The role of vitamin D in dogs with *Spirocerca lupi*-associated neoplastic transformation

By

Chantal Teixeira Rosa

Submitted to the Faculty of Veterinary Science, University of Pretoria in partial fulfilment of the requirements for the MMedVet(Med) degree

Pretoria, November 2013

DISSERTATION

The role of vitamin D in dogs with *Spirocerca lupi*-associated neoplastic transformation

Chantal Teixeira Rosa, DVM, BVSc(Hons)

Research conducted in the Section of Small Animal Medicine
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria

Supervisor:
Prof. E Dvir, DVM, BVSc(Hons), MMedVet(Med), PhD, Dip. ECVIM-CA
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria

Co-supervisor:
Prof. Prof JP Schoeman, BVSc, MMedVet(Med), PhD, DSAM, Dip. ECVIM-CA
Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria

DECLARATION

I, Chantal Teixeira Rosa, hereby declare that this dissertation, submitted for the MMedVet(Med) degree, to the University of Pretoria, is original and that neither the whole work or any part of it has been, or is being or is to be submitted for another degree at this or other University, Tertiary Education Institution or Examining Body.

Chantal Teixeira Rosa

May 2014

TABLE OF CONTENTS

SUMMARY

The role of vitamin D in dogs with *Spirocerca lupi*-associated neoplastic transformation Rosa CT. University of Pretoria, 2013

Spirocercosis in dogs is characterized by oesophageal nodules that readily undergo neoplastic transformation, but the pathogenesis of this neoplastic transformation is poorly understood. Vitamin D has putative anti-proliferative, pro-differentiating, immunomodulatory and anti-neoplastic effects through gene modulation. Hypovitaminosis D is associated with the development of many human neoplasias.

The objective of this study was to measure and compare vitamin D status assessed by serum 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] concentrations in non-neoplastic (n=25) and neoplastic (n=26) spirocercosis dogs and healthy dogs (n=24). We hypothesized that hypovitaminosis D was present in *Spirocerca lupi*-infected dogs; that lower concentrations were present in the neoplastic form of the disease; and finally that hypovitaminosis D could be a potential risk factor for neoplastic transformation.

From 119 dogs presenting to our hospital over a three year period, 51 dogs were selected and further divided into a non-neoplastic or a neoplastic group, based on histopathology of the nodules, macroscopic endoscopic appearance, computed tomography findings, response to therapy, and/or a combination of these findings. Dogs were excluded if they were less than 1 year of age, had concurrent diseases, had received corticosteroids in the previous 6 months or if they were treated prophylactically for spirocercosis. Serum 25(OH)D and 1,25-dihydroxyvitamin D (1,25$(OH)_2$D) concentrations were measured by high-performance liquid chromatography. Spirocercosis dogs' appetites were recorded as normal or abnormal (inappetence or anorexia) and the absolute number of dogs with normal and abnormal appetites were compared between the spirocercosis groups and to the 25(OH)D concentrations. The influence of age and serum albumin on serum 25(OH)D concentrations were also evaluated. The interaction and significance of sex and spay neuter status, body weight and appetite (independent variables) with serum 25(OH)D concentrations (dependent variable) were assessed. Statistical significance was set at $p<0.05$. Serum 25(OH)D and 1,25$(OH)_2$D concentrations were significantly different among all groups (Kruskal-Wallis test, $p<0.001$). Dunn's multiple comparison test showed that median 25(OH)D concentrations were significantly lower in the neoplastic

group [30.7 nmol/l (range 14.7-62.2)] compared to the non-neoplastic [52.7 nmol/l (range 19.1-129.7, ($p<0.05$)] and the healthy groups [74.6 nmol/l (range 37.4-130.5, $p<0.001$)]. Median 25(OH)D concentrations were significantly lower in the non-neoplastic spirocercosis group compared to the healthy group ($p<0.05$). Dunn's multiple comparison test also showed that median $1,25(OH)_2D$ concentrations were significantly lower in the neoplastic group (56.5 pmol/l (range 18-130)] compared to the healthy group [94.5 pmol/l (range 46-142, $p<0.001$)]. No significant difference in the median $1,25(OH)_2D$ concentrations were detected between the neoplastic and the non-neoplastic spirocercosis [70 pmol/l (range 34-127), $p>0.05$] groups and between the non-neoplastic spirocercosis group compared to the healthy group ($p>0.05$). Neoplastic and non-neoplastic spirocercosis dogs had similar appetite classification (Fisher´s Exact test, $p=1.0$). Mann-Whitney U test showed that in the neoplastic spirocercosis groups the median 25(OH)D ($p=0.087$) and $1,25(OH)_2D$ ($p=0.94$) concentrations were not significantly different between dogs with normal and abnormal appetites. In the non-neoplastic spirocercosis groups, the median 25(OH)D ($p=0.125$) and $1,25(OH)_2D$ ($p=0.08$) concentrations were also not significantly different between dogs with normal and abnormal appetites. Analysis of covariance (ANCOVA) demonstrated a significant difference in serum 25(OH)D concentrations among the three groups of dogs independent of either albumin or age ($p<0.05$). No statistical significance was detected for breed ($p=0.84$), sex ($p=0.32$) and spay neuter status ($p=0.58$) among the three groups, using the Chi-square test. Median body weight compared among the groups was not statistically significant (Kruskal-Wallis test, $p=0.27$). Multivariable linear regression analysis demonstrated that all independent variables (sex and spay neuter status, body weight, appetite) were independent of each other, and that there was no linear relationship between serum 25(OH)D concentration and either body weight ($p=0.08$, $r=0.24$) nor appetite ($p=0.16$, $r=-0.49$). Neutered and female dogs had higher serum 25(OH)D concentrations than intact and male dogs.

Hypovitaminosis D is present in dogs with spirocercosis and is lower in the neoplastic dogs compared to non-neoplastic spirocercosis and healthy dogs. Further studies are warranted to determine the potential therapeutic use of vitamin D in spirocercosis and explore the role of hypovitaminosis D in the pathogenesis of malignant transformation. Additional studies focusing on the calcium-regulatory role of vitamin D is also warranted.

LIST OF ABBREVIATIONS

1α(OH)$_{ase}$	1α-hydroxylase
1,25(OH)$_2$D	1,25-dihydroxyvitamin D or calcitriol
24(OH)ase	24-hydroxylase
25(OH)ase	25-hydroxylase
25(OH)D	25-hydroxyvitamin D or calcidiol
ALP	Alkaline phosphatase
ALT	Alanine transaminase
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
CBC	Complete blood count
CRP	C-reactive protein
CT	Computed Tomography
DBP	Vitamin D-binding protein
DEQAS	Vitamin D external quality assessment scheme
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FGF$_{23}$	Fibroblast growth factor 23
HO	Hypertrophic osteopathy
HPLC	High-performance liquid chromatography
IBD	Inflammatory bowel disease
IFA	Indirect fluorescent antibody
IQR	Interquartile range
ISO	International Organization for Standardization
OVAH	Onderstepoort Veterinary Academic Hospital
PTH	Parathyroid hormone
RI	Reference interval
RIA	Radioimmunoassay
RTKs	Receptor tyrosine kinases
S. lupi	*Spirocerca lupi*
TEG	Thromboelastography
VDR	Vitamin D receptor
Vitamin D$_2$	Ergocalciferol (plant origin)

Vitamin D_3 Cholecalciferol (animal origin)

VEGF Vascular endothelial growth factor

LIST OF FIGURES

represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

Figure 6 Box plot of the comparison between the serum 1,25$(OH)_2$D concentrations of dogs with normal or abnormal appetite in the non-neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

Figure 7 Box plot of the comparison between the serum 25(OH)D concentrations of dogs with normal or abnormal appetite in the neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

Figure 8 Box plot of the comparison between the serum 1,25$(OH)_2$D concentrations of dogs with normal or abnormal appetite in the neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to thank the following people:
Eran Dvir, my supervisor and mentor, for his guidance, wisdom and support though this project and my 3 years of residency in small animal medicine.

Prof. Johan Schoeman, my co-supervisor and ECVIM-CA supervisor, for his mentorship and meticulous assistance with the editing of the manuscript and dissertation, and who's experience made a valuable contribution to this project.

Dr Richard Mellanby, my co-worker, for providing objective and important insights into the research project. His knowledge in the importance of vitamin D in inflammatory and neoplastic diseases was fundamental.

Dr Jacqueline Berry for her technical support and expertise in measuring the serum vitamin D.

The Faculty of Veterinary Science, the Department of Companion Animal Clinical Studies, Duncan Campbell Memorial Fund of the South African Veterinary Foundation (SAVF) and the *Spirocerca lupi* research fund/project for financial and technical support that made this research possible.

All owners of the healthy dogs used as controls in this study.

All my friends in Portugal and South Africa, for all their support and encouragement.

My amazing parents, sister and grandparents, for their enduring love, support and belief in me.

CHAPTER 1: Literature review

1.1. Life cycle of *Spirocerca lupi*

Spirocerca lupi (*S. lupi*) is a nematode infecting mainly dogs, with high prevalence in South Africa[1]. The *S. lupi* adult worm resides in a nodule within the oesophageal wall, from which the females shed small embryonated eggs into the oesophageal lumen, that are excreted in the faeces of the host. The eggs are ingested by an intermediate host (coprophagus beetles) and they develop to an infective larva (L3 stage) within 2 months. The beetles can be ingested by parathenic hosts such as lizards, hedgehogs, mice, rabbits and birds. The dog becomes infected when it ingests infected intermediate or paratenic hosts. The L3 penetrates the gastric mucosa of the final host and migrates through the gastric arteries and abdominal aorta wall to the caudal thoracic aorta and into the oesophagus. In the oesophagus, the larva forms a fibroblastic nodule and matures to the adult form[1-7]. The oesophageal nodules enlarge and can undergo neoplastic transformation into an osteosarcoma, a fibrosarcoma or an undifferentiated sarcoma[1,5,8-9]. The underlying pathogenesis of this neoplastic transformation remains poorly understood[2,5,7,10].

The identification and differentiation of neoplastic and non-neoplastic spirocercosis cases is fundamental as it has major therapeutic and prognostic implications. Non-neoplastic spirocercosis can be successfully treated with doramectin[11], milbemycin[12] or imidacloprid and moxidectin spot-on and generally carries a good prognosis, while neoplastic spirocercosis requires surgical resection of the neoplasia followed by chemotherapy[1].

Spirocercosis seems a good model to study helminth-induced neoplasia and factors associated with neoplastic transformation, based on the presence of two groups of dogs with the same disease, presenting with identical clinical signs, differing only by the neoplastic stage of disease progression and the high prevalence (25%) of neoplastic transformation[7].

1.2. Clinical signs in dogs with spirocercosis

Clinical signs in spirocercosis relate to the presence of oesophageal nodules, normal and aberrant larvae migrations and possible complications[1]. The most common signs are regurgitation and/or vomiting, weight loss and pyrexia[13]. Respiratory signs are commonly associated with bronchial compression, tracheal or bronchial displacement, mediastinum

or pleural effusion, aspiration pneumonia and pyothorax[1,3-5,13-14]. Dysphagia and hypersalivation can occur due to sialoadenitis[1,3-5,14]. Aortic aneurisms can lead to sudden death if they rupture[1,4,10,13]. Paresis and paralysis can occur secondary to abnormal worm migrations through the spinal cord[1,3], extramedullary spinal migration[15] or thromboembolism[13-14]. Inflammation of the sites of migration occurs resulting in mediastinitis, pneumomediastinum, pleuritis, pyothorax and/or potentially spondylytis of the caudal thoracic vertebra[1,4,13,16]. Lameness secondary to hypertrophic osteopathy (HO) in neoplastic spirocercosis and polyarthritis in both forms of spirocercosis can also be seen[1,4,13].

1.3. Diagnosis of spirocercosis in the dog

Diagnostic modalities are usually complementary in the final diagnosis of spirocercosis and can be made through one or a combination of the following techniques:

1.3.1. Faecal evaluation

A definitive diagnosis of spirocercosis can be made based on the detection of larvated worm eggs on faecal evaluation[1,17-18], but the diagnosis by faecal evaluation has shown to have a sensitivity of only 67%[19]. The results of faecal analysis for spirocercosis diagnosis can be influenced by several factors such as: intermittent egg shedding, very small sized eggs that can be missed and required diagnostic expertise[1-2,6].

1.3.2. Serology

An indirect fluorescent antibody (IFA) test for *S. lupi* has been described in a single Turkish study with 100% sensitivity and 80% specificity[1], however to date this study has not materialised in an available diagnostic kit.

1.3.3. Radiography

Thoracic radiographs have up to 86% sensitivity in diagnosing spirocercosis[13]. The three complementary thoracic radiographic signs for spirocercosis diagnosis include: a soft tissue opacity mass in the caudodorsal mediastinum, thoracic vertebral spondylitis in the region of T6-T12 (pathognomonic sign), and undulation of the left lateral border of the proximal descending aorta due to aneurysm formation on a dorsoventral thoracic radiograph view[1-2,4,13,20]. Radiography however cannot differentiate neoplastic from non-neoplastic spirocercosis.

1.3.4. Computer Tomography

Computer Tomography (CT) has been used to evaluate the exact size and shape of *S. lupi* masses for surgery planning purpose, early detection of metastasis and other complications arising from the larva migration[1,13].

1.3.5. Oesophageal endoscopy

Endoscopy is the gold standard technique to evaluate the oesophageal mucosa, with direct visualization of the nodules[5,21]. Non-neoplastic nodules tend to be small, smooth and can have a pink nipple-like protuberance, while the neoplastic ones are cauliflower-like, can obscure the entire oesophageal lumen, and are often ulcerated and necrotic[1-3,7,10,13]. Mediastinum masses, aberrant migration and early manifestation will, however, be missed with this method.

1.4. Diagnosis of neoplastic transformation in spirocercosis

Confirming neoplastic transformation in canine spirocercosis can be challenging and requires invasive or expensive diagnostic tools. The endoscopic appearance of the nodules[1,5]; poor treatment response[11]; presence of HO[13]; anaemia due to nodule ulceration and blood loss, leucocytosis, thrombocytosis, hyperglobulinaemia and hypoalbuminaemia[1-2,10,13] have been suggested as indicators of neoplastic transformation, yet their sensitivity is poor.

