sarcoma metastasizes commonly to the lung but also to a variety of abdominal organs (Bailey, 1963; Dvir et al., 2001).

Benign spirocercosis is treated successfully with avermectins [doramectin (Dectomax, Pfizer, France) 400 µg/kg SC at 2-week intervals] (Lavy et al., 2002). However,

malignant neoplasms can only be treated by surgical excision with or without chemotherapy and the success rate is lower (Ranen et al., 2004). This difference in prognosis emphasizes the need to improve diagnostic and prognostic markers for the antemortem diagnosis of the oesophageal nodule and the need for a better understanding of the malignant transformation that may improve treatment for the malignant cases.

While spirocercosis has a few pathognomonic lesions, the ante-mortem differentiation of malignant neoplasm-bearing cases and benign cases has not been studied. The objectives of the present study were to identify clinical ante-mortem differences between malignant and benign spirocercosis cases to assist in diagnosis, treatment and prognostication.

4.3 Material and Methods

Medical records of 297 dogs diagnosed with spirocercosis at the Onderstepoort Veterinary Academic Hospital, University of Pretoria, South Africa, during 1995-2006, were retrospectively reviewed. From these records two groups of cases were selected: confirmed benign cases and confirmed malignant oesophageal nodule cases.

The inclusion criteria for the benign group were: An endoscopic diagnosis of spirocercosis with an obvious response to treatment within at least 6 weeks or a histological diagnosis of a benign nodule after surgical resection of the whole nodule or diagnosis of benign spirocercosis at necropsy, including histological appraisal of the entire nodule. A diagnosis of benign nodule based only on endoscopic guided biopsy was judged to be unsatisfactory as it has been shown to be highly insensitive in a few studies (Dvir et al., 2001; Mazaki-Tovi et al., 2002; Ranen et al., 2004). The inclusion criteria for the malignant group were: histological diagnosis of malignancy of an oesophageal nodule obtained either by endoscopic-guided biopsy, surgical

resection or necropsy. Cases with radiographic diagnosis of caudal oesophageal nodules, spondylitis and thoracic metastases with no other obvious primary neoplasm elsewhere in the body without histological analysis of the oesophageal nodule or the metastasis were also included in the malignant group.

The following clinical parameters were compared: age, breed, gender, body weight, duration of illness and the prevalence of weight loss, vomiting/regurgitation, anorexia, pyrexia (≥ 39.5 ºC), lameness, hypertrophic osteopathy (HO), respiratory signs and salivary gland enlargement. The clinicopathological parameters that were compared included: haematocrit (Ht), mean corpuscular volume (MCV), white blood cell count (WBC), mature and immature neutrophil, monocyte and eosinophil counts and serum albumin and globulin concentrations.

Thoracic radiographic evaluation and measurements were done by one board-certified radiologist (RK) on cases having at least dorsoventral and right lateral thoracic films. The following radiological parameters were compared: mass location (relative to thoracic vertebrae) and mass size (length, width, height). The presence of the following radiological findings were recorded and their prevalence was compared between the two groups: mass mineralization, oesophageal gravel sign (ingested mineralized debris) and air (indicating partial obstruction), spondylitis, pulmonary parenchymal changes, fissure lines/pleural effusion, thoracic lymph node visualization, tracheal displacement, bronchial displacement/compression and aortic changes.

Differences in the above stated categorical parameters (age, breed, gender and prevalence of clinical signs and clinicopathological and radiological abnormalities) were then compared between the two groups using the chi-square (χ^2) test. Continuous parameters (body weight, duration of illness, clinicopathological values and

radiological measurements) are presented as mean ± standard deviation and were compared between the two groups using the t-test. The level of significance for both tests was determined as $p < 0.05$.

4.4 Results

Sixty-two dogs had adequate data to fulfil the inclusion criteria. Thirty one were included in the benign group; 19 were based on endoscopic diagnosis and response to treatment, 12 were based on histology of the entire oesophageal nodule, ten of which were necropsy cases, and two were surgical resection cases. Thirty one dogs were included in the malignant group; 27 were diagnosed histologically and four cases were selected according to the combination of a caudal oesophageal mass, spondylitis and thoracic metastasis.

4.4.1 Signalment

There was a significant difference in the gender distribution between the groups ($p=0.03$) with more females, especially sterilised ones, in the malignant group and more males, especially intact ones, in the benign group (Table 1). The age of the dogs differed significantly between the two groups ($p=0.02$) being 4.93 ± 2.87 years in the benign group and 6.40 ± 1.91 years in the malignant group. There were no significant differences between the average body weight of the two groups (23.61 ± 9.90 kg vs. 26.27 ± 11.10 kg in the benign and malignant group, respectively).

4.4.2 Clinical presentation

Hypertrophic osteopathy was the only clinical sign with a significantly different prevalence between the two groups (Fig. 1), as it presented only in the malignant group (38.7% prevalence $p= 0.0001$). The prevalence of the remaining clinical signs:

weight loss, vomiting/ regurgitation, anorexia, pyrexia, lameness, respiratory signs and salivary gland enlargement, did not differ significantly between groups (Table 2). There were also no significant group differences on average body temperature (39.13 ± 0.73ºC vs. 39.19 ± 0.69ºC) and average duration of illness (7.89 ± 7.92 weeks vs. 8.83 ± 20.58 weeks) between the benign and malignant groups, respectively.

4.4.3 Haematology

Complete blood counts were available for 27 dogs in each group (Table 3). Although the haematocrit was significantly lower in the malignant group (p=0.002), there was a substantial overlap in the range of both groups. Anaemia, defined as a haematocrit < 37l/l, was diagnosed with significantly higher prevalence in the malignant group (p=0.003) and was normocytic in most cases (50% vs. 64.71 % in the benign and malignant groups, respectively). The prevalence of leukocytosis was significantly higher in the malignant group (p=0.03), but there was a substantial overlap in the range of the counts. The increase in cells consisted of mature neutrophils and monocytes. Eosinophil counts were significantly higher in the benign group (p=0.04), however the overlap in the range between the two groups was marked. The thrombocyte count and prevalence of thrombocytosis was significantly higher in the malignant group (p<0.001 and p=0.002, respectively).

4.4.4 Serum proteins

Serum protein concentrations were available for 19 cases in each group. Serum albumin was higher in the benign group compared to the malignant group (2. 87 ± 0.77 vs. 2.5 ± 0.53 mg/dl, normal range 2.7-3.5 mg/dl), but there was a marked overlap in the range and the difference between the groups was not significant (p=0.09). Serum globulin was significantly higher in the benign group (5.09 ± 1.60 vs.

4.11 ± 0.91 mg/dl in the benign and malignant group, respectively, p=0.03, normal range 2–3.7 mg/dl) but again, the overlap was substantial.

4.4.5 Radiology

Thoracic radiographs were available for 25 dogs from the benign group and for 28 dogs in the malignant group (Table 4).

In the benign group, 24 dogs had a confirmed oesophageal mass diagnosed on endoscopy or necropsy, but the mass was only radiologically visible in 21 dogs. In the malignant group 27/28 masses were visible radiologically (Fig. 2). In both groups the masses were located between T5 and T12. Comparing the length, height and width of the oesophageal masses between the groups revealed significant differences in the height and width only (higher values, p = 0.006 and 0.0006, respectively) with substantial overlap. Mass mineralization was a relatively rare sign, more likely to be seen with malignant transformation. Bronchial displacement was significantly more common in the malignant group (p=0.008), as was spondylitis (p=0.03).

4.5 Discussion

Previous studies that discussed spirocercosis-associated malignancy hypothesized that genetic and environmental factors may play a role in carcinogenesis (Bailey, 1972). In the current study, the number of sterilised females with malignant transformation was significantly higher compared to those with benign disease. Intact males were more prevalent in the benign group. This higher prevalence of malignant transformation in sterilised females has also been described in a previous study evaluating only malignant *S. lupi* cases (Ranen et al., 2007; Ranen et al., 2004). This might indicate a predisposition of sterilised female dogs with spirocercosis to undergo

malignant transformation of the oesophageal nodule and a possible resistance in intact males. This may indicate protective effect of sex steroids, especially androgens.

The mean age of the group with malignant transformation is similar to the mean age previously described in *S. lupi*-induced sarcoma cases (Ranen et al., 2004). The difference in age between the two groups, observed in the current study, may be partially explained by the time taken for the malignant neoplasm to develop and be diagnosed, but may also indicate an increase predisposition to malignant transformation with advanced age.

A comparison of the clinical presentation of the two groups revealed only one clinical sign which appeared to be highly specific for malignant cases, namely HO. Hypertrophic osteopathy is often reported in conjunction with *S. lupi*-induced sarcomas (Bailey, 1963; Brodey et al., 1977). It is characterised by irregular thickening of the parosteal tissue and exostotic proliferation of bone and cartilage in the distal limbs (phalanges, metatarsal and metacarpal) and vascularisation (Brodey, 1979; Seibold et al., 1955). Radiologically, HO is described as periosteal new bone formation (Brodey, 1971). Hypertrophic osteopathy has been linked to intrathoracic, especially pulmonary, masses (Brodey, 1971). Pulmonary neoplasia is the most common cause of HO. However, before 1944 the most common aetiology was tuberculosis, indicating that inflammatory–induced masses can also cause HO (Brodey, 1971). It is not clear from the literature if only spirocercosis-induced malignant neoplasms can induce HO or if benign nodules can also induce it (Brodey, 1971). In the current study, with its limited case numbers, only malignant spirocercosis-induced nodules were associated with HO. We therefore encourage clinicians to look for signs of HO on those parts of the thoracic limb seen on thoracic

radiographs as a possible clue for malignancy and to perform distal limb radiographs in any suspected swollen limbs.

Four theories have been proposed for the pathogenesis of HO: vagal nerve stimulation, pulmonary arterio-venous shunting, the production of a humeral substance by neoplastic cells and factors secreted by megakaryocytes or platelet clumps lodged in blood vessels of distal limbs (Dunn et al., 2007). Vagotomy caused dramatic reversal of HO (Brodey, 1979). This finding formed the basis of the theory that vagal stimulation is responsible for the development of HO. Bailey observed involvement of the vagus nerve within some of the malignant *S. lupi*-induced neoplasms (Bailey, 1963), and mass infiltration within the oesophageal vagus has been postulated as the major cause of HO in spirocercosis. Increased limb blood flow was proposed as a major contributor to the development of HO. It might be induced by the vagal stimulation and can be reduced by resection of the affected lung lobe and vagotomy (Brodey, 1971). Experimentally created right-to-left shunts can cause HO (Brodey, 1979). It was proposed that shunting allows humoral substances that are normally inactivated by the lungs to escape and reach the distal limbs (Martinez-Lavin, 1992). Later it was shown that platelet clumps are commonly lodged in the blood vessels of the extremities in HO cases, and it was hypothesized that megakaryocytes that escape the pulmonary capillary beds via the shunts facilitate the production of the platelet clumps that induce HO (Atkinson and Fox, 2004). The same might happen in diseases that release platelet aggregates from the left side of the heart, as happens in mitral or aortic vegetative endocarditis (Dunn et al., 2007). In the early form of HO in humans, also called digital clubbing, increased platelet derived growth factor and vascular endothelial growth factor expression was observed in tissue samples from digits stained immunohistochemically (Atkinson and Fox, 2004).

These growth factors were postulated to be released from the platelet clumps, inducing the tissue proliferation, increased vascularity and capillary permeability seen in HO (Atkinson and Fox, 2004). In a unifying theory for the pathogenesis of HO, platelet derived growth factor was proposed as the humeral substance inducing HO (Martinez-Lavin, 1992). Vagal stimulation was suggested to be only a contributing factor, which presumably causes increased blood flow in distal limbs and therefore facilitate platelets lodging and increased circulation of the humeral agent, which would explain the improvement in some patients following vagotomy. The concept of a humeral factor causing HO in spirocercosis seems very attractive. Spirocercosis appears to induce connective tissue proliferation throughout the course of the disease (spondylitis, oesophageal nodule, sarcoma and HO). It may be postulated that a circulating oncogenic or growth factor induced by the worm infection, which induces connective tissue proliferation, might provide a unifying pathogenesis for the development of the different forms of connective tissue proliferation in spirocercosis.

A few reports have claimed that spondylitis is more common in malignant cases (Brodey et al., 1977; Ranen et al., 2004), as shown in the current study. However, the prevalence of spondylitis in the benign cases was 38.26%, indicating that spondylitis starts early in the disease process and is progressive. The finding of larvae in the muscles between the aorta and the spine led to the theory that aberrant migration may be responsible for the spondylitis (Bailey, 1972). This explanation is under investigation in our institute and it appears to be at best only partially true, because often the malignancy appears to progress long after the worms have disappeared and they therefore can no longer play a role in the development of more overt spondylitis. It can be postulated that the worm may induce the spondylitis by stimulating changes

that later become independent of its presence or further changes could be induced by the malignant tumour.

Other than HO, no other presenting clinical signs or complications, or period of illness before presentation occurred significantly more frequently in either group. In a recent publication describing only spirocercosis associated oesophageal sarcoma cases (Ranen et al., 2004), the prevalence of vomiting and/or regurgitation (94%) was higher than what we observed in any of the groups and was reported in prior spirocercosis studies that investigated malignant and benign cases together (Dvir et al., 2001; Mazaki-Tovi et al., 2002). Thus, an increased prevalence of vomiting or regurgitation might be more frequent in malignant cases but we cannot support it comparing the 2 groups.

Anaemia related to spirocercosis has been described in benign (Brodey et al., 1977) as well as malignant cases (Ranen et al., 2004). In the current study, anaemia was proved to be more severe and more prevalent in malignant cases, but the overlapping range was quite broad. Comparing our results with those of the other study of *S. lupi*-induced sarcoma (Ranen et al., 2004), the current study showed fewer microcytic anaemia cases (35% vs. 63% in Ranen's study) and the current study also revealed no statistical difference from the benign group. Comparing the mean MCV in both studies reveals similar results, making the differences between the studies negligible. The most common explanation for microcytic anaemia is chronic blood loss, which can easily be explained by the predisposition of the *S. lupi* nodule to ulcerate. However, considering the high prevalence of dogs with normocytic anaemia, there are probably other factors that play a role in *S. lupi*-associated anaemia, such as anaemia of chronic disease or possibly paraneoplastic effects.

In the current study, leukocytosis was significantly more severe and prevalent in the malignant cases, as has been reported in another study (Ranen et al., 2004). Leukocytosis and eosinopenia has also been reported in malignant spirocercosis cases in an earlier study based only on 3 dogs (Brodey et al., 1977). In the current study, we confirmed these observations using a larger number of cases and by comparing values between malignant and benign cases. In the current study, thrombocytosis was also more common in the malignant group. This set of abnormalities, anaemia, leukocytosis and thrombocytosis, could be caused by continuous oesophageal irritation and blood loss from the malignant neoplasm. However, these haematological abnormalities may have a paraneoplastic origin and may provide a hint about the role that thrombocytes and leukocytes may play in the malignant transformation of the oesophageal nodule. Further research is required to explore this issue.

The search for radiological parameters to differentiate malignant from benign cases revealed substantial overlap in most measurements excluding HO. It may seem surprising that the mass length was similar for benign and malignant nodules, but this is probably due to the number of nodules, which can range from 1 to 8 and may coalesce longitudinally on radiographs. The height and width of the nodules are more reliable parameters, reflecting the larger size of malignant nodules. Bronchial displacement was also more common in the malignant group, and is probably secondary to the mass size.

Mass mineralization was assumed to be a relatively rare but specific marker for malignant transformation; however we did detect 1 benign case with mineralization on radiographic examination and 2 additional benign cases with foci of osseous metaplasia within a nodule (seen histologically), which could provide a pathophysiological mechanism for the presence of mineralization in benign nodules.

The presence of ingested mineralised debris should not be mistaken for mineralization of the nodule. The presence of osseous metaplasia may be another indication of the slow progression from benign to malignant nodules in spirocercosis. The gradual transition to sarcoma and the evidence of large numbers of embryonic fibroblasts in early benign nodules (Bailey, 1972), may be at least partially responsible for the big overlap or lack of significant differences in most of the parameters compared between benign and malignant groups. Computed tomography with its greater sensitivity to detect mineralization and to assess nodule perfusion after contrast medium administration may provide more clues in future about nodule characteristics and is currently being investigated by our institution.

Endoscopy was reported to be the most sensitive tool for spirocercosis diagnosis (Dvir et al., 2001; Mazaki-Tovi et al., 2002) and Ranen and others (2004) reported that they were able to make a tentative diagnosis of *S. lupi* – induced sarcoma on all the 15 cases they have scoped. We did not perform a detailed retrospective evaluation of our endoscopic and gross pathology changes as no consistent descriptions were used. However, in going through the available material various descriptions of irregularity, proliferation, necrosis and ulceration were common in the malignant cases (Fig. 3A), but 2 cases were described as smooth, which was an unexpected finding in malignant nodules. Benign nodules were very often described as typical (with a nipple-like protuberance), small and smooth (Fig. 3B); however in few cases inflammation, ulceration and necrosis were reported, which could raise the index of suspicion for malignant transformation. These lesions could be secondary to mechanical irritation of the partially obstructed oesophagus. Currently we are prospectively investigating the predictability of endoscopy to determine malignancy. Endoscopy-guided biopsy can only help if it is positive for malignancy as it is specific

but not sensitive (Dvir et al., 2001). Therefore, although we find endoscopy a reliable tool, equivocal cases need to be monitored carefully for their response to treatment. In case of uncertainty following biopsy and a short treatment course, resection is indicated for both treatment and diagnosis of malignant vs. benign nodule.

The search for antemortem indicators of malignant transformation of the oesophageal nodules did not yield any highly sensitive and specific marker. Hypertropic osteopathy was highly specific for malignancy but it is a relatively rare finding (38.7%). Female gender, anaemia, leukocytosis, thrombocytosis, spondylitis and bronchial displacement are more sensitive and less specific parameters that should be evaluated as a constellation of parameters, and, if found together in a specific case, should increase the index of suspicion for malignancy in a diagnosed spirocercosis case. They may also provide clues about the pathogenesis of the malignant transformation, which requires further investigation.

4.6 Tables

Table 1

Gender differences between benign and malignant groups (p=0.03, chi-square test)

Gender	Benign group *n*=31	Malignant group *n*=31
Intact female	38.7%	41.9%
Sterilized female	9.7%	32.3%
Intact male	41.9%	12.9%
Sterilized male	9.7%	12.9%

Table 2

Prevalence differences of clinical signs between the benign and malignant groups

Sign	Prevalence (%)	
	Benign group *n*=31	Malignant group *n*=31
Weight loss	58.06%	77.42%
Vomiting / regurgitation	67.74%	67.74%
Anorexia	45.16%	48.39%
Pyrexia	32.26%	41.94%
Lameness	12.9%	19.35%
Respiratory signs	33.26%	35.48%
*Hypertrophic osteopathy	0%	38.71%
Salivary glands enlargement	22.58%	25.81%

* p = 0.0001 (chi-square test)

Table 3

Haematology differences between the benign and malignant groups

Parameter	Benign group *n*=27	Malignant group *n*=27	Reference Interval
*Haematocrit (l/l)	0.41 ± 0.07	0.34 ± 0.08	0.37-0.55
*Prevalence of anaemia	22.22%	62.96%	
Mean corpuscular volume (fl)	63.67 ± 6.01	61.11 ± 7.62	60-77
Prevalence of microcytosis within the anaemic cases	50%	35.29%	
*White blood cell count ($x10^3/\mu l$)	18.03 ± 12.71	31.60 ± 27.84	6-15
*Prevalence of leukocytosis	44.44%	81.48%	
*Mature neutrophil count ($x10^3/\mu l$)	12.16 ± 9.81	26.06 ± 26.08	3-11.5
*Prevalence of mature neutrophilia	33.33%	70.37%	
Immature neutrophil count ($x10^3/\mu l$)	1.35 ± 3.04	1.06 ± 2.46	0-0.5
Prevalence of immature neutrophilia	29.63%	33.33%	
Monocyte count ($x10^3/\mu l$)	1.49 ± 1.47	2.20 ± 1.35	0.15-1.35
*Prevalence of monocytosis	33.33%	66.67%	
*Eosinophil count ($x10^3/\mu l$)	0.86 ± 0.79	0.49 ± 0.50	0.1-1.25
Prevalence of eosinophilia	25.93%	11.11%	
Prevalence of eosinopaenia	14.81%	29.63%	
*Thrombocyte count ($x10^9/\mu l$)	313.27 ± 128.54	493.15 ± 151.61	200-500
*Prevalence of thrombocytosis	7.41%	37.04%	

* $p < 0.05$ (t-test for absolute values and chi square test for prevalence)

Table 4

Radiological differences between the benign and malignant groups

Thoracic radiological findings	Benign group *n*=25	Malignant group *n*=28
Oesophageal mass length (mm)	81.90 ± 41.79	91.67 ± 28.86
*Oesophageal mass height (mm)	46.43 ± 23.62	62.59 ± 15.15
*Oesophageal mass width (mm)	49.29 ± 25.56	73.93 ± 20.94
Prevalence of oesophageal mass mineralization	4.76%	22.22%
Prevalence of oesophageal gravel sign	0%	11.11%
Prevalence of oesophageal air	52.38%	48.15
Prevalence of pulmonary parenchyma changes	16.00%	14.29%
Prevalence of fissure lines / pleural effusion	12.00%	21.43%
Prevalence of lymph nodes visualization	8.00%	0%
Prevalence of tracheal displacement	8.00%	17.86%
*Prevalence of bronchial displacement	16.00%	53.57%
Prevalence of aortic changes	48.00%	42.86%
Spinal radiological findings	*n*=26	*n*=28
*Prevalence of spondylitis	38.46%	67.86%
Number of spondylitis vertebrae	3.40 ± 1.78	3.55 ± 1.83

* $p < 0.05$ (t-test for absolute values and chi square test for prevalence).

4.7 Figures

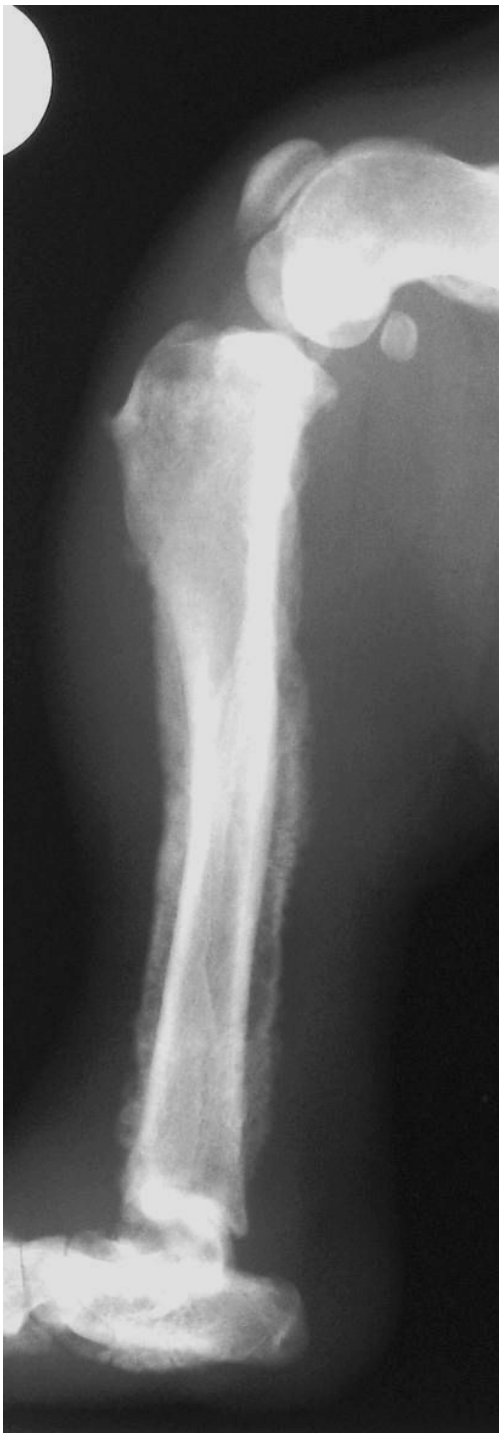

Figure 1: Mediolateral view of the tibia of a six year old Staffordshire bull terrier with hypertrophic osteopathy. Note the thick brush-like periosteal reaction on the tibia, distal femur and caudal aspect of calcaneus. This reaction was present extensively on all the limbs.

