AN ABSTRACT OF THE THESIS OF

Jesse Terry for the degree of Master of Science in Veterinary Sciences presented on April 14, 2016.

Title: Factors Affecting Histologic Tumor Free Margins in Widely Excised Feline Injection Site Sarcomas

Abstract approved:

______________________________________________________

Milan Milovancev

ABSTRACT

Feline injection site sarcomas (FISS) are frequently encountered cutaneous and subcutaneous neoplasms of cats. Significant patient morbidity is related to the invasiveness these tumors display, typically requiring extensive local excision or amputation. As with many veterinary tumors, survival time and tumor recurrence for FISS is highly influenced by histologic margin status.

A general reduction between surgical and histologic margins is expected, but the magnitude, timing and potential factors involved remain poorly described in the veterinary literature. In the first study presented, we begin to define the degree and timing of lateral margin shrinkage in widely excised feline injection site sarcoma specimens. Lateral margins were measured around spontaneously occurring FISS at 1) the time of surgery from grossly palpable tumor 2) immediately following excision 3) following formalin fixation from specimen CT scans and 4) histologic margins.
Consistent with other reports, our findings indicate that the majority of clinically relevant shrinkage occurs prior to formalin fixation (29% decrease from surgically measured margins, p = 0.005). In total, histologic margins represented only 50% of surgically planned margins (p = 0.0012).

In the second report, we showed that FISS tumors themselves do not shrink to the same degree as the normal surrounding tissue. This was done using both standard two dimensional CT scans before and after excision of FISS tumors and three dimensional computed tomography software. Tumor volume was calculated using the ellipsoid formula and compared to volume reported using 3D software. We showed that FISS tumor dimensional shrinkage following formalin fixation was small (<7%) and less than that reported for normal feline tissues. The complexity of FISS peritumoral projections was highlighted and proposed reason why volume calculations using the ellipsoid formula was less than 3D imaging for both in vivo (mean 25.8% smaller, p = 0.038) and ex vivo (mean 24.8% smaller, p = 0.017).

Taken together, these data begin to define and further explore the discrepancy between surgical and histologic margins for FISS. It is reasonable to suspect similar margin reductions around other feline cutaneous and subcutaneous neoplasms, making this data more globally applicable. Future investigation on additional factors that affect margin size and interpretation (eg section number, inking protocol) are needed. Ultimately, with a better understanding of the influences on margin reduction, we may be able to have a more evidence based approach to surgical planning and thereby potentially reduce patient morbidity. Similarly, with additional data on the above topics, we may begin to further correlate the magnitude of
histologic margin with prognosis and survival time for FISS and other veterinary neoplasms.
Factors Affecting Histologic Tumor Free Margins in Widely Excised Feline Injection Site Sarcomas

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Dean of the College of Veterinary Medicine

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Jesse Terry, Author
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Chapter 1

1. Introduction

1.1 Feline Injection Site Sarcoma Background

1.1.1 General

First reported in the literature in 1991, feline injection site sarcomas (FISS) are now a commonly recognized neoplasm in cats, representing between 7 and 21% of cutaneous and subcutaneous neoplasms in this species.\textsuperscript{1,2} These tumors occur at the site of previous injection with a prevalence ranging from 0.32 to 13 per 10,000 vaccinations.\textsuperscript{3,4} The average age at which these tumors are diagnosed is typically younger than cats with non-injection site sarcomas with no reported breed, sex or neuter status predilection.\textsuperscript{5,6} Previously, FeLV, rabies and vaccines that included aluminum or other adjuvants were thought to be the primary drive behind malignant transformation.\textsuperscript{5,7,8} In one study, the risk of tumor development increased with the number of injections administered at the same site.\textsuperscript{5} FISS, however have now been documented following microchip, meloxicam and cisplatin injections as well.\textsuperscript{9-11} While not definitively proven, it has been postulated that chronic inflammation acts as a promoter for neoplastic transformation at these injection sites.\textsuperscript{12}

1.1.2 Changes in Vaccination Guidelines

In response to the increased awareness of FISS, the Vaccine-Associated Feline Sarcoma Task Force was formed in 1996 with the goals of promoting research, treatment and prevention of FISS. This was followed by official vaccination guidelines released by the American Association of Feline Practitioners in cats which were first released in 1998 and are routinely updated.\textsuperscript{13} Included, are
recommendations on vaccination sites and frequency. It is no longer recommended to
vaccinate cats near the shoulder or interscapular region or in the flank/thigh.
Vaccination at the tail base or distal hind limb is now advocated, as these areas lend
for easiest surgical excision (e.g. tail or limb amputation). Similarly, vaccinations
such as FeLV and FIV are now only suggested for adult cats which are deemed “at
risk”, including those in areas with high endemic disease rates, those living in
multiple cat households and those have access to outdoors. These recommendations
have shown an effect on location of FISS, with more recent reports showing a
decrease of these tumors at the interscapular region (53.4 to 39.5%). Following the
release of these guidelines however, the incidence of FISS has not changed in the past
twenty years.

1.1.3 Biologic Behavior of FISS

Biologically, FISS are typically fibroscarcomas, however chondrosarcomas,
rhabdomyosarcomas, liposcarcomas all reported with much less frequency. When
compared to non-injection site tumors, FISS tend to be more commonly located in the
hypodermis, are less differentiated, show more extensive intra-tumoral necrosis, have
higher mitotic rates and have peri-tumoral lymphocytic inflammation. Rapid
growth, marked local invasion and aggressive biologic behavior is a hallmark of
FISS. Like other malignant mesenchymal neoplasms, these tumors are thought to
grow along the path of least resistance (e.g. fascial planes), leading to multiple
projections into the surrounding tissue. This growth pattern along with altered
regional mesenchymal staining and abnormal gene expression support the theory of
“field cancerization” for FISS (disease is considered present throughout an anatomical area as opposed to a discrete mass). Along these same lines, masses adjacent but not contiguous with the primary mass have been documented in FISS and have been termed “skip metastasis.” Collectively, these characteristics have all influenced the current treatment recommendations for FISS, with the theme of aggressive treatment required in the hopes to gain local control.

1.1.4 Treatment of FISS

This aggressive local biologic behavior of FISS has led to wide surgical excision (ie 5 cm lateral margins with two deep fascial planes) with or without adjuvant therapy as the current standard of care. Cats undergoing such radical excision have showed improved survival times (901 days) and lower local recurrence rates (14%). In contrast, reports with more conservative margins have shown survival times of 395 – 804 days and local recurrence of 35 - 59% for FISS. Completeness of surgical margins in cats undergoing wide excision for FISS has been shown to correlate with decreased recurrence rate and greater median survival time. One recent study showed margin status was significantly associated with local recurrence of FISS, with recurrence in 16% of non-infiltrated margins and 69% of infiltrative margins. In that study, tumors with neoplastic cells at the inked margin were ten times more likely to recur when compared to non-infiltrated margins.

In 1986, Enneking described a preoperative staging system that considered tumor type, anatomic location and histologic grade. This system considered anatomic regions as compartments with aggressive surgery entailing complete compartment...
excision (e.g. hemipelvectomy). This paradigm is vastly different than the typical metric approach to veterinary neoplasm excision in which a defined distance of normal tissue (typically in cm) is measured from the mass and included in excision. This concept has recently been breached in the veterinary literature and may ultimately play a role in FISS surgical treatment. For example, Bray et al described a compartmental hemipelvectomy approach in dogs and cats in 2014.27,28 In that retrospective study, 16 feline tumors were included (presumed to be injection site related), with the only factor associated with improved survival being clean histologic margins on multivariate analysis.

1.2 Factors Influencing Margin Interpretation
As touched on previously, curative intent oncologic surgery entails the goal of completely removing a tumor which typically requires the excision of a margin of normal tissue around the neoplasm. This “cuff” of normal tissue defines the histologic tumor free margin (HTFM) and can be reported as a binary result (ie. complete excision vs incomplete) or measured by pathologist. In human literature, the magnitude of HTFM has been linked to various outcome measures for different tumors and is sometimes referenced as the histologic safety zone. This topic is yet to be evaluated for veterinary species however. Currently, only one veterinary study has commented specifically on HTFM and showed a discrepancy of 35 to 42% between HTFM and grossly normal tumor free margin (GNSM) for canine mast cell tumors.29 This is consistent with the general experiences of most surgeons where HTFM is typically less than of GNSM. Understanding the importance of margin status (i.e.
complete vs. incomplete) for FISS, any factors that may influence histologic margin are extremely important for prognosis and treatment recommendations. Intuitive points where discrepancy between HTFM and GNSM may be introduced include inaccuracies in tumor boundary identification on preoperative imaging, tumor cells beyond grossly palpable disease, tissue shrinkage following excision, tissue shrinkage following formalin fixation and specimen processing errors (e.g. inking protocol, sectioning number and location, experience of pathologist).