Endoscopic appearance of the nodules generally correlates well with either non-neoplastic or neoplastic status[5,21], but it remains a subjective tool where few smooth malignant masses and few ulcerated and necrotic non-malignant masses have been misdiagnosed[10]. Response to therapy can be used to determine if neoplastic transformation has occurred, with regression of non-neoplastic nodules and neoplastic ones remaining unchanged[11], but this is time consuming and may not be indicated in compromised patients. The presence of HO is associated with neoplastic transformation, occurring in only 38.7% of neoplastic spirocercosis cases[13]. Although HO has 100% specificity, its poor sensitivity (38.7%) makes it an insensitive marker for neoplastic transformation[13]. A microcytic hypochromic anaemia, leucocytosis, thrombocytosis, hyperglobulinaemia and hypoalbuminaemia have been associated with the neoplastic form of the disease, but a great overlap between neoplastic and non-neoplastic spirocercosis cases is seen[1-2,10,13]. These findings are thought to result from continual oesophageal injury and blood loss from the neoplasm, associated with chronic disease or a paraneoplastic syndrome[4].

Serum alkaline phosphatase (ALP)[22] and C-reactive protein (CRP)[23] concentrations have also been evaluated in spirocercosis, with ALP being unable to differentiate neoplastic from non-neoplastic spirocercosis[19,22], but CRP being useful in the differentiation and as a monitoring tool for evaluation of treatment response[23]. Plasma and serum vascular endothelial growth factor (VEGF) concentrations have also been shown to potentially differentiate between neoplastic and non-neoplastic spirocercosis, with higher concentrations in the neoplastic form[24]. False positive results can be obtained, because VEGF can be produced by other concurrent neoplasias[25] or be induced by systemic hypoxia resulting from chronic anaemia[26]. Thromboelastography (TEG) can be useful in the differentiation of neoplastic from non-neoplastic spirocercosis, with a more hypercoagulable state being associated with the neoplastic transformation (96% specificity for neoplastic transformation when maximum amplitude of the TEG >76mm)[27]. Major limitations of TEG for the diagnosis of neoplastic spirocercosis relate to the fact that other diseases can also result in hypercoagulability, thus TEG results should not be viewed in isolation, and the poor sensitivity of the test (73%) may result in false negative results for neoplastic transformation[27].

Histopathology remains the gold standard for the definitive diagnosis of neoplastic transformation[7]. Endoscopic-guided biopsies can confirm the diagnosis of neoplastic spirocercosis, but its sensitivity can be compromised by a non-representative sample, especially when necrotic or non-diagnostic material from the tumour periphery is obtained[1-5,7,21]. Histopathology of the entire nodule/mass removed surgically or on post-mortem remains the best sample for histopathology.

In light of the difficulties and expenses involved in the ante-mortem diagnosis of neoplastic spirocercosis, there is a need for a relatively easy, non-invasive and inexpensive method to determine if neoplastic transformation has occurred.

1.5. Inflammation and neoplastic transformation

Spirocercosis is a very attractive model to investigate helminth-induced neoplasia and the factors associated with malignant transformation. *Spirocerca lupi* infection has been associated with neoplastic transformation into an osteosarcoma[7,28], anaplastic sarcoma or fibrosarcoma[13]. Neoplasia has been reported closely linked with infectious agents and inflammation[29], with chronic inflammation favouring the initiation and progression of neoplasia[30]. Persistent inflammation increases the probability of genomic instability and mutations, leading to neoplasia development[30-32]. It is hypothesized that the mechanism

related to this neoplastic development is the sustained generation of free radicals and proteases that cause oxidative damage and nitration of deoxyribonucleic acid (DNA) bases, increasing the risk for non repairable DNA mutations and breaks[30-32].

Hypotheses explaining the complex infectious-induced neoplastic transformations include: (1) chronic inflammation leading to increased epithelial cell turnover; (2) ineffectual Th1 response and affection of the immunosurveillance, with changes of the phenotype and genotype (chronic modulation of the immune system), resulting in genomic instability and secondary malignant transformation[33-34]; (3) production of helminth substances (free radicals, nitrogen species) that interact with the host cells, modifying its homeostasis and cell-cell communication, increasing the risk of neoplastic transformation[35]; (4) the parasite itself being directly responsible for neoplastic transformation by secretion/excretion of pro-neoplastic substances[36-38]; and (5) increased local and systemic proportions of T-regulatory cells[39-44].

The exact pathogenesis of the neoplastic transformation in *S. lupi* infection has not been elucidated, but an intense local inflammatory process in non-neoplastic and neoplastic spirocercosis has been described and is evidenced by local neutrophilic and lymphocytic infiltrate[7,45]. The presence of pyrexia (32-51%), leucocytosis with immature neutrophilia (30-81%)[4-5,10,13], raised serum CRP concentrations and IL-8 blood concentrations, in both neoplastic and non-neoplastic spirocercosis[24,46], indicates the presence of systemic inflammation in dogs with spirocercosis. Other hypotheses proposed include genetic predisposition and oncogenic stimuli[47] and release of growth factors by the worm[46].

Fibroblast growth factors (FGF) form a large group of potent mitogens for fibroblasts and epithelial cells[46]. The role of FGF in tumour progression in small animals has been poorly studied, but an increased FGF expression has been described in several tumours, including in *S. lupi*-induced sarcomas[2]. The expression of FGF in spirocercosis has been demonstrated in both the non-neoplastic and the neoplastic forms of the disease, with higher expression in the neoplastic form. Its expression in the non-neoplastic nodules has been hypothesised to originate from a local production from the worm or secondary to the chronic inflammation and potentially plays a role in its malignant transformation. The FGF over-expression in neoplastic spirocercosis is thought to be a consequence of existing neoplasia, functioning to promote the proliferation of tumour cells[2].

1.6. Vitamin D and its role in neoplastic transformation

Vitamin D is a secosteroid hormone, well known for its role in calcium regulation[48]. It also plays an important non-calcaemic role in the regulation of the immune function, cell proliferation and differentiation, and modulation of gene expression[49]. Vitamin D is ingested in the diet as either vitamin D_2 (ergocalciferol - plant origin) and/or vitamin D_3 (cholecalciferol - animal origin) and absorbed in the gastrointestinal tract[50-52]. Once absorbed, it is transported by the vitamin D-binding protein (DBP) to the liver and other target sites[53]. In the liver, this biologically inactive form undergoes activation by hydroxylation to 25-hydroxyvitamin D [25(OH)D], the major circulating form of vitamin D and the one most widely used to assess vitamin D status[50-51,53-54]. The circulating concentration of 25(OH)D reflects vitamin D intake, liver function, fibroblast growth factor 23 (FGF_{23}) and 24-hydroxylase [24(OH)ase] concentrations and reclamation of 25(OH)D-DBP following glomerular filtration[55]. The circulating 25(OH)D-DBP complex undergoes glomerular filtration followed by megalin-receptor-mediated endocytosis and once intra-cellular the DBP complex is degraded and 25(OH)D interacts with 1α-hydroxylase [1α(OH)ase] being converted to 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], the most biologically active form[50-51,53-55]. Serum $1,25(OH)_2D$ concentrations rely on many factors including serum 25(OH)D adequacy, kidney and liver function, serum calcium and phosphorus concentrations, parathyroid hormone (PTH) status, and on the production of extra-renal tissue enzymes such as 1α(OH)ase, FGF_{23} and 24(OH)ase[50-51,53-55]. Regarding catabolism, FGF_{23} production activates the 24(OH)ase and inhibits the 1α(OH)ase activities resulting in reduction of 25(OH)D and $1,25(OH)_2D$ concentrations[50].

Regarding the calcaemic effects of vitamin D, $1,25(OH)_2D$ interacts with vitamin D receptors (VDR), targeting mainly the intestine, kidney and bone, regulating calcium and phosphate homeostasis[50-51,56]. The non-calcaemic vitamin D effects were discovered when VDRs were identified in many other normal[57] and neoplastic cells[50-51,53-54,58] and when extra-renal tissues were additionally found to produce $1,25(OH)_2D$[51,53-54,59-60]. It was found that vitamin D could activate VDR promoting the transcription of specific targeted genes[57,61], having an anti-inflammatory[55], anti-fibrotic[55] and anti-neoplastic effects, inhibiting cell proliferation and angiogenesis, and stimulating cell differentiation and apoptosis[62]. The activity of $1,25(OH)_2D$ was found to be far more active in initiating this response than 25(OH)D.

There are many studies linking hypovitaminosis D to a higher neoplastic risk in humans[51,54,58,62-63] and in dogs[64-67]. Stimulation/inhibition of vitamin D functions is initiated and maintained by the continued activity of several growth factors[63] which mediate their action through receptor tyrosine kinases (RTKs)[68]. Anti-neoplastic calcitriol effects are related to (1) its direct effect on the regulation of the cell cycle, either by binding to the VDR and/or through direct regulation of cell cycle proteins, arresting cell growth and differentiation; (2) the induction of apoptosis of neoplastic cells; (3) the inhibition of angiogenesis (inhibiting VEGF and IL-8 expression) and the regulation of the expression of key molecules; and (4) its anti-inflammatory effects by down-regulating the prostaglandin pathway and cyclooxygenase-2[51,58,62,68].

Experimental and human studies have demonstrated that people with higher vitamin D concentrations are less predisposed to develop colon, prostate, breast, ovarian, stomach, pancreatic, rectal, kidney, lung and uterus neoplasia and non-Hodgkin's lymphoma[51,54, 63,69-71]. A reduction of 77% of breast and colon neoplasias[54,72-73] was noted when humans were supplemented with vitamin D. Although the promising results of some studies, there is still controversy among all studies that investigated vitamin D supplementation. In addition, different doses of vitamin D have been administered in clinical human studies, with the initial vitamin D status of the individuals included not always known, making it unclear whether vitamin D supplements restored existing deficiencies or augmented circulating vitamin D in already sufficient individuals[71].

In vivo, vitamin D studies in animal models of neoplasia have shown that vitamin D reduced cancer volume, metastasis and suppressed tumour growth[70], leading to the hypothesis that hypovitaminosis D might predispose to neoplastic development and that vitamin D could be a potential adjuvant treatment in several tumours. *In vitro*[51,73-74] and *in vivo*[73-75] studies have demonstrated a reduction of the prevalence and size of tumours with vitamin D treatment[51,53-54,59-60,63,69,76]. Therefore, hypovitaminosis D may potentially play a role in spirocerca-induced neoplastic transformation, be used as a biomarker of the neoplastic transformation and potentially as an adjuvant treatment in neoplastic cases.

1.7. Measurement of vitamin D metabolites

The vitamin D metabolites are chemically identical across all species, so measurement of 25(OH)D and 1,25$(OH)_2$D using human assays is possible in dogs[56]. The different assays for vitamin D measurement have progressed from a competitive protein binding assay to a radioimmunoassay (RIA), high-performance liquid chromatographic (HPLC) or liquid

chromatography coupled with mass spectrometry techniques[77]. The vitamin D external quality assessment scheme (DEQAS) has determined that the physical chemical gold standard method for vitamin D measurement is HPLC[77]. The HPLC methods separate and quantitate circulating 25(OH)D_2 and 25(OH)D_3 and are very accurate, but are cumbersome, require a relatively large sample and an internal standard, and the equipment is very expensive[77].

The major circulating form of vitamin D is 25(OH)D and it is widely used to assess vitamin D status[50-51,53-55]. Normal concentrations are defined as the minimum required to maintain skeletal integrity and bone density, cancer and infections prevention, cardiovascular health, diabetes and autoimmune control[77]. In humans, 25(OH)D concentrations should be above 50 nmol/l, but the cut-off point between vitamin D insufficiency and optimal vitamin supply is still debatable. The same type of studies looking at the minimum 25(OH)D concentrations in dogs are lacking. Human plasma 25(OH)D concentrations below 10–12 nmol/l, stimulate 1,25$(OH)_2$D production from 25(OH)D in the kidneys, where the reserves are limited and this concentration leads to rickets or osteomalacia. When plasma 25(OH)D serum concentrations are above 10–12 nmol/l, the kidney produces enough 1,25$(OH)_2$D to maintain systemic mineral ion homeostasis, but availability of 25(OH)D at extra-renal sites may still be too low to guarantee production of 1,25$(OH)_2$D in sufficient amounts to maintain autocrine/paracrine control of cellular homeostasis and function. Thus, vitamin D insufficiency does not cause a disease but rather constitutes a clinically asymptomatic condition, which nevertheless bears an increased risk of multiple aetiologically unrelated chronic diseases[78]. Circulating 1,25$(OH)_2$D is useful as a diagnostic tool for several clinical conditions, including vitamin D–dependent rickets types I and II, lymphoma, hypoparathyroidism, hyperparathyroidism, and chronic renal failure[77].

CHAPTER 2: Research

2.1. Objectives

- To measure 25(OH)D and 1,25$(OH)_2$D concentrations in non-neoplastic and neoplastic spirocercosis dogs and healthy dogs.
- To investigate if the vitamin D status allows the clinical differentiation between neoplastic and non-neoplastic spirocercosis in dogs, potentially to be used as a biomarker of neoplastic transformation of *S. lupi*-associated nodules.

2.2. Research questions

- Are there differences in serum 25(OH)D and 1,25$(OH)_2$D concentrations between non-neoplastic and neoplastic spirocercosis and healthy dogs?
- Does low 25(OH)D and/or 1,25$(OH)_2$D concentration are associated with the neoplastic transformation in canine spirocercosis?

2.3. Research hypothesis

- Hypovitaminosis D will be present in spirocercosis in the non-neoplastic and neoplastic stage, with neoplastic cases showing lower vitamin D concentrations.

2.4. Benefits arising from the study

- To establish whether vitamin D can be used as a biomarker to determine neoplastic transformation of *S. lupi*-associated nodules. This will have implications in the treatment and monitoring of clinical decisions and prognosis.
- To contribute to the understanding of spirocercosis-induced cancer/ leading to more detailed cause and effect studies between vitamin D concentrations and spirocercosis-induced neoplasia.

CHAPTER 3: Materials and Methods

3.1. Study design

A prospective clinical study was performed on client-owned dogs diagnosed with naturally occurring spirocercosis that presented to the Onderstepoort Veterinary Academic Hospital (OVAH) in South Africa over a 3 year period. Fifty one dogs were selected from a total of 119 cases diagnosed with spirocercosis. Owner consent was given for all cases included in the study. Twenty five dogs were diagnosed with non-neoplastic spirocercosis and 26 with neoplastic spirocercosis. Twenty four healthy client-owned dogs were used as a control group. The study was approved by the institutional Animal Use and Care Committee (protocol number V061/11).

3.1.1. Inclusion criteria for dogs diagnosed with spirocercosis:

- Informed consent by the owners in all cases (Appendices A and B)
- Dogs older than 1year of age
- Positive spirocercosis diagnosis based on one or a combination of the following:

a) Positive faecal float or modified $NaNO_3$ centrifugal faecal floatation for *S. lupi* ova.

b) Thoracic radiographs with at least 2 radiographic signs consistent with spirocercosis infection: a caudodorsal mediastinal mass, spondylitis of the caudal thoracic vertebrae or undulation of the lateral border of the descending aorta (Figure 1).

c) Thoracic CT confirming the presence of an oesophageal mass with/without the presence of lung metastasis.

d) Visual identification of *S. lupi* nodules by upper-gastrointestinal endoscopy.

