# Identification of the Presence and Activity of the JAK-STAT Pathway in Canine Tumors

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ABSTRACT (Academic)

**Background:** The JAK-STAT pathway is a cellular signaling pathway, which acts normally in humans and animals in the control of multiple important functions. Dysregulation of this pathway has been identified in human cancers, as well as a limited number of veterinary cancers.

**Objectives:** The aims of this study were to identify the presence and tentative activity of components of the JAK-STAT pathway in selected canine tumors.

**Methods:** Formalin-fixed, paraffin-embedded samples from mast cell tumors (MCT), hemangiosarcomas (HSA), thyroid carcinomas, and apocrine gland anal sac adenocarcinomas (AGASACA) were obtained from the Diagnostic Histopathology Laboratory at the Virginia Maryland College of Veterinary Medicine. Immunohistochemistry was performed to evaluate protein levels of JAK1, phospho-JAK1, JAK2, phospho-JAK2, STAT3, and phospho-STAT3. Signalment, treatment information, and survival information was obtained from the medical record for each case.

**Results:** Tumor samples were scored for percent positive neoplastic cells. Positive staining was seen for all antibodies in all tumor types, with expression of JAK1, STAT3, and pSTAT3 being highest overall for all tumor types. Significant associations were seen between JAK1 and survival time in MCT ($p = 0.03$), pJAK1 and survival time in HSA ($p = 0.009$) and MCT ($p = 0.04$), and pSTAT3 and metastasis in MCT ($p = 0.0008$).

**Conclusions:** The finding of positive staining for the components of the JAK-STAT pathway in the tumor samples evaluated indicates presence and tentative activity of this pathway in the studied cancers. Further study of JAK1, pJAK1, and pSTAT3 should be pursued to evaluate their potential as therapeutic targets.

ABSTRACT (Public)

The JAK-STAT pathway is a cellular signaling pathway which acts in humans and animals to control functions, such as development of the immune system, and development of the mammary glands during pregnancy. This pathway can become dysregulated, and contribute to development of cancer in both humans and animals. Development of cancer drugs that can target this pathway when dysregulated may aid in controlling further growth of spread of cancer, and may help to prolong survival in patients affected.

In our study, four different cancer types were investigated in dogs for the presence and activity of components of the JAK-STAT pathway. Evidence of presence and activity was identified in the cancers evaluated, suggesting that more work should be done to determine if the JAK-STAT pathway is activated in other canine tumor types, and whether the pathway can be targeted as a cancer treatment.

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### A. The JAK-STAT Pathway Overview

#### a. General Signaling Pathway

The JAK-STAT pathway is a highly conserved cellular signaling pathway. The pathway is activated upon cytokine binding to a cytokine receptor. Binding results in multimerization of the receptor subunits. Some cytokines, including erythropoietin and growth hormone, cause homodimerization of subunits, whereas others, including interferons and interleukins, cause heteromultimerization of subunits.[1] JAKs, non-receptor tyrosine kinases, are associated with the intracellular domains of these subunits, and multimerization of the subunits activates the associated JAKs. This activation results in a conformational change in the receptor subunits that brings two or more JAKs into close proximity, allowing for auto- and trans-phosphorylation of JAKs.[2]

Activated JAKs phosphorylate intracellular tyrosine residues on the associated cytokine receptor, which creates docking sites for STATs. STATs, cytoplasmic transcription factors, are recruited to the receptor, and bind to the docking sites via their Src homology 2 (SH2) domain.[2] STATs then become substrates for JAKs, being phosphorylated at a conserved tyrosine residue, which leads to homo- and heterodimerization of STATs.[3] STAT dimers are transported from the cytoplasm to the nucleus via importin α-5 and the Ran nuclear import pathway.[1]

Once in the nucleus, STATs bind to specific DNA sequences in the promoter region of target genes. These sequences are called GAS (gamma activated sequence) elements, and

are 8 to 10-base pair inverted repeat DNA elements, with a consensus sequence of 5'-TT($N_{4-6}$)AA-3'.[4] This initiates gene transcription of the downstream target gene.

The pathway is complete when STAT dimers detach from DNA, and are exported back to the cytoplasm. STATs must be dephosphorylated to detach and exit, a process that is completed via the nuclear export signal (NES) of STATs, and the nuclear export factor chromosome region maintenance 1 (CRM1).[2] Figure 1[5] demonstrates an overview of the JAK-STAT pathway, showing cytokine signaling through to gene transcription, as well as negative pathway regulation, which will be discussed in a later section.

b. Janus kinases

Janus kinases, or JAKs, are non-receptor kinases. There are four JAKs in mammals: JAK1, JAK2, JAK3, and tyrosine kinase 2 (Tyk2). There are seven JAK homology (JH) regions, numbered from the carboxyl terminus to the amino terminus of the JAK protein.[6] It begins with a tyrosine kinase domain (JH1), followed by a pseudokinase domain (JH2), an SH2-like domain (JH3-4), and a FERM domain (JH4-7).

The JH1 domain is a typical bi-lobed tyrosine kinase domain, and is related to the kinase domains of the epidermal growth factor receptor family.[2,6] JH2, the pseudokinase domain, was thought to be catalytically inactive, but has now been shown that it is a dual specificity kinase. To maintain JAKs in their inactive state, Ser523 and Tyr570 in the JH2 domain are autophosphorylated.[2] This suggests that these residues may need to be de-phosphorylated to cause activation of JAKs. These tandem kinases, located on the carboxyl terminus, are what gives JAKs their name, after the two-faced Roman god Janus.

Making up the rest of the JAK protein are JH3-7, which are located on the amino terminus and are made up of approximately 600 amino acid residues. The SH2-like domain is similar to classic SH2 domains, but is missing some of the typical residues found in conserved SH2 domains.[2] It is present for structural purposes. The FERM domain (Band-4.1, erzin, radixin, moesin) is involved with transmembrane proteins, including cytokine receptors, and facilitates binding to these receptors.[6]

JAK1, JAK2, and Tyk2 are universally expressed in mammalian cells, whereas JAK3 is principally expressed in hematopoietic cells.[7] JAK1, JAK2, and Tyk2 genes have been mapped to the chromosomes 1p13.3 and 19p13.2 in humans, with JAK3 mapped to 19p13.1. In mice, JAK1 is localized to chromosome 4, JAK2 to chromosome 19, and JAK3 to chromosome 8.[7] JAK proteins are generally localized to the plasma membrane, due to their close association with cytokine receptor subunits, but have been experimentally expressed in the cytosol.[6]

The majority of cytokines and cytokine receptors are preferential in the JAK combination they use. For example, erythropoietin (EPO) and thrombopoietin (TPO) along with prolactin, growth hormone (GH), and granulocyte-monocyte colony stimulating factor (GM-CSF), use JAK2.[8,9] Interferon-γ (IFN-γ) uses JAK1 and JAK2, and interferon-α/β (IFN-α/β) uses JAK1 and Tyk2. Many interleukins, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21, use JAK1, JAK2 and JAK3.[8,10] JAK1 and JAK2 are also necessary for a family of cytokines that use a receptor subunit gp130, including IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNF). [6,11]

c. Signal Transducers and Activators of Transcription

Signal transducers and activators of transcription, or STATs, are latent cytoplasmic transcription factors. There are seven mammalian STATs: STAT1-4, STAT5a and 5b, and STAT6. STATs map to three chromosomal regions in mice. STAT1 and 4 map to chromosome 1, STATs 3, 5a and 5b map to mouse chromosome 11, and STATs 2 and 6 map to mouse chromosome 10.[12]

There are seven conserved regions that make up STAT proteins. These include an N-terminal domain (NT), a coiled-coil domain, a central DNA-binding domain, a linker region, an SH2 domain, a single conserved tyrosine residue, and a C-terminal transactivation domain.[2] The NT domain is necessary in the formation of tetramers from activated STAT dimers, while the SH2 domain and tyrosine residue are involved in the dimerization of STAT proteins.[3] The C-terminal domain is involved in the transcriptional activity of STATs.[3]

STATs become activated when the single tyrosine residue, located at residue 700 in STAT proteins, is phosphorylated.[12] This phosphorylation can occur through ligand-activated receptors with tyrosine kinase activity, such as epidermal growth factor receptor and platelet derived growth factor receptor, as well as through JAK activation. Resulting from phosphorylation of this tyrosine residue is the formation of STAT dimers through the interaction between the phosphotyrosine residue and the SH2 domain.[2] Some STATs form homodimers, including STAT1, 3, 4, 5, and 6, while some can form heterodimers, including STAT1, 2, and 3.[2,12]

Upon dimerization, STATs translocate to the nucleus. To gain entrance into the nucleus, STATs must traverse the nuclear pore complex (NPC). The NPC is made up of 30 different proteins, called nucleoporins. Nucleoporins line the NPC and interact with

importins and exportins, both members of the karyopherin-β family of proteins, to traffic proteins, such as STAT dimers, into and out of the cell.[13] The protein of interest must have an amino acid sequence, called a nuclear localization signal (NLS) or nuclear export signal (NES), to interact with importins and exportins and traverse the nuclear membrane. Importins and exportins can bind to the protein of interest directly, or indirectly through interactions with adaptor proteins.[13] The direction of nuclear transport, either import or export, depends on the gradient of Ran-GTP from the cytoplasm to the nucleus.[13,14] Ran, a GTPase, is bound to GTP or GDP. There is high concentration of Ran-GTP in the nucleus as compared to the cytoplasm. Importins bind to Ran-GTP once in the nucleus, which causes a conformational change to the importin, resulting in release of the STAT dimer.[13,14] For nuclear export, the NLS on the dephosphorylated STAT is recognized by an exportin, which binds to Ran-GTP. Once in the cytoplasm, the GTP is hydrolyzed to GDP, releasing the STAT protein.[13,14] The activation-inactivation/phosphorylation-dephosphorylation cycle of a STAT protein is approximately 20 minutes, with almost no DNA binding activity noted 30 minutes after cessation of kinase activity.[12,15]

STAT1 dimers are recognized by importin-α5. Importin-α5 does not recognize STAT1 monomers, indicating that a conformational change due to dimerization exposes the NLS.[13] A leucine-rich area in the DNA-binding domain of STAT1 is important for transport into, as well as export, from the nucleus. Without this region, STAT1 dimers cannot bind importin-α5 to enter the nucleus from the cytoplasm, nor leave the nucleus once there.[13,16] The specific residue in the NLS required for import is Leu407, and is masked in unphosphorylated STAT monomers and dimers.[13] Interestingly, the NLS for STAT1 appears to bind to a distinct area of importin-α5, at a site on the carboxyl region

containing armadillo (ARM) repeats 9-10, which is different from other proteins using this importin for access through the NPC.[13] Importin-α5 can bind to a protein with a typical NLS, as well as STAT1, allowing STAT1 to gain entrance to the nucleus without concern for limited quantities of importin-α5. The export process for STAT1 involves the chromosome region maintenance 1 (CRM1) export protein. CRM1 identifies the leucine-rich area in the DNA-binding domain only when STAT1 is not bound to DNA, and when it is not phosphorylated.[17]

STAT2 does not form phosphorylated homodimers; it must heterodimerize with STAT1. Through interactions with STAT1 and importin-α5, phosphorylated STAT2 gains access to the nucleus.[13] However, unphosphorylated STAT2 can be found in the nucleus through its interactions with interferon-regulatory factor 9 (IFN9). There is an NLS in IRF9 that interacts with importin-α3, -α4, and -α7 to cause nuclear import of IRF9 and STAT2.[13] Export of STAT2 from the nucleus depends on the NES located in the C terminus of the protein, which binds to CRM1.[13]

STAT3 nuclear import is not dependent on tyrosine phosphorylation. STAT3 is phosphorylated at tyrosine 705, which causes dimerization of two STAT proteins. STAT3 homo- and heterodimers are present in the nucleus, but unphosphorylated STAT3 also shuttles continuously from the cytoplasm to the nucleus and back.[14] This is achieved through a constitutive NLS located in the coiled-coil domain of STAT3, specifically amino acids 150-162.[18] This NLS is recognized by importin-α3 mainly, with a small contribution from importin-α6 limited to the testes.[19] Functional SH2 and N-terminal domains do not appear to be necessary for nuclear import.[20] While STAT3 is unable to bind to DNA in the unphosphorylated state, it can interact with other transcription factors

in the nucleus to bind DNA, such as c-Fos and c-Jun, and initiate gene transcription.[21,22] There are three NES elements in STAT3 to facilitate nuclear export. One of these NES elements, located on residues 306-318, is involved in the export of STAT3 in the post-activated form, once it has been phosphorylated and consequently dephosphorylated.[23] However, the other two NES elements, located on residues 404-414 and 524-535, are involved in nuclear export of latent STAT3, that has not been phosphorylated.[23]

STAT5 can also enter the nucleus in its unphosphorylated form, through an NLS located in the N terminus.[13] CRM1, similarly to other STATs, controls the nuclear export of STAT5.

In the nucleus, STATs must bind to DNA to complete their role as transcription factors. They bind to conserved sequences of DNA in the promoter region, termed GAS elements, so named from their discovery as a γ-interferon activation sequence recognized by STAT1.[4] The consensus sequence of GAS elements is 5'-TT($N_{4-6}$)AA-3', with STAT1-4, STAT5a, and STAT5b all recognizing five nucleotides, and STAT6 recognizing six.[4] A lysine residue at position 567 on STAT1 has been identified that is necessary for recognizing GAS elements on DNA.[24] The amino acid sequence in which this lysine residue is located is highly conserved among STATs.[24]

d. Negative Regulation

i. Protein Tyrosine Phosphatases

One of the mechanisms for negative regulation of the JAK-STAT pathway is through protein tyrosine phosphatases (PTPs). PTPs function to remove phosphate groups from tyrosine residues on JAKs and STATs. They consist of approximately 100 genes, and have conserved motifs, especially the motif in the catalytic center.[25] PTPs can act on all

aspects of the JAK-STAT signaling cascade, from the ligand receptor at the cytoplasmic membrane, to the receptor-associated JAKs, to both cytoplasmic and nuclear STATs.