1.2.1 Identifying Tumor Boundary

1.2.1.1 Imaging Defined Tumor Boundary

Advanced imaging studies of FISS have been consistent with the described local biologic behavior. In 2013, a computed tomography (CT) based study by Travetti et al described tumor projections, strong contrast uptake and local infiltration for FISS.\textsuperscript{30} In a similar magnetic resonance imaging (MR) based study, tumor projections and local infiltration were also described for FISS.\textsuperscript{31} In these studies and many others describing imaging characteristics in a variety of veterinary tumors, it is assumed that tumor boundary is noted by the junction of contrast enhancing tissue with surrounding (non-contrast enhancing) tissue. While this seems reasonable, the boundary of the contrast enhancement is influenced by peritumoral edema and tumor “reactive zone” and may not always correlate to neoplastic infiltrate.\textsuperscript{32} The interface between tumor and normal tissues, may vary in thickness and contain inflammatory cells, blood vessels and compressed tumor or connective tissue cells.\textsuperscript{33} Importantly,
tumor boundary on preoperative advanced imaging is commonly used as a tool for surgical planning.

To address the question of tumor boundary delineation using advanced imaging (and specifically for FISS), our group recently attempted to correlate tumor projections noted on preoperative CT angiography (CTA) and MR with a histologic diagnosis.\(^{34}\) We developed a fiducial marker which could be seen on both CT and MR to act as a common reference point between preoperative imaging, surgery and histologic processing.\(^ {35}\) Peritumor lesions identified on advanced imaging were then evaluated on histology and labeled as either neoplastic, concerning or non-neoplastic. To date, this is the first veterinary study attempting to describe the nature of the projections surrounding a mass. Interestingly, we found that preoperative contrast enhancing peritumor lesions on both CTA and MR were neoplastic or concerning in a reasonable subset of samples (41.4%) with the remaining found to be reactive or inflammatory. 44 lesions were found on CTA, and 43 lesions were found on MRI. 15.9% of peritumor lesions identified on CTA were neoplastic whereas 20.5% were concerning compared to 23.3% neoplastic and 23.3% concerning for peritumoral lesions identified with MRI. As both CT and MR routinely used for preoperative surgical planning for FISS, the study highlights the inherent flaws in these modalities and offers one contributing factor to the HTFM to GNSM discrepancy.

1.2.1.2 Tumor Measurement Methods
Tumor size has been proposed to carry prognostic value for FISS, however this has yet to be shown in the literature. One study evaluating tumor dimensions (via preoperative CT or caliper) failed to show that tumor size had an influence on survival however the trend was that cats with larger (>5 cm) tumors had a lower survival time (184 days) vs. cats with smaller (<2cm) tumors (929 days, \( p = 0.15 \)).

In another MRI based study, tumor volume (as estimated by the ellipsoid volume formula) was not associated with survival time or predictive of postoperative margin status. Another CT based study reporting on FISS volume used this same formula but did not correlate this to clinical outcome. The ellipsoid formula for volume calculation is based off of the radius measurement in the three axis (often from advanced imaging) and has been used to estimate tissue volume in a variety of circumstances and organs. The accuracy of this protocol is intuitively influenced by the correlation between the shape of the tissue in question and a true ellipsoid and has been proven accurate for certain organs. When tissues with more complex shapes (ie masses with multiple projections or cavitations as shown with FISS) are measured however, the ellipsoid formula is less accurate. When compared to the gold standard of water displacement, the ellipsoid formula has been shown recently to underestimate the volume of the human prostate. This is mentioned to illustrate one of many flaws in tissue measurement which again may influence GNSM to HTFM discrepancy.

**1.2.1.3 Palpation Defined Tumor Boundary**
Commonly, surgical margins are drawn onto the patient by the surgeon with a
knowledge of preoperative imaging findings but ultimately dictated by palpable
tumor. Intuitively, tumors that are more superficial and more discrete may be more
easily palpable and thus have more accurately drawn margins. This concept of
“palpability” is interesting and yet unexplored in veterinary medicine to date.
Furthermore, inter or intraobserver variation in palpation of cutaneous and
subcutaneous masses and margin determination has yet to be described in the
veterinary literature. Previously, Euhus et al found a mean interobserver variation of
15% of the mean calculated volume for tumors on nude mice measured with calipers.
Intraobserver variation showed an error of 7% for large masses and 27% for small
masses. While yet to be explored, it is reasonable to suspect that palpation of FISS
would be less accurate than more “encapsulated” tumors. Until further research is
done, the clinician must accept that at least some degree of inaccuracy is present in
palpation identified and true tumor margin. The magnitude and ultimate clinical
importance of this concept are unknown at this time.

1.2.2 Tissue Shrinkage

1.2.2.1 Normal Tissue Shrinkage

It has long been observed that tissue will shrink immediately following surgical
excision. This is thought to occur due to the intrinsic contractile nature of various
tissues (e.g. connective tissue, muscle) and the release of tissues from surrounding
supportive structures. In humans, reported tissue shrinkage immediately following
excision is considerable and well documented for a variety of tissues. In 1997,
Johnson et al reported a 38.3% decrease in tissue length immediately following excision of normal canine labiobuccal mucosa and a 24.8% length reduction of canine tongue mucosa.\textsuperscript{42} In 2005, Reimer et al. showed a 21-32% reduction in normal canine skin samples.\textsuperscript{47} In that study, samples showed a statistically significant increase in thickness and the inclusion of muscle lead to a length reduction of lower magnitude. Clarke et al. showed a 28.3% decrease in canine small intestinal specimens immediately following excision.\textsuperscript{48} In 2014, Upchurch et al described 13.7% shrinkage for canine skin samples immediately following excision. In that study, 216 samples were measured in four planes and at three time points. Effect of original sample size orientation and body location were all assessed to degree of shrinkage with no significant effect found for any.\textsuperscript{49}

Currently, only two peer reviewed reports provide data on tissue shrinkage in cats and is limited to normal (non-neoplastic) tissues.\textsuperscript{50,51} In the first study by Miller and Dark, tables provided in the manuscript indicate that feline skin shrinkage was less than 10\% following excision however an exact value is not reported. This is in contrast to the recent findings of Risselada et al. who showed a mean closest margin shrinkage of 42.4\% or original length following excision in a study using gelatin to simulate subcutaneous neoplasms.

\textbf{1.2.2.2 Tumor Shrinkage}

In human medicine, the importance of tumor shrinkage includes mis-staging of cancers in which measurements are taken ex vivo. It was recently shown that post
excisional tumor shrinkage could result in under-staging of renal cell carcinomas (pT1b to pT1a) and non-small cell lung tumors (T2-T1) in people. This concept is perhaps most documented for prostate tumors, in which prostate tumor shrinkage correction factors are well described and commonly used. Broadly speaking, postoperative tumor shrinkage may influence pathologist reported HTFM, particularly if tumor changes are different or not in proportion to that of normal tissue found in the lateral margins. This has been documented for human basal cell tumors (or other) in which the tumor free margin shrank proportionally more than that containing tumor (19 vs 11%). Degree of tumor shrinkage following excision has yet to be described for any veterinary neoplasm.

1.2.2.3 Effects of Formalin

The additional influence of formalin fixation on tissue shrinkage has been investigated in several studies. Small but statistically significant tissue shrinkage has been documented for human head and neck tissues. This differs from other studies showing the effect from formalin was insignificant for the skin, kidney and bowel. Of the veterinary studies assessing tissue shrinkage as listed above, four showed no significant changes in tissue size following formalin fixation. In the study by Johnson et al., an additional decrease of 10.5% and 7.6% was seen following formalin fixation for canine labiobuccal and tongue mucosa respectively. Interestingly, Clarke et al actually showed a small increase in tissue size following formalin fixation when compared to measurements taken immediately after surgery, however this difference was not statistically significant. Minimal data exists on the effects of
formalin on feline tissues with both current studies mentioned above showing no statistical effect.

1.2.3 Additional Factors

Following formalin fixation, there remain several potential factors that may influence the ability of the pathologist to determine HTFM or may influence the magnitude of HTFM. For example, inking the surgical margin (as is common practice) may be incomplete or non-representative of true surgical margins due to ink dissection along fascial planes. Irregularities in tumor boundary make the location of radial section key in HTFM length. Furthermore, number of sections assessed is often low and only represent a small portion of the tumor free margin. Number of tissue sections evaluated has been shown to increase the likelihood of a positive margin in human desmoid-type fibromatosis. During slide evaluation, discrepancies between pathologist interpretation of tumor boundary (e.g. reactive stromal cells vs. neoplastic cells) adds further discrepancy. Other influences on margin status not mentioned include cautery/tissue handling artifact and tissue folding.

1.3 Statement of Objectives and Investigational Rationale

1.3.1 Objectives

Presently, no reports describe the magnitude and processing steps at which shrinkage of tumor bearing tissues in the feline species occurs or the relationship between GNSM and HTFM for FISS. Using a population of client-owned cats undergoing wide excision for spontaneously occurring FISS, the objectives of the current project
include was 1) to assess the overall degree of surgical margin length reduction and 2) assess tumor dimension and volume reduction at specific processing steps from surgery to histopathologic evaluation. Tumor volume calculations based on the standard ellipsoid equation were compared to 3D CT software as described above.