3.1.2. Differentiation between non-neoplastic and neoplastic spirocercosis dogs:

The dogs infected with *S. lupi* were placed into two groups: non-neoplastic and neoplastic. Differentiation between non-neoplastic and neoplastic spirocercosis dogs was based on the following findings:

- Macroscopic findings during endoscopy, where non-neoplastic nodules were expected should be smooth with/without and operculum and neoplastic ones ulcerative, necrotic and cauliflower-like (Figure 2).

- Positive response to doramectin therapy in suspected non-neoplastic spirocercosis with subsequent endoscopic confirmation of clinical cure denoted by absent oesophageal nodules post-treatment. If no nodules were detected, clinical cure was assumed and prophylactic therapy instituted. If the nodules were still present, but smaller or reduced in number, therapy was continued for another 6 weeks and oesophagoscopy performed a second time to assure clinical cure.
- Thoracic CT evidence of oesophageal mass(es) and compatible lung metastatic lesions in neoplastic spirocercosis dogs.
- Histopathology of nodules/masses removed either surgically or obtained by endoscopic biopsy (only neoplastic cases) or during post-mortem examination.

3.1.3. Inclusion criteria for healthy dogs (control group):

- Owner consent (Appendixes B and C)
- Dogs more than 1 year of age
- No history of illness or drug administration up to 6 months prior time of blood sample collection.
- Thoracic radiographs to rule out canine spirocercosis or any other systemic disease that could be detected on thoracic radiographs.

3.1.4. Exclusion criteria for all dogs diagnosed with spirocercosis and healthy dogs:

- Dogs less than 1 year of age.
- Dogs with evidence of liver or renal disease based on clinical signs, serum biochemistry, urinalysis, abdominal ultrasound and/or post-mortem and histopathology.
- Dogs with any concurrent systemic or inflammatory diseases.
- Dogs treated with medications that could influence vitamin D concentrations such as corticosteroids, anticonvulsants, calcium channel blockers or diuretics during the past month.
- Dogs treated prophylactically for canine spirocercosis (macrolytic lactones or milbemycin) in the past 6 months – exclusion criteria only for the dogs diagnosed with non-neoplastic or neoplastic spirocercosis.

3.1.5. Appetite of dogs diagnosed with spirocercosis:

- All dogs diagnosed with spirocercosis had their appetite classified as either normal or abnormal (decreased appetite or total anorexia) based on information obtained from the owners.
- The type of diet (commercial, home cooked or mixed) was also obtained from owners for all dogs.

3.2. Data collection

Upon admission a full history, clinical examination, peripheral blood smear evaluation, urinalysis, faecal flotation (using $NaNO_3$), CBC and serum biochemistry [total proteins, albumin, globulins, creatinine and alanine transaminase (ALT)] were performed in all dogs (Appendixes D, E, F, I). In the spirocercosis dogs, any other detected abnormalities (including those not previously described in spirocercosis) resulted in further diagnostics appropriate for each case, which may have resulted in exclusion of the patient from the trial. If any abnormalities were detected in the healthy dogs, they were also excluded.

3.2.1. Sampling

After diagnosis, prior to any treatment, serum and ethylenediaminetetraacetic acid (EDTA) samples were collected from the jugular vein of each patient with a 21-gauge needle by careful venipuncture with minimum stasis. In accordance with the specifications and approval of the Faculty Research Committee and the Animal Ethics Committee of the University of Pretoria, the volume of blood collected always amounted to less than 0.5% of the body weight.

The EDTA sample was used to perform a CBC and the serum sample centrifuged at 2100g for 8 minutes using the Hettich Universal 320 Centrifuge [Labotec (PTY) Ltd, South Africa] and then serum removed and used to measure serum albumin, globulins, total proteins, creatinine and ALT concentrations (appendix F). Remaining serum was stored frozen at -80ºC (Freezer Forma Scientific, Laboteca, South Africa) until sent on dry ice to a Specialist Assay Laboratory CSB3 (Vitamin D Research Laboratory, Manchester Royal Infirmary, Manchester, United Kingdom) accredited by the International Organization for Standarization (ISO) 9001:2008 and ISO 13485:2003 for vitamin D metabolites concentration measurements.

3.2.2. Assay methodologies

- The CBC was performed on an Advia 2120 Haematology System (Siemens, South Africa) and additional manual differential leukocyte counts.
- Serum total protein, albumin, globulin, creatinine and ALT were determined using the Cobas Integra 400 Plus (Roche, South Africa).
- Serum concentrations of 25(OH)D and $1,25(OH)_2D$ were measured and validated as described in detail elsewhere[48,79]. Briefly, samples were extracted using acetonitrile and applied to C18 Silica Sep-paks (Waters Ltd, Elstree, United Kingdom). Metabolites were separated by straight phase HPLC (Waters Associates, Milford, United States of America) using a Hewlett-Packard Zorbax-Sil Column (Hichrom, Reading, United Kingdom) eluted with hexane:propan-2-ol:methanol (92:4:4). Serum $25(OH)D_2$ and $25(OH)D_3$ were measured separately by application to a second Zorbax-Sil Column (Agilent Technologies, Stockport, United Kingdom), eluted with hexane:propan-2-ol (98:2) and quantified by UV absorbance at 265 nm (radioimmuno assay) and corrected for recovery (sensitivity 5 nmol/L, intra- and inter-assay coefficients of variation 3.0% and 4.2%, respectively)[80]. Results were expressed as total 25(OH)D. Following separation by HPLC, $1,25(OH)_2D$ ($1,25(OH)_2D_2$+$1,25(OH)_2D_3$) was quantified by radioimmunoassay as described in detail (sensitivity 3 pmol per assay tube, intra and inter-assay coefficients of variation 7·8% and 10·5%, respectively)[79].

3.2.3. Diagnostics

3.2.3.1. Radiography

The standard right lateral and dorsoventral thoracic radiographs were performed under general anaesthesia (appendix H) and manual positive pressure ventilation in all dogs diagnosed with spirocercosis and healthy dogs. Radiographs were obtained using a digital x-ray generator (Siemens Polymat 50 FFD/SID 107cm, Potter Bucky table with an 8:1 moving focused grid, Simens, South Africa). The x-ray cassettes used were Fujifilm FCR IP cassette (type CC) (Fujifilm, Japan Radiographs) and they were developed with a Fuji Film Axim FCR Capsula XL (Fujifilm, Japan). Radiographs were evaluated by a qualified radiologist.

3.2.3.2. Upper gastrointestinal endoscopy

Oesophageal and gastric endoscopy was performed under general anaesthesia in all dogs with suspected spirocercosis, according to the set standard at the department of small

animal medicine, by the principle investigator or other qualified medicine clinician. The anaesthetic protocol used is described in appendix H. The procedure was performed using a video endoscopy unit, in order to confirm the diagnosis of spirocercosis and aid in the differentiation of neoplastic from non-neoplastic cases. An endoscopic examination was performed at the time of initial diagnosis and a follow-up in all cases not euthanised approximately 6 weeks later to determine response to treatment. The number and macroscopic aspects of the nodules were evaluated in all cases and biopsies were taken in all suspected neoplastic spirocercosis (cauliflower-like aspect, ulcerated and necrotic). All endoscopic findings were recorded (Appendix G). Upon follow-up of non-neoplastic spirocercosis cases, response to treatment was indicated by an absence of nodules or a reduction in the size and number of the previously diagnosed nodules. If complete resolution was seen, clinical cure was assumed and prophylactic therapy instituted, but if nodules persisted, yet smaller or reduced in number, therapy was continued for another 6 weeks and endoscopy performed a second time to assure clinical cure.

3.2.3.3. Thoracic CT angiography

A thoracic CT angiography was performed under general anaesthesia (appendix H) in some dogs diagnosed with spirocercosis using a dual slice CT scanner (Siemens Emotion Duo, Erlangen, Germany). The images were evaluated by a qualified radiologist.

3.2.3.4. Post-mortem and histopathology

Histopathology of the entire nodule was performed if the dogs were subjected to surgical removal of the oesophageal nodules/masses and histopathology of biopsies were performed if samples were obtained by endoscopy. A standard post-mortem examination was performed on all dogs euthanised due to neoplastic spirocercosis, regardless of the ante-mortem diagnostics performed. All organs, including the liver, and the *S. lupi*-induced oesophageal nodules were histopathologically evaluated in these dogs.

3.3. Treatment

Treatment of the animals included in the study for spirocercosis was initiated after all sample collections and diagnostics, including thoracic radiography and endoscopy, were completed. Treatment of non-neoplastic spirocercosis consisted of 400 ug/kg doramectin (Dectomax®, Pfizer, South Africa) administered subcutaneously every two weeks for three treatments[11]. Neoplastic spirocercosis dogs were offered: 1) surgical excision of the nodules/masses followed or not by chemotherapy; 2) symptomatic therapy (anti-ulcerative

and anti-emetic therapy and/or application of a percutaneous endoscopic gastrotomy feeding tube); or 3) euthanasia followed by a post-mortem and histopathology.

3.4. Data analysis

The data was captured using Microsoft Excel 2010® spreadsheet (Microsoft, United States of America) and analysed by the principle investigator. The data was evaluated for normality using a Kolmogorov-Smirnov test (SPSS software, SPSS 17.0 for Microsoft Windows, SPSS Inc., Chicago, United States of America) followed by the most appropriate statistical test. Vitamin D metabolites were analysed separately and compared between the different groups, in order to identify the potential role of these metabolites in the process of the malignant transformation of the *S. lupi*-associated oesophageal nodules. The following statistical analyses were performed:

- Kruskal-Wallis test to compare serum 25(OH)D, 1,25$(OH)_2$D, body weights and albumin concentrations and ages among all groups, and a p*ost-hoc* Dunn's multiple comparison test to assess differences between the *S. lup*i-infected groups and healthy dogs (GraphicPad Prism 5, GraphPad Software Inc., California, United States of America).
- Fisher´s exact test to compare the absolute numbers of dogs with normal and abnormal appetite between the non-neoplastic and neoplastic spirocercosis groups (Microsoft Excel 2010, Microsoft, United States of America).
- Mann-Whitney U test to compare serum 25(OH)D and 1,25$(OH)_2$D concentrations and the appetite in each spirocercosis group. This statistical analysis was performed with a commercial software package (SPSS Statistics 20.0® software, SPSS Inc., United States of America).
- An homogeneity of slopes test followed by an analysis of covariance (ANCOVA) to evaluate the influence of age and serum albumin concentration on serum 25(OH)D concentrations in all groups (Statistica 10, StatSoft Inc., Oklahoma, United States of America).
- Chi-square test to compare breeds, and sex and spay/neuter status among the 3 groups (Microsoft Excel 2010, Microsoft, United States of America).
- A multivariable linear regression analysis combining all 75 dogs as one dataset to evaluate the interaction and significance of sex and spay neuter status, body weight and appetite (independent variables) with serum 25(OH)D concentrations (dependent

variable) (GraphicPad Prism 5, GraphPad Software Inc., California, United States of America).

A P-value of <0.05 was considered statistically significant in all statistical tests performed.

CHAPTER 4: Results

4.1. Study population

A total of 119 client-owned dogs naturally infected with *S. lupi* (39 neoplastic and 80 non-neoplastic) were diagnosed in the OVAH over a three year period. From these, only 51 *S. lupi*-infected dogs fulfilled the inclusion criteria described earlier and were enrolled in the study. Twenty five dogs were diagnosed with non-neoplastic spirocercosis and 26 with neoplastic spirocercosis. Twenty four healthy client-owned dogs were used as a control group.

All dogs in the neoplastic group had neoplastic transformation of the *S. lupi* nodule confirmed on necropsy and/or histopathology. The histopathological diagnoses of neoplastic spirocercosis were divided into 23 osteosarcomas, 2 anaplastic sarcomas and 1 fibrosarcoma. Ten neoplastic cases had metastatic lesions on necropsy and 5 had hypertrophic osteopathy observed on radiographs or at necropsy. No dogs developed neoplasia after being classified as non-neoplastic. No non-neoplastic cases were euthanised. In the group of dogs diagnosed with neoplastic spirocercosis, surgical removal of the neoplastic nodule/mass was performed in 1/26 dogs but not followed by chemotherapy, 2/26 dogs were given symptomatic therapy for a total 1-2 months and the remaining 23/26 dogs were euthanised on the day or within a few days post-diagnosis.

4.2. Serum 25(OH)D and 1,25$(OH)_2$D concentrations

The medium serum 25(OH)D and 1,25$(OH)_2$D concentrations were significantly different among the 3 groups ($p<0.001$) (Figure 3 and 4). Median serum 25(OH)D concentrations were significantly lower in the neoplastic spirocercosis group [30.7 nmol/l (range 14.7-62.2, n=26)] compared to the non-neoplastic spirocercosis [52.7 nmol/l (range 19.1-129.7, n=25) ($p<0.05$)] and healthy groups [74.6 nmol/l (range 37.4-130.5, n=24) ($p<0.005$)] (Figure 3). A significant difference was also observed in the median 25(OH)D concentrations between the healthy group and the non-neoplastic spirocercosis group ($p<0.05$). Median 1,25$(OH)_2$D concentrations were significantly lower in the neoplastic group (56.5 pmol/l (range 18-130)] compared to the healthy group [94.5 pmol/l (range 46-142, $p<0.001$)], but no significant differences were detected between either the neoplastic and the non-neoplastic spirocercosis [70 pmol/l (range 34-127), $p>0.05$]

groups nor between the non-neoplastic spirocercosis and the healthy groups ($p>0.05$) – Figure 4.

4.3. Appetite in canine spirocercosis

No significant differences in the proportion of dogs with normal and abnormal appetite (Table 1) were detected between the two spirocercosis groups using the Fisher's Exact Test ($p=1.0$).

All dogs were fed a mixture of home cooked and commercial diet (various types of grocery store diets). The proportion of each diet could not be accurately assessed. Therefore, calculation of total dietary vitamin D content ingested was not possible.

4.4. Serum 25(OH)D and 1,25$(OH)_2$D concentrations and appetite

In the non-neoplastic spirocercosis group, no significant differences were observed in the median serum 25(OH)D ($p=0.125$, Figure 5) and 1,25$(OH)_2$D ($p=0.08$, Figure 6) concentrations of dogs with normal or abnormal appetites. In the neoplastic spirocercosis group, no significant differences were also observed in the median serum 25(OH)D ($p=0.0869$, Figure 7) and 1,25$(OH)_2$D ($p=0.94$, Figure 8) concentrations of dogs with normal or abnormal appetites.

