Pathogenesis and Treatment of
Canine Thyroid Tumors

Miguel Campos

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Pathogenesis and Treatment of Canine Thyroid Tumors

Miguel Fonseca e Campos

Dissertation submitted in fulfillment of the requirements for the degree of Doctor of Philosophy (PhD) in Veterinary Sciences

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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ABCB1</td>
<td>ATP-binding cassette, sub-family B, member 1</td>
</tr>
<tr>
<td>ABCC1</td>
<td>ATP-binding cassette, sub-family C, member 1</td>
</tr>
<tr>
<td>AKT1</td>
<td>v-Akt murine thymoma viral oncogene homolog 1</td>
</tr>
<tr>
<td>AKT2</td>
<td>v-Akt murine thymoma viral oncogene homolog 2</td>
</tr>
<tr>
<td>ALARA</td>
<td>As Low As Reasonably Achievable</td>
</tr>
<tr>
<td>APES</td>
<td>3-Aminopropyltriethoxysilane</td>
</tr>
<tr>
<td>BRAF</td>
<td>v-Raf murine sarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>bTSH</td>
<td>Bovine thyrotropin</td>
</tr>
<tr>
<td>c-Kit</td>
<td>Stem cell factor receptor</td>
</tr>
<tr>
<td>CALCA</td>
<td>Calcitonin-related polypeptide alpha</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3’,5’-cyclic monophosphate</td>
</tr>
<tr>
<td>Cox</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Cox-1</td>
<td>Cyclooxygenase-1</td>
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<tr>
<td>Cox-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>CSF1R</td>
<td>Colony stimulating factor 1 receptor</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>DFS</td>
<td>Disease-free survival</td>
</tr>
<tr>
<td>dFTC</td>
<td>Differentiated follicular cell thyroid carcinoma</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FF-PE</td>
<td>Formalin-fixed paraffin-embedded</td>
</tr>
<tr>
<td>FLT-3</td>
<td>Fms-like tyrosine kinase 3</td>
</tr>
<tr>
<td>FTC</td>
<td>Follicular cell thyroid carcinoma</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>Guanosine triphosphatase</td>
</tr>
<tr>
<td>H-RAS</td>
<td>Harvey rat sarcoma viral oncogene homolog</td>
</tr>
<tr>
<td>HE</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
</tr>
<tr>
<td>123I</td>
<td>Radioiodine-123</td>
</tr>
<tr>
<td>131I</td>
<td>Radioiodine-131</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>K-RAS</td>
<td>Kirsten rat sarcoma viral oncogene homolog</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MNG</td>
<td>Multinodular goiter</td>
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<tr>
<td>MST</td>
<td>Median survival time</td>
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<tr>
<td>MTC</td>
<td>Medullary thyroid carcinoma</td>
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<tr>
<td>N-RAS</td>
<td>Neuroblastoma RAS viral (v-ras) oncogene homolog</td>
</tr>
<tr>
<td>NIS</td>
<td>Sodium/iodide symporter</td>
</tr>
<tr>
<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>NTI</td>
<td>Non-thyroidal illness</td>
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<tr>
<td>OS</td>
<td>Overall survival time</td>
</tr>
<tr>
<td>P-gp</td>
<td>Permeability glycoprotein</td>
</tr>
<tr>
<td>PAX8</td>
<td>Paired-box 8 – peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>PPARG</td>
<td>Platelet derived growth factor receptor alpha</td>
</tr>
<tr>
<td>PDGFR α</td>
<td>Platelet derived growth factor receptor beta</td>
</tr>
<tr>
<td>PDPK1</td>
<td>3-phosphoinositide dependent protein kinase-1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PIK3CA</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha</td>
</tr>
<tr>
<td>PIK3CB</td>
<td>Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>RAIU</td>
<td>Radioactive iodine uptake</td>
</tr>
<tr>
<td>RAS</td>
<td>Rat sarcoma</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>rhTSH</td>
<td>Recombinant human thyrotropin</td>
</tr>
<tr>
<td>RPS5</td>
<td>Ribosomal protein S5</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
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<tr>
<td>T3</td>
<td>Triiodothyronine</td>
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<tr>
<td>T4</td>
<td>Thyroxine</td>
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<tr>
<td>TCC</td>
<td>Transitional cell carcinoma</td>
</tr>
<tr>
<td>Tg</td>
<td>Thyroglobulin</td>
</tr>
<tr>
<td>THW</td>
<td>Thyroid hormone withdrawal</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine kinase inhibitor</td>
</tr>
<tr>
<td>TM</td>
<td>Time to distant metastases</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyrotropin</td>
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<tr>
<td>TR</td>
<td>Time to loco-regional recurrence</td>
</tr>
<tr>
<td>TT3</td>
<td>Total thyroxine</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Vascular endothelial growth factor receptor-1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular endothelial growth factor receptor-2</td>
</tr>
<tr>
<td>WBS</td>
<td>Whole-body scintigraphy</td>
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Chapter 1

GENERAL INTRODUCTION

Part of this review has been published:

RECOMBINANT HUMAN THYROTROPIN IN VETERINARY MEDICINE: CURRENT USE AND FUTURE PERSPECTIVES

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Adapted from

Journal of Veterinary Internal Medicine 2012; 26:853-62

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Thyroid cancer represents 1.2-3.8% of all neoplasms in the dog.\textsuperscript{1} The Beagle, Golden Retriever and Boxer are predisposed breeds, there is no gender predilection and dogs over 7 years old are more commonly affected.\textsuperscript{2}

In people, thyroid cancer accounts for 2% of all diagnosed cancers and is the most common form of endocrine neoplasia.\textsuperscript{3} Thyroid cancer is nearly 3 times more frequent in women than men and although it can occur at any age, it is more common in middle aged women and men over the age of 60.\textsuperscript{3}

Ninety percent of canine thyroid tumors detected clinically are carcinomas and can be classified as either follicular cell thyroid carcinomas (FTCs), which arise from thyroid follicular cells, or medullary thyroid carcinomas (MTCs), which arise from the parafollicular cells (C-cells) and have a neuroendocrine origin.\textsuperscript{2} In a study including 38 dogs with thyroid carcinoma, FTC accounted for 64% of carcinomas while MTC had a prevalence of 36%.\textsuperscript{4} The morphological, cytochemical and immunohistochemical features of canine MTC resemble human MTC.\textsuperscript{5}

According to the World Health Organization (WHO), canine FTC can be classified as well-differentiated (follicular, compact, follicular-compact, papillary), poorly differentiated, undifferentiated or carcinosarcoma, while human FTC can be classified as well-differentiated (papillary, follicular), poorly differentiated or undifferentiated.\textsuperscript{6,7} Follicular, compact and follicular-compact carcinomas represent 86% of canine FTCs and are remarkably similar in terms of histology and biological behavior to human follicular thyroid carcinoma.\textsuperscript{8,9} The most frequent form of thyroid carcinoma in humans is papillary thyroid carcinoma with a prevalence of 80%, followed by follicular (14%), medullary (4%) and undifferentiated (2%) carcinoma.\textsuperscript{10} Unlike in humans, papillary thyroid carcinoma is rare in dogs.\textsuperscript{9}

The research expounded in this manuscript will focus on the pathogenesis and treatment of canine thyroid tumors. In the general introduction, we review the current knowledge of the aspects of thyroid cancer investigated in this PhD: genetic events, prognostic markers and treatment optimization. A detailed review of all other aspects of thyroid neoplasia is beyond the scope of this introduction.

In the past decade, the major advances made in understanding the molecular pathogenesis of human thyroid cancer have helped to develop new diagnostic,
prognostic and treatment tools, providing an interesting perspective for veterinary medicine.\textsuperscript{11} Given the similarity between human and canine thyroid carcinoma, the current knowledge of the pathogenesis and treatment of human thyroid cancer will also be discussed.
1 PATHOGENESIS

1.1 Genetic events leading to thyroid cancer

There is a variety of genetic alterations described in human thyroid cancer, including gene mutation, gene amplification, gene translocation, aberrant gene methylation and abnormal number of chromosomes (aneuploidy). In human follicular thyroid carcinomas, the most important genetic events are PAX8-PPARG (paired-box 8 – peroxisome proliferator-activated receptor-\(\gamma\)) gene rearrangement, with a prevalence of 60%, and point mutations in the RAS genes, with a prevalence of 20-52%. The RAS genes (N, K, and H) encode membrane-bound intracellular proteins involved in cellular signal transduction. These proteins act as on/off switches, relaying extracellular signals to the cytoplasmic signaling cascades of the mitogen-activated protein kinase (MAPK) and phosphoinositol-3-kinase (PI3K)/Akt pathways, which control cellular proliferation and differentiation (Fig. 1). In many human cancers, point mutations in exon 1 (codons 12 and 13) or exon 2 (codons 59 and 61) fix RAS proteins in a permanently activated form, promoting uncontrolled cellular division and malignant transformation. Although RAS is a classical dual activator of both pathways, RAS mutations seem to preferentially stimulate the PI3K/Akt pathway in thyroid gland tumorigenesis.

RAS mutations are generally uncommon in canine tumors. However up to 25% of canine non-small cell lung tumors and 80% of canine pancreatic tumors have been reported to harbor K-RAS mutations, and 25% of dogs with acute myeloid or lymphoid leukemia harbor N-RAS mutations. RAS mutations have not been investigated in canine thyroid cancer.

Point mutations in the tumor suppressor gene phosphatase and tensin homologue (PTEN) and in the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PIK3CA) gene are also reported in human follicular carcinomas with a prevalence of 6% and 13%, respectively. Because PTEN has an inhibitory effect on the PI3K/Akt pathway, inactivating mutations or deletions in PTEN lead to PI3K/Akt pathway activation and promote tumorigenesis. Activating point mutations in the PIK3CA gene lead to a constitutively activated protein which also activates PI3K/Akt
pathway. Mutations in \textit{PTEN} and \textit{PIK3CA} have not been evaluated in canine thyroid cancer.

\textbf{Fig. 1:} The main signaling pathways involved in thyroid gland carcinogenesis are the MAPK and PI3K/Akt pathways. These pathways are involved in propagation of signals from various cell membrane receptor tyrosine kinases into the nucleus, and they regulate multiple cell processes including proliferation, differentiation and survival. Activation of the MAPK pathway by oncogenic stimuli such as mutated BRAF or the chimeric fusion proteins rearranged during transfection (RET)/PTC is a common tumor initiating event in human papillary carcinoma. Mutations involving the effectors of the PI3K/Akt pathway such as the PIK3CA, AKT1 and PTEN are found more frequently in follicular carcinomas and in less differentiated types of thyroid cancer. \textit{Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Endocrinology]}\textsuperscript{21}, copyright (2011)

\textbf{Abbreviations:} BRAF, serine/threonine protein kinase B-RAF; MEK, mitogen-activated protein kinase (MAPK)/ERK kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3-kinase; AKT, protein kinase B; PTEN, phosphatase and tensin homolog; mTOR, mammalian target of rapamycin;
The most important genetic alterations in human papillary thyroid carcinoma are RET/PTC gene translocation, present in 36% of cases, and activating point mutations of the v-raf murine sarcoma viral oncogene homolog B (BRAF), which have a prevalence of 45%.\textsuperscript{2,23} The most prominent example of such mutations is the transverse point mutation T1799A (exon 15) which leads to expression of a constitutively activated mutant protein (BRAF-V600E) that activates MAPK signaling pathway.\textsuperscript{24} The prevalence of BRAF mutations in canine thyroid tumors is unknown.

In humans, a thyroid follicular cell tumorigenesis model has been proposed associating genetic alterations to pathway activation and tumor type. The MAPK pathway is mainly associated with papillary carcinoma while the PI3K/Akt pathway is the major signaling pathway involved in follicular carcinoma. Activation of both pathways becomes more frequent as the grade of thyroid tumors increases (Fig. 2).

**Fig. 2.** Model of the progression of thyroid follicular cell tumorigenesis driven by the MAPK and PI3K/Akt pathways. Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer]\textsuperscript{12}, copyright (2013)

**Abbreviations:** ATC, anaplastic (undifferentiated) thyroid carcinoma; FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma;
In dogs, constitutive activation of the PI3K/Akt pathway has been recently demonstrated in cell lines of canine lymphoma, hemangiosarcoma, mammary carcinoma, glioma and mast cell tumors. These findings suggest the major importance of this signaling pathway in the pathogenesis of canine cancer.

Mutations in the tumor suppressor gene *p53* are rare in human differentiated FTC (dFTC) (papillary and follicular) occurring mainly in poorly differentiated and undifferentiated carcinomas. In dogs, a study where part of the coding region of *p53* was analyzed revealed a somatic mutation of *p53* in 1 of 23 canine FTCs.

The principal molecular mechanism underlying human MTC is aberrant activation of the receptor tyrosine kinase (RTK) rearranged during transfection (*RET*). Germ line *RET* mutations are responsible for the hereditary form of MTC (eg, multiple endocrine neoplasia types 2A and 2B) while somatic *RET* mutations are present in approximately 50% of patients with sporadic MTC. RET signaling leads to activation of the PI3K/Akt and MAPK pathways and has key roles in cell growth, differentiation, and survival. *RAS* mutations have also been reported in up to 68% of *RET*-negative MTC. In a recent study, exonic sequencing revealed that 90% of human MTCs had mutually exclusive mutations in *RET, H-RAS* or *K-RAS*. In the only case report of canine familial MTC, no mutation was found after complete sequencing of *RET*. To present date, the genetic events underlying canine sporadic MTC have not been investigated.

Oncogenic gene amplification or copy-number gain is an additional prominent genetic mechanism causing thyroid gland tumorigenesis in humans. This is particularly the case for genes involved in the PI3K/Akt pathway. Prominent examples (and respective prevalence in human follicular thyroid carcinoma) include *EGFR* (32.2%), *VEGFR-1* (44.1%), *VEGFR-2* (3.8%), *PIK3CA* (23.8%), *PIK3CB* (45.5%), *AKT1* (8.1%), *AKT2* (22.4%) and *PDPK1* (24.1%). An important and expected consequence of gene amplification or pathway activation is increased mRNA and protein expression and consequent aberrant activation of downstream signaling. The mRNA expression of RTKs and effectors involved in PI3K/Akt signaling may provide valuable information regarding gene amplification and pathway activation and has not yet been investigated in canine thyroid tumors.
Several reports have suggested that the PI3K/Akt signaling pathway regulates the expression of cyclooxygenase-2 (Cox-2).\textsuperscript{33,34} It has been demonstrated that Cox-2 functions downstream of Akt, and that elevated Akt activity is crucial for Cox-2 overexpression in apoptotic-resistant cells.\textsuperscript{33} Cox-2 mRNA expression could therefore also be used to infer on the activity of the PI3K/Akt pathway.

Abnormal number of chromosomes (aneuploidy) is another genetic event described in human and canine thyroid cancer. Approximately 60\% of human follicular thyroid carcinomas are aneuploid which is comparable to what is observed in dogs.\textsuperscript{35,36} However, while in dogs more than 80\% of aneuploid thyroid tumors are hypodiploid, in humans hypodiploidy is only rarely found.\textsuperscript{35,36} Furthermore, ploidy abnormalities (DNA-index variation) are less extensive in the dog compared to those in humans, which could indicate either a fundamental difference in carcinogenesis or that the human genome needs stronger destabilization to promote tumor development.\textsuperscript{36,37}

The differentiation between canine MTC and FTC of compact type may be difficult based on microscopic observation alone.\textsuperscript{4} Immunohistochemistry (IHC) for thyroglobulin, calcitonin or markers of neuroendocrine tissue aids the diagnosis.\textsuperscript{5} mRNA expression of the gene encoding calcitonin (calcitonin-related polypeptide alpha - \textit{CALCA}) has not yet been evaluated in dogs with thyroid tumors and may also help differentiating FTC from MTC.

1.2 Prognostic markers

The median survival time (MST) for dogs with thyroid carcinoma left untreated is only 3 months.\textsuperscript{38} However, prognosis can be good with appropriate treatment.\textsuperscript{39} The reported MST for dogs with freely moveable thyroid tumors treated with thyroidectomy is > 36-50 months, in the absence of metastatic disease.\textsuperscript{40,41} Dogs with unresectable invasive tumors can be treated with radioiodine-131 (\textsuperscript{131}I) therapy or external beam radiation. When no distant metastases are present, \textsuperscript{131}I therapy is associated with a MST of 28-30 months and external beam radiation is associated with a 3-year progression-free survival of 72\%.\textsuperscript{38,42,43} The role of chemotherapy in the treatment of canine thyroid carcinoma has not been fully elucidated.\textsuperscript{44} In dogs with unresectable thyroid carcinoma, partial responses have been described to doxorubicin
and cisplatin in 44-54% of patients, yielding MSTs of 3-8 months.\textsuperscript{45,46} The studies published to present date on adjuvant chemotherapy following thyroidectomy have serious limitations but do not suggest it provides a survival benefit.\textsuperscript{47,48}

Prognostic markers help assessing the biological behavior of cancer identifying tumors with high potential for local recurrence and metastases. In humans, well established prognostic factors of thyroid carcinoma include age, gender, tumor size, stage, histologic type, histologic grade, vascular invasion and extrathyroidal tumor extension.\textsuperscript{49,50} Low-risk patients undergo a follow-up strategy that is considerably different from that of high-risk patients.\textsuperscript{3}

In dogs, thyroid tumor volume > 20 cm\textsuperscript{3}, bilateral thyroid neoplasia and cervical vascular invasion have all been associated with high metastatic rates.\textsuperscript{8,9,42} However, these associations were often based on necropsy studies or studies in dogs with unresectable thyroid tumors. In fact, few studies have investigated prognostic predictors for dogs with operable thyroid tumors. Breed, sex, tumor size, histologic subtype and tumor vascular density did not appear to affect prognosis after surgical resection, while bilateral disease and histological grade of malignancy were prognosticators.\textsuperscript{8,40,41,47,51} Prognostic markers for dogs with thyroid tumors undergoing thyroidectomy are lacking.

The majority of dogs with thyroid neoplasia are euthyroid.\textsuperscript{52,53} It has been suggested that although thyroid carcinoma tissue has a decreased capacity to synthesize thyroid hormones, the contralateral thyroid lobe may function in a compensatory state.\textsuperscript{54} Around 30-40% of dogs with thyroid tumors have decreased circulating total thyroxine (TT\textsubscript{4}) concentrations due to euthyroid sick syndrome or hypothyroidism, and 22-31% of patients are hyperthyroid.\textsuperscript{8,38,52,53} It can be hypothesized that functional tumors (in dogs with hyperthyroidism or with preserved scintigraphic uptake) are more differentiated and, therefore, carry a better prognosis. Although a prospective evaluation of 23 dogs with thyroid tumors undergoing thyroidectomy did not indicate an effect of thyroid function or tumor scintigraphic uptake on prognosis, further studies are warranted.\textsuperscript{41}

In humans, MTC is more aggressive than dFTC.\textsuperscript{10} In most veterinary studies, the prevalence of MTC is likely underestimated as these tumors may be difficult to
distinguish from compact dFTC by microscopic observation alone. IHC for calcitonin or for markers of neuroendocrine tissue is required for their identification. In a study with relatively low number of dogs, MTC represented 36% of all canine thyroid tumors and was suggested to be more amenable to complete surgical resection and have lower metastatic potential than FTC. However, it is still not clear whether canine dFTC and MTC differ with respect to prognosis following thyroidectomy.

E-cadherin is a transmembrane adhesion glycoprotein of epithelial tissues and plays a role in neoplastic cell behavior as a suppressor of invasion and metastasis. In human thyroid carcinomas, loss of E-cadherin expression is an independent prognostic indicator associated with a higher degree of dedifferentiation and higher metastatic potential. In dogs with mammary carcinoma, loss of E-cadherin expression was also found to be related to prognosis. The prognostic significance of E-cadherin expression in canine thyroid carcinoma has not been investigated.

Ki-67 is a cellular proliferation marker expressed in the cell nuclei during all active phases of the cell cycle (G1, S, G2 and mitosis) but not in G0. In human dFTC, high Ki-67 labeling index is associated with higher metastatic rates at diagnosis and shorter disease-free survival (DFS). Although the use of Ki-67 as a marker for prognosis was shown to have limitations in certain canine tumors, its value is well established in mast cell tumors. The prognostic relevance of Ki-67 expression has not yet been examined in canine thyroid tumors.
2 TREATMENT OPTIMIZATION

In dogs, thyroidectomy is the preferred treatment modality for thyroid tumors that are freely moveable at palpation, yielding a MST beyond 3 years if no metastases are present.\textsuperscript{40,48} However, 38\% of dogs present with pulmonary metastases at the time of diagnosis and approximately half of dogs undergoing thyroidectomy experience recurrence or metastatic disease within 2 years of surgery.\textsuperscript{4,8}

To present date, research on adjuvant treatment of canine thyroid carcinoma remains scarce and no single treatment modality has been shown to be effective in the treatment of metastatic disease, which occurs mainly to the lungs and regional lymph nodes (mandibular, retropharyngeal and cranial cervical).\textsuperscript{44} The few studies evaluating post-operative chemotherapy have serious limitations but did not suggest an improvement in outcome.\textsuperscript{47,48} Therefore, it is important to investigate new treatment modalities for the large number of dogs with metastatic disease.

Up to 45\% of canine thyroid tumors are unresectable due to local invasiveness and these patients can still have a good prognosis with external beam radiation or \textsuperscript{131}I therapy. However, these treatment modalities are expensive and are not widely available.\textsuperscript{4,42,43} Furthermore, \textsuperscript{131}I is only expected to be effective in a subset of thyroid tumors with adequate \textsuperscript{131}I uptake. Recently, tyrosine kinase inhibitors (TKIs) and continuous low-dose (metronomic) chemotherapy have shown promising results for palliative treatment of dogs with unresectable thyroid tumors.\textsuperscript{61,62} Given the high percentage of thyroid tumors that are unresectable in dogs, it is vital to continue searching for new ways to improve the treatment of these patients.

The progress made in understanding the pathogenesis of human thyroid cancer has led to increased interest in the development of targeted therapies, including angiogenesis suppression, restoration of wild-type \textit{p53}, Cox-2 inhibition, P-glycoprotein (P-gp) modulation, and inhibition of aberrant intracellular signaling of the MAPK and PI3K/Akt pathways. Additionally, thyrotropin (TSH) suppression and the use of recombinant human TSH (rhTSH) to optimize \textsuperscript{131}I therapy have also allowed a significant improvement of the treatment of human thyroid cancer and have not yet been adequately investigated in dogs.
2.1 Therapeutic targets

Vascular endothelial growth factor (VEGF) is a potent stimulator of endothelial cell growth and can stimulate both physiological and pathological angiogenesis. VEGF mediates its biological effects by binding to 2 main RTKs, vascular endothelial growth factor receptor-1 (VEGFR-1) and vascular endothelial growth factor receptor-2 (VEGFR-2), expressed mainly on endothelial cells. VEGFR-2 is responsible for signaling while VEGFR-1 is thought to play a regulatory role by decreasing the availability of VEGF to VEGFR-2. VEGF is the main stimulator of angiogenesis in the thyroid gland and overexpression VEGF, VEGFR-1 and VEGFR-2 has been demonstrated in human thyroid cancer. When VEGF secreted by cancer cells binds to VEGFR-2 on the surface of endothelial cells and thyrocytes, it promotes signaling through the MAPK and PI3K/Akt pathways stimulating cellular proliferation, migration and survival. This activation of angiogenesis is fundamental for tumor growth and development of metastasis. In people, VEGFR-2 inhibition with TKIs is the most effective new therapeutic strategy developed to date in the treatment of advanced thyroid cancer. VEGF, angiogenesis and VEGF-induced pathway activation may play an important role in the progression of canine thyroid cancer and constitute an important therapeutic target.

Toceranib phosphate (Palladia®; Pfizer Animal Health, Madison, NJ, USA) is a TKI which targets VEGFR-2, platelet-derived growth factor receptor alpha and beta (PDGFRα/β), stem cell factor receptor (c-Kit), Fms-like tyrosine kinase 3 (FLT-3) and colony-stimulating factor 1 receptor (CSF1R), and has been reported to yield a clinical benefit rate of 80% in 15 dogs with FTC (partial remission n=4; stable disease n=8). In a recent study evaluating the major RTKs targeted by toceranib phosphate in 15 canine FTCs, expression of VEGFR-2 was demonstrated in a subset of tumors although no phosphorylation was observed. This suggests that VEGFR-2 is not in an aberrant state of continuous activation and that the clinical response of canine FTCs to toceranib phosphate may be related to inhibition of RTKs on the vascular endothelium and stroma rather than on tumor cells. Overexpression of VEGF by canine thyroid tumors.
may be driving activation of stromal RTKs and has not been investigated in canine thyroid cancer.

Tumor suppressor gene p53 encodes a nuclear phosphoprotein that mediates cell cycle regulation and induces apoptosis in response to DNA damage.\(^7^2\) Mutations that result in loss of normal p53 function lead to loss of cell cycle control and increased risk of malignancy. In humans, p53 mutations have been described in 40-62% of undifferentiated thyroid carcinomas and 5-10% of other thyroid carcinomas.\(^2^6\) Research in human thyroid cancer shows that restoration of wild-type p53 expression by gene therapy is associated with inhibition of tumor cell growth and enhanced sensitivity to chemotherapy and radiation.\(^7^2\) As previously stated, investigations of the p53 gene coding region identified a somatic mutation in 1 of 23 canine FTCs.\(^2^7\) Thus, p53 tumor suppressor gene may be a potential molecular target of modest weight in canine thyroid cancer.

Cyclooxygenases (Cox) are the major cellular targets of non-steroidal anti-inflammatory drugs (NSAIDs). There are 2 Cox isoforms, cyclooxygenase-1 (Cox-1) and Cox-2, and both are involved in prostaglandin biosynthesis. While Cox-1 is expressed constitutively in many tissues, Cox-2 is inducible by growth factors, inflammation and several oncogenes.\(^7^3\)

Cyclooxygenases, particularly Cox-2, may play a critical role in tumor development and progression. In particular, epithelial neoplasms are prone to express large amounts of the inducible form of Cox-2. In dogs, Cox-2 overexpression has been described, for example, in transitional cell carcinoma (TCC) of the urinary bladder and in prostatic carcinoma.\(^7^4\) Several studies have shown that Cox-2 or Cox-1/Cox-2 inhibitors have antitumor and chemopreventive effects, presumably through induction of apoptosis, reduction of angiogenic growth factors and suppression of regulatory T-cells.\(^7^5,^7^6\) Cox-2 is an appealing therapeutic target and its expression has not yet been investigated in canine thyroid tumors.

One study in 44 dogs with surgically excised thyroid carcinoma failed to demonstrate a clinical benefit of adjuvant chemotherapy.\(^4^7\) Moreover, the reported survival times for dogs with unresectable thyroid tumors treated with chemotherapy are disappointing.\(^4^5\) One of the major mechanisms for resistance to chemotherapy is high
expression of ATP-binding cassette (ABC) transporter proteins such as P-gp and multi-drug resistance-related protein 1 (ABCC1). These ATP-dependent membrane efflux pumps decrease the intracellular concentration of chemotherapeutic agents, thereby limiting cytotoxicity at their target site. Expression of P-gp could be one of the mechanisms responsible for the resistance of canine thyroid cancer to chemotherapy. Recent research shows that TKIs and Cox-2 inhibitors can reverse multi-drug resistance by decreasing the expression and function of P-gp. P-gp expression has been identified in several canine tumors and may constitute an attractive molecular target.

2.2 TSH-suppressive therapy

The standard treatment for human dFTC is total or near-total thyroidectomy, followed by destruction of all remaining thyroid cells with $^{131}$I therapy (thyroid remnant ablation) and long-term TSH-suppressive therapy with levothyroxine. Levothyroxine supplementation is not only given to treat iatrogenic hypothyroidism but also to suppress endogenous TSH, which constitutes a potential stimulus for tumor growth. In humans and dogs, FTC cells contain TSH receptors and endogenous TSH may stimulate neoplastic cell growth and tumor progression. In people with dFTC, TSH-suppressive therapy with levothyroxine significantly reduces recurrence rates and cancer-specific mortality. The American and European Thyroid Associations currently recommend adapting the target level of TSH suppression to the patient’s risk for tumor recurrence and mortality.

In a recent retrospective study of 15 dogs with bilateral thyroid neoplasia undergoing thyroidectomy, the dogs receiving levothyroxine replacement therapy after surgery had a significantly longer survival time. These results are encouraging but further studies are needed to corroborate these findings in a larger number of dogs.
2.3 Optimization of $^{131}$I therapy

TSH is a glycoprotein secreted by thyrotrophs in the adenohypophysis. It is composed of $\alpha$ and $\beta$ subunits. The $\alpha$ subunit has 2 oligosaccharide chains and is common to other glycoprotein hormones; the $\beta$ subunit has 1 oligosaccharide chain and is hormone-specific.\(^8^4\) Although the exact amino acid sequence of the $\beta$ subunit varies among species, there is biological cross-reactivity, such that the TSH of 1 species will stimulate the thyroid follicular cells of another species.\(^8^5,8^6\) The sequences of the $\alpha$ and $\beta$ subunits of canine TSH are 96 and 94% homologous to feline TSH and 60 and 91% homologous to human TSH.\(^8^7\)

TSH binds to a membrane TSH G protein-coupled receptor on the surface of thyroid follicular cells and constitutes the most important stimulus for proliferation, differentiation and metabolic activity in these cells. This binding activates adenyl cyclase, causing an increase in the intracytoplasmic concentrations of adenosine 3’,5’-cyclic monophosphate (cAMP), with subsequent phosphorylation of protein kinases.\(^8^8\) This interaction triggers a cascade of reactions, leading to increased iodide uptake and synthesis and secretion of triiodothyronine ($T_3$), thyroxine ($T_4$) and thyroglobulin ($Tg$). 3-6 hours after TSH binds, endocytosis of colloid increases and pre-formed thyroid hormone is released into the bloodstream; hormone concentrations reach a maximum after 24 hours.\(^8^9\) When TSH stimulation persists, increased expression and functionality of the Na/I symporter (NIS) leads to increased uptake and organification of iodine, which peaks after 72 hours (Fig. 3).\(^8^9-9^1\)
1. TSH, consisting of α and β subunits, binds to and activates the TSH receptor (TSHR).

2. Stimulation of the cAMP pathway results in enhanced iodide uptake, growth, differentiation and hormone synthesis.

3. Iodide is actively transported into thyroid follicular cells by the sodium-iodide symporter (NIS) at the basolateral membrane. At the apical membrane, pendrin mediates iodide efflux into the follicular lumen.

4. Organification – thyroid peroxidase (TPO) oxidizes iodide and subsequently iodinates tyrosil residues of thyroglobulin (Tg) in the presence of hydrogen peroxide. The iodotyrosines, mono- and diiodothyrosil, are coupled to T₄ and T₃; this reaction is also catalyzed by TPO (coupling).

5. Tg is internalized into the follicular cell, hydrolyzed in lysosomes and the thyronines T₃ and T₄ are released into the blood stream.

**Abbreviations**: TSH, thyrotropin; TSHR, thyrotropin receptor; cAMP, adenosine 3’,5’-cyclic monophosphate; NIS, sodium/iodide symporter; TPO, thyroid peroxidase; Tg, thyroglobulin; T₄, thyroxine; T₃, triiodothyronine.
Follow-up of human dFTC is based on cervical ultrasonography, measurement of plasma Tg concentrations and $^{131}$I whole-body scintigraphy (WBS). As previously stated, TSH-suppressive therapy with levothyroxine is an important component of the standard treatment of human dFTC. However, high concentrations of TSH are temporarily required during follow-up for appropriate $^{131}$I uptake by normal and neoplastic thyroid cells and, consequently, a sensitive WBS. High TSH levels also increase the sensitivity of Tg determination as a marker for tumor persistence and recurrence. To achieve high concentrations of endogenous TSH (>30 mIU/mL), levothyroxine supplementation must be discontinued for 4 to 6 weeks. However, thyroid hormone withdrawal (THW) typically results in severe hypothyroidism which has serious impact on the patients’ quality of life. Alternatively, levothyroxine supplementation may be continued. In this case, exogenous TSH administration would be used before WBS and plasma Tg determination. Initially, bovine TSH (bTSH) and extractive human TSH (obtained from cadaver pituitary glands) were used for this purpose.

In the early 1990s, the use of bTSH in humans had been largely abandoned because of allergic reactions and the use of extractive TSH was discarded due to its limited availability and potential for transmission of Jacob-Creutzfeldt disease. The need created by these difficulties led to the development of rhTSH. The genes encoding the α and β subunits of TSH were identified and cloned in the 1980s. This success was followed by the expression of an intact human TSH heterodimer in Chinese hamster ovary cells. The amino acid sequence of rhTSH is identical to that of endogenous human TSH, although there is less glycosylation and more sialic acid.

The efficacy of the treatment of human dFTC is increased by $^{131}$I ablation of thyroid remnants after thyroidectomy which destroys all remaining thyroid gland tissue that may contain tumor cells. Not only does this allow a better treatment, but additionally measurement of plasma Tg concentration during follow-up becomes a highly specific marker for recurrent or persistent disease. Preparation of patients for thyroid remnant ablation after thyroidectomy traditionally involved THW which resulted in clinical hypothyroidism and impaired quality of life. Several studies
demonstrated that rhTSH could stimulate $^{131}$I uptake by the thyroid remnants after thyroidectomy, facilitating complete ablation. Preparation of thyroid remnant ablation with rhTSH is associated with a higher quality of life, lower radiation exposure to the blood and lower radiotoxicity whereas ablation rate and tumor recurrence are comparable to THW.

rhTSH can also be used as an adjuvant to $^{131}$I treatment of metastatic and persistent dFTC. If patients with metastatic dFTC cannot achieve adequate plasma TSH concentrations after THW or experience serious depression associated with iatrogenic hypothyroidism, rhTSH may be used compassionately to increase $^{131}$I uptake by tumor tissue.

Recombinant human TSH also is valuable in the treatment of multinodular goiter (MNG). Toxic MNG is characterized by multiple nodular enlargements in the thyroid gland and hyperthyroidism, resembling feline hyperthyroidism. $^{131}$I is the treatment of choice and recent studies have shown that pretreatment with rhTSH allows increased thyroidal uptake of $^{131}$I, reduction of the therapeutic dose of $^{131}$I and decreased radiation delivery to extrathyroidal tissues.

Until recently, research on the use of rhTSH in dogs has focused on the diagnosis of hypothyroidism. Initial studies in healthy Beagles showed rhTSH has a biological effect on the canine thyroid gland and that the IV route allows a maximal increase in TT$_4$ concentrations 4 and 6 h after injection. Different studies have investigated the optimal dosage of rhTSH for TSH stimulation. Most studies have shown there is a dose dependent effect of rhTSH on post-stimulation TT$_4$ concentration which seems to be independent of body weight. The most recent study comparing 2 dosages of rhTSH (75 µg/dog and 150 µg/dog) in healthy and suspected hypothyroid dogs concluded that use of 150 µg/dog provides higher discriminatory power to differentiate hypothyroidism from nonthyroidal illness (NTI).

