# A TRANSLATIONAL STUDY EVALUATING THE USES OF DIAGNOSTIC AND THERAPEUTIC PRACTICES ESTABLISHED IN HUMAN MALIGNANT MELANOMA IN EQUINE MALIGNANT MELANOMA

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## ABSTRACT

Malignant melanoma is a neoplasm of melanocytes. It typically originates in skin, but may metastasize to other body systems. It is a relatively common neoplasm in both humans and horses, with striking similarities across both species. Heritable genetic factors associated with melanoma have been identified in both human malignant melanoma (HMM) and equine malignant melanoma (EMM). This work investigates similarities and differences of EMM and HMM through comparative protein expression using immunohistochemical staining. Nestin, Pax-3/7, B-Raf, and SOX-10 are commonly expressed in HMM tissues. Expression of these proteins is not noted in normal human melanocytes, is associated with decreased melanocytic differentiation, and with increased proliferation leading to tumorigenesis. My findings demonstrate similar expression of these proteins in EMM. Aberrant protein expression patterns may signal underlying genetic mutations. Similar abnormal expression patterns suggest that EMM and HMM may share common genetic abnormalities.

Treatment of malignant melanoma presents similar challenges in both horses and humans. In general, early stages of the disease can be successfully treated with surgical excision; however, advanced stages of EMM and HMM are often refractory. Therefore, development of novel therapies for advanced stages of melanoma is essential in both species, with the horse representing a useful model for this process.

One novel therapy, frankincense oil (FO) is a resin distillate from trees of the genus *Boswellia*. Studies have demonstrated cytostatic and apoptotic-modulating properties of FO in various human cancer cell lines. No studies have evaluated FO as a therapy for skin neoplasms. In my work, the apoptotic properties of FO from *Boswellia frereana* were verified in a HMM cell line (SK-Mel-5). The cytotoxicity and therapeutic efficacy of FO were also studied by evaluating the effects of FO in cases of perianal EMM. FO was found to be consistently cytotoxic when injected directly into EMM tumors, but largely inconsequential when applied topically. FO was found to substantially decrease the size of injected masses. These findings suggest that FO could be useful for debulking large masses in late stage dermal EMM.

The combined results of these studies support further investigation of EMM as a translational model for HMM.

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Common Abbreviations: Equine Malignant Melanoma (EMM), Human Malignant Melanoma (HMM), Frankincense Oil (FO), Cancer Stem Cell (CSC)

Multimedia Objects

## Introduction

Neoplasia is the pathologic process in which an abnormal mass of tissue accumulates as a result of persistent, excessive, and uncoordinated growth of mutated, abnormal cells [1]. Neoplasms are typically categorized as either benign or malignant, with the capacity for local tissue invasion and systemic metastasis of malignant neoplasms representing the most notable distinctions [2]. Malignant melanoma is a neoplasm that results from the uncontrolled growth of mutated melanocytes. It most commonly originates in the skin. In the United States, an estimated 76,690 new cases and 9,480 deaths will be attributed to human malignant melanoma (HMM) in 2013, making it the least common but most deadly common skin neoplasm in people [3]. These statistics have been steadily increasing for more than 30 years, with an increasing incidence in younger individuals[3]. Increased risk of HMM has been associated with exposure to UV radiation, the presence of nevi/moles (a small growth resulting from a well-circumscribed clustering of melanocytes), a family or personal history of melanoma, immune suppression, fair skin that freckles, light hair color, light eye color, and xeroderma pigmentosum (a rare, inherited condition causing extreme sensitivity to ultraviolet (UV) radiation) [4].

- Diagnosis of HMM is primarily achieved through histopathologic evaluation of biopsied lesions, with lymph node biopsies, imaging studies, and blood tests providing additional information for staging of more advanced cases [4]. The American Joint Committee on Cancer (AJCC) “Tumor Node Metastasis [TNM] System” is the most commonly used system for pathological staging of HMM. This system scores the tumor, degree of lymphatic involvement, and the extent of

metastasis and then classifies the melanoma based on the combination of these scores. The overall stage is represented by Roman numerals I through IV, with lower numbers representing less advanced disease. Subsets of each stage, indicating variations in mitotic rate, tumor thickness, ulceration, and the extent of metastasis, are designated by capital letters. A Stage 0 HMM also exists, in which the neoplasm is present in the epidermis but has not spread into the dermis.

- Stage I melanomas are less than 1 mm thick;
- Stage II melanomas are thicker than 1 mm, but have not infiltrated the lymphatics;
- Stage III melanomas have metastasized to the local lymphatic system, but have not metastasized to distant organs; and
- Stage IV melanomas are those that have metastasized to distant sites.

For stage I-II HMM, surgical excision is often curative [4], resulting in an 85% average 5-year survival rate for stage IA through stage IIB [5]. However, once HMM has invaded the lymphatic system or spread to distant organ sites, treatment becomes much more difficult and 5-year survival (a measurement of treatment efficacy) drops. Other therapies, such as chemotherapy, immunotherapy, and radiation, are often used in more advanced stages of disease, but response rates are low. For stage III HMM, the average 5-year survival rate is 59% and drops to 45% after 10 years [5]. Stage IV HMM has a 15-20% survival rate at 5-years and only a 10-15% survival rate at 10 years [5].

