Evaluation of Expression and Function of VEGFR2, PDGFRα, PDGFRβ, KIT, and RET in Canine Apocrine Gland of the Anal Sac Adenocarcinoma and Thyroid Carcinoma

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Bridget Karen Urie, BS, MA, DVM

Graduate Program in Comparative and Veterinary Medicine

The Ohio State University

2012

Thesis Committee:

Cheryl A. London, DVM, PhD, DACVIM, Co-Advisor

William C. Kisseberth, DVM, PhD, DACVIM, Co-Advisor

Ducan S. Russell, BMVS [Hons], DACVP
Abstract

Biological responses to toceranib phosphate have been reported in both canine apocrine gland anal sac adenocarcinomas (AGASACA) and thyroid carcinomas (TC). However, little is known about the molecular biology of either of these tumor types and the mechanism of the biological activity of toceranib. In the present study, we investigated the presence and expression of known targets of toceranib and interrogated AGASACA and TC samples for the expression of VEGFR2, PDGFRα/β, KIT and RET at both the message and protein level to begin to dissect the molecular basis for this observed activity.

Samples from 24 primary AGASACA, 11 with paired metastatic lymph nodes, and 15 TC were evaluated using reverse-transcriptase polymerase chain reaction for detection of mRNA, phosphoprotein arrays to screen for evidence of protein phosphorylation, and tissue microarrays for immunohistochemical evaluation of protein expression.

Messenger RNA from VEGFR2, PDGFRα/β, KIT and RET was detected in all AGASACA samples. Messenger RNA for VEGFR2, PDGFRα/β, and Kit was detected in all TC samples, while mRNA for Ret was amplified in 10/15 TC samples. Evidence of
phosphorylation of VEGFR2, PDGFRα/β, and KIT was not detected on phosphoprotein arrays in either tumor. Evidence of phosphorylation of RET was detected in 54% of the primary AGASACA and 20% of TC. Positive immunoreactivity for VEGFR2 was noted in 19/24 primary and 6/10 metastatic AGASACA and 6/15 TC samples. Positive immunoreactivity for KIT was noted in 8/24 primary and 3/10 metastatic AGASACA and 9/15 TC samples. PDGFRα was noted in all primary and metastatic AGASACA and all of the TC samples. PDGFRβ expression was noted in 4/24 primary and 1/10 metastatic AGASACA and 4/15 TC tumor cells, while intense stromal staining was noted in all tumor samples.

Known targets of toceranib are present in both AGASAC and TC at both the level of message and protein expression. Our findings of VEGFR2 and PDGFR protein expression and phosphorylation of RET merit further investigation as to their roles in the biology of AGSACA and TC as well as to their contribution to the observed response to toceranib.
Dedicated to Michael Doornink and Rachel Keller Vaughan for their love, friendship, and support throughout my residency; to D. Michael Tillson, DVM, MS, DACVS, for his guidance, tutelage, and unwavering support; and for being a role model for what all clinician scientists should be; and to Tyson and Wyatt for making me a better veterinarian and person.
Acknowledgments

I would like to articulate my appreciation for my mentors for their contributions to both the graduate program that generated this thesis as well clinical training. The guidance, knowledge, and experience of Drs. Sandra Barnard, C. Guillermo Couto, William Kisseberth, Cheryl London, and Duncan Russell contribute to making The Ohio State University College of Veterinary Medicine’s Medical Oncology residency one of the premier programs in the country, and I am blessed to have had the opportunity to be trained under their tutelage.

I would especially like to thank Drs. Cheryl London, Duncan Russell, and William Kisseberth for their support and guidance with my research. Each played a different, yet instrumental, role in my education as a scientist and the successful completion of the research to allow for generation of this document. I would also like to express my gratitude to Misty D. Bear and Dr. Joelle Fenger for their knowledge, patience, and technical expertise during data accumulation.

To my resident mates (Drs. Matthew Sherger, Joelle Fenger, and Megan Breit) and the oncology technicians (Stacey Gallant, RVT; Ashley DeFelice Smith, RVT; Kentee Warner, RVT): thank you for the friendship, moral support, sense of humor, and for making my time at The Ohio State University one that I will cherish always.
Vita

2002.......................................................... Bachelor of Science, Auburn University, Auburn, AL
2004 .......................................................... Master of Agriculture, Auburn University, Auburn, AL
2008.......................................................... Doctor of Veterinary Medicine, Auburn University, Auburn, AL
2008-2009 .................................................... Small Animal Rotating Internship, The Ohio State University, Columbus, OH
2009 to present .............................................. Medical Oncology Resident and Graduate Research Associate, Department of Veterinary Clinical Sciences, The Ohio State University

Publications

1. Miller J, Urie BK, Green EM. What’s your diagnosis: Infiltrative lipoma in cat. Journal of the American Veterinary Medical Association, accepted for publication.


Field of Study

Major Field: Comparative and Veterinary Medicine
# Table of Contents

Abstract ................................................................................................................................. i

Acknowledgments........................................................................................................ iii

Vita...................................................................................................................................... iv

List of Tables ......................................................................................................................... ix

List of Figures ........................................................................................................................ x

Chapter 1: Literature Review ............................................................................................. 1

1.1 Targeted cancer therapy ................................................................. 1

1.2 Receptor Tyrosine Kinase Activation and Signaling ...................... 2

1.3 Receptor Tyrosine Kinase Dysregulation in Cancer ......................... 7

1.4 Toceranib and Canine Cancer ......................................................... 8

  1.4.1 Vascular Endothelial Growth Factor Receptor-2 ..................... 10

  1.4.2 Platelet Derived Growth Factor Receptors .............................. 11

  1.4.3 Stem Cell Factor Receptor .................................................. 13

  1.4.4 Rearranged During Transfection Receptor ............................. 15

1.5 Thyroid Neoplasia ........................................................................... 16

  1.5.1 Human Thyroid Neoplasia .................................................. 17

  1.5.2 Canine Thyroid Neoplasia .................................................... 20

1.6 Canine Apocrine Gland of the Anal Sac Adenocarcinoma .......... 25
Chapter 2: Materials and Methods

2.1 Tissue Sample Procurement and Processing

2.2 Proteome-Profiler® Phosphoprotein Array

2.3 RNA Isolation and Reverse-transcriptase PCR

2.4 Tissue Microarray and Immunohistochemistry

2.5 Immunoreactivity Scoring

Chapter 3: Results

3.1 Sample Demographics

3.2 Phosphoprotein Screening Arrays

3.3 Reverse-transcriptase Polymerase Chain Reaction

3.4 Immunohistochemistry

Chapter 4: Discussion

4.1 Limitations

4.2 Future Directions

4.3 Conclusions

References
List of Tables

Table 1. RT-PCR primer sets for canine RTKs .................................................................32
Table 2. Phosphoprotein screening results from AGASACA and TC tumor samples .....37
Table 3. Phosphoprotein screening results from paired AGASACA samples ..............37
Table 4. RTK expression in primary AGASACA samples by IHC ...............................39
Table 5. RTK expression in metastatic AGASACA samples by IHC ..........................39
Table 6. RTK expression in TC samples by IHC .................................................................40
Table 7. Stromal RTK expression in AGASACA and TC samples by IHC...............40
List of Figures

Figure 1. Phospho-RTK array profiling for canine AGASACA and TC tumors ..........36
Figure 2. Tissue microarray IHC of canine AGASACA and TC tumors ......................41
Chapter 1: Literature Review

Chapter 1.1: Targeted Cancer Therapy

Molecular characterization of tumors has contributed to a better understanding of tumor biology and provided insight pertaining to the clinical course of disease, as well as identification of novel therapeutic targets. Targeted cancer therapy is defined as drugs and biological agents that alter the function of specific molecules involved in tumor growth and progression. Small molecule inhibitors and monoclonal antibodies that bind specific growth factors or cell signaling proteins comprise most of the current categories of targeted anticancer therapy. One area of intense research involves alterations in the expression of receptor tyrosine kinases (RTKs), which have proven to be important prognostic factors as well as therapeutic targets in numerous human and canine cancers [1-4].

The therapeutic targeting of RTKs in managing human and veterinary malignancies continues to grow. Toceranib phosphate is a multi-targeted tyrosine kinase inhibitor (TKI) approved for the treatment of canine mast cell tumors (MCTs) based on a single agent response rate of approximately 43% in dogs with recurrent or non-resectable grade 2 or 3 MCTs [5]. A recent report described the clinical benefit of toceranib in solid tumors, where greater than 80% of dogs with progressive thyroid carcinomas (TCs)
and apocrine gland anal sac adenocarcinomas (AGASACAs) experienced either an objective response to therapy or stable disease for over 6 months [6]. The mechanism for the observed response to toceranib in solid tumors is unknown and warrants further investigation.

Chapter 1.2: Receptor Tyrosine Kinase Activation and Signaling

RTKs are transmembrane proteins that bind growth factors resulting in phosphorylation of tyrosine residues on target proteins through transfer of the gamma phosphate from adenosine triphosphate (ATP) to the hydroxyl group on the tyrosine residues and subsequent activation of downstream effector pathways [3, 7, 8]. To date, there are 58 known RTKs in mammals [9]. Receptor tyrosine kinases share a similar structure consisting of an amino-terminal extracellular ligand-binding domain, a hydrophobic transmembrane domain, and a cytoplasmic domain with carboxy-terminal tyrosine kinase site [1, 4, 10, 11]. The extracellular N-terminal region is variable and unique to the ligand binding capabilities of each receptor. The most conserved region of RTK is the intracellular carboxy-terminus, and it is this region that regulates the kinase activity of the receptor [9, 11].

RTKs are categorized based on structure, ligand recognition, and induced biological changes upon activation. With the exception of insulin-like growth factor family disulfide-linked dimer structure, all other RTK are monomers. One large family of RTKs is the split kinase family and is unique in that the ATP-binding pocket and catalytic domain are separated by a kinase insert [2]. Members of the this family include
vascular endothelial growth factor receptors (VEGFRs), platelet-derived growth factor receptors (PDGFRs), stem cell factor receptor (KIT) and fibroblast growth factor receptors (FGFRs) [10]. The role of RTKs in normal homeostasis is essential for numerous fundamental cellular processes and has been implicated in not only cancer but also developmental abnormalities, immunodeficiencies, atherosclerosis, and neurological disorders [1, 3].

Under normal circumstances, activation of most monomeric RTKs is contingent upon growth factor binding induced alteration in receptor confirmation, as both the intracellular and extracellular domains are “locked” rendering the RTK quiescent when unbound [3]. Upon ligand binding induced dimerization, the receptors undergo a conformational change that releases autoinhibition as well as brings the catalytic domains of each RTK in close proximity to each other, where the kinase domain of one receptor can transphosphorylate tyrosine residues on the dimer partner receptor’s cytosolic domain. Once activated, the RTK can elicit a cascade of downstream intracellular signaling that ultimately leads to alterations in gene transcription. The signaling pathways employed by RTKs are extraordinarily complex and integrate several major pathways: Ras/MAPK, phosphatidylinositol 3-kinase (PI3K), phospholipase C (PLC), nuclear factor kappa B (NF-κB) and signal transducer and activation of transcription proteins (STATs).