In dogs, $^{131}$I can be an excellent treatment option for unresectable FTC when external beam radiation is not available. Two retrospective studies showed that dogs with unresectable thyroid tumors treated with $^{131}$I therapy experience a MST of approximately 2.5 years. High therapeutic doses of $^{131}$I (555-1850 MBq) are required and this usually implies a prolonged hospitalization period and high doses of
radiation eliminated to the environment through the excreta. Use and exposure to radiation should be kept “As Low As Reasonably Achievable” (ALARA principle) to minimize risks for patient and human health. Exposure of nonthyroidal tissues to high doses of radiation may cause treatment complications such as fatal myelosuppression, reported at $^{131}$I dosages higher than 160 MBq/kg. Although $\frac{3}{4}$ of canine thyroid tumors trap sufficient $^{131}$I to be visualized by scintigraphy, major limitations of $^{131}$I therapy include its selected effectiveness in differentiated follicular cell tumors with adequate $^{131}$I uptake and the potential need of multiple treatments for tumor control. 

The obvious benefits of the use of rhTSH to optimize the treatment of human thyroid cancer with $^{131}$I provide an interesting perspective for the optimization of $^{131}$I therapy of canine FTC. On the one hand, by increasing the uptake of $^{131}$I by the thyroid tumor, rhTSH may improve $^{131}$I treatment efficacy and decrease the need for multiple treatments. On the other hand, rhTSH may allow a decrease of the therapeutic dosage of $^{131}$I, thereby improving radioprotection, limiting radiotoxicity and complying with the ALARA principle. Furthermore, $^{131}$I dose reduction could potentially reduce the required hospitalization period and costs.

TSH receptors have been demonstrated in canine neoplastic thyroid cells; both in primary tumors and metastases. Furthermore, earlier reports have already suggested the potential of exogenous TSH to increase thyroid radioactive iodine uptake (RAIU) in dogs. However, in these studies the effect of TSH stimulation on thyroid $^{131}$I uptake was described in a small number of healthy and hypophysectomised dogs and no statistical analysis was performed. Our group recently evaluated the effect of rhTSH on thyroid RAIU in hyperthyroid cats and a small but statistically significant increase in thyroid RAIU was observed. The effect of rhTSH on the uptake of $^{131}$I in dogs with thyroid tumors has not been investigated.

The use of $^{131}$I for diagnostic imaging in clinical research has several limitations. Its half-life (8 days) makes it impractical for repeated RAIU determinations within a reasonable period. Furthermore, the emission of limited tissue penetrating beta particles during the decay of $^{131}$I causes a higher localized radiation dose and due to the presumed radiotoxicity may have a deleterious effect on the uptake of the actual $^{131}$I
therapeutic dosage, a controversial phenomenon named thyroid stunning.\textsuperscript{132} Unlike \textsuperscript{131}I, \textsuperscript{123}I has a much shorter half-life (13 h), decays by emitting gamma rays and has been shown to be equal or even superior to \textsuperscript{131}I as a scanning agent.\textsuperscript{133} Hence, in this research \textsuperscript{123}I was chosen as an imaging agent, despite its high cost.

### 2.4 Safety of rhTSH

Before evaluating any human drug in veterinary medicine it is paramount to verify its safety. The use of rhTSH in dogs with thyroid cancer raises 2 major concerns: hypersensitivity reactions and effect on the volume of the primary tumor and tumor metastases.

Differences in amino acid sequences make the β subunit of TSH immunogenic in a non-homologue species. This leads to the risk of antibody induction and hypersensitivity reactions when rhTSH is administered repeatedly. A review of the veterinary literature indicates that at least 547 dogs and 93 cats already have received rhTSH.\textsuperscript{134} Furthermore, 87 of the 547 dogs and 7 of the 93 cats underwent repeated rhTSH administration. To date, no anaphylactic reactions have been recorded and the only adverse effect was transient pain after IM injection.\textsuperscript{125} Since 2006, approximately 30 dogs have undergone TSH stimulation with rhTSH every year at our clinic and this test also is frequently used in other clinics around the world. To our knowledge, no serious adverse reactions have been reported. The adverse reactions previously observed with bTSH (ie, anaphylactoid reactions) most likely were a result of the unpurified chemical grade of bTSH rather than its amino acid sequence.\textsuperscript{135} It is therefore not surprising that the use of a purified pharmaceutical grade rhTSH does not yield similar adverse reactions.

In humans, rhTSH increases the volume of the thyroid gland in healthy subjects\textsuperscript{136,137} and causes expansion of primary thyroid tumors and thyroid tumor metastases.\textsuperscript{138,139} Therefore, rhTSH should be used carefully in patients with large thyroid tumors or central nervous system, spinal, lung or bone metastases because tumor expansion may compress adjacent structures and lead to complications.\textsuperscript{140} This effect is thought to be related to intravascular and interstitial fluid accumulation rather than regular growth of the thyroid tissue.\textsuperscript{136} Evidence of peritumoural edema,
hemorrhage and response to glucocorticoid administration also support this hypothesis.\textsuperscript{138,139} No information is available on the effect of rhTSH on thyroid gland volume in dogs and it seems prudent that this is investigated before rhTSH is used in dogs with thyroid tumors.

Research on pathogenesis and optimization of treatment of canine thyroid cancer is still very limited. Many dogs with thyroid tumors already have metastases at the time of diagnosis and almost half of dogs treated with thyroidectomy develop recurrent or metastatic disease within 2 years of surgery. Given the disappointing results of chemotherapy, it is imperative to investigate new ways to improve treatment. The discovery of the main genetic events leading to human thyroid cancer and research on therapeutic targets has led to the development of novel treatment strategies for patients that are refractory to conventional therapy. Furthermore, stratification of patient risk with prognostic markers, TSH-suppressive therapy and enhancement of $^{131}$I uptake with rhTSH have allowed a significant optimization of the treatment of humans with dFTC. The molecular pathogenesis and expression of potential therapeutic targets is largely unknown in canine thyroid cancer. Furthermore, prognostic markers, TSH suppression and optimization of $^{131}$I therapy with rhTSH have not yet been adequately investigated.
Chapter 2

SCIENTIFIC AIMS
In the last 10 years, major advances have been made in unveiling the molecular pathogenesis of human thyroid cancer and this has allowed the development of new diagnostic, prognostic and treatment tools. A deeper knowledge of the pathogenesis of canine cancer has already allowed the development of targeted therapy (eg, cox-2 inhibitors in canine TCC; TKIs in canine mast cell tumors) and significant improvements in patient outcome. In humans, the use prognostic markers to adapt treatment to the patients’ risk, the benefit of TSH suppression, and the use of rhTSH to optimize $^{131}$I therapy provide an interesting perspective for optimization of treatment of canine thyroid cancer. The general aim of this research was to provide new insights into the *pathogenesis* and *treatment* of canine thyroid tumors.

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The specific aims of our research on *pathogenesis* were:

1) To investigate mutational hotspots in *RAS (N, K, H), PIK3CA PTEN, BRAF* and *RET* genes, and to study the mRNA expression of *VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDK1, PTEN, AKT1, AKT2, COX-2* and *CALCA* in canine FTC and MTC.

2) To identify clinical, pathological and immunohistochemical (calcitonin, Ki-67 and E-cadherin) prognostic factors in dogs with thyroid tumors.

The specific aims of our research on *treatment optimization* were:

3) To evaluate the immunohistochemical expression of VEGF, p53, Cox-2 and P-gp in canine thyroid tumors and to assess their potential as therapeutic targets.

4) To evaluate the effect of levothyroxine therapy and TSH suppression on survival of dogs with thyroid tumors undergoing different treatment modalities.

5) To evaluate the short-term effect of rhTSH on thyroid volume and echogenicity, measured by ultrasonography in healthy Beagles.

6) To evaluate the effect of rhTSH, administered 24 h or 48 h before $^{123}$I, on thyroid RAIU in healthy Beagles.

7) To evaluate the effect of rhTSH, administered 24 h before $^{123}$I, on tumor RAIU in dogs with thyroid tumors.
Chapter 3

*K-RAS MUTATIONS AND INVOLVEMENT OF THE PI3K/AKT PATHWAY IN CANINE THYROID CARCINOMA*

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ABSTRACT

**Background:** Information on the genetic events leading to canine thyroid cancer is lacking.

**Hypothesis/Objectives:** To investigate mutational hotspots in candidate genes and mRNA expression of PI3K/Akt pathway related genes in canine thyroid carcinomas

**Animals:** 59 dogs with thyroid carcinoma and 10 healthy controls.

**Methods:** Mutation analysis was performed for known hotspots of *RAS (N, K, H), PIK3CA, BRAF, RET* and for the entire coding region of *PTEN*. Quantitative RT-PCR was performed for *VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2 and CALCA*.

**Results:** 43 dogs (73%) had follicular cell thyroid carcinomas (FTC) and 16 dogs (27%) had medullary thyroid carcinomas (MTC). Mutation analysis of *K-RAS* revealed 2 activating missense mutations, which have also been described in human thyroid cancer. A G12R substitution was present in 1 FTC and an E63K substitution was present in 1 MTC. No functional mutations were found in the sequenced regions of *H-RAS, N-RAS, PIK3CA, BRAF, RET* and *PTEN*.

The mRNA expressions of *VEGFR-1* (*P*<0.001), *VEGFR-2* (*P*=0.002), *PDPK1* (*P*<0.001), *AKT1* (*P*<0.009), and *AKT2* (*P*<0.001) were increased in FTCs, and those of *EGFR* (*P*<0.001), *VEGFR-1* (*P*=0.036), and *PIK3CA* (*P*=0.019) were increased in MTCs when compared to normal thyroid glands.

**Conclusions and clinical importance:** The mutations most frequently associated with human thyroid neoplasia are rare in canine thyroid cancer. An increased expression of several genes associated with PI3K/Akt signaling pathway was identified, indicating the involvement of this pathway in the pathogenesis of canine thyroid carcinomas, and warranting further research on gene amplification.
INTRODUCTION

Thyroid cancer represents 10-15% of all head and neck neoplasms in the dog.\(^1\) Ninety percent of canine thyroid tumors detected clinically are malignant and can be classified as either follicular cell thyroid carcinomas (FTCs), which arise from thyroid follicular cells, or medullary thyroid carcinomas (MTCs), which arise from the parafollicular C cells.\(^2\)

Information on the genetic pathogenesis of canine thyroid cancer is scarce. In agreement with human studies showing that mutations in the tumor suppressor gene P53 are uncommon in differentiated thyroid cancer, a somatic mutation in P53 has been identified in one of 23 canine FTCs.\(^{26,27}\) It has also been shown that approximately 60% of canine thyroid tumors are DNA-aneuploid which is comparable to what is observed in humans.\(^{35,36}\) However, in dogs more than 80% of aneuploid thyroid tumors are hypodiploid, while in humans hypodiploidy is rare.\(^{35,36}\)

In humans, follicular cell-derived thyroid carcinomas represent a wide spectrum of lesions, ranging from differentiated (follicular and papillary) to poorly differentiated and undifferentiated (anaplastic) carcinomas. Canine differentiated FTC (follicular, compact, follicular-compact, papillary) is remarkably similar in terms of histology and biological behavior to follicular thyroid carcinoma in humans.\(^8\) Likewise, the morphological, cytochemical and immunohistochemical features of canine MTC also resemble human MTC.\(^5\)

One of the most important genetic events described in human follicular thyroid carcinoma are point mutations in one of the three RAS genes, with a prevalence of 20-52%.\(^{13,14}\) The RAS genes (\(N, K,\) and \(H-RAS\)) encode membrane-bound intracellular proteins involved in cellular signal transduction. These proteins act as on/off switches, relaying extracellular signals to the cytoplasmic signaling cascades of the mitogen-activated protein kinase (MAPK) pathway and phosphoinositol-3-kinase(PI3K)/Akt pathway, which control cellular proliferation, differentiation and survival (Fig. 1).\(^{15}\)
Fig. 1. Simplified schematic illustration of PI3K/Akt and MAPK signaling pathways in thyroid cancer. These pathways are involved in propagation of signals from various cell membrane receptor tyrosine kinases into the nucleus, and they regulate multiple cell processes including proliferation, differentiation and survival. Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Endocrinology]\textsuperscript{21}, copyright (2011)
In many human cancers, point mutations in exon 1 (codons 12 and 13) or exon 2 (codons 59 and 61) fix RAS proteins in a permanently activated form, promoting uncontrolled cellular division and malignant transformation.\textsuperscript{16} Although RAS is a classical dual activator of both PI3K/Akt and MAPK signaling, RAS mutations seem to preferentially activate the PI3K/Akt pathway in thyroid gland tumorigenesis.\textsuperscript{14} RAS mutations overall are uncommon in canine tumors, however up to 25\% of canine non-small cell lung tumors and 80\% of canine pancreatic tumors have been reported to harbor \textit{K-RAS} mutations, and 25\% of dogs with acute myeloid or lymphoid leukemia harbor \textit{N-RAS} mutations.\textsuperscript{15,17-19} RAS mutations have not yet been investigated in canine thyroid tumors.

Point mutations in the tumor suppressor gene phosphatase and tensin homologue (\textit{PTEN}) and in the phosphatidylinositols-4,5-bisphosphate 3-kinase catalytic subunit alpha (\textit{PIK3CA}) gene are also reported in human follicular thyroid carcinoma with a prevalence of 6\% and 13\%, respectively.\textsuperscript{12} Because \textit{PTEN} has an inhibitory effect on the PI3K/Akt pathway, inactivating mutations or deletions in \textit{PTEN} lead to PIK3/Akt pathway activation and promote tumorigenesis.\textsuperscript{20} Activating point mutations in the \textit{PIK3CA} gene lead to a constitutively activated protein which also activates PI3K/Akt pathway. Mutations in \textit{PTEN} or \textit{PIK3CA} have not been evaluated in canine thyroid cancer.

The most important genetic alterations in human papillary thyroid carcinoma are activating point mutations of \textit{\textit{v}}-raf murine sarcoma viral oncogene homolog B (\textit{BRAF}), which have a prevalence of 45\%.\textsuperscript{22,23} The most prominent example is the transverse point mutation T1799A in exon 15 which leads to expression of a constitutively activated mutant protein (BRAF-V600E) that activates the MAPK signaling pathway.\textsuperscript{24} Mutations in \textit{BRAF} have not been investigated in canine thyroid tumors.

The principal molecular mechanism underlying human MTC is aberrant activation of the receptor tyrosine kinase (RTK) rearranged during transfection (\textit{RET}) which signals through the PI3K/Akt and MAPK pathways.\textsuperscript{28,141} Germ line \textit{RET} mutations are responsible for the hereditary forms of human MTC, such as multiple endocrine neoplasia type 2, while somatic \textit{RET} mutations are present in approximately
50% of patients with sporadic MTC. Additionally, RAS mutations have been reported in up to 68% of human MTCs without RET mutation. In the only case report of canine familial MTC, no mutation was found after complete genomic sequencing of RET. The genetic events underlying canine sporadic MTC have not yet been investigated.

Oncogenic gene amplification or copy-number gain are additional prominent genetic mechanisms causing thyroid gland tumorigenesis in humans. This is particularly the case for genes involved in the PI3K/Akt pathway. Prominent examples of such genes (and respective prevalence in human follicular carcinoma) include epidermal growth factor receptor (EGFR; 32.2%), vascular endothelial growth factor receptor-1 (VEGFR-1; 44.1%), vascular endothelial growth factor receptor-2 (VEGFR-2; 3.8%), PIK3CA (23.8%), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (PIK3CB; 45.5%), v-akt murine thymoma viral oncogene homolog 1 (AKT1; 8.1%), v-akt murine thymoma viral oncogene homolog 2 (AKT2; 22.4%) and 3-phosphoinositide dependent protein kinase-1 (PDPK1; 24.1%). EGFR, VEGFR-1 and VEGFR-2 are important regulators of both MAPK and PI3K/Akt signaling pathways, however copy number gains in these RTK genes are particularly associated with PI3K/Akt pathway activation in human thyroid cancer. An important and expected consequence of gene-amplification or pathway activation is increased mRNA and protein expression and consequent aberrant activation of downstream signaling. The mRNA expression of RTKs and effectors involved in PI3K/Akt signaling may provide valuable information regarding gene amplification and pathway activation and has not yet been investigated in canine thyroid tumors.

Several reports have suggested that the PI3K/Akt signaling pathway regulates the expression of cyclooxygenase-2 (Cox-2). It has been demonstrated that Cox-2 functions downstream of Akt, and that elevated Akt activity is crucial for COX-2 overexpression in apoptotic-resistant cells. COX-2 mRNA expression could therefore also be used to infer on the activity of the PI3K/Akt pathway.

The differentiation between canine MTC and FTC of compact type may be difficult based on microscopic observation alone. Immunohistochemistry (IHC) for thyroglobulin, calcitonin or markers of neuroendocrine tissue aids the diagnosis.
mRNA expression of calcitonin gene (calcitonin-related polypeptide alpha or CALCA) has not yet been evaluated in dogs with thyroid tumors and may also help differentiating FTCs from MTCs.

The goals of our study were to investigate mutational hotspots in RAS (N, K and H), PIK3CA PTEN, BRAF and RET, and to study the mRNA expression of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2 and CALCA in canine FTC and MTC.
MATERIALS AND METHODS

Case selection

The medical record databases of the Companion Animal Clinics of Ghent and Utrecht Universities were searched for dogs diagnosed with thyroid carcinoma from 1986 to 2013. Patients from which frozen (-80°C) tumor samples were not available were excluded.

Thyroid specimens

In total 59 thyroid tumors (43 FTC and 16 MTC) and 10 normal thyroid glands (whole tissue explants) were analyzed. Tumor samples were collected from the Department of Pathology, Bacteriology and Poultry Diseases of Ghent University and the Department of Pathobiology of Utrecht University. Samples were collected immediately after surgical or necropsy removal, slit and either formalin-fixed paraffin-embedded (FF-PE) or snap-frozen in liquid nitrogen and conserved at -80°C until total RNA extraction.

Histopathology

All HE slides were reviewed by a board-certified pathologist (RD). All tumors were classified according to World Health Organization classification of canine thyroid tumors. The distinction between adenoma and carcinoma was based on the histologic evidence of either capsular invasion, vascular invasion or metastases. Classification of medullary thyroid tumors was also based on positive immunohistochemistry for calcitonin, as previously described.

Immunohistochemistry

Five-μm sections from each FF-PE block were prepared on 3-aminopropyltriethoxysilane (APES)-coated slides. After dewaxing and rehydration, antigen retrieval was performed by immersion in citrate-buffered (0.01 M, pH 6) distilled water and microwaving in a pressure cooker for 15 min at 850 W and 15 min at 300 W. Slides were then allowed to cool for 20 min. Endogenous peroxidase was blocked with hydrogen peroxide 0.03% for 5 minutes followed by rinsing with water and phosphate-buffered saline (PBS pH 7.4). For calcitonin immunohistochemistry,
sections were incubated overnight with the primary antibody (rabbit polyclonal antibody A0576\textsuperscript{a} diluted 1:400) in a humidity chamber at 4°C. Preliminary evaluation of the optimal concentration of the primary antibody was performed with serial antibody dilutions using normal canine thyroid gland as positive control. Incubation with a polymer-based secondary antibody (EnVision\textsuperscript{TM}) was performed at room temperature for 30 min. After each incubation step, sections were rinsed with PBS. 3,3’ diaminobenzidine\textsuperscript{a} in substrate buffer solution served as chromogen and was allowed to react for 5 min. The sections were then counterstained with hematoxylin, rinsed in tap water, dehydrated and mounted with cover slips. Immunohistochemistry was performed in 2 batches.

All sections were examined by the same observer (MC) who was blinded to the clinical information and outcome of each patient. Thyroid tumors positive for calcitonin were classified as MTCs and thyroid tumors negative for calcitonin were classified as FTCs.\textsuperscript{5} To verify our classification, the subset of tumors positive for calcitonin was also stained for thyroglobulin (rabbit polyclonal antibody A0251\textsuperscript{a} diluted 1:800) in an automated immunostainer (Dako, S/N S38-7410-01). Calcitonin and thyroglobulin immunolabeling were not quantified. In both stains, the tumors were considered positive when the cytoplasm of neoplastic cells exhibited a fine granular staining pattern with cell-to-cell variation. Normal thyroid gland was used as control in each batch.

**RNA isolation and reverse transcription**

Frozen tissue samples were disrupted and homogenized using a rotor-stator homogenizer and total RNA was isolated using an RNeasy mini kit\textsuperscript{b} according to the manufacturer’s instructions. A DNase step was performed to avoid genomic DNA contamination. Purified RNA was quantified on a NanoDrop ND-1000 Spectrophotometer\textsuperscript{c} and, in 12 samples (first 10 patients of inclusion period and two normal thyroid glands sequenced), its integrity was assessed on a Bioanalyzer Micro RNA Chip.\textsuperscript{d} Total RNA was reverse transcribed into cDNA using the iScript cDNA synthesis kit\textsuperscript{e} according to the manufacturer’s instructions.
**Primer design**

All PCR amplification primers (Table 1) were designed with Perl-primer v1.1.21 according to the parameters of the Bio-Rad iCycler manual and were ordered from Eurogentec. All PCR primers were also used as sequence primers. When the region of interest could not be amplified in 1 stretch, overlapping primer pairs were used (Table 2). For quantitative RT-PCR (qPCR), temperature gradients were performed to determine the optimal annealing temperature of each primer pair and primer specificity was confirmed by melting curve analysis and sequence analysis of the PCR products (Table 3).
Table 1. PCR primers for amplification of canine RAS (K, N, H), BRAF, PIK3CA, RET and PTEN. All positions are based on the mRNA sequence published on NCBI.

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Sequence (5'-3’)</th>
<th>Location</th>
<th>Exons</th>
<th>T_a (°C)</th>
<th>Product length (bp)</th>
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<tbody>
<tr>
<td>K-RAS Fw18</td>
<td>ATAAAACCTTGGTAGTTGGAGGC</td>
<td>18/39</td>
<td>1–3</td>
<td>62°C</td>
<td>463</td>
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<tr>
<td>K-RAS Rv480</td>
<td>GTATAGAAGGCACATCGTCAACAC</td>
<td>459/480</td>
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</tr>
<tr>
<td>N-RAS Fw12</td>
<td>GGTCTCCAACCTTTCTCC</td>
<td>12/29</td>
<td>1–5</td>
<td>55°C</td>
<td>660</td>
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<tr>
<td>N-RAS Rv672</td>
<td>AGTGTCTTGTTACATCACCA</td>
<td>653/672</td>
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<td></td>
<td></td>
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<tr>
<td>H-RAS Fw54</td>
<td>CCATGACGAGTATAAGCTG</td>
<td>54/73</td>
<td>1–2</td>
<td>55°C</td>
<td>253</td>
</tr>
<tr>
<td>H-RAS Rv306</td>
<td>ATGGCAATAACACAGAAAAG</td>
<td>286/306</td>
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<tr>
<td>BRAF Fw1555</td>
<td>CGACAGACTGCACAGGGCATGG</td>
<td>1555/1573</td>
<td>13–16</td>
<td>55°C</td>
<td>372</td>
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<tr>
<td>BRAF Rv1926</td>
<td>CCGTACCTTACTGAGATCGGAG</td>
<td>1904/1926</td>
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<tr>
<td>PIK3CA Fw1504</td>
<td>TGCCTGAAACCCTATTGGTG</td>
<td>1504/1521</td>
<td>8–12</td>
<td>55°C</td>
<td>450</td>
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<tr>
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<td>19–21</td>
<td>55°C</td>
<td>554</td>
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<tr>
<td>PIK3CA Rv3425</td>
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<td>3408/3425</td>
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<td></td>
</tr>
<tr>
<td>RET Fw1600</td>
<td>AAGTGCAGAGTGAGACAG</td>
<td>1600/1617</td>
<td>9–15</td>
<td>62°C</td>
<td>1055</td>
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<td>RET Rv2654</td>
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<td>2635/2654</td>
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<tr>
<td>PTEN Fw76</td>
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<td>PTEN Fw719</td>
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<td>719/735</td>
<td>6–8</td>
<td>55°C</td>
<td>742</td>
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<tr>
<td>PTEN Rv1460</td>
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Accession numbers: K-RAS: XM_003433561.2, N-RAS: XM_001287065.1, H-RAS: XM_001287069.1, BRAF: XM_532749, PIK3CA: XM_545208.4, RET: NM_001197099.1, PTEN: NM_001003192.1; Abbreviations: K-RAS, Kirsten rat sarcoma viral oncogene homolog; N-RAS, neuroblastoma RAS viral (v-ras) oncogene homolog; H-RAS, Harvey rat sarcoma viral oncogene homolog; BRAF, v-raf murine sarcoma viral oncogene homolog B; PIK3CA, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha; RET, ret proto-oncogene; PTEN, phosphatase and tensin homolog; Fw, forward; Rv, reverse; T_a, optimal annealing temperature; bp: base pairs
Table 2. Sequencing primers for canine \textit{RAS} (\textit{K, N, H}), \textit{BRAF}, \textit{PIK3CA}, \textit{RET} and \textit{PTEN}. All positions are based on the mRNA sequence published on NCBI.

<table>
<thead>
<tr>
<th>PCR primers</th>
<th>Sequence (5’-3’)</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{K-RAS} Rv355</td>
<td>ATTTCTACTAGGACCATAGGT</td>
<td>334/355</td>
</tr>
<tr>
<td>\textit{N-RAS} Fw217</td>
<td>AAACAGTTGTTATAGACGG</td>
<td>217/236</td>
</tr>
<tr>
<td>\textit{N-RAS} Rv524</td>
<td>GTTTCAATGAATGGAATCCC</td>
<td>505/524</td>
</tr>
<tr>
<td>\textit{H-RAS} Fw167</td>
<td>GACTCTATCGGAAGCAAG</td>
<td>167/185</td>
</tr>
<tr>
<td>\textit{H-RAS} Rv230</td>
<td>CTGTGTCAGGATGTCCAG</td>
<td>212/230</td>
</tr>
<tr>
<td>\textit{PIK3CA} Fw1549</td>
<td>CTCCATGCTTAGGAGTGGAAG</td>
<td>1549/1568</td>
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<tr>
<td>\textit{PIK3CA} Rv1815</td>
<td>CACAATAGTGCTGTGGCTC</td>
<td>1796/1815</td>
</tr>
<tr>
<td>\textit{PIK3CA} Fw3029</td>
<td>GATTAAGAGAGCAGCCAGG</td>
<td>3029/3048</td>
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<tr>
<td>\textit{PIK3CA} Rv3336</td>
<td>CATGCTGCTTAATGGTGGAAG</td>
<td>3317/3336</td>
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<tr>
<td>\textit{RET} Fw1906</td>
<td>GTGCTCTTCTCCTCATCGT</td>
<td>1906/1925</td>
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<td>\textit{RET} Fw2221</td>
<td>AAAGGCAAGCAGGATACAC</td>
<td>2221/2240</td>
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<td>\textit{RET} Rv2278</td>
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<td>2259/2278</td>
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<td>\textit{PTEN} Fw475</td>
<td>CACTGTAAAGCTGGAAAGGG</td>
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<td>\textit{PTEN} Rv249</td>
<td>CCTGTATACGCCCTCAAGTC</td>
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<td>\textit{PTEN} Rv595</td>
<td>TGTCCTCGTTCTCCTACTTCC</td>
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<td>\textit{PTEN} Fw1158</td>
<td>TGTAAGGAGCCATCAAACC</td>
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<td>\textit{PTEN} Rv1285</td>
<td>CAAAGGTTTCATTCTCTGGG</td>
<td>1266/1285</td>
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\textbf{Abbreviations:} \textit{K-RAS}, Kirsten rat sarcoma viral oncogene homolog; \textit{N-RAS}, neuroblastoma \textit{RAS} viral (v-ras) oncogene homolog; \textit{H-RAS}, Harvey rat sarcoma viral oncogene homolog; \textit{PIK3CA}, phosphatidylinositol-4,5-biphosphate 3-kinase, catalytic subunit alpha; \textit{RET}, ret proto-oncogene; \textit{PTEN}, phosphatase and tensin homolog; Fw, forward; Rv, reverse.
Table 3. Quantitative RT-PCR primers for canine VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2, CALCA, RPS5 and HPRT. All positions are based on the mRNA sequence published on NCBI.

<table>
<thead>
<tr>
<th>qPCR primers</th>
<th>Sequence (5’-3’)</th>
<th>Location</th>
<th>T&lt;sub&gt;a&lt;/sub&gt; (°C)</th>
<th>Product length (bp)</th>
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<tr>
<td>VEGFR-1 Fw189</td>
<td>GGCTCAGGCAAAACCACAC</td>
<td>189/206</td>
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<tr>
<td>VEGFR-1 Rv378</td>
<td>CCGGCAGGGGATGACGAT</td>
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<tr>
<td>VEGFR-2 Fw3606</td>
<td>GGAAGAGGAAGTGTGTGACCC</td>
<td>3606/3627</td>
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<tr>
<td>VEGFR-2 Rv3786</td>
<td>GACCATACCCTGTCCGTCTGG</td>
<td>3765/3786</td>
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<tr>
<td>EGFR Fw2078</td>
<td>CTGGAGCATTGGGCA</td>
<td>2078/2092</td>
<td>53</td>
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<td>EGFR Rv2185</td>
<td>TGGCTTGGGAGAGACG</td>
<td>2171/2185</td>
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<tr>
<td>PIK3CA Fw1269</td>
<td>CCTTGTTCTAATCCAGGTCG</td>
<td>1269/1288</td>
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<td>PIK3CA Rv1402</td>
<td>GGACAGTGTTCTCTTCTAC</td>
<td>1383/1402</td>
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<td>PIK3CB Fw2978</td>
<td>CCTTCAACAAAGATGGCC</td>
<td>2978/2995</td>
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<td>PIK3CB Rv3119</td>
<td>CTATGTCTATACATCCCAT</td>
<td>3099/3119</td>
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<td>PDPK1 Fw667</td>
<td>AGGTCTGACTGTTTACC</td>
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<td>PDPK1 Rv865</td>
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<td>PTEN Fw1209</td>
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<td>AKT1 Fw717</td>
<td>CACCGTGTGACCATGAGAC</td>
<td>717/737</td>
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<td>AKT1 Rv799</td>
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<td>AKT2 Fw71</td>
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<td>TTCCAGACAGCGAGGCTAAT</td>
<td>971/990</td>
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<td>COX-2 Rv1082</td>
<td>GCAGTCTGCTGGGTCAAACC</td>
<td>1063/1082</td>
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<td>CALCA Fw157</td>
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<td>157/176</td>
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<td>CALCA Rv254</td>
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<td>RPS5 Fw405</td>
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<td>AGCTTGCTGTTGAAAGGAC</td>
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<td>HPRT Rv587</td>
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Accession numbers: VEGFR-1: AF262963.1, VEGFR-2: NM_001048024.1, EGFR: XM_533073.4, PIK3CA: XM_545208.4, PIK3CB: XM_534280.4, PDPK1: XM_005621677.1,
K-RAS mutations & PI3K/Akt pathway

**Abbreviations:** VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor 2; EGFR, epidermal growth factor receptor; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha; PIK3CB, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta; PDPK1, 3-phosphoinositide dependent protein kinase-1; PTEN, phosphatase and tensin homolog; AKT1, v-akt murine thymoma viral oncogene homolog 1; AKT2, v-akt murine thymoma viral oncogene homolog 2; COX-2, cyclooxygenase-2; CALCA, calcitonin-related polypeptide alpha; RPS5, ribosomal protein S5; HPRT, hypoxanthine phosphoribosyltransferase; Fw, forward; Rv, reverse; T_a, optimal annealing temperature; bp: base pairs;

**PCR amplification**

PCR amplification was performed using the Phusion Hot Start Flex DNA Polymerase on a C-1000 Touch thermal cycler (BioRad). PCR products were evaluated by agarose gel electrophoresis to confirm expected product length.

**Sequencing**

Sequencing was performed for human mutation hotspots of N-, K- and H-RAS (exons 1 and 2)\(^{145}\), BRAF (exon 15)\(^{146}\), PIK3CA (exons 9 and 20)\(^{147}\), RET (exons 8, 10, 11, 13-16)\(^{32}\) and for the entire coding region of PTEN (hotspot exons 5-8)\(^{148}\) on all 59 tumor samples and two normal thyroid glands.

PCR products were amplified for sequencing using the BigDye Terminator version 3.1 Cycle Sequencing Kit and filtrated using Sephadex G-50 Superfine. Sequencing was performed using the ABI3130XL Genetic Analyzer according to the manufacturer’s instructions. The obtained sequences were compared to the consensus mRNA sequence using DNASTAR Lasergene core suite 11. All mutations affecting the amino acid sequence were confirmed by repeating RNA extraction, reverse transcription and sequencing.

**Quantitative RT-PCR**

After reverse transcription, qPCR analyses were performed on cDNA to determine and compare the levels of expression of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2 and CALCA in thyroid tumors and normal thyroid glands. To correct for differences in sample input, the expression levels were normalized to the expression of the reference genes ribosomal protein S5.
(RPS5) and hypoxanthine phosphoribosyltranferase (HPRT), already proven to be stable in other canine tissues. Furthermore, the stability (M-value) of the reference genes was verified.

Quantitative RT-PCR was performed on a CFX384 real-time PCR detection system (Bio-Rad). Each qPCR reaction mixture consisted of 4 μL cDNA (diluted 50x), 0.4 μL of both the forward and the reverse primers, 5 μL iQ SYBR Green Supermix and 0.2 μL MilliQ, adding up to a final reaction volume of 10 μL. The thermal cycles were performed as previously described. A fourfold reference standard dilution series using 10× diluted cDNA was included in every plate to assess reaction efficiency, and negative controls to assess the specificity of the reaction and check for contamination. Data were analyzed with CFX manager version 3.0. Relative mRNA expression levels were calculated by relative quantitation and the fold-expression changes were determined by means of the \(2^{-\Delta\Delta CT}\) method. The maximum allowed cycle threshold (CT) value for calculations was 45.

Statistical analysis

Relative expression levels of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2 and CALCA were compared between thyroid tumors (FTCs and MTCs) and normal thyroid glands with an independent-sample Kruskal-Wallis test. Significance level was set at 5%. The Dunn’s test was used for multiple comparisons (adjusted \(P\) values shown in the results section). The same procedure was done to compare the relative expression of reference genes between tumor samples and normal thyroid glands.

According to the mRNA expression of target genes, patients were grouped by means of unsupervised hierarchical clustering. Unsupervised clustering was implemented by Pearson correlation for genes, and by Spearman correlation for samples.
RESULTS

All 59 thyroid tumors were classified as carcinomas. Forty-three dogs (73%) had FTC and 16 dogs (27%) had MTC. Histologic subtypes of FTC included follicular (n=8, 13%), follicular-compact (n=11, 19%), compact (n=18, 30%), papillary (n=1, 2%), follicular-papillary (n=1, 2%), and carcinosarcoma (n=4, 7%).

Mutation analysis

Mutation analysis of K-RAS revealed 2 amino acid changing (missense) point mutations in 2 different tumors and a splice variant present in all thyroid samples, including the normal thyroid glands. A G12R substitution (GGT>CGT) was present in one FTC of compact type and an E63K substitution (GAG>AAG) was observed in one MTC (Fig. 2). In the K-RAS splice variant, exon 2 was missing.

Fig. 2. Mutations found in codon 12 of K-RAS;
A: G12R substitution in a canine FTC;  B: E63K substitution in a canine MTC.
Abbreviations: WT, wild type
Mutation analysis of \textit{N-RAS} showed 1 silent mutation in codon 138 (GGG\textgreater GGA) in 2 FTCs of compact type, occurring in heterozygous form.