The increasing incidence and mortality associated with HMM demonstrates the need for novel treatment options, particularly for stage III and IV melanomas.

Melanoma has been noted in various other species and proposed as models for the study of HMM. These animal species include fish, dogs, pigs, and horses [6-9]. Among these, the Platyfish-Swordtail hybrid and Sinclair swine have received the most attention as potential translational models for melanoma [8, 10]. A translational model of human disease is a naturally occurring or experimentally induced disease state in a surrogate animal, with sufficient similarity to human disease to produce high predictive values in experimental research. Animal models are commonly used for translational research investigating the disease pathogenesis and efficacy of potential therapies [11]. Key features of a good translational animal model include reliable and consistent expression of relevant manifestations of disease and long-term survivability sufficient for extended study [11]. The validity of animal models is most commonly limited by inadequate similarity between the disease model and human disease due to the induction of disease in otherwise healthy animals or inherent differences in naturally occurring animal models [12].

*Based on our work, I hypothesize that the horse is a valid translational animal model for HMM that is not the result of exposure to excessive amounts of ultraviolet (UV) radiation*. As such, the study of melanoma in horses may provide valuable insights into the pathobiology of malignant melanoma and provide a vehicle for developing and testing effective therapies for horses and humans alike.

This doctoral dissertation reviews the published, relevant literature on EMM and then describes a series of laboratory and clinical studies directed to:

- Establish that mutation (evaluated by changes in the expression of selected genes through the use of immunohistochemical staining techniques) occurs in EMM,
- Analyze the homology of immunohistochemical changes in EMM and HMM as a means of supporting the value of EMM as a translational model of HMM, and,
- Evaluate a novel therapeutic, frankincense oil, in both tissue culture studies of HMM and in clinical studies in horses with EMM, showing similar cytolytic effects of the agent on tumor cells *in vitro* and *in vivo*, and
- Test a presumed heritable link for the development of EMM in a related population of Thoroughbred horses.

The document is organized in chapters formatted as a series of publications, either currently in print or in draft format for submission.

**Literature Review (Melanoma in horses: Current perspectives)**

J.S. Moore, C. Shaw, E. Shaw, V. Buechner-Maxwell, K. Scarratt, M. Crisman, M. Furr and J. Robertson

(Moore, J.S., Shaw, C., Shaw, E., Buechner-Maxwell, V., Scarratt, W.K., Crisman, M., Furr, M., Robertson, J., *Melanoma in horses: Current perspectives.* Equine Veterinary Education, 2013. **25**(3): p. 144-151.)

## Abstract

Debate surrounding the nature of equine melanoma has resulted in an underestimation of its life-threatening potential. Contrary to popular dogma, the variable, often slow, rate of growth commonly associated with equine melanoma does not warrant benign classification. Equine melanoma is a malignant neoplasm with the capacity for local invasion and metastasis. A classification scheme has been proposed by Valentine (1995); however, this system does not address the progressive nature of equine malignant melanoma (EMM). Additionally, frustration with conflicting therapeutic recommendations has led many practitioners to inappropriately advocate benign neglect. This article addresses the need for a clinically applicable, standardized classification system, provides a review of current therapies and recommendations for equine practitioners, and comments on the future directions of EMM research.

## Introduction

Melanoma in horses is a common, variably pigmented (grey/brown/black), infiltrative neoplasm which often presents in advanced stages as a multicentric malignancy. It is most commonly seen in grey and white-coated horses and, according to one widely-cited reference, reaches approximately 80% prevalence in aged populations [13]. The

author of this report also predicts that virtually all grey horses will develop "melanocytosis" if they live long enough [13].

The clinical and pathological nature of equine melanoma has been debated for over 225 years. Early authors suggested that equine melanoma was either non-neoplastic (i.e., a mole/pigmented nevus), benign pigment cell dysplasia or a pigment storage disorder [14-19]. Others reported melanoma as a true neoplasm; however, debate over classification of melanoma as a benign or malignant neoplasm continued throughout the 20$^{th}$ century [9, 20-22]. More recently, the concept of equine melanoma as a form of malignant neoplasia- or neoplasia with malignant potential- has become more accepted, with some sources claiming that at least 66% of equine melanocytic tumors eventually become malignant [23].

The purpose of this article is to review the current state of knowledge on equine malignant melanoma (EMM), present a new system for staging EMM, discuss current therapies and comment on the future direction of EMM research.

**Gross and Microscopic Pathology**

Valentine [21] wrote an extensive review of the pathological characteristics of equine melanoma based on a retrospective study of 53 cases. She proposed that there are at least four manifestations of equine melanotic disease: melanocytic nevus, discrete dermal melanoma, dermal melanomatosis, and anaplastic malignant melanoma.

Discrete dermal melanoma and dermal melanomatosis, collectively referred to as dermal melanoma, represent the large majority of melanoma diagnoses in grey horses.