The RAS family of proteins is composed of guanine nucleotide triphosphate (GTP) binding proteins: H-Ras, K-Ras, and N-Ras. These proteins are attached to the intracellular surface of the cell membrane by farnesyl residues, and like other G proteins,
Ras cycles between an activated and inactivated state based on binding to either GDP or GTP [12]. Ras proteins can be activated following RTK phosphorylation, linking extracellular signals to the activation of a multitude of effector molecules culminating in the activation of transcription factors that promote cell cycle entry and progression, motility and survival [4, 10, 13, 14]. The major downstream effector of Ras is the activation of the mitogen-activated protein kinase (MAPK) pathway, although PLC, Ras-related guanine nucleotide dissociation stimulator (RAL/GDS), and PI3K, also can be activated [13]. Mutations in RAS typically alter either the GTP-binding domain or GTPase domain, and result in chronic stimulation of downstream targets, genomic instability, DNA mutations, and malignant transformation [15].

The MAPK pathway is a chain of consecutive serine/threonine kinases that communicate extracellular signals to the nucleus following activation of several RTKs including EGFR, FGFR, and PDGFR [16]. While the MAPK pathway has multiple downstream effectors, phosphorylation of extracellular signal-regulated kinase (ERK) results in the activation of numerous cytosolic, cytoskeletal, and nuclear targets that regulate cell proliferation, survival, migration, and angiogenesis [16, 17]. Dysregulation of ERK activity at any step in the sequence can promote uncontrolled cell growth and proliferation, and thus promote tumorigenesis. Other downstream effectors of MAPK include p38 and JNK/SAPK, although their roles are less clearly defined in tumorigenesis.

The PI3K/Akt signaling pathway influences a variety of cellular and molecular functions, including those associated with cell growth and proliferation, motility and
survival [18, 19]. While there are 3 classes of PI3K, class I is the best characterized with regard to tumorigenesis [19]. Activation of PI3K can occur as the direct result of RTK ligand binding as well as indirectly through activation of Ras [19]. Once activated, PI3K converts phosphatidylinositol-4,5-phosphate (PIP2) to phosphatidylinositol-3,4,5-phosphate (PIP3), which in turn leads to phosphorylation and activation of the primary downstream effector, Akt (protein kinase B/PKB) [18-20]. The serine/threonine kinase AKT promotes cell survival, proliferation, and motility through activation of the mammalian target of rapamycin (mTOR) and a myriad of other effectors including NF-κB, Bcl-2 proteins, and murine double minute 2 (MDM2) that promote cell proliferation and inhibit apoptosis [18, 21, 22]. Under normal circumstances, PI3K signaling is antagonized by the tumor suppressor PTEN [18, 21]. Mutations in PI3K-α have been reported in many human cancers[23]. PI3K-α catalyzes the production of PIP3 from PIP2, and alterations favoring the production of PIP3 result in an elevation of PIP3 and activation of downstream targets [24].

The NF-κB family of rapid acting transcription factors are located in the cytoplasm of all cells in an inactive state bound to an inhibitory protein, IκBα. IκBα must be phosphorylated and dissociate from NF-κB to permit its translocation into the nucleus [25]. While NF-κB family members are typically activated as part of an inflammatory response to infectious agents, they can be activated by Ras and Myc and contribute to tumorigenesis [25]. Numerous growth factors associated with cellular proliferation are regulated by NF-κB, including IL-1 and TNF as well as cyclin D1[19, 25]. NF-κB also mediates local invasion and angiogenesis through transcriptional
regulation of matrix metalloproteinases as well as growth factor secretion [25]. In summary, NF-κB promotes the transcription of a multitude of genes that promote cell proliferation and tumorigenesis, evasion of apoptosis, metastasis, and angiogenesis [25, 26].

The STAT proteins are activated typically through signals initiated by cytokine or growth factor receptors. These stimuli induce phosphorylation of one of four Janus kinases (JAKs) that then bind STAT proteins, resulting in their phosphorylation, dimerization, and translocation into the nucleus [27, 28]. Once inside the nucleus, STAT proteins recognize and bind to specific DNA sequences of target genes resulting in induction or enhancement of transcription. Downstream effector genes of STATs include regulators of cell survival (i.e. Bcl family members) and cellular proliferation (i.e. cyclins and Myc), as well as genes that promote angiogenesis, like VEGF [28]. STAT proteins are not typical oncoproteins, and to date, no mutations in STAT genes have been reported to be responsible for tumorigenesis. However, constitutive activation of JAK leading to continued activation of wild type STAT has been reported to promote tumorigenesis in human malignancies [28].

PLC is a cytoplasmic protein that regulates the levels of PIP$_2$ in response to cellular stimuli [29]. PLC is preferentially degraded into PIP2, and further cleavage of PIP$_2$ results in diacylegylcerol (DAG) and IP$_3$. DAG alters downstream effectors when it phosphorylates protein kinase C. Ultimately, DAG is cleaved into arachidonic acid. IP$_3$ induces the release of calcium from the endoplasmic reticulum, resulting in the activation of several signaling pathways [29]. Ultimately, PLC signaling regulates cell
proliferation, differentiation, gene expression, and motility along with other homeostatic functions.

1.3: Receptor Tyrosine Kinase Dysregulation in Cancer

Evidence continues to accumulate demonstrating the involvement of RTK dysregulation and dysfunction in cellular signaling pathways that contribute to tumor development [3, 30]. Mechanisms of RTK dysregulation include gain of function mutations leading to constitutive phosphorylation of the receptor, generation of fusion proteins resulting in constitutively active kinase activity, gene amplification resulting in RTK overexpression, autocrine-paracrine stimulation of RTKs by overexpression of ligand, or inappropriate expression of the RTK [1, 3, 4]. The net result of RTK dysregulation is promotion of tumor growth and metastasis through angiogenesis, proliferation, avoidance of apoptosis, and loss of appropriate negative regulation [2, 3, 31, 32]. There are numerous examples of tyrosine kinase dysregulation in human cancer that now impact prognosis and influence therapy. Pivotal discoveries include c-KIT mutations in gastrointestinal stromal tumors (GISTs) [33], HER2/Neu overexpression in breast cancer [34, 35] and EGFR overexpression in several types of carcinomas [36].

The impact of RTK dysregulation has been investigated in several canine tumors, and has provided insight into prognosis and an avenue for targeted therapy. Activating mutations in the RTK KIT have been identified in both canine mast cell tumors (MCTs) and GISTs [37-39]. Recent publications also suggest that KIT may be involved in the pathogenesis of canine renal cell carcinoma and some testicular tumors [40, 41].
Expression of the RTKs MET, EGFR and RON in canine osteosarcoma (OSA) has been evaluated [42-44]. Both OSA cell lines and primary tumors express mRNA for EGFR and RON, and increased RON expression was found to correlate inversely with disease free interval while there was no association with MET expression and outcome in the same tumor samples [42]. McCleese and colleagues further demonstrated evidence for EGFR, RON and MET receptor cross-talk in OSA [42]. While aberrant expression and autophosphorylation of MET was not found to impact either survival time or disease free interval of dogs with OSA, MET was speculated to be involved with lymphatic metastasis although the sample number was too low to confirm this theory [44].

In addition to sharing biological behavior, imaging features, and histological characteristics with human primary brain tumors, canine primary brain tumors express mRNA for VEGFR-1, VEGFR-2, EGFR, PDGFR-α, MET, EGFR and PDGFR-α suggesting that similarities between the two species extend to the molecular level providing a unique opportunity for comparative research using dogs with spontaneous cancers [45, 46]. EGFR expression has also been studied using immunohistochemistry (IHC) in canine nasal [47], mammary [48, 49], lung tumors [50], and brain tumors [46], but further work is needed to investigate the link between EGFR and these tumors [51].

1.4 Toceranib and Canine Cancer

Toceranib phosphate (Palladia®; Pfizer Animal Health, Madison, NJ, USA) is an oral oxindole multi-targeted RTK inhibitor (TKI) that blocks the activity of VEGFR2, PDGFRα/β, FMS-like tyrosine kinase 3 (FLT-3), KIT, and colony stimulating factor
receptor (CSFR1) by competitively binding the ATP binding pocket of the receptor [52]. It was approved for the treatment of canine MCTs based on a single agent response rate of approximately 43% in dogs with recurrent or non-resectable grade 2 or 3 MCTs [5].

Toceranib also exhibited activity against multiple tumor types in the original phase 1 study, suggesting that the action of toceranib against receptors other than KIT may play a role in the responses observed in solid tumors [53]. In support of this, toceranib’s kinome mirrors that of sunitinib (Sutent®, Pfizer Inc, New York, NY), a very closely related multi-targeted TKI that has demonstrated activity against renal cell carcinoma, gastrointestinal stromal tumors, thyroid carcinoma, and pancreatic neuroendocrine tumors in humans [54-56]. The biologic activity of sunitinib in these solid tumor settings has been attributed to its inhibition of VEGFR2, RET and likely PDGFR-α and PDGFR-β.

A retrospective analysis of the use of toceranib in solid tumors provided further support for possible biologic activity against multiple tumor types including AGASACA, TC, metastatic osteosarcoma, carcinoma of the head and neck, and nasal carcinoma [6]. In that study, dogs achieving a complete response (CR), partial response (PR) or stable disease (SD) were defined as experiencing a clinical benefit. Of the 32 dogs with AGASACA that were treated with toceranib, a PR to therapy as defined by RECIST criteria was noted in 8/32 (25%) dogs and 20/32 dogs had SD (62.5%) for a clinical benefit (CB) rate of 87.5%. Of the 15 dogs with TC, PR was observed in 4/15 (26.7%) dogs and 8/15 (53.5%) experienced SD for a CB rate of 80%. These data support the notion that targets of toceranib may be expressed and functional in these tumor types.
1.4.1: Vascular Endothelial Growth Factor Receptor 2

Vascular endothelial growth factor (VEGF) is thought to be the most potent regulator of neovascularization and mediates cell proliferation, migration, survival, cell-to-cell communication, and differentiation as well as alters vascular permeability [57, 58]. There are 3 types of VEGF-receptors: VEGFR-1 (Flt-1), VEGFR2 (FLK-1/KDR), and VEGFR-3 (Flt-4), which share the general structure of other split-kinase RTKs but are characterized by a 7 extracellular immunoglobulin-like domains [59]. There is ~80% homology among the Ig-like domains of the VEGFRs and these subtle differences result in differences in preferential ligand binding, kinase activity, and transduced biological responses [58, 60]. VEGFR-1 and VEGFR2 are localized to both vascular and lymphatic endothelial cells, and VEGFR-3 is primarily present in lymphatic endothelium [61]. While VEGFRs are also expressed on a variety of other non-endothelial cells, VEGFR2 is the earliest marker for endothelial development and is considered to be the primary driver of tumor angiogenesis [62]. Activation of VEGFR2 triggers a full range of responses that regulate endothelial cell proliferation, survival, migration, and alterations in permeability [58, 63]. The downstream targets of VEGFR2 signaling include PLC, PIK3, Akt, and MAPK that promote cell proliferation, migration and survival [58, 60]. VEGFR2 overexpression with either paracrine or autocrine activation is the most common RTK dysregulation found in cancers, although isolated reports of somatic mutations involving deletions and point mutations exist [9].