SHP-1 is a PTP expressed in hematopoietic cells.[26] It is located in the cytoplasm, and has two SH2 domains located at the amino terminus, with the phosphatase domain located at the carboxyl terminus.[26] The SH2 domain allows SHP-1 to interact with phosphorylated proteins, and the phosphatase domain effects dephosphorylation. JAKs are substrates of SHP-1, specifically JAK1 and JAK2. It has been shown that SHP-1 does not require a functional SH2 domain to interact with JAK2.[26] The binding domain for JAK2 is located in the amino terminus, predominately in the N1 portion of SHP-1 (amino acids 1 to 106).[26] This amino region is also present in the remainder of the JAK family members, which may indicate a conserved binding site for SHP-1 on the JAK family.[26] SHP-1 also has activity in the dephosphorylation of STAT5 and STAT6.[25]

Mice deficient in SHP-1 are called motheaten mice, and show increased phosphorylation in hematopoietic cells, hypersensitivity to antigen stimuli, and increased myelopoiesis.[26] JAKs from these mice are also hyperphosphorylated.[26,27] These mice suffer from autoimmune diseases and chronic inflammation, and die early.[25] Promoter methylation of SHP-1 is associated with leukemias and lymphomas, as well as myelomas in people.[28]

Similar to SHP-1, SHP-2 also has two SH2 domains at the amino terminus, and a phosphatase at the carboxyl terminus. The SH2 domains prevent the phosphatase domain from accessing substrate, a phosphorylated tyrosine residue on a protein (pTyr), in the basal state. SHP-2 undergoes a conformational change on binding a pTyr, which exposes the phosphatase domain, allowing for dephosphorylation of the protein.[28] SHP-2

functions normally in hematopoiesis and lymphopoiesis, and mutations in SHP-2 are associated with the development of various leukemias.[28]

Targets of SHP-2 include STAT1 and STAT2. Hyperphosphorylation of STAT1 and STAT2 was seen in mouse fibroblasts with mutated, non-functional SHP-2, with reduction in cell viability.[29] When wild-type SHP-2 was introduced to these mutated cells, this hyperphosphorylation was reversed. SHP-2 has also been shown to dephosphorylate STAT3.[25]

There is also evidence that in addition to negative regulation of the JAK-STAT pathway, SHP-2 actually promotes signaling through this pathway. STAT5 is activated by JAK2 in mammopoiesis and lactogenesis.[30] Stat5a deficient mice lack growth of the mammary glands during pregnancy, and do not lactate after pregnancy.[30] Deletion of SHP-2 in mice suppressed activity of STAT5, which caused reduction in lobuloalveolar growth of the mammary gland and reduced lactation.[30] This promotion of signaling comes from the ability of SHP-2 to dephosphorylate a tyrosine residue, Tyr1007, on JAK2, which prevents the association of a negative regulatory protein with JAK2, preventing JAK2 from being ubiquinated and degraded.[30] Interestingly, STAT3 is negatively regulated by SHP-2 to slow involution of the mammary glands after pregnancy, showing reciprocal activity of SHP-2.[30]

CD45 is a transmembrane phosphatase with a role in B and T cell antigen-receptor signaling. CD45 also has activity in dephosphorylation of JAKs. CD45 interacts directly with all JAKs in murine cells to cause dephosphorylation.[25,28] In CD45-negative cells, hyperphosphorylation was seen on tyrosine residues Tyr1022 and Tyr1023 of JAK1,

Tyr1007 and Tyr1008 on JAK2, and Tyr1054 and Tyr1055 on TYK2; these are the same residues that CD45 has been shown to dephosphorylate.[31]

TC-PTP is a PTP that is present in both the cytoplasm and nucleus, and is a major factor in the dephosphorylation of phosphorylated nuclear STAT1.[15,25,32] When activity of TC-PTP is inhibited, STAT1 remains in the nucleus in the phosphorylated state for hours.[15] Additionally, in TC-PTP negative cells, cytoplasmic dephosphorylation of STAT1 is also inhibited.[32] TC-PTP is not the only PTP that can cause nuclear dephosphorylation, but does appear to be the major PTP and most efficient.[32] Nuclear TC-PTP also is involved in the negative regulation of STAT3, causing dephosphorylation of this protein.[33]

PTPRT is another PTP, and has been shown to directly dephosphorylate STAT3 at Tyr705.[28] PTPRT is a transmembrane protein, with two phosphatase domains, and an extracellular and juxtamembrane domain.[34] Being a transmembrane domain, it does not have ability, as a complete protein, to gain access to the nucleus and cause dephosphorylation. One hypothesis as to its ability to dephosphorylate STAT3 is that it does so when STAT3 is activated by JAKs or other receptor-associated kinases, because this localizes phosphorylated STAT3 to the cell membrane.[34] Another hypothesis is that PTPRT can be cleaved, with the phosphatase domains traveling to the nucleus and enacting dephosphorylation.[34] Studies investigating mutated PTPRT in human colorectal cancer have shown it to be a tumor suppressor gene, and it commonly is mutated in this disease, causing reduction in phosphatase activity.[35]

There are a number of other PTPs with activity in the JAK-STAT pathway. PTP-BL, a non-transmembrane PTP, has shown activity in dephosphorylation of STAT4 and

STAT6.[25,28] VHR is a dual specificity phosphatase, meaning it can dephosphorylate both tyrosine and serine/threonine residues.[36] It has activity in dephosphorylating STAT5, but requires phosphorylation itself by Tyk2, and must bind to the SH2 domain of one STAT5 protein to effect dephosphorylation.[37]

ii. Suppressors of Cytokine Signaling

Suppressors of cytokine signaling (SOCS) are cytokine-inducible intracellular proteins that regulate the JAK-STAT pathway as part of a negative feedback pathway. There are eight members of this family, SOCS1-7 and CIS, with SOC1-3 and CIS being the most studied, and SOCS1 and 3 being best characterized with regards to inhibition of the JAK-STAT pathway. These proteins share similar structure, all containing an SH2 region, a variable N-terminal region, and a region at the C-terminus termed the SOCS box.[38] The SOCS box consists of approximately 40 amino acids with conserved sequences between the members of the SOCS family.[39] While the SOCS family of proteins is highly conserved among mammalian species, the specific proteins themselves are not similar amongst one another. Certain pairs are more similar than others; such pairs include CIS and SOCS2, SOCS1 and SOCS3, SOCS4 and SOCS5, and SOCS6 and SOCS7.[39] This suggests that certain SOCS have developed to perform specific functions.

SOCS proteins are not found constitutively in the cytoplasm, but are induced upon cytokine receptor binding. On stimulation with IL-6, SOCS1 and SOCS3 can be detected in murine liver within 20 minutes, returning to base levels after four hours.[38,40] SOCS2 and CIS expression is more prolonged, being detected within 24 hours after stimulation.[38] SOCS5 expression is detected within the murine liver eight to twelve hours after cytokine stimulation.[40]

SOCS gene transcription is promoted by members of the STAT family. The *Socs1* gene has a promoter that contains binding sites for both STAT3 and STAT6, and cells with mutated STAT3 do not express SOCS1 on cytokine stimulation.[38,41] CIS expression is promoted on cytokine stimulation with Epo and IL-3, both of which signal primarily through STAT5, and the promoter for the CIS gene has STAT5 binding sites called MGF boxes.[39,42] SOCS expression also differs within different anatomic sites, even when stimulated by the same cytokine. On cytokine stimulation with growth hormone (GH), STAT5b promotes expression of SOCS2 and SOCS3 in murine liver, and STAT5b deficient mice did not show expression of SOC2 or SOCS3.[43] However, STAT5b deficiency did not have any effect on the ability of GH stimulation to cause expression of SOCS2 in the murine mammary gland.[43] Additionally, neither mice with wild-type STAT5b nor mutated STAT5b demonstrated any expression of SOCS3 in the mammary gland.[43]

SOCS effect inhibition of the JAK-STAT pathway in multiple ways. SOCS1 in particular can interact with all four known JAKs to cause direct inhibition. SOCS3 interacts with all but JAK3.[44] Inhibition is through association of the phosphorylated tyrosine kinase domain on JAKs with the SH2 domain on SOCS, bringing both proteins into close proximity.[41] The effector of inhibition is a region of 24 amino acids N-terminal to the SH2 domain, known as the kinase inhibitory region.[41,44] Both SOCS1 and SOCS3 have a sequence of 12 amino acids within this domain that is similar to the JAK activation loop, suggesting that this region may act as a pseudosubstrate for proteins, such as STATs, normally phosphorylated by JAKs.[41]

SOCS also cause inhibition through interaction with the phosphorylation sites on the cytokine receptors. CIS blocks the phosphorylation site on Epo and IL-3 receptors to prevent activation through these receptors.[41] Sites such as Tyr401 on the Epo receptor are blocked from STAT5 binding; however, it has been shown that high levels of STAT5 can overcome this inhibition, suggesting that the CIS competitively inhibits STAT5 in this setting.[41] SOCS1 and SOC3 also bind to phosphorylation sites on cytokine receptors, blocking binding sites for STAT proteins.[45]

SOCS proteins can also target proteins for proteosomal degradation. Through the SOCS box, SOCS proteins associate with another family of proteins called the elongins, specifically elongins B and C.[41] The SOCS-elongins complex binds to an E3 ubiquitin ligase called cullin-2. Through interaction of the SH2 domain on SOCS, an activated protein can be brought in close proximity to the ubiquitin ligase complex, and target the protein for ubiquitination and degradation.[41,46]

SOCS2 has a unique function, in which it can inhibit or promote growth signaling. At low levels of expression, SOCS2 inhibits signaling through the GH receptor and prolactin receptor, as well as other cytokine receptors.[47] At high levels of expression, SOCS2 can enhance signaling through these receptors.[47] This function may be due to the ability of SOCS2, at high concentrations, to block signaling inhibition from and promote degradation of SOCS1 and SOCS3.[47]

iii. Protein Inhibitor of Activated STATs

Protein inhibitor of activated STATs (PIAS) are a family of proteins that regulate cytokine signaling through their inhibition of STATs. There are four members with different isoforms: PIAS1, PIAS3 and PIAS3β, PIASxα and PIASxβ, and PIASy.[48]

These proteins are constitutive, and interact with phosphorylated STATs in the nucleus. PIAS1 is the largest protein in the family, having a length of 651 amino acids, and with 500 amino acids, PIASy is the smallest.[49]

PIAS proteins are highly conserved in mammals, and are also highly similar between the four family members, with the exception of a variable C-terminus. Located at the N-terminus is a 35-amino acid long domain called the SAP module. This term comes from the three proteins that make up this domain, scaffold attachment factor (SAP), acinus, and PIAS.[49] This SAP domain permits binding of PIAS to chromatin, and recognizes A-T rich sequences present in matrix attachment regions of DNA.[50] Also at the N-terminus is the PINIT (proline, isoleucine, asparagine, isoleucine, threonine) domain, which promotes interaction between PIAS and STATs, and may aid in retaining PIAS in the nucleus.[50,51] The central domain of PIAS is the most conserved domain, and contains a zinc-binding domain called Miz-zinc finger or SP-RING domain, which is related to the classical zinc-binding RING motif.[49] This zinc-binding domain functions as a small ubiquitin-like modifier (SUMO) E3 ligase.[48] The C-terminus of PIAS is the least conserved, and contains serine-threonine rich sequences in all PIAS members except PIASy.[50]

There are multiple ways in which PIAS can inhibit STATs. One way is in the prevention of DNA binding of STATs. PIAS1 inhibits the DNA binding of STAT1, and PIAS3 inhibits the DNA binding of STAT3 and STAT5.[48] A requirement for PIAS1 binding is STAT1 phosphorylation on Tyr701.[52] Another way is through the recruitment of co-regulators, such as histone deacetylases (HDACs). PIASx and PIASy block STAT4 and STAT1 signaling, respectively, through recruitment of HDACs, and do not interfere

with DNA binding of the transcription factors.[48] PIAS1 can also sumoylate STAT1 on Lys703, although the significance of this in STAT inhibition is not known.[48,50]

Although sumoylation is similar to ubiquitination, it does not target proteins for degradation. Instead, sumoylation targets proteins involved in nuclear functions, such as DNA replication and transcription, and modifies protein-protein interactions, aids in cellular and nuclear localization of proteins, and regulation of transcription factors.[50,53] It may also prevent proteins from being degraded.

B. Normal Functions of the JAK-STAT Pathway

a. Immune System Development and Hematopoiesis

JAK-STAT signaling is crucial to differentiation of B and T lymphocyte populations. Th1 and Th2 cells develop from naïve $CD4^+$ lymphocytes, and their differentiation is controlled by a number of cytokines, IL-12, IL-23, IL-13, and IL-4. IL-12 and IL-23 promote differentiation into Th1 cells, which promote cell-mediated immunity, and IL-4 and IL-13 into Th2 cells, which mediate humoral immunity.[54] Cytokine signaling requires JAK and STAT activation to complete differentiation. IL-12 and IL-23 signal through JAK2 and Tyk2, which causes STAT4 phosphorylation and Th1 differentiation.[5] IL-4 and IL-13 signal through JAK1 and JAK3, which causes STAT6 phosphorylation, leading to Th2 differentiation.[5,54] Studies of knockout mice show that without IL-12 signaling, due to lack of STAT4, mice fail to develop Th1 cells, and conversely, without IL-4 signaling, due to lack of STAT6, fail to develop Th2 cells.[3,54] Because STAT4-deficient mice do not develop Th1 cells, they do not present with autoimmune diseases such as arthritis, because many autoimmune diseases require Th1 mediation.[55] However, STAT4-deficient mice are more susceptible to intracellular infection.