1.3.2 Rationale and Hypothesis

In summary, FISS are characterized by their highly aggressive and locally invasive nature. This growth pattern has led to a variety of imaging based studies, all of which comment on the irregular margins and various projections noted from the primary mass. Wide surgical excision remains the gold standard of treatment, with specimen margin status highly predictive of tumor recurrence and MST. Any additional information on factors influencing GNSM to HTFM margin discrepancy may allow more precise and patient specific surgical planning and HTFM interpretation.

Extrapolating from available veterinary and human literature, we hypothesized that 1) HTFM would represent a consistent fraction of GNSM, 2) the majority of this margin length discrepancy would be due to immediate (pre-formalin) tissue shrinkage 3) a difference between normal and tumor tissue shrinkage would be identified and 4) volumetric tumor measurements would differ between the two measurement protocols.
Chapter II

Quantification of Lateral Surgical Margin Length Reduction after Excision of Feline Injection Site Sarcomas – A Pilot Study

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ABSTRACT

OBJECTIVE: To evaluate 1) the degree of lateral surgical margin length reduction at various stages of processing for histological examination and 2) quantify the change in length between grossly normal surgical margins (GNSM) and pathologist reported histologic tumor-free margin (HTFM) in widely resected feline injection site sarcoma (FISS) specimens.

STUDY DESIGN: Prospective clinical study

ANIMALS: 5 client-owned cats with injection site sarcomas

METHODS: All cats underwent wide surgical excision (35-55 mm lateral margins, 2 fascial planes deep). Grossly normal lateral margin measurements from tumor edge were recorded in four directions (e.g. cranial, caudal, dorsal, and ventral) at four time points: intra-op (in vivo GNSM), immediately following excision (ex vivo GNSM), following formalin fixation (post fixation GNSM), and HTFM during histopathology. Percent tissue shrinkage from in vivo measurements was quantified at each time point and compared using a one-way repeated measures ANOVA.

RESULTS: Mean percent shrinkage from in vivo to ex vivo GNSM was 29.2%. Formalin fixation did not result in additional significant change. Mean HTFM was significantly reduced compared to in vivo GNSM (50% of in vivo GNSM length).

CONCLUSIONS: Surgical margin length reductions in FISS specimens were accounted for by post-excision tissue shrinkage (without significant changes caused by formalin fixation). We propose that the presence of microscopic neoplastic infiltrates beyond the gross tumor at any processing stage may account for the further reduction in HTFM.
INTRODUCTION

Feline injection site sarcomas (FISS) are well described in the veterinary literature and comprise 7 to 21% of skin neoplasms in cats. These tumors are typically non-painful and may be freely movable or attached to deeper tissues. The current standard-of-care treatment of feline injection site sarcomas includes wide surgical resection with or without adjuvant therapies. The locally invasive nature of these tumors, paired with the common sites at which they are encountered, often results in significant surgical morbidity with amputation frequently required. In 2011, Phelps et al showed that surgical margins larger than historical standards yielded improved local recurrence rates and median survival times (14% and 901, respectively). In that study, local recurrence was a predictor of survival (499 days vs. 1461 days). Another recent study showed margin status was significantly associated with local recurrence in cats with injection site sarcomas, with recurrence in 16% of non-infiltrated margins and 69% of infiltrative margins. In that study, tumors with neoplastic cells at the inked margin were ten times more likely to recur when compared to non-infiltrated margins.

Invariably, histologic tumor-free margins (HTFM) are reported to be smaller than in vivo grossly normal surgical margins (GNSM) measured by the surgeon intraoperatively. The exact mechanisms behind this length reduction remain unknown. Factors influencing this length reduction have been postulated to include physical shrinkage due to tissue elasticity along with microscopic disease beyond the palpable gross tumor edge (Fig 1). For example, it has been shown that 25% of canine grade
II mast cell tumors have neoplastic mast cells that extend 1 cm beyond the visible edge of the mass. This is further supported by a recent retrospective case series by Risselada et al where mean histologic margins of cutaneous and subcutaneous canine mast cell tumors were 35%-42% smaller than surgically measured margins. Tissue shrinkage and microscopic disease beyond palpable tumor were both suggested as potential contributors to the GNSM to HTFM discrepancy in that study. A considerable amount of human literature is available describing these concepts as well. Presently, tissue shrinkage factors following excision and fixation are well described for prostatic tumors Similarly, several studies on oral tumors report variation in the discrepancy between histologic lateral margins when compared to those measured at the time of surgery. Interestingly, location (e.g. buccal mucosa vs. palate) and stage (T1/T2 vs T3/T4) both influence the degree of margin discrepancy for human oral squamous cell carcinoma. One study on human basal cell carcinomas indicated that dermatologic surgeons accurately identified the tumor edge to within 1 mm at all four margins in only 26% of cases. While tumor boundary underestimation was low in this study (4%), clinically undetected lateral extension of tumor was proposed as a possible Explanation for inaccurate identification of the tumor edge. When assessing literature across species and tumor types, the general consensus is that a better understanding of the factors that may influence margin reduction following excision is needed for both more informed surgical margin planning and interpretation of reported HTFM. This approach has the potential to reduce patient morbidity by reducing surgical dose, when appropriate, or improve outcome via evidence-based surgical planning.
Limited veterinary literature exists describing tissue shrinkage during the process of surgical resection and formalin fixation and has been confined to prospective reports using normal (i.e. tumor-free) tissue and a single retrospective case series in dogs with mast cell tumors. One study of normal canine skin samples showed a decrease in size (combined percentage change in length and width) and an increase in thickness. In that study, histologic processing-associated changes were also influenced by sample origin and tissue type (e.g. inclusion of muscle). In another study evaluating longitudinal shrinkage in normal canine small intestinal specimens after 10% formalin fixation, mean shrinkage from pre-excisional state was 28.3% immediately after excision with no significant changes following 24 hours of formalin fixation. Based on mean shrinkage from initial resection to final microscopic assessment in normal canine oral mucosa samples, Johnson et al concluded that to obtain 5 mm of HTFM, an in vivo GNSM resection of at least 8 to 10 mm is required. To date, the largest veterinary study evaluating ex vivo tissue changes showed that the majority of canine skin sample shrinkage occurred immediately after excision but prior to formalin fixation. Mean overall shrinkage was 15.6% in that study and surgeon, body side or region, and skin tension lines did not significantly correlate to percentage of skin shrinkage. Currently, only two studies evaluating ex vivo tissue shrinkage of healthy feline tissue samples showed all statistically significant tissue shrinkage occurring immediately after excision but prior to formalin fixation.
Presently, no attempts to describe the relationship between GNSM and HTFM have been performed in any described neoplasm in cats. Similarly, no studies are available evaluating the shrinkage of complex feline tissue samples (e.g. samples that include muscle or bone). To begin to address this gap in knowledge, the objectives of the present prospective clinical pilot study were (1) to assess the overall degree of surgical margin length reduction in FISS cases undergoing wide surgical resection at specific processing steps from surgery to histopathologic evaluation and (2) characterize the relationship between GNSM at each processing step with HTFM. Extrapolating from available veterinary and human literature, our hypothesis was that most reductions in surgical margin size would occur after surgical excision, prior to formalin fixation and that HTFM size would represent a consistent fraction of in vivo GNSM.
MATERIALS AND METHODS

Case selection

Cats with pre-operative cytological or tissue biopsy confirmation of FISS and tumors in locations that lent themselves to obtaining wide surgical margins circumferentially around the tumor without necessitating amputation were prospectively enrolled in the study. Exclusion criteria included any pre-operative neoadjuvant therapy (e.g. radiation therapy or chemotherapy) or prior tumor excision. Cats in this study represented a subpopulation of clinical cases enrolled in a concurrent study evaluating preoperative imaging characteristics of FISS with Institutional Animal Care and Use Committee Approval.20

Pre-surgical planning

After anesthetic induction, a previously described triangular-shaped fiducial marker was sutured to the overlying skin above the tumor of each cat.35 Care was taken to suture the marker in a neutral position to ensure it did not distort the skin overlying the tumor. The marker provided a standardized reference point to allow consistency in gross margin length comparison for in vivo GNSM, ex vivo GNSM, and post-fixation specimens. For the purposes of this study, we define “grossly normal” margin length as those margins which could easily be distinguished from tumor based on palpation or measurements from CT. All cats underwent preoperative imaging for surgical planning using a 64 detector helical CT scanner (Toshiba Aquilion, Tochigi, Japan). Under general anesthesia, dual phase CT angiogram studies (CTA) were performed in which images were acquired both pre and post administration of
intravenous iodinated contrast medium (Isovue/Iopamidol, 300 mg I/ml, Princeton, NJ, USA) with image acquisition timed to obtain post contrast images in arterial and venous phases. Helical images were acquired as a volume with 0.5mm voxels, 0.5sec rotation speed, 512 x 512 matrix, 250 mA, 120kVp and pitch of 0.83. The volume data were reconstructed in bone and soft tissue algorithms, and in isovolumetric transverse, sagittal, and dorsal planes at 2mm slice thickness.