Discrete dermal melanomas are seen in grey horses and generally exist as single masses in typical or atypical locations. Afflicted horses are mature, but not aged, with an average age of 13 years. Discrete dermal melanomas are further differentiated into benign and malignant forms, with surgical excision appearing to be curative in most circumstances; this finding was supported by studies of Rowe and Sullins [24].

Dermal melanomatosis is a condition seen in grey horses involving multiple cutaneous masses, with at least one of the masses presenting in a “typical” location. These typical sites include the undersurface of the tail, anal, perianal and genital regions, perineum, and lip commissures [25]. Like discrete dermal melanoma, dermal melanomatosis is most frequently seen in mature horses. However, dermal melanomatosis is associated with a slightly older average age of 17 years, a figure at variance with our studies in which the mean age was less than 10 years [22]. According to Valentine, these masses are not amenable to surgical resection and are very likely to be associated with visceral metastasis. This condition is considered to be potentially fatal.

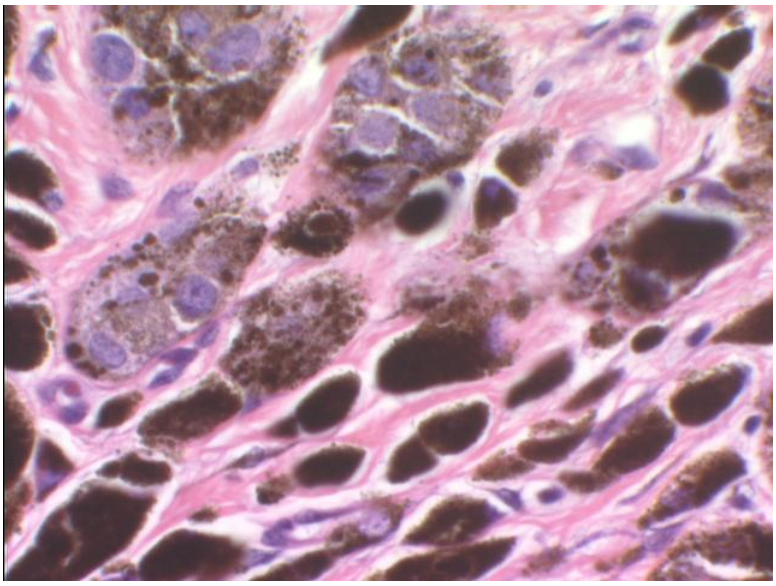

Figure 1: An H&E stained section of typical EMM, showing pleomorphic and variably pigmented neoplastic cells infiltrating connective tissue.

Discrete dermal melanomas and dermal melanomatosis are generally histologically indistinguishable, presenting as indistinct, heavily-pigmented tumor cells in the deep dermis (**Figure 1**). Whilst Valentine described these manifestations of melanotic disease as distinctly separate, it seems equally probable that these diagnoses exist as a continuum.

Discrete dermal melanoma may represent a malignancy *in situ* - a "condition in which one or several cells have acquired the ability to invade and metastasize, but have not yet exercised these options [26]." This has yet to be confirmed and further studies to identify predictive markers for metastasis would need to be performed. However, assuming that discrete dermal melanoma and melanomatosis exist as a continuum, progression from discrete dermal melanoma would depend upon additional genetic or epigenetic events occurring. If one considers the relatively small overall incidence of all

types of neoplasms in horses, it is possible that, as a species, horses may be resistant to genetic mutation. This hypothesized innate genomic stability may be important, since progression from discrete melanoma to melanomatosis would not occur without additional, sequential mutations. This has important implications for tumor management – early removal of discrete tumors may well preclude development of more aggressive forms.

**Clinical Presentation and Staging of EMM**

There is an extensive body of veterinary literature describing the clinical presentation of EMM (**Table 1**). In a retrospective study of the medical records of 67 cases of EMM from Virginia, Pennsylvania and Kentucky, 'typical' horses with EMM ranged in age from less than two years to over 15 years, with a median age of 7.6 years [22]. There was no clear sex predilection, or obvious effect of castration on the development or progression of EMM. As noted by many authors, grey horses were significantly overrepresented in this study. This demographic data is in agreement with that of Valentine [21] and unpublished data from Steier [27]. Taken together, these three studies include nearly 200 horses with EMM.