VEGFR2 expression has been evaluated in canine hemangiosarcoma (HSA), mammary carcinoma, MCT, canine cutaneous fibrosarcoma (FSA), and oral and
cutaneous melanomas [64-71]. Although the role of VEGFR2 in angiogenesis is undisputed, much of the literature reports tumor and/or stromal expression but fails to elucidate the mechanism of activation of VEGFR2. Co-expression of VEGF and VEGFR2 was reported in canine mast cell neoplasia but was not believed to be the primary mechanism of tumor progression and angiogenesis [66]. Another study found that canine mammary carcinomas expressed both VEGF and VEGFR2 in not only tumor cells, but also in endothelial and stromal compartments of invasive mammary tumors, suggesting autocrine and/or paracrine mechanism of angiogenesis [71]. Because VEGFR2 is believed to be expressed predominantly during active angiogenesis and is not normally expressed in mature, quiescent vasculature, it has has been viewed by many as an attractive target for antiangiogenic therapy.

1.4.2: Platelet Derived Growth Factor Receptors

PDGFRs are another group of split-kinase RTKs and are in the same cysteine knot containing superfamily as VEGFR. The primary role of PDGFR signaling is mediating wound healing through angiogenesis and collagen deposition as well as regulating interstitial fluid pressure although it is also implicated in several pathologic diseases and processes including atherosclerosis, pulmonary fibrosis, and glomerulonephritis [59, 72]. PDGF binds to one of 2 receptors, PDGFR-α or PDGFR-β [73] resulting in receptor dimerization and autophosphorylation with subsequent activation of downstream effectors including the PI3K, PLC, and MAPK pathways, among others [72]. Activation of these pathways results in a constellation of biological
responses that stimulate alterations in the cytoskeleton as well as promote chemotaxis, cell proliferation, and transcription of a number of genes such as the oncogenes c-fos and c-myc [72].

Compared to other RTKs, PDGFR mutations are uncommon in human hematologic and solid tumors. Activating mutations in PDGFR-α are identified in 30-50% of human GIST patients who lack c-KIT mutations [74]. TEL/ PDGFR-β fusion proteins resulting in constitutive kinase activation have been identified in humans with chronic myelomonocytic leukemia and in BCR-ABL negative chronic myeloid leukemia [75].

PDGFR signaling has been implicated in autocrine tumor growth and angiogenesis in several tumors. Amplified expression of PDGFR-α and PDGF was found in 5-25% of human glioblastomas tumor cells and surrounding stroma, although activating mutations were not detected in these tumors [76, 77]. More recently, overexpression and gene amplification were reported in low grade gliomas [78-80]. While biologically reasonable based on the mitogenic and chemo-attractant impact PDGF has on fibroblasts and myofibroblasts, there is limited evidence suggesting either an autocrine or activating relationship between PDGF/PDGFR and soft tissue sarcomas [81].

As with VEGFR, evidence supporting the role of PDGFR in tumor angiogenesis and manipulation of the tumor microenvironment is mounting [72, 81]. PDGFR-β mediates pericyte recruitment to tumor vessels as well as recruitment of fibroblasts to the tumor stroma [72]. Upregulation of PDGFR-β expression has also been detected on
tumor endothelial cells in metastatic lesions, although the clinical implications of these findings have yet to be fully elucidated [82, 83].

One other interesting finding with regard to PDGFR signaling is the impact on tumor interstitial pressure. It has been postulated that increased tumor interstitial pressure prevents adequate drug delivery, and inhibition of PDGR-β leads to a transient reduction in interstitial pressure [84]. Co-treatment of cell cultures with a PDGFR inhibitor and cytotoxic drugs resulted in increased intracellular drug concentrations [72].

Investigation of the roles of PDGFR-α and PDGFR-β is limited in the veterinary literature to canine brain tumors, MCTs, HSA and AGASACA; and feline vaccine associated sarcomas [45, 85-87]. Upregulation of PDGFR-α has been documented in canine oligodendrogliomas [45], and there is evidence suggesting a role for PDGFR-β in feline vaccine-associated sarcoma proliferation [88]. Recently, expression of PDGFR-β was evaluated in canine AGASACA in which 15 of 77 samples exhibited positive staining by IHC but expression was not detected in normal anal sac tissue samples [85]. Expression of PDGFR-α and PDGFR-β has recently been evaluated in canine HSA and cutaneous hemangiomas using IHC. The expression of PDGFR-β was significantly higher in HSA while PDGFR-α was higher in hemangiomas [87].

1.4.3: Stem Cell Factor Receptor

The oncogene c-KIT is in the same RTK family as PDGFR, sharing the five immunoglobulin-like regions in the extracellular ligand binding site and split catalytic domain in the cytoplasm. The ligand for KIT is stem cell factor (SCF), and binding of
SCF leads to dimerization of the receptor and phosphorylation of tyrosine residues which ultimately induces downstream signaling of numerous pathways, including MAPK, JAK/STAT, and PI3K/Akt [89, 90].

Gain of function mutations in c-KIT have been identified in human and canine cancers. Activating mutations have been detected in the extracellular and intracellular domains. In both species, mutations within the juxtamembrane domain (exon 11) result in the loss of autoinhibition typically maintained by this region and this is believed to be pivotal in neoplastic transformation [3, 89, 90]. Canine and human GISTs, canine MCTs, and human melanomas have been shown to possess gain of function mutations in this region. Human seminomas, systemic mastocytosis and acute myelogenous leukemia have been associated with mutations in the activating loop of KIT [89-91]. Mutations in the ligand-binding region of the extracellular domain (exons 8 and 9) likely disrupt the inhibitory dimerization motif of KIT [3]. Ultimately, these mutations result in ligand-independent constitutive activation and subsequent downstream signaling driving cellular proliferation.

Both autocrine and paracrine activation of Kit have also been postulated in human small cell lung carcinomas, colorectal carcinoma, breast carcinoma, gynecological tumors, and neuroblastomas based on IHC detected co-expression of KIT and SCF, although the functional relevance of these findings has not been demonstrated [92].
1.4.4: Rearranged During Transfection (RET) Receptor

One of the first RTKs found to be influential in the development of neoplasia was RET, a protein encoded for by the Rearranged during Transfection (RET) proto-oncogene. Like other RTKs, RET is a transmembrane protein composed of an extracellular ligand binding domain, juxtamembrane domain, and intracytoplasmic kinase domain [93]. The extracellular domain is composed of four calcium dependent adhesion like domains that are instrumental in inducing and stabilizing conformational changes needed for interaction with both ligands and co-receptors [93]. RET can be activated by one of several ligands including members of the glial cell line-derived neurotrophic factor (GDNF) like neururnin, artemin, and persephim, as well as glycosylphosphatidylinositol-anchored GDNF-family alpha receptors (GFRαs). Activation requires for one of the GDNF ligands to bind to one of four GFRαs results in phosphorylation. Downstream effectors of RET include IP3, PI3K/Akt, MAPK, and STAT contributing to alterations in cell motility, proliferation, differentiation, and survival [93, 94].

Activating mutations in RET have been incriminated in tumorigenesis in human thyroid neoplasia, type 2 multiendocrine neoplasia, and sporadic pheochromocytoma [93, 94]. The chimeric proteins composed of RET/PTC are the result of either chromosomal inversions or translocations and are characterized by the tyrosine kinase domain fusing to protein N-terminus of the other genes. The promoter sequence of the other gene drives the constitutive expression of RET, and the constitutive kinase activation results due to dimerization [95]. While many chimeric proteins have been reported, RET/PTC1 is the
most common rearrangement in the general population and RET/PTC3 in tumors associated with Chernobyl radiation exposure [95, 96].

Familial medullary thyroid carcinomas and MEN2 syndromes result from autosomal dominant inheritance of germline activating mutations in either the extracellular or kinase domains of RET [93, 95, 97]. Overexpression of RET has been reported in pancreatic neoplasia, prostate cancer, bile duct carcinoma, and lung cancer but the importance of these findings with regard to the pathogenesis of these tumors is unknown [94, 98]. Aside from exploring if a mutation in RET was responsible for familial medullary thyroid cancer in litter of Alaskan malamute mixed breed dogs and screening for mutations in canine mast cell tumor lines, RET has not been evaluated in other canine neoplasms [86, 99].

1.5: Thyroid Neoplasia

The incidence and clinical significance of thyroid neoplasia varies greatly among species, but humans, cats and dogs all share an increased risk with age [21, 100-102]. Thyroid nodules are frequently encountered in both humans and cats, and in these two species benign adenomas and hyperplasia predominate. In contrast, thyroid nodules are infrequently encountered in dogs and, when found, are more likely to be malignant.

Thyroid tumors in all three species most commonly arise from colloid follicular epithelium, more specifically, from the cells that are involved in iodine concentration and thyroid hormone production, although tumors arising from the parafollicular C cells are occasionally encountered [101]. As with other epithelial neoplasms, adenomas and
carcinomas are distinguished from each other histopathologically by degree of
differentiation and invasion into the capsule and/or vasculature and lymphatics. Clinical
features such as degree of local invasiveness and presence of metastatic disease impact
prognosis in both humans and dogs [21, 101, 102].

1.5.1 Human Thyroid Neoplasia

Thyroid carcinoma is the most common endocrine cancer in people, with an
estimated incidence of 7.7 per 100,000 [102, 103]. This rate has increased over the last
20 years, associated with a surge in the incidence of papillary carcinoma [104, 105]. As
with dogs, a majority of thyroid tumors arise from follicular epithelium and are referred
to as differentiated thyroid carcinomas (DTCs). While 80-98% of people with DTC
enjoy a 10 year survival, the poor response to therapy observed in those individuals who
develop metastatic disease has pushed for development of novel therapeutic options [21, 106].

In stark contrast to the dog, much is known about the molecular biology of human
thyroid neoplasia and this has translated into improved therapeutic options. Four
mutation types have been found to constitute the majority of papillary and follicular
thyroid carcinomas (PTC and FTC, respectively): point mutations in BRAF and RAS,
and rearrangements in RET/PTC and PAX/peroxisome proliferator-activated receptor γ
(PPAR γ) [107]. Greater than 70% of PTC harbor BRAF, RAS, or RET/PTC mutations
that activate the MAPK pathway, while ~75% of FTC harbor either RAS or PAX8/
PPARγ mutations [107]. Less commonly, genetic alterations in the PI3K/AKT signaling pathway are encountered as are TRK rearrangements [108, 109].