Th2 cells are the major cells involved in allergic stimulation, and their differentiation is promoted by STAT6. Th2 cells produce numerous cytokines which then stimulate production of IgE, which is important in the sensitization of mast cells. In mouse models of allergic airway disease, mice deficient in STAT6 show reduced pulmonary inflammation, with lower amounts of Th2 cytokines and eosinophils.[56] Additionally, B cells lacking STAT6 cannot undergo immunoglobulin class switching, and do not produce IgE.[55]

Th17 cells require cytokine stimulation, via IL-6, IL-21, and IL-23, causing STAT3 activation, to undergo differentiation.[54] Knockout mice for STAT3 show a reduced Th17 response, and mice with STAT3 overexpression show increased Th17 response.[54] STAT5 is important in the development of regulatory T cells (Tregs). JAK1 and JAK3 are phosphorylated upon IL-2 cytokine receptor binding, which in turn phosphorylates STAT5.[57] STAT5 regulates transcription of FOXP3, which is essential for development and function of Tregs.[58]

JAK1/3 and STAT5 promote B and T cell maturation through IL-7. IL-7 binds to its receptor, which causes activation of JAK1 or JAK3, leading to STAT5 activation.[54,59] In mice lacking only one isoform of STAT5 (STAT5a or STAT5b), B cell development is normal, although NK cell activity is reduced, especially in mice lacking STAT5b.[3] However, in mice lacking both isoform, T and B cells, as well as NK cells, fail to develop, and perinatal lethality is seen due to reduced maturation and numbers of T and B cells.[54,58] JAK3 is vital for lymphoid development, as evidenced by the development of X-linked severe combined immunodeficiency (X-SCID) in humans and mice that have

mutations in JAK3 or in a cytokine receptor chain, $\gamma_c$, of which JAK3 is associated.[3] Mice lacking JAK1 die soon after birth, with diminished lymphopoiesis.[3]

The JAK-STAT pathway has an essential role in the regulation of hematopoiesis. Both STAT5a and 5b are activated by IL-3, IL-5, erythropoietin (EPO), thrombopoietin, stem cell factor (SCF) and granulocyte-monocyte colony stimulating factor (GM-CSF) to promote production of platelets, monocytes, granulocytes, basophils, eosinophils, and mast cells.[59,60] Many of these ligands appear to primarily cause JAK2 activation, although other JAKs have also been shown to be phosphorylated. The binding of SCF to its receptor, c-kit (CD117) on mast cells, causes JAK2 activation. This causes phosphorylation of STAT5 and STAT6, with STAT5 being important in the development, survival, and proliferation of mast cells.[5] IL-3 also activates JAK2 in mast cells, and may have similar functions to SCF in mast cells.[5] JAK2 is also important in activation of STAT3 and STAT5 when thrombopoietin binds its receptor, Mpl.[60] STAT3 and STAT5, activated via thrombopoietin, promote transcription of genes for cyclin D1, p21, p27, and Bcl-xL.[60]

JAK2 knockout mice die in utero, due to lack of erythropoiesis. These mice fail to respond to erythropoietin and thrombopoietin, as well as other cytokines important in hematopoiesis, including IL-3 and GM-CSF.[3] In STAT5 knockout mice, there are significant defects in the lymphoid and myeloid lineage. Bone marrow derived mast cells lacking STAT5 have been shown to have reduced to absent levels of anti-apoptotic proteins Bcl-2 and Bcl-x(L), and increased activation of caspases-9 and -3 in vitro, with prevention of mast cell development in vivo in STAT5-deficient mice.[61] The ability of STAT5a deficient mice to respond to treatment with GM-CSF is much reduced compared

to wild type mice, based on the reduced expression of CIS and A1, both genes known to be activated by GM-CSF, in STAT5a deficient mice.[62]

STAT3, and to a lesser degree STAT5, appears to be important to signaling mediated through the granulocyte-colony stimulating factor (G-CSF). In mice with mutant G-CSF receptors, STAT3 activity is absent, and STAT5 activity is decreased. These mice show severe neutropenia with increased numbers of undifferentiated myeloid precursors in the bone marrow.[63] STAT3 is also important in response to the cytokines IL-2, IL-6, and IL-10, and mice lacking STAT3 die in utero.[3]

b. Mammary Gland Development

STAT5 is crucial to the developing mammary glands. STAT5 was originally named MGF (mammary gland factor), due to its role as the transcription factor for prolactin-mediated cytokine signaling.[64] It is a JAK2/STAT5 interation, upon prolactin binding to its receptor, that mediates signaling and gene transcription of β-casein.[65,66] JAK2 inhibition blocks differentiation of prolactin-stimulated mammary epithelial cells.[66] Both STAT5a and STAT5b have been shown to bind to the GAS element within the β-casein gene to ultimately cause increased expression of milk protein in mice.[67] In addition to the importance of STAT5 in production of milk protein, STAT5 is also important in the development of the mammary epithelium during pregnancy. In mice in which STAT5 is deleted during pregnancy, the secretory component of the mammary glands failed to proliferate, and dams were unable to produce milk to feed their pups.[68] Additionally, deleting STAT5 after differentiation of the mammary gland occurred resulted in de-differentiation and apoptosis of the mammary gland epithelium, indicating that STAT5 is also important in the survival of the mammary epithelium.[68]

At weaning, there is inactivation of STAT5, and increased expression of STAT3 to cause involution of the mammary gland.[69] Milk stasis, through the production of leukemia inhibitory factor (LIF), and glucocorticoids, through an unknown mechanism, mediate the activation of STAT3.[69]

JAK2 has an important role in regulating growth of the mammary epithelium. In mammary epithelium in which JAK2 is deleted, cells show increased growth rates and mitosis compared to cells with normal JAK2 expression.[66] Additionally, cells lacking JAK2 show reduced rates of apoptosis, as well as constitutive STAT3 activation, which has been shown to be an oncogene and constitutively activated in human breast carcinoma cells.[66,70,71]

c. Body Growth and Sexual Dimorphism

Growth hormone (GH) and glucocorticoid receptor (GR) both mediate growth through interaction with STAT5. GH binding to the growth hormone receptor (GHR) causes activation of JAK2, leading to phosphorylation and nuclear translocation of STAT5.[58] STAT5b interacts with GR in the nucleus to initiate transcription of insulin growth factor-1 (IGF-1), specifically in hepatocytes and myocytes, which is very important in body growth.[58] STAT5b-negative mice have smaller body sizes than wild-type mice, with STAT5 deletions in hepatocytes having a more marked difference in growth than in those mice with deletions in myocytes.[72-74] Growth retardation is also seen in humans with STAT5b deletions, and people with STAT5b deletions also show reduction in circulating IGF-1.[58]

It appears that STAT5b is very important in sexual dimorphism, as STAT5b-negative male mice had sex-dependent gene expression similar to that of female mice, and these

levels of gene expression were also significantly decreased compared to those of wild-type male mice.[72] Significantly less fat deposition was seen in STAT5b negative male and female mice, as compared to their wild-type counterparts.[72]

d. Other Functions

The JAK-STAT pathway has functions both in pain and inflammation, and in anti-inflammatory properties. IL-6 is a pro-inflammatory cytokine that activates JAK1 and JAK2 associated with the IL-6 cytokine receptor, which leads to STAT3 activation.[75] The anti-inflammatory cytokine IL-4 also uses the JAK-STAT pathway to exert its effects, with activation of STAT6 being the endpoint. IL-4 deficiency has been linked to increased inflammation and arthritis in mouse models.[75]

STAT3, along with other components of the JAK-STAT pathway, has effects on bone metabolism and skeletal development. STAT3 is the most important, being a positive regulator of bone growth and development. STAT3 promotes the differentiation of osteoblasts. Mice with mutations in or deletions of STAT3 show lower bone mineral density, as well as increased activity of osteoclasts and bone resorption.[76] STAT1 negatively regulates bone mineralization, as shown in mice with STAT1 inactivation, which have osteopetrotic bone, or bone with a higher mineral density than mice with functional STAT1.[76] Mice with STAT5 deletions also show defects in bone development, which seems to be associated with decreased levels of IGF-1 in bone.[76]

JAK1, STAT1 and STAT3 are involved in the differentiation of astrocytes from neural stem cells and neural progenitor cells.[77] The JAK-STAT pathway is also involved in protection of the spinal cord after injury. The interaction of IL-6 with its receptor appears to activate JAK1 and STAT3 in neurons after injury, and IL-6 knockout mice showed

increased damage and death of spinal cord neurons following injury as compared to wild-type mice.[77]

JAK2 and Tyk2 are phosphorylated through angiotensin II (Ang II) binding to the angiotensin II type I receptor ($AT_1$), leading to phosphorylation of STATs 1, 2, and 3.[78] Blocking STAT1 and STAT3 signaling inhibited proliferation of vascular smooth muscle cells after stimulation with Ang II, showing an important role for the JAK-STAT pathway in response of the vessel walls to Ang II.[78]

C. Dysregulation of the JAK-STAT Pathway and Neoplasia in Humans

Dysregulation and activation of the JAK-STAT pathway has been demonstrated in multiple human cancers and cancer cell lines. STAT3 and STAT5 are the most well-recognized and well-studied STAT proteins with regards to activation in human cancers. STAT1 expression is noted in many human tumors, but this protein seems to function as a tumor-suppressor, instead of in malignant transformation, as the deletion of STAT1 promotes tumor cell survival.[79]

Much of the time, the activation of STATs seen in human cancers is because of constitutive activation events upstream of STATs, involving tyrosine kinases such as Src, JAKs, epidermal growth factor receptor (EGFR) and others, or overexpression of these upstream tyrosine kinases. In a study examining STAT3 activation in human breast cancer cell lines, STAT3 was shown to be activated due to a cooperation between c-Src and EGFR on cells over-expressing both of these receptors.[71] In head and neck squamous cell carcinoma (HNSCC) in humans, STAT3 activation is extremely common, but activating mutations in STAT3 have not been discovered, suggesting the activation must come upstream from STAT3, or be due to reduction in negative regulation of STAT3.[80]

However, there is evidence to show that STAT3 can act as an oncogene, without the need for constitutive activation upstream. Substituting two cysteine residues in the SH2 domain of STAT3 results in dimerization without the need for tyrosine phosphorylation.[70] When constitutively activated, STAT3 causes transformation of immortalized fibroblasts, as well as tumor formation on nude mice.[70]

STAT3 plays an important role in the process of malignant transformation. In v-src transformed cells, STAT3 is required for the transformation, as well as the maintenance of transformed cells. This has been demonstrated in vitro, in which STAT3 knock-out fibroblast colonies showed significant reduction in transformation after being infected with a v-src retrovirus, as compared to wild-type STAT3 fibroblasts.[81] This was also demonstrated in vivo, in which mice with wild-type STAT3 v-src transformed cells inoculated in nude mice grew larger tumors more rapidly compared to STAT3 knock-out transformed cells.[81] Interestingly, in this same study, STAT3 was not necessary for normal fibroblast growth.

The activation status of STAT3 also carries prognostic information. In a study of human patients with anaplastic large cell lymphoma, both with and without expression of anaplastic lymphoma kinase (ALK), a kinase shown to be associated with STAT3 activation, activation of STAT3 was examined for correlation with survival. In the ALK-negative patients, those without STAT3 activation had a significantly improved prognosis than those that with STAT3 activation.[82] In ALK-positive patients, the same statistical significance was not seen, but all five patients in that group without STAT3 activation survived without treatment failure in the follow-up period.[82] In another study of human patients with osteosarcoma, expression of phosphorylated STAT3 (pSTAT3) was

associated with survival, with those patients with increased expression of pSTAT3 having a worse prognosis than patients with low levels of pSTAT3 expression.[83]

STAT3 activation is also seen in genitourinary tumors, including prostatic carcinoma, urothelial carcinoma, and renal cell carcinoma. In these tumors, STAT3 promotes tumor angiogenesis, progression from carcinoma *in situ* to invasive disease, and epithelial-to-mesenchymal transition (EMT).[84] In one study examining the activation of STAT3 in human prostate tumor tissues and cell lines, the majority of the tissues showed expression of phosphorylated STAT3 in tumor tissues, as well as all cell lines examined. In this study, blocking STAT3 signaling resulted in decreased growth and increased levels of apoptosis in prostate tumor cell lines.[85] In urothelial carcinomas, STAT3 expression is seen in high grade, invasive tumors.[86,87] STAT3 has been shown to be prognostic in renal cell carcinomas, and is associated with development of metastatic disease.[88]

Activation of STAT3 also has a role in evasion of the immune system, which is a common feature among tumors. STAT3 can diminish tumor cell production of pro-inflammatory cytokines, including interferon-β (IFN-β), tumor necrosis factor-α (TNF-α), and IL-6, as well as proinflammatory chemokines RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) and IP-10.[89] In one study using tumor cell lines, deletion of STAT3 from tumor cells induced expression of these various pro-inflammatory proteins. Additionally, the introduction of constitutively active STAT3 in cells lacking STAT3 caused inhibition of these inflammatory mediators.[89] In this study, it was also shown that STAT3 inhibits dendritic cell maturation. Dendritic cells exposed to supernatant from tumor cells lacking STAT3 expressed larger amounts of maturation markers, including IL-12, MHC class II, and CD40.[89] These dendritic cells were also

better at activating antigen-specific T cells. Inhibition of dendritic cell maturation appeared to be in part due to expression of vascular endothelial growth factor (VEGF) and IL-10 from tumor cells.[89]

In another study looking at the effect of STAT3 and antitumor immunity, the ablation of STAT3 from hematopoietic cells significantly improved antitumor immunity. Increased activation of tumor infiltrating dendritic cells, neutrophils, and NK cells was seen in STAT3-negative mice, as well as increased infiltration of tumor-associated T cells into tumor tissue.[90] In this study, mice with STAT3-negative hematopoietic cells also showed decreased tumor growth when inoculated with mouse melanoma and bladder carcinoma cell lines, whereas significant tumor growth was seen in mice without STAT3 deletion.[90]

Mutation of PTPs is a common finding in many human cancers. PTPRT has been shown to function as a tumor suppressor, but is also the most commonly mutated PTP in human cancers, including colorectal cancer, lung tumors, HNSCC, and gastric cancer.[34,35,80] STAT3 is a substrate of PTPRT, and the mutation of PTPRT provides another way STAT3 can function to promote cancer development.[34]

STAT3 contributes to evasion of apoptosis through interaction with a transcription factor from the AP-1 family, c-Jun. In both mouse and human melanoma cell lines, STAT3 and c-Jun, in cooperation, cause decreased transcription and cell surface expression of Fas, a cell surface receptor that is integral to the formation of the death-inducing signaling complex and promotion of programmed cell death.[22] Inhibition of STAT3 and/or c-Jun expression in melanoma cells resulted in the cell surface expression of Fas, and programmed cell death was able to proceed.[22]

Dysregulation and activation of STAT5 is seen in many hematopoietic neoplasms in people, including chronic myeloid leukemia (CML), acute myeloid leukemia (AML), acute lymphocytic leukemia (ALL), and T-cell leukemias and lymphomas.[91,92] STAT5 is in fact a target for BCR/Abl, which is a constitutively active tyrosine kinase that is important in the development of CML.[93] In one study evaluating the effect of STAT5a and STAT5b suppression on CML and AML cells, proliferation of both cell lines was reduced and apoptosis was increased, demonstrating the importance of STAT5 for growth in these neoplasms.[94] STAT5 also appears to play a role in IL-9 induced proliferation of lymphoid cells. IL-9 is a cytokine that has little effect on normal T cells, but lymphomas appear to proliferate on stimulation with IL-9.[95] In one study, mutation in the binding site for STAT5 on the IL-9 receptor caused significant reduction in proliferation of a pro-B cell line BaF3, and STAT5 activation was also significantly reduced.[95] Additionally, tumorigenic clones of the pro-B cell line as well as a T-helper cell line, TS1, showed constitutive activation of JAK1 and STAT5.[95]

STAT5 is also involved in solid tumors, including breast cancer. Treatment with progestin, a synthetic progestogen that has effects similar to progesterone, causes upregulation of STAT5 in breast cancer cells, and promotes its translocation to the nucleus.[96] In another study using a human breast cancer cell line, treated with recombinant human prolactin, increased expression of phosphorylated JAK2 and STAT5 were seen.[97] To determine if STAT5 contributes to carcinogenesis, another study was performed in which a mouse model of mammary cancer was used to determine if reduced STAT5a expression was associated with reduced survival of cancer cells. Mice with reduced STAT5a expression showed delayed progression of mammary tumors compared

with wild-type mice, with 100% of the wild-type mice showing development of tumors by 7 months old, in comparison to 67% of mice heterozygous for STAT5a.[98] Additionally, the mean age of tumor development was older for heterozygous mice, and they had a lower tumor burden compared to wild-type mice. Comparison of tumor grades between the mice populations did not differ significantly.[98] In a study looking at STAT5 activation in human breast cancer specimens, STAT5 activation was shown to be prognostic in disease-free survival. Interestingly, activation of STAT5 was associated with a better prognosis in the cases examined, especially in those cases with lymph nodes negative for metastatic disease.[99]

STAT5b activation is also implicated in the proliferation of HNSCC. A study looking at STAT5 activation in HNSCC showed that the levels of STAT5a and STAT5b phosphorylation were higher in HNSCC cell lines as compared to normal epithelial cell lines.[100] In this same study, deletion of STAT5a had no effect on proliferation of HNSCC cell lines, whereas deletion of STAT5b caused growth inhibition, demonstrating the need for STAT5b in proliferation.