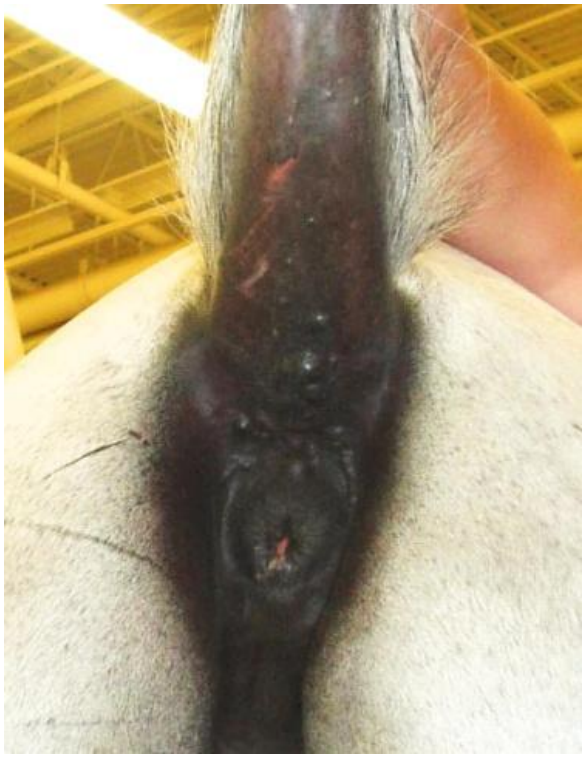

Figure 2: Photograph of multiple, small, distinct melanoma nodules on the perineum and ventral tail of an 11-year-old Thoroughbred gelding (photo courtesy of Ms. Karen Witter).

Many melanomas initially present as single, small, raised nodules on the perineum. Owners of horses may see small melanomas during grooming or veterinarians may note them incidentally during routine physical examinations. In some horses, multiple nodules may be seen at once. Nodules commonly occur on the perineum, tail base, sheath, commissures of the lips, in the parotid/jugular furrow, and subauricular lymph nodes [28, 29] (**Figure 2**).


Figure 3: Photograph of multiple coalescing nodular and plaque-like melanomas in the area of the parotid salivary glands and subauricular lymph nodes of an 11-year-old Thoroughbred gelding.

During stages of the disease in which there is little growth, lesions may exist for many years and cause no clinical problems for either horse or owner. However, this changes when lesions enlarge and coalesce. In some cases, tumor bulk is so substantial in the area of the throat latch that affected horses are unable to flex at the poll, turn their heads from side to side, or eat and drink in comfort (**Figure 3**). Similarly, coalescing perianal tumors frequently become large enough to limit defecation and result in fecal impaction. Lesions may become quite large and develop necrotic cores capable of ulceration, leading to secondary bacterial infection. Surgical debulking of advanced lesions is difficult and, many times, unrewarding.

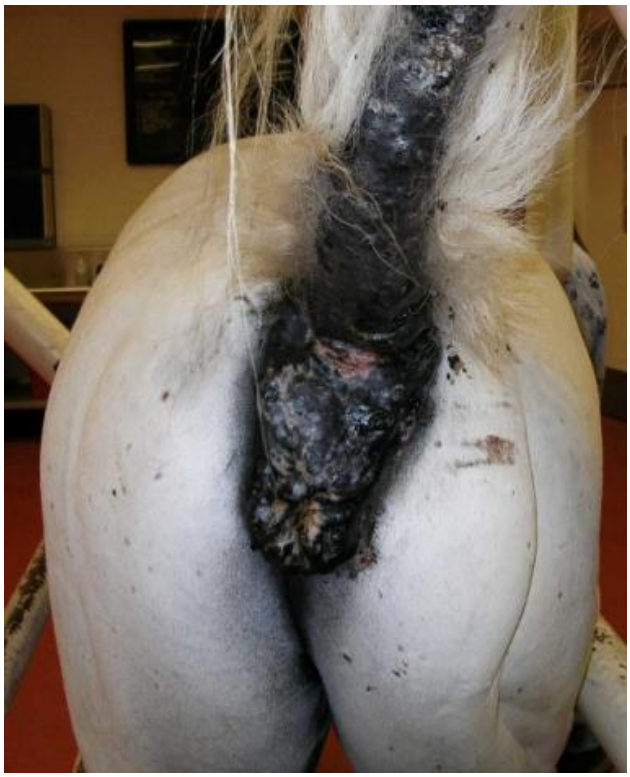

Figure 4: Photograph of large confluence of nodular and plaque-like melanomas on the ventral tail and perineum with ulceration and fissure formation at the junction of the tail and perineum in a 13-year-old Thoroughbred gelding.

Expansile tumor growth deeply infiltrates musculature and may compromise blood supply, leading to the development of fissures penetrating deeply into soft tissue and, at times, to bone. **Figure 4** is an example of such a large, complex mass on the perineum of a 13-year old Thoroughbred gelding. Tumor growth around the anus was extensive and the horse experienced intermittent discomfort associated with rectal fecal impaction. This was somewhat controlled through dietary modification, but was a persistent problem. At the junction of the tail and the perineum, a fissure developed within the mass that terminated at the adjacent vertebral body. Attempts to debride this fissure and promote healing by granulation were unproductive and the horse was euthanized due to pain and incipient osteomyelitis.

Almost invariably, tumor nodules that are expanding are quite firm, strongly suggesting the activation of fibroplasia by tumor cells or in response to tumor cell growth. Likewise, we have seen many tumors that are well-vascularized and this may reflect enhanced angiogenesis as a result of tumor cell proliferation.

There is currently no consensus on whether multiple lesions on a single horse are metastatic or arise spontaneously as multicentric, separate neoplasms. However, there are many references that provide evidence of implantation, lymphatic, and hematogenous metastasis [30-37] (**Figure 5**).