Among the known Raf kinases, B-type Raf kinase (BRAF) is the most potent activator of the MAPK pathway [110]. Somatic mutations in the BRAF gene represent the most common mutations in PTC with 30-70% of tumors being affected [21, 107, 111]. The most common BRAF point mutation results in replacement of valine with glutamic acid (V600E) leading to constitutive activation of BRAF kinase and continued activation of the MAPK pathway that drives tumorigenesis [107, 112]. Numerous reports have demonstrated an association between BRAF mutations and higher risk phenotypes due to local tumor invasion, metastasis and recurrence as well as loss of radioiodine avidity [21, 110, 112]. The loss of TC ability to incorporate radioactive iodine is the result of BRAF-mutant promoted gene silencing of the sodium-iodide symporter, rendering the tumor nonresponsive to that treatment modality and negatively impacting long term prognosis [112].

Rearrangements in RET/PTC are another genetic mutation recognized in PTC. While several translocations have been reported, all RET rearrangements retain the tyrosine kinase domain of RET that enables the RET/PTC fusion protein to activate the RAS-RAF-MAPK signaling pathway [107]. RET mutations occur at a higher frequency in patients with a history of radiation exposure and in PTC occurring in children and young adults [93, 95, 113]. An unique attribute of the RET/PTC rearrangement is the distribution within tumor cells, as it can be detected in <1% of cells in some tumors [114].
Point mutations in RAS genes occur in ~15-20% of PTC, 40-50% FTC and 20-40% of adenomas [107]. The recognized RAS mutations alter the activation state either by increasing affinity for GTP or by inactivating GTPase function, and both types of mutations result in chronic stimulation of the MAPK and PI3K/AKT pathways. Because RAS mutations have been detected throughout the continuum of thyroid neoplasia, it is believed that RAS activation represent an early event in thyroid tumorigenesis [115]. Poorly differentiated and anaplastic TC often harbor multiple RAS mutations and the detection of RAS mutations predicted a poor outcome in well differentiated TC regardless of stage [116].

Rearrangement in the PAX8 gene and PPARγ results in a fusion protein. Under normal circumstances, PAX8 encodes a transcription factor that is requisite for thyroid follicular cell genesis and regulation of thyroid specific gene expression, while PPARγ is a member of the nuclear hormone receptor family that includes thyroid hormone, retinoic acid, and androgen and estrogen receptors [117]. Evidence suggests that the PAX8-PPARγ fusion protein inactivates the wild type PPARγ and abrogates tumor suppressor activity [118]. Approximately 30-40% of all FTCs exhibit PAX8-PPARγ rearrangements and are associated with TC in younger patients, tumors smaller in size at the time of diagnosis, and with vascular invasion [107, 117]. Interestingly, the rearrangement has also been detected in follicular adenomas [119].

Other mutations believed to contribute to either TC tumorigenesis or progression have been characterized. Activation of PI3K signaling in TC is the result of genomic copy gain and amplification of PIK3Cα in FTC and anaplastic TC; this has been
implicated in the pathogenesis of FTC and anaplastic TC, but not PTC [23, 117]. Loss of function mutations in p53 are typically restricted to poorly-differentiated TC and anaplastic TC, suggesting that this occurs late in the progression of thyroid neoplasia [117].

1.5.2: Canine Thyroid Neoplasia

Although thyroid tumors comprise less than 4% of all tumors in dogs, they are the most commonly diagnosed endocrine neoplasm in this species [101, 120]. The most recent update of descriptive statistics based on cases submitted to the Veterinary Medical Database found that thyroid tumors represented 1.1% of all canine neoplasms between January 1995 and December 2005 [121]. The majority of thyroid tumors are malignant, with carcinomas and adenocarcinomas representing 90% of the tumors [101, 121-124]. Metastatic disease is present in 16 to 38% of dogs at the time of diagnosis and 60 to 80% of dogs with thyroid carcinomas have evidence of metastatic disease at the time of death [101, 122, 124, 125]. The most common sites of metastasis are the lungs and regional lymph nodes; the risk of metastasis increases proportional to tumor size, and in one report metastasis was 16 times more likely in dogs with bilateral thyroid neoplasia [124, 126]. Most clinically relevant tumors are malignant, yet two studies reported that 30-50% of the thyroid tumors diagnosed at necropsy were benign adenomas [122, 124].

Thyroid tumors are reported most frequently in older, large breed dogs and Beagles [101, 120, 121, 125]. A recent review of 638 cases of canine thyroid neoplasia supported previous findings and reported that 89% of dogs diagnosed with thyroid cancer
were between 7 and 15 years of age, and 43% were medium to large breed dogs [121]. Historically, Boxers, Golden Retrievers, and Beagles have been considered to be at higher risk for developing thyroid neoplasia [101, 122, 124, 125, 127-130]. Although some reports failed to identify a breed predisposition, others have confirmed the increased risk of Golden Retrievers and Beagles, and also found Siberian Huskies and mixed-breed dogs to be at increased risk of developing thyroid neoplasia [121, 127, 131].

The most common clinical presentation is detection of a ventral cervical mass, although other complaints include clinical manifestations of local disease such as cough, tachypnea, dyspnea, dysphagia, dysphonia, or cranial vena caval syndrome. Presenting complaints referring to local disease are not surprising, as the majority of thyroid tumors are nonfunctional; less than 25% of thyroid tumors in dogs in the United States are hyperfunctional [101, 124, 132]. Interestingly, hyperfunctional tumors represented a larger percentage of canine thyroid tumors in a retrospective study of dogs from Australia, where 31% of the dogs with thyroid neoplasia were hyperthyroid [133]. In a retrospective report of 39 dogs that presented for radioactive iodine (\(^{131}\)I) treatment, 21 were hyperthyroid, but this likely is an overrepresentation, as dogs were referred for treatment under the pretense that hyperfunctional thyroid tumors would be more effectively treated with \(^{131}\)I [134].

The treatment of choice for freely moveable, non-metastatic thyroid neoplasms is surgical excision; it is associated with low morbidity and a median survival time of over 3 years [126, 131]. Unfortunately, more than half of thyroid tumors are not amenable to surgical excision at the time of diagnosis due to local invasiveness [126, 127, 131]. For
nonresectable thyroid tumors, external beam radiation therapy is a therapeutic option for local disease control and palliation of clinical signs. While thyroid tumors were long thought to be radiation resistant, fractionated (definitive intent) radiation therapy is effective in long term control of local disease, resulting in a mean progression free survival of 45 months with 1- and 3-year progression free intervals of 80% and 72%, respectively, in dogs with fixed and invasive thyroid carcinomas [126, 135]. Another report of 8 dogs with invasive thyroid tumors treated with fractionated radiation therapy corroborated these findings [135]. Hypofractionated radiation therapy in a series of 13 dogs resulted in an overall median survival time (MST) of 24 months. While the presence of metastatic disease did not statistically impact survival in these 13 dogs, the growth rate of the tumor was found to significantly impact survival, with a MST of 127 weeks in dogs with lower growth fractions compared to 44 weeks in dogs with rapidly growing tumors [136].

Radioactive iodine has been used to treat primary thyroid tumors as well as metastatic disease, and survival times achieved in 3 studies totaling 121 dogs were similar to those obtained with definitive external beam radiation therapy [133, 134, 137]. Based on the experience of treating hyperthyroid cats and people with $^{131}$I, this modality has been thought to be the best option for hypersecreting thyroid tumors in dogs.

In veterinary medicine, the literature describing hyperfunctional thyroid neoplasia in dogs [138-146] is composed mostly of case reports, two necropsy studies [147, 148] and three case series where a portion of the dogs with thyroid tumors were hyperfunctional [125, 133, 134]. In one case report, thyrotoxic heart disease was
documented, but long-term survival was not evaluated as the dog was lost to follow-up [138]. A recent report described systemic hypertension with evidence of end-organ damage that resolved following thyroidectomy in a dog with a hyperfunctional thyroid adenocarcinoma [145]. In two other reports, resolution of clinical signs and no recurrence following thyroidectomy for hyperfunctioning thyroid adenomas was described [139, 140]. Rijnberk et al were the first to thoroughly investigate and describe hyperthyroidism resulting from thyroid neoplasia in dogs, using nuclear scintigraphy to confirm the altered iodine metabolism compared to normal dogs, and reporting response to therapy based on resolution of clinical signs and normalization of iodine metabolism following either treatment with $^{131}$I or surgical removal of the neoplasm [143, 144, 149].

In one study, thyroid levels did not correlate with response to treatment with $^{131}$I nor did it impact survival times in dogs treated with $^{131}$I [134]. Another study compared surgery, $^{131}$I, and a combination of surgery and $^{131}$I for canine thyroid carcinomas. Eleven of 65 dogs in this study were hyperthyroid. Three of the 11 hyperthyroid dogs underwent surgery alone, and the remaining 8 dogs were treated with $^{131}$I as the sole treatment modality. The MST for dogs receiving surgery alone was not reached, and hyperthyroid status did not have an impact on survival in this cohort. The difference in MST between dogs with elevated and those with normal T4 concentrations receiving $^{131}$I as sole therapy were 31 months and 19 months, respectively, but this difference was not found to be statistically significant [133].

While the veterinary literature provides much information regarding the clinical features of canine thyroid cancer, there is much to be learned about the underlying
molecular biology. In the 1990’s, a canine model to evaluate the timing and dose of radiation therapy for the induction of thyroid neoplasia was developed [150]. This group found that dogs exposed to high doses of radiation at 2 days and 70 days of age had an increased risk of developing follicular thyroid carcinoma, although the molecular mechanism was not investigated. The same group evaluated the development of thyroid neoplasia in a colony of 276 beagle dogs that were allowed to live their natural life [151]. In this cohort of dogs, hypothyroid dogs exhibited an increased risk for follicular thyroid neoplasia compared to euthyroid dogs. Hypothyroidism was the result of lymphocytic thyroiditis and dogs were not supplemented for documented thyroid deficiency; based on this the authors speculated that chronic excessive TSH stimulation played an influential role in thyroid carcinogenesis [152]. This hypothesis prompted another group to evaluate the binding of TSH by thyroid carcinomas. This group found that thyroid carcinomas bind TSH in a similar manner as normal thyroid tissue, suggesting that TSH could continue to act as a growth factor for thyroid carcinomas [153]. Another study evaluated dogs with familial medullary thyroid carcinoma for RET dysregulation similar to that found in human familial tumors but failed to identify any mutations in the gene after complete sequencing [154]. One report in dogs detected a p53 mutation in 1 of 23 thyroid carcinomas evaluated [155], while aneuploidy was found in more than half of the primary thyroid carcinomas evaluated by another group [156].
1.6: Canine Apocrine Gland of the Anal Sac Adenocarcinoma

Apocrine gland of the anal sac adenocarcinoma represents 2% of all skin tumors, and 17% of all perianal tumors in dogs [157]. AGASACA is locally invasive and has high metastatic potential with 36-96% of dogs having evidence of metastatic disease at the time of diagnosis [157-161]. Clinical presentation may be referable to signs associated with the primary tumor or enlarged sublumbar lymph nodes, or clinical signs associated with hypercalcemia, which is reported in 25-50% of dogs with AGASACA [157-161]. Complete staging, including abdominal ultrasonography and thoracic radiographs, is essential to provide the best prognosis and guide therapy.