Mutation analysis of \textit{H-RAS} showed 1 silent mutation in codon 47 (GAC\textgreater GAT) in 21 FTCs and 5 MTCs occurring in both homozygous and heterozygous form.

Mutation analysis of \textit{BRAF} did not reveal any point mutations. However, a splice variant in which exons 13 and 14 were missing was present in all samples, including normal thyroid glands.

Mutation analysis of \textit{PIK3CA} did not show any abnormalities in the sequenced region.

Mutation analysis of the entire coding region of \textit{PTEN} showed 1 silent mutation in codon 325 (CTC\textgreater CTT) in 5 FTCs and 3 MTCs, occurring in both homozygous and heterozygous form.

\textbf{\textit{mRNA expression of PI3K/Akt pathway related genes}}

Relative mRNA expression was evaluated in 41 FTCs, 15 MTCs and 10 normal thyroid glands (Fig. 3). Relative expression levels of reference genes were not significantly different between thyroid tumors and normal thyroid glands.

The relative expression levels of \textit{VEGFR-1} (\(P<0.001\)), \textit{VEGFR-2} (\(P=0.002\)), \textit{PDPK1} (\(P<0.001\)), \textit{AKT1} (\(P=0.009\)), and \textit{AKT2} (\(P<0.001\)) were significantly higher in FTC than in normal thyroid glands. The relative expression levels of \textit{EGFR} (\(P<0.001\)), \textit{VEGFR-1} (\(P=0.036\)), \textit{PIK3CA} (\(P=0.019\)), and \textit{CALCA} (\(P<0.001\)) were significantly higher in MTC than in normal thyroid glands. Relative expression levels of \textit{PTEN}, \textit{PIK3CB} and \textit{COX-2} were not significantly different between thyroid tumors and normal thyroid glands. The relative expression levels of \textit{CALCA} did not overlap between FTCs (range 0 – 3.6) and MTCs (range 3.9 – 156.1).
Fig. 3. Dot plots representing the relative mRNA expression levels of VEGFR-1, VEGFR-2, EGFR, PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2 and CALCA in canine normal thyroid gland (n=10), FTC (n=41) and MTC (n=15). Significant differences between tumors and normal thyroid gland tissue are indicated with asterisks.
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Abbreviations: EGFR, epidermal growth factor receptor; VEGFR-1, vascular endothelial growth factor receptor 1; VEGFR-2, vascular endothelial growth factor receptor 2; PDPK1, 3-phosphoinositide dependent protein kinase 1; AKT1, v-akt murine thymoma viral oncogene homolog 1; AKT2, v-akt murine thymoma viral oncogene homolog 2; PTEN, phosphatase and tensin homolog; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase, subunit alpha; PIK3CB, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit beta; COX-2, cyclooxygenase-2; CALCA, calcitonin-related polypeptide alpha; * P<0.05; ** P<0.01; *** P<0.001.

Unsupervised hierarchical clustering of the samples showed an almost perfect branching of normal thyroid gland, FTC and MTC when all genes were included in the analysis (Fig. 4). After omitting the relative expression of CALCA, clustering based on the remaining genes showed a more elaborate branching with normal thyroid gland enrichment in one branch, MTC enrichment in subsequent branching, and a final branch enriched in FTC.

Unsupervised hierarchical clustering of the genes showed separate branching for CALCA, COX-2, EGFR and PTEN, while VEGFR-1 and VEGF-2 were grouped together and closely positioned to all effectors of the PI3K/Akt pathway (PIK3CA, PIK3CB, PDPK1, AKT1 and AKT2).
Fig. 4. Heat maps illustrating the results of the unsupervised hierarchical clustering in canine normal thyroid gland (n=10), follicular cell thyroid carcinoma (n=41) and medullary thyroid carcinoma (n=15). A: All genes included in the analysis. B: Relative expression of CALCA omitted from the analysis.
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DISCUSSION

Information on the genetic events leading to canine thyroid cancer is scarce. In our study, we investigated mutational hotspots and relative expression of candidate genes commonly involved in tumorigenesis of the human thyroid gland, in 59 canine thyroid carcinomas. Missense mutations in $K$-RAS were found in 1 FTC and 1 MTC. Furthermore, the expression of several genes involved in the PI3K/Akt signaling pathway was increased in canine thyroid carcinomas, when compared to normal canine thyroid gland.

The 2 missense mutations in $K$-RAS identified in our study have also been reported in human thyroid cancer with a similar prevalence. The G12R substitution observed in a compact FTC has been described in 1 of 24 follicular thyroid carcinomas and in 3 of 108 sporadic MTCs without RET mutation in humans.\textsuperscript{151,152} In veterinary medicine, 1 of 5 dogs with pancreatic carcinoma, 1 of 126 dogs with pulmonary carcinoma and 1 of 19 dogs with gastric epithelial tumors were also reported to harbor this $K$-RAS mutation.\textsuperscript{19,153,154}

Regulation of RAS protein function occurs through intrinsic guanosine triphosphatase (GTPase) activity, which in the wild-type RAS switches the protein from an active (guanosine triphosphate[GTP]-bound) to an inactive (guanosine diphosphate [GDP]-bound) state. The substitution of an amino acid without a side chain (glycine) in position 12 by another amino acid with a side chain (arginine), interferes with the geometry of the protein impeding GTP to be hydrolyzed by GTPase.\textsuperscript{155} Such mutations in RAS lead to a permanently activated protein and downstream signaling, facilitating uncontrolled cell division and tumor growth.\textsuperscript{156}

The E63K substitution observed in a MTC has also been described in 1 of 16 MTCs without RET mutation in humans.\textsuperscript{157} This mutation affects an evolutionary conserved amino acid residue identical in all RAS proteins. Similarly to what is described for G12R substitution, the change of glutamic acid in position 63 by lysine also has been demonstrated to abolish GTPase activity, leading to constitutive activation of RAS and potentiating cellular transformation.\textsuperscript{158}
In our study, no amino acid changing mutations were found in the sequenced regions of H-RAS, N-RAS, BRAF, PIK3CA and RET nor in the entire coding sequence of PTEN. These results demonstrate that the mutations most commonly involved in human thyroid tumorigenesis are rare and do not play a major role in the pathogenesis of canine thyroid cancer.

A splice variant of K-RAS in which exon 2 was missing was observed in all thyroid tumors and, to the authors’ knowledge, no equivalent splice variant has been described in humans. Given its presence in normal thyroid gland tissue, it is unlikely that this splice variant is a causal factor in thyroid gland tumorigenesis.

A BRAF splice variant, in which exons 13 and 14 were missing, was identified in all samples. Aberrant BRAF splicing has been described in humans. Splice variants that do not have the N-terminal auto-inhibitory domain lead to formation of truncated proteins, containing only the C-terminal kinase domain, which are constitutively activated. These splice variants may function as an alternative mechanism for oncogenic BRAF activation. However, given that this splice variant was also present in normal thyroid gland tissue, it seems unlikely that it plays a role in canine thyroid gland tumorigenesis.

No mutations were found in the sequenced region of RET in 16 MTCs. This is in agreement with a case report of canine familial MTC in which no mutations were found after complete sequencing of genomic RET. Further research is needed to investigate the genetic events involved in the pathogenesis of canine MTC.

In humans, gene amplification can lead to the activation of tumorigenesis-related signaling pathways and plays an important role in thyroid gland tumorigenesis. In our study, overexpression of VEGFR-1, VEGFR-2, PDPK-1, AKT1 and AKT2 in canine FTC, and overexpression of VEGFR-1, EGFR and PIK3CA in canine MTC suggests activation of PI3K/Akt pathway, particularly in FTC. PI3K/Akt pathway could therefore play an important role in canine thyroid tumorigenesis promoting cell proliferation, resistance to apoptosis and malignant transformation.

The involvement of EGFR, VEGFR-1, VEGFR-2 and PI3K/Akt pathway in the pathogenesis of canine thyroid carcinoma suggests these may constitute promising therapeutic targets. The importance of PI3K/Akt pathway activation and the value of
targeting this pathway have been recently demonstrated in several canine cancer cell lines.\textsuperscript{25} Furthermore, a preliminary study in dogs with solid tumors showed that toceranib phosphate, a multi-targeted TKI which targets VEGFR-2, was associated with a clinical benefit rate of 80\% in 15 dogs with thyroid carcinoma.\textsuperscript{62} In humans with unresectable, radiiodine-refractory thyroid cancer, TKIs and inhibitors of PI3K/Akt signaling have shown encouraging results in recent clinical trials.\textsuperscript{161}

Despite the overexpression of many PI3K/Akt related genes suggesting pathway activation, the relative expression of \textit{COX}-2 was not increased in canine thyroid tumors. Similar findings have been reported in human follicular carcinoma, where PI3K/Akt pathway activation is of major importance.\textsuperscript{162} This suggests that mRNA expression of \textit{COX}-2 may not reflect activation of PI3K/Akt signaling in thyroid cancer.

As expected, canine FTC and MTC showed distinct mRNA expression profiles of PI3K/Akt pathway related genes. These differences were confirmed by unsupervised clustering and suggest that these tumor types probably arise from different molecular mechanisms. The fact that the mRNA expression of \textit{CALCA} did not overlap between FTC and MTC confirms the accuracy of tumor classification based on immunohistochemistry for calcitonin.

Limitations of our mutational analysis include not sequencing normal tissue in patients where mutations were found, to differentiate germ line from somatic mutations, and only sequencing part of the coding region for most genes. Absence of mutations in these regions does not rule out genetic events in the remaining coding region or in the non-coding region of these genes.

Our findings of increased expression of RTKs and intracellular effectors involved in PI3K/Akt signaling urgently ask for further research on phosphorylation of Akt (pAkt) and other members of this pathway to verify pathway activation. Moreover, future studies should also evaluate gene amplification and altered promoter activity of the genes found to be overexpressed in our research.

In conclusion, 2 missense mutations in \textit{K-RAS} were identified in a FTC and a MTC which are likely to be relevant for thyroid gland tumorigenesis. The mutations most frequently associated with human thyroid neoplasia are rare in canine thyroid
cancer. Overexpression of *VEGFR-1, VEGFR-2, PDPK-1, AKT1* and *AKT2* in canine FTC and *VEGFR-1, EGFR* and *PIK3CA* in canine MTC suggests PI3K/Akt signaling pathway is activated and likely involved in the pathogenesis of canine thyroid cancer, particularly in FTC. Further studies are needed to investigate if gene amplification or altered promoter activity is responsible for the increased mRNA expression of these genes.
ENDNOTES

a Dako, Glostrup, Denmark
b Qiagen, Hilden, Germany
c NanoDrop Technologies, Wilmington, DE
d Agilent Technologies, Santa Clara, CA
e Bio-Rad, Hercules, CA
f Eurogentec, Maastricht, The Netherlands
g New England BioLabs Inc, Ipswich, MA
h Applied Biosystems
i Amersham, Buckinghamshire, UK
j AB Applied Biosystems, Carlsbad, CA
k DNASTAR, Madison, WI
l GraphPad Prism 6.03, GraphPad Software Inc., La Jolla, CA
m R 3.0.2

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The authors thank the Department of Pathobiology of Utrecht University for providing all paraffin embedded tumor samples of patients diagnosed and treated at Utrecht University and the Department of Morphology of Gent University for their collaboration in sectioning the paraffin embedded tumor samples. The authors also thank Prof. Luc Duchateau of the Department of Comparative Physiology and Biometrics of Ghent University for his assistance in the statistical analysis.
Chapter 4

CLINICAL, PATHOLOGICAL AND IMMUNOHISTOCHEMICAL PROGNOSTIC FACTORS IN DOGS WITH THYROID CARCINOMA

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ABSTRACT

Background: Prognostic markers for dogs with thyroid tumors are lacking.

Hypothesis/Objectives: To identify clinical, pathological and immunohistochemical prognostic factors for dogs with thyroid tumors.

Animals: 70 dogs with thyroid neoplasia.

Methods: Retrospective study. Dogs with thyroid neoplasia were included when follow-up information and formalin-fixed paraffin-embedded tumor samples were available. Immunohistochemistry (IHC) was performed for thyroglobulin, calcitonin, Ki-67 and E-cadherin. Correlation of tumor variables (diameter, volume, localization, scintigraphic uptake, thyroid function, IHC) with local invasiveness and metastatic disease was performed on all tumor samples. Forty-four dogs treated by thyroidectomy were included in a survival analysis.

Results: 50 dogs (71%) had differentiated follicular cell thyroid carcinoma (dFTC) and 20 (29%) had medullary thyroid carcinoma (MTC). At diagnosis, tumor diameter ($P=0.007; P=0.038$), tumor volume ($P=0.020$), Ki-67 ($P=0.038$), ectopic location ($P=0.002$) and follicular cell origin ($P=0.044$) were positively associated with local invasiveness; tumor diameter ($P=0.002$), tumor volume ($P=0.023$) and bilateral location ($P=0.012$) were positively associated with presence of distant metastases.

Forty-four dogs (28 dFTCs, 16 MTCs; stage I-III) underwent thyroidectomy. Outcome was comparable between dogs with dFTC and MTC. Histologic ($P=0.046$) and macroscopic ($P=0.038$) vascular invasion were independent negative predictors for disease-free survival. Although time to presentation, histologic vascular invasion, and Ki-67 were negatively associated with time to metastases, and time to presentation was negatively associated with time to recurrence, no independent predictors were found. E-cadherin expression was not associated with outcome.

Conclusions and clinical importance: Prognostic factors have been identified which provide relevant information for owners and clinicians.
INTRODUCTION

Thyroid cancer represents 10-15% of all head and neck neoplasms in the dog. Ninety percent of canine thyroid tumors detected clinically are carcinomas and up to 38% of dogs with carcinomas present with metastases at the time of diagnosis. Thyroidectomy is the preferred treatment modality for tumors that are mobile and well circumscribed, while unresectable invasive tumors may be treated with external beam radiation or radioactive iodine-131 (¹³¹I) therapy.

In humans, well established prognostic factors for thyroid carcinoma include age, gender, tumor size, stage, histologic type and grade, vascular invasion and extrathyroidal tumor extension. Low-risk patients undergo a follow-up strategy that is considerably different from that of high-risk patients.

In dogs, tumor volume > 20 cm³, bilateral disease and cervical vascular invasion have been associated with high metastatic rates. However, these associations were often based on necropsy studies or studies in dogs with unresectable tumors and there are few published studies on prognostic predictors for dogs with operable thyroid tumors. Breed, sex, histologic type, and tumor size did not appear to affect prognosis after surgical resection, while bilateral disease and histological grade of malignancy were prognosticators. Prognostic markers are lacking for dogs with thyroid tumors undergoing thyroidectomy.

Thyroid carcinoma can arise from thyroid follicular cells (follicular cell thyroid carcinoma - FTC), or from the parafollicular C cells (medullary thyroid carcinoma - MTC). According to the World Health Organization (WHO) histologic classification of canine thyroid tumors, FTC can be classified as well differentiated (dFTC) (follicular, compact, follicular-compact, papillary), poorly differentiated, undifferentiated or carcinosarcoma. In humans, MTC is more aggressive than dFTC. In most veterinary studies, the prevalence of MTC is likely underestimated as these tumors may be difficult to distinguish from dFTC of compact type by microscopic observation alone; immunohistochemistry (IHC) for calcitonin or for markers of neuroendocrine tissue is required for their identification. In one study, MTC represented 36% of all canine thyroid tumors and was suggested to be more amenable
to complete surgical resection and have lower metastatic potential than FTC.\textsuperscript{4} However, it is still not clear whether canine dFTC and MTC differ with respect to prognosis following thyroidectomy.

E-cadherin is a transmembrane adhesion glycoprotein of epithelial tissues and plays a role in neoplastic cell behavior as a suppressor of invasion and metastasis.\textsuperscript{55} In human thyroid carcinomas, loss of E-cadherin expression is an independent prognostic indicator associated with a higher degree of dedifferentiation and higher metastatic potential.\textsuperscript{55} In dogs with mammary carcinoma, loss of E-cadherin expression was also found to be related to prognosis.\textsuperscript{56} The prognostic significance of E-cadherin expression in canine thyroid gland carcinoma has not been investigated.

Ki-67 is a cellular proliferation marker expressed in the cell nuclei during all active phases of the cell cycle (G1, S, G2 and mitosis) but not in G0.\textsuperscript{57} In human dFTC, high Ki-67 labeling index is associated with higher metastatic rates at diagnosis and shorter disease-free survival (DFS).\textsuperscript{58,59} Although the use of Ki-67 as a marker for prognosis was shown to have limitations in certain canine tumors, its value is well established in mast cell tumors.\textsuperscript{57,60} The prognostic relevance of Ki-67 expression has not yet been examined in canine thyroid tumors.

The goals of the present study were to identify clinical, pathological and immunohistochemical (calcitonin, Ki-67 and E-cadherin) prognostic factors for dogs with thyroid tumors.
MATERIALS AND METHODS

Case selection

The medical record databases of the Companion Animal Clinics of Ghent and Utrecht Universities were searched for dogs diagnosed with thyroid neoplasia from January 1, 1986 to January 1, 2012. For inclusion in the study the diagnosis had to be confirmed by histopathology. Patients with no follow-up information and patients for which the paraffin-embedded tumor samples were not available were excluded.

Medical records review

The medical records of the dogs that met the inclusion criteria were reviewed. When the complete follow-up information was not available in the medical record, referring veterinarians and clients were contacted by phone. The information retrieved from medical records included signalment, time to presentation (from detection of cervical mass to diagnosis), owners impression of tumor progression rate, clinical signs, physical examination, tumor mobility (determined via palpation), tumor measurements, medical imaging results (thoracic radiographs, cervical and thoracic scintigraphy, cervical ultrasonography, computed tomography (CT) scan), WHO tumor stage, treatment, surgery report, outcome and necropsy report. Whenever possible, dimensions of the tumor were based on measurements taken immediately after surgical or necropsy excision and alternatively based on measurements taken during CT scan, cervical ultrasonography or physical examination. The volume of each thyroid tumor was estimated by the use of an ellipsoid formula.

Thyroid volume (mL or cm\(^3\)) = length (cm) x width (cm) x height (cm) x \(\pi/6\)

When more than one thyroid lobe was affected, the sum of both lobes and/or ectopic tissue was used.

Staging was performed according to the WHO staging system (Table 1), based on tumor measurements, thoracic radiographs, cervical and thoracic scintigraphic examination and, when available, cytology and histopathology of regional lymph nodes (LNs) (mandibular and retropharyngeal). In most cases, regional LNs were aspirated or excised (for patients undergoing thyroidectomy) when found to be enlarged at physical examination or surgical exploration; in a subset of patients, cervical ultrasonography
and CT-scan also aided the evaluation of regional LNs. Regional LN metastases were confirmed by histolopathology or by obvious macroscopic evidence of LN invasion during surgery.

Thyroid function status at the time of diagnosis was determined based on basal circulating total thyroxine (TT₄) and thyrotropin (TSH) concentrations and, when available, results of TSH stimulation test.

Surgical and necropsy reports were reviewed to determine if there was macroscopic evidence of vascular or local invasion by the primary tumor. Macroscopic vascular invasion was defined as evidence of tumor emboli in the cervical blood vessels. Macroscopic local invasion was defined as evidence of growth of the primary tumor into neighboring tissues (eg, cervical muscles, esophagus and trachea).

**Table 1.** World Health Organization’s clinical staging system for dogs with thyroid tumors. (Reproduced, with the permission of the publisher, from Owen LN, *TMN classification of tumors in domestic animals*. Geneva: World Health Organization, 1980)³

<table>
<thead>
<tr>
<th>Stage</th>
<th>Primary tumor</th>
<th>Regional LN</th>
<th>Distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T₁a,b</td>
<td>N₀</td>
<td>M₀</td>
</tr>
<tr>
<td></td>
<td>T₀</td>
<td>N₁</td>
<td>M₀</td>
</tr>
<tr>
<td>II</td>
<td>T₁a,b</td>
<td>N₁</td>
<td>M₀</td>
</tr>
<tr>
<td></td>
<td>T₂a,b</td>
<td>N₀ or N₁a</td>
<td>M₀</td>
</tr>
<tr>
<td>III</td>
<td>T₃</td>
<td>Any N</td>
<td>M₀</td>
</tr>
<tr>
<td></td>
<td>Any T</td>
<td>N₁b or N₂b</td>
<td>M₀</td>
</tr>
</tbody>
</table>

**Abbreviations:** T₀, microscopic residual disease; T₁, < 2cm; T₂, 2-5cm; T₃ >5cm; N₀, no lymph node involvement; N₁, ipsilateral lymph node involvement; N₂, bilateral lymph node involvement; M₀, no evidence of distant metastases; M₁, evidence of distant metastases; a, freely movable; b, fixed.
Overall survival (OS), DFS, time to loco-regional recurrence (TR) and time to distant metastases (TM) were determined for dogs treated with thyroidectomy. Overall survival was defined as time from thyroidectomy to death caused by thyroid neoplasia; patients still alive at the last observation time and patients who died due to other causes were censored. If the cause of death could not be determined, it was assumed to be related to thyroid neoplasia. Disease-free survival was defined as TR, TM or time to death caused by thyroid neoplasia, whichever came first. Patients disease-free at the last observation time and patients who died due to other causes were censored. Time to loco-regional recurrence was defined as time from thyroidectomy to local recurrence or regional LN metastases, with dogs censored at the last follow-up whenever physical examination revealed no recurrence or at the time of death if necropsy revealed no tumor recurrence or LN metastases. For TM, distant metastasis was the event of interest, with dogs censored at the last observation time when thoracic radiographs showed no signs of metastatic disease, or at the time of death if necropsy revealed no distant metastases.

**Tumor specimens**

Formalin-fixed paraffin-embedded (FF-PE) tissue blocks for each patient were collected from the Departments of Pathology of Ghent and Utrecht Universities and, in some dogs, multiple blocks from one tumor site and/or blocks from multiple sites (local, regional lymph node, distant metastases) were available. All samples were obtained at surgery or necropsy. In total, 304 FF-PE blocks from 74 patients (52 from Utrecht University, 22 from Ghent University) were available.

**Histopathology**

Five-μm sections from each FF-PE block were stained with hematoxylin and eosin (HE). All HE slides were reviewed by a single board-certified pathologist (RD) blinded to clinical information and previous histopathology report.

The distinction between adenoma and carcinoma was based on the histologic evidence of either capsular invasion, vascular invasion or metastases. The histologic type of primary tumors was classified according to the WHO classification as tumors of follicular cell origin (follicular, compact, follicular-compact, papillary, poorly
differentiated, undifferentiated, carcinosarcoma) or C cell (medullary) origin.\textsuperscript{7} Classification of medullary thyroid tumors was also based on IHC for calcitonin. Follicular cell carcinomas classified as poorly differentiated, undifferentiated or carcinosarcoma were excluded due to their aggressive biological behavior.

Primary thyroid tumors were characterized histologically by local invasion (tumor growth into neighboring tissues observed or not observed), vascular invasion (tumor growth into blood vessels observed or not observed), capsular invasion (not observed, invasion into tumor capsule, invasion beyond tumor capsule), necrosis (0, 1-25%, 26-50%, >50%), hemorrhage (0, \leq 50%, >50%), nuclear pleomorphism (0, 1-25%, 26-50%, >50%) and mitotic index.\textsuperscript{166} Estimation of percentage necrosis and percentage hemorrhage was based on the observation of the entire section at 200x magnification. Percentage nuclear pleomorphism and mitotic index were estimated after observation of at least 10 random fields at 400x magnification.

**Immunohistochemistry**

Five-µm sections from FF-PE blocks from each primary tumor were prepared on 3-aminopropyltriethoxysilane-coated slides. After dewaxing and rehydration, antigen retrieval was performed by immersion in citrate (0.01 M, pH 6) buffered distilled water and microwaving in a pressure cooker for 15 min at 850 W and 15 min at 300 W. Slides were then allowed to cool for 20 min. Endogenous peroxidase was blocked with hydrogen peroxide 0.03% for 5 minutes followed by rinsing with phosphate-buffered saline (PBS pH 7.4). Sections were incubated overnight with the primary antibodies in a humidity chamber at 4ºC (Table 2). Preliminary evaluation of the optimal concentration of each primary antibody was performed with serial antibody dilutions using the respective positive controls (Table 2); canine normal thyroid tissue was used as negative control. Incubation with a polymer-based secondary antibody (EnVision\textsuperscript{TM}, Dako, Glostrup, Denmark) was performed at room temperature for 30 min. After each incubation step, sections were rinsed with PBS. 3,3’ diaminobenzidine (DAB, Dako) in substrate buffer solution (Dako) served as chromogen and was allowed to react for 5 min. The sections were then counterstained with hematoxylin, rinsed in tap water, dehydrated and mounted with cover slips.
The subset of tumors positive for calcitonin was also stained for thyroglobulin in an automated immunostainer (Dako, S/N S38-7410-01).

**Table 2.** Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody</th>
<th>Antibody type</th>
<th>Dilution</th>
<th>Positive control</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thyroglobulin</td>
<td>A 0251(^a)</td>
<td>Rabbit polyclonal</td>
<td>1:800</td>
<td>Normal canine thyroid gland (follicular cells)</td>
<td>Normal canine thyroid (C cells)</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>A 0576(^a)</td>
<td>Rabbit polyclonal</td>
<td>1:400</td>
<td>Normal canine thyroid gland (C cells)</td>
<td>Normal canine thyroid gland (follicular cells)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1(^a)</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
<td>Normal canine small intestine</td>
<td>Normal canine thyroid gland</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>NCH-38(^a)</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
<td>Normal canine thyroid gland</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Dako, Glostrup, Denmark
Quantification of immunoreactive cells

All slides were examined by the same observer (MC), blinded to the clinical information and outcome of each patient. Quantification of Ki-67 labeling index was performed evaluating each slide through an optical grid at 400x magnification. In the region of highest positivity of the section, the fields were chosen randomly with a minimum of 10 fields per section and counting at least 500 cells per section. Only neoplastic cells with nuclear staining were recorded as positive. The labeling index was calculated as the number of positive cells divided by the number of positive plus negative cells (Fig. 1).

Quantification of E-cadherin immunolabeling was performed examining the entire section at 200x magnification and estimating the percentage of neoplastic cells with labeling of membranous E-cadherin. Tumors were classified according to membranous immunolabeling in 0-5%, 6-30%, 31-60%, 61-90% and >90% of positive cells (Fig. 2).

Thyroid tumors positive for calcitonin were classified as medullary tumors and thyroid tumors negative for calcitonin were classified as follicular cell tumors. To ensure the accuracy of this classification, the subset of tumors positive for calcitonin was also stained for thyroglobulin. Calcitonin and thyroglobulin immunolabeling were not quantified. The tumor was considered positive when the cytoplasm of neoplastic cells exhibited a fine granular staining pattern with cell-to-cell variation (Fig. 3).
Fig. 1. Immunohistochemical staining of Ki-67 in a canine medullary thyroid carcinoma with a labeling index of 28.4% (400x).

Fig. 2. Immunohistochemical staining of E-cadherin in a canine medullary thyroid carcinoma with >90% of positive cells (400x).

Fig. 3. Immunohistochemical staining of calcitonin in a canine medullary thyroid carcinoma (400x).
Prognostic factors

Statistical analysis

Correlating tumor variables with local invasiveness and metastases at time of diagnosis

For binary outcomes, the statistical analysis was based on the logistic regression model using exact tests and an exact odds ratio calculation. The binary response variables corresponded to surgical/necropsy evidence of local invasion, histologic evidence of local invasion and evidence of distant metastases at diagnosis. Tumor maximum diameter, tumor volume, tumor localization, scintigraphic uptake, thyroid functional status, histologic type, Ki-67 labeling index and E-cadherin expression, were introduced in the binary regression model as covariates. Significance level was set at 5%. For tumor localization, the significance level was adjusted for multiple comparisons (Bonferroni correction).

Comparison between follicular cell and medullary tumors with respect to the Ki-67 labeling index and E-cadherin expression was based on the Mann-Whitney U test.

Survival analysis

The effect of each variable on OS, DFS, TM and TR was evaluated with the Cox proportional hazards model. Each variable was incorporated in a univariate way, either as categorical or continuous. The variables analyzed included: age, time to presentation, weight loss, owners impression of tumor progression rate, body condition (determined during physical examination), tumor mobility, tumor maximum diameter (cm), tumor volume (cm$^3$), thyroid function, tumor scintigraphic uptake, WHO tumor stage, surgical evidence of vascular invasion, histologic features (histologic type, evidence of vascular invasion, capsular invasion, local invasion, percentage necrosis, percentage hemorrhage, nuclear pleomorphism and mitotic index), Ki-67 labeling index, E-cadherin expression, and levothyroxine supplementation. Variables found to have an effect on outcome at the 5% significance level were incorporated in a multivariate analysis to rule out associated effects.
RESULTS

Seventy dogs were included. Relevant clinical data is summarized in Table 3. All hyperthyroid dogs for which clinical signs were recorded (13 of 14 dogs) had clinical signs compatible with hyperthyroidism. Total calcium was measured in 5 dogs with MTC and was normal in all dogs.

Distant metastases were most frequently located in the lungs (12 dFTCs, 2 MTCs) and liver (2 FTCs). One dog with MTC had signs of metastasis or ectopic thyroid tissue in the mediastinum.

Eighteen dogs had macroscopic and/or histologic evidence of local invasion; 7 dogs had only macroscopic local invasion and 7 dogs had only histologic local invasion.

All thyroid tumors were carcinomas; 50 (71%) tumors were classified as dFTCs and 20 (29%) as MTCs following calcitonin immunostaining (Fig. 3). The dFTCs were classified as follicular (n=13, 19%), compact (n=19, 27%), follicular-compact (n=17, 24%) and follicular-papillary (n=1, 1%). Thyroglobulin staining was negative in all MTCs except in one dog considered to have a rare variant of MTC with mixed expression of calcitonin and thyroglobulin.\textsuperscript{167}
Table 3. Summary of clinical data organized by histologic tumor type in 70 dogs with thyroid tumors.

<table>
<thead>
<tr>
<th>Histologic type</th>
<th>dFTC</th>
<th>MTC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>n=50</td>
<td>n=19</td>
</tr>
<tr>
<td>Range</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>n=50</td>
<td>n=20</td>
</tr>
<tr>
<td>Male</td>
<td>30 (60%)</td>
<td>8 (40)</td>
</tr>
<tr>
<td>Female</td>
<td>20 (40%)</td>
<td>12 (60%)</td>
</tr>
<tr>
<td><strong>Weight loss</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>n=48</td>
<td>n=19</td>
</tr>
<tr>
<td>Present</td>
<td>18 (38%)</td>
<td>4 (21%)</td>
</tr>
<tr>
<td>Absent</td>
<td>30 (62%)</td>
<td>15 (79%)</td>
</tr>
<tr>
<td><strong>Body condition</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Emaciated</td>
<td>n=43</td>
<td>n=16</td>
</tr>
<tr>
<td>Emaciated</td>
<td>10 (23%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>Ideal</td>
<td>27 (63%)</td>
<td>13 (81%)</td>
</tr>
<tr>
<td>Obese</td>
<td>6 (14%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td><strong>Stridor</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>n=50</td>
<td>n=19</td>
</tr>
<tr>
<td>Present</td>
<td>5 (10%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td>Absent</td>
<td>45 (90%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td><strong>Dyspnea</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>n=49</td>
<td>n=19</td>
</tr>
<tr>
<td>Present</td>
<td>5 (10%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td>Absent</td>
<td>44 (90%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td><strong>Tumor localization</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unilateral</td>
<td>n=50</td>
<td>n=19</td>
</tr>
<tr>
<td>Unilateral</td>
<td>38 (76%)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>Bilateral</td>
<td>8 (16%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>Ectopic (ventral larynx)</td>
<td>4 (8%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor mobility</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile</td>
<td>n=48</td>
<td>n=19</td>
</tr>
<tr>
<td>Mobile</td>
<td>21 (55%)</td>
<td>11 (69%)</td>
</tr>
<tr>
<td>Fixed</td>
<td>17 (45%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td><strong>Tumor diameter (cm)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>n=47</td>
<td>n=19</td>
</tr>
<tr>
<td>Median</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Range</td>
<td>1.8 – 120</td>
<td>2.5 – 8.5</td>
</tr>
<tr>
<td><strong>Tumor volume (cm³)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>n=47</td>
<td>n=16</td>
</tr>
<tr>
<td>Median</td>
<td>25.7</td>
<td>23.2</td>
</tr>
<tr>
<td>Range</td>
<td>2 – 290</td>
<td>4 – 117</td>
</tr>
<tr>
<td><strong>Thyroid function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>n=43</td>
<td>n=14</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>1 (2%)</td>
<td>1 (7%)</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>17 (40%)</td>
<td>9 (64%)</td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>12 (28%)</td>
<td></td>
</tr>
<tr>
<td>Eu/hypothyroid</td>
<td>13 (30%)</td>
<td>4 (29%)</td>
</tr>
<tr>
<td><strong>Tumor scintigraphy</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Decreased</td>
<td>n=38</td>
<td>n=13</td>
</tr>
<tr>
<td>Decreased</td>
<td>8 (21%)</td>
<td>7 (54%)</td>
</tr>
<tr>
<td>Normal/increased</td>
<td>30 (79%)</td>
<td>6 (46%)</td>
</tr>
<tr>
<td><strong>Macroscopic local invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>n=38</td>
<td>n=17</td>
</tr>
<tr>
<td>Present</td>
<td>9 (24%)</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>Absent</td>
<td>29 (76%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histologic local invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>n=50</td>
<td>n=20</td>
</tr>
<tr>
<td>Present</td>
<td>8 (20%)</td>
<td>20 (100%)</td>
</tr>
<tr>
<td>Absent</td>
<td>42 (80%)</td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>n=50</td>
<td>n=19</td>
</tr>
<tr>
<td>I</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>21 (42%)</td>
<td>10 (52%)</td>
</tr>
<tr>
<td>III</td>
<td>17 (34%)</td>
<td>6 (32%)</td>
</tr>
<tr>
<td>IV</td>
<td>11 (22%)</td>
<td>3 (16%)</td>
</tr>
</tbody>
</table>

**Abbreviations**: dFTC, differentiated follicular cell thyroid carcinoma; MTC, medullary thyroid carcinoma
Correlating tumor variables with local invasiveness and metastases at time of diagnosis

The analysis was performed in all 70 dogs (50 dFTCs, 20 MTCs) irrespective of treatment modality (Table 4).

Tumor diameter ($P=0.007$), tumor volume ($P=0.020$), follicular cell origin ($P=0.044$) and Ki-67 ($P=0.038$) were positively associated with macroscopic local invasion. Tumor diameter ($P=0.038$) and ectopic location ($P=0.012$) were positively associated with histologic local invasion. Tumor diameter ($P=0.002$), tumor volume ($P=0.023$) and bilateral location ($P=0.012$) were positively associated presence of distant metastases at diagnosis.

Presence of distant metastases at diagnosis was not significantly different between dogs with dFTC and MTC ($P=0.743$).

E-cadherin expression, tumor scintigraphic uptake and thyroid functional status were not associated with either local invasiveness or distant metastases at diagnosis.

E-cadherin expression was significantly higher ($P=0.003$) in MTCs (median immunolabeling 91-100%) compared to dFTCs (median immunolabeling 31-60%) (Fig. 2). Ki-67 labeling index was not significantly different between dFTCs and MTCs ($P=0.668$).
Table 4. Correlating tumor variables with local invasiveness and distant metastases at diagnosis in 70 dogs with thyroid tumors.