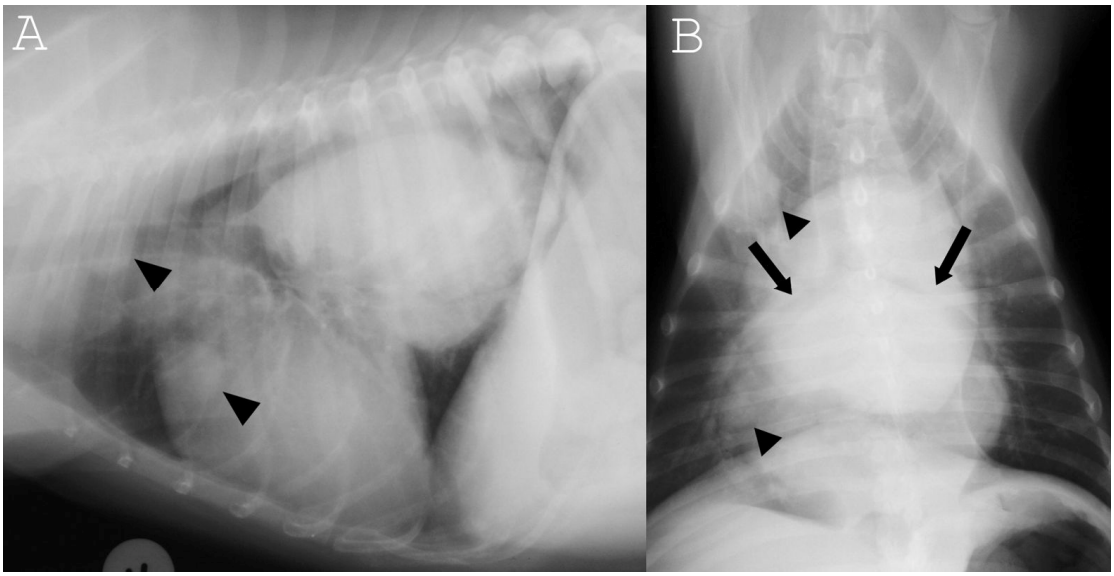

Figure 2: Medial-lateral (A) and ventro-dorsal (B) thoracic radiographs of the same dog as in Fig. 1. Note the large soft tissue mass superimposing on the caudal cardiac silhouette and cranial diaphragm. The mass displaces and compresses the main stem bronchi (arrows). Poorly defined metastatic nodules are visible (arrow heads).

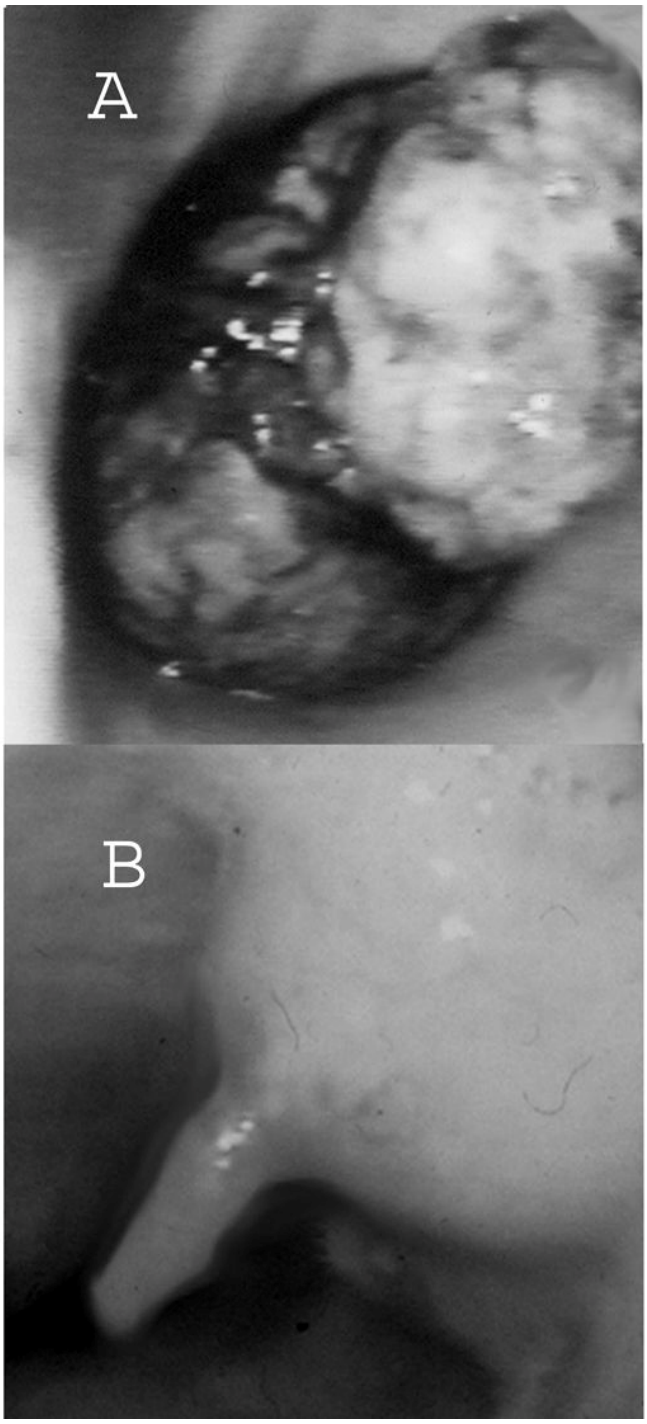

Figure 3: Oesophageal endoscopic pictures of neoplastic nodule (A) and benign nodule (B). In the neoplastic nodule, note the lobulated proliferation, area of black colouration indicating ulceration and necrosis and the size of the nodule occupying most of the lumen. In the benign nodule, note the smooth, round appearance with typical protuberance, the healthy mucosa surrounding it and the small size in relation to the oesophageal lumen.

5 PROPOSED HISTOLOGICAL PROGRESSION OF THE *SPIROCERCA LUPI*-INDUCED OESOPHAGEAL LESION IN DOGS

This chapter was published as a research paper:

Proposed histological progression of the Spirocerca lupi-induced oesophageal lesion in dogs

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5.1 Abstract

This study aims to outline the histological progression of the *Spirocerca lupi* nodule from infection to neoplastic transformation. Sixty two spirocercosis-induced nodules, 42 non-neoplastic and 20 neoplastic, were stained with HE. Ten non-overlapping high power fields per nodule were examined and non-neoplastic and neoplastic nodules were compared. Inflammation was scored 0-3 and revealed a score of 1.91±0.52 in the non-neoplastic and 0.97±0.5 in the neoplastic cases ($p<0.01$). In most non-neoplastic cases the inflammatory infiltrate was lymphoplasmacytic and in the neoplastic cases neutrophils predominated. Necrosis was scored 0-3 and revealed a score of 0.88±0.41

in the non-neoplastic and 1.47±0.5 in the neoplastic cases ($p<0.01$). The average number of mitoses over 10 high power fields per nodule was 1.31±1.55 in the non-neoplastic compared to 42.85±30.79 in the neoplastic cases ($p<0.01$). The average number of multinucleated giant cells over 10 high power fields per nodule was 0.9±1.45 in the non-neoplastic compared to 13.9±14.66 in the neoplastic cases ($p<0.01$). In the non-neoplastic cases, collagen, immature fibroblasts and fibroblast activation (excessively plump euchromatic nuclei with single or multiple prominent nucleoli) were scored 0-3 and a combined score, fibroblasts + activation score - collagen was calculated. The non-neoplastic cases were divided into a combined score of ≤1 (n=15) or >1 (n=27). The 2 groups had similar scores for inflammation and necrosis, but were significantly different ($p<0.01$) in mitotic index (0.26±0.46 vs. 1.89±1.65) and number of multinucleated cells (0 vs. 1.4±1.6). These results indicate 2 stages in the non-neoplastic nodules: early inflammation, characterized by fibrocytes and abundant collagen, and a pre-neoplastic stage, characterized by activated fibroblasts and reduced collagen.

Keywords: Spirocercosis, fibrosarcoma, osteosarcoma, undifferentiated sarcoma, oesophageal nodule

5.2 Introduction

Spirocerca lupi (S. lupi) is a spiurid nematode of carnivores, particularly Canidae, of worldwide distribution but most prevalent in the tropics and subtropics. Spirocercosis in dogs was comprehensively reviewed recently (van der Merwe et al., 2008). Dogs become infested with the worm when they ingest either the coprophagous beetle intermediate hosts or a paratenic host. Following ingestion of the intermediate or paratenic host *S. lupi* L3 larvae are liberated in the gastric lumen. Larvae penetrate the

gastric mucosa and migrate in the wall of the gastric and coeliac arteries to the caudal thoracic aorta. Larvae spend up to three months in small nodules in the aortic wall, where they moult to L4 and finally to adults. Young adult worms then migrate from the aorta to the oesophagus below. Groups of from three to six worms cluster together in the oesopageal submucosa and induce the formation of nodules. Over time, some of the oesophageal nodules may undergo malignant neoplastic transformation, with subsequent metastasis to other sites.

Spirocerca lupi-induced oesophageal nodules are invariably incorrectly referred to as granulomas in the literature (Bailey, 1972; Stephens et al., 1983). Initially the worms are surrounded by highly vascularised loose connective tissue, which contains an exudate consisting of fibrin-rich fluid and neutrophils and foci of necrotic tissue. Bailey (1963) likens it to granulation (repair) tissue. Later, the tissue surrounding the worms is composed mostly of actively proliferating fibroblasts. These fibroblasts often have an embryonal appearance, sometimes resembling those seen in fibrosarcomas (Bailey, 1963; Hu and Hoeppli, 1936). At no stage of the genesis of the nodule is there a predominance of macrophages, which is the hallmark of granulomatous inflammation (Dvir et al., 2008; van der Merwe et al., 2008). The temporal histological progression of the oesophageal lesion, from early nodule to malignant neoplasm has not been reported.

The association between spirocercosis and oesophageal sarcoma was first described in 1955 (Seibold et al., 1955). This association was based on the finding of *S. lupi* worms in oesophageal nodules close to the sarcoma or the pathognomonic findings of spondylitis or aortic aneurysms in conjunction with the oesophageal tumour. The spirocercosis-associated sarcoma has been described in detail (Bailey, 1963, 1972;

Seibold et al., 1955; van der Merwe et al., 2008). Histologically the sarcoma has been classified as fibrosarcoma, osteosarcoma or anaplastic sarcoma (Ranen et al., 2007; Ranen et al., 2004). Interestingly, foci of chondroid and/or osseous metaplasia have also been observed within neoplastic and non-neoplastic nodules (Dvir et al., 2008; Ribelin and Bailey, 1958). The histological characteristics of the *S. lupi*-induced fibrosarcoma include: short or long interwoven bundles of pleomorphic cells that may vary from highly undifferentiated (anaplastic), roughly spindle-shaped cells with round or ovoid nuclei, to interlacing bundles of elongated cells resembling immature connective tissue; variable amounts of intercellular collagenous matrix, and a high mitotic index (Seibold et al., 1955). The histological characteristics of the *S. lupi*-induced osteosarcoma include: foci of closely-packed, short, spindle- or polygonal/triangular-shaped osteoblasts (with plump, ovoid nuclei) oriented to point in various directions and ostensibly filling the intertrabecular spaces; variable numbers of multinucleated cells (both osteoclasts and tumour giant cells can be identified), and variable quantities of osteoid matrix and/or woven bone, with or without foci of chondroid differentiation. In the better-differentiated osteosarcomas, conspicuous osteoid as well as spicules or trabeculae of mature mineralized bone are identified amidst solid foci of neoplastic osteoblasts (Bailey, 1963). Anaplastic sarcomas are characterized histologically by the presence of obviously neoplastic, plump, roughly spindle-shaped cells, usually in an interwoven or interlacing pattern, without the presence of clearly identifiable intercellular matrix, and numerous mitoses. In areas where spirocercosis does not exist, malignant neoplasms of the oesophagus are extremely rare (Ridgway and Suter, 1979), making spirocercosis the major cause of malignant oesophageal neoplasia in dogs.

Studying the temporal morphological changes in a lesion may help to understand the pathogenesis of the lesion as well as possibly establishing cause-and-effect relationships and stage specific prognostication and treatment. In *Helicobacter pylori*-induced gastric carcinoma in humans, another malignancy that is known to be caused by an infectious organism, the earliest defined change involves lymphocytic inflammation (gastritis). This progresses to mucosal atrophy, characterized by the introduction of fibrous stroma in the stomach mucosa. The latter change is thought to be responsible for the subsequent metaplasia, dysplasia and finally neoplastic transformation of the gastric mucosal epithelium (Correa and Houghton, 2007).

Feline vaccine-associated sarcoma is another disease condition that has some similarities to spirocercosis-induced sarcoma. The condition has been described in a large number of cats that received a killed aluminium-adjuvanted feline leukemia virus or rabies vaccination (McEntee and Page, 2001). The vaccine-induced nodule shares certain histological features with *S. lupi* nodules, including the presence of a necrotic core and peripheral inflammatory reaction. It has been assumed that vaccine-associated sarcomas arise from the overzealous inflammatory reaction within the nodule (McEntee and Page, 2001). These fibrosarcomas have been well-described staged and graded (Couto et al., 2002). Grading has also been done on a large number of cases of canine osteosarcoma for prognostication purposes (Kirpensteijn et al., 2002), as well as on oesophageal sarcomas (all of them probably *Spirocerca*-induced) (Ranen et al., 2007).

This study aims to outline the histological progression of the spirocercosis-associated oesophageal nodule from the early stage of infection to fibroblastic nodule and, finally, to sarcoma. Thus, the objective of the present study was to clarify the nature

of the inflammatory response within the *Spirocerca*-induced oesophageal nodule and to describe any morphological changes in the fibrocyte/fibroblast cell population and collagen stroma throughout the progression of the nodule. Another objective of the current study was to examine any potential similarities to other infections that progress to cancer such as *Helicobacter pylori.* We also aimed to apply the widely-accepted grading criteria for canine osteosarcoma to spirocercosis-induced neoplastic nodules and to find prognostic indicators.

5.3 Materials and Methods

Sixty two paraffin blocks containing *Spirocerca*-induced non-neoplastic or neoplastic oesophageal nodules, collected between 1998-2008, were retrieved from the archives of the Section of Pathology, Faculty of Veterinary Science, University of Pretoria. Per block, one 5-µm-thick hematoxylin and eosin–stained section was examined under a light microscope. A Martius, Scarlet and Blue (MSB) stain was applied to a duplicate section in order to detect fibrin within the nodules (Jones, 2002). In addition, 10 sections of normal distal third of oesophagus were evaluated and compared with the *Spirocerca*-induced nodules.

Only one nodule was selected per dog and if a dog had more than one nodule, the nodule that was most mature or advanced in relation to the progression of the nodule toward malignancy was selected for evaluation. If a nodule was sectioned more than once, the section with the most advanced fibroplasia was selected.

The following parameters were recorded as present or not per slide examined: worms, worm eggs, worm tract(s) (with or without debris) and metaplasia (osseous, chondroid or myxomatous). The inflammatory infiltrate within or at the periphery of the nodule

was evaluated and characterized as to its intensity. Ten non-overlapping low power fields (x100) were examined and the inflammation was scored 0-3 (0 = scant or absent; 1 = inflammatory cells obviously present but markedly less than other cells; 2 = inflammatory cells roughly equal to fibrocytes; 3 = predominantly inflammatory cells). Inflammatory cells included lymphocytes/plasma cells, macrophages, eosinophils and neutrophils. The predominant inflammatory cell type was recorded per case. It was also recorded whether macrophages were present. It was noted separately if neutrophils were specifically associated with the worm, worm tract and debris or to the peripherally ulcerated rim of neoplastic nodules.

Ten non-overlapping high power fields (x400) were examined and scored (0-3) for necrosis, (0 = no necrosis; 1= small foci of necrosis or widespread single cell necrosis that required careful perusal of the section; 2 = obvious presence of necrosis, but in <50% of the field; 3 = necrosis in >50% of the field). The same scheme was used to score haemorrhage on 3 non-overlapping x40 fields. The quantity of collagen was scored 0-3 on 3 non-overlapping x40 fields (0 = no or normal amount of collagen; 1 = obvious collagen present but occupying less area than cells; 2 = roughly equal amount of collagen and cells; 3 = obviously more collagen than cells).

For the non-neoplastic nodules, 10 non-overlapping x400 fields were scored (0-3) pertaining to the quantity of fibroblasts within the nodule (0 = no or scant fibroblasts; 1= fibroblasts obviously present but definitely less than fibrocytes; 2 = roughly equal to fibrocytes; 3 = fibroblasts definitely more than fibrocytes). If the fibroblasts looked activated (characterized by excessively plump euchromatic nuclei, often with prominent single or multiple magenta nucleoli) the degree of activated fibroblasts was scored using the same scheme.

Neoplasms were identified based on poor differentiation and anaplastic changes including pleomorphism, nuclear hyperchromasia, multiple nucleoli and prominent nucleoli and or extensive mitoses. The cases were initially selected based on the archived histopathology reports from 6 trained veterinary pathologists in the Pathology Section, Department of Paraclinical Sciences, Faculty of Veterinary Science, Onderstepoort. Two experienced pathologists (SJC and MCW) then selected appropriate cases (using the light microscope) with sufficient tissues and minimal autolysis. The selected tissue sections were then further evaluated under the light microscope by 2 researchers (ED and SJC) and the final decision regarding scoring of various histological criteria was made by SJC, based on her experiential knowledge. Notably, in all ambiguous cases, MCW was again consulted and a consensus was reached. The neoplasms were firstly defined phenotypicaly as fibrosarcoma, osteosarcoma or undifferentiated sarcoma based on the criteria outlined in the introduction. Neoplasms were scored according to their degree of differentiation. Mitotic index was determined by counting the number of mitoses per 10 non-overlapping high power fields (x400). Mitotic index was further scored 0-3 as follows: 1 = 1–9 mitotic figures per ten 400x fields; 2 = 10–19 mitoses per ten 400x fields; 3 = 20 or more mitoses per ten 400x fields. Tumour matrix was scored (0-3) according to the ratio between matrix and neoplastic cells: relatively less matrix than cells (3), equal (2) or more (1). Nuclear pleomorphism (NP) was examined in 10 non-overlapping x400 fields and scored 0–4 (0 = all nuclei identical; 1 = < 25% NP; 2 = 25–50% NP; 3 = 50–75% NP; 4 = > 75% NP). When present, multinucleated giant cells (MNGC) were counted per 10 non-overlapping high power fields (x400). The MNGC were scored on a scale of 0 to 3 (0 = no MNGC; 1 = 1-20 MNGC; 2 = 21-40; 3 = >41 MNGC). The density of neoplastic cells within tumour nodules was

evaluated on a scale of 1 to 4 (1 = less than 25% neoplastic cells; 2 = 25–50% neoplastic cells; 3 = 50–75% neoplastic cells; 4 = more than 75% neoplastic cells).

Each section was also graded according to a scheme adapted, with minor alterations, from a previous publication (Couto et al., 2002), combining the necrosis score, degree of neoplastic differentiation and the mitotic score. Final scores of 3 or 4 were designated grade I; scores of 5 or 6 were designated grade II; scores of 7, 8, or 9 were designated grade III.

5.3.1 Data Analysis

For each score assigned, the average score per case was calculated from the different field's scores. The average scores for non-neoplastic and neoplastic cases were calculated and compared by student t-test. For parameters that were only evaluated as present or absent, the prevalence in each group (non-neoplastic or neoplastic) was calculated and compared by chi-square analysis.

5.3.2 Further Analysis and Grading

In the non-neoplastic cases, a combined score of fibroblasts plus activation score minus collagen was calculated. The non-neoplastic cases were divided into cases with a combined score of ≤1 (n=15) or >1 (n=27), respectively, and compared for the different parameters.

5.4 Results

Of the 62 paraffin blocks that were evaluated, 42 were classified as non-neoplastic oesophageal nodules and 20 were classified as malignant neoplasms. In the non-

neoplastic group, evidence of worm migration was observed in almost all cases; a worm or worms were present in 69% of cases, eggs in 71% of cases and a migratory tract or tracts in 95% of cases. In the neoplastic cases, a worm or worms were present in 15% of cases, eggs in 25% of cases and a migratory tract or tracts in 55% of cases (significantly lower than was observed in the non-neoplastic nodules, $p<0.01$). In the non-neoplastic cases, inflammation was very prominent and scored 1.91±0.52, indicating that roughly half of the cell population in non-neoplastic nodules comprised inflammatory cells, compared to 0.97±0.5 in the neoplastic cases ($p<0.01$). In 40% of non-neoplastic cases the inflammatory infiltrate was predominantly lymphoplasmacytic in nature (Fig. 1), in 24% of cases, lymphocytes and neutrophils were mixed, and in 21% of cases, neutrophils predominated, compared to 25%, 5% and 70%, respectively in the neoplastic cases ($p=0.02$). Neutrophils were often distributed diffusely in a nodule, but in a few cases purulent foci were observed immediately adjacent to worm tract(s) and their associated tissue debris (Fig. 2, 3 and 4) or subadjacent to the ulcerated rim of the nodule. Macrophages were usually engorged with haemosiderin and in only two cases were macrophages organized in multifocal granulomata within a nodule. Necrosis scored 0.88±0.41 in the non-neoplastic cases and 1.47±0.5 in the neoplastic cases ($p<0.01$). Haemorrhage was relatively rare within nodules and there was no difference in the haemorrhage score between the neoplastic and non-neoplastic groups (0.68±0.71 vs.0.81±0.68). Fibrin was present in 70% of the neoplastic cases, predominantly in association with ulcerated areas, and significantly more ($p<0.01$) than in the non-neoplastic cases (29%).

The average mitotic index per nodule was 1.31±1.55 in the non-neoplastic compared to 42.85±30.79 in the neoplastic case group ($p<0.01$). The average number of

multinucleated giant cells per nodule was 0.9±1.45 in the non-neoplastic compared to 13.9±14.66 in the neoplastic group ($p<0.01$).

Fibroplasia was very prominent in the non-neoplastic cases. In this group, the fibroblast score was 2.16±0.7, the fibroblast activation score was 0.82±0.55 and collagen scored 1.91±0.7. Two typical patterns were observed within the non-neoplastic nodules regarding fibrocytes/fibroblasts and the preponderance of collagen: one group was characterized by the presence of occasional inactive fibrocytes and a relatively large amount of collagen and the other group exhibited significant fibroblastic activity and a relatively small amount of collagen. The fibroblast score plus the fibroblast activation score minus the collagen score was calculated per non-neoplastic nodule. The non-neoplastic cases were subsequently divided into cases with a combined score of ≤1 (-0.72±1.18, n=15) or >1 (2.04±0.52, n=27), respectively. These two non-neoplastic groups were significantly different ($p<0.01$) in all 3 parameters: collagen (2.66±0.52 vs. 1.51±0.36), fibroblasts (1.48±0.67 vs. 2.52±0.35) and fibroblast activity scores (0.45±0.6 vs. 1.02±0.41), with minimal overlap. However, these two groups had similar scores for inflammation, necrosis, haemorrhage and fibrin, but they were significantly different ($p<0.01$) with respect to mitotic index (0.26±0.46 vs. 1.89±1.65) and number of multinucleated giant cells (0 vs. 1.4±1.6). The group with fewer active fibroblasts and relatively more collagen was classified as the “early nodule” / stage 1 (Fig. 5) and the group with many more active fibroblasts, more marked cellular atypia, and more numerous mitoses was classified as the “pre-neoplastic nodule” / stage 2 (Fig. 6) (Table 1). In the pre-neoplastic group osseous metaplasia was present in three cases. This lesion was not observed in the early nodule, but was also observed in two osteosarcoma cases, in foci well-separated from the neoplastic osteoid matrix and spicules of new bone. In one of the 15 early

nodules, myxomatous metaplasia was seen. In the neoplastic cases 4/17 osteosarcoma cases showed chondroid/chondrous differentiation.

Of the 20 tumours, 17 were osteosarcoma (Fig. 7), and 3 were classified as fibrosarcoma (Fig. 8). No anaplastic sarcomas were identified.

The neoplastic nodules exhibited a high mitotic index score (2.6±1.01) and cell density (3.45±0.83), moderate nuclear pleomorphism (1.64±1.09), extracellular matrix (1.65±0.82) and necrosis (1.46±0.5) and relatively few tumour giant cells (i.e. osteoclasts were not counted) (scored 1.1±0.79) (Table 2). Eighteen cases (90%) showed 13.9±14.66 multinucleated cells (1-58) per 10 high power (400x) fields. This is also reflected by the prevalence of the individual grades within the neoplastic group (Table 2). Using a combined grading system, 11 cases were graded 2 (55%) and 9 were graded 3 (45%).