Meuten et al were the first to describe hypercalcemia associated with tumors arising from the anal sacs in dogs as well as one of the first to describe AGASACA as a clinically relevant entity [162-164]. In that initial study of 36 dogs, 22 of 23 dogs who were evaluated at necropsy had evidence of regional lymph node metastasis and 9 had evidence of distant metastases. Twenty of the dogs were found to be hypercalcemic at initial diagnosis; however, their hypercalcemia resolved following surgical excision of the tumor and recurred with tumor regrowth. This finding, coupled with parathyroid atrophy, suggested that the tumor was secreting a substance driving the hypercalcemia. Parathyroid hormone related protein (PTHrP) was subsequently identified as the underlying mechanism of hypercalcemia of malignancy observed in AGASACA [165].

The expression of E-cadherin was evaluated in canine AGASACA and its association with survival in 36 canine tumor samples [166]. E-cadherin is a transmembrane protein that mediates intraepithelial adhesion and is essential for the
maintenance of cell polarity and intracellular interactions. Reduced expression of E-cadherin is reported to be prognostic in human thyroid, gastric, colorectal and renal cell carcinomas [167]. As with reports in human epithelial tumors, AGASACA tumors that had reduced expression of E-cadherin (<75%) were associated with shorter overall survival times compared to tumor samples that had >75% expression on IHC.

Treatment of AGASACA is two tiered: addressing local disease and slowing the progression of metastatic disease. The literature unanimously supports the role of surgery to address the primary tumor as well as metastatic lymph nodes and dogs that undergo surgical extirpation of the primary tumor and metastatic lymph nodes enjoy longer survival compared to dogs that do not have surgery as part of their therapy [157-160, 168, 169]. Other historical negative prognostic factors include primary tumor size exceeding 10cm$^2$ and the presence of pulmonary metastases. Some reports found a statistical impact of hypercalcemia on survival, while others failed to identify hypercalcemia as a negative prognostic factor [157-160, 168, 169].

Much like TC, there is no consensus regarding how to treat or slow the progression of metastatic disease. Numerous chemotherapy agents have been used, but studies that report uniformly treated patients are generally lacking. Carboplatin, mitoxantrone and melphalan have been reported as adjuvant or sole therapy with the most frequency [158, 160, 168, 170]. A 33% response rate has been associated with carboplatin, but only 7 dogs were treated in the gross disease setting in this report [159]. Furthermore, a significant difference in MST or time to recurrence was not identified in dogs that received adjuvant chemotherapy combined with surgery (14.4m) compared to
dogs that received surgery (7.9m) or chemotherapy alone (8.7m) [159]. In that study, a variety of chemotherapy agents were employed, and only 2 PRs were observed in the 20 dogs that received chemotherapy and both of which occurred in dogs that received platinum containing drugs (n=1 cisplatin, n=1 carboplatin) [159].

Another report advocated the benefit of neoadjuvant carboplatin, in that it enabled removal of 2 of 4 tumors that previously were not considered to be surgical candidates [160]. A clinical benefit was also observed in 12 of 40 dogs who experienced relief of metastatic lymph node related obstruction after receiving carboplatin. The activity of melphalan in AGASACA was investigated in 14 dogs [168]. Seven of the dogs had metastatic lymph nodes that were also surgically excised, and seven had no evidence of metastatic disease at the time of excision of the primary tumor. The median survival time of all dogs was 20 months with a range of 5 to 42 months, and there was no statistical difference between the dogs with and without evidence of lymph node metastasis at the time of diagnosis. Eleven of the 12 dogs died due to tumor progression.

The longest median survival time for dogs diagnosed with AGASACA was reported in dogs receiving post-operative radiation therapy combined with mitoxantrone chemotherapy. The 15 dogs in the study achieved an overall median survival time of 956 days but a median event free survival of only 287 days. Eleven of the 15 dogs experienced treatment failure with 6 metastasizing to the lungs, 4 to the regional lymph nodes, and one to the contralateral anal sac. Only 11 of the 15 dogs received the prescribed 5 doses of mitoxantrone because of development of progressive disease during
the course of chemotherapy. Eight deaths were the result of tumor progression, and 2 because of late radiation therapy complications [163].

A large retrospective study of 113 cases of canine AGASACA [158] corroborated many of the findings of previous studies. The MST in this cohort was 544 days with a range of 0 to 1,873 days. Dogs who received chemotherapy as sole treatment modality exhibited the shortest survival times (212 days versus 584 days) and dogs that had surgery as part of their treatment had significantly longer survival than dogs that did not (402 days versus 548 days). As with other reports, tumor size was inversely correlated with survival (>10cm$^2$ = 292d versus 10cm$^2$ = 584d), hypercalcemia was associated with statistically shorter survival (256d versus 584d), and the presence of pulmonary metastasis negatively impacted survival (219d versus 548d). As is common with retrospective reports, limitations of this study included lack of uniform treatment groups and small number of dogs in each treatment group.

Toceranib appears to have significant clinical activity in AGASACA based on a clinical study where 32 of 85 solid tumors treated with toceranib were AGASACAs [6]. Twenty-five of the 32 dogs with AGASACA had failed prior therapy. Twenty-eight of the 32 had evidence of metastatic disease in the regional lymph nodes (n=25), lungs (n=9), liver (n=2) as well as other sites (n=11). The clinical benefit of 32 dogs with AGASACA was 87.5% with objective PR in 25% and SD in 62.5%. The median duration of dogs who experienced a PR was 22 weeks (mean 23.1 weeks, range: 10-40 weeks) and the median duration for dogs exhibiting SD was 30.5 weeks (mean 30.9 weeks, range: 10-47 weeks).
Based on observed clinical responses of AGASACA to toceranib, the expression of two known targets of toceranib, PDGFR-β and Kit, in AGASACA was evaluated in 77 tumors using IHC [85]. Two of the 77 samples exhibited positive Kit expression in the cytoplasm of tumor cells. Fifteen of the tumors were positive for PDGFR-β with the labeling being localized to the cytoplasmic membrane and peripheral cytoplasm. Twelve of the 15 samples that expressed PDGFR-β in the tumor cells also expressed stromal staining. The same groups also evaluated five normal anal sacs, which failed to reveal expression of either PDGFR-β or Kit.

For both AGASACA and TC, there is no consensus regarding the role of chemotherapy in slowing the progression of recurrent or metastatic disease or in the setting of minimal residual disease. As with many other malignancies, the response of gross disease to maximum tolerated dose chemotherapy has been disappointing. In contrast, the reported biological activity of toceranib against canine AGASACA and TC not only provides promise with respect to improved management of both malignancies, but also offers insight concerning the molecular biology of these diseases. As such, the purpose of this body of work was to begin to investigate the known targets of toceranib for potential dysregulation in both AGASACA and TC using a variety of molecular techniques.
Chapter 2: Materials and Methods

Chapter 2.1: Tissue Samples

Tumor tissue samples were collected from clinical cases that presented to the Ohio State University Veterinary Medical Center (OSU-VMC). Consent for tissue collection was obtained from all owners in accordance with an approved Institutional Animal Care and Use Committee protocol (2010A0015) and collected by the OSU-VMC Biospecimen Repository. A total of 24 primary AGASACA with 11 paired metastatic regional lymph nodes and 15 primary TC were identified. Surgical and post-mortem collected tumor samples were snap frozen in liquid nitrogen and stored at -80°C. Tissue samples were also placed in formalin and processed for routine paraffin embedding and sectioning for histopathology and stained with hematoxylin and eosin. The medical records of all dogs were reviewed and data pertaining to signalment, staging, treatment, and survival were abstracted.

Chapter 2.2: Proteome-Profiler® Phosphoprotein Arrays

To assess relative phosphorylation of 42 different RTKs in tumor samples, the Proteome Profiler™ Human Phospho-RTK Array Kit was used (R&D Systems, Minneapolis, MN, USA). The methodology and lysate and buffer solutions accompanied
the arrays and the instructions provided by R&D Systems were followed. Briefly, frozen tumor samples were pulverized using a frozen mortar and pestle. The resulting powder was resuspended in liquid nitrogen, and transferred to 1.5mL microcentrifuge tube. Once the liquid nitrogen evaporated away, the samples were allowed to thaw on ice and resuspended in tissue lysis buffer containing 20mM Tris-HCl, 2mM EDTA, 137mM NaCl, 10µg/mL aprotinin, 10µg/mL leupeptin, 1mM sodium orthovanadate, 1% IGEPAL® CA 630, and 10% glycerol. Samples were rocked for 1 hour at 4°C, centrifuged for 15 minutes at 14,000 RPM at 4°C, and supernatants collected. Bradford protein quantification assay was performed on the extracts using BioRad Reagent (BioRad, Hercules, CA, USA). 100µg of protein lysate was used to perform the RTK array following the manufacturer’s instructions.

Chapter 2.3: RNA Isolation and Reverse-transcriptase Polymerase Chain Reaction

Powdered samples of homogenized tissue (described above) was placed in 500uL of TRIzol (Invitrogen Corporation, Carlsbad, CA) and stored at -80°C until RNA isolation according to the manufacturer’s protocol. RNA was quantitated with the Nanodrop-100 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). SuperScriptIII (Invitrogen Corporation, Carlsbad, CA) reverse transcriptase was used for reverse transcription as previously described [171]. For the PCR reactions, primers directed against canine genes of interest (Table 1) were designed to span an intron and were used to amplify product from the cDNA. The program for each PCR reaction was unique for each primer set and was designed to generate a single PCR product. Taq
polymerase (Promega, Madison, WI) was used for each reaction. The amplified PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light. All PCR products were verified by sequencing at The OSU Shared Resources Nucleic Acid Core Laboratory.