Another solid tumor that has shown overexpression of STAT5 is melanoma. In one study, STAT5 was overexpressed in all melanoma cell lines examined, and expression of STAT5 also correlated with resistance to IFNα-mediated reduction in proliferation. In addition, in an IFNα resistant melanoma cell line, depletion of STAT5 resulted in reduced cell proliferation, demonstrating its importance in cell growth of the melanoma cell lines.[101]

In addition to STAT3 and STAT5, STAT6 has also been shown to be activated in hematopoietic neoplasms. STAT6 is predominantly activated by two cytokines, IL-4 and

IL-13.[102] IL-13 is also a cytokine that is produced in many Hodgkin lymphomas (HL), from Hodgkin and Reed-Sternberg cells (HRS).[103] In a study looking at cell lines and tissues from patients with HL, phosphorylated STAT6 was expressed in all cell lines and most tissues evaluated, but expression was seen infrequently in non-Hodgkin lymphomas.[103] This suggested that STAT6 is important in the pathogenesis of HL. The blockage of IL-13 signaling in HL cell lines in this study prevented STAT6 phosphorylation, and following treatment with IL-13 in these cell lines, STAT6 phosphorylation was restored. STAT3 was shown to be activated in HL cell lines and tissues as well, but was not specific to HL, showing activation in NHL tissues, inflammatory cells, and normal lymphoid tissues.[103]

STAT6 is also activated in human prostatic carcinoma. A study looking at STAT6 activation in human prostate tissues showed that, compared to normal prostatic tissue, and tissues adjacent to cancerous prostate tissue, STAT6 activation is significantly higher in carcinomas.[104] STAT4 is also shown to be activated in prostate carcinoma, but shows no difference in activation between neoplastic and normal prostate tissues. STAT1, STAT2, and STAT5 were also evaluated in this study, and none showed evidence of activation within prostate carcinoma tissues.

JAK proteins have implications in human neoplasia as well. Probably one of the most well studied JAK mutations, JAK2V617F, is a mutation present in the JH2 pseudokinase domain of JAK2, and is seen in myeloproliferative neoplasms (MPN). The mutation in the JH2 domain results from a valine to phenylalanine substitution, which interferes with the inhibitory function of the JH2 domain, conferring constitutive activation to the JH1 kinase domain.[105,106] In a study with human and murine cell lines, transfection with the

JAK2V617F mutant allowed cells to grow in the absence of growth factors for three weeks, and maintain proliferation rates comparable to stimulated wild-type cells, indicating the constitutive activation of mutated JAK2.[107] This mutation has been shown to be present in the majority of patients with polycythemia vera (PV), 80% to 90% in some studies.[108,109] JAK2V617F is also seen in 30% to 50% of patients with primary myelofibrosis (PMF) and essential thrombocytosis (ET).[108,109] These patients have increased STAT3 and STAT5 activity, suggesting activation conferred from mutated JAK2. The JAK2V617F mutation is also found less commonly in other myeloid neoplasms, including acute myeloid leukemia (AML) preceded by PV or PMF, Philadelphia-chromosome negative chronic myelogenous leukemia, megakaryocytic AML, and chronic myelomonocytic leukemia.[110]

JAK2 is activated through other ways, in addition to the JAK2V617F mutation. A fusion protein consisting of JAK2 and TEL, a transcription factor, has been shown to cause constitutive activation to leukemia cells from childhood T cell acute lymphoblastic leukemias, as well as a childhood B-precursor acute lymphoblastic leukemia (ALL) and an adult atypical CML.[111,112] A model of TEL-JAK2 induced leukemia was created using transgenic mice for this fusion gene. All mice developed CD8+ T-cell leukemia between 4 to 22 weeks of age, and displayed splenomegaly, lymph node and thymic enlargement, and a moderate to marked leukocytosis.[113] Other JAK2 fusion proteins identified in hematopoietic neoplasms in humans include BCR-JAK2, PCM1-JAK2, and RPN1-JAK2.[91,114,115]

In addition to JAK2, other members of the JAK family are implicated in the pathogenesis of cancer. In one study, JAK1, JAK3, and Tyk2 were all investigated for

evidence of mutations in both adult hematopoietic and solid tumor tissues.[116] JAK1 mutations were found in four acute leukemias, three of which were T-cell leukemias, one breast carcinoma, and one non-small cell lung cancer. JAK3 mutations were seen in two breast carcinomas and one gastric carcinoma. No mutations in Tyk2 were detected in any of the 494 tumor tissue samples investigated. Three of the JAK1 mutations in this mutation were identical, being a V658F mutation. This mutation is homologous to the JAK2V617F mutation.[116] The JAK1V658F mutation, along with a mutation in Tyk2 (V678F), has been shown in a study to confer constitutive activation to these proteins.[117] In this study, a homologous mutation on JAK3 did not result in constitutive activation. In another study looking at JAK1 mutations in adult T-ALL and B-ALL, 18.4% of T-ALL cases had mutations in JAK1, whereas only 3.4% of B-ALL cases had mutations.[118] The patients with T-ALL carrying JAK1 mutations showed statistically significant reductions in disease free survival and overall survival, compared to those patients without JAK1 mutation.

D. JAK-STAT Inhibition in Human Medicine

Due to the significant amount of research identifying activating mutations and constitutive activation of the JAK-STAT pathway in numerous cancer types, interest in therapies targeting this pathway developed. Investigation into inhibition of the components of this pathway has yielded many inhibitors that may play a role in anti-cancer treatment.

Ruxolitinib and tofacitinib are two JAK inhibitors that have gained FDA approval for the treatment of myelofibrosis and rheumatoid arthritis, respectively. Tofacitinib was originally designed as a selective JAK3 inhibitor, but through trials, was found to also

cause JAK1 inhibition, and JAK2.[119] It gained FDA approval in 2012, and is currently being investigated for efficacy in the treatment of a number of immune-mediated diseases, including ulcerative colitis, alopecia, keratoconjunctivitis sicca, transplant rejection, and psoriasis.[119,120] The efficacy of tofacitinib in neoplastic diseases has not been studied.

Ruxolitinib inhibits JAK1 and JAK2, and was FDA approved in 2011. It is used for the treatment of myelofibrosis and polycythemia vera, although there are other trials currently investigating the use of this drug in other malignancies. Its mechanism of action is through competitive inhibition at the ATP binding site on the JH1 kinase domain, and results in downstream hypophosphorylation of STAT proteins, especially STAT3.[120-122] This drug has similar efficacy in both the wild-type JAK2, as well as the JAK2V617F mutation.[123] Adverse events with ruxolitinib include thrombocytopenia and neutropenia as dose-limiting toxicities.[120,123] In trials with human patients affected with MPNs, ruxolitinib has shown reduction in spleen volume and length, hematocrit, as well as reduction in pro-inflammatory cytokines and improvement in clinical signs and symptoms.[120,121] Recently, treatment with ruxolitinib has been investigated in patients with AML, ALL, and CLL. Most patients in these studies had relapsed leukemia that was refractory to previous treatments. In a phase II study looking at patients with relapsed leukemias, modest responses were seen, and the drug was well tolerated, with two patients out of 38 achieving complete remission.[124] In another study in patients with refractory acute leukemias, complete remission was not seen in any of the 27 patients treated, and the trial was stopped early due to lack of efficacy.[125] These patients had all been heavily pretreated with other anti-neoplastic agents, which may have been a reason

for failure of the trial. In a case report of a 17-year-old girl with ALL and a JAK mutation, treatment with ruxolitinib and a modified chemotherapy protocol resulted in complete remission prior to stem cell transplant.[126] In a clinical trial currently enrolling patients with CLL, both treatment-naïve and pre-treated, patients have experienced improvement in clinical signs with ruxolitinib treatment, including reduction in fatigue.[127]

Additional JAK inhibitors include pacritinib, fedratinib, momelotinib, and lestaurtinib. Pacritinib is a JAK2 inhibitor that also functions to inhibit other kinases, including FLT3, IRAK1, and CFS1R.[128] In a phase I/II study investigating pacritinib in patients with myelofibrosis and AML, the overall clinical benefit was 86%.[128] The dose limiting toxicity in this study was diarrhea and cardiac abnormalities. Fedratinib is a JAK2 inhibitor that also showed reduction in STAT3 activity, but development was discontinued when patients being treated developed encephalopathy.[120] Momelotinib is JAK1 and JAK2 inhibitor, which has shown promise in patients with PMNs, and studies of this drug are currently ongoing.[120] Lestaurtinib inhibits JAK2, JAK3, and FLT3, and has been investigated in patients with PMNs.[108] Thrombosis has been seen with this drug, and is a concern for treatment.

Development of specific STAT inhibitors has been more difficult due to the high drug concentrations necessary to cause clinical effect *in vivo*. One way to cause STAT inhibition is through JAK-STAT pathway inhibition upstream of STAT activation at the level of the JAK proteins. Other tactics include blocking dimerization and/or phosphorylation of STAT proteins through interference at the SH2 domain, inhibition of STAT-DNA binding, and inhibition of STAT transcription.[80] Blocking IL-6 cytokine

signaling at the cytokine receptor is another tactic, as IL-6 is known to activate STAT3 through JAK1/2 activation.[80,129]

Treatment of cancer lines with small molecule inhibitors of STAT3, which interfere with the SH2 domain, has been shown to inhibit cell proliferation *in vitro*. LLL12 is one such small molecule inhibitor, the use of which has been investigated in multiple human tumor cell lines, including pancreatic cancer, breast cancer, hepatocellular carcinoma, meduloblastoma, and glioblastoma.[130-133] In these studies, inhibition of STAT3 in cancer cell lines resulted in growth inhibition and reduced cell viability, as well as inhibition of the expression of STAT3 downstream target genes. In the studies looking at breast cancer and hepatocellular carcinoma, mouse xenograft models were used to test LLL12 *in vivo*, with successful suppression of tumor growth in both models.[131,132] FLLL32, derived from curcumin, is another small molecule inhibitor of STAT3. It has been shown to inhibit STAT3 phosphorylation in human hepatocellular carcinoma, pancreatic carcinoma, breast cancer and melanoma cell lines, as well as cause tumor cell apoptosis and inhibition of gene expression of downstream targets of STAT3 in melanoma, breast cancer, and pancreatic carcinoma cell lines.[133-136] STX-0119 is a small molecule inhibitor of STAT3 that has been investigated in xenogenic mouse models of lymphoma.[137] Treatment with STX-0119 caused inhibition of tumor growth and tumor apotosis in the xenogenic mice, as well as inhibition of expression of downstream targets of STAT3 in these mice.

A number of phase I clinical trials have been performed with small-molecule STAT3 inhibitors, with varied results. One phase I trial of patients with solid tumors refractory to other treatments received OPB-51602.[138] Dose-limiting toxicities included hyponatremia and dehydration, with other common toxicities including fatigue and GI signs. Two

patients, both with non-small cell lung cancer, experienced partial responses. In another phase I study, the same inhibitor, OPB-51602, was investigated in patients with refractory hematological malignancies.[139] No clinical responses were seen in this patient group, and long-term administration was difficult due to side effects; therefore, further development of OPB-51602 was discontinued. Another STAT3 small molecule inhibitor, OPB-31121, was investigated in a phase I trial in patients with solid tumors.[140] In these patients, dose limiting toxicities were vomiting and diarrhea, and no clinical responses were seen in any of the thirty patients treated.

Another tactic for targeting the SH2 domain of STATs is through peptide mimetics, which act through preventing STATs from binding to activated cytokine receptors, therefore preventing phosphorylation of STATs and inhibiting downstream gene transcription.[80] PM-73G is such a peptide mimetic that was studied in mice xenografts of human breast cancer.[141] In these mice, PM-73G inhibited tumor growth, and inhibition of STAT3 was seen in tumor samples from the mice. Golotimod is another peptide mimetic that inhibits STAT3 phosphorylation, and while it has not been studied for anticancer properties, has been shown to cause reduction in oral mucositis in animal models and people post-radiation.[80]

Cryptotanshinone is an herbal product that has shown ability to interact with the SH2 domain of STAT3 to prevent phosphorylation and activation. In a study using prostate cancer cell lines, cryptotanshinone inhibited tumor cell growth through 96 hours of treatment, and this inhibition was found to be JAK2-independent.[142] Downstream proteins of STAT3, including cyclin D1 and Bcl-xL, showed reduced expression after treatment with cryptotanshinone in this study.

Use of an oligonucleotide STAT3 decoy, which bind to phosphorylated STAT3 and prevent binding to DNA, has been examined in a phase 0 study in people with HNSCC.[143] Patients were given intratumoral injections of this decoy, compared to saline, and tumors were biopsied prior to treatment and post-treatment (at time of surgery). Reduced protein expression of STAT3 downstream target genes was seen in the STAT3 decoy patients as compared to the saline group, and no toxicities were reported in this study.

Antisense oligonucleotides (ASO) have been designed to inhibit STAT3 mRNA to cause decreased expression. AZD9150 is an ASO that has been investigated in a phase I trial in human patients with advanced lymphoma and solid tumors.[144] Thrombocytopenia was the dose-limiting toxicity, and commonly seen toxicities were liver enzyme elevations. 44% of patients in this study experienced a partial response or stable disease.

Sunitinib, a receptor tyrosine kinase inhibitor, has also been shown to cause STAT3 inhibition in a human renal cell carcinoma line, causing inhibition of STAT3 phosphorylation as soon as two hours into treatment.[145] Additionally, in this study, xenogenic mice for renal cell carcinoma showed reduced tumor growth after treatment with sunitinib.

E. JAK-STAT Pathway in Veterinary Medicine

While there is a significant amount of research on the JAK-STAT pathway in both normal function, dysfunction, and inhibition in human medicine, this type of information is lacking in veterinary medicine.

