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

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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?

## *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

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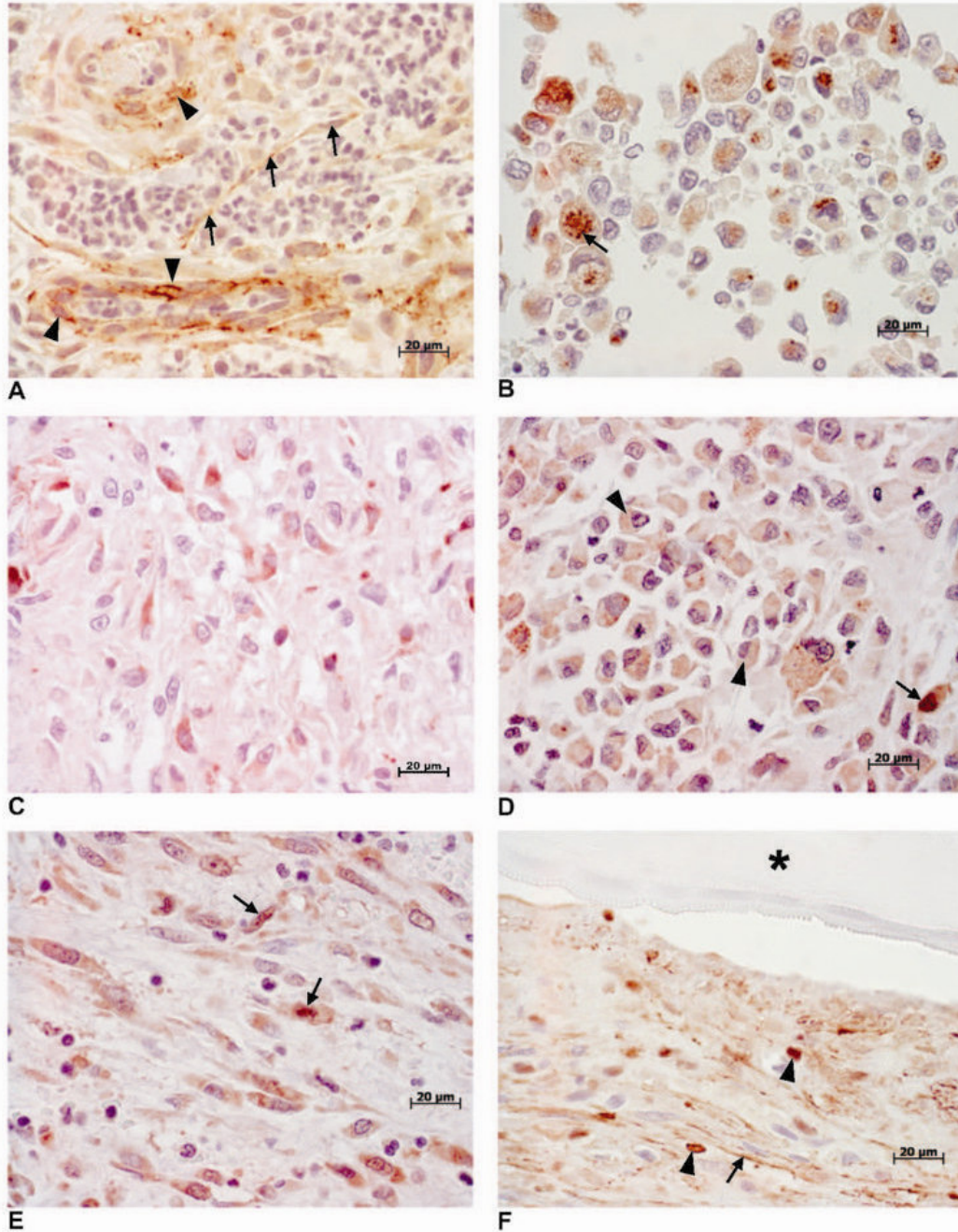
A. Positive control; granulation tissue in a dog. VEGF-specific granular cytoplasmic immunolabelling of microvascular endothelial cells (arrowheads) and a fibroblast (arrows). Streptavidin-peroxidase complex method with NovaRED as the chromogen and Mayer's haematoxylin counterstain.