### Table 1. RT-PCR primer sets for canine RTKs

<table>
<thead>
<tr>
<th>RTK</th>
<th>Primers</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Kit</td>
<td>232F: 5’ -- GAG AAC ACA CAC AAC GAA TG – 3’ 414R: 5’ -- GCA GCG GAC CAG CGT ATC ATT G – 3’</td>
<td>185</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>1917F: 5’ -- GCT CTC ATG TCG GAA CTG AAG – 3’ 2152R: 5’ -- GTG TGC TGT CAT CAG CAG G – 3’</td>
<td>237</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>2235F: 5’ -- GAC GAG TCA GTG GAT TAC GTG – 3’ 2562R: 5’ -- GTC TCT CAT GAT GTC ACG AGC CAG – 3’</td>
<td>329</td>
</tr>
<tr>
<td>c-Ret</td>
<td>206F: 5’ -- GCC ACT GTG ATG CTG TAG AGA GCA G – 3’ 259R: 5’ -- GTG CGG CAG AGC TCA TCA CAC AGT GG – 3’</td>
<td>210</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1537F: 5’ -- GTA AGT ACC CTT GTT ATC CAA GCA GCC – 3’ 1728R: 5’ -- CGT AGT TCT GTC AGT GCA CCA C – 3’</td>
<td>195</td>
</tr>
<tr>
<td>GAPDH</td>
<td>526F: 5’ -- GTC CAT GCC ATC ACT GCC ACC CAG – 3’ 718R: 5’ -- CTG ATA CAT TGG GGG TGG GGA CAC – 3’</td>
<td>196</td>
</tr>
</tbody>
</table>

**Chapter 2.4: Tissue Microarray Construction and Immunohistochemistry**

Representative areas of tumor tissue were identified on hematoxylin-eosin (H&E) stained sections by a single pathologist (DR) for triplicate 4μm core sampling. Cores were extracted from the corresponding areas of paraffin embedded blocks and inserted into predetermined sites on the TMA recipient block. Immunohistochemical staining was performed for VEGFR2 (Santa Cruz Biotechnology; 1:10 dilution)[64], PDGFRα (Santa Cruz Biotechnology; 1:50)[46], PDGFRβ (Biogenex Labs; 1:200)[85], and KIT (Dako;
1:400), on all AGASACA and TC samples. Negative controls consisted of irrelevant isotype matched antibody at matched dilutions and choice of positive controls were based on previously published reports. Both the construction of the TMA bloc and immunohistochemical staining were performed by the OSU-CVM Veterinary Biosciences Histopathology Laboratory.

Chapter 2.5: Scoring of Immunoreactivity

Subjective quantitative scoring of positive or negative immunoreactivity of cells and stroma within the cores were performed by three of the investigators (WK, DR, BU). Percentage of neoplastic cells staining positive was scored as follows: <5% = 0, 5-25% = 1, 26-50% = 2, and >50% = 3. Location of staining was also noted as cytoplasmic (C), membranous (M), nuclear (N), and stromal (S).
Chapter 3: Results

Chapter 3.1: Sample Demographics

Tumor samples from primary and metastatic AGASACA were collected from a heterogenous population of dogs that were presented to the OSU-VMC. The mean age was 9.9 years (range 7.4 to 13.9, median of 10.6 years). Eleven of the dogs were spayed females, and 13 were castrated males. Mixed breed dogs were overrepresented (n=11) with the remaining 13 dogs being defined as a discernable breed and included Labrador retriever (n=4), and one dog representing each of the following breeds: Shetland sheepdog, Siberian husky, German shepherd dog, boxer, Lhasa apso, miniature schnauzer, cocker spaniel, golden retriever and English setter. Ten of the 24 dogs were hypercalcemic at the time of diagnosis. Nine dogs staged free of gross metastatic disease while 15 dogs had evidence of regional lymph metastasis at the time of diagnosis. Of the 15 dogs with lymph node metastasis, one dog had bilateral AGASACA and one dog also had evidence of pulmonary metastatic disease.

TC tumor samples were collected from 15 dogs. The mean age was 9.5 years (range 6 to 14, median of 10.0 years). Ten of the dogs were castrated males, four were spayed females, and one was an intact male. The most commonly represented breeds included Labrador retriever (n=6) and mixed breed (n=4), with one dog representing the
following breeds: Golden retriever, pit bull terrier, bichon frise, blue tick hound, and a Norfolk terrier. Five of the tumors were hypersecretory, and all were diagnosed as follicular thyroid carcinoma based on histopathology. Two dogs had pulmonary metastasis and one dog had a metastatic lymph node at the time of diagnosis. All 15 tumors were deemed amenable to surgical removal.

Chapter 3.2: Phosphoprotein Screening Array Results

The Proteome Profiler™ Human Phospho-RTK Array Kit provided a platform to assess phosphorylation of 42 different receptor tyrosine kinases in primary and metastatic AGASACA tissue specimens and primary thyroid carcinoma samples using the available flash frozen tumor specimens. Representative examples of the RTK arrays are shown in Figure 1 and a summary of the array results for the AGASACA and TC samples is provided in Table 2 and Table 3. Greater than 50% of the primary AGASACAs demonstrated phosphorylation of EGFR (67%), Dtk/TYRO3 (87%), Ron (54%), RET (54%) ROR1 (92%), ROR2 (62%), while phosphorylation of FGFR3 (38%) insulin-R (50%), Tie-1 (21%) and Tie-2 (25%) was observed in a smaller percentage of samples. All of the metastatic lymph node specimens showed phosphorylation of ROR1 and Tie-1, with phosphorylation of EGFR (64%) also observed in a majority of the samples. Table 2 summarizes differences in RTK phosphorylation between paired primary and metastatic AGASACA samples. The TC samples demonstrated phosphorylation of primarily EGFR (100%), Dtk/TYRO3 (53%), ROR1 (100%) and Tie-1 (100%), with insulin-R (40%), and RET (20%), Tie-2 (40%) and Ron (30%) occurring less frequently.
Figure 1. Phospho-RTK array profiling of canine AGASACA and TC tumors.

Shown are representative examples of phosphoprotein arrays of paired primary and metastatic AGASACA and TC using the Proteome Profiler Human Phospho-RTK Array Kit. This platform allowed simultaneous screening of 42 different RTKs. Determination of phosphorylation was based on comparison of capture antibody of interest to positive controls located on the periphery of the array. On these sample arrays, positive controls, EGFR, RET, ROR-1, and Tie-1 have been identified for comparison.
### Table 2. Phosphoprotein screening array results for AGASACA and TC tumor samples

<table>
<thead>
<tr>
<th>RTK</th>
<th>Primary AGAGASACA n (%)</th>
<th>Metastatic AGAGASACA n (%)</th>
<th>Thyroid Carcinoma n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>16 (67)</td>
<td>7 (64)</td>
<td>14 (100)*</td>
</tr>
<tr>
<td>Dtk/TYRO3</td>
<td>21 (87)</td>
<td>3 (27)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Insulin-R</td>
<td>12 (50)</td>
<td>1 (9)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>ROR-1</td>
<td>22 (92)</td>
<td>11 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>ROR-2</td>
<td>15 (62)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ret</td>
<td>13 (54)</td>
<td>1 (9)</td>
<td>3 (20)</td>
</tr>
<tr>
<td>Tie-1</td>
<td>5 (21)</td>
<td>11 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Tie-2</td>
<td>6 (25)</td>
<td>1 (9)</td>
<td>6 (40)</td>
</tr>
<tr>
<td>Ron</td>
<td>13 (54)</td>
<td>1 (9)</td>
<td>5 (30)</td>
</tr>
<tr>
<td>FGFR3</td>
<td>9 (38)</td>
<td>1 (9)</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 3. Phosphoprotein screening array results for AGASACA and TC tumor samples

<table>
<thead>
<tr>
<th>RTK</th>
<th>Primary AGAGASACA n (%)</th>
<th>Metastatic AGAGASACA n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>5 (45)</td>
<td>7 (64)</td>
</tr>
<tr>
<td>Dtk/TYRO3</td>
<td>10 (91)</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Insulin-R</td>
<td>4 (36)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>ROR-1</td>
<td>11 (100)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Ret</td>
<td>5 (45)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Tie-1</td>
<td>1 (9)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Tie-2</td>
<td>1 (9)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Ron</td>
<td>7 (64)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>FGFR3</td>
<td>4 (63)</td>
<td>1 (9)</td>
</tr>
</tbody>
</table>
Chapter 3.3: Reverse-transcriptase Polyermase Chain Reaction

While the phosphorylation array represents a useful screening tool to assess tissue samples for activation of key RTKs, it does not detect total protein, and therefore will not give a complete picture as to the extent of tumor RTK expression. To provide an initial assessment of expression of the RTKs, total RNA was extracted from normal anal sac and thyroid glands, primary and metastatic AGASACA and primary TC tissue samples, and RT-PCR was performed for canine VEGFR2, KIT, RET, PDGFRα and PDGFRβ. Message for all of these RTKs was detected in all AGASACA tissue samples and paired metastatic lymph nodes. Interestingly, message for RET was not detected in the normal anal sac tissue. Similar to AGASACA, message for VEGFR2, Kit, PDGFR-α and PDGFR-β was detected in all TC samples, while message for RET was detected in only 10/15 tumors. Normal thyroid tissue samples expressed message for all of the RTKs.

Chapter 3.4: Immunohistochemistry

Core samples from all tumor tissues except one metastatic AGASACA lymph node were available for evaluation, and tissue microarrays were constructed to evaluate expression of receptors of interest in all samples (Tables 3-6, and Figure 2). Positive immunoreactivity for VEGFR2 was noted in the cytoplasm of tumor cells in 19/24 primary and 6/10 metastatic AGASACA and 6/15 TC samples. Positive immunoreactivity for KIT in the cytoplasm of tumor cells was noted in 8/24 primary and 3/10 metastatic AGASACA and 9/15 TC samples. Strong cytoplasmic and stromal staining for PDGFRα was noted in all primary and metastatic AGASACA and all of the

38
TC samples. Cytoplasmic PDGFRβ expression was noted in 4/24 primary and 1/10 metastatic AGASACA and 4/15 TC tumor cells, while intense stromal staining was noted in all tumor samples. The normal anal sac samples exhibited positive immunoreactivity for VEGFR2 and PDGFRα, while normal thyroid samples displayed positive reactivity for VEGFR2, PDGFRα, and KIT. Unfortunately, RET expression could not be evaluated on this platform due to the lack of a validated antibody for use in canine formalin-fixed paraffin embedded samples.

Table 4. RTK expression in primary AGAGASACA samples by IHC.

<table>
<thead>
<tr>
<th>RTK</th>
<th>Negative</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Predominant Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>4 (17)</td>
<td>6 (25)</td>
<td>2 (8)</td>
<td>11 (46)</td>
<td>C</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>24 (100)</td>
<td>N, C</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>20 (83)</td>
<td>3 (13)</td>
<td>1 (4)</td>
<td>0</td>
<td>M</td>
</tr>
<tr>
<td>Kit</td>
<td>16 (67)</td>
<td>3 (13)</td>
<td>2 (8)</td>
<td>3 (13)</td>
<td>C</td>
</tr>
</tbody>
</table>

C: cytoplasmic; M: membranous; N: nuclear

Table 5. RTK expression in metastatic AGAGASACA samples by IHC.