Figure 5: Photograph of multiple coalescing tumor nodules in the mesentery of a horse subjected to euthanasia for progressive weight loss and colic.

There are also reports of primary visceral masses. Primary visceral melanoma has been documented in the gastrointestinal tract, vertebral canal, skeletal muscles, guttural pouches, and salivary glands of grey horses [36, 38-40]. Tarrant *et al.* [41] report one

such case in which a 22 year old, grey, Arabian gelding presented for acute colic and was found to have melanotic masses in the liver, spleen, bone marrow, and superficial cervical and tracheobronchial lymph nodes, but not the skin. The pattern of organ infiltration was consistent with hematogenous, lymphatic and transcoeloemic metastasis and the masses were found to be compatible with dermal melanomatosis, but a primary dermal tumor was not located. Although the authors admit that a primary cutaneous mass may have been overlooked, they also present two proposals for the origin of primary visceral melanotic disease. The first of these speculations relies upon the embryological rest theory, which suggests that melanocytes become retained in an embryological rest and experience malignant transformation. Alternatively, these authors propose that abnormal cell migration could result in melanocytes in atypical sites. There is little evidence to support or refute either hypothesis and further studies are necessary if the origin of visceral melanotic disease is to be identified.

Additionally, there are three reports of congenital melanoma. All three cases involved masses believed to be of dermal origin. The first of these cases involved a 30x20x10 cm mass on the dorsal midline of a 6-month-old chestnut Arabian colt. This case resulted in humane euthanasia due to the expansile, infiltrative and metastatic nature of the mass [42]. The remaining two cases involved a 3-month-old gray Quarter Horse filly with a 1 cm ulcerated perineal mass and a 2-week-old (gray at maturity) Appaloosa colt with an 18x7 mm mass in the skin of the left lateral thorax. Both were successfully treated with surgical excision [43].

## Table 1: Case Report Data

| Author(s), Title | Number of Cases | Diagnosis and Staging |
|---|---|---|
| Baptiste *et al, Three Cases of Tumors Associated with the Equine Guttural Pouch* | 2 | Stage 3 Dermal Melanoma with Visceral Metastases |
| Caston and Fales-Williams, *Primary Malignant Melanoma in the Oesophagus of a Foal* | 1 | Primary Visceral Melanoma |
| Covington *et al, Recurrent Esophageal Obstruction and Dysphagia Due to a Brainstem Melanoma in a Horse* | 1 | Stage 3 Dermal Melanoma with Visceral Metastases |
| Cox *et al*, *Congenital Malignant Melanoma in Two Foals* | 2 | Stage 1 Congenital Dermal Melanoma |
| Fleury *et al, The Study of Cutaneous Melanomas in Camarague-Type Gray-Skinned Horses: Clincal-Pathological Characterization* | 181 | Stage 0 Dermal Melanoma |
| | 83 | Stage 1-4 Dermal Melanoma |
| Hamilton and Byerly, *Congenital Malignant Melanoma in a Foal* | 1 | Congenital Melanoma (non-grey) |
| Kirker-Head *et al, Pelvic Limb Lameness Due to Malignant Melanoma in a Horse* | 1 | Stage 2 Dermal Melanoma with Visceral Metastases |

| | | |
|---|---|---|
| MacGillivray *et al, Metastatic Melanoma in Horses* | 3 | Primary Visceral Melanoma |
| | 11 | Stage 1-4 Dermal Melanoma with Visceral Metastases |
| Murray *et al, Signs of Sympathetic Denervation Associated with Thoracic Melanoma in a Horse* | 1 | Primary Visceral Melanoma |
| Patterson-Kane *et al, Disseminated Metastatic Intramedullary Melanoma in an Aged Grey Horse* | 1 | Stage 4 Dermal Melanoma with Visceral Metastases |
| Robertson, Melanoma In: *Equine Pathology* | 40 | Stage 1-2 Dermal Melanoma |
| | 27 | Stage 3-4 Dermal Melanoma |
| Rodriguez *et al, Metastatic Melanoma Causing Spinal Cord Compression in a Horse* | 1 | Stage 3 Dermal Melanoma with Visceral Metastases |
| Schott *et al, Melanoma as a Cause of Spinal Cord Compression in Two Horses* | 2 | Stage 3 Dermal Melanoma with Visceral Metastases |
| Seltenhammer *et al, Equine Melanoma in a Population of 296 Grey Lipizzaner Horses* | 148 | Stage 0 Dermal Melanoma |
| | 83 | Stage 1-2 Dermal Melanoma |
| | 65 | Stage 3-4 Dermal Melanoma |
| Steier, unpublished personal communication | 55 | Stage 1-2 Dermal Melanoma |
| | 23 | Stage 3-4 Dermal Melanoma |

| | | |
|---|---|---|
| Tarrant *et al, Diagnosis of Malignant Melanoma in a Horse from Cytology of Body Cavity Fluid and Blood* | 1 | Primary Visceral Melanoma |
| Traver *et al, Epidural Melanoma Causing Posterior Paresis in a Horse* | 1 | Primary Visceral Melanoma |
| Valentine, *Equine Melanocytic Tumors: A retrospective study of 53 horses* | 16 | Melanotic Tumors of Non-Greys (Melanocytic Nevi and Anaplastic Malignant Melanoma) |
| | 17 | Stage 1-2 Melanocytic Nevi and Discrete Dermal Melanoma |
| | 15 | Stage 3-4 Discrete Dermal Melanoma and Dermal Melanomatosis |
| | 5 | Unstaged Melanocytic Nevi and Discrete Dermal Melanoma |