<table>
<thead>
<tr>
<th></th>
<th>Macroscopic local invasion</th>
<th>Histologic local invasion</th>
<th>Distant metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>P value</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>Tumor diameter</td>
<td>1.29</td>
<td>0.007</td>
<td>1.13</td>
</tr>
<tr>
<td>Tumor volume</td>
<td>1.02</td>
<td>0.020</td>
<td>1.01</td>
</tr>
<tr>
<td>Tumor localization*</td>
<td>0.156</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Unilateral vs. Bilateral</td>
<td>0.15</td>
<td>0.214</td>
<td>0.07</td>
</tr>
<tr>
<td>Unilateral vs. Ectopic</td>
<td>0.30</td>
<td>0.728</td>
<td>0.02</td>
</tr>
<tr>
<td>Bilateral vs. Ectopic</td>
<td>1.81</td>
<td>1.000</td>
<td>0.23</td>
</tr>
<tr>
<td>Scintigraphic uptake</td>
<td>1.24</td>
<td>1.000</td>
<td>1.23</td>
</tr>
<tr>
<td>Thyroid function</td>
<td>2.80</td>
<td>0.660</td>
<td>2.01</td>
</tr>
<tr>
<td>Histologic type</td>
<td>6.89</td>
<td>0.044</td>
<td>5.03</td>
</tr>
<tr>
<td>Ki-67</td>
<td>1.06</td>
<td>0.038</td>
<td>1.05</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>1.03</td>
<td>1.000</td>
<td>1.05</td>
</tr>
</tbody>
</table>
Survival analysis

Forty-four dogs were treated with thyroidectomy, 3 dogs underwent debulking, 4 dogs were treated with thyroidectomy and $^{131}$I, 2 dogs were treated with thyroidectomy and chemotherapy, 1 dog was treated with thyroidectomy and external beam radiation, 6 dogs received no treatment and 10 dogs were euthanized at diagnosis.

The 44 dogs undergoing thyroidectomy were included in the survival analysis. Tumor mobility was recorded in 36 dogs; 27 dogs had freely-movable tumors and 9 had fixed tumors. Dogs were staged as stage I (n=1), stage II (n=26) and stage III (n=16). In 1 dog, although there was no evidence of metastases on medical imaging, tumor stage (I-III) could not be determined because tumor measurements were not recorded. Thyroidectomy was unilateral in 42 dogs, bilateral in 1 dog and 1 dog underwent surgical excision of an ectopic thyroid tumor ventral to the larynx. Surgical reports were available for 42 dogs and no dog had macroscopic evidence of local invasion. Median follow-up time at the clinic was 11 m (range, 0-60 m).

Of the 44 dogs treated with thyroidectomy, 28 dogs had dFTC (follicular n=12; follicular-compact n=9; compact n=7) and 16 dogs had MTC. Thirteen dogs with dFTC received lifelong TT$_4$ replacement therapy after thyroidectomy. Given that histologic tumor type (dFTC vs. MTC), and levothyroxine therapy had no significant effect of on outcome, all 44 dogs were analyzed together.

After thyroidectomy, 4 of 19 dogs with dFTC (21%) and 5 of 12 dogs with MTC (42%) developed distant metastases. Furthermore, 4 of 25 dogs with dFTC (16%) and 3 of 14 dogs with MTC (21%) developed loco-regional recurrence: locally (1 dFTC, 1 MTC), in regional LNs (3 dFTCs, 1 MTC), locally and in regional LNs (1 MTC).

Overall survival, DFS, TM and TR, were not significantly different between dogs with dFTC and MTC (Table 5).
Table 5. Comparison of outcome between 28 dogs with differentiated follicular cell thyroid carcinoma and 16 dogs with medullary thyroid carcinoma treated with thyroidectomy.

<table>
<thead>
<tr>
<th></th>
<th>dFTC</th>
<th>MTC</th>
<th>Overall</th>
<th>n</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS (m)</td>
<td>Median</td>
<td>17</td>
<td>42</td>
<td>22</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1 – 60</td>
<td>0.3 – 57</td>
<td>0.3 – 60</td>
<td></td>
</tr>
<tr>
<td>DSF (m)</td>
<td>Median</td>
<td>17</td>
<td>15</td>
<td>17</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.3 – 60</td>
<td>0.3 – 45</td>
<td>0.3 – 60</td>
<td></td>
</tr>
<tr>
<td>TM (m)</td>
<td>Median</td>
<td>60</td>
<td>32</td>
<td>42</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>1 – 60</td>
<td>2 – 42</td>
<td>1 – 60</td>
<td></td>
</tr>
<tr>
<td>TR (m)</td>
<td>Median</td>
<td>&gt; 60</td>
<td>&gt; 42</td>
<td>&gt; 60</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>0.3 – 60</td>
<td>0.5 – 42</td>
<td>0.3 – 60</td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** dFTC, differentiated follicular cell thyroid carcinoma; MTC, medullary thyroid carcinoma; OS, overall survival; DSF, disease-free survival; TM, time to distant metastases; TR, time to loco-regional recurrence; m, months
Results of the univariate analysis revealed that macroscopic vascular invasion was negatively associated with OS ($P=0.011$, Table 6). Macroscopic ($P=0.001$) and histologic ($P=0.037$) vascular invasion were negatively associated with DFS. Time to presentation ($P=0.040$), histologic vascular invasion ($P=0.018$) and Ki-67 labeling index ($P=0.004$) were negatively associated with TM. Each month delay in presentation and each 1% increase in Ki-67 labeling index increased the risk for distant metastases by 14% and 24%, respectively. Time to presentation was negatively associated with TR ($P=0.038$). Each month delay in presentation increased the risk for loco-regional recurrence by 14%.

Given their significant effect on outcome, the above mentioned variables were included in a multivariate analysis (Table 6; Fig. 4). Macroscopic ($P=0.007$) and histologic ($P=0.046$) vascular invasion were independent negative predictors for DFS. No independent predictors were found for OS, TM and TR.

Age, weight loss, tumor progression rate, body condition, stridor, dyspnea, tumor mobility, tumor diameter, tumor volume, stage (I-III), thyroid function, tumor scintigraphic uptake, histologic type, histologic local invasion, capsular invasion, necrosis, hemorrhage, nuclear pleomorphism, mitotic index and E-cadherin expression had no significant effect on OS, DSF, TM or TR.
Table 6. Summary of univariate and multivariate survival analyses in 44 dogs with thyroid gland tumors treated by thyroidectomy. Hazard ratios, 95% confidence intervals and $P$ values are given.

<table>
<thead>
<tr>
<th></th>
<th>OS</th>
<th>DFS</th>
<th>TM</th>
<th>TR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>$P$</td>
<td>HR (95% CI)</td>
<td>$P$</td>
</tr>
<tr>
<td><strong>UNIVARIATE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time to presentation $n=38$</td>
<td>1.01 (0.94-1.09)</td>
<td>0.763</td>
<td>1.03 (0.96-1.11)</td>
<td>0.453</td>
</tr>
<tr>
<td>Macroscopic vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present $n=4$ of 37</td>
<td>4.37 (1.39-13.7)</td>
<td><strong>0.011</strong></td>
<td>10.0 (2.62-38.5)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Histologic vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present $n=24$ of 44</td>
<td>1.89 (0.87-4.11)</td>
<td>0.109</td>
<td>2.36 (1.05-5.31)</td>
<td><strong>0.037</strong></td>
</tr>
<tr>
<td>Ki-67 labeling index $n=44$</td>
<td>1.02 (0.98-1.07)</td>
<td>0.261</td>
<td>1.04 (1.00-1.07)</td>
<td>0.066</td>
</tr>
<tr>
<td><strong>MULTIVARIATE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Macroscopic vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histologic vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** OS, overall survival; DFS, disease-free survival; TM, time to distant metastases; TR, time to loco-regional recurrence; *Not performed because only 1 dog in the analysis had macroscopic evidence of vascular invasion
Fig. 4. Kaplan-Meier survival curves for 44 dogs with thyroid gland tumors treated with thyroidectomy

A

Macroscopic vascular invasion
n=4, median DFS 2.5 m

No vascular invasion
n=33, median DFS 23 m

B

Histologic vascular invasion
n= 24, median DFS 12 m

No vascular invasion
n=20, median DFS 29 m
DISCUSSION

Prognostic markers are lacking for dogs with thyroid tumors undergoing thyroidectomy. In our exploratory analysis, macroscopic and histologic vascular invasion were independent negative predictors for DFS.

In our study, the prevalence of hyperthyroidism in dFTC (28%) was similar to previous reports.\textsuperscript{52} It can be hypothesized that functional tumors (in dogs with hyperthyroidism or with preserved scintigraphic uptake) are more differentiated and, therefore, carry a better prognosis. However, patient thyroid function and tumor scintigraphic uptake had no significant effect on outcome.

Time to presentation was negatively associated with TR, which may be a result of delayed diagnosis and treatment. In a recent retrospective study in dogs with thyroid tumors, the effect of duration of clinical signs on survival approached statistical significance.\textsuperscript{47} In people, time to therapy has been shown to be independently associated with thyroid cancer mortality.\textsuperscript{168}

In agreement with an earlier study, tumor diameter was positively associated with incidence of distant metastases at diagnosis.\textsuperscript{9} However, following thyroidectomy tumor diameter was not associated with outcome as previously reported.\textsuperscript{41,47} In humans, the risk of thyroid tumor recurrence and cancer-related mortality increases linearly with tumor size.\textsuperscript{168}

Macroscopic vascular invasion was negatively associated with OS and was an independent negative predictor for DFS. This is in agreement with earlier reports and is not surprising given the massive degree of neoplastic vessel infiltration necessary for macroscopic observation.\textsuperscript{8} In humans with FTC, extensive vascular invasion is rare but is also reported to have a poor prognosis.\textsuperscript{169}

Histologic vascular invasion was negatively associated with TM and was an independent negative predictor for DFS. Our results are in agreement with an earlier study showing the prognostic value of histologic grade of malignancy in dogs with thyroid cancer.\textsuperscript{41} In that study, vascular invasion was one of the most important histologic criteria used for the overall grade of malignancy. Although in our study histologic vascular invasion was negatively associated with TM and DFS, no
association was found with OS. This may be due to the fact that thyroid cancer metastases can have an indolent progression and are not always associated with rapid clinical deterioration. The fact that the overall median TM was approximately double than median OS supports this possibility. In humans with dFTC, histologic vascular invasion is an independent predictor of cancer-related mortality. Our study suggests that dogs with this histologic feature are at high risk for metastatic disease after thyroidectomy. Further research is necessary to determine if post-operative adjunctive therapy (eg, $^{131}$I) can improve the outcome of these patients.

In the current study, 29% of canine thyroid tumors were MTC based on IHC for calcitonin. This is in accordance with previous studies where 16-36% of thyroid carcinomas were MTCs. Medullary thyroid carcinoma may be difficult to distinguish from dFTC of compact type by microscopic observation alone and earlier studies likely underestimated their prevalence. In fact, 18 of 20 MTCs in our study were provisorily classified as dFTC of compact type and 2 as dFTC of follicular-compact type, prior to IHC for calcitonin. This underlines the importance of routine IHC for identification of MTC.

One of the goals of our study was to investigate the prognostic relevance of differentiating dFTC from MTC with IHC. A previous study suggested that canine MTC may have a less malignant biological behavior with a higher rate of complete surgical excision and lower incidence of metastases at diagnosis compared to dFTC. In accordance with this report, we found that MTCs were significantly less likely to be locally invasive at presentation. However, we found no difference in the incidence of metastatic disease at diagnosis. More importantly, after thyroidectomy, OS, DFS, TM and TR were not significantly different between dogs with dFTC and MTC. This suggests that, although MTC is less invasive and thus more amenable to complete surgical resection, after thyroidectomy the outcome is comparable to dFTC.

Ki-67 labeling index was negatively associated with TM in our study, although only in the univariate analysis. In humans, Ki-67 is associated with clinical stage and survival in both dFTC and MTC. It has been proposed that human thyroid carcinomas with Ki-67 labeling index < 5% have a more benign clinical course and that tumors with labeling index > 15% have a more aggressive biological
behavior.\textsuperscript{173} Interestingly, at the moment of diagnosis Ki-67 was positively associated with local invasiveness but not with distant metastases.

In human thyroid cancer, loss of E-cadherin protein expression is a negative prognostic indicator, however we found no association between E-cadherin expression and outcome.\textsuperscript{55} Furthermore, no correlation was observed between E-cadherin expression and local invasiveness or distant metastases at diagnosis. This suggests that other factors are more important in the development of local invasion and metastatic disease in canine thyroid cancer. Still, it was interesting to note that E-cadherin expression was significantly higher in MTCs compared to dFTCs.

The benefit of TSH-suppressive therapy with levothyroxine is well established in high-risk human dFTC and we recommend it routinely in the treatment of canine FTC.\textsuperscript{82} In our study, as levothyroxine was mainly administered as substitution therapy for dogs with low TT\textsubscript{4} concentrations after thyroidectomy, the lack of significant effect on survival might be due to an insufficient TSH suppression.

Limitations of our study include its retrospective and exploratory nature. Although review of IHC slides was only performed by one observer, which may decrease accuracy of scoring, it maximizes consistency of comparative scoring between slides.

In conclusion, our study suggests that macroscopic and histologic evidence of vascular invasion are independent negative predictors for DFS in dogs with surgically excised thyroid carcinoma. Canine dFTC and MTC seem to have comparable outcome after thyroidectomy.
ENDNOTES

a SAS 9.3, Cary, NC, USA.
b SPSS 20, Chicago, IL, USA.

ACKNOWLEDGMENTS

The authors thank the Department of Pathobiology of Utrecht University for providing all paraffin embedded tumor samples of patients diagnosed and treated at Utrecht University. We also thank the Department of Morphology of Ghent University for their collaboration in sectioning the paraffin-embedded tumor samples.
Chapter 5

IMMUNOHISTOCHEMICAL EXPRESSION OF POTENTIAL THERAPEUTIC TARGETS IN CANINE THYROID CARCINOMA

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ABSTRACT

**Background:** Thyroid carcinoma is a common endocrine tumor in the dog. Local invasive growth frequently precludes surgical excision, and in up to 38% of dogs, the tumor has already metastasized by the time of diagnosis. Therefore, it is important to investigate new treatment modalities that may be useful for the large number of dogs with inoperable tumors or metastatic disease.

**Hypothesis/Objectives:** To investigate the immunohistochemical expression of potential therapeutic targets in canine thyroid tumors.

**Animals:** 74 dogs with thyroid neoplasia.

**Methods:** Immunohistochemistry was performed for thyroglobulin, calcitonin, vascular endothelial growth factor (VEGF), p53, cyclooxygenase-2 (Cox-2) and P-glycoprotein (P-gp).

**Results:** Fifty-four (73%) tumors were classified as follicular cell thyroid carcinomas (FTCs) and 20 (27%) as medullary thyroid carcinomas (MTCs). Eighty percent of FTCs and all MTCs had a high percentage (76-100%) of neoplastic cells immunopositive for VEGF. Thirteen percent of FTCs and 50% of MTCs expressed Cox-2. Seven percent of FTCs and 70% of MTCs expressed P-gp. No tumor was immunopositive for p53 expression. Expression of VEGF ($P=0.034$), Cox-2 ($P=0.013$) and P-gp ($P<0.001$) was significantly higher in MTCs compared to FTCs.

**Conclusions and clinical importance:** VEGF is a potential therapeutic target in both FTC and MTC in dogs. Cox-2 and P-gp may be useful molecular targets in canine MTC.
INTRODUCTION

Thyroid cancer represents 10-15% of all head and neck neoplasms in the dog, and 90% of thyroid tumors detected clinically are carcinomas.²,¹⁶³ Thyroid carcinomas can be classified as follicular cell thyroid carcinomas (FTCs), which arise from follicular thyroid cells, and medullary thyroid carcinomas (MTCs), which arise from the parafollicular C cells and have a neuroendocrine origin. Although thyroidectomy is the preferred treatment modality, invasive non-resectable thyroid tumors are common in dogs, and in up to 38% of dogs the tumor has already metastasized by the time of diagnosis.⁴,⁸ Furthermore, almost 50% of dogs undergoing thyroidectomy experience recurrence or metastatic disease within 2 years of surgery.⁸ Therefore, it is important to investigate new treatment modalities for the large number of dogs with inoperable tumors or metastatic disease.

Vascular endothelial growth factor (VEGF) is the main stimulator of angiogenesis in the thyroid gland, and VEGF overexpression has been found in human thyroid cancer.⁶⁷ VEGF is secreted by cancer cells and binds to VEGF tyrosine kinase receptors on the surface of endothelial cells and thyrocytes. In people, vascular endothelial growth factor receptor-2 (VEGFR-2) inhibition with tyrosine kinase inhibitors (TKIs) is the most effective new therapeutic strategy developed to date in the treatment of advanced thyroid cancer.⁷⁰ VEGF, angiogenesis and VEGF-induced pathway activation may play important roles in the progression of canine thyroid cancer and constitute important therapeutic targets.

Tumor suppressor gene p53 encodes a nuclear phosphoprotein that mediates cell cycle regulation and apoptosis in response to DNA damage.¹⁷⁴ Mutations that result in loss of normal p53 function lead to loss of cell cycle control and increased risk of malignancy. In normal cells, p53 protein has a short half-life and is undetectable with immunohistochemistry (IHC). However, some p53 gene mutations lead to expression of an altered p53 protein with longer half-life that is detectable by IHC.¹⁷⁵ In humans, p53 mutations have been described in 40-62% of undifferentiated thyroid carcinomas and in 5-10% of other thyroid carcinomas.¹⁷⁶ Research in human thyroid cancer shows that restoration of wild-type p53 expression by gene therapy is associated
Therapeutic targets

with inhibition of tumor cell growth and enhanced sensitivity to chemotherapy and radiation.\(^\text{174}\) Earlier investigations of the \textit{p53} gene coding region identified a somatic mutation in 1 of 23 canine FTCs.\(^\text{27}\) Thus, the \textit{p53} tumor suppressor gene may be a potential molecular target in canine thyroid cancer.

Cyclooxygenases (Cox), particularly Cox-2, may play a critical role in tumor development and progression. In particular, epithelial neoplasms are prone to express large amounts of the inducible form of Cox-2. In dogs, Cox-2 over-expression has been described in transitional cell carcinoma (TCC) of the urinary bladder and in prostatic carcinoma.\(^\text{177}\) Several studies have shown that Cox-2 or Cox-1/Cox-2 inhibitors have antitumor and chemopreventive effects, presumably by induction of apoptosis, reduction of angiogenic growth factors and suppression of regulatory T-cells.\(^\text{75,76}\) Cox-2 is an appealing therapeutic target and its expression has not yet been investigated in canine thyroid tumors.

One study in 44 dogs with surgically excised thyroid carcinoma failed to demonstrate a clinical benefit of adjuvant chemotherapy.\(^\text{47}\) Moreover, the reported survival times for dogs with unresectable thyroid tumors treated with chemotherapy are disappointing.\(^\text{45}\) One of the major mechanisms for resistance to chemotherapy is high expression of ATP-binding cassette (ABC) transporter proteins such as P-glycoprotein (P-gp; ABCB1) and multi-drug resistance-related protein 1 (ABCC1).\(^\text{77}\) These ATP-dependent membrane efflux pumps decrease the intracellular concentration of chemotherapeutic agents, thereby limiting cytotoxicity at their target site. Recent research shows that TKIs and Cox-2 inhibitors can reverse multi-drug resistance by decreasing the expression and function of P-gp.\(^\text{78,79}\) P-gp expression has been identified in several canine tumors and may constitute an attractive molecular target.\(^\text{80}\)

The goal of this study was to evaluate the immunohistochemical expression of VEGF, \textit{p53} protein, Cox-2 and P-gp in canine thyroid tumors and to assess their potential as therapeutic targets.
MATERIALS AND METHODS

Case selection
The medical record databases of the Companion Animal Clinics of Ghent and Utrecht Universities were searched for dogs diagnosed with thyroid neoplasia from 1986 to 2012. Patients for which paraffin-embedded tumor samples were no longer available were excluded.

Tumor specimens
Formalin-fixed paraffin-embedded (FF-PE) tissue blocks of each patient were collected. In some dogs several blocks from 1 tumor site or blocks from multiple sites (local, regional node, distant metastases) were available. All samples were obtained at surgery or necropsy. In total, 304 FF-PE blocks from 74 patients (52 from Utrecht University, 22 from Ghent University) were available.

Histopathology
Five-μm sections from each FF-PE block were stained with hematoxylin and eosin (HE). All HE slides were reviewed by the same board-certified pathologist (RD) blinded to the clinical information and previous histopathology report.

When neoplastic thyroid tissue was identified, the section was classified as primary tumor, lymph node metastasis or distant metastasis. The distinction between adenoma and carcinoma was based on histologic evidence of either capsular invasion, vascular invasion or metastases. The histologic type of primary thyroid tumors was classified according to the World Health Organization classification as tumors of follicular cell origin (follicular, compact, follicular-compact, papillary, poorly differentiated, undifferentiated and carcinosarcoma) or C-cell (medullary) origin. Classification of medullary thyroid tumors also was based on positive IHC for calcitonin, as previously described.
Immunohistochemistry

Five-μm sections from each FF-PE block were prepared on 3-aminopropyltriethoxysilane-coated (APES) slides. After dewaxing and rehydration, antigen retrieval was performed by immersion in citrate-buffered (0.01 M, pH 6) distilled water and microwaving in a pressure cooker for 15 min at 850 W and 15 min at 300 W. Slides then were allowed to cool for 20 min. Endogenous peroxidase was blocked with 0.03% hydrogen peroxide for 5 minutes followed by rinsing with water and phosphate-buffered saline (PBS pH 7.4). Sections were incubated overnight with the primary antibodies (Table 1) in a humidity chamber at 4°C. Validation and evaluation of the optimal concentration of each primary antibody were performed with serial antibody dilutions using the respective positive controls (Table 1); normal canine thyroid gland tissue was used as negative control. Incubation with a polymer-based secondary antibody was performed at room temperature for 30 min. After each incubation step, sections were rinsed with PBS. 3,3’ diaminobenzidine, in substrate buffer solution served as chromogen and was allowed to react for 5 min (10 min for p53 antibody). The sections then were counterstained with hematoxylin, rinsed in tap water, dehydrated and mounted with cover slips. Immunohistochemistry for each antibody was performed in 2 batches given the large number of slides that had to be stained.

Table 1. Antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antibody name</th>
<th>Antibody type</th>
<th>Dilution</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>SPM225&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Mouse monoclonal</td>
<td>1:25</td>
<td>Canine mammary carcinoma</td>
</tr>
<tr>
<td>P53</td>
<td>Clone PAb 240&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Mouse monoclonal</td>
<td>1:50</td>
<td>Canine mammary carcinoma known to harbor p53 gene mutation</td>
</tr>
<tr>
<td>Cox-2</td>
<td>Clone 33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Mouse monoclonal</td>
<td>1:800</td>
<td>Normal canine kidney (macula densa)</td>
</tr>
<tr>
<td>Pgp</td>
<td>C494&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Mouse monoclonal</td>
<td>1:200</td>
<td>Normal canine liver</td>
</tr>
<tr>
<td>Pgp</td>
<td>JSB-1&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Mouse monoclonal</td>
<td>1:100</td>
<td>Normal canine liver</td>
</tr>
<tr>
<td>Calcitonin</td>
<td>A0576&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Rabbit polyclonal</td>
<td>1:400</td>
<td>Normal canine thyroid gland</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>A0251&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Rabbit polyclonal</td>
<td>1:800</td>
<td>Normal canine thyroid gland</td>
</tr>
</tbody>
</table>

<sup>a</sup>Santa Cruz Biotechnology, Inc., Dallas, TX, USA; <sup>b</sup>Thermo Fisher Scientific, Loughborough, UK; <sup>c</sup>BD Transduction Laboratories™, San Jose, CA, USA; <sup>d</sup>Enzo Life Sciences, Inc., Farmingdale, NY, USA; <sup>e</sup>Covance, Princeton, NJ, USA; <sup>f</sup>Dako, Glostrup, Denmark;
The subset of tumors positive for calcitonin also was stained for thyroglobulin in an automated immunostainer. 

**Quantification of immunoreactive cells**

All sections were examined by the same investigator (MC) who was blinded to the clinical information and outcome of each patient. For each marker, immunohistochemistry also was performed on normal canine thyroid gland to allow comparison with neoplastic tissue. Quantification of VEGF immunolabeling was performed by evaluating the entire section at 200x magnification and estimating the percentage of neoplastic cells positive for VEGF. Only neoplastic cells with cytoplasmic granular immunolabelling were considered positive. The tumors were classified according to the immunolabelling as 0%, 1-25%, 26-50%, 51-75% and 76-100% positive cells, as previously described. In each batch, endothelial cells were used as internal positive controls and normal thyroid gland was used as negative control.

Quantification of p53 immunolabeling was performed by evaluating each section using an optical grid at 400x magnification. The fields were chosen randomly with a minimum of 10 fields per section and counting at least 500 cells per section. Only neoplastic cells with nuclear staining were recorded as positive. The labeling index was calculated as the number of positive cells divided by the number of positive plus negative cells. Only sections with a labeling index ≥5% of positive neoplastic cells were considered positive for p53 expression, as previously described. In each batch, normal thyroid gland was used as negative control.

Quantification of Cox-2 immunolabeling was performed by calculating the labeling index as described for p53. Only neoplastic cells with cytoplasmic granular staining were recorded as positive. Based on the labeling index and staining intensity (absent, weak, moderate or strong), an overall IHC score (0-12) was calculated as previously described. In each batch, normal kidney was used as positive control and normal thyroid gland was used as negative control.

As recommended, 2 monoclonal antibodies recognizing 2 different epitopes of P-gp were used to improve the reliability of IHC (Table 1). Quantification of P-gp
immunolabeling was performed by evaluating the entire section at 200x magnification and estimating the percentage of neoplastic cells with membranous labeling of P-gp. The tumors were classified as 0-10%, 11-25%, 26-50%, 51-75% and 76-100% positive cells, as previously described.\textsuperscript{182} Additionally, the intensity of the staining was recorded as absent, weak, moderate or strong. Only sections with immunolabelling of \( \geq 11\% \) of neoplastic cells with both antibodies (C494 and JSB-1) were considered truly positive for P-gp expression.\textsuperscript{181,182} In each batch, normal liver was used as a positive control and normal thyroid gland was used as negative control.

Thyroid tumors positive for calcitonin were classified as MTCs and thyroid tumors negative for calcitonin were classified as FTCs.\textsuperscript{5} To ensure the accuracy of this classification, the subset of tumors positive for calcitonin was also stained for thyroglobulin. Calcitonin and thyroglobulin immunolabeling were not quantified. The tumor was considered positive when the cytoplasm of neoplastic cells exhibited a fine granular staining pattern with cell-to-cell variation. Normal thyroid gland was used as control in each batch.

**Statistical analysis**

Expression of VEGF, cox-2 labeling index, cox-2 IHC score and expression of P-gp were compared between FTCs and MTCs. For VEGF, Cox-2 labeling index and Cox-2 IHC score the analysis was based on the Wilcoxon rank sum test. For P-gp expression, the analysis was based on the Fisher exact test.\textsuperscript{4} Significance level was set at 5\%.
RESULTS

Thyroid tumor tissues from 74 dogs, with a mean age of 9.3 years (range 4-16 years) were reviewed. All thyroid tumors were classified as carcinomas. From the 74 patients included in this study, 54 (73%) had FTC and 20 (27%) had MTC. Histologic subtypes of FTC included follicular (n=13, 18%), follicular-compact (n=17, 23%), compact (n=19, 26%), follicular-papillary (n=1, 1%), undifferentiated (n=1, 1%) and carcinosarcoma (n=3, 4%).

Twelve dogs with FTC (22% of FTCs) and 3 dogs with MTC (15% of MTCs), or 20% of all patients, had evidence of distant metastases at the time of diagnosis. Eight dogs with FTC (15% of FTCs) and 4 dogs with MTC (20% of MTCs), or 16% of all patients, had evidence of regional lymph node metastases at the time of diagnosis.

VEGF expression

Immunohistochemistry for VEGF in normal canine thyroid gland indentified staining of endothelial cells and clusters of parafollicular cells, whereas follicular cells were negative with rare exceptions. In contrast, 85% of all tumors (80% FTCs, 100% MTCs) exhibited a high percentage of VEGF-positive tumor cells (76-100%). Expression of VEGF was significantly higher in MTCs than in FTCs ($P=0.034$; Table 2; Fig. 1).

P53 expression

Immunohistochemistry for p53 in normal canine thyroid tissue did not identify positive nuclei. Likewise, all sections of thyroid carcinoma tissue had $<5\%$ positive nuclei, and therefore no tumor was considered positive for p53 expression. Eighty-five percent of all thyroid tumors (87% FTCs, 80% MTCs) had $<1\%$ positive nuclei.

Cox-2 expression

Immunohistochemistry for Cox-2 in normal canine thyroid gland did not identify positive cells. Twenty-three percent of all primary tumors (13% FTCs, 50% MTCs) exhibited Cox-2 expression (Table 2, Fig. 2). Cox-2 labeling index in MTCs (median, 0.5%; range, 0-22.4%) was significantly higher ($P=0.002$) than in FTCs.
Therapeutic targets

(median, 0%; range, 0-7%). Likewise, Cox-2 IHC score of MTCs (median 1; range 0-6) was significantly higher ($P=0.013$) than in FTCs (median, 0; range, 0-3).

**P-gp expression**

Normal canine thyroid tissue did not have cells with membranous immunolabeling using monoclonal antibody JSB-1. With C494, weak membranous immunolabeling was observed in follicular cells (especially apical membrane), endothelial cells and occasionally parafollicular cells.

Twenty-four percent of all primary thyroid tumors (7% FTCs, 70% MTCs) were positive for C494 and JSB-1 (Table 2, Fig. 3) and therefore were considered truly positive for P-gp expression. The proportion of MTCs expressing P-gp was significantly higher ($P<0.001$) than that of FTCs. The membranous staining intensity for C494 was considered mild in 4 (5%), moderate in 7 (9%) and strong in 7 (9%) primary tumors. The membranous staining intensity for JSB-1 was mild in 11 (15%), moderate in 4 (5%) and strong in 3 (4%) cases.
Table 2. Immunohistochemical expression of VEGF, cox-2 and P-gp (C494, JSB-1) in primary thyroid tumors of 74 dogs.

<table>
<thead>
<tr>
<th></th>
<th>FTC count (%)</th>
<th>MTC count (%)</th>
<th>Total count (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1-25%</td>
<td>1 (2%)</td>
<td>0</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>26-50%</td>
<td>2 (4%)</td>
<td>0</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>51-75%</td>
<td>8 (14%)</td>
<td>0</td>
<td>8 (11%)</td>
</tr>
<tr>
<td>76-100%</td>
<td>43 (80%)</td>
<td>20 (100%)</td>
<td>63 (85%)</td>
</tr>
<tr>
<td><strong>Cox-2 IHC score</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>47 (87%)</td>
<td>10 (50%)</td>
<td>57 (77%)</td>
</tr>
<tr>
<td>1</td>
<td>2 (4%)</td>
<td>2 (10%)</td>
<td>4 (5%)</td>
</tr>
<tr>
<td>2</td>
<td>3 (5%)</td>
<td>5 (25%)</td>
<td>8 (11%)</td>
</tr>
<tr>
<td>3</td>
<td>2 (4%)</td>
<td>0</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>3 (15%)</td>
<td>3 (4%)</td>
</tr>
<tr>
<td><strong>P-gp (C494 &amp; JSB-1)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>50 (93%)</td>
<td>6 (30%)</td>
<td>56 (76%)</td>
</tr>
<tr>
<td>positive</td>
<td>4 (7%)</td>
<td>14 (70%)</td>
<td>18 (24%)</td>
</tr>
<tr>
<td><strong>n</strong></td>
<td><strong>54 (73%)</strong></td>
<td><strong>20 (27%)</strong></td>
<td><strong>74 (100%)</strong></td>
</tr>
</tbody>
</table>

**Abbreviations:** Cox-2, cyclooxygenase-2; FTC, follicular cell thyroid carcinoma; MTC, medullary thyroid carcinoma; P-gp, P-glycoprotein; VEGF, vascular endothelial growth factor
**Therapeutic targets**

**Fig. 1.**

A: Immunohistochemical expression of VEGF in a FTC of compact type with 76-100% of positive neoplastic cells (400×).

B: Immunohistochemical expression of VEGF in a MTC with 76-100% of positive neoplastic cells (400×).

**Fig. 2.**

A: Immunohistochemical expression of Cox-2 in a FTC of follicular-compact type with labeling index of 6.8% (400x).

B: Immunohistochemical expression of Cox-2 in a MTC with a labeling index of 22.4% (400×).
Fig. 3.  
A: Immunohistochemical expression of P-gp (C494) in a FTC of compact type (400×).
B: Immunohistochemical expression of P-gp (C494) in a MTC (400×).
C: Immunohistochemical expression of P-gp (JSB-1) in a FTC of compact type (400×).
D: Immunohistochemical expression of P-gp (JSB-1) in a MTC (400×).
DISCUSSION

Local invasive growth frequently precludes surgical excision of canine thyroid tumors and in up to 38% of dogs the tumor has already metastasized by the time diagnosis. Furthermore, approximately half of the patients treated with thyroidectomy experience local recurrence or metastatic disease within 2 years of surgery. Therefore, it is important to search for new treatment modalities. In this study, we found high expression of VEGF in both FTC and MTC, and common expression of Cox-2 and P-gp in canine MTC, indicating that these may represent valuable therapeutic targets for dogs with inoperable thyroid tumors or metastatic disease.

In our study, 85% of all thyroid tumors (80% FTCs, 100% MTCs) expressed VEGF in ≥76% of tumor cells. In human thyroid carcinoma, VEGF expression also is up-regulated. The high expression of VEGF observed in our study suggests it may play an important role in the progression of canine thyroid cancer. Consequently, the VEGF system seems to be an attractive target for the treatment of both FTC and MTC in dogs. In a preliminary study in dogs with solid tumors, a multi-targeted TKI, targeting VEGFR-2, induced partial remission in 4 of 15 dogs and stable disease in 8 of the 15 dogs with thyroid carcinoma.

Loss of function mutation of p53 often leads to accumulation of p53 in the nucleus which becomes readily detectable by IHC staining. In our study, no section was considered positive for p53 protein. A low prevalence of p53 expression was expected because p53 mutation only was found in 1 of 23 canine FTCs in a previous study examining part of the coding region of the p53 gene. In humans, p53 mutations have been described in 40-62% of undifferentiated thyroid carcinomas and in 5-10% of other thyroid carcinomas. A study investigating the immunohistochemical expression of p53 in canine malignant tumors with and without p53 mutations indicated that antibody PAb240 (used in our study) had a sensitivity of 36% and a specificity of 94% for detection of p53 mutations on FF-PE sections. Although in that study another antibody (CM1) was reported to have the highest sensitivity (55%) for detection of p53 mutations in FF-PE tumor samples, in our laboratory it repeatedly stained nuclei of the negative control (normal thyroid gland) showing lack of
specificity. Given the reported low sensitivity of IHC with PAb240, our results may underestimate the true prevalence of p53 mutation in canine thyroid carcinoma. Nevertheless, taking our results and results from previous studies into account, the p53 tumor suppressor gene does not seem to be a realistic therapeutic target for most cases of canine thyroid carcinoma.