The most research into this pathway has been in canine and feline mammary carcinoma. In a study from 2001 looking at the effect of growth hormone (GH) in a

canine mammary carcinoma cell line, treatment with GH was shown to induce phosphorylation of STAT5a and STAT5b in tumor cells, in a dose-dependent manner.[146] A study from 2006 evaluated the expression of STAT3 in feline mammary gland tumors.[147] Correlation between STAT3 expression and spay status, tumor grade, mitotic index, tubular score, and nuclear pleomorphism was evaluated in each sample. There was no significant correlation between spayed or intact cats and STAT3 expression. Significant correlations were seen between STAT3 expression and tubule formation, nuclear pleomorphism, mitotic index, and tumor grade. In a follow up study in 2007, the expression of STAT3, phosphorylated specifically at the amino acid residue tyr705, was evaluated in feline mammary tumors for relationship to tumor grade.[148] The majority of tumor cells (80%) showed positive cytoplasmic and nuclear staining. Significant correlations were seen between nuclear STAT3-p-tyr705 expression and tubule formation, mitotic index, and tumor grade. In this study, hyperplastic mammary tissues were also evaluated. STAT3-p-tyr705 expression was significantly higher in tumor tissues as compared to hyperplastic lesions. Another study in 2007 of feline mammary tumors evaluated expression of STAT3 phosphorylated specifically at the amino acid residue ser 727.[149] Significant correlation was seen between nuclear expression of STAT-p-ser727 and nuclear pleomorphism, mitotic index, and tumor grade.

A study from 2011 looked at canine mammary tumors and correlation between pSTAT3 expression and the likelihood of a tumor to metastasize.[150] Higher pSTAT3 expression was seen in the tissue samples from metastatic mammary carcinomas, as compared to those from non-metastatic mammary carcinomas. In this study, cell lines from metastatic and non-metastatic tumors were also examined for expression patterns,

and the metastatic lines showed nuclear and cytoplasmic expression, whereas only cytoplasmic staining was seen in the non-metastatic cell lines. A study in 2016 showed similar results regarding pSTAT3 expression in canine mammary tumor metastasis.[151] In this study, expression of pSTAT3 was higher in primary tumors that had metastasized, compared to mammary tumors in which metastasis had not occurred. In 2015, a study using equine mammary tumors showed STAT3 nuclear expression in three of seven samples evaluated.[152]

A relatively large amount of research into the JAK-STAT pathway, as well as STAT inhibition, has been performed in canine osteosarcoma (OSA). A study in 2009 examined the expression and activation of STAT3 in canine and human OSA.[153] In this study, STAT3 was shown to be phosphorylated in both cell lines and tissues from canine and human OSA, while normal canine osteoblasts did not show STAT3 phosphorylation. A small molecule inhibitor of Src, SU6656, was also used to determine if this could reduce STAT3 phosphorylation in this study. Concurrent with Src inhibition, downregulation of STAT3 phosphorylation was also seen after treatment with SU6656, and use of this inhibitor also caused reduction in DNA binding of STAT3. Direct blocking of STAT3 with another small molecule inhibitor, LLL3, also caused inhibition of STAT3 DNA binding. Use of either of these small molecule inhibitors in canine and human OSA cell lines caused reduced proliferation and increased apoptosis.[153]

A study in 2009 looking at an inhibitor of heat shock protein 90 (HSP90), STA-1474, in canine and human OSA cell lines, showed reduced cell viability and proliferation, as well as increased apoptosis in cell lines.[154] In this study, reduced expression of a number of proteins, including pSTAT3, was seen after treatment with STA-1474. A phase I study

of STA-1474 in dogs with a variety of tumor types, including one OSA, showed a 24% response rate and a 36% overall biological activity.[155] The dog with OSA experienced a partial response to treatment with STA-1474 for 20 weeks.

In a 2011 study, the role of Oncostatin M (OSM) in STAT3 activation of canine and human OSA cell lines was studied.[156] OSM is a cytokine produced by inflammatory cells, as well as some tumor cells in human cancers, including osteosarcoma. OSM receptor binding causes JAK2 activation, which leads to STAT3 activation. In canine and human cell lines stimulated with OSM, JAK2 and STAT3 phosphorylation increased within five minutes of treatment. In another study from 2011, looking at a small molecule inhibitor of STAT3, FLLL32, OSA canine and human cell lines showed growth inhibition and increased apoptosis after treatment.[135] FLLL32 also decreased STAT3 DNA binding, and decreased pSTAT3 and STAT3 expression in OSA cell lines in this study.

A 2012 study looked at the use of LLL12, a small molecule inhibitor of STAT3, in canine OSA cell lines.[157] LLL12 inhibited proliferation and increased apoptosis in OSA cell lines, as well as decreased expression of pSTAT3 and downregulated STAT3-induced gene transcription. LLL12 was also combined with doxorubicin, which improved the anti-proliferative effects of doxorubicin on the OSA cell lines.

STAT3 expression and phosphorylation has been examined in a number of other canine and feline tumor types. In a study looking at STAT3 expression in hemangioma and hemangiosarcoma (HSA) in dogs, significant differences were seen in the percentage of positive cells between the two tumor types, with HSA having a higher percentage of positive cells.[158] STAT3 nuclear expression was also common in the HSA cells, whereas

nuclear expression was uncommon in hemangioma cells. Phosphorylated STAT3 has been identified in canine HSA cell lines in another study.[159]

In a study of canine prostate cancer, tissues from dogs with prostatic carcinoma showed a higher percentage of cells positive for STAT3, as well as a stronger staining intensity for STAT3, as compared to tissues from dogs with benign prostatic hypertrophy (BPH).[160] In this study, solid prostatic carcinoma tissues had a greater number of positive cells compared to cells with a more differentiated, tubular histologic pattern. In a study of canine mast cell tumors before and after treatment with prednisone, STAT3 was expressed in all pre- and post-treatment mast cell tumors (MCT), while pSTAT3 expression was significantly reduced in post-treatment MCT.[161]

Tissues from cats with injection site sarcomas (ISS), both untreated and treated with doxorubicin, were examined for expression of STAT3.[162] Those cats that had been treated with doxorubicin showed small reductions in STAT3 expression as compared to those cats that did not receive doxorubicin treatment. Tumor tissues from cats receiving doxorubicin treatment also demonstrated lack of nuclear STAT3 expression, whereas tissues from untreated cats did show STAT3 nuclear expression. Mitotic index was correlated with STAT3 expression in all tumors in this study. In a study of feline oral squamous cell carcinoma (SCC), expression of pSTAT3 was variable, with some cell lines showing increased expression compared to others, which also correlated with response to LLL12, a STAT3 small molecule inhibitor.[163] Those cell lines with high expression of pSTAT3 showed the most reduction in cell proliferation. Cell lines with high expression of pSTAT3 also showed more apoptosis after treatment with LLL12.

There has been a small amount of research in canine malignancies and JAK expression. In one study of canine primary polycythemia, a mutated JAK2 was identified in one dog, with amino acid modifications at V617F, and C618L.[164] These mutations in JAK2 conferred constitutive activation of hematopoietic cells in this dog. JAK2V617F is the same mutation seen consistently in polycythemia vera in humans. In a study on tyrosine kinases in canine malignant melanoma, JAK1 expression was identified using RT-PCR.[165]

## Chapter II: Identification of the Presence and Activity of the JAK-STAT Pathway in Multiple Canine Tumors

### A. Introduction

The JAK-STAT pathway is a highly conserved cellular signaling pathway. There are four Janus kinases (JAKs): JAK1, JAK2, JAK3, and Tyk2, and seven signal transducers and activators of transcription (STATs): STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6. Ligand binding activates receptor associated JAKs, leading to autophosphorylation. Latent cytoplasmic STATs are recruited to the activated receptor-JAK complex, are phosphorylated and dimerize via phosphotyrosine-SH2 interactions[119,166]. Phosphorylated STATs translocate to the nucleus, binding to specific promoter sequences on DNA-response elements to initiate transcription of genes involved in differentiation and the regulation of the cell cycle and apoptosis, including cyclin D1, c-myc, p21, and Bcl family members[2,12,166].

Dysregulation of the JAK-STAT pathway has been implicated in human malignancies, including myeloproliferative disorders, acute leukemias, lymphomas, breast cancer, head and neck squamous cell carcinoma, melanoma, lung carcinoma, prostate carcinoma, meningioma, and glioma [82,119,129,166]. More than 90% of patients with polycythemia vera, and many patients with essential thrombocytosis and primary myelofibrosis, have an acquired recurrent mutation in the JAK2 gene V617F which confers constitutive activity [119]. Aberrant STAT3 and STAT5 signaling have been found in both solid and hematologic cancers, with a high incidence of abnormal STAT3 activation [83,119,166]. Clinically, pSTAT3 expression has been associated with a poorer prognosis in multiple tumor types [82,83].

The JAK-STAT pathway holds promise as a therapeutic target in tumors with aberrant signaling. Small molecule inhibitors of the various JAKs and STATs have been developed in human medicine for the treatment of multiple tumor types [83]. In human tumor cell lines, STAT3 inhibition led to decreased expression of anti-apoptotic proteins and chemotherapy sensitization [166]. Additionally, inhibition of STAT3 *in vitro* inhibited v-src mediated fibroblast transformation but did not appear to affect the proliferation of normal fibroblasts [81].

The existing literature suggests that the JAK-STAT pathway may be of importance in veterinary medicine, as well. STAT3 has been shown to have constitutive activity in canine osteosarcoma, and small molecule inhibitors of STAT3 have *in vitro* activity against canine osteosarcoma cell lines [135,153,154,156,157]. STAT3 and STAT5 expression have been identified in canine and feline mammary tumors [146-150], and aberrant expression of STAT3 has been reported in canine mast cell tumors, canine hemangiosarcoma, canine prostate carcinoma, feline injection site sarcoma, and feline oral squamous cell carcinoma [160-162,167,168]. JAK1 expression has been demonstrated in canine malignant melanoma, and the V617F mutation has been identified in a dog with primary polycythemia [164,165].

The purpose of this study was to determine the presence and activity of JAK1, JAK2, and STAT3 as part of the JAK-STAT pathway in four different canine tumors: splenic hemangiosarcoma (HSA), mast cell tumor (MCT), apocrine gland anal sac adenocarcinoma (AGASACas), and thyroid carcinoma. The hypothesis was that the JAK-STAT pathway would be present and active in each tumor type investigated. A secondary

objective was to determine if activity of the JAK-STAT pathway was related to treatment response or prognosis.

## B. Materials and Methods

### Case selection

Tumor specimens were obtained from Virginia Tech Animal Laboratory Services (ViTALS). Case logs from ViTALS were searched from June 2013 to January 2016 for cases with a histological diagnosis of splenic hemangiosarcoma, high grade mast cell tumor, apocrine gland anal sac adenocarcinoma, or thyroid carcinoma. High grade mast cell tumors in this study were defined as either a diagnosis of Kiupel high grade and/or Patnaik grade III mast cell tumor. Cases were considered eligible if all of the following criteria were met: they had one of the four aforementioned diagnoses, the tumor specimen was obtained surgically through the Virginia Maryland College of Veterinary Medicine's Veterinary Teaching Hospital (VMCVM VTH) in Blacksburg VA, or through the VMCVM satellite clinic in Roanoke VA, and formalin-fixed paraffin-embedded tissues were available. Patient signalment, date of diagnosis, tumor progression, and death, as well as treatment data, including history of chemotherapy and steroid administration, were collected from the medical record at the VMCVM VTH or Roanoke satellite clinic. Referring veterinarians were contacted via telephone for information not available in the medical record. High grade mast cell tumor samples and hemangiosarcoma samples were re-evaluated by one pathologist (TL) to confirm diagnosis.

### Antibodies and Controls

Rabbit anti-JAK1 monoclonal (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-JAK2 monoclonal (Cell Signaling Technology, Danvers, MA, USA), mouse

anti-STAT3 monoclonal (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-pJAK1-pY1022 polyclonal (MyBioSource, Inc., San Diego, CA, USA), rabbit anti-pJAK2-tyr1007 polyclonal (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), and rabbit anti-pSTAT3-tyr705 monoclonal (Cell Signaling Technology, Danvers, MA, USA) primary antibodies were used for all procedures. Formalin-fixed paraffin embedded HeLa cells (Cell Signaling Technology, Danvers, MA, USA) were used as positive controls.

Immunohistochemistry Procedure

All tumor sections were cut at 5 microns, placed on Precleaned Plus Slides, placed into the low temperature oven at 40 degrees Celsius for 30 minutes and then placed into the high temperature oven at 65 degrees Celsius for 30 minutes.

All samples were deparaffinized on the Ventana Benchmark XT (Ventana Medical Systems, Inc., Tucson, AZ, USA) using Ventana EZ Prep Pre-Dilute solution (Roche Diagnostics GmbH, Mannheim, Germany) and rehydrated in Ventana Reaction Buffer (Roche Diagnostics GmbH, Mannheim, Germany). Antigen retrieval was performed with Ventana's Cell Conditioning One Solution (Roche Diagnostics GmbH, Mannheim, Germany) for 60 minutes at 96 degrees Celsius. Samples were then incubated with JAK1 at a dilution of 1:100, pJAK1 at a dilution of 1:25, JAK2 at a dilution of 1:100, pJAK2 at a dilution of 1:10, STAT3 at a dilution of 1:475, and pSTAT3 at a dilution of 1:100, all for 32 minutes at 37 degrees Celsius. Bound antibody was evaluated using the Ventana Amplification Kit (Roche Diagnostics GmbH, Mannheim, Germany), which was applied for 16 minutes at 37 degrees Celsius. Secondary antibody was then applied, using biotinylated goat anti-mouse/anti-rabbit antibody (Discovery Universal Secondary Antibody, Roche Diagnostics GmbH, Mannheim, Germany), for 30 minutes. This was

followed by application of Ventana's Enhanced Alkaline Phosphatase Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) for 40 minutes at room temperature. This kit included incubation with a streptavidin-alkaline phosphatase conjugate in Tris buffer with MgCl2 and ZnCl, and application of a naphtol substrate and fast-red chromogen. Ventana Reaction Buffer (Roche Diagnostics GmbH, Mannheim, Germany) was used as a rinse between each of the above steps. Sections were counterstained with Richard-Allan hematoxylin and air dried. Negative controls were performed by substituting the primary antibody with Ventana Antibody Dilution Buffer (Roche Diagnostics GmbH, Mannheim, Germany).

All tumor samples were stained with JAK1, pJAK1, JAK2, pJAK2, STAT3, and pSTAT3, and evaluated by one pathologist (TL). Positive staining for JAK1, JAK2, and STAT3 indicated presence of the JAK-STAT pathway, and positive staining for pJAK1, pJAK2, and pSTAT3 indicated activity of the pathway. Immunohistochemical labeling for each antibody in each tumor sample was assessed in ten high power fields (400x). The percentage of positive cells was scored as follows: 1 = 0% - 25% of tumor cells positive; 2 = 26% - 50% of tumor cells positive; 3 = 51% - 75% of tumor cells positive; and 4 = 76% - 100% of tumor cells positive [160]. The presence of nuclear and cytoplasmic staining was also recorded.