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.
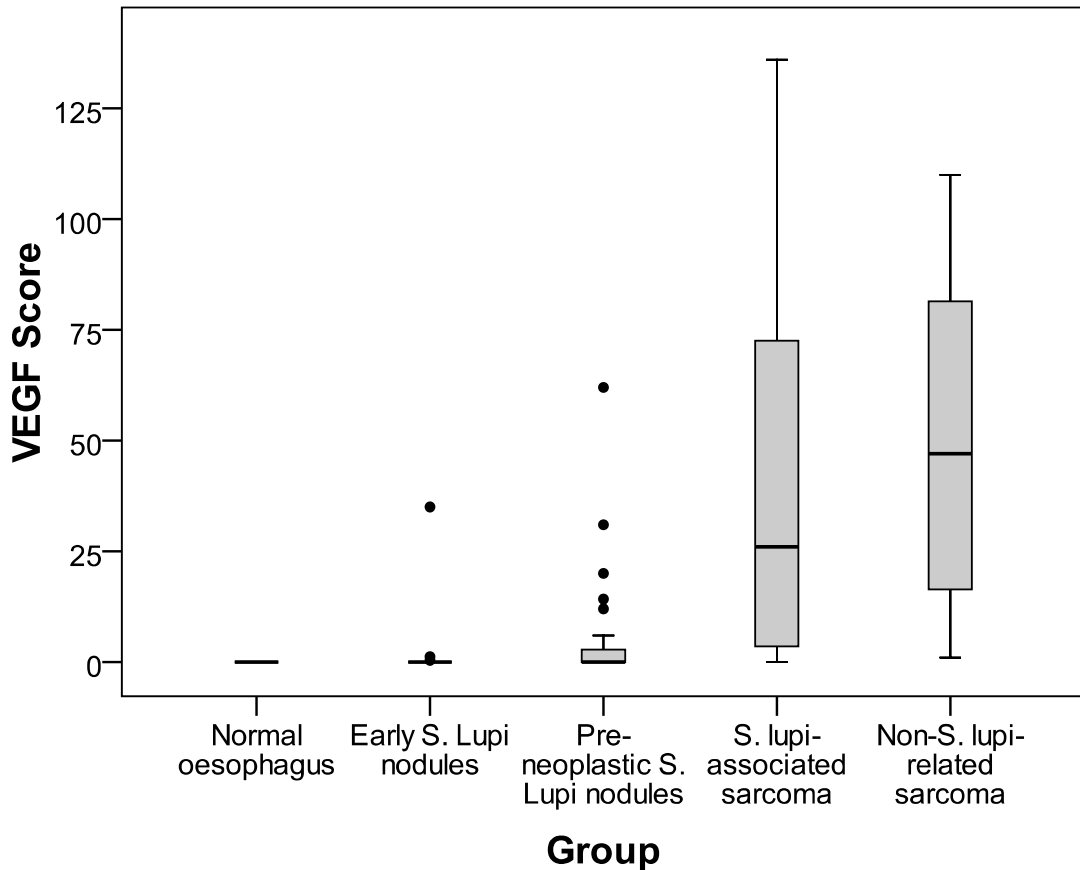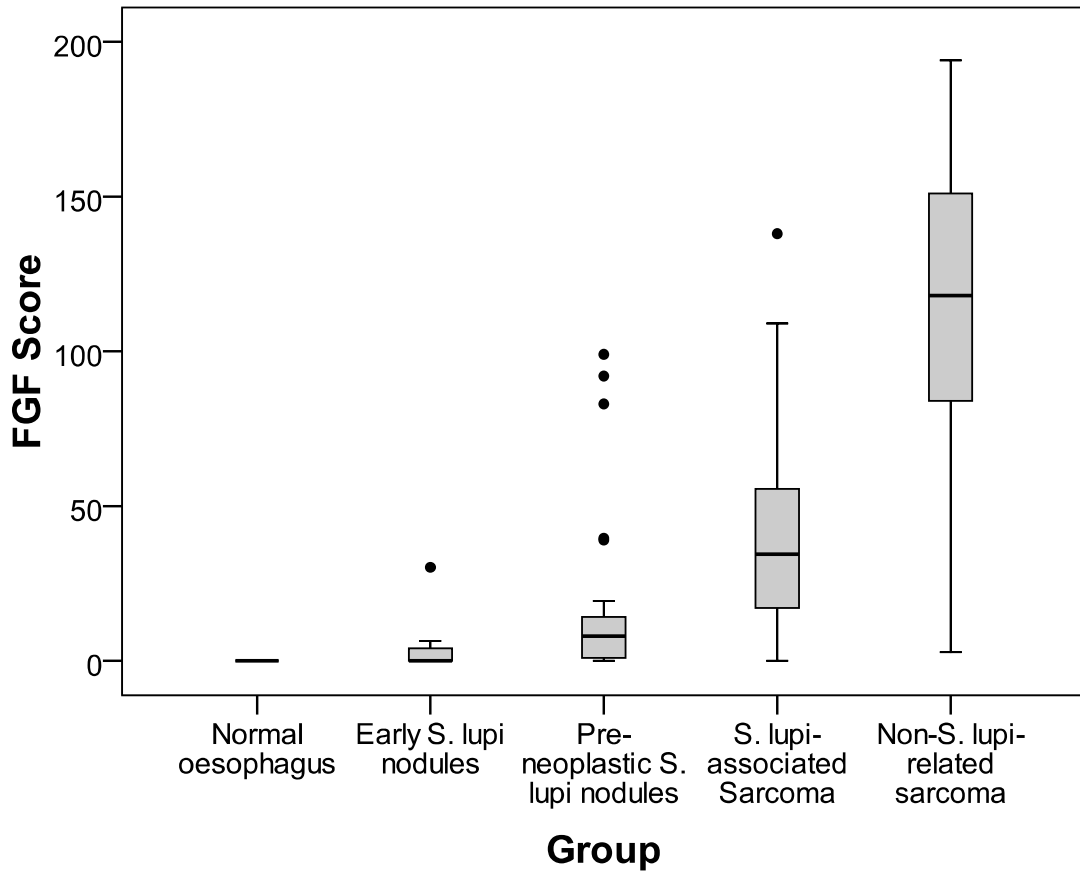
Figure 2: Box Plot of the VEGF expression score in the different groups. The overall comparison between the groups was statistically significant (p<0.001). All groups, except the early nodules, exhibited significantly