In this study, Cox-2 expression was observed in 50% of MTCs and in only 13% of FTCs. The higher prevalence of Cox-2 expression in MTC is in agreement with reports in humans, where 26-41% of FTCs and 75-82% of MTCs have been shown to express Cox-2.\textsuperscript{184} In a study investigating Cox-2 expression in canine invasive TCC of the urinary bladder, the percentage of positive tumor cells at diagnosis ranged from 1 to 22%, comparable to that found in our study.\textsuperscript{185} Interestingly, although in that study all tumors were positive for Cox-2 expression, no significant association was found between the level of Cox-2 immunolabeling and tumor remission with piroxicam, observed in 12 of 18 dogs. This suggests that clinical benefit may be observed even in cases of low Cox-2 expression. Our results suggest that Cox-2 is an interesting molecular target in canine thyroid carcinoma, particularly in MTC.

In our study, P-gp expression with both antibodies was observed in 7% of FTCs and 70% of MTCs. These results suggest that expression of P-gp in canine MTC is common and could explain multi-drug resistance in these patients. Literature on the expression of P-gp in human thyroid carcinoma is scarce. In vitro studies have shown expression of the ABCB1 gene in tumor cells from 12 patients with MTC and in several MTC cell lines.\textsuperscript{79,186} In humans, MTC is refractory to conventional chemotherapy which yields partial responses in only 10 to 20% of patients.\textsuperscript{187} Experimental evidence suggests that multi-drug resistance is 1 of the mechanisms for this highly chemoresistant phenotype and that by targeting P-gp, chemoresistance can be reversed.\textsuperscript{79,186} Our study suggests that P-gp is an interesting molecular target for the treatment of canine MTC. Inhibition of P-gp using specific P-gp inhibitors (eg, verapamil) or TKIs could increase tumor sensitivity to chemotherapy and improve outcome.

Medullary thyroid carcinoma may be difficult to distinguish from compact FTC by light microscopy alone.\textsuperscript{4} The different expression of potential molecular targets
between these tumor types observed in our study underlines the importance of their adequate differentiation using routine IHC. The higher expression of Cox-2 and P-gp in MTC is in agreement with clinical and experimental studies in human thyroid cancer.

In fact, there is experimental evidence of a direct causal relationship between Cox-2 expression and P-gp regulation. Overexpression of Cox-2 leads to increased expression and function of P-gp in a dose-dependent manner and this effect can be blocked by specific Cox-2 inhibitors. Furthermore, *in vitro* studies in MTC cells have shown the ability of Cox-2 inhibitors to reverse multi-drug resistance in these cells by inhibiting the expression of P-gp. In an *in vivo* model of human colorectal cancer, Cox-2 expression was correlated with chemoresistant phenotype, and the most tumor regression was achieved with a combination of cox-2 inhibitors and chemotherapy.

An estimation of the percentage of immunopositive tumor cells in each section was performed for VEGF and P-gp because often all tumor cells in each section were either positive or negative, unlike for Cox-2 or p53. We therefore considered that it would be more time-efficient to provide an estimation of the immunopositivity in the entire section, as previously described, instead of counting cellular fields with identical immunopositivity. Limitations of our study include the relative low number of MTCs and the fact that review of IHC was performed by only 1 observer. Although this may decrease the accuracy of scoring, it maximizes consistency of comparative scoring among sections. Additional studies are needed to confirm if protein expression, as identified by IHC, correlates with *in vivo* protein function.

In conclusion, our study shows that the VEGF system is a potential therapeutic target in both FTC and MTC in dogs. Cox-2 and P-gp seem to be attractive molecular targets in canine MTC. p53 does not seem to be a potential molecular target for canine thyroid carcinoma.
ENDNOTES

a EnVision™, Dako, Glostrup, Denmark
b DAB, Dako, Glostrup, Denmark
c S/N S38-7410-01, Dako, Glostrup, Denmark
d SAS 9.3, Cary, NC, USA.

ACKNOWLEDGMENTS

The authors thank the Department of Pathobiology of Utrecht University for providing paraffin-embedded tumor samples of patients diagnosed and treated at Utrecht University. We also thank the Department of Morphology of Ghent University for their collaboration in sectioning the paraffin-embedded tumor samples.
Chapter 6

EFFECT OF LEVOTHYROXINE THERAPY ON SURVIVAL OF DOGS WITH THYROID TUMORS

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In preparation

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  Faculty of Veterinary Medicine, Ghent University, Belgium
ABSTRACT

Hypothesis/Objectives: To investigate the effect of levothyroxine therapy on survival of dogs with thyroid tumors undergoing different treatment modalities.

Design: Retrospective study.

Animals: 42 dogs with thyroid neoplasia.

Methods: Medical records of Ghent University were reviewed (2001-2012). Treatment groups included thyroidectomy with or without levothyroxine therapy (n=17); radioactive iodine-131 with or without levothyroxine therapy (n=11); no treatment or levothyroxine therapy alone (n=14).

The effect of levothyroxine therapy, thyrotropin (TSH) suppression (TSH < 0.1 ng/mL), tumor localization, tumor mobility, tumor maximum diameter, metastases at diagnosis, tumor stage and thyroid function, on survival was evaluated with the Cox proportional hazards model with stratification for treatment at the 5% significance level.

Results: Levothyroxine therapy (P=0.716), thyrotropin suppression (P=0.597), tumor localization (P=0.496), tumor mobility (P=0.939) and hyperthyroidism (P=0.08) had no significant effect on survival. Large tumor diameter (P=0.003) and evidence of metastases at diagnosis (P=0.019) were negatively associated with survival. Dogs with stage IV disease had 4.8 times higher risk for death at any given time than dogs with stage II (P=0.008).

Conclusions and clinical importance: Levothyroxine therapy does not seem to provide a survival benefit in dogs with thyroid tumors. Large tumor diameter and evidence of distant metastases at diagnosis are negatively associated with prognosis.
INTRODUCTION

Thyroid cancer represents 1.2-3.8% of all neoplasms in the dog. Ninety percent of thyroid tumors detected clinically are carcinomas and up to 38% of patients with carcinomas have evidence of metastatic disease at the time of diagnosis. Thyroidectomy is considered the preferred treatment modality for non-invasive tumors while fixed invasive tumors can be treated with external beam radiation or radioiodine-131 ($^{131}$I). To present date, research on optimization of treatment of canine thyroid cancer remains scarce. The few studies investigating post-operative chemotherapy did not show an improvement in outcome and our group recently investigated the use of recombinant human thyrotropin (TSH) to optimize radioiodine uptake, which also did not show a clear benefit with the protocol used. Tyrosine kinase inhibitors and metronomic chemotherapy have been reported for palliative treatment of canine thyroid tumors but these treatment modalities have not yet been investigated for adjunctive therapy. Further research on optimization of treatment of canine thyroid cancer is needed.

Thyroid carcinoma can be classified as follicular cell thyroid carcinoma (FTC), which arises from thyroid follicular cells, or medullary thyroid carcinoma (MTC), which arises from the parafollicular C cells. In humans and dogs, FTC cells contain TSH receptors and endogenous TSH may stimulate neoplastic cell growth and tumor progression. In people with differentiated FTC undergoing classical treatment, ie, thyroidectomy followed by $^{131}$I, lifelong TSH-suppressive therapy with levothyroxine significantly reduces recurrence rates and cancer-specific mortality. The American and European Thyroid Associations currently recommend adapting the target level of TSH suppression to the patient’s risk of tumor recurrence and mortality. A recent study in 15 dogs with bilateral thyroid neoplasia treated by thyroidectomy revealed that the dogs receiving levothyroxine therapy after surgery had a significantly longer survival time. These results are encouraging and further studies are needed to corroborate these results in a larger number of dogs. Furthermore, as TSH suppression is the main goal of levothyroxine therapy, the effectiveness of suppression should also be evaluated during follow-up.
The aim of this study was to retrospectively evaluate the effect of levothyroxine therapy on survival of dogs with thyroid tumors undergoing different treatment modalities. Additionally, we also aimed to evaluate the effect of TSH suppression on survival.
MATERIALS AND METHODS

Case selection

The medical record database of the Small Animal Clinic of Ghent University was searched for dogs diagnosed with thyroid neoplasia from January 1, 2001 to July 1, 2012. For inclusion in the study the diagnosis of thyroid neoplasia had to be confirmed by cytology, histopathology or scintigraphic examination. Furthermore, dogs had to be treated with thyroidectomy, $^{131}$I, levothyroxine therapy or receive no treatment. Dogs undergoing other treatment modalities, dogs diagnosed with MTC and dogs for which no follow-up information was available were excluded. To study the effect of levothyroxine therapy on survival in each treatment group, the 52 dogs meeting the initial inclusion criteria were divided according to treatment: thyroidectomy with or without levothyroxine therapy (n=17); $^{131}$I with or without levothyroxine therapy (n=11); thyroidectomy, $^{131}$I and levothyroxine therapy (n=10); no treatment or levothyroxine therapy alone (n=14). As all dogs treated with a combination of thyroidectomy and $^{131}$I also received levothyroxine therapy this group was excluded.

Medical record review

The medical records of the dogs included were reviewed. When the complete follow-up information was not available in the medical record, referring veterinarians and clients were contacted by phone. The information retrieved from medical records included signalment, clinical signs, physical examination findings, tumor characteristics (maximum diameter, localization, mobility, cytology, histologic examination), blood hematology and routine biochemistry, medical imaging (thoracic radiographs, cervical and abdominal ultrasound, scintigraphic examination, CT scan), WHO tumor stage, treatment, follow-up TSH concentrations and date of death. Thyroid function at the time of diagnosis was determined based on circulating basal total thyroxine (TT$_4$) and TSH concentrations. For patients receiving levothyroxine therapy, effective TSH suppression was defined as circulating TSH concentrations < 0.1 ng/mL (reference < 0.5 ng/mL) during follow-up. Survival time was defined as the time from treatment to death of any cause; patients still alive at the last observation time were censored.
**Statistical analysis**

To ascertain if levothyroxine therapy was more likely to be prescribed to high-risk patients, which would need to be taken into account interpreting the results of this study, the effects of tumor localization, tumor mobility, tumor size, WHO stage and thyroid function, on survival were also evaluated and subsequently compared between dogs that received levothyroxine therapy and dogs that did not.

The effect of each variable on survival was evaluated separately with the Cox proportional hazards model with stratification for treatment group (no treatment or levothyroxine alone; thyroidectomy with or without levothyroxine; $^{131}$I with or without levothyroxine). Each variable was incorporated in a univariate way, either as categorical or continuous. The variables analyzed included: tumor localization (unilateral or bilateral), tumor mobility (mobile or fixed), tumor maximum diameter (cm), evidence of distant metastases at diagnosis (absent or present), WHO tumor stage (I-IV), thyroid function (hyperthyroid or euthyroid/hypothyroid), levothyroxine therapy (yes or no) and TSH suppression (yes or no). Dogs receiving levothyroxine therapy with unknown follow-up TSH concentrations were excluded from the analysis of TSH suppression. Significance level was set at 5%. When multiple comparisons were performed, the significance level was adjusted (Bonferroni correction). a

Dogs that received levothyroxine therapy were compared to dogs that did not receive this treatment with respect to tumor localization, tumor mobility, evidence of metastases at diagnosis and thyroid function based on a logistic regression model using exact tests and exact odds ratio calculation; with respect to tumor diameter and tumor stage based on the Mann Whitney U test. a
RESULTS

Forty-two dogs with a mean age of 9.7 years (range 5-15 years) were included. There was a similar number of males (n=20) and females (n=22). Twenty-eight dogs (67%) had unilateral thyroid tumors, 12 (28%) had bilateral tumors and 2 dogs (5%) had ectopic tumors ventral to the larynx. Tumor mobility at palpation was recorded in 25 dogs; 9 dogs (36%) had mobile tumors and 16 (64%) had fixed tumors. Tumor maximum diameter (range 2.6-15 cm), measured during physical examination, cervical ultrasound or CT, was recorded in 37 dogs.

Thyroid function results were available in 35 dogs; 21 dogs (60%) were euthyroid, 6 (17%) were hyperthyroid, 6 (17%) were hypothyroid and in 2 dogs (6%) hypothyroidism could not be differentiated from euthyroid sick syndrome.

Tumor cytology was performed in 30 dogs and was diagnostic for neuroendocrine tissue in 26 dogs (87%). In 4 dogs (13%) fine-needle aspiration cytology was not considered diagnostic.

Information regarding local invasiveness was available for 28 of 33 dogs that underwent cervical ultrasound; in 24 dogs (86%) tumors were considered non-invasive and in 4 dogs (14%) there were signs of local invasion. Thoracic radiographs were performed in 37 dogs, revealing pulmonary metastases in 7 dogs (19%). A CT scan of the neck and thorax was performed in 6 dogs, showing a well circumscribed mass in 2 dogs (33%) and local invasion in 4 dogs (67%). One dog with an infiltrative thyroid tumor also had evidence of pulmonary metastases at CT scan. Cervical and thoracic scintigraphy were performed in 28 dogs, revealing homogenous tumor uptake in 15 dogs (53%), heterogeneous tumor uptake in 12 dogs (43%) and no uptake in the remaining dog (4%). Ectopic uptake foci in the thorax, consistent with ectopic thyroid tissue or tumor metastases, were identified in 12 dogs (46%).

Fifteen dogs (38%) had stage II disease, 10 (26%) had stage III and 14 (36%) had stage IV. In 3 dogs tumor stage could not be determined because tumor measurements were not recorded. Distant metastases were most frequently located in the lungs (n=13) and liver (n=4).
Levothyroxine therapy

Tumor histopathology was performed in 19 dogs (45%). One dog was diagnosed with an oxyphilic thyroid adenoma and 18 dogs were diagnosed with FTC.

Treatment

Seventeen dogs (41%) underwent thyroidectomy as main treatment modality (MST 29 m); 11 dogs (26%) were treated with $^{131}$I therapy (MST 15 m); 14 dogs (33%) were either treated palliatively with levothyroxine alone (n= 6, MST 7.5 m) or not treated (n=8, MST 3 m).

Lifelong levothyroxine therapy was prescribed to 7 of 17 dogs undergoing thyroidectomy, to 8 of 11 dogs undergoing $^{131}$I therapy and to the 6 dogs treated palliatively. In one dog undergoing bilateral thyroidectomy, levothyroxine was prescribed as replacement therapy for hypothyroidism. In the remaining 20 dogs levothyroxine therapy was prescribed with the intent of suppressing endogenous TSH. The clinicians’ decision to prescribe levothyroxine depended mainly on the period during which the patient was treated. Eighteen of 21 patients receiving levothyroxine therapy were treated during the last 5 years of the study when TSH suppression became a routine component of the treatment of canine follicular cell thyroid neoplasia at our clinic.

The average starting dose of levothyroxine was 11.3 ±4.7 µg/kg q12h. TSH suppression was recorded in 5 dogs at an average levothyroxine dosage of 15 ±7.3 µg/kg q12h, after a median of 0.5 dose adjustments (range, 0-2). In 4 of these 5 dogs, follow-up TSH values were below the detection limit of the TSH assay used by the laboratory at the time (< 0.1 ng/mL n=2; < 0.03 ng/mL n=2). In the remaining dog, TSH concentration was 0.07 ng/mL at the last follow-up appointment. When TSH suppression was achieved, the average TT$_4$ 3 h post-pill was 38.2±12.7 nmol/L.

Tumor mobility ($P=0.423$), evidence of distant metastases at diagnosis ($P=0.514$), tumor diameter ($P=0.199$), tumor stage ($P=0.342$) and thyroid function ($P=0.817$) were not significantly different between dogs that received levothyroxine therapy and dogs that did not. The group of dogs receiving levothyroxine therapy had a significantly ($P=0.049$) higher proportion (43%) of patients with bilateral disease than the group not receiving levothyroxine therapy (14%).
**Outcome**

Levothyroxine therapy ($P=0.716$), TSH suppression ($P=0.597$), tumor localization ($P=0.496$), tumor mobility ($P=0.939$) and hyperthyroidism ($P=0.08$) had no significant effect on outcome (Table 1). Tumor diameter had a significant effect on survival ($P=0.003$); each cm increase in tumor diameter increased the risk for death at any given time by 16% (Table 1). Evidence of distant metastases at diagnosis was also negatively correlated with survival ($P=0.019$); dogs with metastatic disease had 3.4 times higher risk for death at any given time than dogs with no evidence of metastases at diagnosis. Dogs with stage IV disease had 4.8 times higher risk for death at any given time than dogs with stage II ($P=0.008$).
Table 1. Survival analysis stratified for treatment in 42 dogs with thyroid tumors. *P*-values and Hazard Ratios (top/bottom group for each variable) are given.

<table>
<thead>
<tr>
<th>Variable</th>
<th>n</th>
<th><em>P</em>-value</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levothyroxine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21</td>
<td>0.716</td>
<td>0.88</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSH suppression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>5</td>
<td>0.597</td>
<td>0.70</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor localization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Uni/Ect</td>
<td>30</td>
<td>0.496</td>
<td>0.76</td>
</tr>
<tr>
<td>Bilat</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor mobility</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile</td>
<td>9</td>
<td>0.939</td>
<td>0.96</td>
</tr>
<tr>
<td>Fixed</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor diameter</td>
<td>37</td>
<td><strong>0.003</strong></td>
<td>1.16</td>
</tr>
<tr>
<td>Distant metastases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>14</td>
<td><strong>0.019</strong></td>
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</tr>
<tr>
<td>No</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage</td>
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<td></td>
</tr>
<tr>
<td>Stage IV vs II</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td><strong>0.008</strong></td>
<td>4.76</td>
</tr>
<tr>
<td>II</td>
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<td></td>
</tr>
<tr>
<td>Stage IV vs III</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td>0.061</td>
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<tr>
<td>III</td>
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<tr>
<td>Hyperthyroidism</td>
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<td></td>
</tr>
<tr>
<td>Yes</td>
<td>6</td>
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<td>0.02</td>
</tr>
<tr>
<td>No</td>
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<td></td>
</tr>
</tbody>
</table>

**Abbreviations**: HR, Hazard Ratio; Uni/Ect, Unilateral/Ectopic
DISCUSSION

In humans, the benefit of TSH-suppressive therapy in the management of high-risk differentiated FTC is well established and for the past years the authors recommend it routinely in the treatment of canine FTC.\textsuperscript{82} However, the results of this study failed to demonstrate that levothyroxine therapy and TSH suppression provide a survival benefit in dogs with thyroid tumors. This is in disagreement with a recent retrospective study in 15 dogs with bilateral thyroid tumors that showed a survival benefit for the dogs that also received levothyroxine replacement therapy after thyroidectomy.\textsuperscript{48} The cause for the disparity of results between these studies is unclear. If levothyroxine therapy would have been preferentially prescribed to high-risk patients in our study, this could have masked its clinical benefit. However, this did not seem to be the case according to our statistical analysis. Although the group receiving levothyroxine therapy had a significantly higher proportion of dogs with bilateral disease, tumor localization was not a prognostic factor in our study. Furthermore, the aforementioned veterinary study reports a prognosis for dogs with bilateral thyroid neoplasia undergoing thyroidectomy comparable to what is the reported for dogs with unilateral disease treated similarly.\textsuperscript{44,48} In humans with differentiated FTC, the beneficial effect of TSH suppression is only proven beyond doubt in high-risk patients.\textsuperscript{83} Unfortunately, our sample size was insufficient to repeat the statistical analysis adequately in a subgroup of high-risk patients.

It is possible that the lack of effect of levothyroxine therapy observed in our study is related to an insufficient power. In humans, retrospective studies showing the clinical benefit of TSH-suppressive therapy involve more than 100 patients treated similarly and the prospective study proving the benefit of high TSH suppression in high-risk patients with differentiated FTC involved nearly 3000 patients.\textsuperscript{83} Although our sample size is small, our study is the largest veterinary study investigating the effect of levothyroxine therapy on survival of dogs with thyroid tumors. Thyroid cancer is not common in dogs and studies with a much larger number of patients are difficult to accomplish.
Levothyroxine therapy

It is also possible that the lack of clinical benefit of levothyroxine therapy observed in our study is due to an overall insufficient degree of TSH suppression in the patients receiving levothyroxine. On one hand, TSH suppression as defined in our study could only be confirmed during follow-up in 5 of 21 patients receiving levothyroxine. On the other hand, although we routinely adapt the dosage of levothyroxine to reach nearly immeasurably low levels of TSH during follow-up of these patients, the ideal target level of TSH suppression in dogs with thyroid tumors is not currently known. During the study period, the detection limit of the canine TSH assay used at our laboratory changed from 0.1 ng/mL to 0.03 ng/mL. We have therefore decided to evaluate 0.1 ng/mL as cut-off for TSH suppression. However, the high sensitivity of human TSH assays allows targeting TSH concentrations to a level that is far below the detection limit of the current canine TSH assays. In humans, the ideal level of TSH suppression in patients with DTC is a topic of much debate and, at present, it is recommended to adapt the target level of TSH suppression to the patients risk for tumor recurrence and mortality based on prognostic factors. Further research is warranted to investigate if TSH-suppressive therapy is beneficial in dogs with FTC and to determine the adequate level of TSH suppression in these patients.

In our study, larger tumor diameter and evidence of distant metastases at diagnosis were negatively associated with prognosis and have already been described as prognosticators in canine thyroid cancer.

Thyroid tumors causing hyperthyroidism have preserved iodine trapping and hormone production, which could be associated with a higher degree of differentiation and a better prognosis. However, in agreement with previous reports, thyroid function was not associated with prognosis in our study. In humans, hyperfunctional thyroid carcinomas have a reported prevalence of only 1-2% and these scarce reports also suggest a comparable prognosis between hyperthyroid and euthyroid patients.

Limitations of our study include its retrospective nature, the limited number of dogs where TSH suppression could be confirmed, and the limited number of patients in each treatment group. Although histopathologic examination was performed in less than half of the dogs, this reflects the clinical dilemma of performing incisional biopsies in patients with unresectable thyroid tumors. In such cases, a presumptive
diagnosis obtained with cytology or scintigraphy is often favored due to potential bleeding complications of biopsying such highly vascularized tumors.

In conclusion, our study suggests that levothyroxine therapy does not provide a survival benefit in dogs with thyroid tumors and that large tumor diameter and evidence of distant metastases at diagnosis are negatively associated with prognosis. Further studies are needed to further evaluate if TSH-suppressive therapy is beneficial to dogs with thyroid tumors.
ENDNOTES

a IBM SPSS 20, Chicago, IL, USA

ACKNOWLEDGMENTS

The authors thank Marieke J. Voorhorst for her help obtaining clinical information from patients followed-up at Utrecht University.
Chapter 7

SHORT-TERM EFFECT OF RECOMBINANT HUMAN THYROTROPIN ON THYROID VOLUME AND ECHOGENICITY IN HEALTHY BEAGLES

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² Department of Veterinary Medical Imaging and Small Animal Orthopaedics
³ Department of Physiology and Biometrics
Faculty of Veterinary Medicine, Ghent University, Belgium
ABSTRACT

Background: In humans, administration of recombinant human TSH (rhTSH) allows radioactive iodine dose reduction and higher efficacy in the treatment of multinodular goiter and thyroid cancer. A similar effect might be expected in dogs with thyroid carcinoma. However, if rhTSH leads to an increase in thyroid volume it must be used carefully in dogs with large thyroid tumors to avoid compression of key anatomical structures.

Hypothesis/Objectives: The aim of the study was to determine the short-term effect of rhTSH on thyroid volume and echogenicity, measured by ultrasonography, in healthy Beagles.

Animals: Seven beagles.

Methods: The dogs were divided randomly in 2 groups in a prospective placebo-controlled blinded cross-over study. On day 1, one group received 100 µg of rhTSH intravenously and the other group received placebo. After a wash-out period of 3 weeks the groups were crossed over. Evaluation of thyroid echogenicity, homogeneity, shape, capsule delineation, and measurement of thyroid length, width and height were performed by the same observer at baseline, and at 6, 24 and 48h after injection of rhTSH and placebo.

Results: There was no significant difference between the effect of rhTSH and placebo on thyroid volume (P=0.397), echogenicity, homogeneity or capsule delineation. Time had a significant effect on thyroid volume (P=0.027). No adverse effects after rhTSH administration were noticed.

Conclusions and clinical importance: The effect of rhTSH on thyroid volume in dogs with thyroid carcinoma needs to be assessed.
INTRODUCTION

In humans with multinodular goiter and thyroid cancer, recombinant human thyroid stimulating hormone (rhTSH) increases radioactive iodine thyroid uptake, allowing \(^{131}\)I dose reduction, lower radiation-absorbed doses by extrathyroidal organs and tissues, and higher treatment efficacy.\(^{121,123,140,191}\) rhTSH also increases radioactive iodine thyroid uptake in the rhesus monkey and in hyperthyroid cats.\(^{131,192}\)

The use of rhTSH in thyroid carcinoma patients raises important safety issues. In humans, rhTSH increases the volume of the thyroid gland in healthy subjects and causes expansion of primary thyroid tumors and thyroid tumor metastases.\(^{136-139}\) Therefore, rhTSH should be used carefully in patients with large thyroid tumors or central nervous system, spinal, lung or bone metastases because tumor expansion may compress adjacent structures, leading to complications.\(^{140}\) Tumor enlargement is thought to result from swelling rather than growth, and glucocorticoids are administered before rhTSH is given to humans.\(^{140,193}\) No information is available on the effect of rhTSH on thyroid gland volume in dogs.

Sonography of the thyroid can be used to measure thyroid gland volume in dogs. Furthermore, gray-scale ultrasonography is a sensitive and quick test for the diagnosis of canine primary hypothyroidism.\(^{194-197}\) Before rhTSH is used in dogs with thyroid carcinoma, the influence of rhTSH on thyroid volume and echogenicity should be characterized. The goal of this study was to evaluate the short-term effect of rhTSH on thyroid volume and echogenicity, measured by ultrasonography, in healthy Beagles.
MATERIALS AND METHODS

Seven healthy Beagles were studied; all were neutered females with mean age of 9.6 ± 0.79 years and mean weight of 12.3 ± 1.5 kg. All dogs were healthy and euthyroid based on routine clinical testing and assays for serum total thyroxin (TT₄) and basal serum thyrotropin (TSH). TT₄ was quantified using a commercially available solid-phase, chemiluminescent competitive immunoassay, and basal serum TSH concentration was quantified with a commercially available solid-phase, two-site chemiluminescent immunometric assay, both previously validated in dogs. No dog had received any medication for at least 8 weeks prior to the study. Environmental conditions were unchanged throughout the study. All dogs were fed a standard commercial diet once daily and water was available ad libitum.

The dogs were divided randomly in 2 groups in a prospective placebo-controlled blinded cross-over study. On day 1, one group received 100 µg of rhTSH IV and the other group received placebo (1 mL of physiologic saline IV). After a wash-out period of 3 weeks, the groups were crossed over. In this manner, during the study each dog received rhTSH and placebo. The echogenicity, maximal length, width and height of both thyroid glands were measured using ultrasonography immediately prior to injection, and at 6, 24 and 48h after injection of rhTSH or placebo.

Each vial of 1100 µg of rhTSH was reconstituted with 5.5 mL of sterile water (200 µg/mL). Individual doses of 100 µg of freshly reconstituted rhTSH in 1 mL plastic syringes with needle and rubber caps and were stored frozen at -20°C for a maximum of 12 weeks, as previously described. Before use, frozen TSH was allowed to defrost at room temperature minutes before administration.

Blood samples for TT₄ measurement were collected by jugular venipuncture immediately before injection, and at 6, 12, 24 and 48h after rhTSH administration. Blood was centrifuged and the serum stored at -20°C until analysis. TT₄ concentration was determined with the aforementioned chemiluminescent immunoassay.

For sonography, the ventral cervical region was clipped from the larynx to the thoracic inlet. The dogs were in dorsal recumbency with the head in extension. Neither sedation nor anesthesia were used; two helpers restrained the dogs. Ultrasound gel was
applied. All ultrasound examinations were performed by the same board certified radiologist (JHS) who was unaware of the treatment administered. All dogs were scanned with the same ultrasound machine (Logiq 7, GE Medical Systems, Wisconsin, USA) connected to a multifrequency (7-14 MHz) linear matrix transducer with the frequency set at 12 MHz. Identical image presets were used for all patients. The overall gain was adjusted for each patient. Scanning was performed according to previously described method. The echogenicity of the thyroid glands was assessed by comparison with the surrounding muscles. The echotexture (homogeneous, heterogeneous), shape (rounded, ovoid, triangular) and delineation (smoothly, roughly) of the thyroid glands as well as the position of the esophagus (left, right) were recorded. The maximum height and the maximum width of each lobe, which were not necessarily located in the same plane, were measured on transverse images by use of electronic calipers. Following this, a longitudinal image of each lobe was obtained by starting transversally and slowly rotating the transducer 90°. The maximum length of each separate lobe, excluding the cranially located external parathyroid gland if visible, was obtained in the longitudinal plane. The volume of each thyroid lobe was estimated by the use the following rotation ellipse formula:

\[ \text{Thyroid gland lobe volume (cm}^3\) = length (cm) \times width (cm) \times height (cm) \times 0.479 \]

Based on a power analysis, the sample size was adequate to detect a 35.5% increase in thyroid gland volume (0.3 cm³ to 0.405 cm³) with a power of 80% for a two-sided test with significance level equal to 0.05. Statistical analysis was based on a mixed model with dog, week and side as random effects and side, treatment, time and the interaction between treatment and time as categorical fixed effects, using F-tests for the fixed effects at the 5% significance level. The change in TT₄ levels over time was analyzed separately for the two treatment groups using a mixed model with dog as random effect and time as a categorical fixed effect. Each time point was compared with time 0 using Dunnett’s multiple comparisons method at a global significance level of 5%. The Pearson correlation coefficients between the TT₄ concentration and the thyroid volume, height, length and width were calculated.
rhTSH & thyroid volume in healthy Beagles

RESULTS

No significant difference was found between the effect of rhTSH vs. placebo on thyroid volume ($P=0.397$) (Table 1). Thyroid volume decreased from baseline to 6h then increased at 24h and then decreased at 48h ($P=0.027$). This volume change did not differ significantly between placebo and rhTSH ($P=0.610$). No significant differences were found between the effect of rhTSH and placebo on thyroid height ($P=0.497$), width ($P=0.554$) or length ($P=0.752$). No significant changes over time were found for thyroid height ($P=0.967$), width ($P=0.211$) or length ($P=0.066$). Also no significant interactions between time and treatment were observed for thyroid height ($P=0.841$), width ($P=0.875$) or length ($P=0.405$).

The relative echogenicity of the thyroid gland compared to the surrounding muscles was unchanged. The gland was homogeneous in all dogs except one at initial examination. The appearance of the heterogeneous gland remained similar during follow-up examinations. At initial examination, the gland was classified as ovoid (n=18), round (n=6) or triangular (n=4). During the follow-up studies, changes in this classification were observed in 20/112 examinations. The esophagus was lying on the left side of the trachea in 47/56 examinations and on the right side in 9/56 examinations. The position of the esophagus changed within the same dog between measurements affecting the shape of the thyroid gland.

rhTSH caused a significant change in serum TT$_4$ concentration over time ($P<0.0001$). TT$_4$ concentration was significantly higher 6h after rhTSH administration ($P<0.0001$) compared with baseline (Fig. 1). Overall no correlation was found between TT$_4$ concentration and thyroid volume, length, height or width.

No adverse effects were observed after rhTSH administration.
### Table 1. Thyroid Gland Ultrasound Measurements in 7 Healthy Beagles after Recombinant Human TSH and Placebo. Mean values (standard deviation) of the estimated thyroid gland volume (mL), observed thyroid gland height (cm), width (cm) and length (cm) for each time point after placebo and rhTSH administration, the differences (95% confidence interval), percentage of change and \( P \) value for each comparison between placebo and rhTSH are given.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Placebo (StdDev)</th>
<th>rhTSH (StdDev)</th>
<th>Difference [95% CI]</th>
<th>% change</th>
<th>( P )-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.325 (0.070)</td>
<td>0.333 (0.079)</td>
<td>-0.008 [-0.058;0.041]</td>
<td>2.462</td>
<td>0.747</td>
</tr>
<tr>
<td>6 h</td>
<td>0.318 (0.077)</td>
<td>0.327 (0.086)</td>
<td>-0.009 [-0.058;0.040]</td>
<td>2.830</td>
<td>0.728</td>
</tr>
<tr>
<td>24 h</td>
<td>0.330 (0.079)</td>
<td>0.369 (0.103)</td>
<td>-0.039 [-0.088;0.010]</td>
<td>11.818</td>
<td>0.140</td>
</tr>
<tr>
<td>48 h</td>
<td>0.303 (0.074)</td>
<td>0.317 (0.091)</td>
<td>-0.014 [-0.064;0.035]</td>
<td>4.620</td>
<td>0.577</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Thyroid Height (cm)</th>
</tr>
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<tbody>
<tr>
<td>Time point</td>
</tr>
<tr>
<td>Baseline</td>
</tr>
<tr>
<td>6 h</td>
</tr>
<tr>
<td>24 h</td>
</tr>
<tr>
<td>48 h</td>
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</table>

<table>
<thead>
<tr>
<th>Thyroid Width (cm)</th>
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<tbody>
<tr>
<td>Time point</td>
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<tr>
<td>Baseline</td>
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<tr>
<td>6 h</td>
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<tr>
<td>24 h</td>
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<td>48 h</td>
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<table>
<thead>
<tr>
<th>Thyroid Length (cm)</th>
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<tr>
<td>Time point</td>
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<tr>
<td>Baseline</td>
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<tr>
<td>6 h</td>
</tr>
<tr>
<td>24 h</td>
</tr>
<tr>
<td>48 h</td>
</tr>
</tbody>
</table>
Fig. 1: Mean serum TT$_4$ concentration (nmol/L), with respective standard deviation, as a function of time after rhTSH administration.
DISCUSSION

In this trial, no significant difference was detected between the effect of rhTSH and placebo on thyroid volume during the first 48h after injection. These results are in contrast with what has been found in humans, where rhTSH stimulation significantly increased thyroid gland volume. Mean thyroid volume increased by 23.3 % 24 hours after 900 µg rhTSH stimulation, and by 35.5 % after 48 hours but returned to baseline values on day 4. In another study in humans, thyroid volume increased by 10% 48 hours after 100 µg rhTSH injection, but this was not placebo-controlled, and sonography was performed by multiple individuals.

We used 100 µg of rhTSH as this is the dose that is considered most appropriate for TSH stimulation in dogs weighing more than 20 kg. If the effect of rhTSH on thyroid volume is dose dependent we may have observed a volume change at higher doses. A dose effect may also explain the disparate results obtained in human studies noted above.

The route of rhTSH administration could also have influenced our results. In humans, rhTSH is administered intramuscularly and in our study it was given intravenously. The intravenous route was chosen to maximize bioavailability, given the high cost of rhTSH. There are no data that support the intramuscular route being better than the intravenous route for rhTSH administration. On the contrary, giving the same dose of rhTSH to healthy Beagles intravenously resulted in a significantly higher serum TT4 elevation compared to subcutaneous and intramuscular administration. Intramuscular vs. intravenous administration of rhTSH was also compared in healthy dogs of different breeds and there was no significant effect on TT4 concentration.

In humans, the marked, fast, transient effect of rhTSH on thyroid gland volume was thought most likely due to intravascular and interstitial fluid accumulation, rather than regular growth of the thyroid tissue. Evidence of peritumoural edema or, less commonly, hemorrhage, and response to glucocorticoid administration also support this hypothesis.