4.5. Age and serum albumin concentration in canine spirocercosis and their influence on serum 25(OH)D concentrations

A significant difference in the ages among all 3 groups of dogs ($p<0.001$) was found. Post-test analysis demonstrated that the median ages were significantly higher in the neoplastic spirocercosis group [72 months (range 36-132 months, n=26)] compared to the non-neoplastic spirocercosis [38.25 months (range 16-150 months, n=25) ($p<0.01$)] and healthy groups [30 months (range 12-120 months, n=24) ($p<0.01$)]. No significant difference was observed in the median age between the healthy group and the non-neoplastic spirocercosis group ($p=1$).

The serum albumin concentration was significantly different among all groups ($p<0.001$). The median serum albumin concentration was significantly lower in the neoplastic spirocercosis group [22.1 g/l (range 12.1-35.9, n=26)] compared to the non-neoplastic spirocercosis [32.1 g/l (range 23.1-37.4, n=25) ($p<0.001$)] and the healthy groups [35.2 g/l (range 24.5-39.2, n=24) ($p<0.001$)]. From the non-neoplastic spirocercosis group, 5/25 dogs had evidence of soft faeces, while 9/26 neoplastic spirocercosis dogs had diarrhoea.

No significant difference was observed in the median serum albumin concentration between the non-neoplastic spirocercosis and the healthy groups ($p>0.05$).

ANCOVA demonstrated a significant difference in serum 25(OH)D concentrations among the three groups of dogs that was independent of either albumin or age ($p<0.05$).

4.6. Breed, body weight, sex and spay neuter status and their influence on serum 25(OH)D concentration

Breeds varied within all groups (total 26 dog breeds) including small, medium, large and giant breed dogs. There was no preponderance of any specific breed in any group ($p=0.84$). No significant differences were detected for body weight ($p=0.27$), sex ($p=0.32$) and spay neuter status ($p=0.58$) among the three groups (Table 2). The non-neoplastic spirocercosis dogs had a median body weight of 30.2 kg (range 6.9-78), followed by the healthy ones with 29.7 kg (range 5.4-70), and the neoplastic spirocercosis dogs with 23.2 kg (range 5.4-50) (Table 2).

Multivariable linear regression analysis demonstrated that serum 25(OH)D concentration was significantly different among all groups ($p<0.001$, $r=0.35$), that all independent variables were independent of each other, and that there was no linear relationship with either body weight ($p=0.08$, $r=0.24$) nor appetite ($p=0.16$, $r=-0.49$). Neutered and female dogs had higher 25(OH)D concentrations than intact and male dogs (data not shown).

CHAPTER 5: Discussion

This study has shown that serum 25(OH)D is low in canine spirocercosis. A reduction of serum 25(OH)D concentrations with progression of the disease from a non-neoplastic to a neoplastic state was evident. These results were independent of appetite, age and serum albumin and the low vitamin D status could thus potentially play a role in the neoplastic transformation of canine spirocercosis.

Causes of reduced serum 25(OH)D concentrations could be attributed to anorexia, reduced hepatic function, over-expression of FGF_{23} or 24(OH)ase activity[50-51,53-54], and losses of 25(OH)D-DBP though the urine or diarrhoea[55]. Firstly, our study showed that appetite was unlikely to account for the difference in serum 25(OH)D concentrations among the spirocercosis groups. However, it is possible that dogs that had inflammatory oesophageal disease or neoplasia were eating less commercial dog food and more table foods (that are typically low in vitamin D) than control dogs, which may have contributed to the vitamin D deficiency in the former group of dogs. No linear relationship was also found between either body weight nor appetite and 25(OH)D concentrations. Secondly, only dogs without evidence of liver disease based on serum liver enzymes evaluation, abdominal ultrasonographic findings and/or histopathology were included in the study. Therefore, hepatic disease was also an unlikely cause of the reduced 25(OH)D concentrations. Thirdly, we speculate that the pathophysiologic mechanism(s) leading to the low serum 25(OH)D concentrations in spirocercosis could relate to increased catabolism due to FGF_{23} over-expression with increase 24-OHase activity[53-54,58] and impaired 1αOHase activity[81], genetic mutations leading to reduced 25(OH)D synthesis or chronic inflammation[81-83]. Neoplastic and non-neoplastic spirocercosis nodules have been previously shown to over-express FGF, with higher expressions in the neoplastic compared to the non-neoplastic cases[84]. The FGF over expression in spirocercosis might be secondary to a local production from the worm or due to chronic inflammation[84]. This could potentially contribute to the low vitamin D status detected in spirocercosis compared to healthy dogs. Genetic mutations leading to over-expression of FGF or genetic polymorphisms in genes encoding VDR, vitamin D-binding protein, 1α(OH)ase (*CYP27B1*), 24(OH)ase (*CYP24A1*) and 25(OH)ase (*CYP2R1*) remain possible[82], although further studies to prove this association are required.

Chronic inflammation caused by parasitic infections is recognized as an important risk factor for neoplastic development[83]. *Spirocerca lupi*-induced systemic inflammation has been demonstrated locally by the presence of neutrophilic and lymphocytic infiltrates of the nodules[7,45], leucocytosis with immature neutrophilia[4-5,10,13] and raised serum CRP concentrations and IL-8 blood concentrations in both neoplastic and non-neoplastic spirocercosis[24,46]. Inflammatory mediators that are produced can induce DNA damage in tumour suppressor genes leading to post-translational modifications of proteins involved in cellular apoptosis, DNA repair, and cell cycle checkpoints[34,83]. The association between neoplasia development, chronic inflammation[34] and vitamin D has been shown in *in vitro* human[51,63,66] and animal[63,67] studies, and in human epidemiologic investigations[51,54]. Chronic inflammation leads to lipid peroxidation and potentially genetic mutations[34]. If these mutations accumulate in key host cell regulatory genes they can eventually change the cell phenotype and lead to neoplasia[34]. Vitamin D seems to modulate the immune system, preventing neoplasia by suppressing inflammation that facilitates tumourigenesis, by activating receptors of cells of the adaptive immune system in the presence of abnormal cells or antigens[63], inhibiting angiogenesis (suppressing VEGF and IL-8 expression)[58,62] and arresting cell growth and differentiation through a direct effect on the regulation of the cell cycle[51,68]. Vascular endothelial growth factor[24] and IL-8[46] are over expressed in canine spirocercosis and more significantly in the neoplastic form of the disease, what could be a consequence of hypovitaminosis D.

In a recent study, induced endotoxaemic dogs were found to have low serum vitamin D concentrations[81]. Hypovitaminosis D in sepsis was attributed to result from an impaired activity of the 1αOHase, reduced DBP and loss of urinary 25(OH)D[81], which could apply to spirocercosis, although not evaluated in our study. The loss of urinary 25(OH)D seems less likely, as no proteinuria was detected on dip stick urinalysis, although minor losses could still occur.

Some of the dogs in this study had low albumin and evidence of soft faeces or diarrhoea. Gastrointestinal albumin loss has been shown to correlate with serum 25(OH)D concentrations in dogs with intestinal bowel disease (IBD). Intestinal loss of DBP was postulated as a potential cause of hypovitaminosis D in dogs with IBD and hypoalbuminaemia[85-88]. In this study, although the serum albumin was significantly lower in the neoplastic group, the 25(OH)D was independently lower, and therefore it can only be regarded as a contributing factor. This phenomenon, although valid, would still not explain the low 25(OH)D detected in the remaining dogs without evidence of diarrhoea,

soft faeces or hypoalbuminaemia. The cause of the hypoalbuminaemia detected in our study could relate to loss from the ulcerated neoplastic lesion, malnutrition, parasitism or chronic inflammation (albumin is a negative acute phase protein). In humans, an association between acute and chronic inflammation and hypovitaminosis D has been demonstrated and correlated with hypoalbuminaemia and increased CRP concentrations. Vitamin D has been implied to be a negative acute phase protein and hypovitaminosis D a consequence of chronic inflammation[89]. In spirocercosis increased serum CRP is also evident[23] and the interlink with hypovitaminosis D require further investigation.

Reduction in serum vitamin D concentrations has been associated with increasing age[90], however this study has demonstrated a trend of reduction of serum 25(OH)D concentrations with progression of the disease from non-neoplastic to neoplastic, independent of age. The 25(OH)D concentrations were lower in the non-neoplastic group versus the healthy group, yet the ages were similar, supporting the fact that age is unlikely to account for the differences in vitamin D concentrations. The relationship between age, vitamin D and neoplasia remains questionable, because many exposures and events accumulate with age, potentially leading to genetic mutations and epigenetic changes associated with neoplasia development[63].

Serum $1,25(OH)_2D$ concentrations were significantly different among all groups, following the same trend as the serum 25(OH)D concentrations. The lowest serum $1,25(OH)_2D$ concentrations were found in the neoplastic group followed by the non-neoplastic and healthy groups, but the only significant difference detected was between the neoplastic and the healthy groups, highlighting the association between low vitamin D and neoplastic transformation and its anti-neoplastic effects. The differences noted between the serum 25(OH)D and $1,25(OH)_2D$ concentrations may relate to other factors that only influence serum $1,25(OH)_2D$ concentrations, such as serum calcium, phosphate and PTH, which were not evaluated in this study.

The anti-neoplastic treatment properties of calcitriol that leads to tumour regression have been shown in *in vitro* and *in vivo* canine neoplasias[64,66-67,91] (transitional cell carcinomas[66], mast cell tumors[64], osteosarcoma[91], hemangiosarcoma[91] and carcinomas[91]) and in humans[54,58,63]. Vitamin D supplementation has been described in the treatment of veterinary patients with renal[92-93], metabolic[81,85] and neoplastic diseases[64,66,91]. Treatment of neoplastic cells with $1,25(OH)_2D$ may inhibit cell tube formation and tumour growth by repressing VEGF and IL-8[58]. Canine neoplastic spirocercosis has also been shown to over-express IL-8[46] and VEGF[82], therefore the use of $1,25(OH)_2D$ as an adjuvant

therapeutic agent in the neoplastic spirocercosis could be considered. Its use in non-neoplastic spirocercosis could also be beneficial for its anti-inflammatory effects on COX-2 expression and the prostaglandin pathway[62], potentially reducing the risk of neoplastic transformation. The major *in vivo* limitation for calcitriol supplementation/treatment is the potential hypercalcaemic effects, especially when used for its anti-neoplastic effects (dose-dependent effect), because high doses would be required[64]. Further studies are warranted to evaluate calcitriol treatment and to determine whether it would decrease the incidence of neoplastic transformation. The therapeutic use of calcitriol should be accompanied by close monitoring of serum calcium concentrations. Limitations of this study include a lack of quantification of the proportion of home cooked diet versus commercial diet, and a lack of body condition scoring. Body condition scoring (although subjective) would have been helpful in our understanding of whether food intake was a factor influencing the 25(OH)D and 1,25$(OH)_2$D concentrations among the groups, as it is thought less subjective than appetite scoring by owners. Furthermore, since this was not a breed-matched study, the use of body weight was a poor proxy for either hyporexia or adiposity. The measurement of urine calcitroic acid or 25(OH)D-DBP in the urine and vitamin D metabolites in diarrhoeic or soft faeces would have been useful for the understanding of the pathophysiology of hypovitaminosis D in spirocercosis, although loss seems an unlikely cause of hypovitaminosis D in spirocercosis as no proteinuria was detected and hypovitaminosis D was present both in dogs with and without diarrhoea or soft faeces.

CHAPTER 6: Conclusions

- Hypovitaminosis D exists in canine spirocercosis, with lower serum vitamin D concentrations in the neoplastic form of the disease.
- Hypovitaminosis D in canine spirocercosis could relate to FGF over-expression or chronic inflammation and immune system suppression, and potentially be a risk factor for neoplastic transformation.
- Low serum 25(OH)D concentrations were found independent of appetite, albumin, age, body weight, breed and sex and spay neuter status.
- Based on this study, once spirocercosis has been diagnosed, serum 25(OH)D concentrations might be useful as an adjuvant test to support the differentiation of non-neoplastic from neoplastic spirocercosis. More importantly, vitamin D might be useful as therapeutic agent for its anti-proliferative, apoptotic and anti-angiogenic effects, in the prevention of neoplastic transformation and as an adjuvant agent in the neoplastic form of the disease, although further studies are warranted.

FIGURES

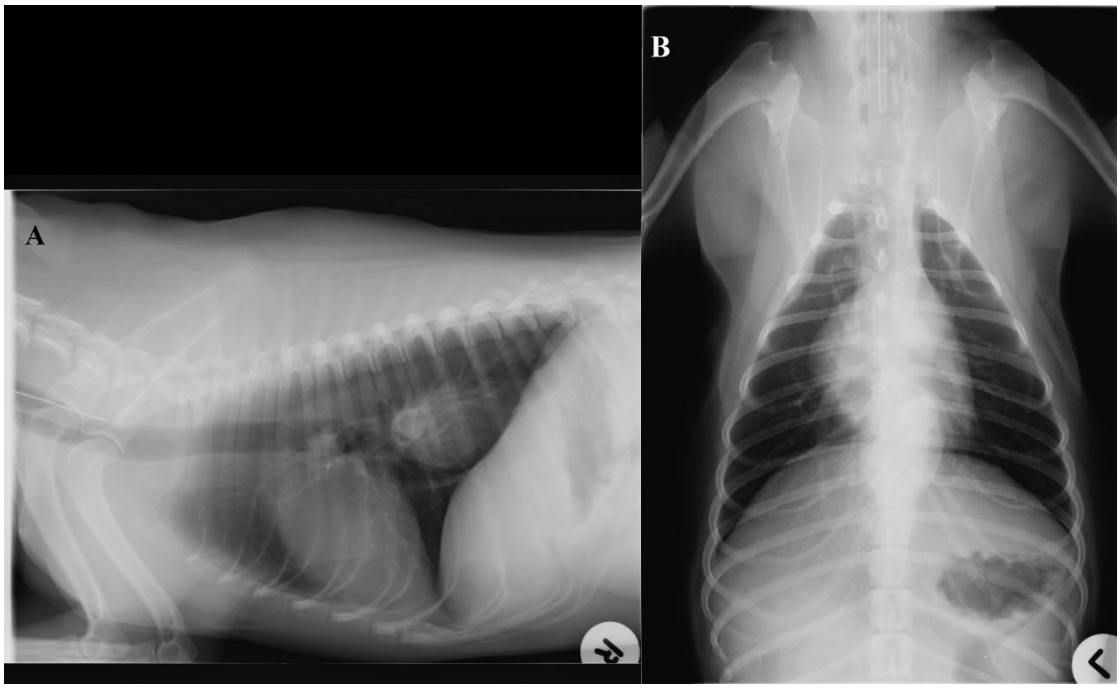


Figure 1 – Right lateral (A) and dorsoventral (B) thoracic radiographs of a dog diagnosed with neoplastic spirocercosis. Caudodorsal mediastinal mass, aortic aneurysms and focal mild spondylitis are noted. R represents right and L left.