## Statistical Analysis

Correlation between IHC scores and variables assessed was evaluated using Spearman's coefficient and Kendall's Tau. Variables assessed included sex, neuter status, whether chemotherapy was administered, and presence of metastatic disease. Overall survival (OS), defined as the time from surgery until death from any cause, was

calculated using the Kaplan-Meier method. The log rank test was used to compare OS between groups. Dogs were censored from survival analysis if lost to follow up or still alive at the time of data collection. Statistical analysis was performed with commercial software. P values of $< 0.05$ were considered significant.

## C. Results

### Clinical Cases

Fifty-five tumors, comprising 55 different canine patients, were eligible for inclusion. One case had originally been diagnosed as AGASACA, but was determined to be malignant melanoma on immunohistochemistry performed for clinical purposes, and was excluded. Three mast cell tumor samples originally diagnosed as high grade mast cell tumors were determined to be low grade mast cell tumors on re-evaluation. The tumor series therefore included 54 total cases: 20 HSA, 15 MCT (12 high grade, 3 low grade), 10 thyroid carcinomas and 9 AGASACA. In all cases where positive immunohistochemical staining was identified, pSTAT3 staining was confined to the nucleus, while all other antibodies included intranuclear and intracytoplasmic staining.

### Hemangiosarcoma

Twenty cases of HSA were identified. There were seven castrated male dogs, two male intact dogs, nine female spayed dogs, and two female intact dogs. Sixteen were purebred dogs, and four were mixed breed dogs. Purebred dogs included four Boxers, four German Shepherds, two Labrador retrievers, and one each of the following breeds: beagle, Golden retriever, collie, Belgian shepherd, miniature poodle, and English springer spaniel. Median age of the 20 dogs was 10 years (range 6 years – 15 years). Median

weight was 30kg (range 3.6kg – 40.1kg). Eleven dogs were treated with chemotherapy; chemotherapy agents administered included doxorubicin, carboplatin, and vinorelbine. One patient had been treated prior to diagnosis with chlorambucil for chronic lymphocytic leukemia. Three dogs were lost to follow up after surgery and were censored. Death was confirmed from referring veterinarian records for one of these three dogs, but date of death was not known. The remaining 17 dogs were known to have died or been euthanized. Median OS for all dogs with HSA was 57 days (range 15 – 426 days). Eight dogs were identified to have intraabdominal metastatic disease either at diagnosis or through the course of treatment. Metastasis was identified via abdominal ultrasound imaging, histopathology, and/or necropsy. Dogs with metastatic disease had a statistically significant decreased median survival of 18 days, compared to 130 days for dogs without metastasis (Figure 2; $P = 0.02$). Three additional dogs had a hemoabdomen identified at the time of euthanasia, but further imaging or necropsy was not pursued in these dogs. One dog was euthanized due to post-operative complications associated with surgical correction of gastric dilatation-volvulus. At the time of euthanasia, this dog was 426 days post-surgery for HSA, with no evidence of metastatic disease on thoracic radiographs or abdominal ultrasound. The remaining eight dogs were either lost to follow up and censored (three dogs), or died of unknown causes (five dogs).

Immunohistochemical scores for HSA are presented in Table 1, and representative examples of IHC staining are presented in Figure 3. The highest scores were seen with STAT3 and pSTAT3 staining. Statistically significant correlations were seen between JAK1 and pJAK1 IHC scores ($P = 0.001$), JAK2 and pJAK2 IHC scores ($P = 0.001$), and STAT3 and pSTAT3 IHC scores ($P = 0.005$). A statistically significant difference in

survival was seen between dogs with pJAK1 scores of 1 and 2 (Figure 4). Median survival of dogs with a pJAK1 score of 1 was 78 days, compared to 15 days for dogs with a pJAK1 score of 2 ($P = 0.009$). There were no significant associations between IHC scores and sex, neuter status, or whether chemotherapy was administered.

## Mast Cell Tumors

Fifteen cases of mast cell tumors were identified; 12 were classified as high grade and three were classified as low grade. There were three male castrated dogs, one male intact dog, and 11 female spayed dogs. Twelve were purebred dogs and three were mixed breed dogs. Purebred dogs included three Labrador retrievers, two each of Boxers, pugs, and Staffordshire terriers, and one each of the following: Golden retriever, English setter, and shih tzu. Median age of the 15 dogs was 8 years (range 3years – 13 years). Median weight was 25.8kg (range 5.4kg – 38.1kg). Seven dogs were treated with chemotherapy; chemotherapy agents administered included vinblastine, lomustine, toceranib phosphate, and masitinib mesylate. Six of the 15 dogs had confirmed cytologic or histopathologic evidence of lymph node and/or visceral metastasis; five of these dogs were diagnosed with high grade MCT, and one dog diagnosed with a low grade MCT had metastasis to the local lymph node. Four dogs were censored; three were lost to follow up, and one dog was alive at the time of last follow up. The remaining 11 dogs were known to have died or been euthanized. Median OS for all dogs with MCT was 229 days (range 17-956). One dog with a high grade MCT that received adjuvant lomustine had a survival of 956 days. At the time of euthanasia, this dog had developed edema and lymphadenopathy in the

same limb as the previously diagnosed mast cell tumor. This was suspected to be due to mast cell disease, but was not confirmed histologically.

Immunohistochemical scores for MCT are presented in Table 2, and representative examples of IHC staining are presented in Figure 5. The highest scores were seen with JAK2 and STAT3 staining. Statistically significant positive correlations were seen between JAK1 and JAK2 IHC scores ($P = 0.0003$), JAK1 and pJAK1 IHC scores ($P = 0.03$), JAK2 and pJAK1 IHC scores ($P = 0.006$), JAK2 and pJAK2 IHC scores ($P = 0.01$), and JAK2 and pSTAT3 IHC scores ($P = 0.008$). pSTAT3 score was significantly associated with presence of metastatic disease ($P = 0.0008$). JAK1 and pJAK1 IHC scores were significantly correlated with survival in dogs with MCT. Median survival of dogs with a JAK1 IHC score of 3 (105 days) was significantly shorter compared to those of dogs with IHC scores of 1 or 2 (229 days and 343 days, respectively; $P = 0.03$, Figure 6). The median survival of dogs with a pJAK1 IHC score of 1 (343 days) was significantly longer than those of dogs with pJAK1 scores of 2 and 3 (124 and 29 days, respectively; $P = 0.04$, Figure 7). There were no significant associations between IHC scores and sex, neuter status, and whether chemotherapy was administered.

## Thyroid Carcinomas

Ten cases of thyroid carcinomas were identified. There were two male castrated dogs, six female spayed dogs, and two female intact dogs. Seven were purebred dogs and three were mixed breed dogs. Purebred dogs included two shih tzus, and one each of the following: beagle, Irish terrier, Jack Russell terrier, Rottweiler, and Briard. Median age of the 10 dogs was 9 years (range 6 years – 13 years). Median weight was 13.5kg (range

5.4kg – 50.1kg). One dog received toceranib phosphate treatment. Three dogs had evidence of pulmonary metastasis based on thoracic imaging with radiographs or CT. Metastatic disease was diagnosed over a year post-surgery in all three cases. The status of metastasis was unknown for the remainder of dogs due to lack of follow-up imaging. One dog died, three dogs were lost to follow up and censored, and the remainder of dogs were known to still be alive at the time of last follow-up and were censored. Median OS in this group of dogs was not reached, with 6/10 dogs being alive two years after surgery.

Immunohistochemical scores for thyroid carcinoma are presented in Table 3, and representative examples of IHC staining are presented in Figure 8. The highest scores were seen with JAK1 and STAT3 staining. A statistically significant correlation was determined between JAK2 and pJAK1 scores ($P = 0.02$). There were no significant associations between IHC scores and sex, neuter status, whether chemotherapy was administered, presence of metastasis, or OS.

## Apocrine Gland Anal Sac Adenocarcinomas

Nine cases of AGASACA were identified. There were five male castrated dogs, and four female spayed dogs. Seven were purebred dogs and two were mixed breed dogs. Purebred dogs included one each of the following: German shepherd, Hungarian pointer, Siberian husky, Brittany spaniel, Australian shepherd, Labrador retriever, and standard poodle. Median age of the nine dogs was 9 years (range 8 years – 13 years). Median weight was 25.2kg (range 20kg – 46.2kg). One dog received only an incisional biopsy; the remainder were excisional biopsies. Six dogs received chemotherapy, including carboplatin, toceranib phosphate, and melphalan. Eight dogs underwent staging

evaluation at the time of surgery. All of these eight dogs had evidence of regional lymph node metastasis at the time of surgery. Pulmonary metastasis was suspected in one of the eight dogs on thoracic radiographs, but was not confirmed histologically. One dog developed confirmed distant metastasis involving the liver, spleen, and ventral lumbar musculature during the course of treatment. Three dogs were censored from survival; one dog was lost to follow-up, the date of euthanasia could not be confirmed in one dog, and one dog was still alive at time of last follow-up. The remainder of dogs were known to have died or been euthanized. Median OS for all dogs was 216 days (range 100 days – 999 days).

Immunohistochemical scores for AGASACA are presented in Table 4, and representative examples of IHC staining are presented in Figure 9. The highest scores were seen with JAK1 and STAT3 staining. Statistically significant correlations were seen between JAK2 and pJAK2 scores ($P = 0.007$) and JAK1 and STAT3 scores ($P = 0.03$). There were no significant associations between IHC scores and sex, neuter status, whether chemotherapy was administered, presence of metastasis, or OS.

D. Discussion

This study demonstrated that the six investigated components of the JAK-STAT pathway, JAK1 and 2, STAT3, pJAK1 and 2, and pSTAT3, are present via immunohistochemical expression in four different canine solid tumors. The presence of phosphorylated JAK1 and 2 and intranuclear pSTAT3 also suggests that this pathway has activity in the tumors evaluated.

STAT3 is a transcription factor that is latent in the cytoplasm until phosphorylation causes dimerization of two STAT3 proteins. Phosphorylated STAT3, or pSTAT3, then

translocates to the nucleus, where it binds to specific sequences within the promoter sequences of DNA, called gamma activated sequences, or GAS elements [4]. This promotes transcription of the target gene. However, there is much evidence showing that this classical pathway of STAT3 activation is not the only way that STAT3 gains access to the nucleus. In addition to JAK activation, the activation of a number of other tyrosine kinases have shown the ability to activate and phosphorylate STAT3, including Src and EGFR [71]. Furthermore, STAT3 phosphorylation is not necessarily required for nuclear import. Unphosphorylated STAT3 is shuttled back and forth from the cytoplasm to the nucleus, and while it is unable to bind to promoter regions on the DNA in its unphosphorylated form, STAT3 can complex with other transcription factors, including c-Fos and c-Jun, in the nucleus, and bind DNA [14,21,22]. For this reason, we cannot say, due to the presence of intranuclear pSTAT3 in this study, that the JAK-STAT pathway is definitely active in these tumor types. However, our findings do identify all components of the pathway in all four tumor types, and can suggest activity of the pathway.

There has been some investigation into STAT3 and its association with neoplasia in the veterinary literature. The majority of the work has been in canine osteosarcoma (OSA), and canine and feline mammary carcinoma. Studies have identified STAT3 expression and phosphorylation in canine osteosarcoma cell lines and tissues [153]. Other studies have identified decreased pSTAT3 DNA binding and increased apoptosis in canine OSA cell lines after treatment with small molecule inhibitors of STAT3 [135,153,154,157]. pSTAT3 expression in canine and feline mammary carcinoma has been shown to correlate with mitotic index, tubule formation, and tumor grade, as well as the likelihood of tumor metastasis [147-150]. While we did not look at mammary carcinomas in

our study, the epithelial tumors we did evaluate, thyroid carcinoma and AGASACA, demonstrated high levels of STAT3 and pSTAT3 expression, suggesting that epithelial tumors may overexpress these proteins, and may be a druggable target for these tumor types.

Some interesting findings regarding JAK1 expression were noted in our study. In dogs with HSA, pJAK1 scores were significantly correlated with survival. Those dogs with scores of 1 had significantly longer median survival times than dogs with pJAK1 scores of 2. In MCT cases, JAK1 and pJAK1 were also associated with survival. Dogs with JAK1 scores of 3 had a significantly shorter median survival time than dogs with scores of 1 or 2. Dogs with pJAK1 scores of 1 had a significantly longer median survival time than dogs with scores of 2 or 3. Very few studies in the veterinary literature have evaluated JAK proteins in canine tumors. One study identified the presence of a JAK2 mutation in dogs with polycythemia vera that is identical to the JAK2 mutation in human polycythemia vera, JAK2617F; another identified JAK1 expression in canine malignant melanoma [164,165]. In humans, mutations in JAK1 have been identified that confer constitutive activation to the kinase [116,117]. Possible mutation of JAK1 may explain some of our findings, but if they were only due to JAK1 constitutive activation, we would not expect pJAK1 expression to have any correlation with survival. Future studies to further investigate the effect of overexpression of JAK1 and pJAK1 in multiple tumor types is warranted.

Significant correlations between JAK1, JAK2, STAT3, and their phosphorylated counterparts, was expected. We would anticipate that a higher score of the unphosphorylated protein would translate to a higher score in the phosphorylated protein.

The significance of the correlations between JAK1 and JAK2 in MCT, and JAK2 and pJAK1 in MCT and thyroid carcinomas is unknown. A possible explanation for these findings could be related to the cytokine receptor family activating these kinases. JAK1 and JAK2 are preferentially activated by a family of cytokines that use a receptor subunit gp130, including IL-6, IL-11, oncostatin M, leukemia inhibitory factor (LIF), and ciliary neurotrophic factor (CNF) [6,11]. It would be interesting to investigate the cytokines and cytokine receptors that activate JAKs in these tumors, to determine if this hypothesis is correct.

JAK2 and pSTAT3 scores were correlated in dogs with MCT, and JAK1 and STAT3 scores were correlated in dogs with AGASACA. This lends support to STAT phosphorylation though JAK activation. Inflammation of the tumor and the local tumor environment may also be an explanation for these findings. Both MCT and AGASACA can have a large inflammatory component. IL-6 is a pro-inflammatory cytokine that activates JAK1 and JAK2 associated with the IL-6 cytokine receptor, which leads to the STAT3 activation [75].

Other significant findings in the current study involved metastatic disease. Dogs with HSA having metastatic disease had a significantly shorter median survival time than those dogs without metastasis. This was not surprising; the majority of dogs with evidence of metastasic disease had lesions identified in the liver, omentum, and body wall, which were not amenable to surgical resection. We also found that pSTAT3 scores were associated with metastatic disease in dogs with MCT. To our knowledge, there is only one other study evaluating pSTAT3 expression in canine MCT [161]. This study did identify expression of pSTAT3, but did not evaluate survival; therefore, we are unable to

corroborate our findings with this study. Further research is necessary to determine if pSTAT3 is truly associated with survival, and whether inhibition of STAT3 may improve survival in dogs with high grade MCT.