5.5 Discussion

The present study describes and analyses the progression of canine spirocercosis, from inflammatory oesophageal nodule, to pre-neoplastic fibroblastic nodule and, finally, to sarcoma. This concept has largely been adapted from a similar scheme which was used to map the progression of *Helicobacter*-induced gastritis to gastric adenocarcinoma in humans. In the latter work, such a classification system created the basis for studies on pathogenesis, prognosis and stage-specific therapy (Correa and Houghton, 2007). We propose that non-neoplastic *S. lupi* nodules can be divided into 2 stages: An early inflammatory stage, where the nodule is characterized histologically by lympho-plasmacytic inflammation, fibrocytes and abundant collagen, and a pre-neoplastic stage, where the nodule is characterized by the presence

of activated fibroblasts and reduced collagen, as well as lympho-plasmacytic inflammation. The latter group is regarded as preneoplastic (and therefore more mature than the first group) because: mitoses were more numerous, and the greater proportion of fibroblasts showed some degree of atypia (including multinucleated cells that were often polygonal in shape, and, in some cases, even plump and fusiform with a fairly linear arrangement of plump ovoid central or paracentral overlapping nuclei; these cells were usually intimately associated with plump fibroblasts and intervening wavy collagen fibres). The combined score of fibroblasts plus fibroblast activation score minus collagen indeed resulted in two such distinctive groups. However, within each of the 3 parameters there was minor overlap between the 2 groups, meaning that there are some early cases with reduced collagen and/or increased numbers of immature activated fibroblasts and vice versa. These cases no doubt represent the intermediate phases, indicating that the proposed stages are just a simplified reflection of a continuous process with characteristic temporal and spatial features, rather than a strictly stage-wise process. A larger study would probably have revealed more intermediate phases between the first 2 stages and the final neoplastic stage. Inflammation in both stages of the non-neoplastic nodule was characterized by a predominance of lymphocytes and plasma cells. However, in the earliest stage of the non-neoplastic nodule, there were 3 cases in which inflammation was characterized by a predominance of eosinophils. These particular cases may represent still earlier infection, or they may be representative of nodules containing dead parasites. If such a stage exists, it is particularly difficult to detect as the nodules are likely to be very small and the disease at this point is subclinical.

In *Helicobacter*-induced adenocarcinoma the following stages have been described: Inflammation, dysplasia, metaplasia and, finally, neoplasia. Similar to the

Helicobacter cases, the current study indicates that *Spirocerca*-induced oesophageal nodules are characterized by an early inflammatory stage followed by fibroplasia, increasing fibroblastic activity with cellular atypia, occasional focal metaplasia and, finally, neoplasia. However, contrary to the *Helicobacter* cases, in the present study, the entire oesophageal lesion was never predominantly metaplastic. Three cases in the pre-neoplastic group had foci of osseous metaplasia and might represent a metaplastic phase somewhere between non-neoplastic and obviously neoplastic cases. Alternatively, the metaplastic change may be an incidental change not related to the progression of the lesion to neoplasia.

Helicobacter pylori is by no means the only pathogen considered to be carginogenic in humans. Several other organisms have been implicated as causing neoplasia in humans by virtue of the chronic inflammatory reaction associated with them. These include Epstein-Barr virus, human papillomaviruses, hepatitis B and hepatitis C viruses, human immunodeficiency virus type 1, *Clonorchis sinensis*, *Opisthorchis viverrini* and *Schistosoma hematobium* (Herrera et al., 2005; Schottenfeld and Beebe-Dimmer, 2006). All of these infectious agents and macroparasites are known to induce chronic inflammation, followed, sometimes, by neoplastic change (Moss and Blaser, 2005). However, in comparison to the abovementioned pathogens, neoplastic transformation appears to be far more prevalent (about 20%) in spirocercosis (Dvir et al., 2001). More importantly, where oesophageal sarcoma (excluding leiomyosarcoma) is almost invariably associated with *S. lupi* in the dog, in other oncogenic pathogen-associated neoplasia, most cases cannot be associated with the pathogen and the pathogenesis remains unknown (Herrera et al., 2005). No doubt, spirocercosis-associated sarcoma provides a very promising model system for the

study of pathogen-induced neoplasia. As such, it certainly warrants further in-depth investigation.

The severity of the inflammatory infiltrate, especially in the early oesophageal nodule, has not been described before. The finding of pockets of neutrophils within nodules has been described, but not the high prevalence and severity of the lympho-plasmacytic infiltrate. Interestingly, in a previous study, 8% of spirocercosis cases showed lymphocytosis in their complete blood count (Mylonakis et al., 2006). Lympho-plasmacytic inflammation has been described in association with *Helicobacter*-induced gastritis, where T helper lymphocyte type 1 (Th1) cells and their associated cytokines are thought to play a significant role in carcinogenesis (Wilson and Crabtree, 2007). Lymphoplasmacytic inflammation has also been described in vaccine-associated sarcomas in cats (Couto et al., 2002). Another hypothesis linking lymphocytes and tumour progression is based on the finding of T lymphocytes responsible for suppression of the anti-tumour immune response, namely T-regulatory cells (Beyer and Schultze, 2006). This reaction has been described in a murine model of fibrosarcomas (Beyer and Schultze, 2006), and it is generally characterized by large numbers of lymphocytes in or around the tumours. The role of the lymphocytic infiltrate in the progression of the spirocercal nodule and subsequent neoplastic transformation may warrant further investigation. The first step would be to characterize the lymphocytes involved in the spirocercosis-associated immune response.

Purulent inflammation seems to be particularly associated with ulceration and necrosis in the neoplastic cases, and with the worms, worm tracts with debris, and worm eggs in the early cases. In the neoplastic cases, fibrin was also more abundant.

However, both fibrin and neutrophils are considered to be indicators of acute inflammation, and in spirocercosis-associated nodules they should be considered predominantly in association with foci of necrosis and acute ulceration.

A competing, but not mutually exclusive, hypothesis for the progression of the spirocercal nodule to malignant neoplasia is that the parasite itself, rather than the inflammatory response it produces, is directly responsible for tumourogenesis. This could conceivably be mediated by the synthesis and release of chemical mediators or analogues of host cell-cycle factors such as fibroblast growth factor by the intra-nodular worms. This hypothesis has not, so far, been tested but we hope to remedy this in the near future.

The spirocercosis-induced sarcoma is an aggressive tumour, much more aggressive than feline vaccine-associated sarcomas (Couto et al., 2002), and even more aggressive than non-spirocercosis-associated canine osteosarcoma (Kirpensteijn et al., 2002). A comparison of the prevalence of each graded variable (Table 2) between spirocercosis-associated canine oesophageal osteosarcoma and previously reported non-spirocercosis-associated canine osteosarcoma (Kirpensteijn et al., 2002), revealed that large amounts of necrosis and tumour matrix, as well as increased tumour cell density and increased numbers of MNGC (specifically tumour giant cells), are more commonly associated with spirocercosis-associated sarcoma. However, nuclear pleomorphism seemed to be less pronounced in spirocercosis-associated osteosarcoma in the present study, compared to previous reports of non-spirocercosis-associated canine osteosarcoma. Tumours of different grades within the types of spirocercosis-associated sarcomas may also represent different stages in cancer progression. Further investigation of a greater number and range of spirocercosis-induced oesophageal

nodules from barely-discernable early nodules through to end-stage sarcoma may improve our understanding of the progression and pathogenesis of the oesophageal lesion. It is hoped that the current study has laid the foundations for such an endeavour.

5.6 Tables

Table 1

Histological parameters in the early and preneoplastic non-neoplastic groups

	Early nodule (n=15)	Preneoplastic nodule (n=27)	P value
Collagen (score 0-3)	2.66±0.52	1.51±0.36	<0.01
Fibroblast (score 0-3)	1.48±0.67	2.52±0.35	<0.01
Activated fibroblast (score 0-3)	0.45±0.6	1.02±0.41	<0.01
Combined score: fibroblast + fibroblast activation - collagen	-0.72±1.18	2.04±0.52	<0.01
Mitotic index score (average number of 10 field)	0.26±0.46	1.89±1.65	<0.01
Multinucleated giant cells (average number of 10 fields)	0±0	1.4±1.6	<0.01
Inflammation score	1.92±1.12	1.89±0.75	0.44
Necrosis score	1.07±0.73	1.14±0.82	0.73

Table 2

Frequencies of histological scores for neoplastic variables in the 20 cases with spirocercosis-induced sarcoma

Variable	0	1	2	3	4
Mitotic index score		10%	20%	70%	
Nuclear Pleomorphism	15%	30%	35%	10%	10%
Necrosis		45%	50%	5%	
Matrix		15%	40%	45%	
Cell density			20%	15%	65%
Multinucleated cells	20%	55%	20%	5%	

5.7 Figures

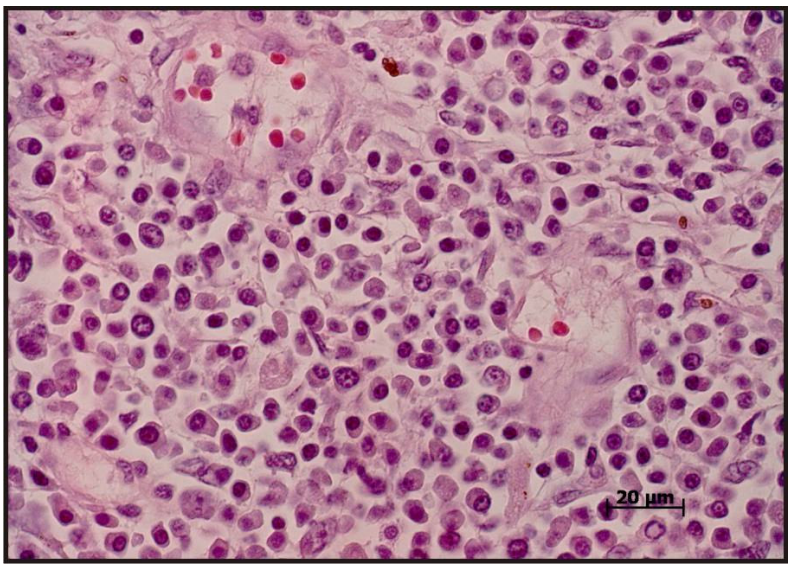

Figure 1: Florid lymphoplasmacytic cell infiltrate within a non-neoplastic oesophageal nodule (pre-neoplastic / stage 2), H&E.

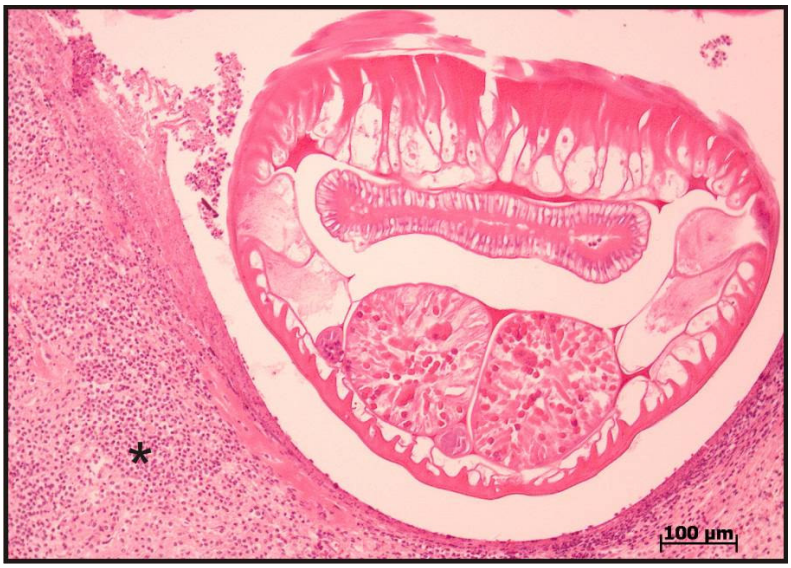

Figure 2: *Spirocerca lupi* larva surrounded by a rim of necrotic cell debris and degenerate neutrophils (asterisk) within a non-neoplastic oesophageal nodule (pre-neoplastic / stage 2). H&E.

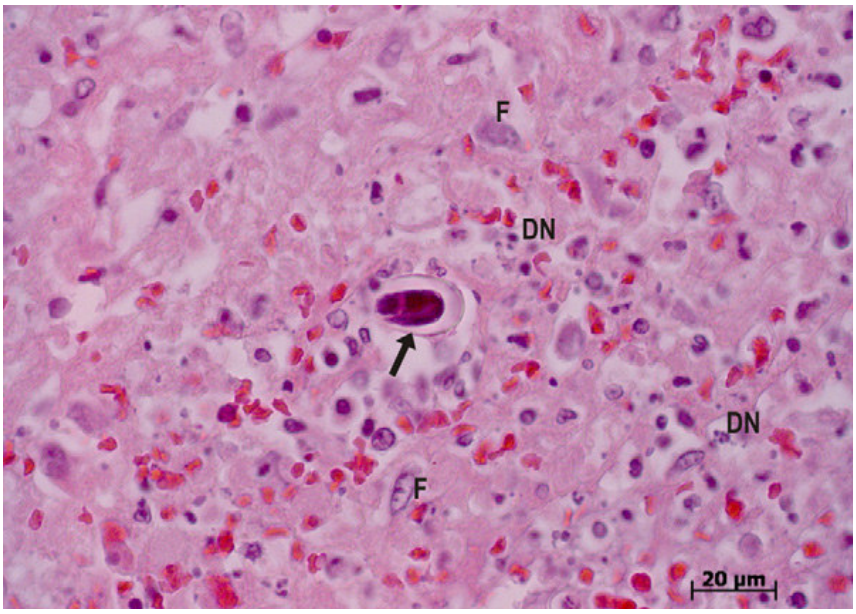

Figure 3: *Spirocerca lupi* egg (arrow) surrounded by degenerate neutrophils (DN), occasional fibroblasts (F) and haemorrhage within a non-neoplastic oesophageal nodule (pre-neoplastic / stage 2). H&E.

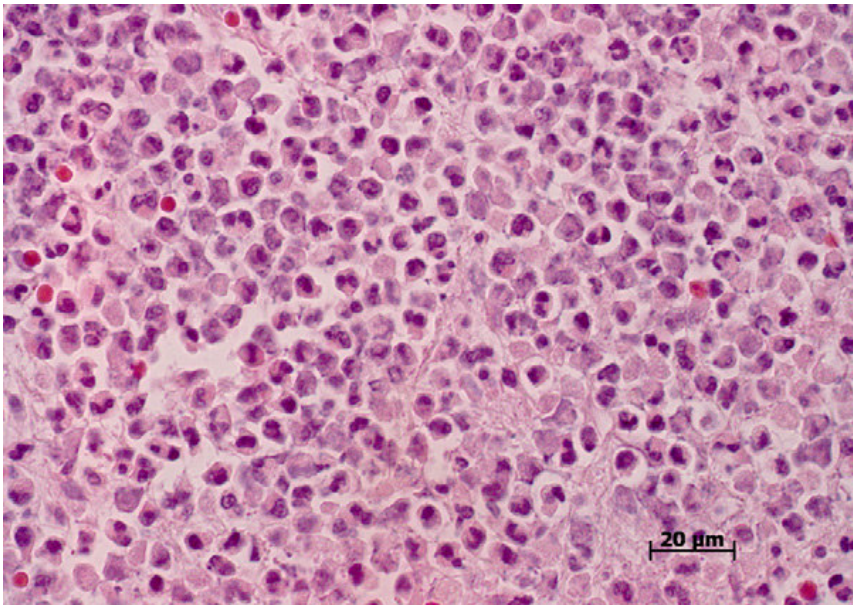

Figure 4: Purulent exudate (associated with *Spirocerca lupi* worm and tract) within a non-neoplastic oesophageal nodule (pre-neoplastic / stage 2). H&E.

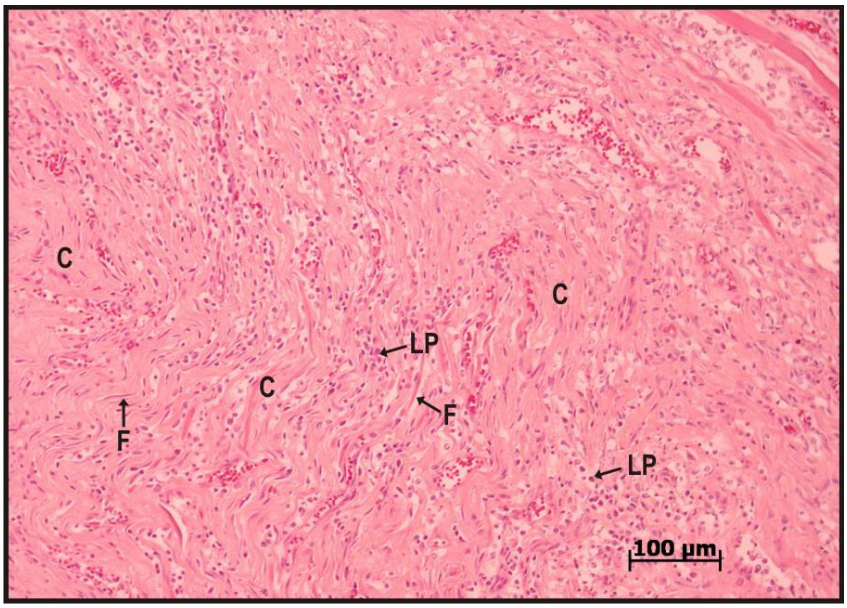

Figure 5: Collagen (C), fibrocytes (F) and intervening lymphoplasmacytic cell infiltrate (LP) within a non-neoplastic oesophageal nodule (early / stage 1). H&E.

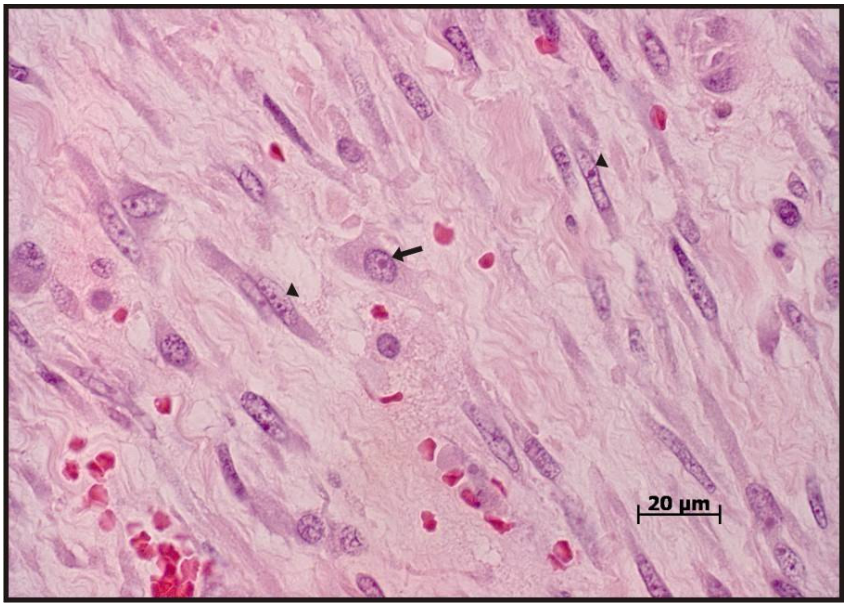

Figure 6: Fibroplasia within a non-neoplastic oesophageal nodule (pre-neoplastic / stage 2). Plump, spindle-shaped (arrowhead) to polygonal (arrow) fibroblasts with a large nucleus to cytoplasmic ratio, basophilic cytoplasm and prominent magenta nucleoli. H&E.

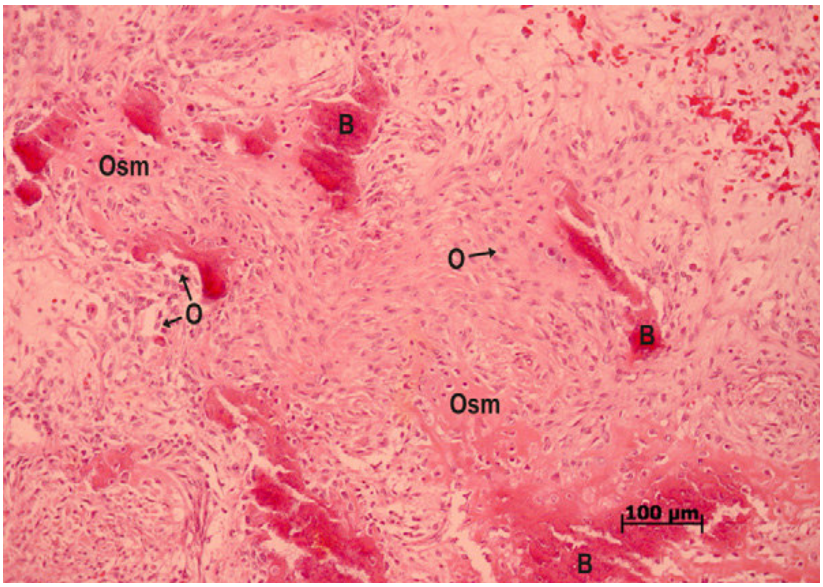

Figure 7: Well-differentiated oesophageal osteosarcoma . Neoplastic pyriform osteoblasts (O) in association with osteoid matrix (Osm) and fragments of mineralized bone (B). H&E.

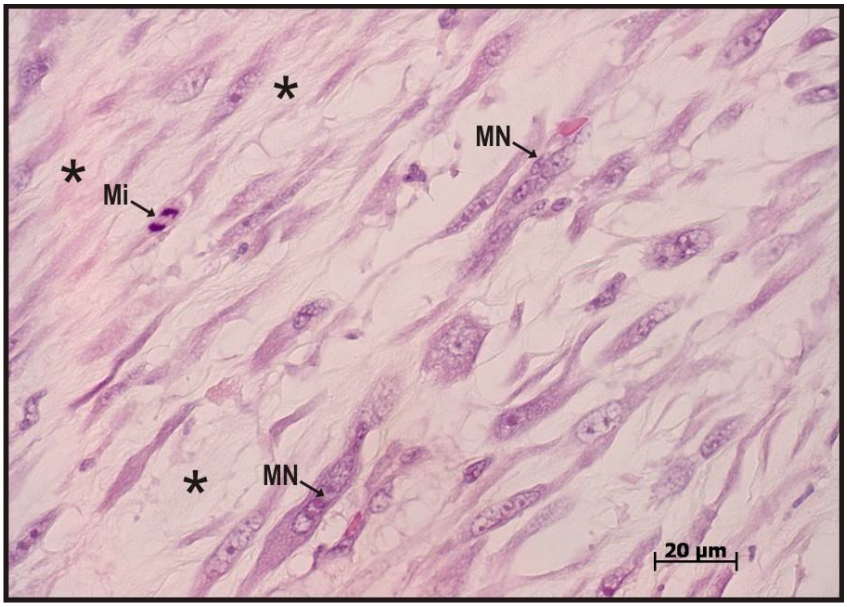

Figure 8: Poorly-differentiated oesophageal fibrosarcoma. Neoplastic spindle-shaped cells showing nuclear atypia, multinucleated cells (MN) and mitoses (Mi), amidst intervening fibrillar collagenous matrix (asterisk). H&E.

6 EVALUATION OF SELECTED GROWTH FACTOR EXPRESSION IN CANINE SPIROCERCOSIS (*SPIROCERCA LUPI*)-ASSOCIATED NON-NEOPLASTIC NODULES AND SARCOMAS

This chapter was published as a research paper:

Evaluation of selected growth factor expression in canine spirocercosis (*Spirocerca lupi*)-associated non-neoplastic nodules and sarcomas

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6.1 Abstract

The study aims to assess the expression of vascular endothelial growth factor (VEGF)-A, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) in the progression of spirocercosis-induced esophageal nodule in the dog from an early, non-neoplastic, inflammatory nodule to sarcomatous neoplasia.

Triplicate 4-µm-thick sections from 62 paraffin blocks containing *Spirocerca*-induced nodules were treated according to the labeled steptavidin-biotin (LSAB) immunohistochemical method using polyclonal goat anti-canine VEGF and anti-human FGF and PDGF antibodies. The nodules were classified as early inflammatory (n=15), pre-neoplastic (n=27) and neoplastic (n=20). Additionally, 10 sections of normal distal third of the esophagus and 21 non-spirocercosis-related sarcomas were evaluated and compared with the *Spirocerca*-induced nodules. Five non-overlapping high power fields per case were evaluated under the light microscope and the fibroblasts were evaluated for percentage of labeled cells. The intensity of labeling was further classified as weak (score 1) or strong (score 2). The intensity score was multiplied by the percentage of labeled fibroblasts to yield a field score and the final score was obtained by calculating an average of the 5 fields. Antigen labeling was compared between the different histological grades and the controls using the Kruskal-Wallis Test followed by the Mann-Whitney Test for comparison between specific groups. The level of significance was set at 0.05.

