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

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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.

8.6 Acknowledgement

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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.