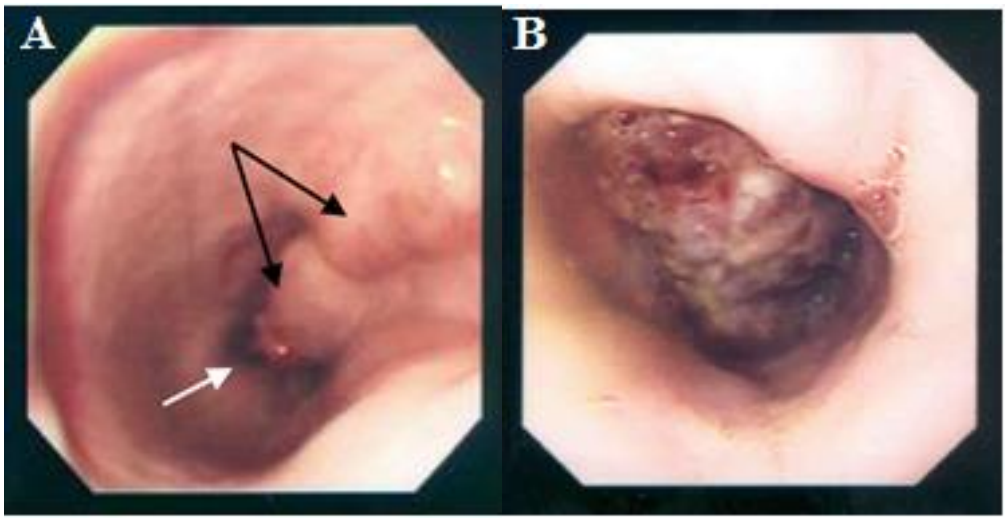
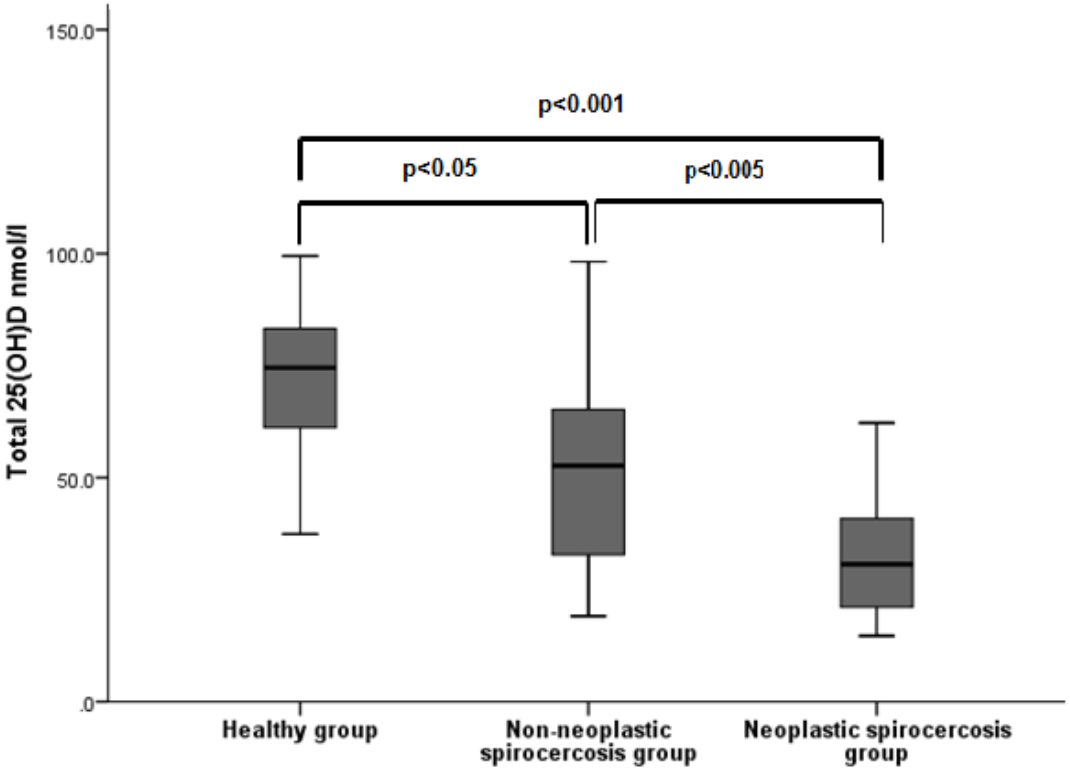
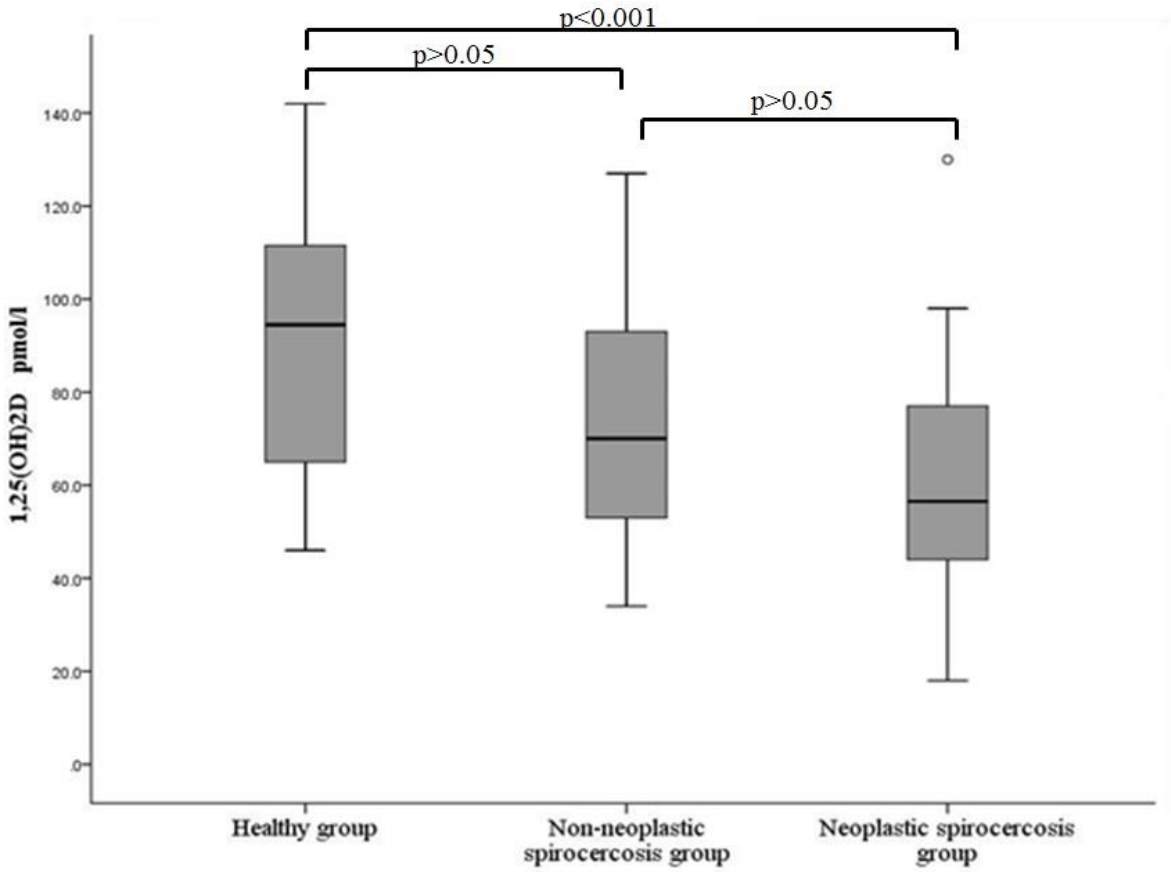


Figure 2 – Oesophageal endoscopic images of non-neoplastic spirocercosis nodules (A) and a neoplastic spirocercosis mass (B) in two different dogs. Note the macroscopic difference between the two forms of the disease. The non-neoplastic spirocercosis nodules (black arrows) are small, smooth and can have and operculum (white arrow), while the neoplastic spirocercosis masses (B) tend to be big, cauliflower-like, ulcerated and necrotic.



Median	**74.6**	**52.7**	**30.7**
Range	**37.4 – 130.5**	**19.1 – 129.7**	**14.7 – 62.2**

Figure 3: Box plot of the comparison between the serum 25(OH)D concentrations in the healthy, non-neoplastic spirocercosis and neoplastic spirocercosis groups (Kruskal-Wallis test, followed by Dunn's multiple comparison test). For each plot, the box represents the interquartile range (IQR), the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.



Median	94.5	70	56.5
Range	46-142	34-127	18-130

Figure 4: Box plot of the comparison between the serum 1,25$(OH)_2$D concentrations in the healthy, non-neoplastic spirocercosis and neoplastic spirocercosis groups. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles. The ° indicates an outlier.

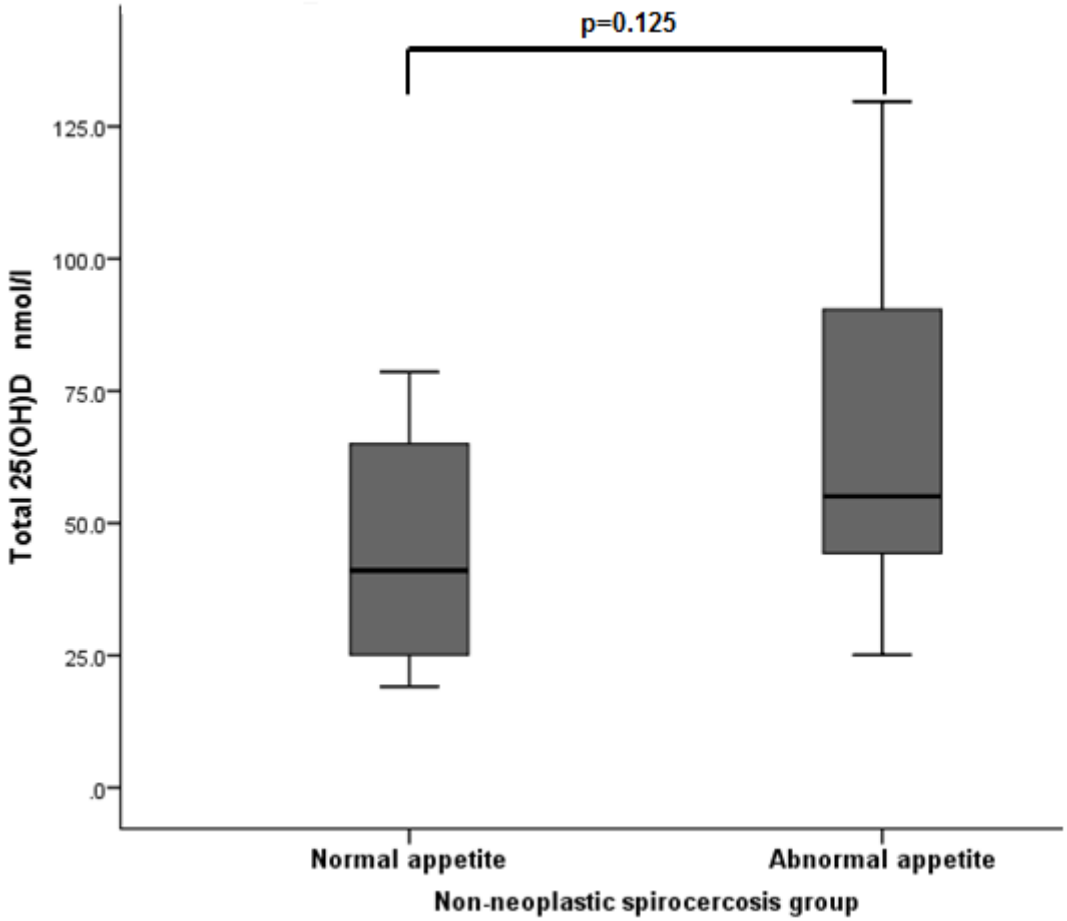


Figure 5: Box plot of the comparison between the serum 25(OH)D concentrations of dogs with normal or abnormal appetite in the non-neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

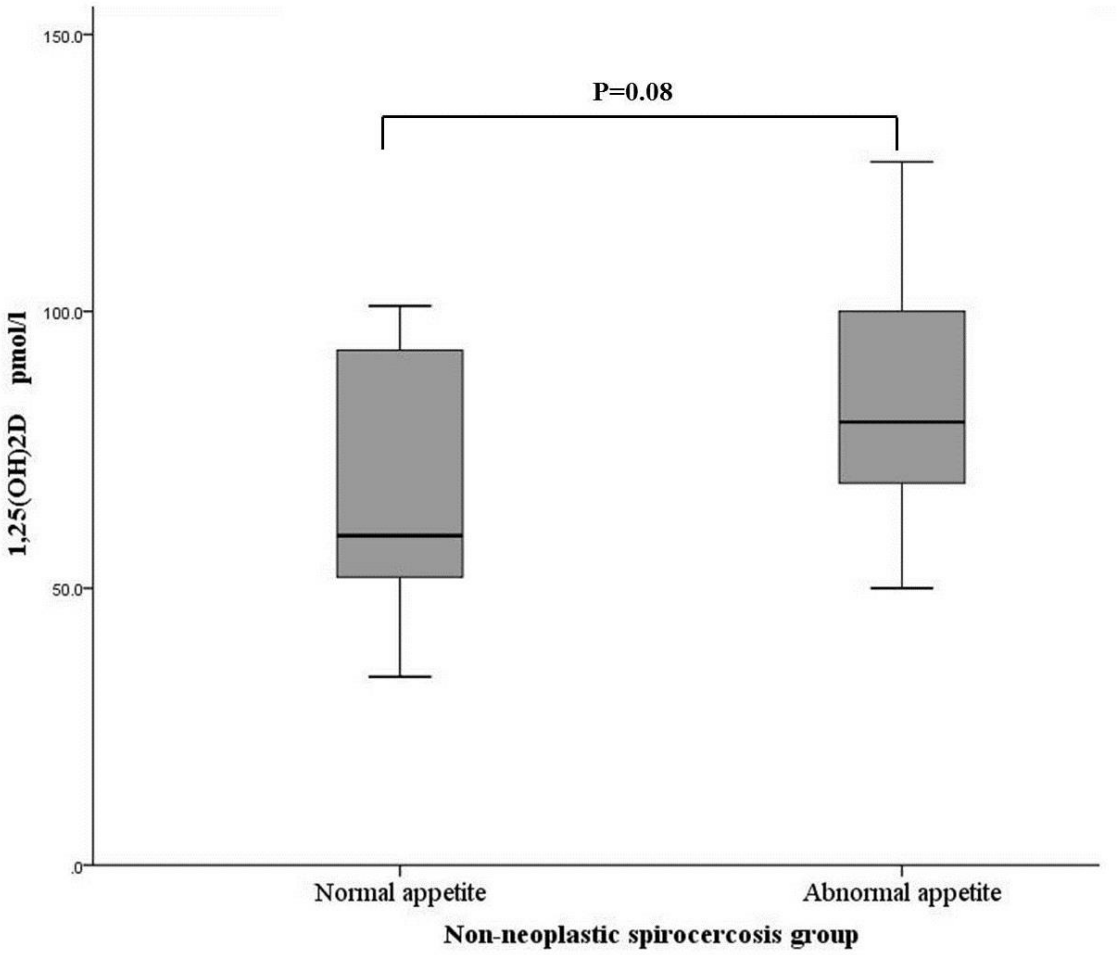


Figure 6: Box plot of the comparison between the serum 1,25$(OH)_2$D concentrations of dogs with normal or abnormal appetite in the non-neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