There were significant differences between the groups' score in all the growth factors that were examined. The normal oesophagus showed no labeling for any of the growth factors. FGF scored highest in the non-spirocercosis-related sarcoma group (median 118, 3-194) followed by the spirocercosis-induced sarcoma (34.5, 0-138), pre-neoplastic nodule (8, 0-99) and early nodule (0, 0-30) groups. All the differences

among the groups were significant. VEGF scored highest in the non-spirocercosis-related sarcoma group (median 47, 1-110) followed by the spirocercosis-induced sarcoma (26, 0-136), pre-neoplastic nodule (0, 0-62) and early nodule (0, 0-35) groups. PDGF scored highest in the non-spirocercosis-related sarcoma group (median 29.2, 0-70) followed by the pre-neoplastic nodule (23.4, 0-95), early nodule (13.6, 0-132) and spirocercosis-induced sarcoma (0, 0-47) groups.

The expression of VEGF and FGF increased as the nodule progressed from early inflammation to sarcoma, but it was not limited to spirocercosis-induced sarcomas. The expression of PDGF in spirocercosis was restricted to the early stages of nodule progression. Further investigation is warranted to establish whether FGF, VEGF or PDGF play a role in the pathogenesis of the neoplastic transformation in canine spirocercosis or are they simply integral to angiogenesis induction?

Keywords: Spirocercosis, *Spirocerca lupi*, vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor, sarcoma

6.2 Introduction

Spirocerca lupi (*S. lupi*) is a nematode of worldwide distribution, but it is most commonly found in tropical and subtropical regions (Bailey, 1972). Dogs are the definitive hosts and become infected by ingesting the coprophagous beetle intermediate hosts (Bailey, 1972). After ingestion, the larvae are liberated in the gastric lumen and migrate through the gastric mucosa, the gastric arteries and through the thoracic aortic wall to the caudal oesophagus. Typically, the worms settle within the oesophageal wall, mature to adults and promote formation of a fibroblastic nodule (Bailey, 1963, 1972; van der Merwe et al., 2008). The oesophageal nodule can progress to sarcomatous neoplasia. The relationship between *S. lupi* infection and

oesophageal sarcoma was first described in 1955 (Seibold et al., 1955). Histologically the tumours are identified as fibrosarcomas, osteosarcomas or undifferentiated sarcomas (Ranen et al., 2007). We recently described different types of non-neoplastic spirocercosis-induced oesophageal nodule, based on histopathology. We also proposed a progression scheme for the nodule from early inflammatory nodule to pre-neoplastic stage and, finally, sarcoma (Dvir et al., 2010).

Non-neoplastic spirocercosis is treated successfully with avermectins [(doramectin[7] 400 µg/kg SC at 2-week intervals (Lavy et al., 2002)], however, neoplastic tumours can only be treated surgically and the success rate is lower compared to non-neoplastic cases (Ranen et al., 2004). This difference in prognosis emphasizes a) the need to improve diagnostic and prognostic markers for the antemortal diagnosis of the oesophageal nodule, and b) the need for a better understanding of the neoplastic transformation of nodules. Such knowledge may improve the treatment of neoplastic cases. The present study aims to address these issues via the evaluation of selected cancer-associated growth factor expression, namely vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF), during the different stages of the S. *lupi*-associated oesophageal nodule. These particular growth factors were selected for the following reasons:

1. Spirocercosis induces hypertrophic osteopathy (HO), which is observed only in neoplastic cases (Dvir et al., 2008). Early HO is well described in human cancer patients where it is called 'digital clubbing'. It was shown that VEGF-A and PDGF expression is significantly increased in the clubbed digits together with increased

[7] Dectomax, Pfizer, France

microvessel density and it was concluded that these parameters play a central role in the pathogenesis of digital clubbing (Atkinson and Fox, 2004).

2. Spirocercosis-induced nodules exhibit fibroblast proliferation with abandoned angiogenesis (van der Merwe et al., 2008). VEGF, FGF and PDGF are novel immunohistochemical (IHC) markers of angiogenesis, making them useful markers to detect malignancy and possible targets for anti-cancer therapy (Craft and Harris, 1994).

3. Vaccine-associated sarcoma in cats is a condition with similar pathology to spirocercosis-induced sarcoma in dogs. The lesion in cats starts with exogenous irritation, develops to form a reactive inflammatory lesion with a central necrotic core that is invaded by granulation tissue, which, in time, becomes predominantly fibroblastic in nature, and ultimately transforms to sarcoma (McEntee and Page, 2001; Nieto et al., 2003). The sarcoma in cats has been found to be immunoreactive for PDGF and its receptor as well as FGF-b. In contrast, non-vaccine-associated fibrosarcomas were only faintly positive or negative for these growth factors (McEntee and Page, 2001; Nieto et al., 2003).

4. Growth factor expression has been studied in a variety of tumours in dogs for diagnostic and prognostic purposes. For example, it has been found that the expression of VEGF increases with reduced differentiation of mammary tumours in dogs (Restucci et al., 2002). Also, in canine haemangiosarcoma, the expression of VEGF, FGF-b, and their receptors (Flt-1, Flk-1, and Flg-1) was found to be elevated, which was not the case with haemangioma, again indicating an association between these factors and tumour malignancy (Yonemaru et al., 2006).

All of the aforementioned canine and feline studies used IHC to detect the growth factors, employing commercially available antihuman polyclonal antibodies.

As previous studies have indicated that growth factors can be used to differentiate between non-neoplastic and malignant neoplasms, we hypothesised that VEGF, FGF and PDGF would be expressed in *S. lupi*–induced nodules and that their level of expression would increase with progression to malignancy.

6.3 Material and Methods

6.3.1 Case selection

Spirocerca-induced oesophageal nodules, collected between 1998-2008, were retrieved from the archives of the Section of Pathology, Faculty of Veterinary Science, University of Pretoria. Per block, one 5-µm-thick hematoxylin and eosin (H&E)–stained section was examined under a light microscope. Only one nodule was selected per dog and if a dog had more than one nodule, the nodule that was most mature or advanced toward neoplastic transformation was selected for evaluation. If a non-neoplastic nodule was sectioned more than once, the section with the greatest number of fibroblasts was selected. If a neoplastic nodule was sectioned more than once, the section that was most representative of tumour phenotype was selected.

On the H&E sections the nodules were classified into 3 stages, early inflammatory, pre-neoplastic and neoplastic nodules, as follows (Dvir et al., 2010). Early *Spirocerca* nodules were characterized by minimal fibroplasia; well-differentiated fibrocytes and more collagen than fibrocytes. Pre-neoplastic nodules showed increased activated fibroblast density and relatively less collagen. To classify the non-neoplastic nodules, we scored (0-3) the quantity of immature fibroblasts within the nodule and their degree of activity (characterized by excessively plump euchromatic nuclei, often with prominent single or multiple magenta nucleoli). The amount of collagen was also scored 0-3. A combined score of fibroblasts plus activation score minus collagen was

calculated and the non-neoplastic cases were divided into cases with a combined score of ≤1 (early nodules) or >1 (pre neoplastic nodules), respectively. Neoplasms were classified phenotypically as fibrosarcoma, osteosarcoma or undifferentiated sarcoma. Tumours were further categorized as well-differentiated, of intermediate differentiation or poorly differentiated based on the level of anaplasia observed within sections. Histological features that were assessed included: Cellular pleomorphism, nuclear hyperchromasia, the presence of multinucleated neoplastic cells, multiple and/or prominent nucleoli and the number of mitoses.

6.3.2 Controls

Immature granulation tissue from a dog was used as a positive-tissue control for all of the selected antibodies. Histologically, the granulation tissue consisted of numerous neocapillaries, intervening plump fibroblasts, occasional neutrophils and macrophages and oedematous collagenous matrix.

For negative-tissue control purposes, 10 sections of normal distal third of dog oesophagus were used. In addition, twenty one non-spirocercosis-related sarcomas of the same phenotypes that have been associated with spirocercosis were evaluated and compared with the *Spirocerca*-induced nodules. The non-*Spirocerca*-associated sarcomas included 4 anaplastic sarcomas, 6 fibrosarcomas and 11 osteosarcomas. All sections were immunolabelled for PDGF, VEGF and FGF-b.

For negative-reagent control purposes, PBS-BSA buffer was applied (instead of the primary antibody) to a section of the positive-tissue control for each batch of cases submitted for immunolabeling. On a separate positive-tissue control section per batch of cases processed per day, an irrelevant polyclonal antibody, e.g. S100 protein (a calcium-flux determinant with a diversity of potentially reactive cell types) was used

at the same dilution as the primary antibodies (1:20) and incubated for (to assess the extent and pattern of non-specific binding of the primary antibody).

6.3.3 Immunohistochemistry (IHC)

Per selected tissue block per dog case, 3-4 µm-thick sections were cut (for VEGF, FGF, PDGF and Factor VIII immunolabelling), mounted on Superfrost-Plus glass slides and dried overnight in an oven at 58ºC to enhance tissue adhesion. The sections were routinely dewaxed in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched by incubating the tissue sections with 0.3% hydrogen peroxide in methanol for 30 minutes at room temperature, followed by rinsing in distilled water and again in phosphate-buffered saline (PBS) containing 0.1 % bovine serum albumin (BSA, Roche Diagnostics, GmbH, Cataloge No. 735 094 FrV) (pH 7.6) for 5 minutes. Heat-induced antigen retrieval was used, whereby sections were immersed in Tris EDTA buffer (pH 9.0) and microwaved for 2 cycles of 7 minutes. After cooling for 15 minutes at room temperature, the buffer was decanted and the sections washed in distilled water, followed by PBS-BSA buffer for 5 minutes. Irrelevant antigens were block by Novocastra Protein Block (Novocastra Laboratories, RE 7102), for 5 minutes, followed by 2% milk powder for 30 minutes. Thereafter the sections were incubated at room temperature with the following polyclonal primary antibodies: Goat-anti-canine VEGF (R&D System, AF1603), Goat-anti-human PDGF-AA (R&D System , AF-221-NA) and Goat-anti- FGF-basic (R&D System , AF-233-NA). All antibodies were diluted 1:20 in PBS-BSA buffer. The VEGF and PDGF antibodies were incubated for 1 hour and the FGF antibody for 2 hours. Sections were then rinsed with distilled water, followed by PBS-BSA buffer. Secondary antibodies were applied using the LSAB-plus kit (Dako, k0679) as

instructed by the manufacturer. Nova Red (Vector, SK-4800) was used as the chromogen with haematoxylin as the counterstain.

6.3.4 Scoring of immunoreactivity

The IHC-stained sections were evaluated under the light microscope (Olympus BH-2, Japan, serial number 028160). Fibroblasts or tumour cells were evaluated in 5 non-overlapping high power/x400 fields (about 200 cells per field, 1000 cells in total) for the percentage of positive cells and the intensity of the labelling (no labelling, weak labelling or strong labelling). Finally a total score per section was calculated by multiplying the percentage of weak positive cells by a factor of 1 and the percentage of strongly positive cells by factor of 2. The sum of the 2 yielded the final score of the field and the mean of the field scores was recorded as the final section score.

6.3.5 Assessment of Microvessel Density (MVD)

An anti-von Willebrand Factor (factor VIII) antibody was used to identify vascular endothelium in the tissues. Sections were prepared and peroxidise activity was blocked as described previously. An enzymatic method was used for antigen retrieval, whereby the sections were immersed in pre-heated (37°C) 50mg Protease XIV (Sigma, P-5147) in 100 ml PBS-BSA. Thereafter, sections were incubated at room temperature with a polyclonal rabbit anti-human factor VIII (Dako, A0082) diluted 1:300 in PBS-BSA for 1 hour. Sections were rinsed with distilled water, followed by PBS-BSA buffer. Secondary antibodies were applied using the LSAB-plus kit (Dako, k0679), as instructed by the manufacturer. Nova Red (Vector, SK-4800) was used as the chromogen followed by haematoxylin as the counterstain.

Only the *S.lupi*-associated nodules and normal oesophagus were labelled with the factor VIII antibody. Each section was scanned on x40 magnification for areas of high

vessel density (vascular "hot spots") at the periphery and at the centre of the nodules. The number of vessels in these "hot spots" were counted in 3 non-overlapping high power fields (x400), as previously described (Weidner, 1995). The average number of vessels per nodule per area (periphery or centre) was compared between the 3 different stages of nodules and the normal oesophagus control group.

6.3.6 Statistical analysis

The data was captured on Excel data sheets. Statistical analysis was performed with SPSS Statistics 17.0 software (SPS Inc., Chicago). Scores of VEGF, FGF, PDGF and Factor VIII were compared between the different *S. lupi* nodule stages, the normal oesophagus and the non-spirocercosis sarcoma groups. The differences between all groups were tested for significance by Kruskal-Wallis Test, followed by Mann-Whitney Test for differences between specific pairs of groups (post-hoc).

6.4 Results

6.4.1 Growth factor immunohistochemistry

The selected growth factors were evaluated and compared in 93 sections, of which 15 were inflammatory nodules, 27 were pre-neoplastic nodules, 20 were *S. lupi*-associated oesophageal sarcomas, 10 were normal oesophaguses and 21 were non- *S. lupi*-associated sarcomas.

6.4.2 Labelling of the positive-tissue control

The positive-tissue control showed weak through to strong cytoplasmic labelling of fibroblasts with the application of all the selected primary antibodies (VEGF, FGF and PDGF). In addition, VEGF generally revealed strongly positive cytoplasmic labelling of endothelial cells lining neocapillaries in the positive control tissue (Fig

1A). The PDGF antibody also labelled occasional neutrophils within inflamed areas, mononuclear cells (macrophages and lymphoid cells) and oedematous tissue in the positive-tissue control (labelling varied from weak through to strong and was most commonly cytoplasmic). In the positive-tissue control, PDGF-specific positive labelling occurred also in the form of long, thin, linear, strands of granules (corresponding to elongated cytoplasmic processes).

6.4.3 VEGF labelling of fibroblasts and tumour cells

Vascular endothelial growth factor expression was significantly different between the groups ($p<0.001$, Fig. 2). The highest expression was observed in the non- *S. lupi*-associated sarcoma group (median score 47, range 1-110), followed by the *S. lupi*-associated oesophageal sarcoma group (median score 26, range 0-136), then the pre-neoplastic *S. lupi* oesophageal nodule group (median 0, range 0-62), followed by the early non-neoplastic *S. lupi* oesophageal nodule group (median 0, range 0-35) and, finally, the normal oesophagus group showed no VEGF expression in any of the cases. All groups, except the early nodules, exhibited significantly higher VEGF expression compared to the control group. Both sarcoma groups had significantly higher VEGF expression compared to the 2 non- neoplastic *S. lupi* nodule groups. The 2 non- neoplastic *S. lupi* nodule groups were not statistically different and the same is true for to the 2 sarcoma groups. The prevalence of positive cases per group followed the same trend as the group score (Table 1). Most positive cases showed labelling of mixed intensity with both strong and weak diffuse cytoplasmic and occasionally nuclear labelling of fibroblasts and/or tumour cells (Fig. 1B and 1C).

6.4.4 FGF labelling of fibroblasts and tumour cells

Scoring of positive cells revealed significantly different FGF expression between the groups ($p<0.001$, Fig. 3). The highest expression was observed in the non- *S. lupi*-associated sarcoma group (median score 118, range 3-194), followed by the *S. lupi*-associated oesophageal sarcoma group (median score 34.5, range 1-138), then the pre-neoplastic *S. lupi* oesophageal nodule group (median 8, range 0-99), followed by the early non-neoplastic *S. lupi* oesophageal nodule group (median 0, range 0-30). Finally, the normal oesophagus group showed no FGF expression in any of the cases. All comparisons between groups were statistically significant. The prevalence of positive cases per group followed the same trend as the group score (Table 1). Again, the fibroblasts/tumour cells exhibited predominantly diffuse cytoplasmic labelling, but a few cases showed granular cytoplasmic or pale nuclear labelling (Fig. 1D and 1E). Most positive cases showed labelling of mixed intensity with both strongly and weakly labelled cells. In a few oesophageal osteosarcomas, multinucleated neoplastic giant cells labelled strongly with the FGF antibody.

6.4.5 PDGF labelling of fibroblasts and tumour cells

Scoring of fibroblast/tumour cell positively revealed significantly different PDGF expression between the groups ($p=0.003$, Fig. 4). The highest expression was observed in the non- *S. lupi*-associated sarcoma group (median score 29.2, range 0-70), followed by the pre-neoplastic *S. lupi* oesophageal nodule group (median 23.4, range 0-95), followed by the early non-neoplastic *S. lupi* oesophageal nodule group (median 13.6, range 0-132) followed by the *S. lupi*-associated oesophageal sarcoma group (median score 0, range 0-47). The normal oesophagus showed no PDGF expression in any of the cases. The pre-neoplastic oesophageal *S. lupi* nodule group,

the early non-neoplastic *S. lupi* oesophageal nodule group and the non- *S. lupi*-associated sarcoma group exhibited significantly higher PDGF expression compared to the control and *S. lupi*-associated oesophageal sarcoma groups. However, the differences between these 3 groups were not significant. There was also no statistically significant difference between the groups with the lowest PDGF expression, namely the *S. lupi*-associated oesophageal sarcoma and normal oesophagus groups. The prevalence of positive cases per group followed the same trend as the group score (Table 1). The PDGF antibody elicited more non-specific labelling compared with the other growth factor antibodies. Non-specific labelling was most pronounced within foci of purulent inflammation and necrosis, e.g. the worm migratory tract and necro-ulcerative foci at the periphery of oesophageal tumours. Some of the positive labelling had a granular strand-like appearance that probably represented cytoplasmic cell processes (i.e. as in the positive-tissue control case). However, most positive cases showed mixed intensity labelling with both strong and weak diffuse cytoplasmic positivity. A few cases exhibited weak to strong diffuse intranuclear labelling (Fig. 1F).

Apart from the scored fibroblast and/or tumour cell positively, all the growth factor antibodies showed some labelling of other cells (Table 2). Occasional endothelial cells and lymphoid cells (especially plasma cells) were labelled by all 3 growth factors.

6.4.6 Microvessel density

Microvessels were evaluated in the S.lupi-associated nodules (early nodules = 15, pre-neoplastic nodules = 27, and neoplastic nodules = 20) and the normal oesophagus (n=10) sections. The mean microvessel count per high power field at the periphery of

the nodules revealed the highest count in the pre-neoplastic oesophageal *S. lupi* nodule group (median 38, range 5.67-62.33), followed by the early non-neoplastic *S. lupi* oesophageal nodule group (median 32.33, range 15.67-62.67), then the *S. lupi*-associated oesophageal sarcoma group (median score 27.5, range 9-65), and, finally, the normal oesophagus group (median score 20, range 10-35) (Fig. 5). The differences between the groups were significant ($p=0.002$). Comparing individual pairs of groups, only the early and pre-neoplastic nodule groups had a significantly higher count compared to the control. However, the difference between the counts in these 2 groups was not significantly different. The count difference between the neoplastic and control groups was also not statistically different. The microvessel count in the neoplastic groups was significantly lower compared to the pre-neoplastic nodules but not compared to the inflammatory nodules.

A comparison of microvessel density at the centre of the nodules between the different groups revealed a very similar pattern to the periphery of the nodule (Fig. 6) with the highest count occurring in the pre-neoplastic *S. lupi* oesophageal nodule group (median 31, range 8.67-45.33), followed by the early non-neoplastic *S. lupi* oesophageal nodule group (median 30, range 11-52.67), then the normal oesophagus group (median score 20, range 10-35), and, finally, the *S. lupi*-associated oesophageal sarcoma group (median score 14.33, range 8.67-43.33). The differences between the groups were significant ($p<0.001$). Comparing individual pairs of groups, only the inflammatory and pre-neoplastic nodule groups had significantly higher counts compared to the control group. The difference between the counts in these 2 groups was not significantly different. The difference in number of microvessels between the neoplastic and control groups was also not statistically different. The microvessel

count in the neoplastic group was significantly lower than in the pre-neoplastic and inflammatory nodule groups.

6.5 Discussion

In areas where spirocercosis does not exist, oesophageal neoplasia is extremely rare (Ridgway and Suter, 1979), making spirocercosis the major cause of oesophageal neoplasia in the dog, and therefore a potential natural model for carcinogenesis. The fact that nematodes can induce cancer is documented in human medicine as well (Mostafa et al., 1999), and the idea that spirocercosis can serve as a model for nematode-induced cancer has also been proposed by others (Herrera et al., 2005; Melendez and Suarez-Pellin, 2001). To date, there is no evidence-based literature pertaining to the pathogenesis of the neoplastic transformation. Bailey, who worked extensively on spirocercosis, had a few hypotheses regarding the neoplastic transformation, including genetic predisposition and oncogenic stimuli (Bailey, 1972). Other researchers have postulated that the worm might release growth factors (Melendez and Suarez-Pellin, 2001). The purpose of the present study was to obtain basic knowledge of the expression of selected growth factors, namely VEGF, FGF and PDGF, during the progression of the *S. lupi*-associated oesophageal nodule.

The present study demonstrated marked VEGF and FGF expression in the spirocercosis-induced nodules; both growth factors increased with progression of the nodule from inflammation to the pre-neoplastic stage and, eventually, to neoplasia. These findings indicate that it may be worth investigating whether VEGF and FGF are elevated in the serum as well; if so, they might serve as diagnostic markers for neoplastic transformation in spirocercosis.

Vascular endothelial growth factor expression has been described in many canine tumours including mammary tumours (Restucci et al., 2002), mast cell

tumours (Rebuzzi et al., 2007), seminomas (Restucci et al., 2003), haemangiosarcomas (Yonemaru et al., 2006), lymphomas (Wolfesberger et al., 2007), squamous cell carcinomas (Maiolino et al., 2000) and various intracranial tumours (Rossmeisl et al., 2007). Interestingly, VEGF expression has been found to increase in more anaplastic (Restucci et al., 2004; Rossmeisl et al., 2007) and aggressive tumours (e.g. inflammatory mammary carcinoma) (Millanta et al., 2010).

Fibroblast growth factors form a large group of potent mitogens for fibroblasts and epithelial cells and, as such, they play an important role in wound repair (Halper, 2009). The role of FGF in tumour progression in small animals has been far less studied than VEGF. Increased FGF expression has been described in haemangiosarcomas (Yonemaru et al., 2006), transitional cell carcinomas (Mohammed et al., 2003) and feline vaccine-associated sarcomas (Nieto et al., 2003).

It is beyond the scope of the present study to determine the role played by selected growth factors in the pathogenesis of *S. lupi*-induced tumours. However, the study clearly indicates that VEGF, FGF and PDGF are expressed not only in *S. lupi*-induced, but also in comparable types of non-*S. lupi*-related sarcoma (osteosarcoma, fibrosarcoma and anaplastic sarcoma). It is thus clearly shown that expression of the selected growth factors is not unique to *S. lupi* oesophageal sarcomas, but they are more likely to be general markers for sarcomas. However, expression of these growth factors may still be significant in the induction of neoplastic transformation by the worm or the inflammation in the nodule. Alternatively, increased growth factor expression may be a consequence of existing neoplasia, their function being to promote the proliferation of tumour cells. For example, VEGF is the most potent inducer of angiogenesis. Angiogenesis is a fundamental process in tumour progression and metastasis. Tumour growth is dependent on angiogenesis to allow exchange of

nutrients, oxygen and waste products, once simple diffusion is no longer able to meet the needs of the rapidly dividing cell population (Weidner, 1995). Tumour metastasis is dependant upon angiogenesis so that the neoplastic cells within a primary tumour can gain access to the circulation and also in order for the metastasized cells to grow within target organs (Weidner, 1995). Tumour cells can both produce VEGF constitutively and respond in an autocrine and apocrine manner to this peptide (Rebuzzi et al., 2007). Also, VEGF receptors (Flk- or Flt-) have been demonstrated in mammary tumours (Restucci et al., 2004), intracranial tumours (Rossmeisl et al., 2007), haemangiosarcomas (Yonemaru et al., 2006) and mastocytomas (Rebuzzi et al., 2007), as has VEGF mRNA (Rebuzzi et al., 2007; Wolfesberger et al., 2007; Yonemaru et al., 2006). The implication is that VEGF also performs functions other than the stimulation of angiogenesis in tumour progression.