Surgery

Each cat underwent routine surgical clipping and aseptic preparation using 4% chlorohexidine scrub (VetOne, Boise, ID). After the final surgical scrub, but prior to application of surgical drapes, the fiducial marker was covered with an antimicrobial drape (Ioban, 3M, St. Paul, MN). The marker remained sutured to the specimen throughout surgery and tissue processing. Using a sterile surgical pen, 35 to 55 mm in vivo GNSM were drawn in all directions surrounding the mass, measuring from the edge of the palpable tumor (Fig 2). Attempts were taken to acquire the widest possible surgical margin, while minimizing patient morbidity based on pertinent regional anatomy (e.g. spinal cord) as judged by the operating board-certified veterinary surgeon. The tumor and associated tissue were removed en bloc with a minimum of two deep fascial planes. Muscles, nerves and vessels that crossed the transection plane were sharply transected and/or ligated. Every effort was made to maintain a consistent lateral margin to prevent narrowing of the margin as dissection was carried deeper.
Post-excision sample processing

Following excision, all cut surgical margins were labeled with commercially available margin inks previously demonstrated to yield consistent performance and high pathologist preference (Fig 3). For one mass in which there was concern that normal anatomic relationships may be distorted (i.e. folding) during formalin fixation and histologic processing, the specimen was carefully sutured to a piece of cardboard to maintain neutral tissue relationships. Particular attention was paid to ensure suturing did not create tissue distortion in itself; sutures were left relatively loose, without stretching the tissue.

All specimens were placed in 10% neutral buffered formalin at a 1:10 tissue to formalin ratio. Samples were allowed fixation for 48 to 72 hours, consistent with standard operating procedures (SOPs) at the Oregon State University Veterinary Diagnostic Laboratory (VDL). After formalin fixation, excised tissue specimens underwent CT scans with the same settings as previously described, but without administration of intravenous contrast medium. All tumors remained easily distinguishable on CT imaging from surrounding soft tissues after excision.

All histologic analyses were performed by a single board-certified veterinary anatomic pathologist (CVL). Following SOPs of the VDL, tissue cassettes were labeled with the VDL accession number and a running number. All tissue samples underwent routine preparation for histologic evaluation using hematoxylin and eosin staining on standard glass slides following VDL SOPs. Particular care was taken to
describe the precise length, in millimeters, of the HTFM for each inked surgical margin based on radial tissue sections using a micrometer. Tangential sections were also taken at each inked surgical margin and were used for a binary (yes/no) indication of whether tumor cells were present at the surgical margin.

Margin measurements

Using the fiducial marker to aid in consistency and orientation, lateral margin measurements were obtained at the following points:

1. *In vivo* GNSM – measured intra-operatively prior to skin incision, from the palpable tumor edge to four lateral surgical margins (e.g. caudal, cranial, dorsal, and ventral; Fig 2).

2. *Ex vivo* GNSM – measured immediately after *en bloc* excision, prior to formalin fixation. Measurements taken from the palpable tumor edge to the four lateral surgical margins (same directions as for *in vivo* GNSM; Fig 3). Measurements were obtained using a combination of physical palpation, immediate post-excision photographs and cross-referenced with those reported in the surgical report. Photographs included a ruler within the field, at the level of the specimen.

3. Post-fixation GNSM – measured after formalin fixation, prior to trimming, using a post-fixation CT scan (as described above). Measurements were again
made from the tumor edge (readily identified by tissue attenuation differences as the masses retained contrast enhancement in all cases) to the four lateral cut surgical margins (same directions as for *in vivo* GNSM; Fig 4). This measurement was obtained using the ruler tool of a commercially available DICOM viewing software program (eFilm, Merge HealthCare, Milwaukee, WI).

4. HTFM – the narrowest HTFM in the four lateral surgical margin directions was measured by a single board-certified anatomic pathologist (CVL) using a combination of radial and tangential tissue sections taken from each inked surgical margin using a micrometer. Radial sections were obtained at the narrowest point from all four surgical margin directions (same directions as for *in vivo* GNSM; Fig 5).

Due to difficulty in reliably quantifying size of deep surgical margins at each time point, they were not included in the present analysis.

*Statistical analysis*

Mean margin measurements were calculated by pooling margin lengths from the four directions measured (e.g. cranial, caudal, ventral, and dorsal) at each time point (i.e. *in vivo* GNSM, *ex vivo* GNSM, post-fixation GNSM and HTFM) for each individual cat. These margin measurements were normalized by expressing them as a percentage of their matched mean *in vivo* GNSM (where *in vivo* GNSM is 100%) from the same cat. The resultant normalized percentages from each of the 5 cats were averaged
within each processing step, yielding an overall mean margin length percentage at the four steps from surgical excision to histopathologic evaluation.

Differences between absolute and normalized mean margin measurements among the different time points were assessed using a repeated-measures ANOVA with a Greenhouse-Geisser correction. A post-hoc Tukey’s test was further used to assess differences between each processing step. All statistical analyses were performed using a commercially available computer software package (GraphPad Prism v6.02 for Windows, GraphPad Software, San Diego, CA). Unless otherwise noted, data are presented as mean ± standard error of the mean (SEM) and with 95% confidence intervals (CI) when appropriate. A p-value less than or equal to 0.05 was considered statistically significant.
RESULTS

Five client-owned cats undergoing wide surgical excision without amputation for FISS at the Oregon State University Veterinary Teaching Hospital between March 2013 and February 2015 were prospectively enrolled in this clinical study. All cats were domestic shorthair with four neutered males and one spayed female. Median age was 9 years and median body weight was 5.2 kg. Surgeries performed included wide excision of the lateral thigh musculature in three cats, partial scapulectomy in one cat, and excision of the dorsal neck musculature including vertebral spinous process ostectomy in one cat (Table 1).

Four lateral margin length measurements were obtained for each of the five specimens, yielding a total of 20 surgical margin measurements at four processing steps (in vivo GNSM, ex vivo GNSM, post-fixation GNSM, and HTFM) for a total of 80 margin length measurements. Postoperative histopathology confirmed FISS in all cats with no neoplastic cells reaching the cut/inked edge of any surgical margin. At the time of last follow up (median 15 months), no gross tumor recurrence of evidence of distant metastasis had been noted for any cat.

Lateral surgical margin length reduction data are summarized in Tables 1 & 2 with the key findings described below. Significant reductions in surgical margin length measurements were found over the processing steps of the study, whether lengths were expressed as absolute measurements or normalized as a percentage of in vivo GNSM (p < 0.01 for both). Significant reductions were observed from in vivo to ex...
*vivo* GNSM (29.2%, p = 0.005); no significant changes were seen from *ex vivo* to post-fixation GNSM (p = 0.47). HTFM represented 50% of *in vivo* GNSM. A significant difference was not found when comparing either *ex vivo* GNSM or post-fixation GNSM to HTFM with p-values of 0.082 and 0.098 respectively.
DISCUSSION

The objectives of the present pilot study were to identify the surgical margin length changes at specific processing steps of FISS cases undergoing wide surgical resection and characterize the relationship between *in vivo* GNSM and HTFM. All measurements were taken from the tumor margin to the inked edge, thereby removing any influence of size changes in the tumor itself during each step. The results indicate that a major factor affecting surgical margin length reduction is physical tissue shrinkage (mean of 29.2%) observed immediately post-excision, prior to formalin fixation. Margin reduction due to tissue shrinkage alone, however, does not explain the even larger reduction observed between *in vivo* GNSM and HTFM (mean of 50%). We propose that other factors influencing margin reduction may include the presence of microscopic neoplastic infiltrates beyond the palpable tumor and potentially additional shrinkage of trimmed tissue sections during tissue processing from formalin to paraffin. While in the present study, a statistically significant difference between post-formalin fixation GNSM and HTFM was not found, the data trended towards significance (p = 0.098) and may represent a type II statistical error. Collectively, these data suggest that most of the tissue dimensional changes surrounding FISS tumor specimens occur immediately post-excision and that HTFM is consistently reduced further due to as of yet unproven factors that warrant further investigation.

The relationship between *in vivo* GNSM, *ex vivo* GNSM, post-formalin GNSM and HTFM have been described in depth for a variety of human neoplasms.\textsuperscript{46,55,64} One
example of such research involves the use of various fluorescent – based intraoperative tumor staining techniques which often identify residual neoplastic disease at the resected surgical site. \(^{65-67}\) This data supports our speculation that margin planning as dictated by visible tumor, palpation or even preoperative imaging comes with an inherent degree of error and represents one contributing factor in \textit{in vivo} GNSM to HTFM discrepancy. Many human studies report on margin status and magnitude of histologic tumor free margin (sometimes referred to as a “histologic safety zone”) as prognostic for a variety of human neoplasms. \(^{68-70}\) A recent retrospective study on canine mast cell tumor was the first veterinary report on the \textit{in vivo} GNSM to HTFM relationship. \(^{29}\) In this study, HTFM was consistently lower (38-43\%) than \textit{in vivo} GNSM. In that study, directionality and patient body condition score was not associated with degree of discrepancy. With the array of human studies and single veterinary retrospective report to extrapolate from, we propose that both physiological and artifactual influences on HTFM are important for surgical planning and HTFM interpretation, yet remain largely unidentified in veterinary medicine.