Based on clinical observations and a review of published case reports, we agree with the aforementioned suggestion that equine melanoma possesses the potential for malignant behavior. In particular, dermal melanoma demonstrates a proclivity for local invasion and metastasis. As such, we propose that all manifestations of dermal melanoma in grey horses should be referred to as equine malignant melanoma (EMM). In addition, we propose that EMM is best classified into stages of increasing expression of malignant behavior (ie- rapid growth, local invasion, metastasis, etc.). As the majority of cases seen by us have presented as more advanced manifestations of disease (ie- not amenable to surgical excision), we recognize the importance of regular, standardized evaluations for detection of significant changes in tumor behavior.

Consequently we have proposed a clinical classification system in which equine malignant melanoma of grey horses occurs in five stages (**Table 2**).

**Table 2: EMM Clinical Staging System**

| Stage | Tumor Burden | Individual Tumor Diameter | Dissemination / Metastasis | Growth Pattern |
|---|---|---|---|---|
| 0 | none | - | - | - |
| 1 | single | < 2 cm | absent | slow to quiescent; maintaining diameter of < 2 cm for months to years |
| 2 | multiple | < 2 cm | absent | slow to quiescent; maintaining diameter of < 2 cm for months to years |
| 3 | multiple | < 4 cm | present | slow; changes in size ≤ 25% over a course of months to years |
| 4 | multiple | > 4 cm | present | rapid; changes in size > 25% over a course of weeks to months |

## Current Therapies for Equine Malignant Melanoma

While many therapies have been tested, there is no widely accepted treatment for EMM. A limited number of early stage malignancies located on the tail or in the perineal region have been surgically excised with apparent success [24]. However, surgical excision is not a realistic option in many advanced cases, particularly those with significant local invasion of tumors in areas, such as the parotid salivary gland, that pose a more complicated surgical approach. It is also important to note that localized excision does not prevent further progression of disease. Nearly half of the horses in the study performed by Rowe and Sullins experienced either an increase in pre-existing

tumor size or number of tumors at distant or local sites following localized surgical excision [24].

Cimetidine, a histamine type II receptor antagonist, has been used as a therapeutic agent for melanoma. A study by Goetz and co-workers [44] showed cimetidine to be effective at reduction of tumor size and number in three adult horses with multifocal melanoma. Diagnosis was confirmed in two of the three horses via histopathology, while the third was diagnosed based on clinical examination alone. Additionally, while all three horses were noted to have multiple masses in multiple anatomic locations, the maximum diameter measured prior to initiation of cimetidine therapy did not exceed 2 centimeters. Treatment consisted of oral administration of 2.5 mg/kg of cimetidine every 8 hours for a period of 4 to 12 months. All 3 horses were reported to have a 50-90% decrease in size and number of tumors [44]. However, a more recent study by Laus and co-workers [45] found cimetidine therapy to be ineffective at reducing tumor size or number in horses with dermal melanomatosis, with only one out of ten treated horses showing a reduction in tumor volume. Histopathology and cytology were used to confirm the diagnosis of melanoma in all of the horses that participated in this study. The trial involved a total of fifteen horses evenly divided into a control group and two treatment groups, one in which the horses were administered 3.5 mg/kg of cimetidine orally every 12 hours for 60 days and a second in which the horses were administered 7.5 mg/kg of cimetidine orally daily for 60 days. The horses were evaluated weekly during treatment and monthly following treatment for a total of 12 examinations. The only horse to show a slight, localized, reduction in tumor size belonged to the second treatment group (7.5

mg/kg PO SID) [45]. The findings of Laus and co-workers [45] are in congruence with similar studies by Bowers and co-workers [46] and Warnick and co-workers [47].

Various mechanisms of action for cimetidine have been proposed, including selective inactivation of suppressor T-cells, enhanced natural killer cell activity, antagonism of histamine type II receptors, and anti-inflammatory effects, but their significance with regard to treatment of EMM has yet to be established [47-50]. Inconsistent results have made cimetidine an unreliable treatment option for horses with EMM. Some propose that these inconsistencies may be related to the variability within EMM itself, suggesting that variations in cellular metabolism, differentiation or receptor activation may be responsible [45], but there is no evidence to substantiate this theory.