The role played by PDGF in tumour progression is varied. It has also been implicated in angiogenesis, itself being induced by VEGF. PDGF is also a potent mitogen which is encoded by the *sis* oncogene and is overexpressed in the canine osteosarcoma cell line (Levine, 2002). Evidence for its expression in small animals is minimal and includes feline vaccine-associated sarcoma (Katayama et al., 2004). Overproduction of PDGF may be involved in autocrine and paracrine growth stimulation of human tumours, especially those of fibroblast origin, where it is considered important in neoplastic transformation (Heldin and Westermark, 1999). Activation of PGDF and its receptors leads to the induction of several oncogenes, including *c-fos* and *c-myc* (Halper, 2009). It is via intracrine activity and the activation of oncogenes such as *c-ras* and *c-myc* that PDGF receptors may play a crucial role in neoplastic transformation (compared to tumour progression via angiogenesis which is the major function of both VEGF and FGF). The implied extensive nuclear activity may in fact

explain the unusual intranuclear labelling seen in a number of cases in the present study. The oncogenic effects of PDGF make it a strong candidate for involvement in the neoplastic transformation in spirocercosis as well. However, PDGF expression in the present study did not follow a clear trend. It was most strongly expressed in the non-neoplastic stages of the *S. lupi* nodule. Of course, it is possible that neoplasia is induced during the earliest stages of nodule development. It is also possible that PDGFs other than PDGF-AA, namely PDGF-AB or PDGFF-BB, were expressed and were therefore not detected in the present study. However, PDGF-AA is the most studied PDGF in the dog and it was strongly expressed in the non-spirocercosis-related-sarcoma group. Thus, the implication might be that PDGF-AA plays a unique role in the pathogenesis of malignancy in spirocercosis. Clearly, further investigation would be necessary to explore the validity of this statement. Unfortunately, it was beyond the scope of the present study.

When all stages of the spirocercosis-associated oesophageal nodule were labelled with the factor VIII antibody, MVD was observed to be greatest in the non-neoplastic stages, especially the inflammatory stage. The MVD of the spirocercosis-induced neoplastic nodules was lower than in the non–neoplastic nodules, and similar to that of the normal oesophagus. This finding contradicts the expected pattern, of increased MVD in more neoplastic tumours (Wolfesberger et al., 2008). In mast cell tumours, both plasma VEGF concentration and MVD were significantly higher in more poorly differentiated tumours. Also, a significantly strong correlation was observed between VEGF and MVD (Patruno et al., 2009). However, no increase in MVD in neoplasia compared with non-neoplastic tissue was reported in a study quantifying MVD in tumours in highly vascular organs (Weidner, 1995). Sarcomas are known to be relatively less vascular than other tumours (Luong et al., 2006) and this might explain

why there is a decrease in MVD as the *S.* lupi-induced nodules progress from inflammatory nodule to sarcoma. On the other hand, there are studies that show that MVD is a powerful indicator of tumour progression in sarcomas as well. In a study of canine soft tissue sarcoma, the histological grade was positively associated with MVD (Luong et al., 2006). In another study, tumours with documented metastasis had higher MVD (Coomber et al., 1998). In spirocercosis it would probably be more useful to use MVD to monitor tumour progression, rather than as a parameter of the nodule progression from inflammatory lesion to neoplasia. The fact that VEGF and FGF were strongly expressed in the neoplastic cells, but not in factor VIII-positive microvessels may imply that: a) Their function is not only associated with angiogenesis, or b) the angiogenesis that takes place is not effective and therefore microvessels do not reach structural maturity (i.e. VEGF and FGF were used to count cells and factor VIII to count microvessels). It is possible that as inflammation regresses and sarcoma develops, increasing hypoxia induces angiogenic stimulators, with microvessels only actually developing a little later. Another possible explanation for low MVD, despite the presence of angiogenic stimulators, might be the counter-activity exerted by angiogenic inhibitors (Jones and Fujiyama, 1999). Lastly, the labelling of factor VIII alone might underestimate the true MVD (i.e. very small vessels might escape labelling) (Wolfesberger et al., 2008).

Expression of VEGF, FGF and PDGF in tumours has been associated with poor tumour differentiation and prognosis. The high level of expression of these growth factors in the present study confirms the aggressive nature of *S. lupi*-induced oesophageal sarcomas, something which was observed in a prior study when criteria for tumour aggression were compared in HE-stained sections from spirocercosis- and non-spirocercosis-associated sarcomas (Dvir et al., 2010). Further assessment of

spirocercosis-related tumour malignancy could involve assessing how immunolabelled neoplastic cases respond to treatment. However, this was clearly beyond the scope of the present study. Another important application of this work might be targeted-therapy against these growth factors in a variety of sarcomas, i.e. using bevacizumab (Avastin, Genentech), a monoclonal anti-VEGF (Halper, 2009). Toceranib phosphate is an inhibitor of tyrosine kinase, VEGF and PDGF receptors and it showed good response in treating mast cell tumours in dogs (London et al., 2009). Imatinib mesylate, a tyrosine kinase inhibitor that inhibits PDGF-BB was used successfully in feline vaccine-associated sarcoma cell lines (Katayama et al., 2004). Presently, these therapies are still cost-prohibitive in medium-sized dogs, but they may prove useful (especially bevacizumab) as adjuvant therapies for sarcomas in the future.

6.6 Conclusion

This study indicates that spirocercosis-associated nodules express high levels of VEGF, FGF and PDGF. However, further investigation is required to ascertain whether: a) The proteins are being produced in the nodules (by measuring mRNA levels) or b), are bound to receptors after being secreted elsewhere, possibly by inflammatory cells, endothelium or the worm (using receptor IHC). Therefore, future studies to investigate the true function of these growth factors in the spirocercosis-associated nodule (using cell cultures) are clearly warranted.

6.7 Tables

Table 1

Expression of VEGF, bFGF and PDGF in Spirocercosis-associated nodule

Antibody	Group	N	Labelling score		Labelling prevalence
			Median	Range	Positive
VEGF	Normal Oesophagus	10	0	0	0%
	Early inflammatory *S. lupi* nodules	15	0	0-35	20%
	Pre-neoplastic *S. lupi* nodules	27	0	0-62	31%
	S. lupi-associated sarcoma	20	26	0-136	85%
	Non-*S. lupi*-related sarcoma	21	47	1-110	100%
bFGF	Normal Oesophagus	10	0	0	0%
	Early *S .lupi* nodules	14	0	0-30	43%
	Pre-neoplastic *S. lupi* nodules	27	8	0-99	81%
	S. lupi-associated sarcoma	20	34.5	0-138	95%
	Non-*S. lupi*-related sarcoma	21	118	3-194	100%
PDGF	Normal Oesophagus	10	0	0	0%
	Early *S. lupi* nodules	15	9.4	0-132	53%
	Pre-neoplastic *S. lupi* nodules	27	23.4	0-95	93%
	S. lupi-associated sarcoma	20	0	0-47	25%
	Non-*S. lupi*-related sarcoma	21	29.2	1-70	90%

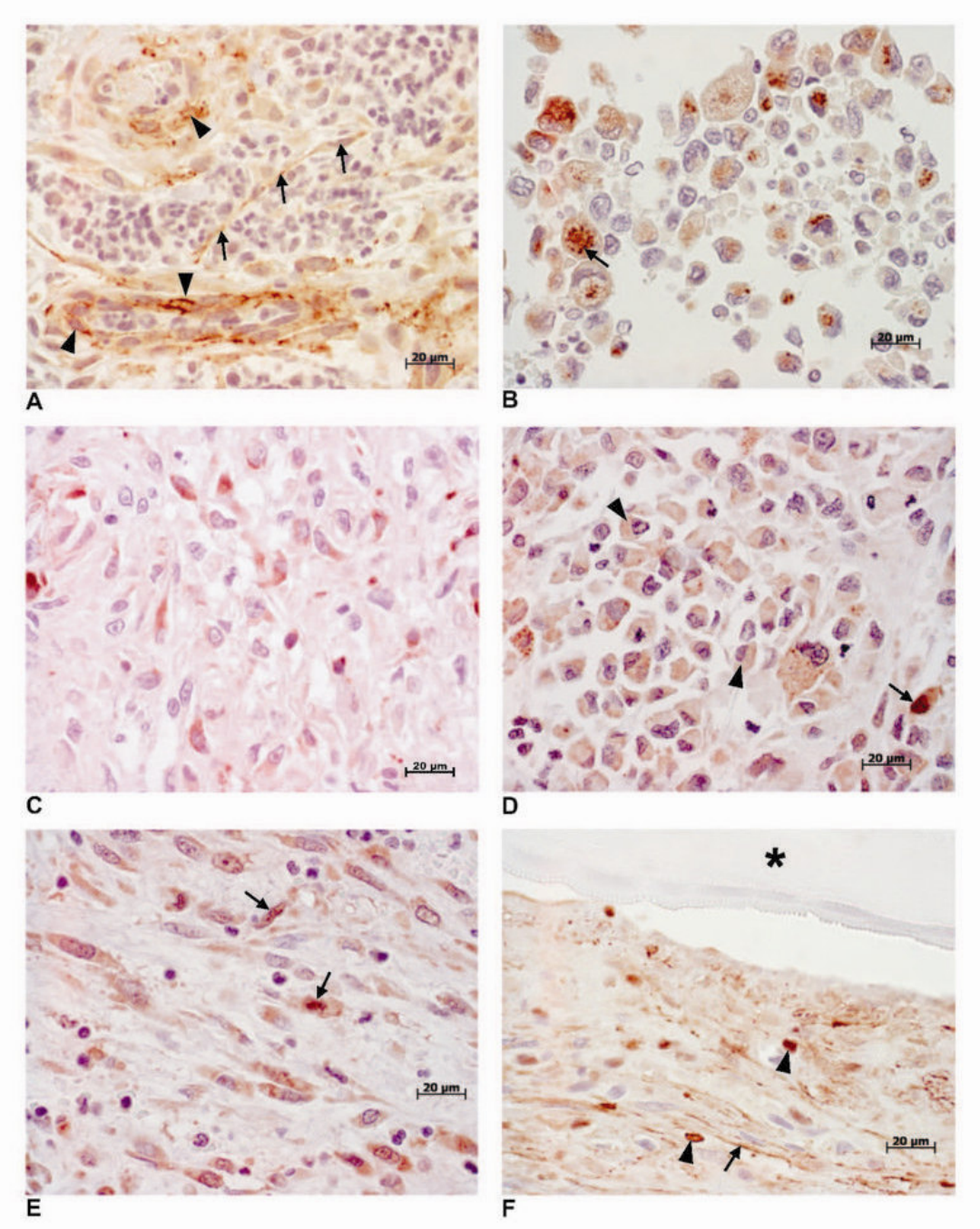
Table 2

Non specific VEGF, FGF and PDGF labelling observed in cells other than fibroblasts and tumour cells

	VEGF	FGF	PDGF
Endothelial cells	+	+	+
Lymphoplasmacytic cells	+	+	+
Neutrophils		+	+
Macrophages	+		
Squamous and glandular oesophageal epithelium	+	+	
Ganglions, neurons and support cells		+	
Skeletal muscles		+	

6.8 Figures

Figure 1:



A. Positive control; granulation tissue in a dog. VEGF-specific granular cytoplasmic immunolabelling of microvascular endothelial cells (arrowheads) and a fibroblast (arrows). Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

B. *S. lupi*-associated oesophageal osteosarcoma in a dog; Pale, diffuse as well as granular cytoplasmic and strong nuclear (arrow) immunolabelling of VEGF antigen in anaplastic tumour cells. Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

C. Pre-neoplastic *S. lupi* -associated oesophageal nodule in a dog; cytoplasmic diffuse VEGF-specific immunolabelling of fibroblasts adjacent to a migratory tract. Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

D. *S. lupi*-associated oesophageal osteosarcoma in a dog; Positive FGF antigen labelling in neoplastic cells; note the pale, diffuse cytoplasmic (arrowhead), strongly granular cytoplasmic and also strong nuclear (arrow) pattern of immunolabelling in tumour cells. Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

E. *S. lupi*-associated pre-neoplastic oesophageal nodule in a dog; FGF-specific diffuse and granular cytoplasmic as well as diffuse nuclear (arrow) immunolabelling in numerous fibroblasts. Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

F. Early inflammatory *S. lupi*-associated oesophageal nodule in a dog; cytoplasmic granular (arrow) and strong, diffuse nuclear (arrowhead) PDGF-specific immunolabelling of fibroblasts adjacent to a worm (asterisk). Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

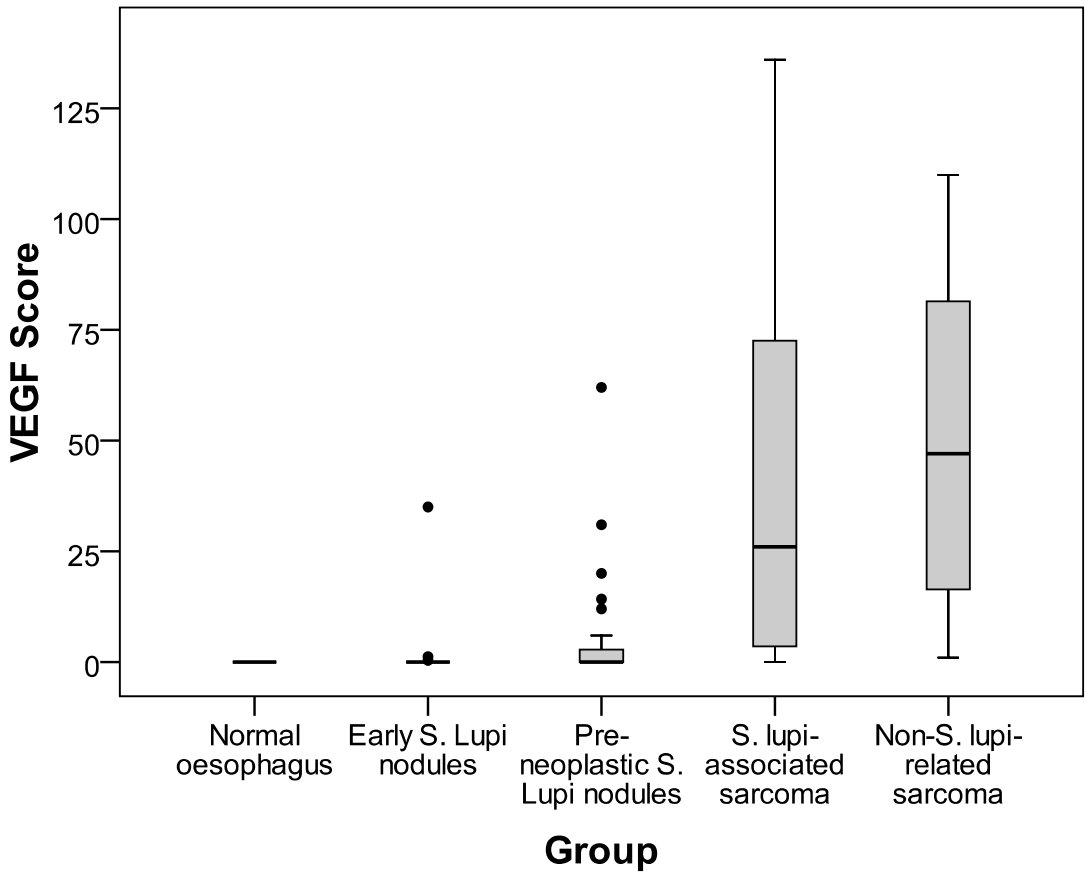
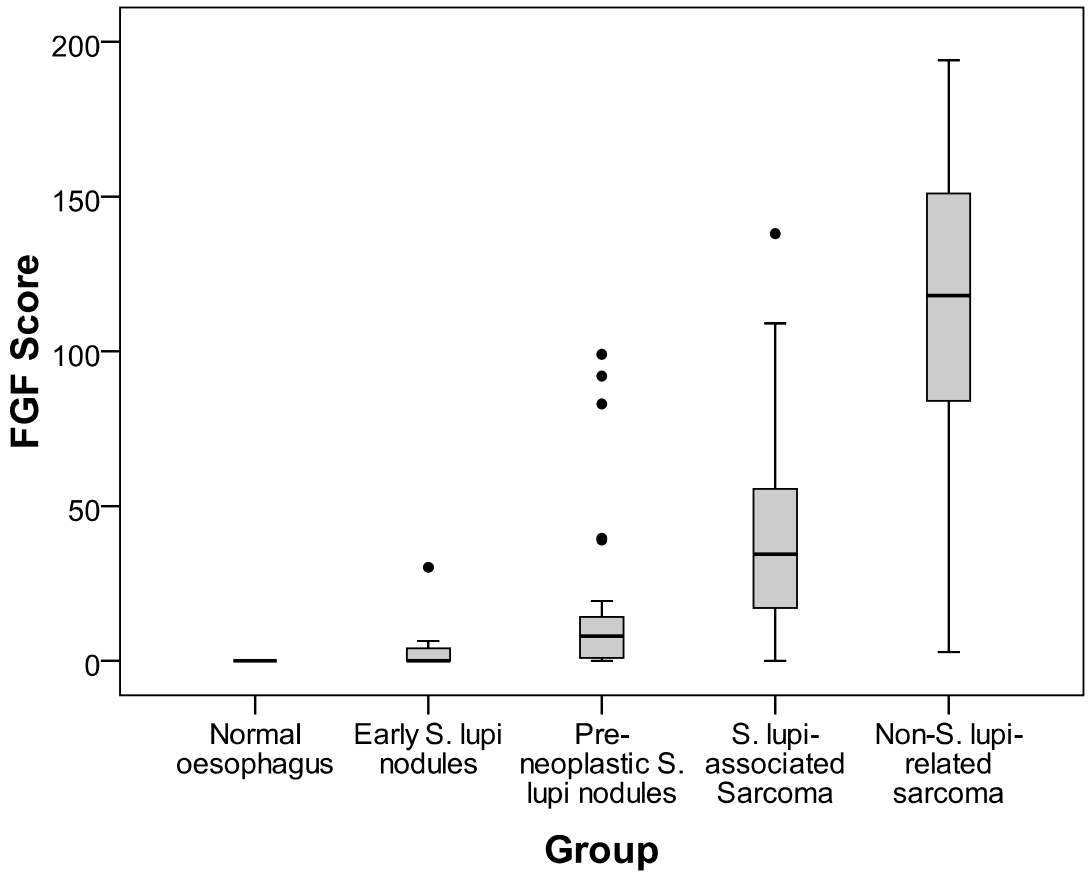


Figure 2: Box Plot of the VEGF expression score in the different groups. The overall comparison between the groups was statistically significant ($p<0.001$). All groups, except the early nodules, exhibited significantly higher VEGF expression compared to the control group ($p<0.05$). Both sarcoma groups had significantly higher VEGF expression compared to the 2 non- neoplastic *S. lupi* nodule groups ($p<0.001$). The 2 non- neoplastic *S. lupi* nodule groups were not statistically different; the same is true for the 2 sarcoma groups.



true for comparisons between the individual groups ($p<0.05$).

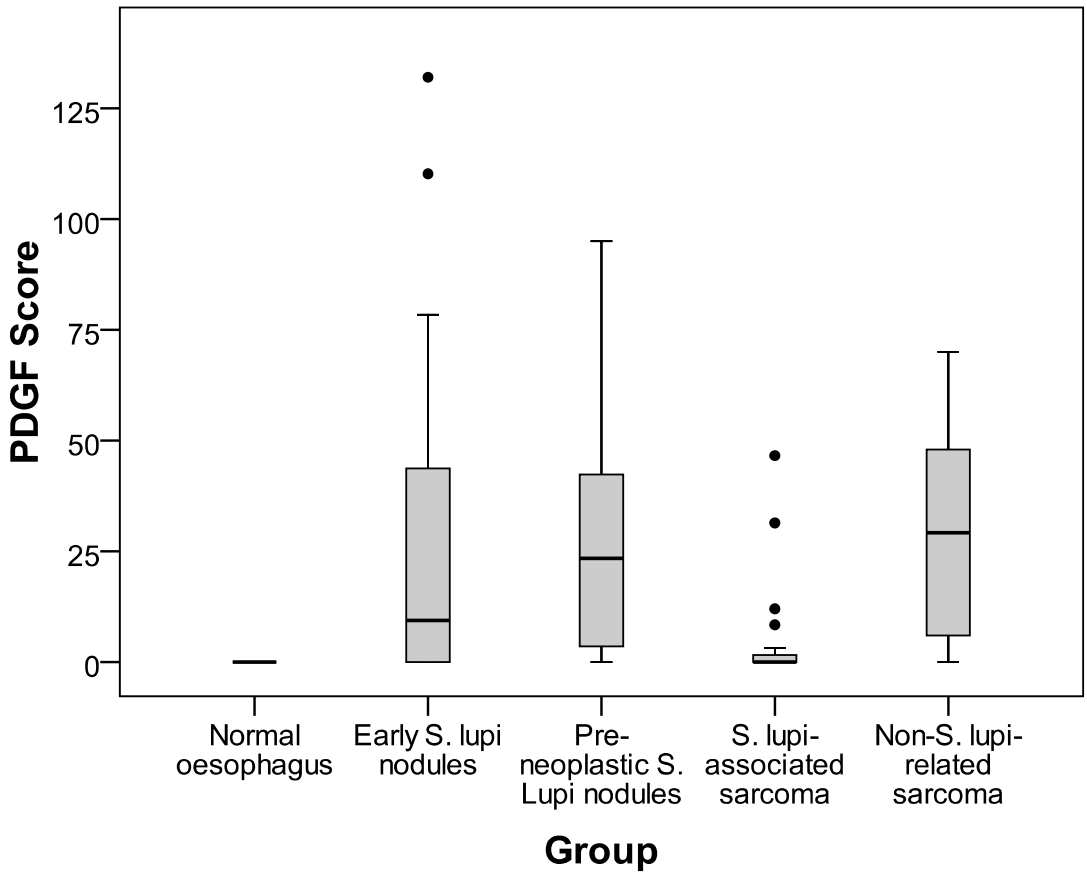


Figure 4: Box Plot of the PDGF expression score in the different groups. The overall comparison between the groups was statistically significant (p=0.003). The pre-neoplastic oesophageal *S. lupi* nodule group, the early non-neoplastic *S. lupi* oesophageal nodule group and the non- *S. lupi*-associated sarcoma group exhibited significantly higher PDGF expression compared to the control and *S. lupi*-associated oesophageal sarcoma groups ($p<0.05$). However, the differences between these 3 groups were not significant. There was also no statistically significant difference between the groups with the lowest PDGF expression, namely the *S. lupi*-associated oesophageal sarcoma and normal oesophagus groups.

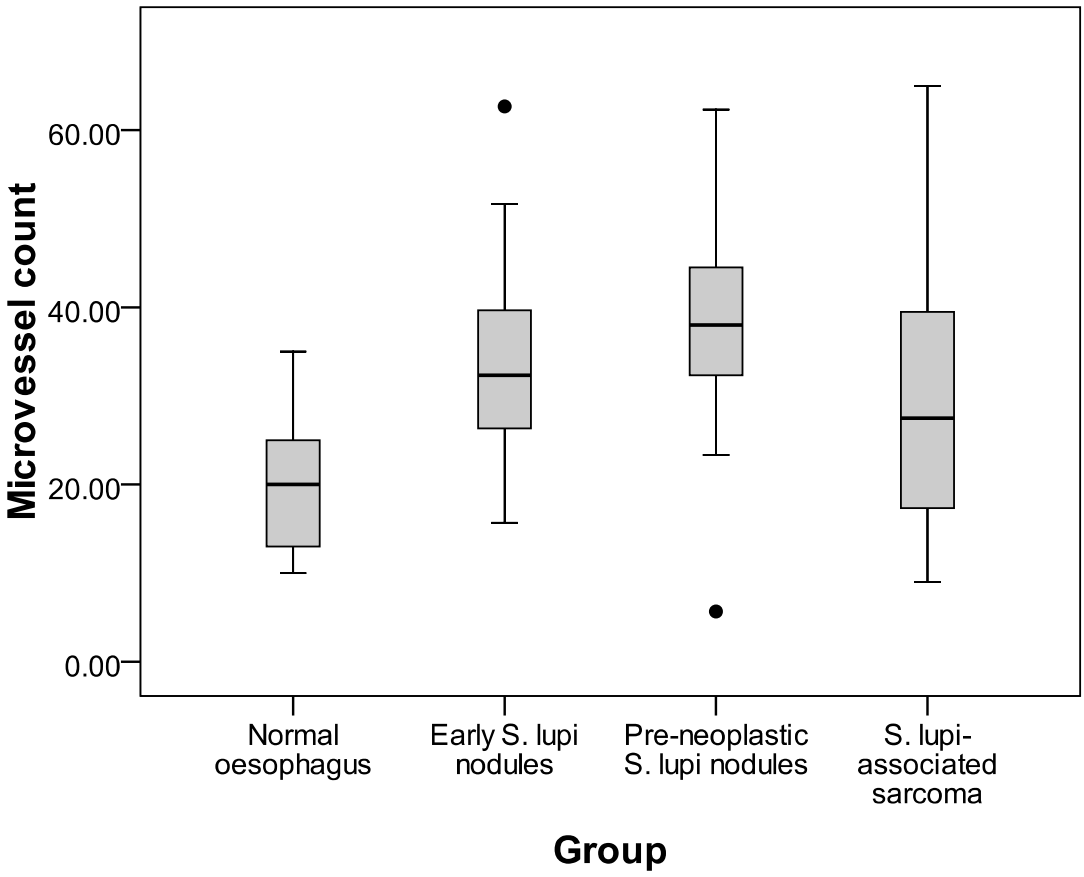


Figure 5: Box Plot of the mean microvessel count per high power field at the periphery of the nodules. The overall comparison between the groups was statistically significant (p=0.002). The pre-neoplastic oesophageal *S. lupi* nodule and the early non-neoplastic *S. lupi* oesophageal nodule groups exhibited significantly higher MVD compared to the control and *S. lupi*-associated oesophageal sarcoma groups ($p<0.05$). However, the differences between these 2 groups were not significant. There was also no statistically significant difference between the the *S. lupi*-associated oesophageal sarcoma and normal oesophagus groups.