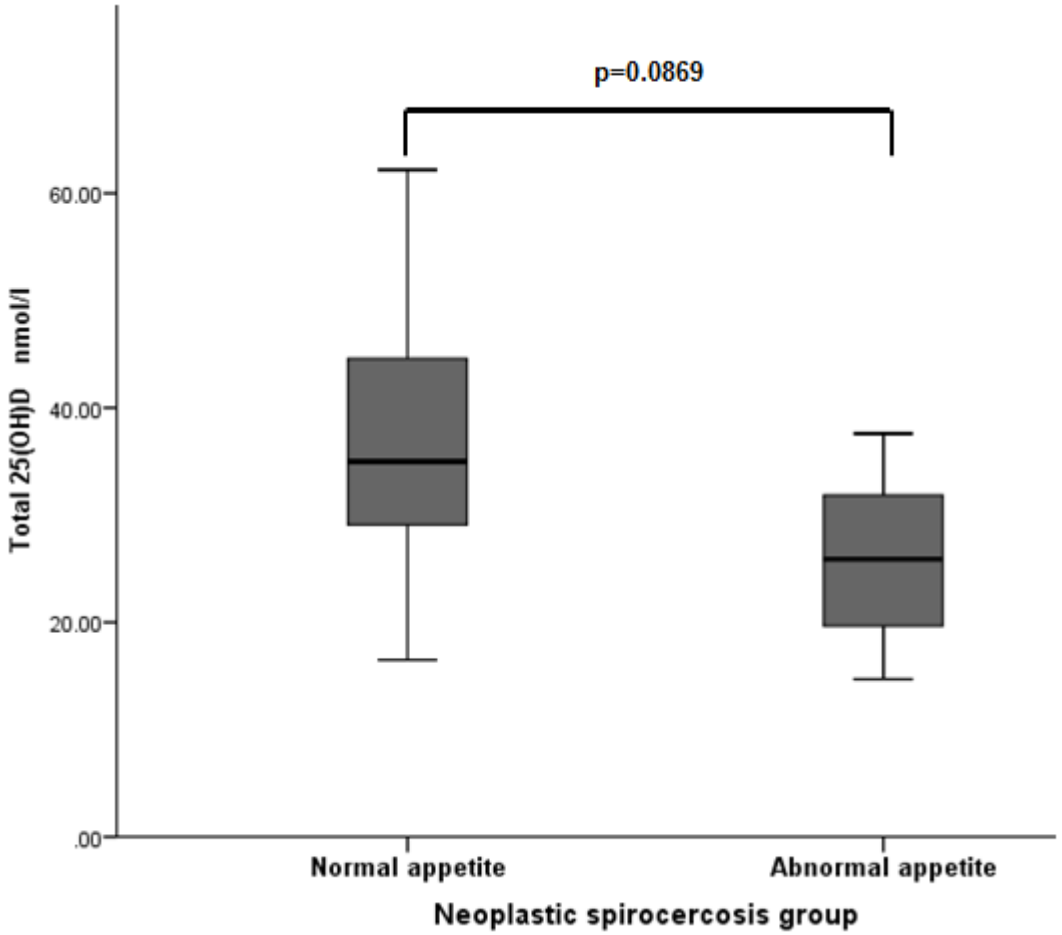


Figure 7: Box plot of the comparison between the serum 25(OH)D concentrations of dogs with normal or abnormal appetite in the neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

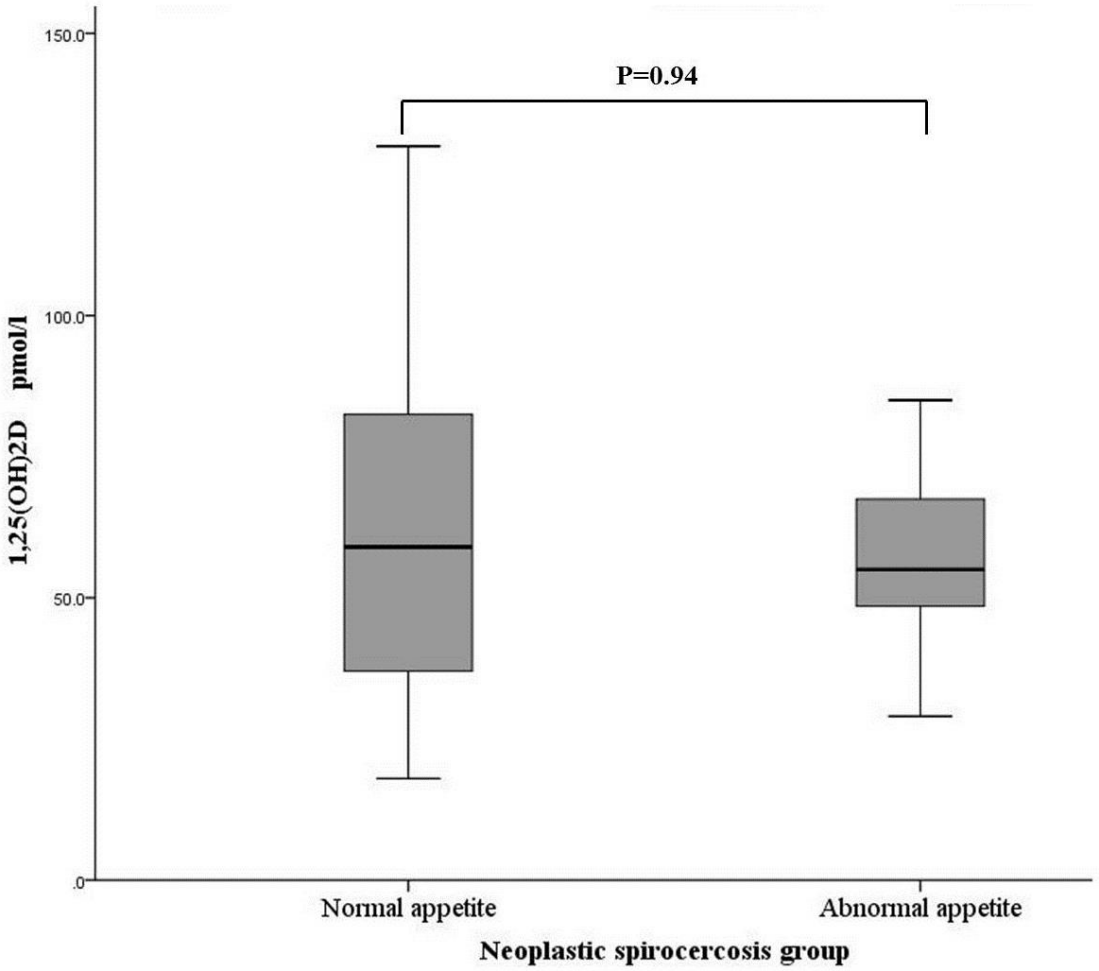


Figure 8: Box plot of the comparison between the serum 1,25$(OH)_2$D concentrations of dogs with normal or abnormal appetite in the neoplastic spirocercosis group using the Mann-Whitney U test. For each plot, the box represents the IQR, the horizontal line in the middle of the box represents the median, and the whiskers denote the range extending to 1.5 times the IQR from the 25th and 75th quartiles.

TABLES

Table 1 – Absolute numbers of dogs with normal and abnormal appetite, within the non-neoplastic and neoplastic spirocercosis groups, respectively.

	Normal appetite	Abnormal appetite
Non-neoplastic spirocercosis (n=25)	14	11
Neoplastic spirocercosis (n=26)	15	11

Table 2 - Body weight, sex and spay neuter status of dogs diagnosed with spirocercosis and healthy control dogs.

	Weight (kg) median and ranges	Sex and spay neuter status			
		Intact male	Neutered male	Intact female	Neutered female
Non-neoplastic spirocercosis (n=25)	30.2 (6.9-78)	6	4	9	6
Neoplastic spirocercosis (n=26)	23.2 (5.4-50)	12	2	7	5
Healthy dogs (n=24)	29.7 (5.4-70)	4	4	11	5

REFERENCES

1. Van der Merwe L, Kirberger RM, Clift S, et al. *Spirocerca lupi* infection in the dog: A review. Vet J 2008;176:294–309.

2. Dvir E, Clift SJ. Evaluation of selected growth factor expression in canine spirocercosis (*Spirocerca lupi*) - Associated non-neoplastic nodules and sarcomas. Vet Parasitol 2010;174:257-266.

3. Baneth G. Canine spirocercosis and associated neoplasia. Proceedings of the 33rd World Small Animal Veterinary Association 14th FECAVA Congress 2007.

4. Mazaki-Tovi M, Baneth G, Aroch I, *et al*. Canine spirocercosis: Clinical, diagnostic, pathologic, and epidemiologic characteristics. Vet Parasitol 2002;107:235-250.

5. Ranen E, Lavy E, Aizenberg I, et al. Spirocercosis-associated esophageal sarcomas in dogs: A retrospective study of 17 cases. Vet Parasitol 2004;119:209–221.

6. Traversa D, Avolio S, Modrý D, *et al*. Copromicroscopic and molecular assays for the detection of cancer-causing parasitic nematode *Spirocerca lupi*. Vet Parasitol 2008;157:108-116.

7. Dvir E, Clift SJ, Williams MC. Proposed histological progression of the *Spirocerca lupi*-induced oesophageal lesion in dogs. Vet Parasitol 2012;168:71–77.

8. Bailey WS. Parasites and cancer: sarcoma in dogs associated with *Spirocerca lupi*. Ann NY Acad Sci 1963;108:890–923.

9. Seibold HR, Bailey WS, Hoerlein BF, et al. Observations on the possible relation of malignant esophageal tumors and *Spirocerca lupi* lesions in the dog. Am J Vet Res 1955;16:5–14.

10. Dvir E, Kirberger RM, Mukorera V, et al. Clinical differentiation between dogs with benign and malignant spirocercosis. Vet Parasitol 2008;155:80–88.

11. Lavy E, Aroch I, Bark H, Markovics A, Aizenberg I, Mazaki-Tovi M, Hagag A, Harrus S. Evaluation of doramectin for the treatment of experimental canine spirocercosis. Vet Parasitol 2002;109:65-73.

12. Kelly PJ, Fisher M, Lucas H, Krecek RC. Treatment of esophageal spirocercosis with milbemycin oxime. Vet Parasitol 2008;156:358-360.

13. Dvir E, Kirberger RM, Malleczek D. Radiographic and computed tomographic changes and clinical presentation of Spirocercosis in the dog. Vet Radiol Ultrasound 2001;42:119-129.

14. Gal A, Kleinbart S, Aizenberg Z, Baneth G. Aortic thromboembolism associated with *Spirocerca lupi* infection. Vet Parasitol 2005;130:331-335.

15. Du Plessis CJ, Keller N, Millward IR. Aberrant extradural spinal migration of *Spirocerca lupi*: Four dogs. J Small Anim Pract 2007;48:275-278.

16. Klainbart S, Mazaki-Tovi M, Auerbach N, *et al*. Spirocercosis-associated pyothorax in dogs. Vet J 2007;173:209-214.

17. Mylonakis ME, Rallis T, Koutinas AF, *et al*. Clinical signs and Clinicopathologic abnormalities in dogs with clinical spirocercosis: 39 cases (1996-2004). J Am Vet Med Assoc 2006;228:1063-1067.

18. Lobetti RG. Survey of the incidence, diagnosis, clinical manifestations and treatment of *Spirocerca lupi* in South Africa. J S Afr Vet Assoc 2007;71:43-46.

19. Christie J, Schwan EV, Bodenstein LL, Sommerville JE, van der Merwe LL. The sensitivity of direct faecal examination, direct faecal floatation, modified centrifugal faecal floatation and centrifugal sedimentation/floatation in the diagnosis of canine spirocercosis. J S Afr Vet Assoc 2011;82:71-75.

20. Fisher MM, Morgan JP, Krecek RC, Kelly PJ. Radiography for the diagnosis of spirocercosis in apparently healthy dogs, St. Kitts, West Indies. Vet Parasitol 2009;160:337-339.

21. Ranen E, Dank G, Lavy E, *et al*. Oesophageal sarcomas in dogs: Histological and clinical evaluation. Vet J 2008;178:78-84.

22. Mukorera V, van der Merwe LL, Lavy E, Aroch I, Dvir E. Serum alkaline phosphatase activity is not a marker for neoplastic transformation of esophageal nodules in canine spirocercosis. Vet Clin Pathol 2011;40:389-392.

23. Mukorera V, Dvir E, van der Merwe LL, Goddard A. Serum C-Reactive Protein Concentration in Benign and Malignant Canine Spirocercosis. J Vet Intern Med 2011;25:963-966.

24. Mukorera V. Expression of vascular endothelial growth factor in dogs with *Spirocerca lupi*-associated neoplastic transformation [Dissertation]. Pretoria: Faculty of Veterinary Science, University of Pretoria;2012.

25. Schmidt T, Carmeliet P. Angiogenesis: A target in solid tumors, also in leukemia? Hematology Am Soc Hematol Educ Program 2011;2011:1-8.

26. Tekin D, Dursun AD, Bastug M, Karaorman G, Ficicilar H. The effects of acute and intermittent hypoxia on the expressions of HIF-1alpha and VEGF in the left and right ventricles of the rabbit heart. Anadolu Kardiyol Derg 2011;11:379-385.

27. Pazzi P. Coagulation abnormalities in canine spirocercosis [Dissertation]. Pretoria: Faculty of Veterinary Science, University of Pretoria;2012.

28. Bailey WS, Cabrera DJ, Diamond DL. Beetles of the Family Scarabaeidae as Intermediate Hosts for Spirocerca Lupi. J Parasitol 1963;49:485-488.

29. Porta C, Larghi P, Rimoldi M, et al. Cellular and Molecular Pathways Linking Inflammation and Cancer. Immunobiology 2009;214:761-777.

30. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-Related Inflammation. Nature 2008;454:436-444.