<table>
<thead>
<tr>
<th>RTK</th>
<th>Negative</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Predominant Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>4 (40)</td>
<td>1 (10)</td>
<td>0</td>
<td>5 (50)</td>
<td>C</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10 (100)</td>
<td>N, C</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>9 (90)</td>
<td>1 (10)</td>
<td>0</td>
<td>0</td>
<td>M</td>
</tr>
<tr>
<td>Kit</td>
<td>7 (70)</td>
<td>0</td>
<td>2 (20)</td>
<td>1 (10)</td>
<td>C</td>
</tr>
</tbody>
</table>

C: cytoplasmic; M: membranous; N: nuclear
Table 6. RTK expression in TC samples by IHC.

<table>
<thead>
<tr>
<th>RTK</th>
<th>Negative</th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>Predominant Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>13 (87)</td>
<td>1 (7)</td>
<td>0</td>
<td>1 (7)</td>
<td>C</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15 (100)</td>
<td>C</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>11 (73)</td>
<td>1 (7)</td>
<td>2 (13)</td>
<td>1 (7)</td>
<td>C</td>
</tr>
<tr>
<td>Kit</td>
<td>6 (40)</td>
<td>1 (7)</td>
<td>4 (270)</td>
<td>4 (27)</td>
<td>C</td>
</tr>
</tbody>
</table>

C: cytoplasmic; M: membranous; N: nuclear

Table 7. Stromal RTK expression in AGASAC and TC samples by IHC.

<table>
<thead>
<tr>
<th>RTK</th>
<th>Primary AGAGASACA n (%)</th>
<th>Metastatic AGAGASACA n (%)</th>
<th>Thyroid Carcinoma n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFR2</td>
<td>0</td>
<td>1 (10)</td>
<td>0</td>
</tr>
<tr>
<td>PDGFRα</td>
<td>24 (100)</td>
<td>10 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>24 (100)</td>
<td>10 (100)</td>
<td>15 (100)</td>
</tr>
<tr>
<td>Kit</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
**Figure 2. Tissue microarray immunohistochemistry of canine AGASACA and TC tumors.** The TMAs constructed for primary and metastatic AGASACA and TC were probed for VEGFR2, KIT, PDGFRα, and PDGFRβ. Positive immunoreactivity was scored and location of staining was noted. Shown are representative images for primary and metastatic AGASACA and TC for each RTK evaluated.
Chapter 4: Discussion and Conclusions

The purpose of this study was to evaluate AGASACA and TC for the expression of VEGFR2, PDGFRα/β, KIT and RET at both the message and protein level to begin to dissect the molecular basis for the observed response of these tumors to toceranib. While message for VEGFR2, KIT, PDGFRα and PDGFRβ was detected in all tumor samples, only PDGFR-α and VEGFR2 expression were detected in all AGASACA and TC tumor cells by IHC. Strong expression of PDGFRβ was observed in the stroma of both tumor sets. While message for RET was identified in all tumor samples, this RTK was found to be phosphorylated in only 54% of AGASACA and 20% of TC samples. Similarly, although VEGFR2, PDGFRα and PDGFRβ were detected in all tumor specimens by IHC, phosphorylation of these was not observed on the arrays. These data suggest that while these RTKs may be present in most AGASACAs and TCs, most probably they do not exist in a state of continual activation/signaling observed in the setting of typical RTK dysregulation associated with mutation, chromosomal translocation, and over-expression. As such, the majority of biological activity in dogs with AGASACA and TC may relate to inhibition of RTKs that are in support of tumor growth (i.e., VEGFR2 and PDGFRβ associated with vascular endothelium and stroma) rather than a direct inhibition of specific RTKs expressed on tumor cells.
To date, no driver mutations or chromosomal alterations have been reported in either canine AGASACA or TC. A report of a canine pedigree with familial medullary TC (MTC) that clinically paralleled MTC in humans failed to demonstrate a mutation in RET [99]. Another study identified the presence of somatic mutations in p53 in canine TC [172]. Aside from the well documented relationship between AGASACA and parathyroid hormone related-protein, limited data exists regarding the genetics and molecular biology of this tumor [162, 165, 173]. With regard to AGASACA, a higher frequency of the disease has been documented in English Cocker Spaniels with the DLA-DQB1 allele [174], and a direct relationship between E-cadherin expression assessed by IHC on formalin fixed samples and survival in dogs with AGASACA has been documented [166]. As previously mentioned, a large study of 77 archival AGASACA samples found 19.5% expressed positive immunoreactivity for PDGFRβ and 2.6% expressed positive immunoreactivity for KIT [85]. Clearly, more detailed investigations of the molecular aberrations in both AGASACA and TC are needed to better characterize the key biological drivers of these diseases.

In contrast to the lack of published data in veterinary medicine, significant strides have been made in understanding the molecular biology of differentiated thyroid cancer (DTC) in people where specific gene signatures and tumor-initiating events have been identified [175]. DTC includes papillary TC (PTC), follicular TC (FTC), and Hurthle cell carcinoma (HCC). Although often grouped together, there are molecular differences among PTC, FTC and HCC that are associated with phenotype and biological behavior. For example, alterations in several RTKs have been identified, with the best described
being dysregulation of RET present in 5-30% of sporadic PTC in adults [176, 177].

Chromosomal rearrangements and mutations resulting in ligand-independent constitutive activation of RET induce neoplastic transformation of thyroid follicular cells through the activation of multiple intracellular signal transduction pathways[178]. Dysregulation of NTRK1, BRAF, Ras, and PAX/PPARγ has also been associated with the development and progression of other thyroid cancers [176, 179].

In the present study, message for RET was detected in the majority of samples and evidence of phosphorylated protein was detected in 20% of TC and 54% of primary AGASACA. In the paired primary and metastatic AGASACA samples, 5 of the primary tumors had evidence of phosphorylated RET as detected by the phospho-RTK arrays, while it was only detected in one of the lymph nodes suggesting down-regulation of the protein or loss of phosphorylation following metastasis. Unfortunately, an antibody for probing of canine RET by IHC has yet to be validated and limited the authors’ ability to assess protein expression on the TMA. Activating mutations in RET have been documented in human PTC and MTC, multiple endocrine neoplasia 2 syndromes, pheochromocytoma, and paragangliomas that are known to drive tumor cell growth [94] [180]. There is also evidence accumulating to support RET activation and signaling in the pathogenesis of a subset of estrogen receptor α positive breast cancer[98] and overexpression of RET has been reported in prostate, pancreatic, neural crest derived tumors, bile duct carcinomas, melanoma, and lung cancers[181-187]. VEGFRs and RET exhibit structural similarity and as such, TKIs that target VEGFRs such as sunitinib, motesanib and vandetanib have been demonstrated to also inhibit RET phosphorylation.
Sunitinib, a TKI with an inhibitory profile very similar to that of toceranib, has been used as therapy for progressive/metastatic DTC in people resulting in modest objective responses but promising CB with prolonged periods of disease stabilization[106, 179, 188]. A recent kinome analysis of toceranib demonstrated that RET is a target of this TKI (London et al, unpublished) suggesting that as is the case with several human carcinomas, phosphorylation of RET in AGASACA and TC from some dogs may be contributing to cell growth and survival and could therefore be responsible for the objective response to therapy noted in a subset of dogs on toceranib.

In humans, the role of PDGFR in the pathogenesis of TC has also been explored [189]. In one study, 8 patients with advanced DTC exhibiting overexpression of PDGFRs were treated with imatinib, a TKI that targets Bcr-Abl, PDGFR and KIT [189]. Partial responses were documented in 2/8 with another 4/8 experiencing SD for a CB of 75% suggesting that PDGFR may represent a target for therapeutic intervention. In the current study, we found PDGFRα to be expressed in the tumor cells of all canine TC samples, while PDGFRβ was primarily expressed in the tumor stroma by IHC. Interestingly, phosphorylation of these receptors was not documented using the phospho-RTK array, indicating that while expressed, PDGFRα/β is likely not driving tumor growth and survival through constitutive activation.

Similar to the case of TC, we identified expression of PGDFRα in the tumor cells of all AGASACA samples and PDGFRβ in the tumor stroma by IHC. Expression of PDGFRβ in tumor cells was restricted to only 16% of tumor samples, which is consistent with a previous study that found 15 of 77 samples to be positive for this receptor [85].
The association of PDGFRβ primarily with the stroma, and not tumor cells, in both AGASACA and TC suggests that toceranib may be exerting much of its biologic activity through effects on the tumor stroma and blood supply, particularly in cases where SD is observed.

In our study, message for KIT was detected in all primary and metastatic AGASACA, as well as TC tumor samples. In contrast, KIT protein immunoreactivity as assessed by IHC was found in only 8/24 primary and 3/10 LN AGASACA samples and 9/15 of the TC samples, with expression being localized to tumor cells, not stroma. The discordant results between message and protein expression may be secondary to detection of KIT message present in mast cells and other inflammatory cells present within the tumor. Our results also differ from a previous study in which only 2/77 AGASACA samples were positive for KIT by IHC. The reason for this discordance is unclear, although differences in tissue processing, primary antibody used and staining protocol may be contributory. Nevertheless, phosphorylation of KIT was not detected on the phospho-RTK arrays suggesting that as with PDGFR, KIT signaling is probably not playing a role in the driving tumor cell growth and proliferation of AGASACA and TC in dogs. This is consistent with the biology of thyroid neoplasia in people in which KIT dysregulation is not believed to be a contributory factor in tumor biology.

Message for VEGFR2 was detected in tumor cells from all AGASACA and TC samples evaluated. While most (19/25 primary and 6/10 LN metastases) expressed VEGFR2 protein as assessed by IHC, only 2/15 TC samples were positive for protein. As with PDGFR and KIT, there was no evidence of VEGFR2 phosphorylation on the
phospho-RTK arrays. While stromal VEGFR2 expression was only noted in one AGASACA LN sample, this may be due to the fact that VEGFR2 is primarily expressed on circulating endothelial precursors and neo-vessels, rather than mature blood vessels, and as such it may be difficult to identify these populations in the formalin-fixed specimens. However, the role of VEGFR2 and angiogenesis in TC is supported by several observations including the high degree of vascularity of thyroid tumors, the correlation between increased VEGF expression and development of metastatic disease, and evidence revealing that VEGFR2 inhibition may contribute biologic responses observed with multi-targeted RTK inhibitor therapy in humans[21].

The phospho-RTK arrays identified evidence of phosphorylation of several other RTKs that may be influential in the development and progression of both AGASACA and TC. It is well recognized that EGFR dysregulation is a powerful oncogenic driver with overexpression and activating mutations documented in numerous tumors of epithelial origin[190]. In this study, 16/24 of primary and 7/11 metastatic AGASACA and all TC had evidence of phosphorylated EGFR on the phosphoprotein arrays. While expression of EGFR has been evaluated in canine mammary tumors, brain tumors, nasal carcinomas, and lung tumors, it has yet to be proven as a driver of tumor proliferation in any canine cancer[51]. A recent report evaluating several markers in human thyroid carcinoma found strong EGFR expression in invasive PTC and FTC [191]. Vandetanib, a TKI that inhibits EGFR as well as VEGFR and RET, is approved for the treatment of advanced MTC but the impact of EGFR inhibition in this disease is unknown and to date, and there is limited data about its activity in the treatment of DTC, although clinical trials
are presently underway [179, 188]. EGFR expression was not evaluated in the present study, but evidence of phosphorylation supports further investigation.