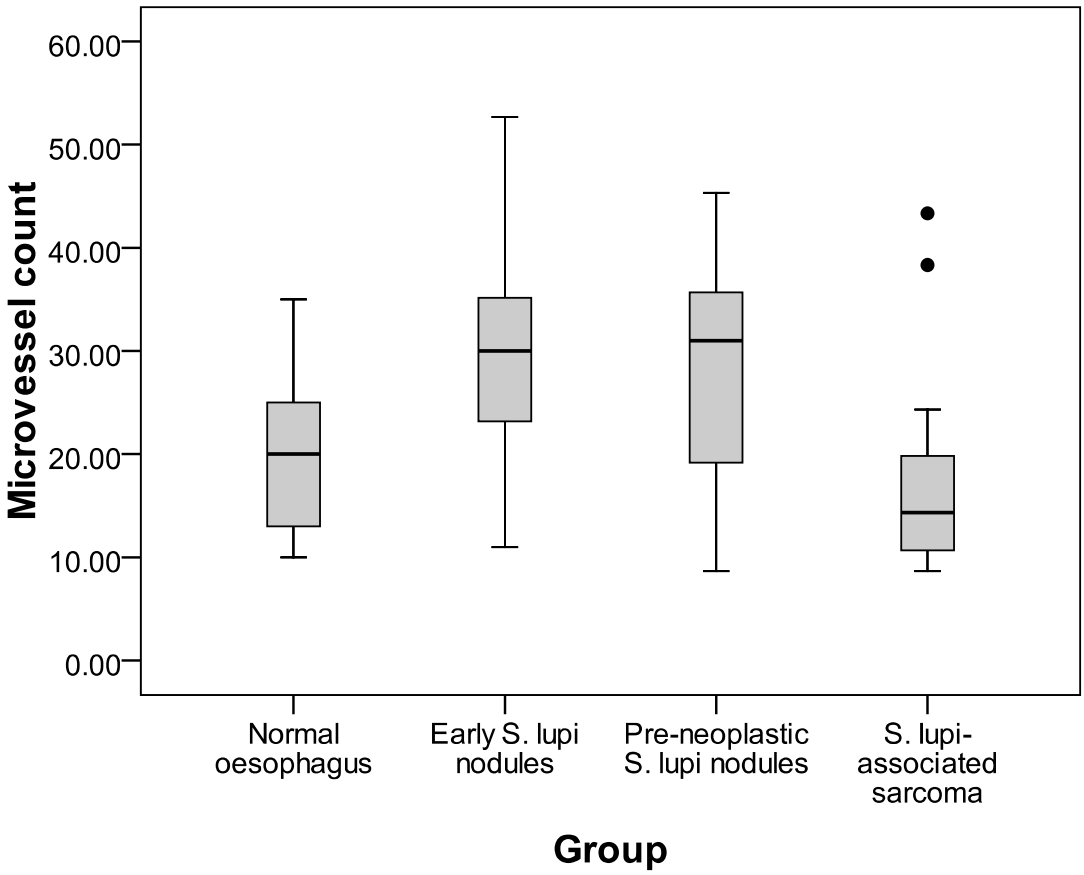


Figure 6: Box Plot of the mean microvessel count per high power field at the centre of the nodules. The overall comparison between the groups was statistically significant ($p<0.001$). The pre-neoplastic oesophageal *S. lupi* nodule and the early non-neoplastic *S. lupi* oesophageal nodule groups exhibited significantly higher MVD compared to the control and *S. lupi*-associated oesophageal sarcoma groups ($p<0.05$). However, the differences between these 2 groups were not significant. There was also no statistically significant difference between the the *S. lupi*-associated oesophageal sarcoma and normal oesophagus groups.

7 IMMUNOHISTOCHEMICAL CHARACTERIZATION OF LYMPHOCYTE AND MYELOID CELL INFILTRATES IN SPIROCERCOSIS-INDUCED ESOPHAGEAL NODULES

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Immunohistochemical characterization of lymphocyte and myeloid cell infiltrates in spirocercosis-induced esophageal nodules

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7.1 Abstract

Spirocerca lupi is a nematode that infects the dog's esophagus and promotes the formation of an inflammatory fibroblastic nodule that progresses to sarcoma in approximately 25% of cases. Spirocercosis-associated esophageal sarcoma is an excellent and under-utilized spontaneous model of parasite-associated malignancy.

The inflammatory infiltrate of paraffin-embedded non-neoplastic esophageal nodules (n=46), neoplastic nodules (n=25) and normal esophagus (n=14) was examined by immunohistochemistry using MAC387 (myeloid cells), CD3 (T cells), Pax5 (B cells) and FoxP3 (T regulatory cells) antibodies. Myeloid cells predominated in 70% of nodules in pockets around the worms' migratory tracts and in necro-ulcerative areas in neoplastic cases. T cells predominated in 23% of cases with a focal or diffuse distribution, in the nodule periphery. No significant differences were observed between neoplastic and non-neoplastic stages. FoxP3+ cells were observed in low numbers, not significantly different from the controls.

The inflammation in spirocercosis is characterized by pockets of pus surrounded by organized lymphoid foci. There was no evidence of a local accumulation of FoxP3+ cells, unlike many previous studies which have reported an increase in Foxp3+ T cells in both malignancies and parasite infections. The triggering factor(s) driving the neoplastic transformation of the spirocercosis-associated chronic inflammatory nodule warrants further investigation.

Key words Spirocerca lupi, sarcoma, T regulatory cells, FoxP3, CD3, Pax5, MAC387

7.2 Introduction

Spirocerca lupi (S. lupi) is a nematode for which the dog is the final host (Bailey, 1972). In the dog the adult nematode resides in the esophagus which results in the formation of an esophageal nodule. Over time up to 25% of these nodules undergo neoplastic transformation (Dvir et al., 2001). Histologically the sarcoma has been classified as fibrosarcoma, osteosarcoma or anaplastic sarcoma (Ranen et al., 2008; Ranen et al., 2004). The different stages of the spirocercosis-induced esophageal nodule have recently been described (Dvir et al., 2010). It was proposed that non-neoplastic *S. lupi* nodules could be divided into 2 stages: an early inflammatory stage, where the nodule is characterized histologically by fibrocytes and abundant collagen, and a pre-neoplastic stage, where the nodule is characterized by the presence of activated fibroblasts (more mitoses and a greater proportion of fibroblasts that showed some degree of atypia) and reduced collagen. Both stages are characterized by lympho-plasmacytic inflammation. Finally the nodule develops into neoplastic sarcoma (Dvir et al., 2010). This study was the first to describe the high prevalence and severity of the lympho-plasmacytic infiltrates in *S. lupi*-induced nodules which have often previously been incorrectly classified as granulomas (Bailey, 1972). Neutrophils were also very common in the non-neoplastic cases, where they were distributed either diffusely or in purulent foci immediately adjacent to the worm tract(s) and their associated tissue debris. The neoplastic cases generally had less inflammation; the inflammation was predominantly suppurative and the foci of suppuration were typically confined to necro-ulcerative areas in the tumour.

The finding that *S. lupi* nodules have a marked lympho-plasmacytic infiltration is important since the association between chronic infection-induced inflammation and cancer is now well-described and is thought to be the mechanism responsible for up to 18% of global cancers (Vennervald and Polman, 2009). In terms of parasite-associated malignancies, three helminth infections have been classified as carcinogenic in humans, namely *Schistosoma haematobium, Clonorchis sinensis* and *Opisthorchis viverrini* and the presence of chronic inflammation induced by parasites or their deposition is considered a key element in their carcinogenesis (Vennervald and Polman, 2009). In dogs esophageal sarcoma (excluding leiomyosarcoma) is almost invariably associated with *S. lupi* infections, whereas in human oncogenic helminth-associated neoplasia the association is limited to only a few of the specific cancer cases (Herrera et al., 2005), making spirocercosis a highly attractive model to study the association between cancer, helminth infection and inflammation.

It is widely accepted that helminths and their antigens induce a Th2 response (Maizels et al., 2009), and although a Th2 response to the parasite is essential for the host to clear the infection, it is imperative that the immune response is well controlled. The Th2 response can be tightly controlled by CD4+ regulatory T cells (Tregs), which are characterized by the expression of CD25 and the intracellular forkhead box P3 (FoxP3) transcription factor, secretion of interleukin (IL)-10 and transforming growth factor β (TGFβ) (Maizels et al., 2009). While Tregs are essential in the prevention of autoimmune and allergic diseases via their inhibition of an autopathogenic immune response, induction of Tregs by helminths can facilitate long-lasting infection (Maizels et al., 2009). Similarly, Tregs can inhibit the anti-tumour immune response (Beyer and Schultze, 2006) and an increase in their number may facilitate tumour development. Numerous clinical studies on human patients with various types of

cancer have shown increased Tregs proportions in the peripheral blood, draining lymph nodes and within the tumours (Curiel et al., 2004; Heimberger et al., 2008; Liyanage et al., 2002; Wolf et al., 2003; Woo et al., 2001).

FoxP3+ Tregs can be identified in the dog using a cross-reactive, directly conjugated murine FoxP3 antibody (Biller et al., 2007). As in humans, tumour-bearing dogs were found to have an increased number and/or proportions of Tregs in the circulation (Biller et al., 2007; O'Neill et al., 2009; Tominaga et al., 2010), draining lymph nodes (Biller et al., 2007) and within the tumour (Tominaga et al., 2010). The fact that the role of Tregs is well described in both helminth infection and cancer may indicate a potential role in helminth-induced cancer such as spirocercosis. However, the role of Foxp3+ Tregs in helminth infections in dogs has not been investigated and the presence of FoxP3+ cells has not been examined by immunohistochemistry in canine tissue.

The primary objective of this study was to characterize the lymphocyte and myeloid infiltrate in *S. lupi* nodules by immunohistochemistry using antibodies against CD3 (T cells), Pax 5 (B cells) and MAC387 (myeloid cells) (Vanherberghen et al., 2009; Willmann et al., 2009). A secondary objective of the study was to investigate the prevalence of FoxP3+ Tregs in the *S. lupi* nodule by immunohistochemistry.

7.3 Materials and Methods

7.3.1 Case Selection

Seventy one formalin-fixed, paraffin-embedded *S. lupi*-induced esophageal nodules, collected between 1998-2009, were retrieved from the archives of the Section of Pathology, Faculty of Veterinary Science, University of Pretoria (retrospective study). The samples were collected during necropsy. In most cases, only one sample was

collected for diagnostic purposes. In the smaller non-neoplastic nodules, a transverse section was taken through the entire nodule. One 5µm-thick tissue section per block was stained with hematoxylin and eosin (H&E) for subsequent histological evaluation. Nodules were classified into neoplastic (n=25) and non-neoplastic (n=46) groups. Only one nodule was selected per dog for subsequent immunohistochemical analyses. If a dog had more than one nodule, the nodule that was most mature or advanced towards neoplastic transformation was selected. In the larger nodules multiple sections were taken and the most diagnostic section was selected.

For negative tissue control purposes, 14 sections of normal distal third of dog esophagus were used. For 9 of the *S. lupi*-induced esophageal nodule cases (5 neoplastic and 4 non-neoplastic), the draining lymph nodes of the distal esophagus (bronchial) and remote lymph nodes (popliteal) were also collected. The entire lymph nodes were collected and a transverse section was fixed in paraffin. Lymph node was the positive tissue control for immunohistochemical labeling.

7.3.2 Immunohistochemical labelling of FoxP3, CD3, Pax5 and Myeloid/Histiocyte antigen MAC387

Four µm-thick serial sections were cut and mounted on Superfrost-Plus glass slides (Thermo), and dried overnight in an oven at 60ºC to enhance tissue adhesion. Following rehydration, antigen-retrieval was performed. For FoxP3, CD3 and Pax5 labeling, heat-induced epitope retrieval was performed by autoclaving at 121^{o}C for 10 minutes in 10mM citrate buffer pH 6.0. For MAC387 labelling, sections were pre-treated with Proteinase K (Dako) for 5 minutes at 25°C. The sections were washed twice in phosphate-buffered saline (PBS) and again in PBS containing 0.5% Tween 80 (PBST80) for 5 minutes. Endogenous peroxidase activity was quenched by incubating the tissue sections with 0.3% hydrogen peroxide in PBST80 for 20 minutes

at room temperature (RT). Following two washes in PBST80, slides were loaded into a Sequenza immunostaining centre (Thermo Scientific). Non-specific tissue antigens were blocked by incubation in 25% normal goat serum (NGS) in PBS/0.5% Tween 80 (PBS/T80) for 1 hr at RT prior to incubation overnight at 4°C with the following primary antibodies: 1:100 dilution of rat anti-mouse/rat FoxP3 monoclonal antibody (mAb) (FJK-16s, eBioscience, San Diego, CA, USA); 1:200 dilution of polyclonal rabbit anti-human CD3 antibody (Dako); 1:50 dilution of mouse anti-human Pax-5 mAb (clone 24, BD Biosciences). MAC 387 antibodies were incubated for 1 hour at 25ºC: 1:400 dilution of mouse anti-human Myeloid/Histiocyte Antigen mAb (clone MAC387, Dako). Control antibodies included: Rat IgG2a isotype control mAb (eBioscience), mouse anti-Border disease virus p125/p80 mAb VPM21 and purified rabbit immunoglobulin (Sigma-Aldrich), for rat, mouse and rabbit primary antibodies, respectively. All antibodies were diluted in PBS/T80 containing 10% normal goat serum (NGS).

Slides were washed twice in PBS and the appropriate secondary antibody (peroxidase-labelled anti-mouse or anti-rabbit EnVision™+ reagent, Dako) was applied to sections for 30 minutes at RT. After a final PBS wash, sections were incubated with 3,3'-diaminobenzidine (DAB) for 7.5 minutes at RT, washed in distilled water, counterstained with haematoxylin, dehydrated and mounted in Shandon synthetic mountant (Thermo Scientific).

7.3.3 Scoring of IHC labelling

Each nodule was scanned under the light microscope. The initial scan was done with a wide-angle lens at low power (x20) and the following data were recorded: The predominant inflammatory cell type, the distribution of the cell infiltrate (diffuse or focal/multifocal) and the location of the infiltrate within the nodule (peripheral,

central or both). CD3+ and Pax5+ cells tended to occur in a focal/multifocal distribution pattern in the sections and the foci of CD3+ and Pax5+ cells were counted in the most active x20 field (the field with the highest number of foci). CD3+ and Pax5+ infiltrates were subjectively scored 0-3 (Table 1). MAC387+ infiltrates were also scored 0-3; however, MAC387+ cells occurred more diffusely in sections, either evenly distributed or in patches and therefore the scoring system was slightly different (Table 2). Numbers of FoxP3+ cells were counted in 10 non-overlapping x400 fields (5 peripheral and 5 central fields per esophageal nodule using a 0.0625mm^2 graticule). In the normal esophagus control group and lymph nodes 5 non-overlapping x400 fields were counted. Counting was confined to CD3+ areas.

7.3.4 Statistical analyses

Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc. CA, USA). The difference in prevalence and distribution of the different proportions of cell types was tested using the Chi square test. The differences between the scores of the different types of infiltrate were tested for significance between all groups using a Kruskal-Wallis Test, followed by Dunn's post-hoc test. *P* values of < 0.05 were considered significant.

7.4 Results

Myeloid cells predominated in 70% of cases, while T cells predominated in 23% of cases. In the remaining 7% of cases the number of T cells and myeloid cells were approximately equal. There was no difference in the proportion of myeloid and T cells between the neoplastic and non-neoplastic groups (p=0.27). When cells were present in normal esophageal sections they were diffusely scattered and myeloid and T cells tended to occur in equal proportions (Table 3). The inflammatory score of all

cell types was significantly higher ($p < 0.05$) in the spirocercosis groups compared to the control group, but was not different between the neoplastic and non-neoplastic groups (Table 4, 5 and 6).

Myeloid cells were most commonly confined to massive diffuse pockets around worm migratory tracts (Figure 1A) and to necro–ulcerative areas, the latter especially in neoplastic cases (Figure 1B). Most cases had massive diffuse areas that could not be counted. To a lesser extent, myeloid cells were diffusely scattered throughout the nodules (Table 4).

T cells occurred diffusely (Figure 1C) or in a focal/multifocal (Figure 1D) distribution pattern, predominantly at the periphery of the nodule (Table 5). The number of foci in the most active x20 field ranged from 0 to 18. B cells followed the same distribution within the nodule as T cells (Table 6), but there were fewer of them (Table 7) and they were more confined to focal/multifocal areas (Figure 1E).

FoxP3+ cells were detected in 30% of nodules (32% of neoplastic cases and 28% of the non-neoplastic cases), especially in T cell foci, but they were not observed in the normal esophagus. In most of the *S. lupi* cases where FoxP3+ cells were detected, the number of cells was very low (Table 8) and was not significantly different from the normal esophagus, where no FoxP3+ cells were detected. However, 3 cases (1 non-neoplastic and 2 neoplastic) contained a high power field with more than 10 FoxP3+ cells (up to 47 cells/0.0625mm^2 in a selected high power field; Figure 1F).

High numbers of FoxP3+ cells were observed in the lymph nodes (Table 9, Figure 1G), but no difference was observed between the bronchial and popliteal nodes and between the neoplastic-draining (86.44±34.39, mean±STD/0.0625mm^2) and non-neoplastic-draining nodes (85.95±54.55). These FoxP3+ cells were confined to CD3+ areas (Figure 1H).

7.5 Discussion

The current study revealed that the predominant inflammatory cells in *S. lupi* esophageal nodules are of myeloid lineage. These cells were identified by a MAC387 antibody, which does not enable differentiation between the different types of myeloid cells. However, based on the histological appearance, the vast majority of myeloid cells were neutrophils. These neutrophils formed pockets of pus around the worm, or they were confined to necro-ulcerative areas in the neoplastic nodules. Alternatively, neutrophils occurred diffusely throughout the nodules. The lymphocytic infiltrates had a prominent focal/multifocal distribution pattern (compared to the myeloid cells) and they were usually peripherally located within nodules. However, in the majority of cases, lymphocytes occurred in a mixed pattern; namely focal/multifocal and diffuse. The relative proportions of leukocytes within *S. lupi* nodules was different to our initial observations in H&E-stained sections (Dvir et al., 2010). This finding shows the importance of further identification and quantification of cells using IHC. There are two possible explanations for the observed difference. Firstly, in the current study plasma cells were not labeled, but plasma cell-rich foci in HE-stained sections would have been incorporated into the lympho-plasmacytic scoring in the previous study. Also, the focal/multifocal distribution pattern of the lympho-plasmacytic reaction, which frequently made it the predominant cell infiltrate in certain fields, may have biased our scoring over the whole slide in the previous study. We could also not demonstrate the difference in the inflammation score and composition of the cell infiltrate between neoplastic and non-neoplastic cases that we previously observed (Dvir et al., 2010).

Myeloid cells and especially neutrophils play a major role in the innate local inflammatory response in the spirocercosis-induced nodule. Myeloid cells can have an

important role in cancer induction by generating proteases, free radical and nitrogen species that can cause oxidative damage to the DNA (Vennervald and Polman, 2009). They can also play a crucial role in establishing cytokine-induced tumour rejection (Di Carlo et al., 2001), and they also play a major part in endothelium-mediated lymphocyte trafficking and antigen presentation. Polymorphonuclear cells have shown both pro- and anti-inflammatory activities. They may participate in the switch to immune suppression by Th2 and Tregs through up-regulation of IL-10 (Di Carlo et al., 2001). More recently neutrophils have been shown to play a pivotal role in the regulation of the inflammatory response against cancer (Mattarollo and Smyth). For instance, neutrophils can be induced by serum amyloid A (SAA)1 to secrete IL-10 which induces suppression of immune surveillance (De Santo et al., 2010).

In the present study, T cells outnumbered B cells. To further differentiate between the different T cell types, especially into $CD4^+$ or $CD8^+$ cells, frozen sections (which were not available in this study) would be necessary. Based on the current knowledge of helminth-associated chronic inflammation these cells are likely to be Th2 CD4+ cells (Maizels et al., 2009). Th2 responses are generally correlated with suppressed cell mediated immune response and with enhanced tumour promotion and progression. B cell response is often associated with Th2 cell response and also with increased risk for neoplastic progression (de Visser et al., 2005; Shah et al., 2005; Tan and Coussens, 2007). Additionally, immunoglobulins and more specifically immune complexes are regarded as tumour promoting (Tan and Coussens, 2007). The humoral response in spirocercosis warrant further investigation for its role in the carcinogenesis in spirocercosis and also for the potential use of serology as a diagnostic tool in this disease.

This study reports for the first time an approach to the identification of FoxP3+ cells in excised diseased canine tissue. We hypothesized that Tregs will be present in high numbers in the spirocercosis-induced nodules and that their numbers will increase as the nodule progressed toward sarcoma, but although Foxp3+ cells were found in large numbers within CD3+ regions of lymph nodes, they were rarely observed in *S. lupi* associated esophageal nodules and when present, were usually in very small numbers. This is surprising considering the wide range of studies which have found increased numbers and proportions of Foxp3+ Tregs within tumours in humans (Carreras et al., 2006; Unitt et al., 2005; Xue et al., 2009) and murine models (Imai et al., 2007) including models of fibrosarcoma (Betts et al., 2007). The only other study to examine Tregs within canine tumours found similar results to the many other studies of human tumours and experimental cancer models. They reported that the percentage of FoxP3+ CD4+ cells in dogs with malignant melanoma was significantly increased in the blood compared with healthy control dogs, and the percentage of FoxP3+ CD4+ cells within tumours compared to blood was also significantly increased (Tominaga et al.). Therefore, this study clearly demonstrates that the developing dogma that Foxp3+ T cells are highly prevalent in tumour-associated inflammation is not universally true and emphasises that neoplastic transformation can still occur in the absence of immunosuppressive Foxp3+ T cells. It is in agreement with the canine literature on sarcoma (O'Neill et al., 2009), especially osteosarcoma (Rissetto et al., 2010). Interestingly, in humans with Ewing's sarcoma, there was also no infiltration of FoxP3+ cells into the tumours, whereas in patients with metastases, the number of FoxP3+ cells only increased in the bone marrow (Brinkrolf et al., 2009). The fact that a large number of positive cells were observed in a few cases, as well as in lymph nodes, but not in the iso- or tissue-controls, excludes technical error. Moreover, all

samples were fixed by the same method (formalin-fixed and paraffin-embedded) and the 9 positive controls (lymph nodes) originate from 9 of the study cases. Therefore, it seems feasible that there is a real difference in the immune response to sarcomas (especially in dogs), compared to other tumours, especially melanomas.