Treatment of EMM using toremifene, a triphenylethylene derivative, has also been attempted. This orally administered compound has primarily been used in the treatment of human patients with metastatic breast cancer [51, 52], but has been shown to exhibit antiestrogenic and nonantiestrogenic inhibitory effects on melanocyte lines *in vitro* [53]. Toremifene was administered transdermally in a topical gel preparation to one horse with malignant melanoma. It was found to provide high local concentrations at the site of administration and a slight reduction in tumor volume, but results were never verified with histopathology and further studies were not performed [54].

Recent reports indicate that cisplatin might be useful in the treatment of equine dermal melanoma. In one such study [55], thirteen horses with 16 subcutaneous and dermal

melanotic tumors were treated with a sesame oil-based cisplatin formulation administered via intratumoral injections. Epinephrine was added to the cisplatin formulation to optimize drug localization and prevent bleeding. Treatment consisted of a series of 4 intratumoral administrations at 2 week intervals, resulting in an 81% success rate. Therapy was less effective in larger, more advanced tumors and in tumors that had previously been treated by other methods, especially cryotherapy. In addition, new tumors continued to develop outside of the treated area during the follow-up period, which ranged from two to six years.

Similarly, a study testing the efficacy of implanting cisplatin-containing biodegradable beads in various equine cutaneous tumors, including melanoma, has also been performed. Each 3mm bead contained approximately 1.6 mg of cisplatin in a commercially available calcium sulfate and dextran sulfate matrix material. Two out of thirteen cases of cutaneous melanoma were treated with beads alone, while the remaining eleven cases received a combination of conventional debulking or CO2 laser debulking in addition to bead implantation. Researchers found that treatment resulted in "successful resolution of the tumor for at least 2 years after treatment" [56].

A preliminary study to evaluate the therapeutic potential of an autochthonous vaccine has also been performed. The vaccine contained whole, autogenous tumor cells combined with adjuvant and was injected subcutaneously over regional lymph nodes. In the study, the vaccine was administered every other week for six weeks and then every six weeks for an undisclosed period of time. The author reported tumor regression in 11

out of 12 horses; however, corroborative data has yet to be presented [57]. In addition, a study examining the efficacy of local suicide gene therapy in combination with a systemic anti-cancer vaccine was evaluated in a single horse with melanoma. In this study, superficial tumors were removed under general anesthesia and used, in combination with live irradiated xenogenic Chinese hamster ovary cells, to prepare a vaccine that was later injected subcutaneously into the flanks. Vaccine administration occurred weekly for 5 weeks, every 2 weeks until day 105 and then every 28 days until day 245. In addition, the surgical margins were infiltrated with lipoplexes carrying the HSV*tk* gene and ganciclovir directly following surgery. Weekly doses of *HSVtk* lipoplexes and ganciclovir were injected into the remaining subcutaneous tumors. After five localized treatments of six masses, four “developed draining fistulae that healed and dissolved completely” and two decreased in size by approximately 50 percent. Three untreated lesions decreased in size until they disappeared completely. Follow-up continued for thirty-three months and the horse was reported to have no local relapses of melanoma in the areas that had been treated surgically, but follow-up regarding other tumor sites was not mentioned [58].

An alternate immunotherapeutic approach that has shown some promise in treating EMM is interleukin therapy. In 2001, a study aimed at evaluating the effects of intratumoral IL-12 injection on gray horses with metastatic melanoma was performed [59]. Twelve masses in a total of seven grey horses received intratumoral injections of IL-12 encoding plasmid DNA in a series of one to four cycles. Each cycle consisted of 3 injections administered every 2 days. Mean tumor size, as evaluated by caliper

measurements, was reduced to 41% of baseline after a single treatment cycle [59]. The results of a follow-up study evaluating the effects of intratumoral injection of IL-18 and IL-12 encoding plasmid DNA on 26 adult grey horses with metastatic melanoma have recently been published [60]. Treatment consisted of two cycles, spaced two weeks apart, of three intratumoral injections of approximately 250 µg of plasmid DNA administered every two days. Changes in tumor size were primarily evaluated by caliper measurements, but ultrasound evaluation was also performed when possible. Sixty-four days after initiation of treatment, tumors injected with IL-12 encoding plasmid DNA demonstrated an average decrease in tumor volume to approximately 80% of baseline caliper measurements. IL-18 was somewhat less effective, with final tumor volumes averaging approximately 90% of baseline measurements. Control masses, injected with empty plasmid DNA, demonstrated a slight increase in tumor volume, averaging approximately 110% of baseline by day 64 [60]. Both studies showed the therapy to be well-tolerated, with occasional, mild, peri-tumoral swelling as the only noted adverse effect [59, 60].

**Genetics and Genomics**

A connection between grey coat color and EMM has long been recognized. A fully dominant, autosomal trait controlled by a cis-acting regulatory mutation, more specifically a 4.6 kb duplication in intron 6 of syntaxin-17, has been proposed as the probable genetic link between grey coat color and EMM. The mutation is associated with four genes: NR4A3 (nuclear receptor subfamily 4, group A, member 3), STX17 (syntaxin 17), TXNDC4 (thioredoxin domain-contain-4'), and INVS (inversin), with

notably higher levels of STX17 and NR4A3 present in melanomas. Horses that were homozygous for the mutation were found to gray more rapidly and were also more homogeneously white with less speckling than heterozygous horses. Homozygotes were also found to exhibit a significantly higher incidence of melanoma [61].