Tie-1 and Tie-2 comprise the Tie family of RTKs and share the angiopoietins as activating ligands. In the present study, evidence of phosphorylation of Tie-1 was noted on the phosphoprotein arrays in 5/24 primary and 11/11 metastatic AGASACA and all TC, while evidence of phosphorylation of Tie-2 was noted in 6/24 primary and 1/11 metastatic AGASACA and 6/15 TC, including three dogs with metastatic disease at the time of diagnosis. Tie-1 is typically expressed on vascular endothelium and has been implicated in tumor angiogenesis[192]. Expression of Tie-1 has been reported in breast, gastric, colon, and thyroid cancers, but constitutive activation has only been proven in a breast cancer cell line [192, 193].

Expression of Tie-1 in 135 human patients with TC has been previously reported [193]. Tie-1 immunoreactivity was observed in 55.7% (39/70) of PTC and 8.3% (2/24) of anaplastic TC but not in normal follicular cells, follicular adenomas, or FTC. There was a significant difference between Tie-1 expression in PTC compared to anaplastic TC, and it was also noted that large tumors had decreased expression [193]. Based on the recognized biological differences between PTC and FTC, Ito and colleagues suggested that the difference in Tie-1 expression may influence intrathyroidal and lymph node metastases that are commonly observed in PTC and may contribute to the early phase of PTC [193]. This hypothesis is interesting and supports findings in the present study with regard to TC as only 3/15 TC samples had evidence of metastatic disease at the time of surgery and majority were considered to be low grade tumors, albeit follicular in origin.
All dogs with evidence of metastatic disease (2 pulmonary, 1 regional lymph node) had evidence of phosphorylation of Tie-1.

The detection of Tie-1 phosphorylation in the primary and metastatic AGASACA could suggest a role of the Tie/Ang pathway for development of metastasis. Rees and colleagues evaluated Tie-1 expression in several breast carcinoma, colorectal carcinoma and lung carcinoma cell lines [192]. Their data not only revealed that Tie-1 was expressed in a number cell lines but that constitutive activation of Tie-1 was present in the MCF-7 breast carcinoma cell line. Others reported that Tie-1 expression in gastric and breast carcinomas was an independent negative prognostic factor, likely due to the role of Tie-1 in angiogenesis [194, 195]. The finding of Tie-1 in only 5/24 primary but all 11 metastatic AGASACA lymph nodes could suggest a role for Tie-1 in lymph node metastasis; however, further work is required to elucidate this speculation.

Tie-2 expression has been shown to be increased with breast cancer and is also expressed in human PTC, FTC and follicular adenomas [196, 197]. A correlation has been demonstrated between Tie-2 expression and increased risk of metastasis and relapse in breast cancer patients, and expression is associated with decreased survival [196]. Although the role of Tie-2 in thyroid cancer has not been clearly delineated, there is evidence demonstrating that Tie-2 and Ang-1, but not Ang-2, are expressed in PTC and FTC as well as adenomas and hyperplastic regions of adenomatous goiters [197]. Loss Tie-2 phosphorylation was noted in the AGASACA samples, as 6/24 primary samples were positive for pTie-2 on the RTK array while only 1/11 metastatic lymph node samples maintained pTie-2. The role of Tie-2 in canine TC remains unknown, but 6/15
TC, including the 3 dogs with metastatic disease, did show evidence of phosphorylation suggesting it may play a role in this tumor.

Another RTK found to be phosphorylated on the RTK array was DTK/TYRO3, a member of the TAM mediated signaling pathway involved in cell survival, proliferation, migration and adhesion, as well as vascular smooth muscle homeostasis, platelet function, and erythropoiesis [198, 199]. Of the tumor samples evaluated, 21/24 primary and 3/24 metastatic AGASACA and 8/15 TC had evidence of DTK/TYRO3 phosphorylation. One report demonstrated constitutive expression of AXL and TYRO3 as well as their shared ligand, GAS6, in PTC and anaplastic TC, providing evidence supporting their role in thyroid cancer [198]. The contribution of TAM receptor signaling in migration, invasion, and angiogenesis may contribute to the biology of both AGASACA and TC, but further work is needed to clarify the role of DTK/TYRO3 in these tumor types. Interestingly, AXL is inhibited by toceranib at biologically relevant levels and the homology as well as relationship between AXL and TYRO3 could represent one of the mechanisms of the observed response of AGASACA and TC to toceranib [6, 52]

Phosphorylation of recepteur d’origine nantais (RON) was detected in 13/24 primary and 1/11 metastatic AGASACA and 5/15 TC, including both dogs with pulmonary metastasis. RON is in the MET proto-oncogene family of RTKs and is activated by macrophage stimulating protein (MSP). Abnormal expression of RON has been implicated in the pathogenesis of breast, colon, lung, thyroid, bladder and pancreatic carcinomas [200, 201]. In colon carcinoma, RON has been found to accumulate in such
high levels that it becomes constitutive phosphorylated [202]. Differences in RON expression between normal thyroid cells, adenomas and PTC and FTC has been reported, and overexpression was noted in PTC and FTC and correlating with advanced stage and lymph node metastasis [201]. Constitutive activation of RON has been demonstrated in thyroid carcinoma cells, providing evidence for the role of RON in progression of thyroid carcinomas [201]. Given the evidence supporting the impact of RON in a subset of epithelial tumors and the detection of the phosphorylated protein in both AGASACA and TC, further investigation of this RTK is warranted.

Chapter 4.1: Limitations

The descriptive nature of this project has inherent limitations. The small number of cases of each tumor type and diversity of treatments prohibited drawing conclusions regarding disease free intervals and survival. Protein expression by IHC does not directly correlate with a causative role in tumor growth and survival, and while phosphorylation of several key RTKs was observed on the arrays, their exact contribution to AGASACA and TC pathogenesis was not investigated. Additionally, the presence of mRNA does not necessarily correlate with protein expression and expression of RET was not investigated due to lack of validated antibody. The tumor samples in this study were not microdissected, and thus both tumor cells and stroma were present in protein lysates and RNA, making it impossible to know if detected mRNA originated from the tumor cells or from stoma and non-neoplastic cell infiltrates such as mast cells and lymphocytes. The discordance between detected mRNA and evidence of phosphorylation on protein arrays
may be the reflect lack of translation of mRNA or lack of phosphorylation of targets, but could be due to the lack of antibody cross reactivity between human and canine proteins. It is possible that phosphorylated RTKs detected on the phosphoprotein array were the result of nonspecific cross-reactivity and binding, and not true evidence of phosphorylation of the RTK.

**Chapter 4.2: Future Directions**

The findings of VEGFR and PDGFR protein expression and phosphorylation of RET merit further investigation as to their roles in the biology of AGSACA and TC as well as to their contribution to the observed response to toceranib. Probing for targeted phosphoproteins by Western blot analysis or evaluating expression of phosphoprotein on the TMAs would provide valuable information regarding the phosphorylation status of VEGFR2, PDGFRs, and RET as well as either corroborate or dismiss phosphoprotein array findings. One benefit of phosphoprotein IHC would be determining if protein phosphorylation is associated with the stroma or tumor cell, as well as cellular location. Evaluating ligand expression in tumor cells and stroma could provide insight regarding autocrine/paracrine activation as a mechanism of RTK dysregulation in AGASACA and TC.

Microdissected tumor samples would allow the determination of the cell of origin of mRNA detected by RT-PCR and assessing variation in mRNA expression via quantitative RT-PCR could also provide valuable knowledge about the role of these RTKs. The limited number of samples precluded the ability to draw clinical conclusions,
and increasing the number of samples of each tumor type may afford an opportunity to identify possible prognostic factors related to RTKs. Additionally, more paired primary and metastatic tumor samples would provide a unique opportunity to identify pathways associated with metastatic phenotype. Cell culture of primary canine AGASACA and TC would allow evaluation of target modulation by toceranib, although it would be challenging to recapitulate the tumor microenvironment to fully elucidate the relationship between toceranib and tumor stroma.

Lastly, the detection of phosphorylated TIE-1, TIE-2, DTK, EGFR, and RON warrants further investigation to confirm the target and to determine if these RTKs impact tumorigenesis and pathogenesis in canine AGASACA and TC.

Chapter 4.3: Conclusions

We demonstrated both mRNA and protein expression of known targets of toceranib in both canine AGASACA and TC. The findings of VEGFR2 and PDGFRα/β protein expression and phosphorylation of RET merit further investigation as to their roles in the biology of AGASACA and TC as well as to their contribution to the observed response to toceranib. Additionally, other RTKs known to be influential in the pathogenesis of other tumors were detected and provide a platform for further work elucidating the role of these RTKs in both canine AGASACA and TC.
References


45. Dickinson PJ, Roberts B, Higgins RJ, Leutenegger CM, Bollen AW, Kass PH, LeCouteur RA: Expression of receptor tyrosine kinases VEGFR-1 (Flt-1),


67. Rawlings NG, Simko E, Bebchuk T, Caldwell SJ, Singh B: Localization of integrin αvβ3 and vascular endothelial growth factor receptor-2 (KDR/Flk-1) in cutaneous and oral melanomas of dog. *Histology and Histopathology* 2003, **18:**819-826.

68. Al-Dissi AN, Haines D, Singh B, Kidney BA: Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor in canine cutaneous fibrosarcoma. *Journal of Comparative Pathology* 2009, **141:**229-236.


70. Al-Dissi AN, Haines D, Singh B, Kidney BA: Immunohistochemical expression of vascular endothelial growth factor and vascular endothelial growth factor receptor associated with tumor cell proliferation in canine cutaneous squamous cell carcinomas and trichoepitheliomas. *Veterinary Pathology* 2007, **44:**832-830.


86. Takeuchi Y, Fujino Y, Watanabe M, Nakagawa T, Ohno K, Sasaki N, Sugano S, Tsujimoto H: **Screening of therapeutic targets for canine mast cell tumors from a variety of kinase molecules.** *Journal of Veterinary Medical Science* 2011, 73(10):1295-1302.


90. Roskoski R: **Structure and regulation of Kit protein-tyrosine kinase -- the stem cell factor.** *Biochemical and Biophysical Research Communications* 2005, 338(3):1307-1215.


110. Garnett MJ, Marias R: **Guilty as charged: B-RAF is a human oncogene.** *Cancer Cell* 2004, 6(313-319).


152. Benjamin SA, Stephens LC, Hamilton BF, Saunders WJ, Lee AC, Angleton GM, Mallinckrodt CH: Association between lymphocytic thyroiditis,