The possible role of Tregs in the pathogenesis of spirocercosis-induced sarcoma is especially intriguing due to the well-documented role of Tregs in helminth infection. In chronic helminth infection, and spirocercosis-induced inflammation is, indeed, chronic, Tregs reduce the intensity of the infection (Maizels et al., 2009). There is evidence that the increased Tregs response facilitate long lasting chronic inflammation that reduce auto-immunity and allergy in infected subjects (Wilson and Maizels, 2004). This notion is part of the proposed mechanism of what is known as the "hygiene hypothesis" that describes the association between of helminth infection and low incidence of autoimmunity (Maizels, 2009). The Tregs-induced increased "self tolerance" may reduce anti-tumour immunity and this could potentially be the link between spirocercosis and tumour formation. It appears, however, that although FoxP3+ cells were circulating in lymphatics around *S. lupi* nodules, "homing" into the nodules did not take place. The low number of FoxP3+ cells does not entirely preclude their potential role in local or systemic immune inhibition in spirocercosis but functionality assays are required. However, it is important to acknowledge that although FoxP3 is the gold standard marker of murine Tregs (Rouse, 2007), there are many types of Tregs that do not express FoxP3, for example the widely described Tr1 cells (Rouse, 2007). These cells also regulate the immune response through secretion of IL-10 and TGFβ and it is possible that they are involved in immunoregulation in spirocercosis.

One weakness of the current study is that tissue sampling was not standardized. Unfortunately, this is the reality when utilizing clinical cases, especially in a retrospective study. The cell counting was also limited to a single section. However, because this is primarily a descriptive study, we believe the results are valid. Moreover, in the search for Tregs, we tried to augment the chances for finding them by limiting the count to areas with high CD3+ cells presence (based on the lymph node findings and pilot observations) and yet we met with limited success. Therefore, the lack of FoxP3+ cells in most of the *S. lupi* nodules seems reliable. The study also provide unique *in situ* morphologic picture of the FoxP3+ infiltrate, which no dog study has reported. The key question in spirocercosis remains: What is the trigger for the transformation from the chronic inflammatory, fibroblastic nodule to sarcoma? This transformation may be triggered by the inflammatory response or, alternatively, via worm excretory / secretory (ES) products. Recent studies have shown that ES products from *Opisthorchis viverrini*, a helminth that induces cholangiocarcinoma in humans, increased fibroblast cell proliferation in cell cultures (Thuwajit et al., 2004). However, the theory of stimulation of cells in the nodule by the worm does not completely exclude the inflammatory mediation hypothesis, because other studies have shown that *Opisthorchis viverrini* ES products up-regulate the expression of TGFβ, which may represent an indirect carcinogenic effect via immunosuppression (Thuwajit et al., 2006). Many studies have elucidated the role played by helminth ES products in the modulation of the immune response, especially via the inhibition of innate cell functions and induction of a Th2 response (Hewitson et al., 2009). Such mechanisms clearly warrant further investigation if we are to understand the pathogenesis of *S. lupi*-induced sarcoma.

7.6 Tables

Table 1

Scoring system for CD3+ and Pax5+ infiltrates

score	Infiltrate intensity (x400 fields)	Number of foci (x20 fields)
0	scant or absent	0
1	positive cells evident but not in all fields	≤ 1
2	positive cells present in all fields but markedly fewer in number than other inflammatory cells	≤ 3
3	Positive cells predominant	≥4

Table 2

Scoring system for MAC387+ infiltrates

score	Infiltrate intensity (x400 fields)
0	scant or absent
1	positive cells evident but not in all fields
2	positive cells present in all fields but markedly fewer in number than other inflammatory cells
3	Positive cells predominant

Table 3

Leukocyte prevalence in the different groups

Group	Predominantly MAC387	Predominantly CD3	Equal CD3 and MAC387
Neoplastic	72% (18/25)	24% (6/25)	4% (1/25)
Non-neoplastic	70 % (32/46)	21.5% (10/46)	8.5% (4/46)
Normal esophagus	43% (6/14)	50% (7/14)	7% (1/14)

Table 4

Nodule distribution and score of MAC387+ cells

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Score	
	Even	Patchy	Mixed	Peripheral	Central	Both		Mean	Median
Neoplastic	88% (22/25)	4% (1/25)	8% (2/25	60% (15/25)	8% (2/25)	32% (8/46)	0/46	2.04 ± 0.98	2 (1-3)
Non-neoplastic	98% (45/46)	2% (1/46)	0/46	6% (3/46)	22% (10/46)	72% (33/46)	0/46	2.37 ± 0.9	3 (1-3)
Normal esophagus	50% (7/14)	1/14 (7%)	0/14	NA	NA	NA	43% (6/14)	0.57 ± 0.51*	1 (0-1)

*The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 5

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Foci number		Score	
	Diffuse	Focal / multifocal	Mixed	Peripheral	Central	Both		Mean	Median†	Mean	Median
Neoplastic	24% (6/25)	12% (3/25)	48% (12/25)	48% (12/25)	0	36% (9/25)	16% (4/25)	3.56 ± 4.69	1 (0-16)	1.56 ± 1,21	1 (0-3)
Non-neoplastic	52% (24/46)	13% (6/46)	33% (15/46)	48% (22/46)	4.5% (2/46)	45.5.5% (21/46)	2% (1/46)	2.68 ± 4.04	1 (0-18)	1.78 ± 0.94	2 (0-3)
Normal esophagus	43% (6/14)	0/14	0/14	NA	NA	NA	57% (8/14)	0	0	0.43 ± 0.51*	0 (0-1)

Nodule distribution and score of CD3+ cells

† 2 cases in the non-neoplastic group had focally extensive areas of cell infiltrate that could not be counted

* The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 6

Nodule distribution and score of Pax5+ cells

Group	Pattern of the infiltrate distribution			Location within the nodule			No cells	Number of foci		Score	
	Diffuse	Focal / multifocal	Mixed	Peripheral	Central	Both		Mean	Median	Mean	Median
Neoplastic	8% (2/25)	20% (5/25)	24% 6/25	44% (11/25)	0/25	8% (2/25)	48% (12/25)	2.44 ± 3.91	1 (0-16)	0.96 ± 1.14	1 (0-3)
Non-neoplastic	28% (13/46)	33% (15/46)	13% (6/46)	45.5% (21/46)	4.5% (2/46)	24% (11/46)	26% (12/46)	1.8 ± 2.62	0 (0-1)	1.15 ± 1.07	1 (0-3)
Normal esophagus	0/14	0/14	0/14	NA	NA	NA	100% (14/14)	0	0	0*	0

* The score of the control was significantly (<0.05) lower compared to the spirocercosis groups' scores using Kruskal-Wallis Test, followed by Dunn's post-hoc test.

Table 7

Lymphocyte prevalence in the different study groups

Group	Predominantly CD3	Predominantly Pax5	Equal prevalence
Neoplastic	80% (20/25)	0/25	20%, 5/25
Non-neoplastic	78% (36/46)	4.5% (2/46)	17.5%, 8/46
Normal esophagus	100% (14/14)	0/14	0/14

Table 8

The number of FoxP3+ cells per 0.0625mm^2 in the different groups

Group	FoxP3+ cells			
	Peripheral		Central	
	Mean±STD	Median (range)	Mean±STD	Median (range)
Neoplastic	0.73±3.65	0 (0-3)	0.13±0.39	0 (0-2.2)
Non-neoplastic	0.69±1.19	0 (0-4.2)	1.34±5.55	0 (0-27.8)
Normal esophagus	Mean:0±0, Median: 0			

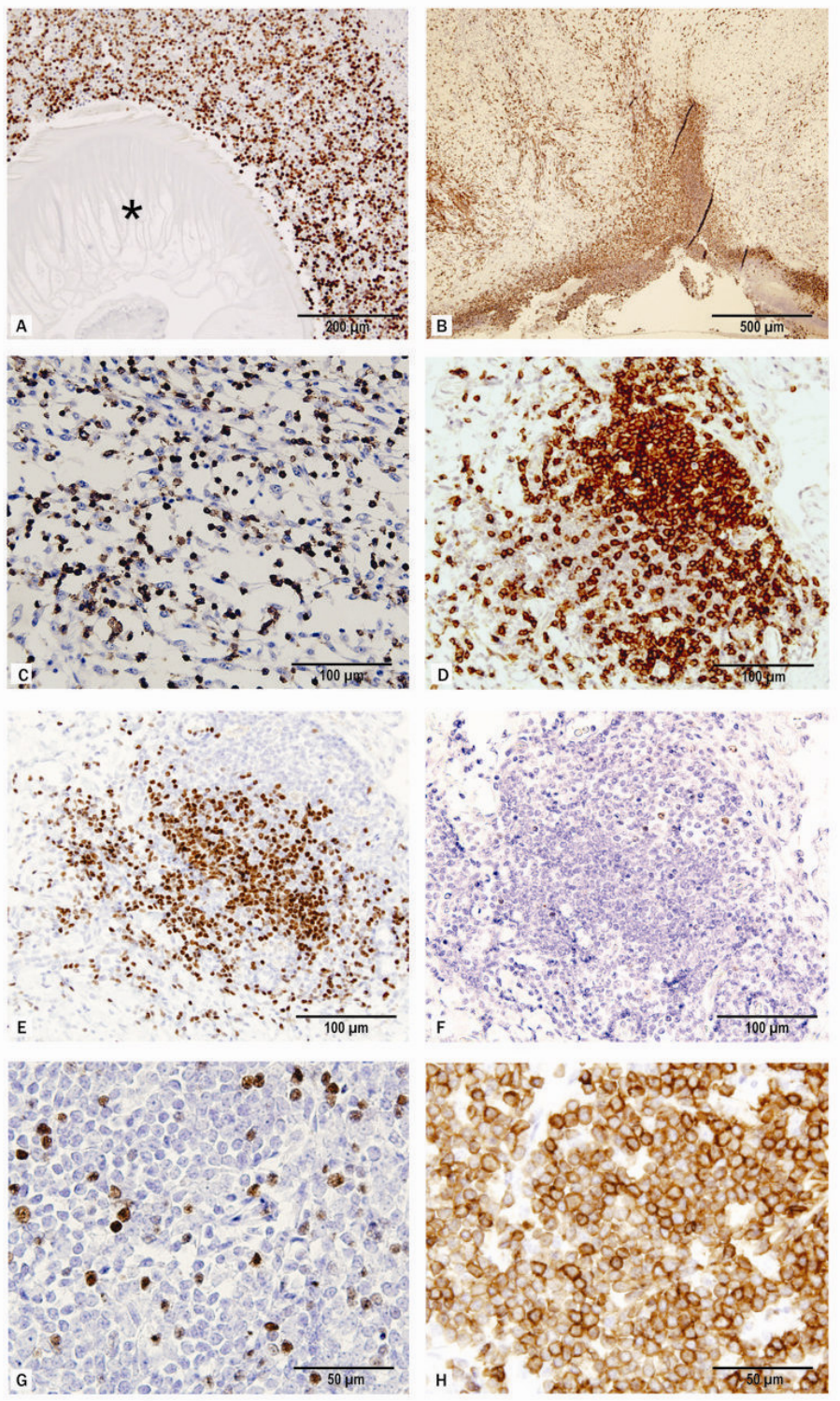
Table 9

Number of T regulatory cells per 0.0625mm^2 in the lymph nodes of the different groups

Group	Bronchial lymph nodes (5 fields)		Popliteal lymph nodes	
	Mean±STD	Median	Mean±STD	Median (range)
Neoplastic (n=5)	86.44±34.39	97 (38-130)	91.5±23.59	84.5 (65-112)
Non-neoplastic (n=4)	85.95±54.55	81 (33-158)	108.35±35.8	100 (74-156)

7.7 Figure Legends

Figure 1



A: MAC387+ leukocytes, predominantly neutrophils, around a *Spirocerca lupi* parasite (asterisk) in a non-neoplastic esophageal nodule.

B: MAC387+ leukocytes, predominantly neutrophils, in an extensive area of ulceration in a *Spirocerca lupi*-induced esophageal osteosarcoma.

C: Diffuse distribution of CD3+ T lymphocytes in a *Spirocerca lupi*-induced esophageal undifferentiated sarcoma.

D: Focal/nodular distribution of CD3+ T lymphocytes in a *Spirocerca lupi*-induced esophageal undifferentiated sarcoma.

E: Pax5+ B lymphocytes in the same lymphoid focus at the periphery of Spirocerca *lupi*-induced esophageal undifferentiated sarcoma as is shown in D.

F: FoxP3+ cells in the same lymphoid focus at the periphery of *Spirocerca lupi*-induced esophageal undifferentiated sarcoma as is shown in D and E.

G: FoxP3+ cells in a bronchial lymph node, draining the distal esophageal osteosarcoma referred to in figure D.

H: CD3+ T lymphocytes in the same area of bronchial lymph node as shown in figure G.

8 PLASMA IL-8 CONCENTRATIONS ARE INCREASED IN DOGS WITH SPIROCERCOSIS

This chapter was published as a research paper:

Plasma IL-8 concentrations are increased in dogs with spirocercosis

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8.1 Abstract

The nematode *Spirocerca lupi* (*S. lupi*) induces sarcoma in the dog oesophagus in about 25% of cases. The aim of this study was to compare the differences in the cytokine milieu between dogs with neoplastic (n=29) and non-neoplastic disease (n=49) and age- and gender-matched healthy controls (n=25). We measured IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, GM-CSF and MCP-1 in a specific canine multiplex immunoassay kit. Cytokine concentrations were compared between the different groups using the Kruskal-Wallis test followed by Dunn's test.

Only IL-8 and IL-18 showed significant differences in their plasma concentration among the three groups. Kruskal-Wallis Test revealed a significant ($p=0.001$) difference in IL-8 concentration between the neoplastic group (634 pg/ml), the non-neoplastic (429 pg/ml) and the control groups (150 pg/ml). Post-test analysis revealed a significance difference between the two *S. lupi* groups and the control group ($p<0.01$). The highest IL-18 concentration was found in the non-neoplastic group (53 pg/ml), followed by the control group (46 pg/ml) and finally the neoplastic group (33 pg/ml). IL-18 concentrations were significantly higher in the non-neoplastic group than in the neoplastic group ($p=0.05$).

The increased IL-8 in the spirocercosis groups is consistent with the neutrophilic infiltrate in spirocercosis lesions and in those of other inflammatory-induced neoplasias such as Barret's oesophagus and *Helicobacter* gastritis. IL-18 showed negative regulatory effect in several worm infections and it is possible that it plays the same role in spirocercosis, allowing the worm to evade the host response and to induce neoplastic transformation.

*Keywords:*Spirocercosis, *Spirocerca lupi*, cytokines, Interleukin 8, Interleukin 18, sarcoma

8.2 Introduction

Spirocercosis is a disease caused by the *Spirocerca lupi (S. lupi)* nematode in dogs (Bailey, 1972). The disease occurs worldwide throughout tropical and subtropical areas, in very high prevalence in certain locations. (Dvir et al., 2001). In South Africa the prevalence in some areas may reach up to 70% (Kok et al., 2010). At the end of the migration route within the dog the adult worm settles in a fibro-inflammatory nodule in the caudal oesophagus (Dvir et al., 2010). These nodules transform to a sarcoma in approximately 25% of cases (Dvir et al., 2001). The initial non-neoplastic nodule shows marked inflammation that involve myeloid cells (predominantly neutrophils) and lymphoplasmacytic cells (with high prevalence of CD3+ T cells and to a lesser degree Pax5+ B cells)(Dvir et al., 2011). There is also evidence of an increased systemic inflammatory response in both the neoplastic and non-neoplastic stages that is reflected as a leukocytosis (Dvir et al., 2008) and an elevated C-reactive protein (CRP) (Mukorera et al., 2011a).

The association between chronic infection-induced inflammation and cancer is now well-described and is thought to be the mechanism responsible for up to 18% of cancers globally (Vennervald and Polman, 2009). In terms of parasite-associated malignancies, three helminth infections have been classified as carcinogenic in humans, namely *Schistosoma haematobium, Clonorchis sinensis* and *Opisthorchis viverrini* (Vennervald and Polman, 2009), while *Schistosoma mansoni* (*S. mansoni*) is suspected to be carcinogenic (Yoshida et al., 2002). In dogs, oesophageal sarcoma (excluding

leiomyosarcoma) is almost invariably associated with *S. lupi* infections, whereas in human oncogenic helminth-associated neoplasia the association is limited to only a portion of the specific cancer cases (Herrera et al., 2005), making spirocercosis a highly attractive model to study the association between cancer, helminth infection and inflammation.

Our central hypothesis, while investigating the neoplastic transformation and the inflammatory response in canine spirocercosis, was that the parasite produces excretory product(s) which diverts the immune response from a T helper 1 (Th1) to Th2 cell response, typical of many nematode infections, and further to an immunoregulatory (immunosuppressive), FoxP3+ regulatory T cell- predominated response which then facilitates neoplastic transformation. This immune response is well-described across species. It is associated with fibroblastic proliferation and has been classified as a delayed hypersensitivity type 3 (Meeusen, 1999). The potential link between switching from Th1 to Th2 response and cancer formation was demonstrated in *S. mansoni*-infected mice that were injected with fibrosarcoma cells. The infected mice had up-regulation of their Th2 responses and consequently had a significantly weaker rejection of the cancer cells compared to the non-infected mice that showed Th1 response and stronger rejection (Yoshida et al., 2002). Increased numbers and proportions of Foxp3+ Tregs within tumours are well described in humans (Carreras et al., 2006; Unitt et al., 2005; Xue et al., 2009) and murine models (Imai et al., 2007), including models of fibrosarcoma (Betts et al., 2007). Surprisingly, Foxp3+ cells are rarely observed in *S. lupi*-associated oesophageal nodules and when present, are usually in very small numbers (Dvir et al., 2011). However, they are found in large numbers within CD3+ regions of the bronchial lymph nodes that are draining these lesions (Dvir et al., 2011). These findings cannot

completely exclude the possibility of Treg-associated immunosuppression during the neoplastic transformation in spirocercosis and one potential mechanism is a systemic response driven by circulating cytokines. It is, therefore imperative to investigate the cytokine milieu in canine spirocercosis and to determine if it is Th1-related, Th2-related, immunosuppressive or pro-inflammatory.

Since cytokines work in networks, several canine plasma cytokines including GM-CSF, IL-2, IL-4, IL-6, IL-8, IL-10, IL-18 and MCP-1 should be measured. Of these cytokines IL-2, IL-6, IL-8 and IL-18 are pro-inflammatory and IL-4 and IL-10 are immunoregulatory. The pro-inflammary cytokines such as IL-2 enhance the cytolytic activity of T lymphocytes and NK cells (Antony and Dudek, 2010). It is a Th1-related-interleukin and has major anti-tumour activity. IL-2 works synergistically with IL-18 (Srivastava et al., 2010). IL-6 is another pro-inflammatory cytokine that is classified as a major pro-tumourogenic cytokine. It serves as a growth and survival factor that stimulates angiogenesis, tumour progression and metastasis, and it is reported to maintain tumour-promoting inflammation (Grivennikov and Karin, 2011). Cancer cells, including tumour-associated fibroblasts, are also capable of IL-6 production and can significantly contribute to the serum concentration of this cytokine (Grivennikov and Karin, 2011). Elevated IL-6 was suggested as a useful biomarker for poor prognosis in dogs with cancer (Itoh et al., 2009), yet it was also successfully used as therapy in dogs with transmissible venereal tumours demonstrating anti-tumour activity (Chou et al., 2009).

Interleukin-8 is another pro-inflammatory cytokine that is expected to be elevated in malignancy. It is regarded as a significant regulatory factor within the tumour microenvironment and is produced by various inflammatory cells, but also by tumour

cells (Waugh and Wilson, 2008). Secretion of IL-8 from cancer cells can enhance their proliferation and survival, promote angiogenesis and induce chemotactic infiltration of neutrophils into the tumour site. Elevated serum IL-8 was correlated with tumour progression in humans with oesophageal squamous cell carcinoma (Diakowska et al., 2006; Krzystek-Korpacka et al., 2008). It was also detected in Barret's oesophagus (Fitzgerald et al., 2002), a human condition that involves oesophageal inflammation due to influx, epithelial dysplasia and metaplasia and eventually neoplastic transformation. The expression of IL-8 increases as the diseases progresses to cancer (Oh et al., 2007).

IL-18, like IL-2, enhances cytolytic activity of natural killer (NK) cells and cytotoxic T lymphocytes. IL-18 is a critical molecule in the activation of the Th1 immune response (Park et al., 2007). Like many cytokines, IL-18 has dual effects in cancer progression; namely enhancing anti-tumour immunity and promoting tumour progression (Park et al., 2007). Higher expression or secretion of IL-18 is detected in various cancer cells in comparison with normal controls and IL-18 is able to induce tumour angiogenesis, migration/metastasis, proliferation and immune evasion (Park et al., 2007). IL-18 stimulates production of vascular endothelial growth factor (VEGF) mRNA and the final protein product leading to angiogenesis (Park et al., 2007). VEGF is highly expressed in spirocercosis-induced neoplasia (Dvir and Clift, 2010). Elevated serum IL-18 was found in humans with oesophageal squamous cell carcinoma and correlated with tumour progression (Diakowska et al., 2006; Krzystek-Korpacka et al., 2008).

IL-4 is a typical Th2-related cytokine and is therefore, not "expected" to have a major anti-tumour effect. In fact, many Th2-related cytokines are regarded as immunosuppressive and "tumour promoting". This approach has, however, been proven

to be over-simplistic, because IL-4 can also contribute to tumour rejection by boosting eosinophil function and increased antibody reaction (Dranoff, 2004). Therefore, it is not surprising that therapy with IL-4 shows an anti-tumour effect (Dranoff, 2004). IL-10 is the typical immunoregulatory cytokine, being a Treg- and Th2-related cytokine. It is widely believed that Treg function in cancer is mainly to suppress protective anti-cancer inflammatory responses (Beyer and Schultze, 2006). The role of Treg and the associated IL-10 is paradoxical, since IL-10 and Treg also reduce inflammation associated with infectious diseases ("hygiene theory"). This ability to reduce inflammation consequently inhibits or suppresses cancer (Erdman and Poutahidis, 2010).

Granulocyte-macrophages colony-stimulated factor (GM-CSF) is important in the process of protection from infection-induced cancer. GM-CSF / INF-γ double-knockout mice developed diverse haematological and solid neoplasms after various chronic infections and inflammations (Dranoff, 2004). GM-CSF is also used as an adjuvant in anti-cancer therapy in a few types of cancer in clinical trials. However, as many other cytokines, GM-CSF has a dual effect in cancer immunity and it also promotes invasion and dissemination of breast carcinoma in a transgenic mouse model.

Monocyte chemotactic protein-1 (MCP-1) is a chemokine that was originally termed 'tumour derived chemotactic protein'. It is secreted by several tumour cell lines and it is a potent chemotactic protein for monocytes, neutrophils, memory T cells and NK cells as well as stimulant for emigration of myeloid cells from the bone marrow (Perry et al., 2010). The early (non-neoplastic) spirocercosis nodules consist of massive pockets of MAC387+ myeloid cells (Dvir et al., 2011). It is likely that this response is a normal innate response to the pathogen. Expression of MCP-1 is a biomarker for poor outcomes

in breast carcinoma and ovarian cancer in humans and it has been associated with a poor prognosis in dogs diagnosed with lymphoma (Perry et al., 2010). Therefore, it is of interest to investigate its level in the different stages of spirocercosis.

In summary, it is of great interest to investigate the cytokine milieu and especially this set of cytokines in spirocercosis. We hypothesise that the cytokines expressed in spirocercosis (an infectious disease that progress to aggressive cancer with marked inflammation) will serve as biomarkers for neoplastic transformation and provide insights into the pathogenesis of this process.

8.3 Material and methods

8.3.1 Study population

The study population comprised of client-owned dogs admitted to the Onderstepoort Veterinary Academic Hospital, at the Faculty of Veterinary Science, University of Pretoria between 2008 and 2011. The study was approved by the faculty's animal use and care committee. The dogs were divided into 3 groups, non-neoplastic, neoplastic and healthy controls.

An initial diagnosis of spirocercosis was made by one of the following criteria: a faecal float that was positive for *S. lupi* worm eggs or radiological signs consistent with *S. lupi* infection, namely caudodorsal mediastinal mass together with spondylitis of the caudal thoracic vertebrae or undulation of the lateral border of the descending aorta (2 pathognomonic radiological signs associated with spirocercosis).

Classifying the mass as non-neoplastic was done by one of the following criteria:

- The masses had the typical smooth appearance of non-neoplastic nodules on endoscopy and responded to treatment monitored by follow-up endoscopy at 6 weeks and again at 12 weeks, if a poor initial response was shown at 6 weeks.
- Histopathological evaluation of the entire oesophageal nodule showed no evidence of neoplastic transformation. The sample was obtained by either surgical excision of the mass or necropsy.

Classifying the mass as neoplastic was performed by one of the following criteria:

- Histopathological diagnosis of neoplastic transformation by endoscopy-guided biopsy or post-necropsy.
- Metastatic lesions in the lungs, together with radiological signs associated with *S. lupi* and no other diagnosed neoplasm.

Cases that could not be classified as non-neoplastic or neoplastic were excluded (n=3).