Limitations of this study include the retrospective nature of the clinical cases. Due to incomplete information in the medical record and from rDVM records, no standardized treatments, and numerous cases lost to follow up, we were unable to evaluate the significance of many variables. Small case numbers in each group also limited our ability to identify significant differences between variables. The primary goal of this study was to identify the different components of the JAK-STAT pathway, and determine if this pathway was active in the four different tumor types we evaluated, so the lack of follow up information was not seen as a significant limitation in this study. Another limitation is the small number of low grade MCT cases included in the MCT group. These cases were initially diagnosed as high grade MCT, but after re-evaluation, were diagnosed as low grade. We included these in the study in the interest of case numbers and comparison of low grade vs high grade tumors. Three dogs out of 15 had low grade mast cell tumors. One of these dogs was lost to follow up shortly after surgery, but out of the other two, one dog was still alive at the time of follow-up, and another was lost to follow up over 1.5 years past surgery, which likely contributed to the long median survival time seen overall for dogs with mast cell tumors. Due to the small number of cases, we were unable to make any valuable comparisons between high grade and low grade MCT.

Another limitation in this study is the method used, immunohistochemistry. IHC is used routinely in research and the clinical setting to identify epitopes of interest in a sample. However, it is not a perfect test, as formalin fixation causes cross-linking of

epitopes. Antigen retrieval aims to restore immunoreactivity, but it is not 100% successful. For this reason, we likely did not identify the total amount of truly positive cells. Additionally, under- or over-fixation of a tissue specimen can interfere with immunoreactivity; we used only tissues collected from cases in-hospital, where surgery was performed by a board-certified surgeon or surgery resident to attempt to control for this, as we expected that fixation times would be standardized at the VTH and under- and over-fixation would be less likely.

Proteins are rapidly dephosphorylated in the body, and dephosphorylation continues after a tissue has lost blood supply. The activation-inactivation/phosphorylation-dephosphorylation cycle of a STAT protein is approximately 20 minutes, with almost no DNA binding activity noted 30 minutes after cessation of kinase activity [12,15]. Therefore, we suspect that the percentage of phospho-protein positive cells in this study is lower than it actually would be, as during surgery, there is time between vessel ligation, tumor removal, and fixation in formalin, during which proteins can be dephosphorylated.

## CHAPTER III: Conclusions

The results of this research demonstrate expression of the different components of the JAK-STAT pathway investigated, those being JAKs 1 and 2, pJAKs 1 and 2, and STAT3 and pSTAT3. These findings suggest that this pathway is present and active in the four tumor types evaluated. There are additional cell signaling pathways that these components of the JAK-STAT pathway may interact with and participate in, which were not evaluated in this research. Therefore, it is possible that some of the results seen in this study are due to activation through other kinases, such as Src or EGFR. Further study investigating presence and activity of this pathway through IHC, expanding on the number of tumor samples and including other tumor types, such as canine osteosarcoma and mammary carcinoma, should be pursued.

The significant associations of JAK1 and survival time in MCT, pJAK1 and survival time in HSA and MCT, and pSTAT3 and metastasis in MCT, are interesting. There is a lack of studies evaluating JAK1 expression and activation in the veterinary literature, so these findings cannot be corroborated with other studies. pSTAT3 has been evaluated for expression in a number of other canine tumors, but, to the author's knowledge, has not been correlated with survival in canine mast cell tumors. These findings indicate potential therapeutic avenues for targeting these proteins. Further study is necessary to determine if JAK1, pJAK1, or pSTAT3 could be therapeutic targets in the tumor types evaluated.

FIGURES

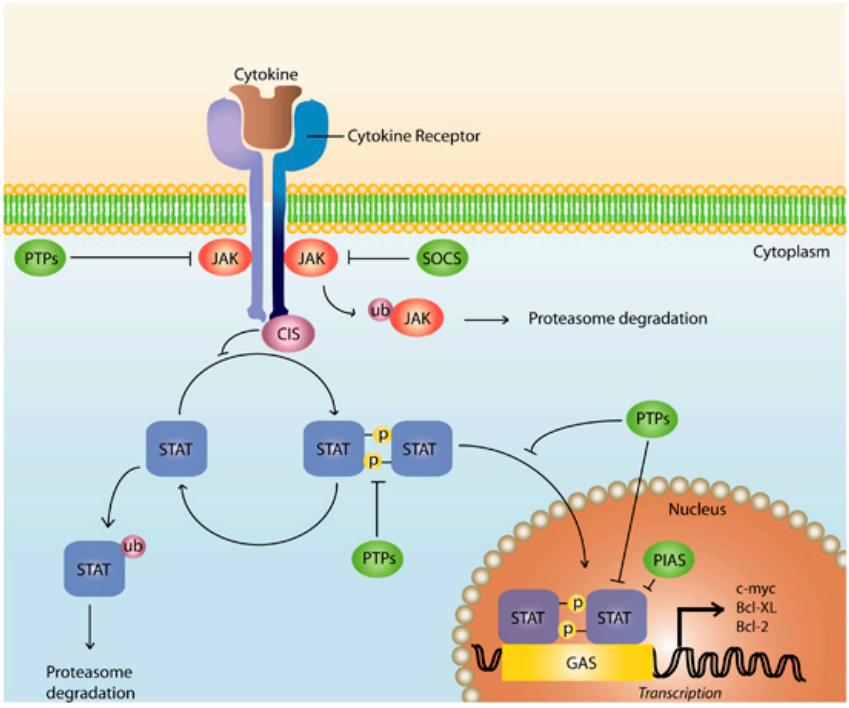


**Figure 1[5]; JAK-STAT pathway.** Upon cytokine binding, receptor dimerization results in JAK activation. This recruits STATs to the cytokine receptor, where they become phosphorylated through receptor-JAK interactions. Phosphorylation results in STAT dimerization and translocation to the nucleus. STAT dimers bind to GAS elements in the promoter region of the DNA, and initiate gene transcription. The pathway can be inhibited at several points, by protein tyrosine phosphatases (PTPs), suppressors of cytokine signaling (SOCS), and protein inhibitor of activated STATs (PIAS).

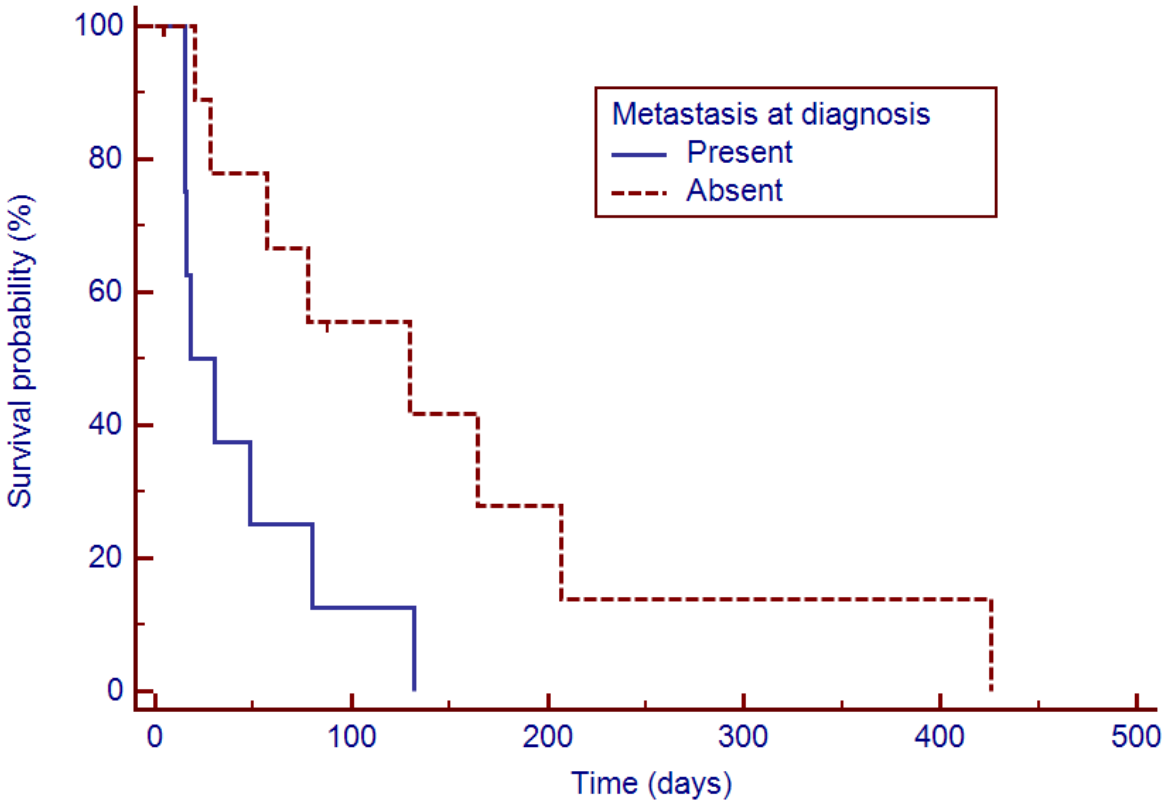


**Figure 2.** Kaplan-Meier curves plotting median survival time in dogs with splenic hemangiosarcoma with and without metastatic disease ($P = 0.02$)

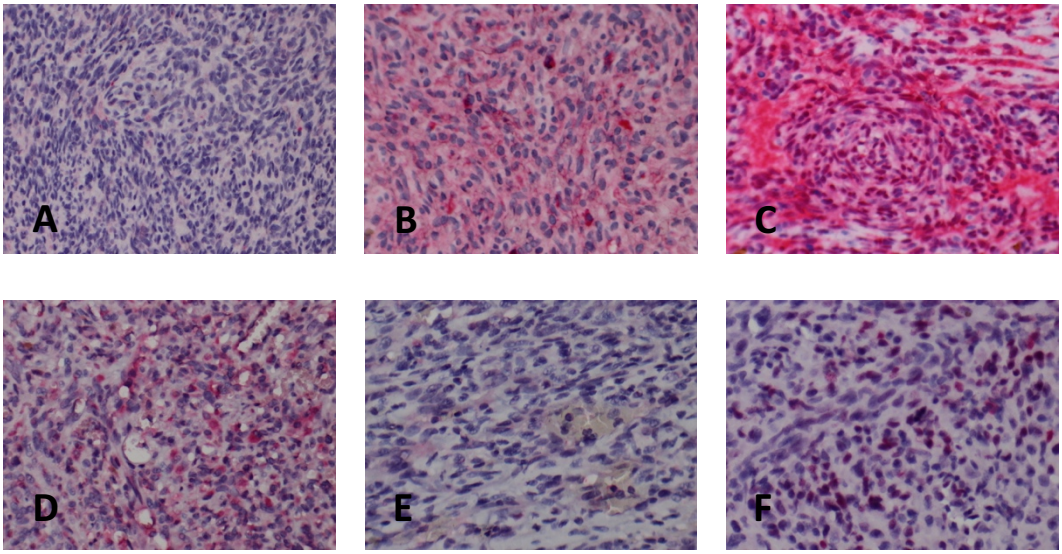


**Figure 3.** HSA, immunohistochemistry for JAK1, score 1 (A); JAK2, score 2 (B); STAT3, score 4 (C); pJAK1, score 1 (D); pJAK2, score 2 (E); and pSTAT3, score 2 (F)

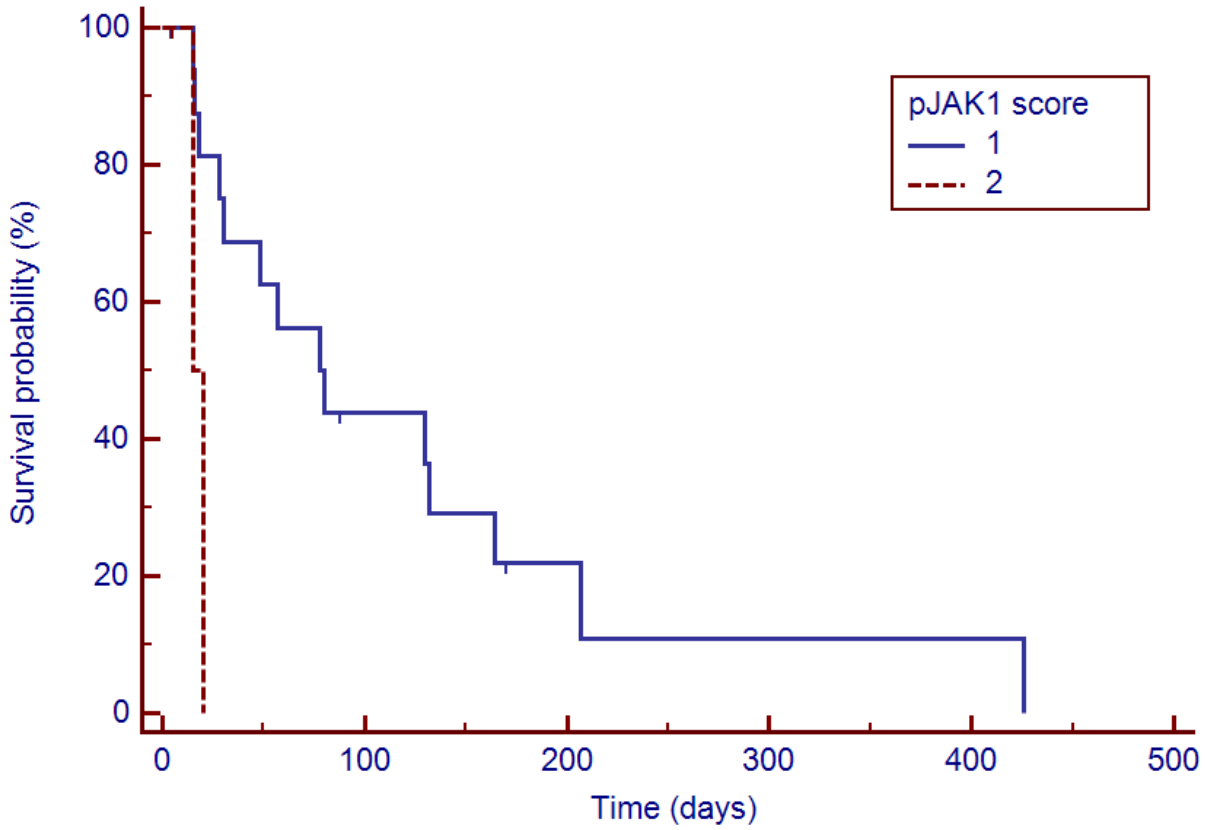


**Figure 4.** Kaplan-Meier curves plotting median survival time for dogs with splenic hemangiosarcoma having pJAK1 IHC scores of 1 and 2 ($P = 0.009$)