In the present study, the average absolute physical length reduction between \textit{in vivo} GNSM and HTFM was a mean of 20.3 mm (95\% CI 15.9 – 24.6 mm). The upper limit of the 95\% CI supports the recent suggestion that a traditional wide excision with an \textit{in vivo} GNSM of up to 30 mm may be inadequate in FISS cases (i.e. they may result in an HTFM of 0 mm; incomplete excision). Stated differently, the larger the surgically planned margins, the less likely tissue shrinkage will affect excision status. This is consistent with a recent study wherein cats undergoing more aggressive
resection (50 mm lateral margins and 2 tissue planes deep) without adjuvant therapy experienced a median survival time 901 days and overall recurrence rate 14%.\textsuperscript{20} In similar studies where less aggressive margins were taken, recurrence rates of 35% to 59% were reported, suggesting these more conservative excisions may have left residual disease at the wound bed which led to local tumor recurrence.\textsuperscript{21-25,71} More broadly, histologic margin status (i.e. “positive” vs “negative”) has been described as a prognostic indicator in a variety of veterinary tumors.\textsuperscript{72-74} With this in mind, it is not unreasonable to propose that further knowledge of any factors which may influence HTFM should be considered during surgical planning and HTFM interpretation.

Tissue shrinkage following formalin fixation was relatively inconsequential in the present study, with all significant gross tissue changes occurring immediately after surgical excision. This supports data from canine studies and the two studies on normal feline tissue specimens in which the majority of significant shrinkage occurs after excision but prior to fixation.\textsuperscript{48,49,51} It has been hypothesized that these tissue changes are a result of myofibril contractility and tissue elasticity as it is released from surrounding opposing structures.\textsuperscript{48,49} Interestingly, one report in cats showed shrinkage to be insignificant for specimens collected from the thorax. This conflicts with the findings of Upchurch et al, where specimen location had no effect on magnitude of tissue shrinkage in dogs.\textsuperscript{49} The limited sample size (two interscapular and three lateral thigh masses) prevented evaluation of location on degree of shrinkage in our study. Additionally, our specimens were a product of aggressive
surgical excision of spontaneously occurring neoplasms which included bone and muscle, precluding direct comparison to the previous feline studies.

Limitations of the present report center on the relatively small sample size, potential inaccuracies in gross and/or histologic margin length measurements, and undetermined contribution of margin shrinkage during tissue processing from formalin to paraffin. Only 80 total measurements were obtained from the time of surgery through histopathological evaluation for 5 FISS tumor specimens. Our sample size is similar to the scope of previous prospective studies evaluating tissue shrinkage in dogs (e.g. 12 specimens were measured for intestinal shrinkage, 6 dogs were used for 36 total measurements for canine skin shrinkage, and 10 dogs were used for 20 total measurements in a study evaluating oral mucosa shrinkage). Challenges associated with accurately measuring gross and histological margins are not well characterized but specific efforts were taken in our study to address this potential source of error. To minimize errors in gross and histologic margin measurement, a fiducial marker was used as a common reference point at each time point, absolute margin measurements were normalized within individual cases by expressing them as a percentage of their corresponding in vivo GNSM and a single board-certified veterinary anatomic pathologist (CVL) personally trimmed all the samples.

As the margin measurements were averaged for each cat and not assessed individually, our study design did not assess shrinkage in specific directions or depth
of tissue. The authors acknowledge that ideally, post-formalin fixation measurements should have been obtained via physical palpation and gross measurement to maintain consistency across all collection points. For two cats in which formalin fixed tissues were still available at the time of manuscript review, additional post-formalin fixation GNSM measurements were taken using physical palpation and gross measurement. These measurements were an average of 3 mm larger than CT-derived post-formalin fixation GNSM lengths (data not shown). This is consistent with recent literature indicating that CT consistently overestimates FISS tumor size, and therefore would underestimate lateral margin length. The net effect of this potential discrepancy would be that the difference between ex vivo GNSM and post-fixation GNSM may be even smaller than reported in this study. We acknowledge that the concept of microscopic non-palpable disease being a factor in reducing HTFM size in veterinary neoplasms remains speculative at this point. Other variables likely worth pursuing in future studies include the overall composition of specimens (e.g. the inclusion of bone), shrinkage associated with histologic slide preparation, and the effect of primary tumor size, location, grade, and/or stage. Given the pilot study design of the present report, our goal was to begin to explore some of the specific factors involved in length discrepancies between in vivo GNSM and HTFM in order to guide potentially important future research efforts directed at informing clinically-relevant surgical margin planning.

In conclusion, we found that the majority of lateral margin shrinkage in FISS excision specimens occurred immediately following excision, prior to formalin fixation. After
accounting for tissue shrinkage up to post-fixation, we propose additional factors that may explain further discrepancies between *in vivo* GNSM and HTFM. As other excised cutaneous or subcutaneous neoplasms would contain similar tissue types (i.e. muscle, fat, connective tissue), it is reasonable to postulate that similar *ex vivo* changes may be encountered in other feline tumor types make these results more broadly applicable. This may provide surgeons with an improved rationale in their pre-operative surgical planning and interpretation of HTFM for cats with FISS, as well as inform future studies on the topic of surgical margin evaluation and accuracy.
ACKNOWLEDGEMENTS

The authors would like to thank the help and technical expertise of Jason Weist and Cynthia Viramontes. This study was funded by an Oregon State University Department of Clinical Sciences Intramural Resident Training grant.
FIGURE LEGENDS

**Figure 1** – Schematic representation of the steps involved in the reduction of lateral surgical margin size as a tumor specimen is surgically excised, formalin fixed, and histopathologically evaluated. The intra-operative measurement of *in vivo* GNSM (quadrant 1), immediately *ex vivo* GNSM (quadrant 2), post-formalin fixation (quadrant 3) and reported histologic tumor free margin (HTFM; quadrant 4). Excision related tissue shrinkage is found by subtracting *ex vivo* GNSM length from *in vivo* GNSM length (distance at position A). Formalin-related shrinkage is found by subtracting post-formalin fixation GNSM from *ex vivo* GNSM (distance at position B). Final reported HTFM by the pathologist may be further reduced due to presence of non-palpable microscopic disease infiltrating the surgical margin (C, stippled).

**Figure 2** – *In vivo* grossly normal surgical margin (GNSM); measured from palpable tumor to four lateral margins (indicated with sterile surgical marking pen as P = proximal; D = distal; Cr = cranial; and Cd = caudal). *In vivo* GNSM ranged from 35 to 55 mm in the current study with a preference for the widest possible margins that did not significantly increase the risk of patient morbidity or mortality based on regional anatomy (e.g. spinal cord).

**Figure 3** – *Ex vivo* grossly normal surgical margin; measured from palpable tumor to margin edges after excision but prior to formalin fixation. The use of a custom fiducial marker which was sutured to the skin prior to surgery served as a reference point and allowed *ex vivo* measurements to mirror those taken *in vivo*.

**Figure 4** – Representative example of post-formalin fixation grossly normal surgical margin; measured after formalin fixation for 48-72 hours as deemed necessary by the participating board-certified veterinary pathologist. Measurements were taken from the tumor edge to the four lateral cut surgical margins. The use of a fiducial marker that was easily identifiable on CT imaging allowed post fixation measurements to mirror those taken both *in vivo* and *ex vivo*.

**Figure 5** – Representative photomicrograph of histologic tumor free margin (HTFM) determination. In the photomicrograph provided, the tumor is seen at the bottom of the slide and is more basophilic than the surrounding skeletal muscle (right) and haired skin (left), with the inked surgical margin at the top of the image consisting primarily of subcutaneous fat. Microscopic neoplastic cells were seen extending from the gross mass toward the cut surgical margin and ending at the black arrow. In this example, the HTFM length was 16 mm (the narrowest distance from neoplastic cells, indicated by the black arrow, to the cut surgical margin).
Figure 1
Figure 3
Figure 4
Table 1 – Primary tumor location, size and margin measurement at each time point described. L = tumor length as measured in the cranial to caudal direction, W = width as measured from dorsal to ventral or left to right when on midline, D = depth as measured from superficial to deep. All initial measurements reported are based on preoperative palpation and contrast enhanced CT.