Thyroid gland volume changed significantly over time after both rhTSH and placebo administration. Although this variation in thyroid volume was statistically
significant, it is too small to be clinically relevant. This is supported by the fact that neither thyroid height, width nor length changed significantly over time. Therefore, the authors consider this observation to be, most likely, a statistical aberration.

There were no changes in the delineation, echogenicity or homogeneity of the thyroid gland, although changes in thyroid gland shape were observed, however characterization of shape is subjective. Additionally, the position of the esophagus or the position of the dog and pressure of the transducer could influence the shape of the gland on a transverse scan.

rhTSH had a significant effect on serum $TT_4$ concentrations and on the variation of serum $TT_4$ concentrations over time. The peak serum $TT_4$ concentration occurred 6 hours after rhTSH injection, as expected. The peak of serum $TT_4$ concentration coincided with the lowest thyroid gland volume. However, a decrease in thyroid volume was also observed in the placebo group, and there was no statistical association between the $TT_4$ concentration and the observed variation in thyroid volume, length, height or width.

The absence of an effect of rhTSH on thyroid volume in healthy dogs cannot be extrapolated to dogs with thyroid carcinoma. Healthy thyroid tissue and neoplastic thyroid tissue differ histologically and functionally. Therefore, although our results suggest no influence of rhTSH on thyroid volume, it is not known that rhTSH will not cause tumor expansion in dogs with thyroid carcinoma. Therefore, rhTSH must be used with caution in thyroid carcinoma patients, especially patients with bulky thyroid masses, respiratory, central nervous system, spinal or bone metastases. Pretreatment with glucocorticoids should be considered if tumor expansion would lead to unacceptable complications.
Chapter 7

ENDNOTES

a IMMULITE 2000 Canine Total T4, Siemens, Los Angeles, CA, USA
b IMMULITE 2000 Canine TSH, Siemens, Los Angeles, CA, USA
c Thyrogen®, Genzyme Corporation, Cambridge, Maine, USA
d SAS Version 9.1.3
Chapter 8

EFFECT OF RECOMBINANT HUMAN THYROTROPIN ON THE UPTAKE OF RADIOACTIVE IODINE ($^{123}$I) BY THE THYROID GLAND IN HEALTHY BEAGLES

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Adapted from

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²Department of Veterinary Medical Imaging and Small Animal Orthopaedics
³Department of Physiology and Biometrics
Faculty of Veterinary Medicine, Ghent University, Belgium
ABSTRACT

**Background:** In human medicine recombinant human TSH (rhTSH) increases the thyroid radioactive iodine uptake (RAIU) allowing radioiodine-131 ($^{131}$I) dose reduction and higher efficacy in the treatment of differentiated thyroid cancer and multinodular goiter.

**Hypothesis/Objectives:** The goal of this study was to evaluate the effect of rhTSH, administered 24 h and 48 h before radioiodine-123 ($^{123}$I), on the thyroid RAIU in healthy dogs.

**Animals:** Seven euthyroid healthy Beagles.

**Methods:** Dogs were randomly divided in three groups (two groups of two dogs and one group of three dogs) in a prospective, blinded, cross-over study. On week 1, one group received $^{123}$I for a baseline RAIU, one group received 100 µg of rhTSH IV 24 h before $^{123}$I and one group received 100 µg of rhTSH IV 48 h before $^{123}$I. All dogs received 37 MBq of radioactive $^{123}$I IV and thyroid RAIU was determined 8 h, 24 h and 48 h thereafter. The study was designed in such a manner that each dog received the three treatments and a wash-out period of three weeks was respected in between. Blood samples were taken for measurement of serum total thyroxine (TT$_4$) and thyrotropin (TSH) concentration at baseline, 6 h, 12 h, 24 h and 48 h after rhTSH administration.

**Results:** Recombinant human TSH caused no significant change on thyroid RAIU. The overall mean thyroid RAIU significantly decreased during the study independent of the treatment. Recombinant human TSH significantly increased serum TT$_4$ concentration, which peaked 6 h after rhTSH administration. Compared to baseline, serum TSH concentration remained higher at 6 h, 12 h, 24 h and 48 h. However, a statistical significant difference was only reached at 6 h and 12 h after rhTSH administration. No adverse effects of rhTSH were observed during the study.

**Conclusions and clinical importance:** Further studies are needed to determine the best timing and dosage of administration of rhTSH in healthy and thyroid carcinoma dogs.
INTRODUCTION

Thyroid tumors are uncommon in dogs, accounting for 1.2 to 3.7% of all neoplasms. Carcinomas are more common than adenomas, representing up to 90% of palpable thyroid tumors at diagnosis. Treatment modalities include surgical excision, external beam radiation, chemotherapy and radioiodine-131 ($^{131}$I). Two recent retrospective studies have shown a high treatment efficacy and prolonged median survival time with $^{131}$I therapy both as a sole therapeutic modality and as an adjunct to surgery.

Several human studies have shown that rhTSH increases the radioactive iodine uptake (RAIU) by thyroid cancer tissue with relatively few side-effects. The same effect has been shown in healthy humans and in patients with multinodular goiter. Recombinant human TSH allows higher efficacy, lower radiation-absorbed doses by extrathyroidal tissues and $^{131}$I dose reduction in the treatment of differentiated thyroid cancer and multinodular goiter.

Recombinant human TSH is currently used in veterinary medicine to aid in the diagnosis of canine hypothyroidism. Recombinant human TSH increases the thyroid RAIU in the rhesus monkey and a recent study has shown that it significantly increases the thyroid RAIU in hyperthyroid cats. This property of rhTSH is particularly interesting in dogs because high doses of $^{131}$I are used for the treatment of canine thyroid carcinoma. Radiation exposure should be kept «as low as reasonably achievable» (ALARA principle) and rhTSH may allow $^{131}$I dose reduction, higher treatment efficacy and reduction of radioactivity delivered to extrathyroidal tissues and to the environment.

Before rhTSH starts being more intensively investigated in the treatment of dogs with thyroid carcinoma, it is important to evaluate its effect on thyroid RAIU. Currently, to the authors’ knowledge, no such studies have been performed in dogs.

$^{131}$I decays emitting beta particles, which cause a higher localized radiation dose with greater potential for impaired thyroid function. $^{123}$I decays by electron capture emitting gamma rays, and has been shown to be equal or superior to $^{131}$I as a scanning agent. Therefore, the goal of this study was to evaluate the effect of rhTSH,
administered 24 h or 48 h before radiiodine-123 ($^{123}$I), on the thyroid RAIU in healthy dogs.
MATERIALS AND METHODS

Animals

Seven healthy Beagles participated in this study. They were all spayed females with mean age of 9.6 ± 0.79 years and mean weight of 12.3 ± 1.5 kg at the start of the study. All dogs were healthy and euthyroid based on their history, thorough physical examination, full hematological and biochemical blood analysis, serum total thyroxin ($TT_4$) concentration and basal serum thyrotropin (TSH) concentration within reference range. Serum $TT_4$ concentration was determined with a commercially available solid-phase, chemiluminescent competitive immunoassay (IMMULITE 2000 Canine Total $T_4$, Siemens, Deerfield, IL, USA) previously validated in dogs, and the reference range used was 6.4-43.8 nmol/L. Basal serum TSH concentration was determined with a commercially available solid-phase, two-site chemiluminescent immunometric assay (IMMULITE 2000 Canine TSH, Siemens, Deerfield, IL, USA) previously validated in dogs, and the reference range used was <0.5 ng/mL. None of the dogs had received any medication for at least eight weeks prior to the study. During weeks 1, 5 and 9 of the study, the dogs were kept in nuclear medicine facilities for radioactivity safety purposes. During the washout period the dogs were kept in the kennel where they usually live. Environmental conditions, such as photoperiod, ventilation, temperature and humidity were kept unchanged throughout the study. All dogs were fed a standard commercial diet once daily and water was available ad libitum. Animal care was in accordance with European guidelines and directives (EC directive 86/609/EEC for animal experiments) and the study was approved by the Ethical committee of the faculty of veterinary medicine of Ghent University, Belgium (approval number EC 2008/051).

Study design

The dogs were divided in three groups in a prospective, cross-over study. On week 1, one group received $^{123}$I for a baseline thyroid RAIU, one group received 100 µg of rhTSH IV 24 h before $^{123}$I and one group received 100 µg of rhTSH IV 48 h before $^{123}$I. All dogs received 37 MBq (1 mCi) of $^{123}$I IV and thyroid RAIU was determined 8 h, 24 h and 48 h thereafter. The study was designed in such a manner that
each dog received the three treatments and a wash-out period of three weeks was respected (Table 1).

**Recombinant human TSH**

Each vial of 1100 µg of rhTSH (Thyrogen®, Genzyme Corporation, Cambridge, Maine, USA) was reconstituted with 5.5 mL of sterile water (200 µg/mL). Individual doses of 100 µg of freshly reconstituted rhTSH were prepared in 1 mL plastic syringes with needle and rubber caps and were stored frozen at -20º C for a maximum of 12 weeks. For TSH stimulation, frozen rhTSH was defrosted at room temperature few minutes before administration.

**Table 1.** Cross-over design of the study. A wash-out period of three weeks was respected between study weeks.

<table>
<thead>
<tr>
<th>Cross-over design</th>
<th>Week 1</th>
<th>Week 5</th>
<th>Week 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>$^{123}$I (baseline RAIU)</td>
<td>rhTSH 24h before $^{123}$I</td>
<td>rhTSH 48h before $^{123}$I</td>
</tr>
<tr>
<td>Group II</td>
<td>rhTSH 24h before $^{123}$I</td>
<td>rhTSH 48h before $^{123}$I</td>
<td>$^{123}$I (baseline RAIU)</td>
</tr>
<tr>
<td>Group III</td>
<td>rhTSH 48h before $^{123}$I</td>
<td>$^{123}$I (baseline RAIU)</td>
<td>rhTSH 24h before $^{123}$I</td>
</tr>
</tbody>
</table>


**RAIU**

Each dog received 37 MBq (1 mCi) of $^{123}$I intravenously. The injected activity of $^{123}$I was calculated by subtracting the activity of the empty syringe from the activity of the full syringe both measured in a dose calibrator. To determine the thyroid RAIU, a static planar ventrodorsal image was obtained with a one head $\gamma$-camera (Toshiba GCA 901) using a low-energy high resolution collimator with the dog in sternal recumbency under general anesthesia. General anesthesia was induced with propofol and maintained with isoflurane. Data were acquired during 5 minutes for the 8h-RAIU, 10 minutes for the 24h-RAIU and 15 minutes for the 48h-RAIU on a 128x128 matrix. A syringe with a known amount of radioactivity (2.5 ± 1.6 MBq) was placed next to the animal and served as the standard activity necessary to calculate RAIU. Regions of interest (ROIs) were manually drawn over the two thyroid lobes (cpm$_{thyroid}$) and over the activity of the standard (cpm$_{standard}$). In order to correct for background activity a ROI was drawn over an area close to, but not overlapping, the thyroid gland (cpm$_{background}$) and another ROI was placed outside the dog (cpm$_{room}$). These ROI’s were placed on one day and by the same person (KP) who was blinded to the dogs’ treatment. RAIU was calculated as a percentage of the administered dose of $^{123}$I corrected for physical decay and background activity using the following formula:

\[
\left( \frac{(\text{cpm}_{thyroid} - \text{cpm}_{background})}{(\text{cpm}_{standard} - \text{cpm}_{room})} \right) \times \frac{\text{MBq}_{standard}}{\text{MBq}_{dose}} \times 100
\]

**Blood sampling**

Blood samples for TT$_4$ and TSH measurement were taken in the two weeks that each dog received rhTSH. Blood was collected by jugular venipuncture at baseline, 6 h, 12 h, 24 h and 48 h after rhTSH administration. Blood was centrifuged and the serum was stored for 3 weeks at -20ºC to reach sufficient decay of radioactivity to be analyzed. To measure and follow up rhTSH serum concentrations, a commercially available two-site sandwich immunoassay (ADVIA Centaur TSH, Siemens, Deerfield, IL, USA) for human TSH determination was used.
Statistical analysis

Data were analyzed with SAS version 9.1 (SAS, Cary, North Carolina, USA). The effect of rhTSH administration on RAIU was analyzed with a mixed model with period, treatment, time and the interaction between treatment and time as categorical fixed effects and dog and the period by dog interaction as random effects. Comparisons were based on the F-test at a global significance level of 5%, using Tukey's procedure for multiple comparisons. The variation of TT₄ and TSH levels over time was analyzed using a mixed model with dog as random effect and time as a categorical fixed effect. Each time point was compared with time 0 using Dunnett’s multiple comparisons method at a global significance level of 5%.
RESULTS

No significant difference was found in thyroid RAIU among the three protocols (no rhTSH, rhTSH 24 h before $^{123}$I and rhTSH 48 h before $^{123}$I, $P=0.424$). Thyroid RAIU increased significantly over time ($P<0.0001$; Fig. 1). There was no significant interaction between treatment and time ($P=0.887$).

In all groups, the thyroid RAIU significantly decreased throughout the weeks of the study ($P<0.0001$) from 0.268 (SD=0.032) on week 1 to 0.129 (SD=0.024) on week 5, and 0.082 (SD=0.018) on week 9.

Recombinant human TSH caused a significant increase in serum TT$_4$ concentration over time ($P<0.001$), reaching a peak 6 h after administration. Serum TT$_4$ concentration after rhTSH stimulation performed 24 h before $^{123}$I and 48 h before $^{123}$I did not differ significantly from each other ($P=0.217$; Fig. 2).

In both groups where rhTSH was administered, rhTSH changed significantly over time ($P<0.0001$), and was significantly higher at time 6 h ($P<0.0001$) and at time 12 h, (when rhTSH was administered 24 h before $^{123}$I: $P=0.015$ and 48 h before $^{123}$I: $P=0.002$), compared to baseline (Table 2).

![Fig. 1: Thyroid RAIU in 7 Healthy Beagles. Overall mean values of thyroid RAIU (%), with respective standard deviation, are shown for each time point for the 3 study protocols (baseline RAIU, rhTSH 24 h before $^{123}$I and rhTSH 48 h before $^{123}$I).](image)
Fig. 2: Serum TT₄ concentrations in 7 Healthy Beagles after recombinant human TSH. Mean serum TT₄ concentrations (nmol/L), with respective standard deviation, are given for each time point after rhTSH (24 h before $^{123}$I and 48 h before $^{123}$I).

Table 2. Serum rhTSH concentration in 7 Healthy Beagles after Recombinant Human TSH (rhTSH) administration. Mean values (standard deviation) of serum rhTSH concentration (mIU/L) for each time point are given.

<table>
<thead>
<tr>
<th>Time point</th>
<th>rhTSH 24h before $^{123}$I</th>
<th>rhTSH 48h before $^{123}$I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0.068 (0.038)</td>
<td>0.076 (0.045)</td>
</tr>
<tr>
<td>6 h</td>
<td>59.543 (27.384)*</td>
<td>50.544 (18.257)*</td>
</tr>
<tr>
<td>12h</td>
<td>19.703 (10.758)*</td>
<td>16.484 (6.011)*</td>
</tr>
<tr>
<td>24 h</td>
<td>5.634 (2.360)</td>
<td>4.960 (1.636)</td>
</tr>
<tr>
<td>48 h</td>
<td>1.059 (0.449)</td>
<td>1.77 (0.394)</td>
</tr>
</tbody>
</table>

* Values significantly different from baseline ($P < 0.05$)
DISCUSSION

Canine thyroid carcinoma can be successfully treated with high doses of $^{131}$I. $^{38,43}$ rhTSH is a potent stimulator of thyroid function and increases thyroid RAIU in humans, rhesus monkey and hyperthyroid cats. $^{131,140,192}$ If, as in humans, rhTSH increases the thyroid RAIU in dogs with thyroid carcinoma it may allow $^{131}$I dose reduction and higher treatment efficacy in these patients, along with delivery of less radioactivity to non-thyroidal tissues and to the environment. In this context, our study may have important clinical implications. Furthermore, this is the first study that investigates the effect of rhTSH on thyroid RAIU in healthy dogs.

In our study, rhTSH administration had no influence on thyroid RAIU. These results contrast with studies performed in healthy humans. In a study comparable to ours, 25 euthyroid healthy volunteers received a baseline 24 h RAIU after $^{123}$I and were then randomly divided in three groups to receive either 100 µg of rhTSH IM 24 h before $^{123}$I (3.7 MBq or 0.1 mCi PO), 48 h before $^{123}$I or 72 h before $^{123}$I. $^{137}$ Thyroid RAIU was determined in all subjects 24 h after $^{123}$I administration. Results revealed an 88% increase in the thyroid RAIU when rhTSH was administered 24 h before $^{123}$I and a 36% increase in the RAIU when rhTSH was administered 48 h before $^{123}$I. In another study, one week after a baseline RAIU, six healthy humans received 900 µg of rhTSH IM 24 h before $^{123}$I (11.1 MBq or 0.3 mCi) and thyroid RAIU was determined 6 h and 24 h after $^{123}$I administration. Results revealed that thyroid RAIU approximately doubled after rhTSH administration. $^{207}$

Several factors may explain why rhTSH had no effect on thyroid RAIU in our study. It is possible that despite having a significant effect on thyroid function, rhTSH does not enhance the trapping and retention of iodine in dogs, and therefore does not increase the thyroid RAIU in this species. Although possible, this hypothesis does not seem likely because rhTSH has been proven to increase thyroid RAIU in other species such as the rhesus monkey and cats. $^{131,192}$

The study in hyperthyroid cats reported a 7% increase in thyroid RAIU after rhTSH administration. $^{131}$ However, the different results obtained in this study in cats may be related to the hyperthyroid state of the patients or to the different dosage and
timing of rhTSH administration (25 µg of rhTSH were administered IV 1 h before $^{123}$I). Huysmans et al. investigated different time intervals between rhTSH injection and $^{131}$I in human patients with non-toxic multinodular goiter, and concluded that this interval was a determinant factor to observe changes in thyroid RAIU. In their study, a 24 h interval between rhTSH administration and iodine injection was significantly more effective than a 2 h interval. Another possibility is that biokinetics of rhTSH are different in dogs and humans and that the effect of rhTSH on iodine accumulation occurs sooner in dogs than in humans. Further studies are warranted to determine the optimal timing for rhTSH administration.

rhTSH was administered 24 h and 48 h before $^{123}$I because studies on FRTL-5 cells (Fischer Rat Thyroid cell Line) revealed that 12 h to 24 h are needed before TSH stimulates iodine accumulation in thyroid cells. In our study, serum TSH concentration remained increased at 48 h, but a statistically significant increase was only observed at 6 h and 12 h, which might not be enough to stimulate iodine accumulation and increase thyroid RAIU. The results of the above-mentioned study by Pena et al. in healthy humans support this hypothesis. In this study, serum TSH concentration remained markedly elevated at 24 h (above 11 mU/L) and at 48 h (above 4 mU/L) after rhTSH IM administration. In this context, the route of rhTSH administration could also have influenced our results. In our study, the intravenous route was chosen to maximize bioavailability, given the high cost of rhTSH. Although there are no reports arguing that IM route is superior to IV route, it could be that if rhTSH is administered intramuscularly, such as in humans, its clearance is slower allowing a longer stimulation of the thyroid cells with a potential increase in thyroid RAIU.

It is possible that the effect of rhTSH on the thyroid RAIU is dose dependent and that 100 µg of rhTSH is not enough to cause a significant increase of thyroid RAIU in dogs. Currently, no studies have shown that the effect of rhTSH on thyroid RAIU is dose dependent. Further studies are warranted to evaluate the effect of different doses of rhTSH on canine thyroid RAIU.

The progressively lower RAIU in all groups throughout the study suggests either a stunning effect or an alteration of the biokinetics of iodine. In human medicine
the topic of stunning of the thyroid gland is controversial. The phenomenon has been related to the diagnostic use of small amounts of $^{131}\text{I}$ before therapy.\textsuperscript{209,210} It is thought to be related to radiotoxic effects of the radionuclide resulting in (temporary) decreased ability of the thyroid cells to trap and retain radioiodine.\textsuperscript{209,211} Stunning is considered to be dose dependent and seems to occur in relation with a longer interval between diagnostic use of $^{131}\text{I}$ and therapy.\textsuperscript{212} This effect is transient due to recovery of the thyroid cells with normalization of trapping and retaining ability after 20-25 days.\textsuperscript{213} Despite the fact that the «stunning» debate in literature is predominantly about $^{131}\text{I}$, the possible occurrence of this effect has been reported in relation with the use of $^{123}\text{I}$ (74 MBq; 200 MBq).\textsuperscript{213,214} This radionuclide, exclusive for diagnostic use, decays primarily through emission of gamma rays and Auger electrons, thereby limiting radiotoxic effects to the thyroid. It was concluded that decreased uptake after diagnostic use of $^{123}\text{I}$ was probably not due to the damaging effects but rather the consequences of differential rates of radioiodine turn-over.\textsuperscript{214} To avoid this stunning effect by the repeated injections of $^{123}\text{I}$ we used a small amount of radioactivity (37 MBq or 1 mCi). However, a wash-out period of 3 weeks was chosen in this study to allow for decay of residual $^{123}\text{I}$ in order not to compromise the imaging results. If stunning would have occurred, this time interval would be borderline for recuperation of the thyroid cells considering the 20-25 days interval for recovery after diagnostic $^{131}\text{I}$. Further studies are mandatory to evaluate whether a longer wash-out period or a lower $^{123}\text{I}$ dose produce similar results.

Differential biokinetics of radioiodine due to its subsequent administration is another theoretical possibility. It is well known that iodine supplementation alters the turn-over of subsequent iodine administration. However, it has long been demonstrated that iodine supplementation up to 0.1 mg does not affect the uptake of $^{131}\text{I}$ nor the wash-out.\textsuperscript{215} The specific activity of the $^{123}\text{I}$ solution is high (1,93 x 10$^6$ Ci/g) with as a consequence the presence of an extremely low amount of iodine (1,004 ng) in the solution. Therefore it is doubtful that this would have any influence on iodine turn-over.

Another factor capable of causing an altered turn-over of radioiodine is an increase in the dietary intake of iodine. However, no diet change occurred during the
study or previously which would explain the consecutive decrease in uptake. A change in the iodine content of the drinking water is also possible although it does not seem likely.

As expected rhTSH had a statistical significant effect on serum TT$_4$ concentrations and on the variation of serum TT$_4$ concentrations over time. The peak of serum TT$_4$ concentration occurred 6 h after rhTSH injection, such as previously described.$^{125}$

Extrapolation of results from healthy dogs to patients with thyroid carcinoma is not possible and this is one limitation of our study. Healthy thyroid tissue and neoplastic thyroid tissue are functionally different. Therefore, it is possible that, despite our results, rhTSH has clinical use in canine patients with thyroid carcinoma.

In conclusion, our results show that 100 µg of rhTSH administered intravenously 24 h and 48 h before $^{123}$I does not affect thyroid RAIU in healthy dogs. Further studies are needed to determine the best timing and dosage of administration of rhTSH in healthy and thyroid carcinoma bearing dogs.
ACKNOWLEDGMENTS

The authors thank Simon Vermeire and Sara Janssens for their collaboration in this research project.
Chapter 9

EFFECT OF RECOMBINANT HUMAN THYROTROPIN ON THE UPTAKE OF RADIOACTIVE IODINE (\(^{123}\)I) IN DOGS WITH THYROID TUMORS

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Adapted from

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ABSTRACT

Background: In humans, recombinant human thyrotropin (rhTSH) enhances radioactive iodine uptake (RAIU) in patients with differentiated thyroid cancer. No studies have been performed in veterinary medicine to optimize radioiodine treatment of thyroid cancer.

Hypothesis/Objectives: To evaluate the effect of rhTSH on the uptake of radioiodine-123 ($^{123}$I) in dogs with thyroid tumors.

Animals: Nine dogs with thyroid neoplasia.

Methods: The dogs were divided in 2 groups in this prospective cross-over study. In one group, $^{123}$I was administered for a baseline RAIU determination in week 1. In week 4 (after a washout period of 2 weeks), these dogs received rhTSH (100 μg IV) 24 h before $^{123}$I injection. In the other group the order of the protocol was reversed. For each scan, the dogs received 37 MBq (1 mCi) of $^{123}$I intravenously (IV) and planar scintigraphy was performed after 8 and 24 h for tumor RAIU calculation.

Results: Overall, rhTSH administration caused no statistically significant change on thyroid tumor RAIU at 8 h ($P=0.89$) or at 24 h ($P=0.98$). A significant positive correlation was found between the effect of rhTSH on tumor 8h-RAIU and rhTSH serum concentrations at 6 h ($τ=0.68; P=0.03$), at 12 h ($τ=0.68; P=0.03$) and at 24 h ($τ=0.78; P=0.02$) after rhTSH injection. This study suggests that IV administration of 100 μg rhTSH 24 h before $^{123}$I has an inconsistent effect on thyroid tumor RAIU.

Conclusions and clinical importance: Further studies are necessary to determine the best protocol of rhTSH administration to optimize thyroid tumor RAIU.
INTRODUCTION

Thyroid tumors account for 10-15% of all head and neck neoplasms in dogs. Ninety percent of canine thyroid tumors are carcinomas and 16-38% of the patients present evidence of metastasis at the time of diagnosis. Surgery is the preferred treatment modality for mobile tumors, while large invasive tumors have a better prognosis with external beam radiation or radioactive iodine-131 (\(^{131}\)I) therapy. Two recent retrospective studies showed prolonged median survival times of 27 and 30 months following \(^{131}\)I therapy. Furthermore, \(^{131}\)I may be the only effective therapy against thyroid cancer metastases. In dogs, high doses of \(^{131}\)I are required and this usually implies a prolonged hospitalization period and high doses of radiation eliminated to the environment through the excreta. Use and exposure to radiation should be kept “as low as reasonably achievable” (ALARA principle) to minimize risks for patient and human health. Exposure of nonthyroidal tissues to high doses of radiation may cause treatment complications such as fatal myelosuppression. Major limitations of \(^{131}\)I therapy include its selected effectiveness in differentiated thyroid tumors exhibiting adequate \(^{131}\)I uptake and the potential need of multiple treatments for tumor control.

In human medicine, recombinant human thyrotropin\(^a\) (rhTSH, Genzyme Corporation, Cambridge, ME, USA) is used to increase \(^{131}\)I uptake by normal and neoplastic thyroid tissue in the treatment and diagnostic follow-up of differentiated thyroid carcinoma. In addition, the use of rhTSH before \(^{131}\)I therapy is associated with a lower whole-body exposure to radiation, limiting treatment complications.

Thyrotropin (TSH) binds to a membrane TSH G protein-coupled receptor on the surface of follicular thyroid cells and triggers a cascade of intracellular reactions leading to synthesis and secretion of triiodothyronine (T\(_3\)), thyroxine (T\(_4\)) and thyroglobulin (Tg). Prolonged TSH stimulation (>24 h) increases the expression and functionality of the Na/I symporter (NIS) and, consequently, leads to an increased uptake and organification of iodine.

In veterinary medicine, rhTSH has been mainly used for the diagnosis of canine hypothyroidism due to the lack of specificity of the current endogenous TSH
However, TSH receptors have already been demonstrated in canine neoplastic thyroid cells; both in primary tumors and metastases. The optimization of radioiodine treatment with rhTSH may offer important clinical advantages. On one hand, by increasing the uptake $^{131}\text{I}$ by the thyroid tumor, rhTSH may improve $^{131}\text{I}$ treatment efficacy and decrease the need for multiple treatments. On the other hand, rhTSH may allow a decrease of the therapeutic dosage of $^{131}\text{I}$, thereby improving radioprotection, limiting radiotoxicity and complying with the ALARA principle. Furthermore, $^{131}\text{I}$ dose reduction could potentially reduce the required hospitalization period and costs.

The use of $^{131}\text{I}$ for diagnostic imaging in clinical research has several limitations. Its half-life (8 days) makes it impractical for repeated radioactive iodine uptake (RAIU) determinations within a reasonable period. Furthermore, the emission of beta particles during the decay of $^{131}\text{I}$ causes a higher localized radiation dose and may have a deleterious effect on the uptake of the actual $^{131}\text{I}$ therapeutic dosage, a phenomenon named thyroid stunning. Unlike $^{131}\text{I}$, $^{123}\text{I}$ has a much shorter half-life (13 h), decays by emitting gamma rays and has been shown to be equal or even superior to $^{131}\text{I}$ as a scanning agent. Hence, in this study $^{123}\text{I}$ was chosen as an imaging agent, despite its high cost. Recent pilot studies performed by our group have already investigated the use of rhTSH to optimize the uptake of radioiodine-$^{123}\text{I}$ in healthy dogs and hyperthyroid cats. The goal of this study was to evaluate the effect of 100 μg rhTSH, administered IV 24 h before $^{123}\text{I}$, on tumor RAIU in dogs with thyroid tumors.
MATERIALS AND METHODS

Sample size

A preliminary power analysis showed that 9 patients included in a prospective cross-over study would suffice to detect a 28% increase in tumor RAIU with a power of 80% at a global significance level of 5%.

Animals

The inclusion criteria of our study were diagnosis of thyroid neoplasia by either cytology, biopsy and/or scintigraphy and tumor uptake of $^{123}$I at scintigraphy. Patients where treatment was deemed urgent due to upper airway obstruction were excluded. The first nine dogs referred to the Small Animal Clinic of Ghent University that met the inclusion criteria and for which owner consent was obtained, were included. Patients were recruited between December 2007 and November 2011. Diagnosis was based on physical examination, cervical mass cytology, cervical scintigraphy and, when available, histopathology from excisional biopsies. Complete hematological and biochemical analysis, including serum total thyroxine (TT$_4$) and TSH, were performed in all patients.

Determination of thyroid functional status was based on clinical signs, basal serum TT$_4$ and TSH concentrations, cervical scintigraphy and TSH stimulation. Results of TSH stimulation were interpreted comparing serum TT$_4$ concentrations at baseline and 6 h after rhTSH administration. Euthyroidism was confirmed when post-stimulation TT$_4$ was $\geq$ 40 nmol/L and hypothyroidism was considered likely when post-stimulation TT$_4$ concentration was $< 20$ nmol/L.$^{126,200}$ Dogs with post-stimulation TT$_4$ between 20 and 40 nmol/L would have been classified as having undefined thyroid function, but such results were not observed. Dogs with baseline TT$_4$ > 43,86 nmol/L and compatible clinical signs were considered hyperthyroid.

All dogs were staged according to the WHO staging system for canine thyroid tumors.$^{164}$ For this purpose, cervical palpation, tridimensional measurement of the tumor, radiographs or computed tomography (CT) of the thorax, cervical and thoracic scintigraphy were performed in all patients. Cervical ultrasound was performed in 6
patients. Cervical and thoracic computed tomography was performed in 4 patients. Abdominal ultrasound was performed in 2 patients.

During the washout period, the dogs stayed at home. Diet and water source were kept unchanged during the study.

**Ethics statement**

Animal care was in accordance with European guidelines and directives (EC directive 86/609/EEC for animal experiments) and the study was approved by the Ethical committee of the Faculty of Veterinary Medicine of Ghent University and by the Belgian Deontological committee (approval number EC 2010/168). Furthermore, an owner consent form was signed by all owners.

**Study design**

The dogs were divided in 2 groups in a prospective cross-over study. In group A, $^{123}$I was administered for a baseline RAIU determination in week 1. In week 4 (after a washout period of 2 weeks), these dogs received rhTSH (100 μg IV) 24 h before $^{123}$I injection. In group B the order of the protocol was reversed (Table 1). For each scan, the dogs received 37 MBq (1mCi) of $^{123}$I IV and planar scintigraphy was subsequently performed at 8 h and 24 h for tumor RAIU calculation.

**Blood samples**

Blood samples were taken for serial measurements of TT$_4$ and rhTSH serum concentrations in the week rhTSH was administered. Blood was collected by jugular venipuncture at baseline, 6, 12, 24 and 48 h after rhTSH injection. Blood was centrifuged and the serum was stored for at least 3 weeks at -20ºC to reach sufficient decay of radioactivity to be analyzed.

The TT$_4$ serum concentration was determined with a commercially available solid-phase, chemiluminescent competitive immunoassay (IMMULITE 2000 Canine Total T$_4$, Siemens, Deerfield, IL, USA) previously validated in dogs, and the reference range used was 6.45-43.86 nmol/L. Basal TSH serum concentration was determined with a commercially available solid-phase, two-site chemiluminescent immunometric
assay (IMMULITE 2000 Canine TSH, Siemens, Deerfield, IL, USA) previously validated in dogs, and the reference range used was <0.5 ng/mL.

rhTSH serum concentrations were measured with a commercially available chemiluminescent microparticle immunoassay for human TSH determination in an immunoassay analyzer (Abbott ARCHITECT i2000SR, Abbott Laboratories, Abbott Park, IL, USA).

**Recombinant human TSH**

Each vial of 900 µg of rhTSH was reconstituted with 4.5 mL of sterile water (200 µg/mL). Individual doses of 100 µg of freshly reconstituted rhTSH were prepared in 1 mL plastic syringes with needle and rubber caps and were stored frozen at -20º C for a maximum of 12 weeks. For TSH stimulation, frozen rhTSH was thawed at room temperature a few minutes before administration.

**RAIU**

Each dog received 37 MBq (1 mCi) of $^{123}$I IV. The injected activity of $^{123}$I was calculated by subtracting the activity of the empty syringe from the activity of the full syringe both measured in a dose calibrator. To determine the tumor/metastases RAIU, a static planar ventrodorsal image was obtained with a one head γ-camera (Toshiba GCA 901) using a low-energy high resolution collimator with the dog in sternal recumbency under general anesthesia. General anesthesia was induced with propofol and maintained with isoflurane vaporized in oxygen using a rebreathing system. Data were acquired during 5 minutes for the 8h-RAIU and 10 minutes for the 24h-RAIU on a 128x128 matrix. A syringe with a known amount of radioactivity (2.5 ± 1.6 MBq) was placed next to the animal and served as the standard activity necessary to calculate the RAIU. Regions of interest (ROI) were manually drawn over the primary tumor/metastases and over the activity of the standard. In order to correct for background activity a ROI with the same dimensions as the ROI over the tumor was drawn over an area close to, but not overlapping, the thyroid tumor (soft tissue background correction) and another ROI with the same dimensions of the ROI over the standard was placed outside the dog (room background correction). The total number of counts in each ROI was recorded and transformed to counts per minute (cpm) for
RAIU calculation, yielding $cpm_{tumor}$, $cpm_{standard}$, $cpm_{background}$ and $cpm_{room}$. These ROI’s were placed on one day and by the same person (EV). RAIU was calculated as a percentage of the administered dose of $^{123}$I corrected for physical decay and background activity using the following formula:

$\left(\frac{(cpm_{tumor} - cpm_{background})}{(cpm_{standard} - cpm_{room})}\right) \times \left(\frac{MBq_{standard}}{MBq_{injected~activity}}\right) \times 100$

**Statistical analysis**

Data were analyzed with SAS version 9.1 (SAS, Cary, North Carolina, USA). The effect of rhTSH administration on RAIU was analyzed with a mixed model with period, treatment, time and the interaction between treatment and time as categorical fixed effects and dog and the period by dog interaction as random effects. Comparisons were based on the F-test at a global significance level of 5%, using Tukey's procedure for multiple comparisons.