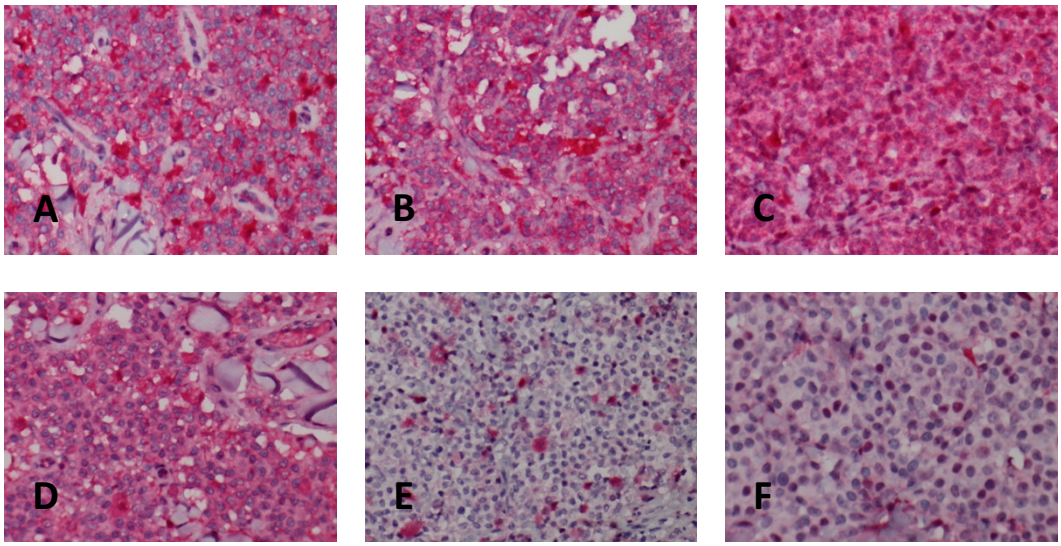


**Figure 5.** MCT, immunohistochemistry for JAK1, score 3 (A); JAK2, score 4 (B); STAT3, score 4 (C); pJAK1, score 3 (D); pJAK2, score 2 (E); pSTAT3, score 2 (F)

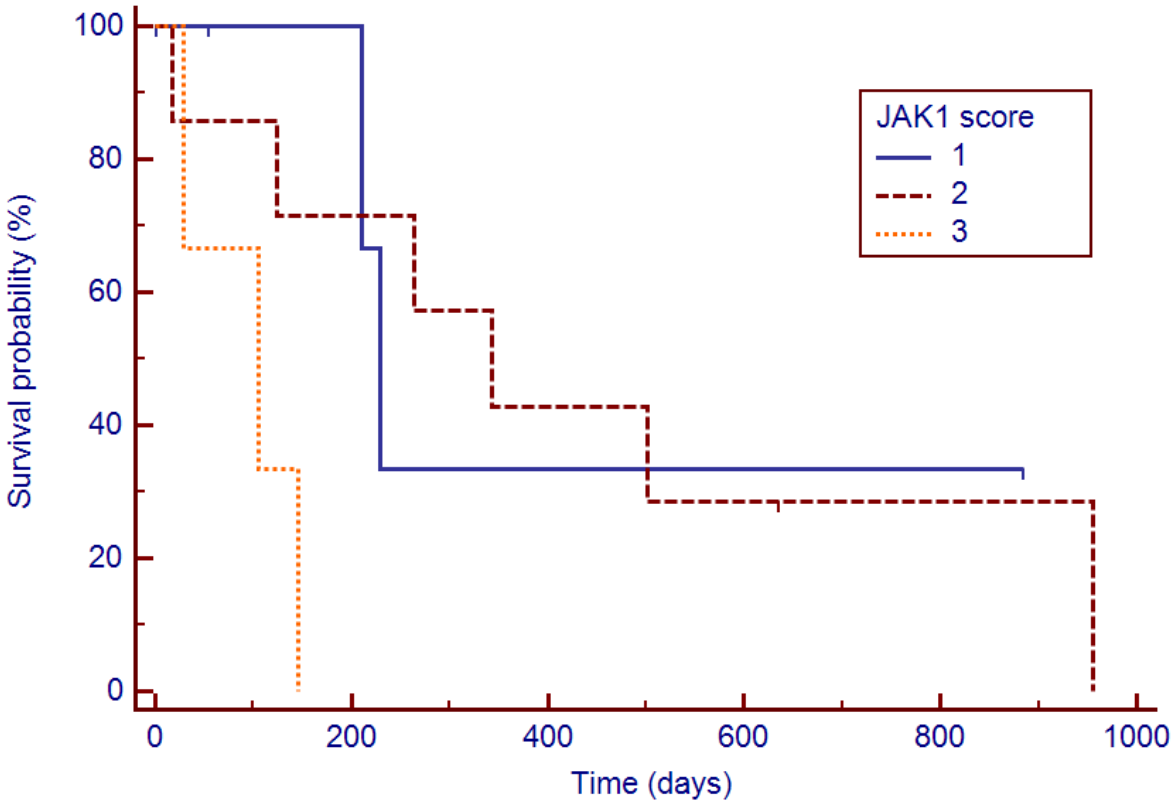


**Figure 6.** Kaplan-Meier curves plotting median survival time for dogs with mast cell tumors having JAK1 IHC scores of 1, 2, and 3 ($P = 0.03$)

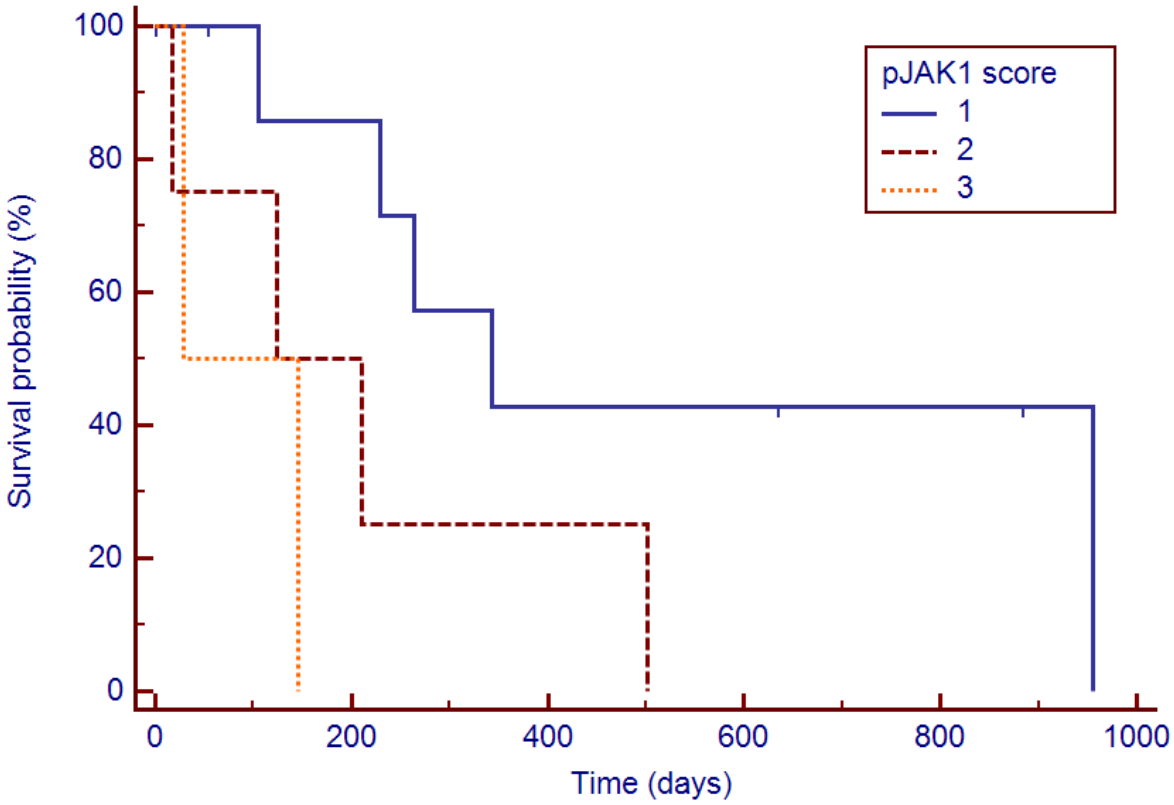


**Figure 7.** Kaplan-Meier curves plotting median survival time for dogs with mast cell tumors having pJAK1 scores of 1, 2, and 3 ($P = 0.04$)

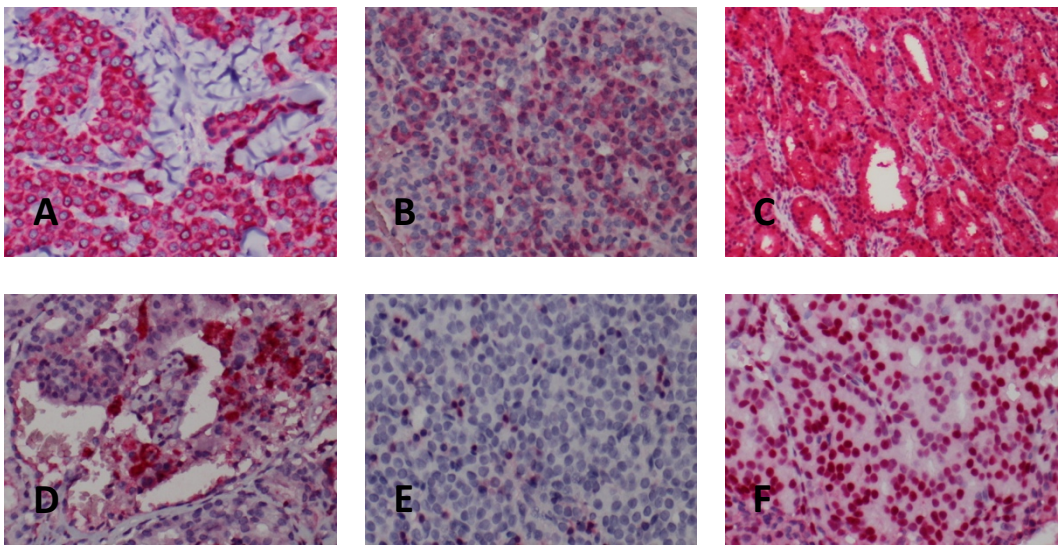


**Figure 8.** Thyroid carcinoma, immunohistochemistry for JAK1, score 4 (A); JAK2, score 2 (B); STAT3, score 4 (C); pJAK1, score 1 (D); pJAK2, score 1 (E); pSTAT3, score 4 (F)

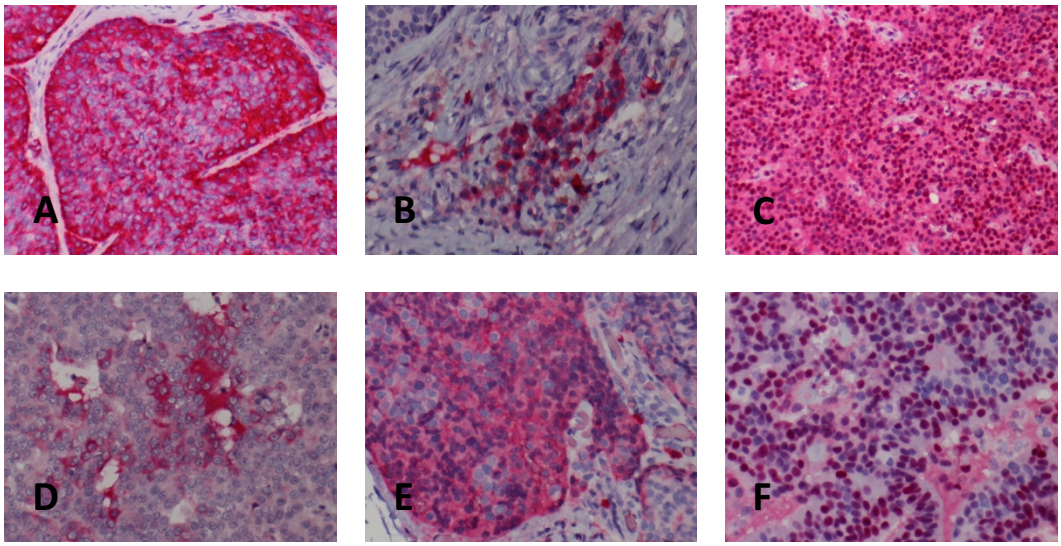


**Figure 9.** AGASACA, immunohistochemistry for JAK1, score 4 (A); JAK2, score 1 (B); STAT3, score 3 (C); pJAK1, score 2 (D); pJAK2, score 1 (E); pSTAT3, score 2 (F)

TABLES

| Score | JAK1 | pJAK1 | JAK2 | pJAK2 | STAT3 | pSTAT3 |
|---|---|---|---|---|---|---|
| 1 | 11 | 17 | 11 | 17 | 0 | 7 |
| 2 | 8 | 3 | 6 | 3 | 1 | 7 |
| 3 | 1 | 0 | 3 | 0 | 5 | 4 |
| 4 | 0 | 0 | 0 | 0 | 14 | 2 |

**Table 1.** IHC scores for hemangiosarcoma samples; 20 cases

| Score | JAK1 | pJAK1 | JAK2 | pJAK2 | STAT3 | pSTAT3 |
|---|---|---|---|---|---|---|
| 1 | 5 | 9 | 4 | 8 | 0 | 10 |
| 2 | 7 | 4 | 7 | 5 | 0 | 5 |
| 3 | 3 | 2 | 3 | 2 | 3 | 0 |
| 4 | 0 | 0 | 1 | 0 | 12 | 0 |

**Table 2.** IHC scores for mast cell tumor samples; 15 cases

| Score | JAK1 | pJAK1 | JAK2 | pJAK2 | STAT3 | pSTAT3 |
|---|---|---|---|---|---|---|
| 1 | 2 | 8 | 6 | 9 | 0 | 3 |
| 2 | 4 | 2 | 4 | 1 | 1 | 5 |
| 3 | 2 | 0 | 0 | 0 | 2 | 1 |
| 4 | 2 | 0 | 0 | 0 | 7 | 1 |

**Table 3.** IHC scores for thyroid carcinoma; 10 cases

| score | JAK1 | pJAK1 | JAK2 | pJAK2 | STAT3 | pSTAT3 |
|---|---|---|---|---|---|---|
| 1 | 0 | 5 | 5 | 7 | 0 | 0 |
| 2 | 0 | 3 | 2 | 2 | 1 | 6 |
| 3 | 2 | 0 | 2 | 0 | 8 | 3 |
| 4 | 7 | 1 | 0 | 0 | 0 | 0 |

**Table 4.** IHC scores for apocrine gland anal sac adenocarcinoma; 9 cases

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