The control group was composed of dogs that were presented for ovariohistorectomy, castration or blood donation. They were healthy by definition, had a full clinical history and had normal clinical examination and haematology. All dogs were negative for spirocercosis on faecal floatation and most of the dogs had thoracic radiographs and all thoracic radiographs were negative for *S. lupi*. They were age- and gender-matched with the other study groups.

8.3.2 Patient sampling

Blood samples were collected at admission by jugular veinpuncture with a 21g needle and a 5 ml potassium EDTA vacutainer syringe. The samples were then immediately centrifuged, separated, aliquoted and frozen at −80°C. The samples were batched and analysed together.

8.3.3 Analyses

Plasma cytokine concentrations were assessed at the department of Small Animal Clinical Sciences, University of Copenhagen, Denmark by a canine-specific multiplex assay (CCYTO-90K, Millipore, Billerica, MA) including internal quality control material with an automated analyzer (Luminex 200, Luminex Corporation, Austin, TX) for interleukin-2 (IL-2), IL-4, IL-6, IL-8, IL-10, IL-18, MCP-1 and GM-CSF (Kjelgaard-Hansen et al., 2011).

8.3.4 Data analysis

The median of each cytokine plasma concentration of each group was calculated and compared between groups using Kruskal-Wallis test, followed by Dunn's test for differences between specific pairs of groups. The level of significance was set at $p \leq 0.05$.

8.4 Results

One hundred and three dogs were enrolled in the study and were divided into 3 groups, non-neoplastic (n=49), neoplastic (n=29) and healthy control (n=25). Seventy six out of the 78 dogs with spirocercosis had oesophageal endoscopy performed and the typical oesophageal *S. lupi* nodule(s) were identified. The 49 non-neoplastic cases had the typical smooth appearance and responded to doramectin treatment. Twenty seven neoplastic cases had the characteristic cauliflower-like appearance on endoscopy and/or necropsy with area of necrosis and ulceration and were diagnosed by histopathology as sarcoma. The remaining 2 cases were neoplastic cases that were diagnosed based on the pathognomonic radiological signs and metastases in the lungs. The neoplastic nature of the lesion was later confirmed by necropsy in 1 of the 2 cases.

Interleukin 2, IL-4, IL-6, IL-10, MCP-1 and GM-CSF concentrations were not significantly different between the three groups. Only IL-8 and IL-18 showed significant differences in their plasma concentrations among the three groups. The highest median IL-8 concentration was in the neoplastic group [634 pg/ml, interquartile range (IR), 309-1230], followed by the non-neoplastic (429 pg/ml, IR 161-1277) and the control groups (150 pg/ml, IR 33-446). Post-test analysis revealed a significance difference between the two *S. lupi* groups and the control group (neoplastic vs. control, p = 0.002 and non-neoplastic vs. control, p = 0.003). The highest IL-18 concentration was in the non-neoplastic group (53 pg/ml, IR 25-156), followed by the control group (46 pg/ml, IR 24-264) and finally the lowest concentration was found in the neoplastic group (33 pg/ml, IR 1.6-79). Post-test analysis revealed that IL-18 concentrations were significantly higher in the non-neoplastic group than in the neoplastic group (p = 0.05). There was a trend toward a low IL-2 in the neoplastic group compared to the non-neoplastic and the control groups (Table 1).

8.5 Discussion

This study investigated key plasma cytokine concentrations in canine spirocercosis. Although this disease is associated with a severe systemic and local inflammatory response (Dvir et al., 2011; Mukorera et al., 2011a), the plasma cytokine milieu has never been investigated. The most significant finding in this study is the increased concentration of IL-8 in the spirocercosis group and especially the neoplastic group. However, despite a marked difference between the median IL-8 plasma concentration between the neoplastic and the non-neoplastic groups (634 pg/ml and 429 pg/ml, respectively), the difference was not significant, most probably due to the wide range within each group and the substantial overlap between the ranges [interquartile range

(IR), 309-1230, and 161-1277, respectively]. IL-8 is a chemoattractant for neutrophils, which in turn can also produce IL-8 (Wiinberg et al., 2005). The high concentrations of IL-8 seen in this study is, therefore, in agreement with previous studies that showed an intense neutrophilic inflammatory reaction within the nodule (Dvir et al., 2010; Dvir et al., 2011), neutrophilia (Dvir et al., 2008) and elevated serum CRP (Mukorera et al., 2011a). Interestingly, a recent publication described an increased activation of canine neutrophils with increased production of IL-8 as a response to *Wolbachia* surface protein (Bazzocchi et al., 2003) *Wolbachia* is an endosymbiont of *Dirofilaria immitis* and *Dirofilaria repens* and the study speculates that these findings can explain some of the inflammatory features of dirofilariosis in the dog. It is possible that *S. lupi* also harbours bacteria that are responsible for the inflammatory response, which may explain why the inflammatory features are different from what is expected in helminth infection (namely elevated Th2- and Treg-associated cytokines). Interestingly, *Wolbachia* was found to be associated with tumour development in a filariae called *Onchocerca volvulus* and treating the bacteria with doxycycline reduced the incidence of tumour development in this filariae (Brattig et al., 2010), emphasizing the oncogenic potential of those endobacteria. The association between up-regulated IL-8 and bacterial infection-induced cancer is well demonstrated in *Helicobacter pylori* (*H. pylori*)-induced chronic gastritis in humans, where it is proposed to play a role in the neoplastic transformation to adenocarcinoma (Wiinberg et al., 2005). IL-8 was also highly expressed in dogs infected with *Helicobacter spp* (Wiinberg et al., 2005), correlating with the intensity of the infection-associated neutrophilic infiltrate (Wiinberg et al., 2005). However, *H. pylori* infection is more associated with lymphocytic hyperplasia, where IL-8 is highly expressed in correlation with the lymphocytic infiltrate (Straubinger et al., 2003), which is the second

most common inflammatory infiltrate in spirocercosis. IL-8 was uniformly over-expressed in dogs with osteosarcoma and its expression was associated with poor outcome in paediatric osteosarcoma patients (Paoloni et al., 2009). This is of specific interest, considering the fact that osteosarcoma is the most common spirocercosis-associated tumour (Dvir et al., 2010).

Another condition, where the cytokine milieu is quite similar to this study, namely increased IL-8 and no change in IL-4 and IL-10, is gastro-oesophageal reflux disease and especially Barrett's oesophagus in humans (Jenkins et al., 2007; Rieder et al., 2010). In Barrett's oesophagus there is also further increases in IL-8 as the disease progresses to adenocarcinoma and it is found to be up-regulated by NF-κb (Jenkins et al., 2007). The possibility that spirocercosis-induced cancer can serve as a model for Barret's oesophagus-induced cancer warrants further investigation, as this is an emerging cause of oesophageal cancer in the western world. In reflux oesophageal diseases in humans, IL-8 is also secreted by fibroblasts, epithelial and endothelial cells and because it is a potent chemoattractant of neutrophils that further secrete IL-8, it creates a spiral of inflammation and damage leading to further injury (Jenkins et al., 2007). This cascade of events can also happen in spirocercosis in the dog, where fibroblasts are abundant (Dvir et al., 2010). The question remains, whether the innate response, and its potentially associated increased IL-8 expression, has a role in the neoplastic transformation.

IL-18, a typical pro-inflammatory cytokine that induces IFN-γ production and stimulates Th1 immune responses, was significantly lower in the neoplastic group compared to the non-neoplastic group. In the current study it is not clear if the reduced IL-18 is related to

spirocercosis infection, because the non-neoplastic group was not different from the control and in the neoplastic cases the worm is often not present any more (Dvir et al., 2010). However, the possibility that the low IL-18 is related to prolonged *S. lupi* infection, evasion of the host response and the neoplastic transformation cannot be excluded. Decreased IL-18 is unusual in cancer patients, but decreased Th1 cytokines such as IL-18 is commonly observed in chronic helminth infections across species. There are, however, a few reports of increased IL-18 in a number of nematodes infections such as *Trichuris muris* (Grencis, 2001) and *Trichinella spiralis* (Helmby and Grencis, 2002) and trematodes infections such as *S. mansoni* (Hogg et al., 2003) and *Schistosoma japonicum* (He et al., 2002). One mechanisms in which IL-18 plays a role in down-regulating the normal anti-helminth response is by inhibiting mast cells function (Helmby et al., 2001), but there is also contrasting evidence showing that IL-18 treatment is associated with prominent mastocytosis and increased expulsion of *Strongyloides venezuelenzis* in mice (Sasaki et al., 2005). Clinical studies, such as ours cannot establish cause and effect relationships, but indicate that the role of IL-18 in canine spirocercosis warrants further investigation.

Chronic helminth infections are often associated with increased Th2- associated cytokines, such as IL-4 and IL-10, a pattern that was also described in dogs with *Dirofilaria immitis* (Morchon et al., 2007). In *Toxocara canis* infection in the dog the production of the Th-2- and Treg-associated cytokine, IL-10, is increased while the production of the Th-1-associated cytokines, IL-18 and INF-γ are decreased (Torina et al., 2005). Such a response is often described as "immunoregulatory" or "immunosuppressive" and because it also promotes or is associated with cancer formation, our central hypothesis was that these cytokines would be increased in the *S.*

lupi cases, especially the ones with the neoplastic transformation. However, this was not the case and IL-4 and IL-10 showed very low concentrations in the *S. lupi* groups as well as the controls. We have considered the possibility that these results might be kit dependent, however, the respective kits' detection limits (1.6pg/ml) is much lower than the IL-10 concentrations that was reported in dogs in a study that was performed in our laboratory (Kjelgaard-Hansen et al., 2007).

Our study aim was also to assess if any of the investigated cytokines may serve as a biomarker for neoplastic transformation. In that respect elevated IL-8 might indicate neoplastic transformation, while high IL-18 indicated non-neoplastic transformation.

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8.7 Tables

Table 1

The different cytokines plasma concentrations (pg/ml) in the different groups

Cytokine	Detection limit (DL)	Neoplastic		Non-neoplastic		Control		P
		Median	Interquartile range	Median	Interquartile range	Median	Interquartile range	
IL-2	6.4	18	7-72	38	17-137	28	9-233	0.18
IL-4	28.8	<DL	<DL to <DL	<DL	<DL to <DL	<DL	<DL to <DL	0.65
IL-6	12.1	<DL	<DL to <DL	<DL	<DL to <DL	<DL	<DL to <DL	0.99
IL-8	20.3	634	309-945	429	161-1277	150	34-446	<0.01
IL-10	1.6	<DL	<DL to 7	<DL	<DL to 4	<DL	<DL to <DL	0.26
IL-18	4.6	33	1.6-79	53	25-156	46	24-264	0.05
MCP-1	8.6	209	100-334	163	116-377	142	101-276	0.43
GM-CSF	14.4	26	<DL to 93	42	18-145	35	17-172	0.62

9 General discussion and conclusions

The primary objective of this study was to search for biomarkers which accurately predicted neoplastic transformation in canine spirocercosis. We have adopted a broader approach to biomarkers (Mishra and Verma, 2010) and included not only circulatory or tissue biomarkers, but also other clinical diagnostic fields such as imaging. The current study revealed a few promising biomarkers including: HO, leukocytosis, thrombocytosis, anaemia, FGF and VEGF tissue expression and plasma IL-8 concentration. No optimal biomarker that has high sensitivity and specificity was found. Hypertrophic osteopathy showed 100% specificity, but only 40% sensitivity to predict neoplastic transformation in a spirocercosis-diagnosed patient. The leukocyte and platelet count, haematocrit, growth factors tissue expression and the cytokine circulatory concentrations showed substantial overlap between non-neoplastic and neoplastic cases. The inability to find an optimal biomarker is a common finding in most studies of this kind (Chatterjee and Zetter, 2005; Polanski and Anderson, 2007) and it is probably due to the fact that neoplastic transformation is a continuous process. A practical solution for this problem is to screen for a particular patient by using a panel of biomarkers and to increase the index of suspicion, which together can serve as a predictor for such transformation. The current study also laid the basis for other studies that search for biomarkers of the neoplastic transformation in spirocercosis. The increase in the lesional expression of VEGF lead to investigating its circulatory concentration in spirocercosis and revealed significantly higher concentrations in neoplastic cases compared to non-neoplastic cases with a minimum overlap (Mukorera et al., 2011b), indicating that it might be a promising practical diagnostic marker as well as a therapeutic target. A similar study examining the use of circulatory FGF is also warranted. The increase in a number of pro-inflammatory

cytokines lead to investigation of serum CRP in spirocercosis and showed great potential to monitor response to therapy and to replace the need for follow up endoscopy, because the serum concentration dropped dramatically after initiation of treatment (Mukorera et al., 2011a).

The second and scientifically more intriguing objective of this study was to use the identified biomarkers for deeper understanding of the pathogenesis of the *S. lupi*-induced sarcoma. Spirocercosis produces three lesions that involve uncontrolled mesenchymal proliferation namely HO, spondylitis and sarcoma. The current theory about the pathogenesis of HO (or the early human form called digital clubbing) is that thoracic masses facilitate the blood shunting away from the and the vagal stimulation enhance the blood supply. The shunted blood contains more megakaryocytes and platelets that contain and release humoral factors at the periosteum (Atkinson and Fox, 2004). The humoral factors that were proposed as being responsible for the periosteal reaction are VEGF and PDGF and to a lesser degree FGF, because their expression was increased in the diseased tissue, using immunohistochemistry (Atkinson and Fox, 2004). Consequently, we have examined the expression of these growth factors in the *S. lupi*-induced nodule and VEGF and FGF showed significantly higher expression in the neoplastic nodules compared to the non-neoplastic ones. These growth factors are common angiogenic factors (Craft and Harris, 1994) and our study could not differentiate whether these factors were a secondary reaction to the neoplastic changes or primary factors in its induction. However, it illustrate that the *S. lupi* has a unique ability to induce massive secretion of growth factors and to perpetuate mesenchymal proliferation. The major question is whether the

worm secretes a product that stimulates such a reaction or it diverts the inflammatory reaction to stimulate the mesenchymal proliferation and carcinogenesis.

When we examined the potential role of the immune system in the neoplastic transformation, our central hypothesis was that the parasite produces excretory product(s) which diverts the immune response from a T helper 1 (Th1) to Th2 cell response, typical of many nematode infections (Maizels et al., 2009), and further to an immunoregulatory (immunosuppressive), FoxP3+ regulatory T cell- predominated response (Maizels, 2009) which then facilitates neoplastic transformation (Beyer and Schultze, 2006). This immune response is well-described not only secondary to helminth infection but also as proneoplastic across species including humans (Carreras et al., 2006; Unitt et al., 2005; Xue et al., 2009), murine models (Imai et al., 2007), including models of fibrosarcoma (Betts et al., 2007) and dogs (Curiel et al., 2004; Heimberger et al., 2008; Liyanage et al., 2002; Wolf et al., 2003; Woo et al., 2001). We have tested this hypothesis by characterizing the nodule inflammatory infiltrate using immunohistochemistry labelling of MAC387 for myeloid cells, CD3 for T cells, Pax5 for B cells and FoxP3 for Tregs and by measuring the plasma concentrations of several cytokines including IL-2, IL-4, IL-6, IL-8, IL-10, IL-18, GM-CSF and MCP-1.

The immunohistochenistry study indicated an intensive innate (neutrophilic) response within the *S. lupi* nodule. These neutrophils formed pockets of pus around the worm, or they were confined to necro-ulcerative areas in the neoplastic nodules. Alternatively, neutrophils occurred diffusely throughout the nodules. The lymphocytic infiltrates had a prominent focal/multifocal distribution pattern (compared to the myeloid cells) and they were usually peripherally located within nodules. However, in the majority of cases, lymphocytes occurred in a mixed pattern; namely focal/multifocal and diffuse. This

innate response is not only observed locally, but also systemically in the form of high neutrophil count on haematology and elevated CRP on serum biochemistry. Regarding the nodular lymphocyte infiltrate, T cells outnumbered B cells and Tregs were very rare. Foxp3+ cells were found in large numbers only within CD3+ regions of lymph nodes. The measurement of a range of plasma cytokine revealed a pro-inflammatory response that was characterized by increased IL-8 and IL-18 concentrations. This unexpected inflammatory response can potentially indicate a symbiotic bacterium that induces a more typical antibacterial pro-inflammatory response, and possibly, a bigger role of the innate response in the carcinogenesis. Interestingly, a recent publication described an increased activation of canine neutrophils with increased production of IL-8 as a response to *Wolbachia* surface protein (Bazzocchi et al., 2003). *Wolbachia* is an endosymbiont of *Dirophilaria immitis* and *repens* and the study speculates that these findings can explain some of the inflammatory features of dirophilariasis in the dog. It is possible that *S. lupi* also harbours bacteria that are responsible for the inflammatory response, which may explain why the inflammatory features are different from what is expected in helminth infection (namely elevated Th2- and Treg-associated cytokines). Similarly, *Wolbachia* was found to be associated with tumour development in a filariae called *Onchocera volvulus* (Brattig et al., 2010), emphasizing the oncogenic potential of those endobacteria.

Neutrophils has a major role in the anti-tumour immune response by initiating cytotoxic response and by direct destruction of tumours (Di Carlo et al., 2001). The question is whether they have a similar role in spirocercosis and if so, how does the *Spirocerca*-induced neoplasia escape this immune mechanism? On the other hand, they can have an important role in cancer induction by generating proteases, free radical and nitrogen species that can cause oxidative damage to the DNA (Vennervald and Polman, 2009).

More recently neutrophils have been shown to play a pivotal role in the regulation of the inflammatory response against cancer (Mattarollo and Smyth). For instance, neutrophils can be induced by serum amyloid A (SAA)1 to secrete IL-10 which induces suppression of immune surveillance (De Santo et al., 2010). As the neutrophilic response is as intensive in the non-neoplastic phase, it might be involved in promoting the neoplastic transformation, a question that warrants further studies. It is also possible that the neutrophilic response is functionally impaired in spirocercosis and as such self-perpetuating, causing more oxidative damage that is known to be tumourogenic. The pro-inflammatory response was also associated with few inflammatory-associated neoplastic conditions. A pro-inflammatory but ineffective reaction T-cell (Th1) response is well described in *Helicobacter pylori*-induced adenocarcinoma, where it is suspected to play a pivotal role in the neoplastic transformation (Straubinger et al., 2003). In this condition, up-regulated IL-8 is proposed to play a role in the neoplastic transformation (Wiinberg et al., 2005). Increased serum IL-8 concentrations were also correlated with tumour progression in humans with oesophageal squamous cell carcinoma (Diakowska et al., 2006; Krzystek-Korpacka et al., 2008). It was also detected in Barret's oesophagus (Fitzgerald et al., 2002), a human condition that involves oesophageal inflammation due to reflux, epithelial dysplasia and metaplasia and eventually neoplastic transformation. The expression of IL-8 increases as the diseases progresses to cancer (Oh et al., 2007). Future studies are warranted to fully understand the functional aspects of the lymphocytic response in spirocercosis and its role in the neoplastic transformation.

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11 APPENDICES

11.1 List of journal publications of work directly related to this thesis

1. Dvir E, Kjelgaard-Hansen M, Mellanby RJ, Schoeman JP. Plasma IL-8 concentrations are increased in dogs with spirocercosis. Accepted to Vet Parasitol.
2. Dvir E, Schoeman JP, Clift SJ, McNeilly TN, Mellanby RJ. Immunohistochemical characterization of lymphocyte and myeloid cell infiltrates in spirocercosis-induced esophageal nodules. Parasite Immunol. 2011. 33:545-553.
3. Dvir E, Clift SJ, Evaluation of selected growth factor expression in canine spirocercosis *(Spirocerca lupi)*-associated non-neoplastic nodules and sarcomas Vet. Parasitol 2010. 174:257–266.
4. Dvir E, Clift SJ, Williams MC. Proposed histological progression of the Spirocerca lupi-induced oesophageal lesion in dogs. Vet Parasitol 2010. 168:71-77.
5. Dvir E, Kirberger RM, Mukorera V, van der Merwe LL, Clift SJ. Clinical differentiation between dogs with benign and malignant spirocercosis. Vet Parasitol 2008. 155: 80-88.

11.2 List of journal publications of work in the same study, but not directly related to this thesis

1. Kirberger RM, Dvir E, van der Merwe L. Canine pneumo-esophagraphy and the appearance of caudal esophageal masses secondary to Spirocercosis. J of the Am Vet Med Assoc. 2012. 240:420-426.
2. Mukorera V, Dvir E, van der Merwe LL, Goddard, A. Serum c-reactive protein concentration in benign and malignant canine spirocercosis. J. Vet. Int. Med. 2011. 25:963-966.
3. Mukorera V, van der Merwe LL, Lavy E, Aroch I, Dvir E. Serum alkaline phosphatase activity is not a marker for neoplastic transformatiom of esophageal nodules in canine spirocercosis. Vet. Clin. Path. 2011. 40:389-392.
4. Dvir E, Kirberger RM, Clift SJ, van der Merwe LL. Review: challenges in diagnosis and treatment of canine spirocercosis. Israel J of Vet Med 2010. 1:5-10.
5. Kirberger RM, Dvir E, van der Merwe LL. The effect of positioning on the radiographic appearance of caudodorsal mediastinal masses in the dog. Vet Radiol Ultrasound 2009. 50:630-634.
6. Dvir E, Perl S, Loeb E, Shklar-Hirsch S, Chai O, Mazaki-Tovi M, Aroch I, Shamir MH. Spinal intramedullary aberrant Spirocerca lupi migration in 3 dogs. J Vet Intern Med 2007. 21:860-864.
7. Dvir E, Kirberger RM, Malleczeck D. Radiographic and computed tomographic changes and clinical presentation of spirocercosis in the dog. Vet Radiol & Ultrasound 2001. 42:119-129.

11.3 List of conference presentations directly related to this thesis

11.3.1 Keynote addresses

1. Dvir, E. Pathological changes with nodule progression. 30th World Veterinary Congress, October 2011, Cape Town, South Africa
2. Dvir, E. Neoplastic transformation in spirocercosis - new research. The 30th World Veterinary Congress, October 2011, Cape Town, South Africa
3. Mukorera, V, **Dvir E.** Benign vs malignant spirocercosis. The 30th World Veterinary Congress, October 2011, Cape Town, South Africa
4. Dvir E. Update on spirocercosis-induced oesophageal sarcoma and *spirocerca lupi* aberrant migration. The 5th South African Veterinary Association (SAVA) Congress, August 2010, Drakensberg, South Africa.
5. Dvir E, Clift SJ. Update on spirocercosis-induced oesophageal sarcoma and *spirocerca lupi* aberrant migration. The 19th Congress of the European College of Veterinary Internal Medicine Companion Animals (ECVIM-CA), September 2009, Porto, Portugal.
6. Dvir E, Kirberger RM, Clift SJ. Spirocercosis-induced oesophageal sarcoma and its clinical complications. The 4th South African Veterinary Association (SAVA) Congress, July 2008, Sun City, South Africa.
7. Williams MC, Clift SJ, **Dvir E**. The oesophageal nodule in canine spirocercosis – a fascinating phenomenon. The 4th South African Veterinary Association (SAVA) Congress, July 2008, Sun City, South Africa

11.3.2 Research abstracts

1. Dvir E, Mellanby RJ, van der Merwe LL, Kjelgaard-Hansen M, Schoeman JP. Differences in the plasma cytokine milieu between dogs with benign and malignant spirocercosis. The 21th Congress of the European College of Veterinary Internal Medicine Companion Animals (ECVIM-CA), September 2011, Seville, Spain.
2. Dvir E, Schoeman JP, McNeilly TN, Clift SJ, Mellanby RJ. Characterisation of regulatory t cell, t and b lymphocyte and myeloid cell infiltrates in spirocercosis-induced oesophageal nodules. The 20th Congress of the European College of Veterinary Internal Medicine Companion Animals (ECVIM-CA), September 2010, Toulouse, France.
3. Dvir E, Clift SJ. Selected growth factor expression in *Spirocerca lupi* esophageal nodules. Congress of the American College of Veterinary Internal Medicine (ACVIM), June 2010, Anaheim, California.
4. Dvir E, Clift SJ. The spirocercosis-induced oesophageal nodule: progression from inflammation to sarcoma. The 19th Congress of the European College of Veterinary Internal Medicine Companion Animals (ECVIM-CA), September 2009, Porto, Portugal.
5. Dvir E, Kirberger RM, Mokarera V, van der Merwe LL, Clift SJ. Clinicopathological differences between dogs with benign and malignant spirocercosis-induced oesophageal nodules. The 17th Congress of the European College of Veterinary Internal Medicine Companion Animals (ECVIM-CA), September 2007, Budapest, Hungary