The change of TT$_4$ serum concentration from baseline to 6 h was analyzed using a mixed model with dog as random effect and time as a categorical fixed effect.

Possible associations between the effect of rhTSH on tumor RAIU (at either 8h or 24h) and rhTSH serum concentration at all time points and results of TSH stimulation were evaluated with the Kendall’s τ correlation coefficient.

The association between the effect of rhTSH on tumor RAIU (at either 8h or 24h) and thyroid function status (euthyroid vs hyperthyroid) was evaluated on a data set excluding the one hypothyroid patient that was in the data set. The analysis was based on a mixed model with period, treatment, time, status and the interactions between treatment, time and status as categorical fixed effects and the dog and the period by dog interaction as random effects.
RESULTS

Two mixed breed dogs, 1 medium-sized Poodle, 1 American Staffordshire Terrier, 1 German Longhaired Pointer, 1 Jack Russell Terrier, 1 Bearded Collie, 1 Belgian Shepherd Malinois and 1 Beagle were included in this study. Six dogs were males, 3 were females, mean age was 9.5 years (range 6 – 12 years).

Six dogs were diagnosed with unilateral thyroid tumors, 1 dog had bilateral thyroid tumors and 2 dogs had ectopic tumors. Three patients were diagnosed with thoracic metastases visible at scintigraphy (n=2) and radiographs (n=1). Histopathology was performed in 6 patients. The only patient for which cytology or histopathology were not performed was diagnosed with an ectopic thyroid tumor clearly visible at scintigraphy. Furthermore, the dog had clinical hyperthyroidism.

Five dogs were euthyroid, 3 dogs were hyperthyroid and 1 dog was hypothyroid. All patients presented for a palpable cervical mass. Additionally, the 3 hyperthyroid dogs presented with PU/PD and weight loss; two of these also presented polyphagia. The hypothyroid dog had been diagnosed several years prior to referral and was being treated with levothyroxine supplementation. One dog had been previously diagnosed and treated for hypothyroidism but was reclassified as euthyroid based on basal TT4 serum concentrations, cervical scintigraphy and TSH stimulation results at the moment of inclusion in our study. In both cases, levothyroxine supplementation was interrupted for at least 3 days before the study week. Four patients were diagnosed with stage II, 2 patients with stage III and 3 patients with stage IV thyroid cancer.

The results of thyroid function status, tumor histopathology and tumor RAIU determination of each dog are summarized in Table 1. After rhTSH administration, the 8h-RAIU increased in 5 tumors and decreased in 5; the 24h-RAIU increased in 4 tumors and decreased in 6. Overall, rhTSH caused no statistically significant change on primary thyroid tumor RAIU at 8 h (P=0.89) or at 24 h (P=0.98) (Fig. 1). The RAIU of thoracic metastases could only be evaluated in 2 of the 3 patients with stage IV disease. After rhTSH administration, the 8h- and 24h-RAIU increased in 1 thoracic metastasis (Table 2).
rhTSH & $^{123}$I uptake in dogs with thyroid tumors

**Table 1.** Thyroid function status, tumor histopathology, primary tumor RAIU and cross-over group in 9 dogs with thyroid tumors. The mean and standard deviation of the 8h- and 24h-RAIU with and without rhTSH stimulation are given.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Thyroid function</th>
<th>Histology</th>
<th>8h RAIU (%)</th>
<th>8h-rhTSH RAIU (%)</th>
<th>24h RAIU (%)</th>
<th>24h-rhTSH RAIU (%)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Euthyroid</td>
<td>FTCc</td>
<td>8.5</td>
<td>7.8</td>
<td>17.4</td>
<td>15.7</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Euthyroid</td>
<td>FTCc</td>
<td>0.2</td>
<td>0.8</td>
<td>0.2</td>
<td>0.6</td>
<td>A</td>
</tr>
<tr>
<td>3</td>
<td>Euthyroid</td>
<td>FTCfp</td>
<td>3.1</td>
<td>3.7</td>
<td>4.9</td>
<td>8.3</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>Hyperthyroid</td>
<td>FTCc</td>
<td>10.6</td>
<td>16.2</td>
<td>27.2</td>
<td>14.8</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>Hyperthyroid</td>
<td>FTCfc</td>
<td>8.9</td>
<td>7.0</td>
<td>10.7</td>
<td>9.9</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>Euthyroid</td>
<td>NA</td>
<td>3.4</td>
<td>9.3</td>
<td>5.6</td>
<td>13.7</td>
<td>A</td>
</tr>
<tr>
<td>7</td>
<td>Hyperthyroid</td>
<td>NA</td>
<td>29.6</td>
<td>29.5</td>
<td>31.1</td>
<td>28.3</td>
<td>B</td>
</tr>
<tr>
<td>8</td>
<td>Euthyroid</td>
<td>MTC</td>
<td>0.7</td>
<td>2.0</td>
<td>0.9</td>
<td>2.8</td>
<td>B</td>
</tr>
<tr>
<td>9R</td>
<td>Hypothyroid</td>
<td>NA</td>
<td>2.1</td>
<td>1.7</td>
<td>1.8</td>
<td>1.6</td>
<td>B</td>
</tr>
<tr>
<td>9L</td>
<td>Hypothyroid</td>
<td>NA</td>
<td>0.8</td>
<td>0.4</td>
<td>0.7</td>
<td>0.5</td>
<td>B</td>
</tr>
</tbody>
</table>

**Abbreviations:** FTCc, follicular cell thyroid carcinoma of compact type; FTCfc, follicular cell thyroid carcinoma of follicular-compact type; FTCfp, follicular cell thyroid carcinoma of follicular-papillary type; MTC, medullary (C cell) thyroid carcinoma; NA, not available; R, right; L, left

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**Fig. 1.** Primary thyroid tumor RAIU in 9 dogs with thyroid tumors. The mean and standard deviation of the 8h- and 24h-RAIU with and without rhTSH stimulation are given.
Table 2. Thyroid function status, metastases RAIU and cross-over group in 2 dogs with thoracic metastases.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Thyroid function</th>
<th>8h RAIU (%)</th>
<th>8h-rhTSH RAIU (%)</th>
<th>24h RAIU (%)</th>
<th>24h-rhTSH RAIU (%)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 – thorax</td>
<td>Euthyroid</td>
<td>0.05</td>
<td>0.15</td>
<td>0.04</td>
<td>0.09</td>
<td>A</td>
</tr>
<tr>
<td>8 – thorax</td>
<td>Euthyroid</td>
<td>0.03</td>
<td>0.01</td>
<td>0.06</td>
<td>0.01</td>
<td>B</td>
</tr>
</tbody>
</table>

Table 3. rhTSH and TT₄ serum concentrations in dogs with thyroid tumors before and after injection of 100 μg rhTSH IV. The mean values (standard deviation) of rhTSH serum concentrations of 6 dogs and TT₄ serum concentrations of 4 euthyroid dogs with thyroid tumors are given for each time point.

<table>
<thead>
<tr>
<th>Time point</th>
<th>rhTSH (mIU/L)</th>
<th>TT₄ (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>0 (0)</td>
<td>24.2 (6.45)</td>
</tr>
<tr>
<td>6 h</td>
<td>26.97 (4.47)</td>
<td>50.97 (7.03)</td>
</tr>
<tr>
<td>12h</td>
<td>9.52 (1.45)</td>
<td>35.79 (10.47)</td>
</tr>
<tr>
<td>24 h</td>
<td>3.30 (0.63)</td>
<td>25.83 (9.1)</td>
</tr>
<tr>
<td>48 h</td>
<td>0.77 (0.27)</td>
<td>22.28 (6.94)</td>
</tr>
</tbody>
</table>

TT₄ and rhTSH serum concentrations were measured immediately before and followed up after rhTSH injection in 4 euthyroid dogs, 1 hyperthyroid dog and 1 hypothyroid dog. The 4 euthyroid patients showed a significant increase in TT₄ serum concentrations 6 h after rhTSH injection compared to baseline (P=0.01) (Table 3). The hyperthyroid dog and the hypothyroid dog did not show meaningful changes in serum TT₄ concentrations at any time point.

A significant positive correlation was found between the effect of rhTSH on tumor 8h-RAIU and rhTSH serum concentrations at 6 h (τ=0.68; P=0.03), at 12 h (τ=0.68; P=0.03) and at 24 h (τ=0.78; P =0.02) after rhTSH injection. When tumor metastases were included in the analysis, a significant positive correlation was also detected between the effect of rhTSH on 24h-RAIU and rhTSH serum concentrations at 6 h (τ=0.59; P=0.04), 24 h (τ=0.59; P=0.04) and 48 h (τ=0.67; P=0.02). No significant correlation was found between the change in TT₄ serum concentrations from
baseline to 6 h and the effect of rhTSH on tumor RAIU, at either 8 h ($P=0.54$) or 24 h ($P=0.13$).

Hyperthyroid dogs had significantly higher RAIU values than euthyroid dogs ($P=0.01$). Furthermore, the effect of the rhTSH on 24h-RAIU differed significantly between euthyroid and hyperthyroid dogs ($P=0.02$) but not at 8 h ($P=0.98$). In euthyroid dogs, the 24h-RAIU increased from 5.8%, at baseline, to 8.2%, after rhTSH. In hyperthyroid dogs, the 24h-RAIU decreased from 23%, at baseline, to 17.7%, after rhTSH.

No adverse effects were observed following the administration of rhTSH.
Thyroid cancer is the most common endocrine neoplasia in dogs and, as in humans, these tumors are mainly of follicular cell origin. In humans, rhTSH stimulates the uptake of $^{131}$I in patients with differentiated thyroid carcinoma and can be used to improve the efficacy of $^{131}$I therapy. Additionally, the use of rhTSH before $^{131}$I therapy is associated with a lower $^{131}$I effective half-life and, consequently, lower exposure of blood and the whole-body to radiation. This limits radiotoxicity without compromising the efficacy of the treatment. The obvious benefits of the use of rhTSH to optimize the treatment of human thyroid cancer with $^{131}$I provide an interesting perspective for the optimization of $^{131}$I therapy of canine thyroid tumors. Increased $^{131}$I uptake by canine thyroid tumors may improve treatment efficacy and decrease the need for multiple treatments; reduced blood exposure to radiation may limit myelosuppression; $^{131}$I dose reduction could improve radioprotection, reduce the hospitalization period and costs. This is the first study to evaluate the effect of rhTSH on radioiodine uptake in dogs with thyroid tumors.

Overall, no significant effect of rhTSH on tumor RAIU was observed with our protocol. These results are in agreement with the results of a recent pilot study performed by our group in healthy Beagles. In that study, rhTSH (100 μg IV, administered 24 h or 48 h before $^{123}$I) also did not cause a significant change in thyroid RAIU. Earlier reports have suggested the potential of exogenous TSH to increase thyroid RAIU in dogs. However, in these studies the effect of TSH stimulation on thyroid $^{131}$I uptake was described in a small number of healthy and hypophysectomized dogs and no statistical analysis was performed. In healthy humans, TSH stimulation with a protocol similar to the one used in this study was shown to approximately double thyroid RAIU.

The inconsistent effect of rhTSH on thyroid tumor RAIU observed in our study raises important issues regarding the dosage, the route and timing of rhTSH administration. The significant increase in TT$_4$ serum concentrations 6 h after rhTSH injection, observed in euthyroid patients, was expected and confirmed the biological activity of rhTSH. The observed correlation between the effect of rhTSH on tumor...
RAIU and rhTSH serum concentrations suggests that higher plasma concentrations of rhTSH may allow an increased uptake of $^{123}$I by thyroid tumors. The plasma concentration of rhTSH at different time points is mainly related to the dose administered. It is possible that doses higher than 100 μg may induce a more consistent increase of thyroid tumor RAIU. In humans, high plasma concentrations of TSH (>30 mIU/mL) are deemed necessary to stimulate sodium-iodide symporters to concentrate iodine, in normal and neoplastic thyroid tissue. Hence, high doses of rhTSH (2x900μg IM 24 h apart) are administered 24 h before $^{131}$I injection for diagnostic follow-up and treatment of differentiated thyroid cancer. Nevertheless, the optimal magnitude of TSH elevation is unknown and differs among patients. It is interesting to note that, in our study, all patients with rhTSH serum concentrations > 30 mIU/mL 6 h after rhTSH injection experienced an increase in thyroid tumor RAIU after rhTSH. The administration of rhTSH doses similar to those given to humans with thyroid cancer is not realistic in the veterinary clinical setting given the high cost of rhTSH. Furthermore, studies in humans with multinodular goiter have demonstrated that rhTSH doses as low as 5 or 100 μg suffice to effectively increase thyroid RAIU. In our study, the dosage of 100 μg was chosen because this dose is considered appropriate for a functional stimulation of the thyroid gland in most dogs. Further studies are necessary to determine the effect of higher doses of rhTSH on thyroid tumor RAIU in dogs.

An important factor influencing the pharmacokinetics of rhTSH is the route of administration. In our study, the IV route was chosen to maximize bioavailability. Although there are no reports arguing that the IM route is preferable, it is possible that if rhTSH is administered IM, such as in humans, its clearance is slower allowing a longer stimulation of the thyroid cells and possibly increasing tumor RAIU more consistently.

In our study, rhTSH was administered 24 h before $^{123}$I because studies on FRTL-5 cells (Fischer rat thyroid cell line) revealed that 12 to 24 h are needed before TSH stimulates accumulation of iodine in thyroid cells and because this is considered the optimal timing to increase thyroid RAIU in humans. The optimal timing of
rhTSH administration to increase thyroid RAIU in dogs with thyroid tumors is currently unknown.

The inconsistent effect of rhTSH on thyroid tumor RAIU may not be related to the protocol of rhTSH administration but rather to intrinsic properties of the neoplastic thyroid tissue. A previous study has shown that the concentration and affinity of TSH receptors in neoplastic canine thyroid cells is variable. In that study, of 22 primary canine thyroid tumors were shown to have fewer TSH-binding sites than the lowest value observed in normal thyroid tissues, suggesting that TSH receptor concentration could be related to the functional variability of thyroid neoplasms. Likewise, studies in humans have demonstrated that the expression of TSH-receptor mRNA may be decreased in thyroid carcinomas. Additionally, in vitro studies have revealed that TSH unresponsiveness in human thyroid carcinomas can also be related to defects in TSH signal transduction or errors in iodine transport.

As expected, our study showed that hyperthyroid dogs had significantly higher RAIU values than euthyroid dogs. In hyperthyroidism, increased thyroid function enhances iodine trapping and organification. Hence, dogs with thyroid tumors and hyperthyroidism frequently present high tumoral uptake and are often ideal candidates for 131I therapy. The significantly different effect of rhTSH on thyroid RAIU between euthyroid and hyperthyroid patients was an interesting finding. In a study of 55 dogs with thyroid tumors, dogs with evidence of autonomous hyperfunction of the goiter had an increased thyroidal iodine turn-over. It is possible that a positive effect of rhTSH on tumor RAIU occurs sooner in hyperthyroid patients and was, therefore, not observed with our protocol (RAIU determination 8 h and 24 h after 123I injection). On the other hand, the lack of effect of rhTSH in hyperthyroid patients may be caused by decreased thyroid functional reserve. This seems, however, less likely because in hyperthyroid cats and in humans with toxic nodular goiter (a condition characterized by nodular enlargement of the thyroid gland and hyperthyroidism), a significant increase in thyroid RAIU is observed after rhTSH administration.

The administration of rhTSH to patients with thyroid carcinoma raises important safety issues. In humans, rhTSH causes expansion of primary thyroid tumors and thyroid tumor metastases. Therefore, rhTSH should be used carefully in
patients with large thyroid tumors or central nervous system, spinal, lung or bone metastases. A pilot study performed in healthy Beagles showed no effect of rhTSH on thyroid gland volume.\textsuperscript{226} Likewise, no adverse effects of rhTSH were observed during our study.

All thyroid tumors for which histopathology was available were malignant and 5 of the 6 tumors were of follicular cell origin. This was expected as 90\% of canine thyroid tumors are malignant and only patients with \textsuperscript{123}I uptake were included.\textsuperscript{44} In humans and dogs, malignant thyroid tumors are predominantly of follicular cell origin, but in humans only 8.1-14.8\% of all thyroid nodules are malignant.\textsuperscript{227} Another important difference resides in the predominant histologic types. In dogs, thyroid carcinomas are predominantly mixed follicular-compact, while in humans 80\% of thyroid malignancies are of papillary type which is rare in dogs.\textsuperscript{10,85} Undifferentiated carcinomas are relatively uncommon in both species, accounting for 2\% of thyroid malignancies in humans and 12\% in dogs.

One dog with a C-cell carcinoma presented \textsuperscript{123}I uptake by the primary tumor and thoracic metastases. To the authors knowledge, there is only one previous report in veterinary medicine and very limited reports in human medicine of medullary carcinomas exhibiting iodine uptake.\textsuperscript{52,228,229} The mechanism underlying the ability of medullary carcinoma cells to trap iodine remains unclear.

In conclusion, our study shows that 100 μg rhTSH administered IV 24 h before \textsuperscript{123}I has no significant effect on thyroid tumor RAIU in dogs. The detected correlation between increased tumor RAIU and rhTSH serum concentrations attained after injection suggests that higher dosages of rhTSH may be necessary. Further studies are needed to determine the optimal protocol of rhTSH administration to increase thyroid tumor RAIU in dogs.
Chapter 10

GENERAL DISCUSSION
Thyroid neoplasia is a common form of endocrine cancer in dogs. Almost all thyroid tumors detected clinically are carcinomas and many patients present with pulmonary metastases at the moment of diagnosis. Approximately 2/3 of canine thyroid carcinomas are of follicular cell origin and 1/3 are of medullary origin. In the absence of metastatic disease, dogs with freely moveable tumors can be treated with thyroidectomy alone while dogs with invasive unresectable tumors can be treated with external beam radiation or $^{131}$I. However, 40-50% of dogs may experience local recurrence or metastatic disease after treatment. Research on genetic alterations, prognostic markers, therapeutic targets, and treatment optimization of this disease is lacking. The aim of this PhD research was to provide new insights into the *pathogenesis and treatment of canine thyroid tumors.*
Chapter 10

1 PATHOGENESIS

To present date, research on the genetic events leading to canine thyroid cancer remains scarce. The major advances recently made in understanding the molecular pathogenesis of human thyroid cancer led to the discovery of new diagnostic tools and to the development of innovative treatments. In humans, the PI3K/Akt pathway is the major signaling pathway involved in follicular thyroid carcinoma and genetic alterations (eg, mutations, gene amplification) in the effectors of this pathway are frequently observed. Research on the molecular pathogenesis of canine thyroid cancer will help understand the process of thyroid gland tumorigenesis in dogs and also aid the development of new treatment strategies in the future.

1.1 Follicular cell and medullary thyroid cancer

In our research, the differentiation between follicular cell and medullary origin of thyroid cancer was based on IHC for calcitonin, in accordance with the WHO classification of canine thyroid tumors. Calcitonin has been shown to be the most sensitive marker for identification of canine MTC. In the investigations described in chapters 3, 4 and 5, 27% of canine thyroid tumors were MTCs in agreement with previous studies reporting a prevalence of 16-36%. In dogs, MTC may be difficult to distinguish from compact FTC by microscopic observation alone and earlier studies lacking IHC likely underestimated its prevalence. In fact, 18 of 20 MTCs in our research were provisorily classified as compact FTC and 2 as follicular-compact FTC prior to IHC for calcitonin. This underlines the importance of routine IHC for identification of canine MTC. The fact that in our research mRNA expression of CALCA did not overlap between FTCs and MTCs supports the accuracy of tumor classification based on IHC.

The potential value of lifelong levothyroxine therapy to suppress endogenous TSH (TSH-suppressive therapy) in FTC is an important reason to routinely differentiate canine FTC from MTC with IHC. The differences observed in mRNA expression of PI3K/Akt related genes (chapter 3) and especially in expression of
potential therapeutic targets (chapter 5), further strengthen the need for differentiation of thyroid cancer origin in dogs.

The definitive diagnosis of canine thyroid cancer is established by histopathologic examination. When excisional or incisional biopsy samples are available, IHC is a simple, easy and fast technique to obtain confirmation of the cellular origin of thyroid cancer. However, in patients with unresectable tumors, clinicians often favor a presumptive diagnosis obtained with cytology or scintigraphy due to potential bleeding complications of biopsying such highly vascularized tumors. In the future, immunocytochemistry may help circumvent this limitation.

1.2 Genetic events

In the study reported in chapter 3, we investigated known mutational hotspots and mRNA expression of genes commonly involved in human thyroid gland tumorigenesis, in 43 canine FTCs and 16 canine MTCs. Activating missense mutations in K-RAS were found in 1 FTC and 1 MTC. These mutations are reported in human thyroid cancer with similar prevalence and are likely to play a relevant role in canine thyroid gland tumorigenesis. The overall low prevalence of RAS mutations in our research is in agreement with a large veterinary study investigating mutational hotspots of RAS (H, N, K) in a large series of canine tumors. In that study, only 1 mutation in H-RAS was found in a canine melanoma.

The absence of amino acid changing mutations in the sequenced regions of H-RAS, N-RAS, BRAF, PIK3CA, RET and in the entire coding region of PTEN, demonstrates that the mutations most commonly involved in human thyroid cancer are rare and do not play a major role in the pathogenesis of canine thyroid carcinoma. Further research is needed to investigate the main genetic events leading to canine thyroid cancer.

The fact that we could not identify mutations frequently associated with canine thyroid carcinoma shows the limitations of the candidate gene approach chosen in our research. Sequencing mutational hotspots in candidate genes is a simple, quick and relatively cost-effective approach to investigate genetic alterations in target regions of the genome. In chapter 3, the candidate genes and target exons were selected based
on mutational hotspots of human thyroid cancer. An alternative approach would be to select candidate genes based on signaling pathways found to be activated in canine thyroid cancer, followed by sequencing of the entire coding region of all genes involved in the activated pathway(s). Additional and broader approaches to investigate the genetic pathogenesis of cancer include gene expression profiling, genome wide association studies (GWAS), next-generation sequencing and whole exome sequencing. However, these comprehensive approaches are more complex, take longer to analyze and involve much higher costs.

In humans, gene amplification also plays an important role in thyroid gland tumorigenesis, and this is particularly the case for genes involved in the PI3K/Akt signaling pathway. Commonly amplified genes in human follicular thyroid carcinoma include EGFR, VEGFR-1, VEGFR-2, PIK3CA, PIK3CB, AKT1, AKT2 and PDPK1.

In the investigation described in chapter 3 we demonstrate increased mRNA expression levels of VEGFR-1, VEGFR-2, PDPK-1, AKT1 and AKT2 in canine FTC and increased mRNA expression levels of VEGFR-1, EGFR and PIK3CA in canine MTC. The distinct mRNA expression profiles of FTC and MTC suggests these tumors probably arise from different molecular mechanisms. Moreover, the increased mRNA expression of the above mentioned genes indicates the involvement of the PI3K/Akt pathway in the pathogenesis of canine thyroid carcinoma and suggests this pathway may be activated. A recent study in canine FTC had already showed mRNA and protein expression of VEGFR-2 and phosphorylation (activation) of EGFR and RET, which are known to signal through the PI3K/Akt and MAPK pathways. Further research is needed to investigate if gene amplification or altered promoter activity is responsible for the increased mRNA expression of the genes found to be overexpressed in our investigation.

Despite the overexpression of many PI3K/Akt related genes, the relative expression of COX-2 was not increased in canine thyroid tumors. Similar findings have been reported in follicular thyroid carcinoma in humans, where PI3K/Akt pathway activation is of major importance. This suggests that mRNA expression of COX-2 may not reflect activation of PI3K/Akt signaling in thyroid cancer. Interestingly, our
immunohistochemical findings in chapter 5 clearly show expression of Cox-2 in 13% of FTCs and 50% of MTCs.

PI3K/Akt pathway activation has been recently demonstrated in several of canine cancer cell lines, including canine lymphoma, hemangiosarcoma, mammary carcinoma, glioma and mastocytoma. The decreased tumor cell viability observed with PI3K and Akt inhibitors suggests that this signaling pathway is a promising therapeutic target in canine cancer.

Knowledge on the pathogenesis and biological behavior of canine thyroid cancer can also be obtained studying the association between tumor features (clinical, pathological, immunohistochemical) and patient outcome. Prognostic markers help ascertain which tumor characteristics are associated with more aggressive phenotypes providing valuable information to owners and clinicians.

1.3 Prognostic markers

In the research detailed in chapter 4, we performed an exploratory analysis investigating clinical, pathological and immunohistochemical prognostic markers in 50 dogs with dFTC and 20 dogs with MTC. A subset of 44 dogs (28 dFTCs, 16 MTCs) treated with thyroidectomy was included in a survival analysis.

One of the main goals of this investigation was to ascertain if there is a prognostic difference between canine dFTC and MTC following thyroidectomy. A previous study suggested that canine MTC may have a less malignant biological behavior with a higher rate of complete surgical excision and lower incidence of metastases at diagnosis compared to FTC. In agreement with this report, we found that MTC was less likely to be locally invasive at diagnosis. However, we found no difference in the incidence of metastatic disease at diagnosis and more importantly, after thyroidectomy the outcome was comparable between dogs with dFTC and MTC.

In the study reported in chapter 4, the prevalence of hyperthyroidism in dogs with dFTC (28%) was similar to previous reports. It can be hypothesized that functional tumors (in dogs with hyperthyroidism or with preserved scintigraphic uptake) are more differentiated and, therefore, carry a better prognosis. However, patient thyroid function and tumor scintigraphic uptake had no significant effect on
outcome. These findings are in agreement with the study described in chapter 6, where hyperthyroidism also did not have a significant effect on survival of 23 dogs with thyroid tumors undergoing different treatment modalities. In humans, hyperfunctional thyroid carcinomas have a prevalence of only 1-2% and these scarce reports also suggest a comparable prognosis between hyperthyroid and euthyroid patients.234-236

Macroscopic vascular invasion was negatively associated with OS and was an independent negative predictor for DFS. This is in agreement with earlier reports and is not surprising given the massive degree of neoplastic vessel infiltration necessary for macroscopic observation.8 In humans with FTC, extensive vascular invasion is rare and is also reported to carry a poor prognosis.169

Histologic vascular invasion was negatively associated with TM and was an independent negative predictor for DFS. Our results are in agreement with an earlier study showing the prognostic value of histologic grade of malignancy in dogs with thyroid carcinoma.41 In that study, vascular invasion was one of the most important histologic criteria used for the overall grade of malignancy. In humans with FTC, histologic vascular invasion is also an independent predictor of cancer-related mortality.170 Our findings suggest that dogs with this histologic feature are at high risk for metastases after thyroidectomy and are, therefore, likely to benefit from intensive follow-up and adjunctive therapy. Further studies are needed to determine if post-operative adjunctive therapy (eg, 131I, chemotherapy, levothyroxine) can improve the outcome of these patients.

In humans, Ki-67 is associated with clinical stage and survival in both FTC and MTC.59,172 In our study, Ki-67 labeling index was positively associated with local invasiveness at diagnosis. However, our multivariate analysis showed that it was not independently associated with outcome following thyroidectomy. Although in humans loss of E-cadherin expression is also a negative prognosticator, we found no association between E-cadherin expression and local invasiveness/metastatic disease at diagnosis, or outcome following thyroidectomy.55

Human thyroid cancer is poorly responsive to chemotherapy and the few studies evaluating its use in dogs with thyroid tumors also showed modest results.237,238 Dogs with unresectable thyroid tumors had partial responses to doxorubicin and
cisplatin in 44-54% of cases however adjuvant chemotherapy after thyroidectomy did seem to improve survival.\textsuperscript{45-48} Chemotherapy is most effective against rapidly dividing tumor cells and in agreement with previous studies the overall mitotic index observed in our research (chapter 4) was very low.\textsuperscript{7,149,150} Together with our findings on P-gp expression (chapter 5), this may help to explain the moderate response of canine thyroid cancer to chemotherapy.

The newly identified prognostic factors provide relevant information and may help to adapt treatment and follow-up to patients’ risk. However, to present date no treatment has been shown to be effective for adjunctive therapy. Given the need to investigate new ways to optimize treatment of dogs with thyroid tumors, our following studies focused on new therapeutic targets (chapter 5), effect of levothyroxine therapy on patient survival (chapter 6) and on the safety and value of rhTSH to optimize \textsuperscript{123}I uptake (chapters 7, 8, 9).
2 TREATMENT OPTIMIZATION

It is important to investigate new treatment modalities for the large number of dogs with unresectable thyroid tumors or distant metastases. Molecular targeted therapy, TSH suppression and the use of rhTSH to optimize $^{131}$I uptake are examples of treatment strategies which have allowed a significant improvement of the prognosis of human thyroid cancer and have not yet been adequately investigated in dogs.

Research on the expression of therapeutic targets may lead to a better understanding of the pathogenesis of canine thyroid cancer and also to the development of new therapeutic approaches. The use of VEGF inhibitors, TKIs and Cox-2 inhibitors in human MTC are success examples of research in this field.

In humans, the benefit of TSH-suppressive therapy is well established for high-risk patients with dFTC and for the past years the authors recommend it routinely in the treatment of canine FTC. In a small clinical study in dogs with bilateral thyroid tumors undergoing thyroidectomy, levothyroxine therapy was associated with a longer survival time. However, larger studies are needed to confirm these results.

rhTSH stimulates the uptake of $^{131}$I in humans with dFTC and can be used to improve the efficacy of $^{131}$I therapy. Additionally, the use of rhTSH is associated with a lower $^{131}$I effective half-life and, consequently, lower exposure of blood and the whole-body to radiation, limiting radiation dose exposure. However, the use of rhTSH in dogs with thyroid cancer raises important safety issues because, in people, rhTSH causes expansion of primary thyroid tumors and thyroid tumor metastases. Before evaluating the effect of rhTSH on $^{131}$I uptake in dogs with thyroid tumors it is important to ensure its safety evaluating its influence on thyroid gland volume and thyroid RAIU in healthy dogs.
2.1 Therapeutic targets

In the study reported in chapter 5, the immunohistochemical expression of VEGF, p53, Cox-2 and P-gp was investigated in 54 canine FTCs and 20 canine MTCs. The high expression of VEGF in 80% of FTCs and all MTCs suggests VEGF may play an important role in the pathogenesis of these highly vascularized tumors. Consequently, the VEGF system seems to be an attractive target for the treatment of both FTC and MTC in dogs. In human thyroid carcinoma, expression of VEGF is also upregulated. Recently, VEGF-inhibitors (eg, cetuximab, thalidomide, lenalidomide) and TKIs that block VEGFR (eg, vandetanib, sunitinib) have shown promising antitumor activity in the treatment of radioiodine-refractory FTC, advanced MTC, and undifferentiated thyroid carcinoma in humans. In a preliminary study in dogs with solid tumors, a multitargeted TKI (toceranib phosphate) targeting VEGFR-2, induced partial remission in 4 of 15 dogs, and stable disease in 8 of the 15 dogs with thyroid carcinoma. It has been suggested that the clinical response of canine thyroid carcinomas to toceranib phosphate may result from inhibition of RTKs on tumor-supporting vasculature and stroma, rather than on tumor cells. Our results indicate that there is an overexpression of VEGF in these tumors capable of a paracrine stimulation of VEGF receptors on stromal endothelial cells.

In chapter 3 we demonstrate the increased expression of VEGFR-2 mRNA in canine FTC. Immunohistochemical expression of this receptor had already been shown in canine FTC cells. Combining these findings with the observed overexpression of VEGF in canine FTC (chapter 5), there is enough evidence to suggest that VEGF may also have an autocrine action on canine FTC cells, stimulating tumor cell growth. On the one hand, this autocrine stimulation could be partially responsible for the involvement of PI3K/Akt signaling pathway in canine FTC, observed in chapter 3. On the other hand, PI3K/Akt pathway signaling can also induce VEGF expression.

VEGF is the most important stimulator of angiogenesis in the thyroid gland and tumor angiogenesis can also be targeted with continuous low-dose (metronomic) chemotherapy. A preliminary study on the effect of metronomic chemotherapy with chlorambucil in different canine tumors showed complete remission in the only dog.
with thyroid carcinoma.\textsuperscript{61} This report is encouraging and larger studies are needed to further evaluate the value of metronomic chemotherapy in the treatment of canine thyroid tumors.

In our study, no tumor expressed p53 protein. A low prevalence of p53 expression was expected because \textit{p53} mutation only was found in 1 of 23 canine FTCs in a study examining part of the coding region of this gene.\textsuperscript{27} In humans, \textit{p53} mutations have been described in 40-62\% of undifferentiated thyroid carcinomas but only in 5-10\% of other thyroid carcinomas.\textsuperscript{26} Our research suggests that \textit{p53} tumor suppressor gene does not play a major role in canine thyroid gland tumorigenesis and does not seem to be a realistic therapeutic target for most cases of canine thyroid carcinoma.

In \textbf{chapter 5} we report Cox-2 expression in 50\% of MTCs and in 13\% of FTCs. The higher prevalence of Cox-2 expression in MTC is in agreement with reports in humans, where 26-41\% of FTCs and 75-82\% of MTCs have been shown to express Cox-2.\textsuperscript{184} In a study investigating Cox-2 expression in canine invasive transitional cell carcinoma of the urinary bladder, the percentage of positive tumor cells in each tumor ranged from 1 to 22\%, comparable to that found in our study.\textsuperscript{185} Interestingly, in that study no association was found between the level of Cox-2 immunolabeling and tumor remission with piroxicam. This suggests that clinical benefit may be observed even in cases of low Cox-2 expression. Our results suggest that Cox-2 is an interesting molecular target for the treatment of canine thyroid carcinoma, particularly MTC.

In contrast to our immunohistochemical findings, \textit{mRNA} expression levels of \textit{COX-2} were not increased in canine thyroid carcinoma (\textbf{chapter 3}). This discordance was an interesting finding and can have several explanations. Firstly, Cox-2 immunolabeling was heterogeneous and while immunohistochemistry allows the evaluation of protein expression a large section of the tumor, \textit{mRNA} expression is only analyzed in a small tumor fragment. Secondly, posttranscriptional factors (eg, rapid \textit{mRNA} degradation, increased protein half-life) are also likely to play a role. In a large study in human lung cancer it was demonstrated that a correlation between protein and \textit{mRNA} expression was only present in 21 of 98 genes.\textsuperscript{244} A similar lack of correlation between protein and \textit{mRNA} expression has also been reported for NIS in human thyroid nodules.\textsuperscript{245}
P-gp expression was observed in 7% of FTCs and 70% of MTCs which could explain multi-drug resistance in canine MTC. Literature on the expression of P-gp in human thyroid carcinoma is scarce. Human MTC is refractory to conventional chemotherapy yielding partial responses in only 10 to 20% of patients. Experimental evidence suggests that multi-drug resistance is one of the mechanisms responsible for this highly chemoresistant phenotype and that by targeting P-gp, chemoresistance can be reversed. Our study suggests that P-gp is an interesting molecular target for the treatment of canine MTC. Inhibition of P-gp with specific P-gp inhibitors (eg, verapamil) or TKIs could increase tumor sensitivity to chemotherapy and improve patient outcome. It must be kept in mind, however, that there is a multitude of other ABC transporters (eg, ABCC1, ABCG2) which can be expressed by cancer cells and that inhibition of P-gp alone, might not suffice to reverse multi-drug resistance.