<table>
<thead>
<tr>
<th>Primary Tumor location and size (L x W x D)</th>
<th>Margin location</th>
<th>In vivo GNSM (mm)</th>
<th>Ex vivo GNSM (mm)</th>
<th>Post fixation GNSM (mm)</th>
<th>HTFM (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cat 1</td>
<td>Cranial</td>
<td>55</td>
<td>57</td>
<td>56</td>
<td>35</td>
</tr>
<tr>
<td>Right cranial thigh (16 x 20 x 7 mm)</td>
<td>Caudal</td>
<td>50</td>
<td>36</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Dorsal</td>
<td>50</td>
<td>30</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>50</td>
<td>36</td>
<td>38</td>
<td>35</td>
</tr>
<tr>
<td>Cat 2</td>
<td>Cranial</td>
<td>40</td>
<td>23</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Left lateral thigh (13 x 14 x 7 mm)</td>
<td>Caudal</td>
<td>40</td>
<td>34</td>
<td>39</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>Dorsal</td>
<td>40</td>
<td>20</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Ventral</td>
<td>40</td>
<td>22</td>
<td>19</td>
<td>17</td>
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<tr>
<td>Cat 3</td>
<td>Cranial</td>
<td>35</td>
<td>18</td>
<td>17</td>
<td>13</td>
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<tr>
<td>Interscapular (21 x 26 x 25 mm)</td>
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<td>35</td>
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<td>29</td>
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</tr>
<tr>
<td></td>
<td>Right</td>
<td>35</td>
<td>29</td>
<td>28</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>35</td>
<td>20</td>
<td>17</td>
<td>08</td>
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<td>Cat 4</td>
<td>Cranial</td>
<td>40</td>
<td>36</td>
<td>32</td>
<td>15</td>
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<tr>
<td>Interscapular (24 x 22 x 23 mm)</td>
<td>Caudal</td>
<td>40</td>
<td>32</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>40</td>
<td>29</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Left</td>
<td>40</td>
<td>33</td>
<td>34</td>
<td>23</td>
</tr>
<tr>
<td>Cat 5</td>
<td>Cranial</td>
<td>40</td>
<td>27</td>
<td>20</td>
<td>10</td>
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<tr>
<td>Right Lateral thigh (12 x 18 x 10 mm)</td>
<td>Caudal</td>
<td>40</td>
<td>28</td>
<td>28</td>
<td>22</td>
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<td>Ventral</td>
<td>40</td>
<td>25</td>
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</tr>
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</table>
Table 2 - Results of Tukey multiple comparisons analysis between all measurement points. Column one indicates each time point comparison with percent lateral margin shrinkage between these two points, 95% confidence intervals and associate p-values in columns two through four. The remaining columns show the same data as absolute values (mm). A significant reduction in lateral margins was noted between in vivo GNSM and ex vivo GNSM with no further significant reductions following formalin fixation. HTFM was approximately half of in vivo GNSM.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>%Margin shrinkage</th>
<th>p-value</th>
<th>Absolute diff (mm)</th>
<th>95% CI</th>
<th>p-value</th>
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<tr>
<td>in vivo GNSM vs. ex vivo GNSM</td>
<td>29.2</td>
<td>0.005</td>
<td>11.9</td>
<td>6.3 to 17.4</td>
<td>0.0034</td>
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<td>in vivo GNSM vs. Post fixation GNSM</td>
<td>31</td>
<td>0.0064</td>
<td>12.6</td>
<td>6.5 to 18.6</td>
<td>0.0037</td>
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<tr>
<td>in vivo GNSM vs. HTFM</td>
<td>50</td>
<td>0.0012</td>
<td>20.3</td>
<td>15.9 to 24.6</td>
<td>0.0002</td>
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<tr>
<td>ex vivo GNSM vs. Post fixation GNSM</td>
<td>1.9</td>
<td>0.4686</td>
<td>0.7</td>
<td>-1.1 to 2.5</td>
<td>0.4777</td>
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<tr>
<td>ex vivo GNSM vs. HTFM</td>
<td>20.9</td>
<td>0.0817</td>
<td>8.4</td>
<td>-0.9 to 17.7</td>
<td>0.0683</td>
</tr>
<tr>
<td>Post fixation GNSM vs. HTFM</td>
<td>19</td>
<td>0.0982</td>
<td>7.7</td>
<td>-1.5 to 16.9</td>
<td>0.0868</td>
</tr>
</tbody>
</table>
Chapter III

Evaluation of feline injection site sarcoma dimension and volume changes following formalin fixation using the ellipsoid volume formula and three dimensional computed tomography software

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Abstract

OBJECTIVE: To evaluate the degree of tumor dimensional and volume changes in surgically excised feline injection site sarcoma specimens after formalin fixation and to compare tumor volume data derived from the standard ellipsoid formula from 2D CT measurements and volume as determined using automated 3D CT software.

STUDY DESIGN: Prospective clinical study.

ANIMALS: Ten client-owned cats with injection site sarcomas.

PROCEDURES: Tumor dimensions (maximum length, width and depth) were measured from 2D CT images obtained preoperatively (in vivo) and after excision and formalin fixation (ex vivo). Measurements were used to calculate tumor volume using the standard ellipsoid formula. 3D CT volumetric data was obtained using a commercially available software package. A Students t-test was used to compare in vivo and ex vivo tumor dimensions and compare ellipsoid estimated volume and 3D CT volume.

RESULTS: Small (<7%) decreases were seen in tumor length, width and depth following formalin fixation. Only decreases in tumor length were statistically significant. Using the ellipsoid formula, average volume shrinkage between in vivo and ex vivo specimens was 12.8% (p=0.029). Using 3D CT software, average volume shrinkage was 12.7% (p=0.04). Ellipsoid volume calculations were significantly smaller than 3D CT volume measurements for both in vivo (mean 25.8% smaller; p = 0.038) and ex vivo (mean 24.8% smaller; p = 0.017).

CONCLUSIONS: Feline injection site sarcoma shrinkage following excision and formalin fixation was small and may be less than that of grossly normal feline tissues.
Ellipsoid formula volume estimation is lower than that of 3D CT volume measurements.
Abbreviations

FISS  Feline injection site sarcoma
CT    Computed tomography
HTFM  Histologic tumor free margin
HU    Hounsfield Units
MRI   Magnetic resonance imaging

Introduction

Feline injection site sarcoma (FISS) is a common soft tissue tumor developing at sites of prior vaccination with a prevalence ranging from 1 - 1.3 per 1,000 vaccinated cats.\(^3,76-78\) These tumors are estimated to represent 7 to 21% of skin neoplasms in cats, with recent evidence that they may occur secondary to injections and not just vaccines.\(^2,9,11\) There is considerable literature documenting the various histologic subtypes of FISS, biologic behavior and suggested treatment modalities.\(^23-25\)

Consistent with other sarcomas, local tissue invasion is a defining feature of FISS. It is thought that such neoplasms grow along the path of least resistance along fascial planes, leading to a non-uniform tumor boundary.\(^18\) This growth pattern and invasiveness may explain peritumoral lesions identified surrounding the tumor as identified on both CT and MRI, described previously for FISS.\(^30,31,34\) Other imaging findings reported for FISS include strong contrast uptake on CT angiograms and hyperintensity relative to surrounding musculature on T1W and T2W MRI.\(^30,31\)

Completeness of surgical margins in cats undergoing wide excision for FISS has been shown to correlate with decreased recurrence rate and greater median survival
In one report, tumor recurrence was 19% for those in which complete excision was reported whereas recurrence was 69% when neoplastic cells were seen at the excision margins. These characteristics of FISS have influenced current recommendations of aggressive local surgical excision (up to 5 cm lateral margins and two fascial planes deep) which led to improved median survival times and recurrence rates, compared to historically recommended, more conservative surgical excision.

Knowledge of all factors influencing pathologist reported histologic tumor-free margin is of upmost importance to the surgeon. Intuitively, these factors may include grossly normal surgical margin shrinkage following excision, tumor shrinkage, microscopic infiltrate beyond palpable disease and additional post-formalin processing changes such as alcohol dehydration of sections taken from paraffin embedded tissue blocks. Unfortunately, few reports of tissue shrinkage following surgical excision are present in the veterinary literature and are limited to grossly normal tissue. Only one peer reviewed report provides data on tissue shrinkage in cats and is limited to normal skin specimens. In that study, all statistically significant skin shrinkage was noted immediately after excision with no further changes after formalin fixation. While the authors did not report an average percent shrinkage, tables provided in the manuscript indicate that shrinkage was less than 10% at all measure points. This is less than is seen in canine samples, with tissue shrinkage of 15 to 32% reported. In recent unpublished work, the authors of the present report demonstrated an average of 29.2% in grossly normal (i.e. appearing
normal based on evaluation using unenhanced surgeon eyesight and palpation) lateral margin shrinkage following surgical excision of FISS specimens with no significant additional effect of formalin fixation. As neoplastic tissue differs from the surrounding tissue types that comprise the surgical margins (i.e. heterogeneity of tissue types, primary extracellular matrix components, inclusion of muscle), it is reasonable to suspect that *ex vivo* tumor shrinkage may differ from that of the grossly normal surgical margin. This phenomenon has been documented in a study of human basal cell carcinoma, where tissue shrinkage was non-uniform across a specimen and disproportionately high in the tumor-free margin.\(^{55}\) In that study, tumor-bearing skin contracted an average of 11%, whereas adjacent (tumor-free) skin contracted an average of 19%. This discrepancy would suggest that estimates of tumor-free margin may be erroneously low if based on an expected uniform tissue contraction. This may have significant implications on prognosis and post-surgical adjuvant therapy recommendations such as radiation or chemotherapy. Currently, no data exists on the degree, if any, of post excisional and post fixation, tumor related changes for any veterinary neoplasm.

