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.