The higher expression of Cox-2 and P-gp in MTC is in agreement with clinical and experimental studies in human thyroid cancer. In fact, there is evidence of a direct causal relationship between Cox-2 expression and P-gp regulation. Overexpression of Cox-2 leads to increased expression and function of P-gp in a dose-dependent manner and this effect can be blocked by specific Cox-2 inhibitors. In an in vivo model of human colorectal cancer, Cox-2 expression was correlated with chemoresistant phenotype, and the most tumor regression was achieved with a combination of Cox-2 inhibitors and chemotherapy. Although a direct correlation between P-gp and Cox-2 expression was not observed in our investigation, P-gp inhibition could represent an additional therapeutic effect of the use of Cox-2 inhibitors in dogs with MTC.

In short, the research expounded in chapter 5 suggested that the VEGF system is an attractive therapeutic target for canine FTC and MTC and that Cox-2 and P-gp are interesting molecular targets for the treatment of canine MTC. We pursued our research on treatment optimization investigating the effect of levothyroxine therapy and TSH suppression on survival of dogs with thyroid tumors.
2.2 Levothyroxine therapy

In the investigation described in chapter 6, we investigated the effect of levothyroxine therapy on outcome of 42 dogs with thyroid tumors undergoing different treatment modalities.

Our study failed to demonstrate that levothyroxine therapy and TSH suppression provide a survival benefit to dogs with thyroid tumors. This is in disagreement with a recent report in 15 dogs with bilateral thyroid tumors showing a significantly longer survival for the dogs that received levothyroxine therapy after thyroidectomy.\textsuperscript{48} The cause for the disparity of results between these studies is unclear. Although in our study the proportion of dogs with bilateral thyroid tumors was significantly higher in the group receiving levothyroxine, bilateral disease was not a prognostic factor.

It is possible that the lack of effect of levothyroxine therapy observed in our research is related to an insufficient power. Although our sample size is small, our study is the largest in veterinary medicine evaluating levothyroxine therapy in dogs with thyroid tumors. Thyroid cancer is not common in dogs and studies with a much larger number of patients are difficult.

It is also possible that the lack of clinical benefit of levothyroxine therapy in our research is due to an overall insufficient degree of TSH suppression in the patients receiving levothyroxine. On the one hand, TSH suppression as defined in our study could only be confirmed during follow-up in 5 of 21 patients receiving levothyroxine. On the other hand, the ideal target level of TSH suppression in dogs with thyroid tumors is not currently known. In humans, the ideal level of TSH suppression in patients with FTC is a topic of debate and, at present, it is recommended to adapt the target level of TSH suppression to the patients’ risk for tumor recurrence and mortality based on prognostic factors.\textsuperscript{83} However, the high sensitivity of human TSH assays allows targeting TSH concentrations to a level that is far below the detection limit of the current canine TSH assays. Further research is warranted to investigate if TSH-suppressive therapy is beneficial to dogs with FTC and to determine the adequate level of TSH suppression in these patients.
Our interest in levothyroxine therapy also resulted from its wide availability, relative low cost and potential benefit to all dogs with FTC, independently of the primary treatment modality. However, we also intended to investigate new ways to improve the treatment of dogs with unresectable thyroid tumors specifically. Hence, in our final investigations (chapters 7, 8, 9) we focused on the use of rhTSH to optimize \(^{131}\text{I}\) therapy.

2.3 Safety of rhTSH

In the study reported in chapter 7, rhTSH (100 µg) showed no significant effect on thyroid gland volume in healthy Beagles for 48h after injection. These results contrast with what has been found in healthy humans, where thyroid gland volume increased by 10% 48 hours after rhTSH (100 µg) injection.\(^{137}\) In another study in healthy humans, a higher dose (900 µg) of rhTSH led to an increase of 23% in thyroid gland volume at 24 hours and 35% at 48 hours.\(^{136}\) A dose effect could explain these different results. In our research, we used 100 µg of rhTSH because this dose is considered appropriate for stimulation of thyroid function in dogs weighing more than 20 kg.\(^{200}\) If the effect of rhTSH on thyroid gland volume is dose dependent, a volume change could be observed at higher doses.

Although our results cannot be extrapolated to dogs with thyroid carcinoma, this preliminary data does not suggest that rhTSH induces swelling or edema of the canine thyroid gland at the dosage used. Still, rhTSH must be used with caution in thyroid carcinoma patients, especially in dogs with bulky thyroid masses, respiratory or central nervous system metastases. Pretreatment with glucocorticoids should be considered if tumor expansion would lead to unacceptable complications.
2.4 Optimization of $^{131}$I therapy

After completing a pilot study in healthy Beagles (chapter 8) we investigated the effect of rhTSH (100 μg) on the uptake of $^{123}$I in 9 dogs with thyroid tumors (chapter 9).

In these studies, rhTSH (100 μg) caused no significant change in thyroid RAIU. Our results contrast with earlier reports suggesting that exogenous TSH can increase thyroid RAIU in dogs. However, in these studies the effect of TSH stimulation on thyroid $^{131}$I uptake was described in a small number of healthy and hypophysectomized dogs and no statistical analysis was performed. In a recent study, our group demonstrated that rhTSH can cause a small but statistical significant increase in thyroid RAIU in hyperthyroid cats. In healthy humans, TSH stimulation with a protocol similar to the one used in our research was shown to approximately double thyroid RAIU.

The significant increase in TT$_4$ plasma concentrations 6 h after rhTSH injection confirmed the biological activity of rhTSH in our investigations. Therefore, the inconsistent effect of rhTSH on thyroid tumor RAIU raises important questions regarding dosage, route and timing of rhTSH administration, which are already discussed in detail in chapter 9.

The observed correlation between the effect of rhTSH on thyroid tumor RAIU and rhTSH plasma concentrations suggests that higher doses of rhTSH may allow a more consistent increase thyroid tumor RAIU and it is interesting to note that all dogs achieving rhTSH plasma concentrations > 30 mIU/mL 6 h post-injection had an increased thyroid tumor RAIU with rhTSH. In humans, high doses of rhTSH (2x900μg IM 24 h apart) are used to stimulate NIS in normal and neoplastic thyroid tissue. However, the use of these dosages in dogs may not be realistic given the high costs involved. Further studies are necessary to evaluate if higher doses of rhTSH allow an increased radioiodine uptake in dogs with FTC.

The significantly different effect of rhTSH on thyroid tumor RAIU between euthyroid and hyperthyroid patients was an interesting finding. In a study of 55 dogs with thyroid tumors, dogs with evidence of autonomous hyperfunction of the tumor had
an increased thyroidal iodine turn-over. It is possible that a positive effect of rhTSH on tumor RAIU occurs sooner in hyperthyroid patients and was, therefore, not observed with our protocol (RAIU determination 8 h and 24 h after $^{123}$I). On the other hand, the lack of effect of rhTSH in hyperthyroid patients may be caused by decreased thyroid functional reserve. This seems, however, less likely because in hyperthyroid cats a significant increase in thyroid RAIU is observed after rhTSH administration. Future studies should evaluate the effect of rhTSH on thyroid tumor RAIU separately in euthyroid and hyperthyroid dogs because the timing of radioiodine injection and RAIU determination may need to be adjusted in the latter group.
3 CONCLUSIONS

The general aim of our research was to provide insight into the pathogenesis and treatment of canine thyroid tumors. For this purpose we investigated mutational hotspots, mRNA expression of PI3K/Akt pathway-related genes, prognostic markers, therapeutic targets, levothyroxine therapy and optimization $^{123}$I uptake with rhTSH.

The main conclusions of our research are:

1. **K-RAS** missense mutations were identified in 2% of FTCs and 6% of MTCs. The mutations most frequently associated with thyroid gland tumorigenesis in humans are rare in canine thyroid carcinoma.

2. mRNA expression levels of VEGFR-1, VEGFR-2, PDK-1, AKT1 and AKT2 were increased in canine FTC and those of VEGFR-1, EGFR and PIK3CA were increased in canine MTC, showing the involvement of the PI3K/Akt pathway in the pathogenesis of canine thyroid carcinoma, particularly in FTC.

3. The VEGF system is an attractive target for the treatment of both FTC and MTC in dogs; Cox-2 and P-gp seem to be interesting molecular targets for the treatment of canine MTC.

4. In dogs with surgically excised thyroid carcinoma, macroscopic or histologic evidence of vascular invasion are independent negative predictors for DFS. dFTC and MTC have comparable prognosis following thyroidectomy.

5. According to our investigation in a limited number of patients, levothyroxine therapy does not seem to improve survival of dogs with thyroid tumors.

6. rhTSH (100 μg) causes no significant change in thyroid gland volume, echogenicity or homogeneity in healthy dogs.

7. rhTSH (100 μg) administered IV 24 h before $^{123}$I has no significant effect on thyroid RAIU in dogs with thyroid tumors or in healthy dogs. The observed correlation between the effect of rhTSH on tumor RAIU and rhTSH plasma concentrations suggests that higher dosages of rhTSH may be necessary.
4 FUTURE DIRECTIONS

Our research described in chapter 3 identifies 2 K-RAS mutations likely to be involved in thyroid gland tumorigenesis in dogs and indicates that the PI3K/Akt signaling pathway is implicated in the pathogenesis of canine thyroid carcinoma, particularly in FTC. Further research is needed to clarify the genetic pathogenesis of canine thyroid cancer.

Gene amplification could be an important genetic alteration in canine thyroid cancer. To investigate if this genetic event is responsible for the increased mRNA expression levels of several genes observed in chapter 3, future studies should evaluate copy number gain in these genes. This can be accomplished performing qPCR in genomic DNA. It would also be interesting to completely sequence the candidate genes selected in our research to definitively rule out point mutations in these genes. Furthermore, to truly investigate PI3K/Akt and MAPK pathway activation, western blot or immunohistochemistry should be performed on frozen tumor samples to evaluated phosphorylation of pathway effectors. The complete coding region of the genes involved in the activated pathway(s) should then be sequenced to search for point mutations. Other important investigations include evaluation of gene rearrangement with fluorescent in-situ hybridization (FISH) and more comprehensive approaches such as GWAS, next-generation sequencing and whole exome sequencing.

In chapter 5 we suggest that the VEGF system is an attractive target for the treatment of both FTC and MTC in dogs, and that Cox-2 and P-gp are interesting molecular targets for the treatment of canine MTC. VEGFR-2 can be targeted with TKIs, Cox-2 can be targeted with Cox-2 inhibitors and P-gp can be targeted with specific P-gp inhibitors, Cox-2 inhibitors or TKIs. The clinical benefit of TKIs in canine FTC has already been shown. However, further research is needed to investigate the value of TKIs, Cox-2 inhibitors and specific P-gp inhibitors in the treatment of canine MTC.

Although we could not demonstrate a clinical benefit of levothyroxine therapy in dogs with thyroid tumors (chapter 6), the value of TSH suppression is well established in humans with high-risk dFTC. Prospective and randomized clinical
studies with follow-up of TSH levels are needed to investigate if TSH suppression is beneficial in dogs with thyroid tumors.

In humans, thyroid remnant ablation with $^{131}$I is a routine component of the treatment of FTC. $^{131}$I remnant ablation is associated with decreased recurrence and disease-specific mortality, and allows using Tg plasma concentrations during follow up as a very specific marker for tumor recurrence and metastatic disease.$^3$ Although this was not the focus of our research, future studies should evaluate the value of adjuvant $^{131}$I therapy after thyroidectomy in dogs with FTC.

In chapter 4, we show that macroscopic and histologic evidence of vascular invasion are independent negative predictors for DFS. Patients with these features seem to be at high risk for metastatic disease and death after thyroidectomy and are likely to benefit from intensive follow-up and additional therapy. Future studies on adjuvant treatment (e.g., $^{131}$I, chemotherapy, levothyroxine therapy) should probably focus on this high-risk group.

In the investigation described in chapters 9, rhTSH did not cause a significant increase thyroid tumor RAIU. However, the correlation observed between the effect of rhTSH on tumor RAIU and rhTSH plasma concentrations achieved after injection suggests that in future clinical studies higher dosages should be investigated. Additionally, repetitive stimulation with rhTSH (2x 24 h apart) could also be explored. Furthermore, the observed difference between the effect of rhTSH on tumor RAIU in euthyroid and hyperthyroid dogs suggests that future studies should probably evaluate these patients separately and that the timing of rhTSH administration and RAIU determination may need to be adjusted in the latter group due to increased thyroidal iodine turn-over in those patients.

In our research, we could not evaluate the effect of diet iodine content on thyroid RAIU because it could interfere with the effect of rhTSH. In human medicine, patient preparation for $^{131}$I therapy includes a low-iodine diet for 2 weeks.$^{247}$ Low-iodine diet increases thyroid RAIU in healthy dogs and may be a cost-effective additional tool to the use of rhTSH to optimize $^{131}$I uptake in dogs with thyroid tumors.$^{248}$ Clinical studies are needed to evaluate the effect of iodine-restricted diet on $^{131}$I uptake in dogs with thyroid tumors.
$^{131}$I therapy takes advantage of the unique iodide-handling machinery of thyroid follicular cells to trap and organify $^{131}$I. However, humans and dogs with thyroid cancer commonly present with iodine non-avid disease which is main cause of thyroid cancer-associated morbidity and mortality in humans.$^{249}$ The loss of the ability of thyroid cancer cells to take up and concentrate $^{131}$I results from an impaired expression of iodide-handling genes and aberrant localization of NIS.$^{250}$ Recent studies have shown that activation of the PI3K/Akt and MAPK signaling pathways is involved in the silencing of thyroid iodide-handling genes and that inhibitors of these signaling pathways can restore the ability of thyroid cancer cells to take up radioiodine.$^{250-252}$ Given that canine and human FTC are remarkably similar, the dog could potentially serve as a model for clinical research in the capability of these drugs to restore $^{131}$I uptake in patients with FTC.

Our research aimed to provide information on the molecular pathogenesis of canine thyroid cancer and identified valuable prognostic markers that can be used to tailor follow-up and adjunctive therapy to patients’ risk. The newly identified therapeutic targets are easy to detect with immunohistochemistry and open the door to personalized medicine in canine thyroid cancer. Although we could not demonstrate a survival benefit with levothyroxine therapy or an improved tumor $^{131}$I uptake with rhTSH, our findings form a base for further research. Future challenges include pursuing the main genetic events leading to canine thyroid cancer, exploring clinically the molecular targets identified in our research, investigating adjuvant therapy (eg, $^{131}$I, chemotherapy, TSH suppression) with randomized prospective studies and continuing searching for ways to optimize $^{131}$I therapy.
In the past decade, major advances have been made in unveiling the molecular pathogenesis of human thyroid cancer. The PI3K/Akt pathway is the major signaling pathway involved in human follicular thyroid carcinoma which is remarkably similar in terms of histology and biological behavior to canine differentiated follicular cell thyroid carcinoma (dFTC). The discovery of the main genetic events involved in thyroid gland tumorigenesis in humans led to the discovery of new molecular targets and to the development of innovative treatments. Furthermore, stratification of patient risk with prognostic markers, TSH-suppressive therapy and increased $^{131}$I uptake with rhTSH, have allowed a significant improvement of the treatment of human dFTC and provide an interesting perspective for optimization of treatment of canine dFTC.

The molecular pathogenesis and expression of therapeutic targets in canine thyroid cancer are largely unknown and, to present date, research on prognostic markers and treatment optimization remains scarce. Up to 38% of dogs with thyroid cancer have metastases at the time of diagnosis and almost half of dogs treated with thyroidectomy develop recurrent or metastatic disease within 2 years of surgery. Given the modest results of chemotherapy, it is imperative to investigate new ways to improve treatment. The general aim of this research was to provide new insights into the pathogenesis and treatment of canine thyroid cancer.

In our 2 first studies we focused on the pathogenesis of canine thyroid cancer investigating genetic alterations and prognostic markers. In our first study (chapter 3) we try to unveil the molecular pathogenesis of canine thyroid cancer by investigating mutational hotspots and mRNA expression of candidate genes in 43 canine follicular cell thyroid carcinomas (FTCs) and 16 canine medullary thyroid carcinomas (MTCs). Mutation analysis of known hotspots of $RAS$ ($H$, $K$, $H$), $PIK3CA$, $BRAF$, $RET$ and of the entire coding region of $PTEN$, revealed 2 activating missense mutations in $K$-$RAS$, also described in human thyroid cancer. A G12R substitution was present in 1 FTC and an E63K substitution was present in 1 MTC. No functional mutations were found in the sequenced regions of $H$-$RAS$, $N$-$RAS$, $PIK3CA$, $BRAF$, $RET$ and $PTEN$ demonstrating that the mutations most frequently associated with human thyroid neoplasia are rare in canine thyroid cancer.
Quantitative RT-PCR was performed for selected receptor tyrosine kinases (RTKs) (VEGFR-1, VEGFR-2, EGFR) and PI3K/Akt pathway members (PIK3CA, PIK3CB, PDPK1, PTEN, AKT1, AKT2, COX-2) known to be commonly amplified in human thyroid cancer. The mRNA expression levels of VEGFR-1, VEGFR-2, PDPK1, AKT1, and AKT2 were increased in FTC, and those of EGFR, VEGFR-1, and PIK3CA were increased in MTC when compared to normal thyroid. The increased mRNA expression of these genes indicates the involvement of the PI3K/Akt signaling pathway in the pathogenesis of canine thyroid cancer, particularly in FTC. Further research is necessary to investigate if gene amplification is responsible for the increased mRNA expression of these genes.

Given the lack of prognostic factors for dogs with operable thyroid tumors, in our second study (chapter 4) we investigated clinical, pathological and immunohistochemical prognostic factors in 50 dogs with dFTC and 20 dogs with MTC. In this retrospective study, IHC for calcitonin, Ki-67 and E-cadherin was performed in all tumor samples and tumor features (diameter, volume, localization, scintigraphic uptake, thyroid function, IHC) were correlated with local invasiveness and metastatic disease at diagnosis. Furthermore, 44 dogs (28 dFTCs, 16 MTCs; stage I-III) treated by thyroidectomy were included in a survival analysis. In agreement with a previous report, we found that MTC was significantly less likely to be locally invasive at diagnosis. However, we found no difference in the incidence of metastatic disease at the time of diagnosis and, more importantly, following thyroidectomy outcome was comparable between dogs with dFTC and MTC. Macroscopic and histologic vascular invasion were independent negative predictors for disease-free survival. In contrast with human reports, E-cadherin expression was not associated with outcome.

The newly identified prognostic factors provide relevant information for owners and clinicians and may help to adapt follow-up and adjunctive therapy to the patients’ risk. However, to present date no single treatment modality has been shown to be effective for adjunctive therapy. Given the need to investigate new ways to improve treatment our following studies focused on treatment optimization, namely on new therapeutic targets (chapter 5), effect of levothyroxine therapy on patient survival.
(chapter 6) and on the safety and value of rhTSH to optimize $^{123}$I uptake in dogs with thyroid tumors (chapters 7, 8, 9).

In the study reported in chapter 5 we investigated the expression of potential therapeutic targets in 54 canine FTCs and 20 canine MTCs. For this purpose, we performed IHC for vascular endothelial growth factor (VEGF), p53, cyclooxygenase-2 (Cox-2) and P-glycoprotein (P-gp) in all tumor samples. 80% of FTCs and all MTCs had a high percentage (76-100%) of neoplastic cells immunopositive for VEGF, suggesting it may play an important role in the pathogenesis of these highly vascularized tumors. Consequently, the VEGF system seems to be an attractive target for the treatment of both FTC and MTC in the dog. 13% of FTCs and 50% of MTCs expressed Cox-2, and 7% of FTCs and 70% of MTCs expressed P-gp, which suggests these could be interesting molecular targets for the treatment of canine MTC. No tumor was immunopositive for p53 expression.

Pursuing our research on treatment optimization (chapter 6), we investigated the effect of levothyroxine therapy and TSH suppression (TSH < 0.1 ng/mL) on survival of 42 dogs with thyroid tumors undergoing different treatment modalities. In this retrospective study, dogs were grouped according to treatment as follows: thyroidectomy with or without levothyroxine therapy (n=17); radioactive iodine-131 with or without levothyroxine therapy (n=11); no treatment or levothyroxine therapy alone (n=14). Although we could not demonstrate that levothyroxine therapy or TSH suppression improve patient survival, randomized clinical studies are needed.

rhTSH may allow a significant optimization of $^{131}$I therapy in dogs with thyroid cancer. However, if as in humans rhTSH leads to an increase in thyroid gland volume it must be used carefully in dogs with large thyroid tumors or distant metastases to avoid compression of key anatomical structures. Therefore, we first evaluated the short-term effect of rhTSH on thyroid gland volume and echogenicity, measured by ultrasonography, in 7 healthy Beagles (chapter 7). In this prospective blinded cross-over study, a single observer evaluated thyroid echogenicity, homogeneity, shape, capsule delineation, and measurement of thyroid length, width and height at baseline, and at 6, 24 and 48h after injection of rhTSH (100 µg IV) or placebo. rhTSH had no significant effect on thyroid gland volume, echogenicity,
homogeneity or capsule delineation and no adverse effects were noticed. Although these results could not be extrapolated to dogs with thyroid tumors, this preliminary data did not suggest that rhTSH induced swelling or edema of the canine thyroid gland at the dosage used.

After concluding a pilot study (chapter 8) on the effect of rhTSH on thyroid scintigraphy in healthy Beagles we investigated the effect of rhTSH on the uptake of $^{123}$I in 9 dogs with thyroid tumors (chapter 9). In this prospective cross-over study, rhTSH (100 μg) administered IV 24 h before $^{123}$I (37 MBq IV) caused no significant change on thyroid tumor radioactive iodine uptake (RAIU) at 8 h or at 24 h. Interestingly, a significant positive correlation was found between the effect of rhTSH on tumor 8h-RAIU and rhTSH serum concentrations 6 h, 12 h and 24 h after rhTSH administration, suggesting that higher dosages of rhTSH may be necessary. Further studies are needed to determine the best protocol of rhTSH administration to optimize thyroid tumor RAIU.

Our research starts to uncover the pathogenesis of canine thyroid cancer identifying 2 activating mutations in K-RAS and showing the involvement of the PI3K/Akt pathway, particularly in FTC. The prognostic markers found in our research provide relevant information to owners and clinicians, and could be used to adapt follow-up and adjunctive therapy to each patient’s risk. The newly identified therapeutic targets are easily detectable with immunohistochemistry and open the possibility of personalized medicine. Although we could not demonstrate an improved survival with levothyroxine therapy or an enhanced tumor $^{123}$I uptake with rhTSH, further research is necessary. Future challenges include pursuing the main genetic events leading to canine thyroid cancer, performing clinical trials to further explore the molecular targets identified in our research, and continuing to search for ways to optimize $^{131}$I therapy and improve patient outcome.
SAMENVATTING
De afgelopen tien jaar is er enorme vooruitgang geboekt in het ontrafelen van de moleculaire pathogenese van schildklierkanker bij de mens. Bij de humane folliculaire schildklierkanker, die op vlak van histologie en biologisch gedrag veel gelijkenissen vertoont met de gedifferentieerde folliculaire-cel schildklierkanker (dFTC) bij de hond, is de PI3K/Akt route de belangrijkste signaalweg. De ontdekking van de belangrijkste genetische processen in het ontstaan van humane schildkliertumoren ging gepaard met nieuwe moleculaire doelwitten en met de ontwikkeling van innovatieve behandelingen. Bovendien heeft een betere inschatting van het patiëntrisico met behulp van prognostische merkers, TSH-suppressieve therapie en toegenomen opname van $^{131}$I met behulp van rhTSH, gezorgd voor een duidelijke verbetering van de behandeling van humaan dFTC. Dit levert belangrijke nieuwe perspectieven ter optimalisatie van de behandeling van caniene FTC.

De moleculaire pathogenese en expressie van therapeutische doelwitten bij caniene schildkliertumoren zijn nog grotendeels onbekend en, tot op heden, is onderzoek naar prognostische merkers en optimalisatie van de behandeling schaars. Tot 38% van de honden met schildklierkanker heeft uitzaaingen op het tijdstip van diagnose en bijna de helft van de honden recidiveren of ontwikkelen uitzaaingen binnen de 2 jaar na thyroïdectomie. Omwille van de teleurstellende resultaten met chemotherapie, is verder onderzoek naar nieuwe mogelijkheden om de behandeling te verbeteren noodzakelijk.

De algemene doelstelling van dit onderzoek was het verkrijgen van inzichten in de pathogenese en behandeling van schildklierkanker bij de hond.

In de eerste 2 studies werd ingegaan op de pathogenese van caniene schildklierkanker door het onderzoeken van genetische veranderingen en prognostische merkers. De moleculaire pathogenese werd bestudeerd in onze eerste studie (hoofdstuk 3) door “hotspots” voor mutaties en mRNA expressie van kandidaat genen te onderzoeken bij 43 honden met FTC (73%) en 16 honden met medullaire schildklierkanker (MTC) (27%). Mutatie analyse in gekende hotspot's van RAS (H, K, H), PIK3CA, BRAF, RET en in de volledige coderende regio van PTEN, toonde 2 activerende missense mutaties in K-RAS, die ook beschreven zijn bij schildklierkanker bij de mens. Een G12R substitutie was aanwezig in 1 FTC, en een E63K substitutie was aanwezig in 1 MTC. Functionele mutaties werden niet gevonden in de regio’s van

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H-RAS, N-RAS, PIK3CA, BRAF, RET en PTEN die gesequenced werden. Dit toont aan dat mutaties die frequent voorkomen bij humane schildklierkanker eerder zeldzaam zijn bij caniene schildklierkanker. Kwantitatieve RT-PCR werd uitgevoerd voor een selectie van tyrosine kinase receptoren (VEGFR-1, VEGFR-2, EGFR) en onderdelen van de PI3K/Akt signaalweg (PIK3CA, PIK3CB, PDK1, PTEN, AKT1, AKT2, COX-2), waarvan de expressie toeneemt tgv gen amplificatie (toename van het aantal genen) bij humane schildklierkanker. In vergelijking met de normale schildklier was er een verhoogde mRNA expressie van VEGFR-1, VEGFR-2, PDK1, AKT1 en AKT2 in FTCs en van EGFR, VEGFR-1 en PIK3CA in MTCs. De verhoogde mRNA expressie van deze genen wijst op betrokkenheid van de PI3K/Akt signaalweg in de pathogenese van schildklierkanker bij de hond, voornamelijk bij FTCs. Verder onderzoek is noodzakelijk om na te gaan of gen amplificatie verantwoordelijk is voor deze verhoogde expressie.

Het gebrek aan prognostische merkers voor honden met operabele schildkliertumoren, leidde tot een tweede studie (hoofdstuk 4) waarin zowel klinische, pathologische als immunohistochemische (IHC) prognostische factoren bij 50 honden met dFTC en 20 honden met MTC werden onderzocht. In deze retrospectieve studie werd IHC voor calcitonine, Ki-67 en E-cadherine toegepast op alle tumor stalen. Verschillende tumor variabelen (diameter, volume, locatie, opname tijdens scintigrafie, schildklierfunctie, IHC) werden gecorreleerd aan het lokaal invasief karakter en de aanwezigheid van metastasen op het moment van diagnose. Daarnaast werd de overleving na thyroïdectomie geanalyseerd bij 44 honden (28 dFTCs, 16 MTCs, stage I-III). De MTCs waren significant minder lokaal invasief op het moment van diagnose, wat overeenkwam met een eerder verschenen studie. Er werd geen verschil waargenomen tussen dFTC en MTC in het initieel voorkomen van metastasen en, nog belangrijker, de uitkomst na thyroïdectomie was vergelijkbaar voor beide tumoren. Macroscopisch en histologisch vastgestelde vasculaire invasie waren onafhankelijke negatieve prognostische factoren voor ziekte-vrije overleving. In tegenstelling tot humane studies, was er geen associatie tussen E-cadherine expressie en prognose.

Deze nieuwe prognostische merkers verstrekken potentieel interessante informatie voor eigenaars en clinici omdat ze de mogelijkheid bieden om de opvolging
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en bijkomende behandeling aan te passem aan het risico van de patiënt. En moet wel gezegd worden dat tot op vandaag, er van geen enkele bijkomende behandeling een bijkomend effect kon aangetoond worden. Gezien de nood aan nieuwe therapeutische opties, concentreren onze volgende studies zich op de behandeling, met name op het bestuderen van nieuwe therapeutische doelwitten (hoofdstuk 5), van de invloed van levothyroxine toediening op de overleving van de patiënt (hoofdstuk 6) en van de veiligheid en waarde van rhTSH om de $^{123}$I opname bij honden met schildklierkanker te verbeteren (hoofdstukken 7, 8, 9).

In hoofdstuk 5 werd de expressie van potentiële therapeutische doelwitten bij 54 caniene FTCs en 20 caniene MTCs onderzocht. In deze tumor stalen werd IHC uitgevoerd voor “vascular endothelial growth factor” (VEGF), p53, cyclooxygenase-2 (Cox-2) en P-glycoproteïne (P-gp). 80% van de FTCs en alle MTCs toonde een zeer hoog percentage (76-100%) VEGF immunopositieve cellen, wat op een mogelijke rol voor VEGF in de pathogenese van deze sterk gevasculariseerde tumoren kan wijzen. Het VEGF systeem lijkt dan ook een interessant doelwit voor de behandeling van zowel FTCs als MTCs bij de hond. Cox-2 expressie werd waargenomen in 13% van de FTCs en 50% van de MTCs, en P-gp expressie in 7% van de FTCs en 70% van de MTCs wat suggereert dat dit aantrekkelijke moleculaire doelwitten kunnen zijn voor de behandeling van caniene MTCs. Geen enkele tumor was immunopositief voor p53.

Als verdere stap om de optimalisatie van de behandeling te onderzoeken (hoofdstuk 6) werd het effect van levothyroxine behandeling en TSH onderdrukking (TSH<0.1ng/mL) op de overleving bestudeerd bij 42 honden met schildkliertumoren die verschillende types behandeling ondergingen. In deze retrospectieve studie werden honden onderverdeeld in 3 groepen naargelang hun behandeling: thyroïdectomie met of zonder levothyroxine supplementatie (n=17); radiojood-131 ($^{131}$I) behandeling met of zonder aanvullende levothyroxine toediening (n=11); geen behandeling of enkel levothyroxine toediening (n=14). Een gunstig effect van levothyroxine toediening op de overleving kon niet worden aangetoond. Bijkomende prospectieve, gerandomiseerde studies zijn echter nodig om hierover definitief uitsluitse te geven.

In de volgende hoofdstukken werd nagegaan of het gebruik van rhTSH de $^{131}$I behandeling van honden met schildklierkanker kan optimalizeren. Indien rhTSH
toediening echter, net zoals bij mensen, leidt tot een toename in het schildkliervolume, is voorzichtigheid geboden bij honden met grote schildkliertumoren of met uitzaaiingen om druk op belangrijke anatomische structuren te vermijden. Daarom evalueerden we eerst, met behulp van echografie, het korte termijn effect van rhTSH op schildkliervolume en echogeniciteit bij 7 gezonde beagles (hoofdstuk 7). In deze prospectieve, geblindeerde, cross-over studie werden echogeniciteit, homogeniteit, vorm, aflijning van het kapsel, lengte, breedte en hoogte van de schildklier vóór en 6, 24 en 48h na injecteren van rhTHS (100 µg IV) beoordeeld door één enkele onderzoeker. rhTSH had in de gebruikte dosis geen significant effect op volume, echogeniciteit, homogeniteit of aflijning van het kapsel van de schildklier. Bovendien werden geen bijwerkingen waargenomen. Deze resultaten kunnen echter niet zomaar geëxtrapoleerd worden naar honden met schildkliertumoren.

Na het uitvoeren van een pilootstudie (hoofdstuk 8) naar het effect van rhTSH op scintigrafisch onderzoek van de schildklier bij 7 gezonde beagles, bestudeerden we het effect van rhTSH op de opname van radiojood-123 (123I) bij 9 honden met schildkliertumoren (hoofdstuk 9). Deze prospectieve cross-over studie toonde geen significant effect aan van IV toediening van 100 µg rhTSH 24u vóór de toediening van 123I (37 MBq IV) op de opname van radiojood (RAIU) in de schildkliertumor na 8 of 24h. Er werd wel een positieve correlatie gevonden tussen het effect van rhTSH op tumor 8u-RAIU en rhTSH serum concentraties 6, 12 en 24h na rhTSH injectie. Dit suggereert dat hogere rhTSH dosissen mogelijk wel een significant effect op tumor RAIU zouden hebben. Verdere studies zijn noodzakelijk om het beste protocol te bepalen om de tumor RAIU te optimaliseren met rhTSH.

Ons onderzoek is een belangrijke eerste stap naar het ontrafelen van de biologische basis van schildklierkanker bij de hond door middel van de identificatie van 2 activerende K-RAS mutaties en de aangetoonde betrokkenheid van de PI3K/Akt signaalweg, voornamelijk bij FTCs. De prognostische merkers die in onze studie gevonden werden, leveren belangrijke informatie aan eigenaars en clinici en kunnen een leiddraad zijn bij de opvolging en het instellen van bijkomende behandelingen in functie van het risico van de individuele patiënt. De nieuwe therapeutische doelwitten zijn eenvoudig aan te tonen met IHC, wat deuren opent naar “gepersonaliseerde”
diergeneeskunde. Hoewel een klinisch voordeel van levothyroxine behandeling en een invloed van rhTSH op de opname van $^{123}$I niet kon worden aangetoond, is verder onderzoek aangewezen. Toekomstige uitdagingen omvatten verder onderzoek naar de genetische processen die leiden tot schildklierkanker bij de hond, klinische onderzoeken gericht op de door ons geïdentificeerde moleculaire doelwitten die ook mogelijks nieuwe bijkomende therapeutische mogelijkheden creeren en het optimalizeren van $^{131}$I behandeling.


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CURRICULUM VITAE
Miguel Fonseca e Campos was born May 14, 1981 in Lisbon.

He studied veterinary medicine at the Technical University of Lisbon, obtaining his degree of Doctor in Veterinary Medicine with distinction in 2005. He then performed a one-year rotating internship at the Frégis Veterinary Hospital Center in France. In 2006 he started a residency in small animal internal medicine at the Faculty of Veterinary Medicine of Ghent University, becoming a diplomate of the European College of Veterinary Internal Medicine – Companion Animals (ECVIM-CA) specialty Internal Medicine in 2010.

After passing the specialty exam, he started his PhD on “Pathogenesis and Treatment of Canine Thyroid Tumors” at Ghent University, pursuing the research initiated during his residency. This research was supported by the Special Research Fund (BOF) of Ghent University, the Department of Medicine and Clinical Biology of Small Animals of Ghent University, the Clinical Studies Fund of ECVIM-CA and the Dutch Cancer Foundation for Animals (NKFD).

During his PhD Miguel attended several courses, including Laboratory Animal Science where he obtained the accreditation of FELASA category C (person responsible for directing animal experiments).

Miguel Campos is author and co-author of several publications in peer-reviewed international journals and he participated in various national and international conferences as delegate and speaker.
Publications in peer-reviewed international journals


Campos M, Baert A, Van de Maele I, Duchateau L, de Rooster H, Steblaj B, Peremans K, Daminet S. Effect of levothyroxine therapy on survival of dogs with thyroid tumors. *In preparation*


Research communications presented at international scientific meetings


Awarded the Royal Canin Resident & Research Award: second prize in fundamental research / PhD data


Awarded the Intervet/Schering-Plough Award 2009 for the best abstract presented from the European Society of Veterinary Endocrinology


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