Tumor volume has been shown to have prognostic value and is historically an important component of staging in a variety of canine and feline tumors. This has been postulated but not definitively demonstrated for FISS.\(^{79}\) In a recent MRI-based study for example, FISS tumor volume was not predictive of postoperative margin status.\(^{31}\) Using similar protocols, another study estimated tumor volume in FISS specimens but did not attempt to correlate this with clinical outcome.\(^{30}\) Both of these
reports estimated tumor volume using the volume formula of an ellipsoid. With this method, volume estimation is based on gross tumor dimension measurements in three dimensions (length, width and depth) using calipers or preoperative imaging with CT or MRI. This approach disregards projections or cavitations of the tumor, but is nonetheless a widely utilized method to estimate tissue volume.

With the increasing availability of 3D volume rendering software which allow the image of a tumor to be displayed and measured in three different planes using multi-detector CT scan images, clinicians now have a potentially more accurate means of tumor assessment prior to surgery. Using such 3D CT software, volume measurements have been validated and shown to be highly accurate. In one recent study, volume rendered three-dimensional reconstructions were made using canine stifle joints after intra-articular injection of a 10% iopamidol (370mg I/ml) and saline solution. A high correlation was found between actual volume infused and that measured on 3D CT by an observer blinded to the original volume ($R^2=0.90$, P<0.003). To the author’s knowledge, no veterinary reports have compared traditional ellipsoid volume estimates to 3D CT reported volumes for any neoplasm.

A detailed understanding of the three dimensional shape of a tumor and how it may change after formalin fixation are important for planning of surgical excision, as is accurate and detailed communication with the pathologist regarding margins of concern. The purpose of this study was to evaluate the degree of tumor dimensional and volume changes in FISS specimens after wide surgical excision using standard
ellipsoid volume calculations from 2D CT measurements derived from a single, displayed planar (and only measurable in two planes), compared to the volume reported by validated 3D CT computer rendering software. We hypothesized that there would be a small, but significant reduction in tumor dimensions after formalin fixation when compared to preoperative dimension measurements; and, that estimated tumor volumes would be statistically different between the ellipsoid formula and those calculated by 3D CT computer software. Cumulatively, we propose that the data herein could influence future CT and histologic margin interpretation, tissue volume measurement protocols and patient staging. This may direct future studies focused on refining surgical planning for cats with FISS.

**Procedures**

**Case selection**

Ten client-owned cats presenting for wide surgical resection of cytologically or histologically confirmed FISS admitted to the Oregon State University Veterinary Teaching Hospital between March 2013 and March 2015. Exclusion criteria included any pre-operative neoadjuvant therapy or prior tumor excision. These cats were concurrently enrolled in an Institutional Animal Care and Use Committee approved study evaluating the histologic diagnoses associated with imaging-identified peritumoral lesions in FISS and written informed consent for participation in the present study was obtained from owners via an approved study consent form.³⁴

**Computed Tomography**
Under the same general anesthetic event as surgical excision, dual phase CT angiogram studies using a 64 detector helical CT scanner\textsuperscript{a} was employed to acquire images both prior to, and following the intravenous administration of a non-ionic, iodinated contrast media\textsuperscript{b} at a dose of 1ml/kg using a power injector. No saline bolus was administered following contrast injection and image acquisition timing was set to obtain post contrast images in both the arterial and venous phases. Helical images were acquired as a volume with 0.5 mm voxels, 0.5 mm reconstruction interval, 0.5 sec rotation speed, 512 x 512 matrix, 120 kVp using a 150-350 mA tube current and a pitch of 0.83. The volume data were reconstructed in bone and soft tissue algorithms, and in isovolumetric transverse, sagittal, and dorsal image planes at a 2mm slice thickness. Images were presented and measured with window width and level for soft tissue (WW=120, WL=40) and bone (WW=2700, WL=350), but observers were allowed to adjust these settings. Following completion of excision, which ranged from three to five hours following initial CT angiogram, specimens were placed in 10% formalin for 48 to 96 hours (as necessary to ensure complete sample fixation, depending on sample size and thickness, as judged by the participating board-certified veterinary anatomic pathologist, CVL). Following fixation, specimens underwent CT imaging again with 0.5 mm voxels, 0.5 mm reconstruction interval, 0.5 sec rotation speed, 512 x 512 matrix, 100-120 kVp, 50-250 mA and a pitch of 0.83.

\textit{2D CT Tumor measurements}

Tumor dimensions (length, width and depth) were taken from the preoperative post-contrast venous phase CT DICOM images (\textit{in vivo}) and from the post fixation
specimen CT DICOM images (ex vivo) using the linear measurement tool of commercially available software. All measurements were acquired by the same observer (JT) and represent the maximum distance in millimeters identified in a particular plane (Figure 6). Tumor width was defined as that in a plane parallel to the cat’s spine, with the length measurement being perpendicular to this. Depth was defined in the plane from superficial to deep. Tumor attenuation (in HU) was measured on the venous phase of all in vivo CT angiograms and on the ex vivo CT images using the software region of interest tool. For each tumor, three separate 0.3 cm² regions that were confined to within the visible tumor boundaries and did not overlap were measured and averaged to obtain attenuation values.

3D CT measurements

Three dimensional reconstructions were made of the in vivo post-contrast venous CT images as well as the ex vivo specimen CT images using commercially available software. Using a previously described methodology, the window width and level were adjusted such that each tumor was easily discernable from surrounding tissues. This attenuation difference allowed three dimensional “trimming” of all non-tumor tissue. The software volume tool was used on the final (tumor only) image (Figure 7).

Statistics

To account for the range of tumor sizes in the study, ex vivo measurements (length, width, depth, ellipsoid volume, and 3D CT volume) were normalized by expressing them as a percentage of their paired in vivo measurements (where in vivo = 100%).
These measurements were then averaged to give an overall percent shrinkage following formalin fixation for each variable (length, width, depth, ellipsoid volume, and 3D CT volume). The ellipsoid volume used was: tumor volume = (length x width x depth) x \( \frac{\pi}{6} \)

A Students paired t-test was used to assess for differences between normalized \textit{in vivo} and \textit{ex vivo} tumor dimensions and volumes. All statistical analyses were performed using a commercially available computer software package.\textsuperscript{e} Unless noted otherwise, data are presented as mean ± standard error of the mean and with 95% confidence intervals (CI) when appropriate. A p-value less than or equal to 0.05 was considered statistically significant.

**Results**

Ten client-owned cats undergoing wide excision for FISS at the Oregon State University Lois Bates Veterinary Teaching hospital were prospectively enrolled in the study. Surgeries performed included wide excision of the lateral thigh musculature in four cats, hind limb amputation via coxofemoral disarticulation in three cats, wide excision of the dorsal neck epaxial muscles in two cats, and forequarter forelimb amputation in one cat.

Average post-contrast \textit{in vivo} tumor attenuation was 74.1 +/- 7.7 HU while average \textit{ex vivo} tumor attenuation was 70.2 +/- 3.8 HU (normal non-contrast soft tissue attenuation 40-60 HU).\textsuperscript{83} No statistical difference was noted in tumor attenuation
between *in vivo* and *ex vivo* measurement points, indicating persistent contrast enhancement following excision (p = 0.61). Mean *in vivo* and *ex vivo* tumor dimensions are summarized in Table 3. Median ellipsoid tumor volume estimate was 5.04 cm$^3$ *in vivo* and 3.99 cm$^3$ *ex vivo*. Median 3D CT volume was 5.40 cm$^3$ *in vivo* and 4.08 cm$^3$ *ex vivo*.

When expressed as a percentage of its paired *in vivo* measurement, the *ex vivo* length, width and depth of the specimen shrank an average of 4.8%, 2.2% and 6.1% respectively; however only shrinkage in length was statistically significant (Table 3). *Ex vivo* tumor volumes were significantly lower than *in vivo* tumor volumes for both the ellipsoid calculation (12.8% reduction; p = 0.029) and the 3D CT volumes (12.7% reduction; p = 0.04). Ellipsoid formula tumor volume calculations were significantly lower than 3D CT tumor volumes both *in vivo* and *ex vivo* (25.8% and 24.8%, respectively; p = 0.038 and p = 0.017, respectively).

**Discussion**

The *ex vivo* formalin fixed tumor length, width and depth shrank an average of 4.8%, 2.2% and 6.1% of the original *in vivo* venous contrast enhanced tissue measurements. Tumor length was the only value that showed a statistically significant change between *in vivo* and *ex vivo* measurement points. In contrast, tumor volume shrinkage was statistically significant when using both the ellipsoid formula to estimate volume and 3D CT volumes. Collectively, these data support our hypotheses that significant tumor dimensional shrinkage would be evident between *in vivo* and *ex vivo* time.
points and that a significant difference would be found between the ellipsoid formula and 3D CT reported tumor volumes. When comparing volume measurements obtained using the ellipsoid formula to those obtained using 3D CT software, the ellipsoid formula was found to result in significantly lower volumes at both the in vivo and ex vivo time points.

Tissue shrinkage following excision has been well described in human medicine and recently documented in the veterinary literature. In one canine study, average lateral dimensional shrinkage of surgically resected skin specimens was 21.1 to 32.0 %.