higher VEGF expression compared to the control group (p<0.05). Both sarcoma groups 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.

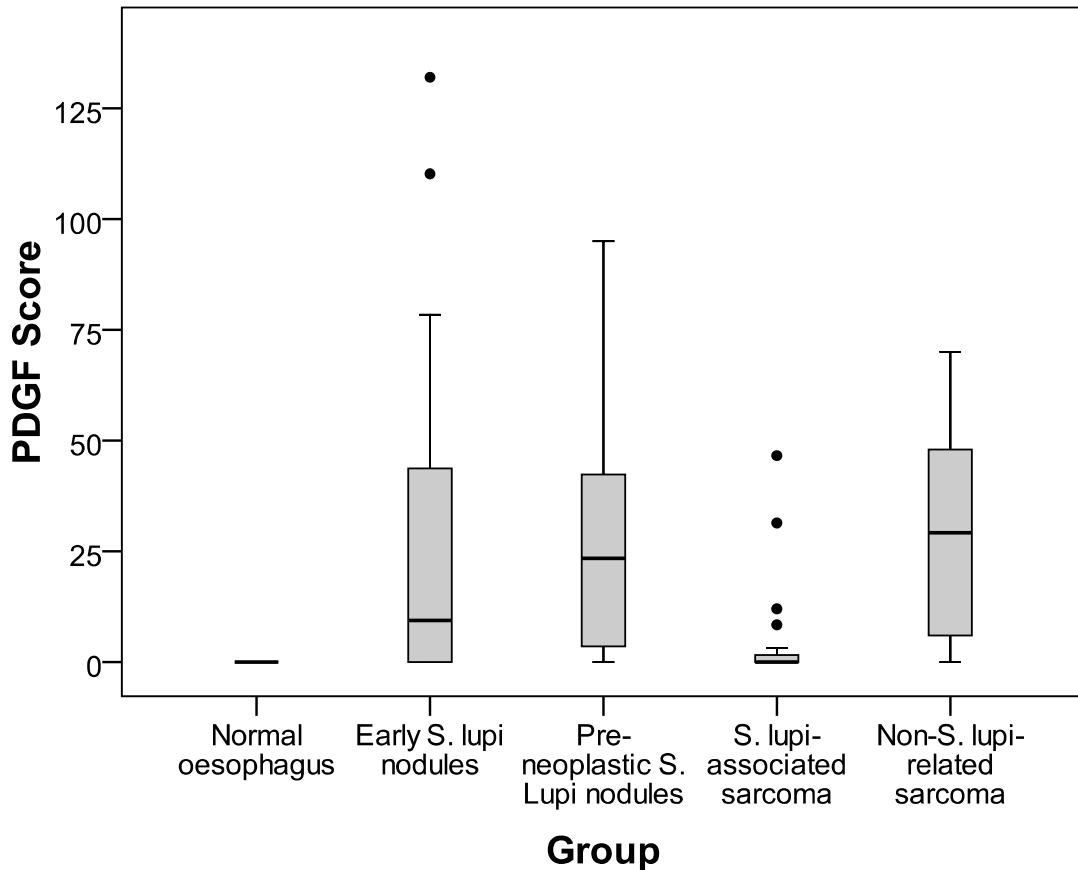true for comparisons between the individual groups ($p < 0.05$).

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.
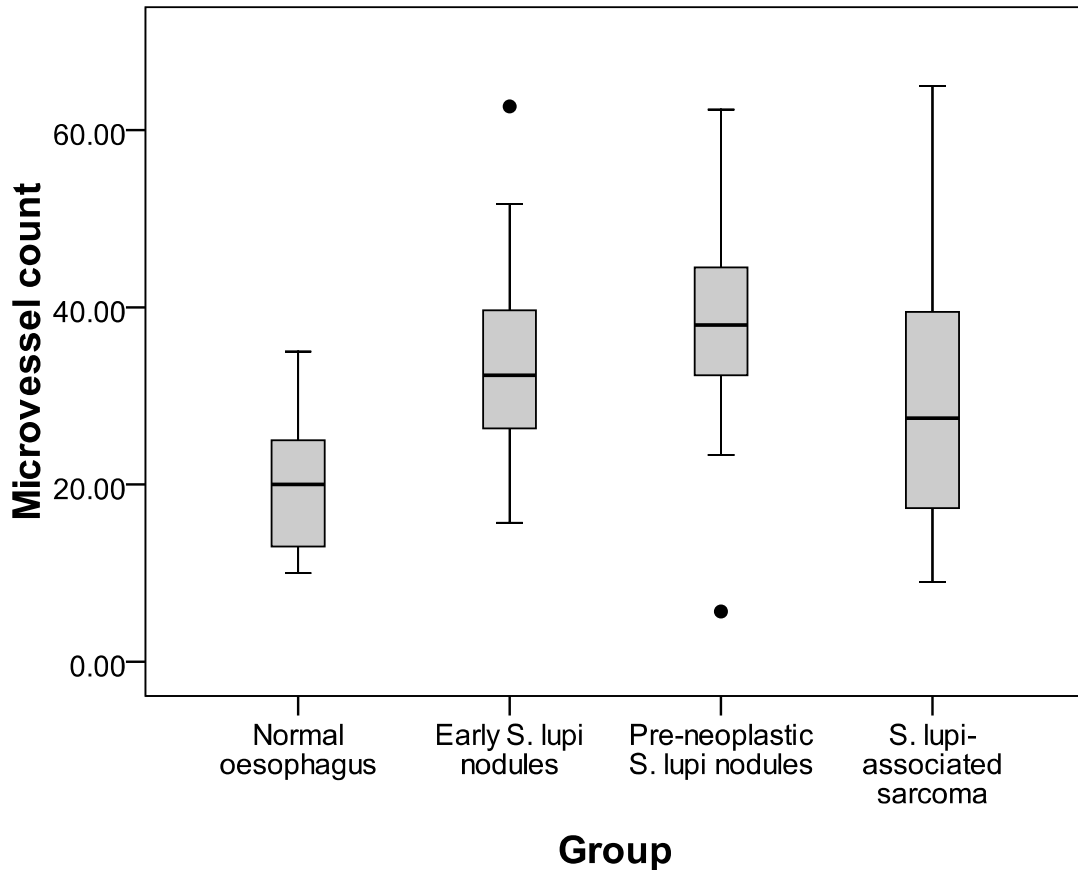
Figure 5: Box Plot of the mean microvessel count per high power field at the periphery of the nodules. The overall comparison between the groups was statistically significant (p=0.002). The pre-neoplastic oesophageal *S. lupi* nodule and the early non-neoplastic *S. lupi* oesophageal nodule groups exhibited 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.