31. Lu H, Ouyang W, Huang C. Inflammation, a Key Event in Cancer Development. Molecular Cancer Research: MCR 2006;4:221-233.

32. Shacter E, Weitzman SA. Chronic Inflammation and Cancer. Oncology 2002;16:217;Feb-226.

33. Herrera LA, Benitez-Bribiesca L, Mohar A, Ostrosky-Wegman P. Role of infectious diseases in human carcinogenesis. Environ Mol Mutagen 2005;45:284-303.

34. Moss SF, Blaser MJ. Mechanisms of disease: inflammation and the origins of cancer. Nat Clin Pract Onc 2005;2:90-97.

35. Vennervald BJ, Polman K. Helminths and Malignancy. Parasite Immunol 2009;31:686-696.

36. Mulvenna J, Sripa B, Brindley PJ, et al. The Secreted and Surface Proteomes of the Adult Stage of the Carcinogenic Human Liver Fluke Opisthorchis Viverrini. Proteomics 2010;10:1063-1078.

37. Smout MJ, Laha T, Mulvenna J, et al. A Granulin-Like Growth Factor Secreted by the Carcinogenic Liver Fluke, Opisthorchis Viverrini, Promotes Proliferation of Host Cells. PloS Pathogens 2009;5:e1000611.

38. Kaewpitoon N, Kaewpitoon SJ, Pengsaa P, Sripa B. Opisthorchis Viverrini: The Carcinogenic Human Liver Fluke. World J Gastroenterology 2008;14:666-674.

39. Curiel TJ, Coukos G, Zou L, et al. Specific Recruitment of Regulatory T Cells in Ovarian Carcinoma Fosters Immune Privilege and Predicts Reduced Survival. Nat Med 2004;10:942-949.

40. Liyanage UK, Moore TT, Joo HG, et al. Prevalence of Regulatory T Cells is Increased in Peripheral Blood and Tumor Microenvironment of Patients with Pancreas Or Breast Adenocarcinoma. J Immunology 2002;169:2756-2761.

41. Wolf AM, Wolf D, Steurer M, et al. Increase of Regulatory T Cells in the Peripheral Blood of Cancer Patients. Clinical Cancer Research 2003;9:606-612.

42. Woo EY, Chu CS, Goletz TJ, et al. Regulatory CD4(+)CD25(+) T Cells in Tumors from Patients with Early-Stage Non-Small Cell Lung Cancer and Late-Stage Ovarian Cancer. Cancer Res 2001;61:4766-4772.

43. Horiuchi Y, Tominaga M, Ichikawa M, et al. Increase of Regulatory T Cells in the Peripheral Blood of Dogs with Metastatic Tumors. Microbiology & Immunology 2009;53:468-474.

44. Beyer M, Schultze JL. Regulatory T Cells in Cancer. Blood 2006;108:804-811.

45. Dvir E, Schoeman JP, Clift SJ, et al. Immunohistochemical Characterization of Lymphocyte and Myeloid Cell Infiltrates in Spirocercosis-Induced Oesophageal Nodules. Parasite Immunol 2011;33:545-553.

46. Dvir E, Mellanby RJ, Kjelgaard-Hansen M, Schoeman JP. Plasma IL-8 concentrations are increased in dogs with spirocercosis. Vet Parasitol 2012;190:185-190.

47. Bailey WS. Spirocerca lupi: a continuing inquiry. J Parasitol 1972;58:3–22.

48. Mawer EB, Hann JT, Berry JL, Davies JL. Vitamin D metabolism in patients intoxicated with ergocalciferol. ClinSci 1985;68:135-141.

49. Schenck PA, Chew DJ, Nagode LA, Rosol TJ. Disorders of calcium: Hypercalcemia and hypocalcemia. In: DiBartola SP (ed). Fluid, electrolyte, and acid-base disorders in small animal practice, 3rd ed. Missouri: Elsevier, Inc.;2006:122-194.

50. Hazewinkel H, Tryfonidou M. Vitamin D_3 metabolism in dogs. Mol Cel Endocrinol 2002;197:23-33.

51. Mullin GE, Dobs A. Vitamin D and its role in cancer and immunity: A prescription for sunlight. Nutr Clin Pract 2007;22:305-322.

52. Greco DS. Endocrine Causes of Calcium Disorders. Topics in Compan An Med 2012;27:150–155.

53. Christakos S, Ajibade D, Dhawan P, et al. Vitamin D: Metabolism. Endocrinol Metab Clin 2010;39:243-253.

54. Bikle D. Nonclassic actions of vitamin D. J Clin Endocrinol Metab 2009;94:26-34.

55. Galvão JFB, Nagode LA, Schenck PA, Chew J. Calcitriol, calcidiol, parathyroid hormone, and fibroblast growth factor-23 interactions in chronic kidney disease. J Vet Emerg Crit Care 2013;23(2):134–162.

56. Barber P. Investigation of hypercalcaemia and hypocalcaemia. In: Mooney, CT, Peterson, ME (eds) BSAVA Manual of canine and feline endocrinology (3rd end). BSAVA publications, England. 2004;5:26-42.

57. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. Current Opinion in Pharmacology 2010;10:482-496.

58. Rosen CJ, Adams JS, Bikle DD, et al. The nonskeletal effects of vitamin D: An endocrine society scientific statement. 2012;33(3):456-492.

59. Borges MC, Martini LA, Rogero MM. Current perspectives on vitamin D, immune system, and chronic diseases. Nutrition 2011;27:399-404.

60. Bikle D. Vitamin D regulation of immune function. Vitam Horm. 2011;1:1-21.

61. Cantorna MT. Why do T cells express the vitamin D receptor? Ann NY Acad Sci 2011;1217:77-82.

62. Krishnan AV, Trump DL, Johnson CS, Feldman D. The role of vitamin D in cancer prevention and treatment. Endocrinol Metab Clin N Am 2010;39:401-418.

63. Fleet JC, Desmet M, Johnsons R, Li Y. Vitamin D and cancer: a review of molecular mechanisms. Biochem J 2012;441:61-76.

64. Malone EK, Rassnick KM, Wakshlag JJ, et al. Calcitriol (1,25-dihydroxycholecalciferol) enhances mast cell tumour chemotherapy and receptor tyrosine kinase inhibitor activity *in vitro* as single-agent activity against spontaneous occurring canine mast cell tumours. Vet Comp Onc 2010;8:209-220.

65. Wakshlag JJ, Rassnick KM, Malone EK, et al. Cross-sectional study to investigate the association between vitamin D status and cutaneous mast cell tumours in Labrador retrievers. Br J Nutr 2011;106:60-63.

66. Kaewsakhorn T, Kisseberth WC, Capen CC, et al. Effects of calcitriol, seocalcitiol, and medium-chain triglyceride on a canine transitional cell carcinoma cell line. Anticancer Res 2005;25:2689-2696.

67. Kunakornsawat S, Rosol TJ, Capen CC, et al. Effects of $1,25(OH)_2D_3$, $25(OH)D_3$, and EB1089 on cell growth and vitamin D receptor mRNA and 1_{α}-hydroxylase mRNA expression in primary cultures of the canine prostate. J Steroid Biochem Mol Biol 2004;89-90:409-412.

68. Chakraborti CK. Vitamin D as a promising anticancer agent. Indian J Pharmacol 2011;43(2):113-120.

69. Campbell FC, Xu H, El-Tanani M, Crowe P, Bingham V. The yin and yang of vitamin D receptor (VDR) signaling in neoplastic progression: Operational networks and tissue-specific growth control. Biochem Pharmacol 2010;79:1-9.

70. van Leeuwen JPTM, Pols HAP. Vitamin D: Cancer and differentiation. In: Feldman, Pike, Glorieux (eds) Vitamin D (2nd end). Elsevier Saunders publications 2005;89:1571-1597.

71. Welsh J. Cellular and molecular effects of vitamin D on carcinogenesis. Arch Biochem Biophys 2012;523:107–114.

72. Lappe JM, Travers-Gustafson D, Davies KM, Recker RR, Heaney RP. Vitamin D and calcium supplementation reduces cancer risk: Results of a randomized trial. Am J Clin Nutr 2007;85:1586–1591.

73. Manson JE, Mayne ST, Clinton SK. Vitamin D and prevention of cancer - Ready for prime time? New Engl J Med 2011;364(15):1385-1387.

74. Kun-Chun C, Tai CC. Vitamin D for the prevention and treatment of pancreatic cancer. World J Gastroentero 2009;15(27):3349-3354.

75. Gorham ED, Garland CF, Garland FC, Grant WB, Mohr SB, *et al*. Vitamin D and prevention of colorectal cancer. J Steroid Biochem 2005;97:179–194.

76. Garland CF, Gorham ED, Mohr SB, Garland FC. Vitamin D for cancer prevention: Global perspective. Ann Epidemiol 2009;19:468-483.

77. Hollis BW, Horst RL. The assessment of circulating 25(OH)D and 1,25(OH)$_2$D: Where we are and where we are going. J Steroid Biochem 2007;103:473-476.

78. Peterlik M. Vitamin D insufficiency and chronic diseases: Hype and reality. Food Funct 2012;3:784-794.

79. Mawer EB, Berry JL, Cundall JP, et al. A sensitive radioimmunoassay using a monoclonal antibody that is equipotent for ercalcitriol and calcitriol (1,25-dihydroxy vitamin D2 and D3). Clin Chim Acta 1990;190:199-209.

80. Berry JL, Martin J, Mawer EB. 25-Hydroxyvitamin D assay kits: speed at cost of accuracy? In: Norman AW, Bouillion R, Thomasset M (eds). Vitamin D endocrine system: Structural, biological, genetic and clinical aspects. University of California: Riverside, California;2000:797-800.

81. Holowaychuk MK, Birkenheuer AJ, Li J, et al. Hypocalcemia and hypovitaminosis D in dogs with induced endotoxemia. J Vet Intern Med 2012;26:244-251.

82. Kitanaka S, Isojima T, Takaki M, et al. Association of vitamin D-related gene polymorphisms with manifestation of vitamin D deficiency in children. Endocrinol J 2012;59:1007-1014.

83. Morrison WB. Inflammation and cancer: A comparative view. J Vet Intern Med 2012;26:18–31.

84. Dvir E, Clift S. Evaluation of selected growth factor expression in canine spirocercosis (*Spirocerca lupi*)-associated non-neoplastic nodules and sarcomas. Vet Parasitol 2010;174:257-266.

85. Gow AG, Else R, Evans H, et al. Hypovitaminosis D in dogs with inflammatory bowel disease and hypoalbuminemia. J Small Anim Pract 2011;52:411-418.

86. Pappa MH, Gordon CM, Saslowsky TM, et al. Vitamin D status in children and young adults with inflammatory bowel disease. Pedriatics 2006;118:1950-1961.

87. Abreu MT, Kantorovich V, Vasiliauskas EA, et al. Measurement of vitamin D levels in inflammatory bowel disease patients reveals a subset of Crohn's disease patients with elevated 1,25-dihydroxivitamin D and low bone mineral density. Gut 2004;53:1129-1136.

88. Lo CW, Paris PW, Clemens TL, et al. Vitamin D absorption in healthy subjects and in patients with intestinal malabsorption syndromes. Am J ClinNutr 1985;42:644-649.

89. Waldron JL, Ashby HL, Cornes MP, *et al*. Vitamin D: a negative acute phase reactant. J Clin Pathol 2013;66:620–622.

90. Tuohimaa P. Vitamin D, aging, and cancer. Nutr Res 2008;66:147-152.

91. Rassnick KM, Muindi JR, Johnson CS, et al. *In vitro* and *in vivo* evaluation of combined calcitriol and cisplatin in dogs with spontaneous occurring tumours. Cancer Chemother Pharmacol 2008;62:881-891.

92. Hostutler RA, DiBartola SP, Chew DJ, et al. Comparison of the effects of daily and intermittent-dose calcitriol on serum parathyroid hormone and ionized calcium concentrations in normal cats and cats with chronic renal failure. Vet Intern Med 2006;20:1307–1313.

93. Galler A, Tran JL, Krammer-Lukas S, et al. Blood vitamin levels in dogs with chronic kidney disease. Vet J 2012;192:226–231.

Appendix A

ONDERSTEPOORT
VETERINARY ACADEMIC HOSPITAL

Patient sticker containing all client and patient details

Client Consent Form:

Dear Client

Your pet _____ has been diagnosed with suspected Spirocercosis. The incidence of this parasitic disease is increasing dramatically and we are trying to improve our ability to diagnose infection earlier in patients, determine if the nodule has become cancerous and determine the best form of treatment and prevention of infection. To achieve these goals we would like to perform some procedures on your pet. These are listed below.

A. Whilst under anaesthetic an oesophageal endoscopy (camera passed down the oesophagus) will be performed. This is considered routine for these cases.

B. Whilst under anaesthetic, a computer tomography (CT) scan will be performed, this will require an additional 20 minutes of anaesthesia.

C. We would collect additional blood samples from your pet (< 20 ml of blood).

The above-mentioned procedures hold no additional risk for your pet.

The above-mentioned CT and additional blood samples will be subsidised by the Veterinary Faculty

D. If your pet undergoes therapy you will undertake to return for re-evaluation by means of endoscopy at a predetermined time-point. The costs for this routine follow up procedure will be calculated at cost of materials used only. The follow up evaluations are routine but often waived by the client due to an improvement in clinical signs, however this does not mean that your dog is healed. We have to insist on a follow up evaluation, as it is vital to assess efficacy of treatment protocols.

E. If at any stage you decide to put your pet to sleep due to poor prognosis for recovery or escalating costs we would request permission to perform a full post mortem examination (autopsy). Disposal options would be arranged as standardly done at the OVAH.

F. CONSENT FOR EXTRA-LABEL DRUG USE

The drug Dectomax® (Doramectin®) has proven efficacy against spirocercosis. It is however not registered in South Africa for use in dogs, necessitating client consent. Side effects are rare and include transient drowsiness and blindness, occurring usually due to accidental over dosage. Approximately 35% of dogs of herding breeds are very susceptible to these side effects and may become very ill and comatose. All such dogs will need to have a blood test performed to determine susceptibility prior to commencing treatment

I Mr/Mrs/Ms/Dr/Prof/_____

Hereby acknowledge that I have received the information brochure on spirocercosis in dogs and I hereby give permission for the additional tests mentioned from A-C to be performed on my pet over the following 3 months.