In that study, tissue composition with regard to the inclusion of muscle or fascia, affected overall ex vivo dimensional changes. In a study of normal canine small intestine specimens, mean shrinkage from pre-excision state was 28.3% immediately after excision and 26.3% after 24 hours of formalin fixation. In another study, mean overall shrinkage of normal canine skin samples was 15.6% with no significant tissue changes noted between immediately excised and post formalin measurement points. Currently, the only published report on feline tissue shrinkage showed small (>10%) but statistically significant changes in skin-only specimens immediately ex vivo with no further changes after formalin fixation. In recent unpublished work, we found a larger mean shrinkage (29.2%) in grossly normal lateral margins from FISS specimens. To the best of our knowledge, no previous veterinary study has specifically assessed tumor dimensional or volume changes following excision. Interestingly, our data indicate that tumor dimensional shrinkage is less than that reported of normal tissues for both cats and dogs. When evaluating
tumor dimensions, we found a significant difference between in vivo and ex vivo measurements only for length, however all dimensions showed a small (<7%) decrease. These changes in tumor dimensions following excision, along with previously reported shrinkage of grossly normal surgical margins, likely represent a key influence in lateral margin shrinkage and partially explain the difference between in vivo surgical margins and those reported by pathologists.

Previously, strong iodinated contrast enhancement has been reported for FISS in vivo. In the present study, we showed persistent contrast enhancement of the tumor following formalin fixation. To the authors’ knowledge, appearance of FISS specimens on ex vivo CT images and continued contrast enhancement of feline tumors ex vivo has never been reported. This characteristic facilitated tumor delineation and measurement of the formalin fixed (ex vivo) specimens, using the same CT-based methods as were used in vivo. Previous studies using the ellipsoid volume formula have reported median FISS tumor volumes ranging from 7.57 – 23 cm³. In our study, we showed that the ellipsoid formula consistently yielded significantly lower volumes when compared to 3D CT reported volumes, at both measurement time points. It should be noted that the current study utilized volume rendering as opposed to surface rendering algorithms for CT calculations. This was chosen due to literature indicating volume rendering is an accurate means by which to estimate tissue volume. The disparity between 3D CT reported volumes and the ellipsoid formula may be due to the three dimensional complexity and irregularity of a FISS tumor which is not accounted for with the ellipsoid formula. Previously, the
ellipsoid formula has shown to be an accurate and simple method to estimate the volume of relatively uniform structures such as normal testicles and renal masses. In contrast, other reports have shown the ellipsoid method to consistently underestimate human renal and prostatic volumes. Our findings highlight the potential limitations of the ellipsoid formula and may discourage researchers from using this method when volume measurements are of particular importance (such as for patient staging), and access to more advance and validated software is available.

Limitations of this study include the relatively small sample size and data collection performed by a single observer. In reviewing the current veterinary literature, many reports on tissue dimensional changes following excision contain similar sample sizes. It is reasonable to believe that the FISS tumors included in the present study are representative of most other FISS tumors. With the current sample size and standard deviations of each parameter measured, our study was appropriately powered (80% power with one-tailed alpha of 0.05) to detect a 4.9% or greater shrinkage in length, 10.7% or greater shrinkage in width, 10.9% or greater shrinkage in depth, 12.3% or greater shrinkage in ellipsoid volume and 13.2% or greater shrinkage in 3D CT volume. This means that differences smaller than those listed could be potentially missed by our study (being erroneously reported as having no significant difference), thus representing a type II statistical error. We based our decision on having data collection performed by a single observer on a previous report demonstrating high inter and intraobserver agreement with 3D CT volumetric measurements. The contrast administration protocol was non-standardized in this
study in relation to catheter size and catheter limb laterality in relation to the primary tumor. It is unknown how these factors may affect imaging measurements. Finally, all measurements in this study were taken from suspected tumor based on contrast medium enhancement. While strong contrast medium enhancement has been documented for FISS, it is quite possible that not all tumor boundaries enhanced equally and lead to partial or inaccurate volume measurements.\textsuperscript{30} To the author’s knowledge, no data is available on post excisional contrast attenuation for FISS and the effect of surgical excision, hemorrhage or postoperative manipulation of the mass on its attenuation characteristics remain unknown. Similarly, it is possible that contrast enhancing tissue at the edge of a FISS mass (the reactive zone) that was included in volume calculations may not actually represent neoplastic tissue.

In conclusion, we found a small but significant decrease in FISS length as well as tumor volumes following surgical excision and formalin fixation, with both volume measurement techniques used (ellipsoid formula and 3D CT software) showing a similar percentage of volume reduction. Furthermore, statistically significant lower tumor volumes were obtained from the ellipsoid formula compared to 3D CT reported volume. These data may add further understanding to the various factors that influence the disparity between grossly normal surgical margins excised \textit{en bloc} with a tumor during surgery and the HTFM reported by the pathologist. Furthermore, our finding may discourage clinicians from using the ellipsoid volume estimate method when volume accuracy is of particular concern and advanced imaging with a multidetector CT scanner is available. Further studies evaluating normal and
neoplastic feline tissue shrinkage and the relationship between surgical margin and histologic margin are warranted.

Footnotes

a. Toshiba Aquilion, Tochigi, Japan
b. Isovue/Iopamidol, 300 mg I/ml, Princeton, NJ, USA
c. eFilm, Merge HealthCare, Milwaukee, WI
d. Vitrea, Toshiba Medical Systems, The Netherlands
e. GraphPad Prism v6.02 for Windows, GraphPad Software, San Diego, CA
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Figure Legends

Figure 6 – Two dimensional transverse computed tomography images of the same feline injection site sarcoma *in vivo* (A) and *ex vivo* (B). Tumor maximum length (L) and depth (D) are labeled in each image. Maximum tumor width was measured in the orthogonal (sagittal) plane (not shown).

Figure 7 – Three dimensional computed tomography software images. Tumors were easily seen at both *in vivo* (A) and *ex vivo* time points. Using 3D computed tomography computer software, all non-contrast enhancing tissue could be removed (B), allowing for volume calculation of contrast enhancing tissue only. Note the multiple irregularities and projections shown in this tumor.
Figure 7
Table 3 - Mean tumor dimensional changes as measured from preoperative contrast enhanced computed tomography images (*in vivo*) and post excision computed tomography images of the tumors after formalin fixation (*ex vivo*). A statistically significant reduction in tumor length, estimated volume (ellipsoid formula) and 3D CT reported volume was found.

<table>
<thead>
<tr>
<th></th>
<th>In vivo</th>
<th>Ex vivo</th>
<th>% Change</th>
<th>95% CI</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (cm)</td>
<td>3.27 ± 0.86</td>
<td>3.15 ± 0.87</td>
<td>-4.8%</td>
<td>-9.2 to -0.36%</td>
<td>0.037</td>
</tr>
<tr>
<td>Width (cm)</td>
<td>3.20 ± 0.77</td>
<td>3.21 ± 0.87</td>
<td>-2.2%</td>
<td>-11.9 to 7.5%</td>
<td>0.600</td>
</tr>
<tr>
<td>Depth (cm)</td>
<td>2.12 ± 0.5</td>
<td>1.98 ± 0.46</td>
<td>-6.1%</td>
<td>-15.9 to 3.8%</td>
<td>0.200</td>
</tr>
<tr>
<td>Median ellipsoid Volume (cm$^3$)</td>
<td>5.04</td>
<td>3.99</td>
<td>-12.8%</td>
<td>-24 to -1.65%</td>
<td>0.029</td>
</tr>
<tr>
<td>Median 3D CT Volume (cm$^3$)</td>
<td>5.40</td>
<td>4.08</td>
<td>-12.7%</td>
<td>-24.7 to -0.7%</td>
<td>0.040</td>
</tr>
</tbody>
</table>
4.1 Summary

Despite changes in vaccination location and frequency, injection site sarcomas remain a common cutaneous and subcutaneous neoplasm in cats. Current treatment recommendations center around wide surgical excision due to the local invasiveness these tumors display. Surgical margin status is of particular importance in predicting recurrence for FISS.

The length of tumor free margin around a surgically excised neoplasm is often different when comparing that measured at surgery and that measured histologically. While this discrepancy is not surprising, the magnitude and potential factors involved remain poorly described for veterinary species. In the studies presented here, we begin to define the degree and processing step of lateral margin shrinkage in the first tumor bearing report in the feline species. This is key as tissue shrinkage is likely a large influence on GNSM to HTFM length discrepancy. Consistent with other reports, our findings indicate that the majority of clinically relevant shrinkage occurs prior to formalin fixation. In the second report we showed that FISS specimens themselves do not shrink to the same degree as the normal surrounding tissue. The complexity of FISS peritumoral projections was shown via 3D CT and suspected to play a role in the differences in volume calculations observed.

Taken together, these data begin to define and further explore the GNSM to HTFM difference. A variety of future studies are needed to define additional areas that affect margin size and interpretation. Further understanding of any influences on GNSM to
HTFM discrepancy may allow more accurate surgical planning and has the potential to reduce patient morbidity.

4.2 References