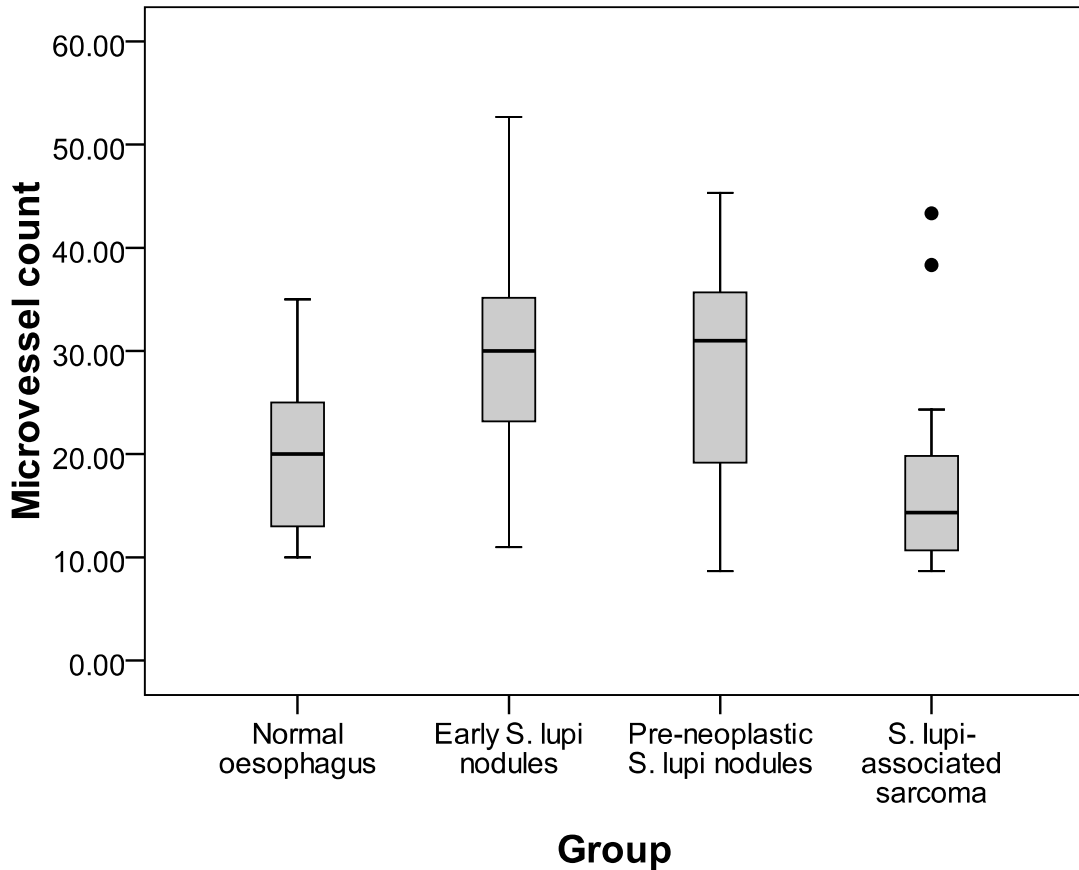
Figure 6: Box Plot of the mean microvessel count per high power field at the centre of the nodules. The overall comparison between the groups was statistically significant (p<0.001). The pre-neoplastic oesophageal *S. lupi* nodule and the early non-neoplastic *S. lupi* oesophageal nodule groups exhibited 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.