Initial: _____

I also give permission for the extra-label use of Doramectin® in my dog as indicated in F above

Initial: _____

Signed at: _____

Date : _____

Signature _____

Duty Clinician :: Print: _____

Sign: _____

Appendix B

Client Spirocercosis information brochure

Spirocercosis in Dogs

Spirocerca lupi is a worm that starts its lifecycle in the dung-beetle which contains infective larvae. Infective larvae are also found in animals which eat the dung beetles, such as mice, lizards and birds. Dogs become infested when eating either the beetle or the so-called transport hosts. The worm larvae are released by the process of digestion and move through the stomach wall from where they migrate via the walls of the major blood vessels to eventually reach the aorta, the largest blood vessel in the body. They then rest and grow to maturity here, a process which takes 100 days. Once mature they then migrate across the aortic wall towards the oesophagus, which lies in between the lungs, together with the aorta, trachea and heart. Once the worms reach the oesophagus, they burrow into the wall and make a small nodule. Here they reach sexual maturity and start laying eggs. Each nodule can contain many worms. When the eggs are passed in the faeces, they once again infect the dung beetles

The worm causes disease in many ways: Fever, infection, lung infection, rupture of the major blood vessels due to aneurysms, obstruction of the oesophagus due the nodule, intractable vomiting due to oesophageal irritation and eventual transformation of the nodule into a cancer with spread to the lungs.

There is, to date, no method of detecting infection with this parasite prior to it settling in the oesophagus and starting to pass eggs. These eggs can be detected in stool examinations, but the test is not very sensitive, as the worms only pass very small eggs, intermittently. Once clinical signs of vomiting have been detected chest x-rays will often show a suspicious area behind the heart. Confirmation of the diagnosis is usually by passing an endoscope (camera) into the oesophagus and visualising the nodule. If there is a suspicion that the nodule may be cancerous a biopsy is taken.

Treatment is initiated and response to treatment is usually established by a follow up endoscopy after 48-56 days.

In complicated cases the diagnostic and treatment protocol are individualised.

Appendix C

Consent form for vitamin D trial (Healthy dogs)

Spirocerca lupi (*S. lupi*) is a worm that infects mainly the dogs and causes a disease called Spirocercosis.

The adult worms of *S. lupi* are located in a nodule within the oesophagus wall where the females shed small eggs that are transferred to the gastrointestinal tract and are excreted in the faeces of dog. These eggs are ingested by beetles or animals like birds, hedgehogs, lizards, mice, and rabbits. The eggs will develop to a larvae stage and be passed to the dog in the food chain, when the dogs eats the beetle or the other animals infected by the larva of *S. lupi*. In the dog, the larvae penetrates the stomach wall and migrates to the aorta through the gastric arteries, were it develops to immature adults. About 3 months post-infection, the larva migrates to the oesophagus and matures to the adult form, leading to the development of a nodule involving the adult worms. The oesophageal nodules grow and a transformation into a tumour can occur.

A study with the measurement of vitamin D in dogs infected with *S. lupi* is being performed and the objectives of this study is to determine if the levels of this vitamin can be used as a indicator value of transformation of the benign nodules into tumours. For this purpose it is necessary for to measure the levels of vitamin D in healthy dogs and compare those levels with the dogs infected with *S. lupi*.

For this study to be reliable we need to screen your pet for other diseases and *S. lupi*, meaning that we will need to perform thoracic radiographs, faecal floatation and routine blood samples for haematology and serum chemistry, which tests will all be free of charge and done before castration or spaying your animal. The same blood samples will be stored for further evaluation of the vitamin D levels.

Do not hesitate to contact Dr Chantal Rosa, on the following numbers, if any queries regarding this study. Office: 012 529 8002, SAM Clinics 012 529 8128/8096, Cell number 0712152162, email: chantal.rosa@up.ac.za

I, (full name) _____hereby give permission for the dog under my care (Dog's name) _____ Breed_____ Age _____ Sex_____

To participate in the clinical study on vitamin D levels in *S. lupi* infected dogs at the Onderstepoort Veterinary Academic Hospital.

The trial has been explained to me and l understand that the study will in no way harm my dog and that the costs of additional testing will be borne by the trial fund.

Signed at_____ on the_____ day of (month) _____ (year) _____

Signature of owner/authorised person_____

Home Tel_____

Office Tel_____

Cell _____

Appendix D

Physical examination and anamnesis at admission

History and Clinical Examination form : *S lupi* prospective study

Unique patient number

Patient sticker

History

Signalment:	Breed:	
	Sex:	
	Neutered:	
	Age:	

Environment

Physical address where the dogs live _____

_____

_____

How many dogs in the household? _____

What breeds: _____

_____

Type of home:

Suburban detached home	Townhouse	Plot/farm

Does the client observe the following in their garden?

Dung beetles	Lizards	Frogs	Small mammals

How would the owner describe the dogs' lifestyle?

Only indoor	Free access indoor and outdoor	Only outdoor	Working dog

If the dog is a working dog, what does it do?

Herding	Hunting	Guard	Agility etc

Other _____

_____

_____

How would the owner describe the dogs' nature

Placid and lazy	Active and inquisitive	Active but not inquisitive

Does the client take the dog for walks or to visit farms over weekends?

Where

How frequently? _____

_____

How does the client manage the dog faeces in their yard?

Removes daily	Removes 2x /week	Removes weekly	Leaves (large property)

Does the client have a compost heap? | |

Does the client feed any fresh raw offal to the dogs?

Beef	Pork	Lamb	Chicken

Where is this offal obtained from?

Abbatoir	Butchery	Plot/farm

Area? _____

History

Why is the client bringing the dog in?

Regurgitation	
Vomiting	
Vomiting/regurgitation	
Weight loss	
Inappetence/anorexia	
Coughing	
Decreased exercise tolerance	
Increased respiratory rate	
Thickened limbs/lameness	

Other | |

Details:

What is the duration of the dogs' clinical signs?

Eating habits:

Is the dog anorexic, not interested in food at all?	Yes	No
Is the dog hungry, but when approaches bowl seems to back off?	Yes	No
Does the dog show excessive swallowing movements when eating?	Yes	No

Clinical Examination

Temperature		Pulse	

Respiratory rate at rest :

RESPIRATORY SYSTEM

Type of respiration:

Abdominal	Costo-Abdominal	Tucked abdomen (paradoxical)

Lung auscultation

Normal	Wheezes	Referred breath sounds	Crackles	Muffled

Is there a pleural effusion	Yes……No

If yes, perform a thoracocentesis, place the sample immediately into an EDTA tube and serum tube and send for cytology and culture

Thoracocentesis performed?	Yes	No

Thoracic radiographs must be requested

HEAD and NECK

Palpate all the salivary glands and lymph nodes on the head. Ensure that you differentiate between lymph nodes and glands.

Salivary glands	Mandibular (L)		Mandibular (R)		Parotid (L)		Parotid (R)	
Enlarged?	Y	N	Y	N	Y	N	Y	N
Palpation causes discomfort?	Y	N	Y	N	Y	N	Y	N
Feel round and very hard? (golfball)	Y	N	Y	N	Y	N	Y	N

Palpate the pharyngeal region? Does it cause discomfort and increased swallowing movements?	Y	N

GASTROINTESTINAL SYSTEM

Faecal Flotation	**Standard Method**	**Ova Found**	
		Identify	
	(see guide below)	**Degree of infestation**	1 + 2+ 3+ 4+
		(to be checked by clinician)	
	Special flotation		
	Day 1		
	Day 2		
	Sample collected for helminthology	Day 1	Day 2
	Any melaena?	**Y N**	

MUSCULOSKELETAL

Is there any thickening of the distal limbs? If yes, which limbs?	Y N
Is there any pain on deep palpation of the distal limbs If yes, which limbs	Y N
Is their any pain on manipulating the joints of the limbs? If yes, which joints?	Y N

	Y	N
Is there any effusion of the joints?		
If Yes, which joints		

If there is an effusion or pain of joint manipulation a joint tap must be performed? The fluid should be placed into EDTA, or, if insufficient, should be smeared onto a slide and sent to clinicopathology unstained

Which samples were taken?

URINARY SYSTEM

Urine to be collected by cystocentesis and UA performed	
Any Evidence of UTI on sediment?	
If yes. Describe	

Proteinuria on dipstick?	1+	2+	3+	4+
pH				

If protein is >2+ with SG < 10.15 or > 3+ with any SG, and the sediment is inactive, request a UPC.

UPC results	
Urine SG	
Hydration status	
Fluid therapy?	

Appendix E

Blood smear protocol

- Hair will be clipped from the ventral surface of the ear near the caudal margin with clippers or curved scissors.
- A 23G hypodermic needle will be used to pierce the skin or the ear near the caudal margin but away from the marginal ear vein.
- Blood will be massaged toward the needle hole.
- The first drop of blood appearing here will be collected with a spreader slide and with this drop a blood smear will be made.
- The smear, if deemed of sufficient quality by the clinician on duty, will be evaluated according to standard protocol.

Urine analysis protocol

- A bladder will be palpated prior to attempting cystocentesis
- The ventral caudal abdomen will be shaved if necessary
- Alcohol swabs will be used to clean the skin prior to cystocentesis
- A 22G hypodermic needle with a sterile 5ml syringe will be used to penetrate the skin and bladder wall and collect the urine
- The needle will then be removed once a sample is obtained
- Urine analysis will include a urine dipstick analysis (Cobur-9; Roche) and urine specific gravity determination using a refractometer. The remaining urine will be spun down and the sediment mixed with a drop of Steinheimer stain and examined for cells, casts, bacteria, crystals. A thick-thin smear will then be made and diff quick stain applied to ensure no bacteria.
- All the above information and findings will be recorded on a standard OVAH urine analysis form

Faecal analysis protocol

- At least 1 gram of faeces from a gloved digital rectal will be used
- The sample will be placed in 5mls $NaNO_3$ flotation fluid (SG 1.22) under go centrifugation (1400G) SG 1.22) for 10 minutes after which 0.1ml of the supernatant will be aspirated from the surface for microscopic examination.
- The cover slip will then be placed onto a clean slide and examined for the presence of ova, including *S. lupi*.

Appendix F

Summary of sample collection and handling

Sample	Sample handling	Procedure	Storage
1. Serum	• Collection prior to Treatment	• Storage • Albumin • Globulin • Total serum proteins • ALT • Creatinine • Total calcium • Inorganic phosphate	• Centrifuge for 8 minutes at 2616 g (4000rpm) • Aliquotation in cryovials with 500uL in each • Stored at -80ºC
2. EDTA	• Collection prior to treatment • Blood smear within 30min • Evaluate sample within 4 hours for full blood count	• CBC • Blood film evaluation	• Room temperature • EDTA to be stored in fridge before sending for PCR • Centrifugation of remaining EDTA for 8 minutes at 2616 g • Plasma to be stored at -80ºC • Store whole blood
3. Faeces	• Collect at least 0.5 gram faeces per rectum	• Faecal flotation • Modified NaNO3 centrifuge faecal evaluation	

Appendix G

***SPIROCERCA LUPI* PATIENT ENDOSCOPY FORMS :**
(NB – Only the video endoscope must be used to enable data capture)

Date: _____ Unique Patient no._____

Patient Sticker

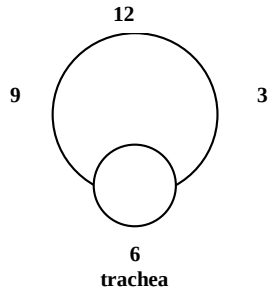
Scope number : _____

NUMBER of nodules: _____

For EACH nodule:

1. Length of nodule (cm): (measure using distance to cranial pole at level of canine and subtract from distance to caudal pole from canine.)

2. Position of nodule :



3. Appearance of nodules (IN THE DOG):

Smooth or Cauliflower like? , Operculum visible?, Necrotic or ulcerated?

Nodule and Nº.	**Length**		**Insufflated (%)**	**Position**	**Appearance (specify)**				
	Cranial margin	**Caudal margin**			**Smooth Cauliflower**	**Operculum or worm visible?**		**Necrotic or ulcerated?**	
1					Smooth Cauliflower	Y	N	Y	N
2					Smooth Cauliflower	Y	N	Y	N
3					Smooth Cauliflower	Y	N	Y	N
4					Smooth Cauliflower	Y	N	Y	N
5					Smooth Cauliflower	Y	N	Y	N
6					Smooth Cauliflower	Y	N	Y	N
7					Smooth Cauliflower	Y	N	Y	N
8					Smooth Cauliflower	Y	N	Y	N

General:

Is oesophageal ulceration or oesophagitis present? Y / N

Are worms visible? Y / N

Do any nodules involve the cardia? (perform J manoeuvre) Y / N

Approximate max % of insufflated oesophageal lumen compromised: _____

Biopsied ?

Y	**N**

Histopathology S number: _____

Procedure recorded on the DVD: ☐

Follow up Scoping dates:

(i) _____

(ii) _____

(iii) _____

Any further description:

_____

_____

_____

_____

_____

_____

_____

_____

_____

_____

Appendix H

Anaesthetic Protocol

Anaesthesia is expected to be approximately 5-10 minutes for thoracic radiographs and 15-20 minutes for oesophageal endoscopy.

Pre-anaesthesia:

Diazepam: 0.2mg/kg, IV, 5 min prior to induction.

Induction:

Propofol: IV, 4mg/kg or to effect to point of intubation and maintenance with isoflurane becomes possible.

Maintenance:

Isoflurane: To maintain stage 3, plane 2 of anaesthesia.

Appendix I

Unique Number

SUSPECTED *S LUPI* PATIENT

DATA COLLECTION CHECK LIST

Patient sticker

Client consent and extra-label forms signed ☐

Client information leaflet handed out ☐

Full clinical examination

- ☐ History form completed
- ☐ Clinical examination form completed

Faecal flotation

- ☐ Performed in student lab with flotation fluid, evaluated by clinician

Urinalysis

- ☐ Student UA performed

Haematology

- ☐ Collect 2 EDTA tubes
- ☐ Request: standard complete blood count
- ☐ Store EDTA

Serum chemistry & Coagulation screen

- ☐ Collect 2 serum tubes AND 1 Citrate tube
- ☐ Request : TSP, alb, glob, ALP, TEG
- ☐ Store serum and citrate for PT/PTT/AT/D-Dimer/Fibrinogen/C-Reactive protein

Diagnostic imaging

- ☐ 2 survey thoracic views

Oesophageal and gastric endoscopy (video-endoscope)

- ☐ Complete specific endoscopy form and record on DVD

Pathology

- ☐ Biopsy of enlarged salivary glands
- ☐ Histopathology of any biopsied material
- ☐ Post Mortem:

Follow up visits

Day 7 or 14 (depending on treatment protocol)

- ☐ Doramectin injection

Day 48-56

- ☐ Follow up endoscopy

Clinician admitting the case:_____

Primary clinician involved with the case:_____

PUBLISHED PAPERS EMANATING FORM THIS DISSERTATION

The results of this study have been published in the Journal of Veterinary Internal Medicine (J Vet Intern Med 2013;27:1159–1164) in 2013 and presented at the 22nd ECVIM-CA Annual Congress, 2012, in Maastricht, the Netherlands.