Further studies are needed to evaluate the association between mutations and EMM to determine whether manifestations of the disease are related to one or a combination of these genes or perhaps others that have yet to be identified. A complete study of the equine genome and further study of the physiologic mechanisms associated with the implicated genetic mutation(s) are necessary. We believe that there must be common mutations leading to the high incidence of EMM, that the acquisition of multiple mutations may account for the progression of clinical stages, and that identification of mutations will be the first step in developing effective targeted therapies.

**Summary and Clinical Implications**

Based on uncertainties and controversy in the veterinary literature, many veterinarians counsel horse owners that most melanomas will follow a benign, lengthy clinical course. Unfortunately, these ‘benign’ tumors may eventually lead to the humane destruction of affected horses, when tumor bulk, location, or infiltrative growth compromises various functions, including eating, drinking, defecation, urination or breeding.

Many veterinarians do not biopsy suspect lesions, feeling that gross appearance of darkly pigmented nodules, alone, is sufficient for diagnosis. These veterinarians may

also feel biopsies are unnecessary, since they consider lesions to be benign. Paradoxically, some equine specialists appear reluctant to either surgically biopsy or resect melanomas for fear of inciting aggressive tumor growth or promoting the development of tumor metastases. This reluctance is not supported by data in published studies, which show that localized lesions can be effectively removed [24].

Practitioners that are presented with a mass they suspect to be EMM are encouraged to perform an excisional biopsy, when possible, and submit the sample for histopathology. Although EMM has a fairly characteristic gross appearance and distribution, it is critically important to get histological confirmation of tumor identity. Proper confirmation and documentation of diagnosis is not only important for guiding treatment, but is also essential if relationships between histopathological characteristics, therapeutic efficacy and prognosis are to be identified.

All owners of grey horses, regardless of the horse's current disease stage, should be educated regarding the probability of this disease occurring and encouraged to perform regular examinations to identify new masses and/or evaluate tumor growth rate. Confirmed cases of EMM should be treated as malignant, regardless of histopathological classification. Surgical excision should be encouraged in cases with masses in Stages 1 or 2 in locations that allow for complete surgical excision. Horses in advanced stages of disease are unlikely to benefit from surgical intervention alone. Cisplatin may be used alone to decrease tumor bulk in large tumors or as an adjunct to

surgical debulking. Additionally, various trials for experimental therapies are ongoing and may represent the best treatment options for horses in Stage 4.

Understanding of the factors involved in the development and progression of EMM is limited, especially in contrast to the knowledge base associated with malignant melanoma in humans and canines. Multiple therapies have been developed and studied, but there are no successful treatments for advanced stages of EMM. Much further study is necessary if we are to understand the genetics and pathophysiology of this disease and ultimately produce successful therapeutic and preventative options.

## Chapter 1: Comparative Expression of Immunohistochemical Markers in Human and Equine Malignant Melanoma

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### Abstract

Heritable genetic and genomic factors associated with melanoma have been identified in both human malignant melanoma (HMM) and equine malignant melanoma (EMM). This work investigates genetic similarities and differences of EMM and HMM through the study of comparative protein expression using immunohistochemical staining. Nestin, Pax-3/7, B-Raf, and SOX-10 are cytoplasmic and nuclear proteins commonly expressed in HMM tissues. Expression of these proteins is associated with mutations producing decreased melanocytic differentiation and increased proliferation leading to tumorigenesis. This work demonstrates overexpression of these proteins in EMM. Aberrant protein expression may signal underlying genetic mutations. Similar abnormal expression patterns in equine and human malignant melanomas suggests that EMM and HMM may share common genomic mutations, further validating EMM as a translational model for HMM.

### Introduction

Malignant melanoma is a neoplastic condition that affects multiple species, including humans and horses. It estimated that nearly 77,000 new cases of HMM will be diagnosed in the United States in 2013, and more than 9,000 people are expected to die as a result of the disease, indicating that the incidence of HMM has been steadily increasing for more than 30 years[3]. These estimations, supplied by the National

Cancer Institute (NCI), are based on epidemiologic data collected from hundreds of thousands of patients in the United States from 1975 through 2009 [3]. The AJCC TNM System for staging and survival rate data for HMM were based on analysis of 38,918 patients followed for a minimum of 10 years [5]. Comparable long-term studies documenting the prevalence, progression, and survival rates in a population of hundreds or thousands of horses with EMM are not present within the current literature. Seltenhammer *et al.* published a study of the occurrence of EMM in 296 grey Lipizzaners over the age of 4 years and found a prevalence of 50% [29]. Fleury *et al.* surveyed 264 Camarague-type grey horses of all ages and reported 31.4% prevalence [28]. These estimates are based on clinical observation of masses consistent with EMM. Both studies showed an increased incidence of EMM in grey horses over the age of 15 years [28, 29]. Based on review of published data, others have estimated that 80% of all grey horses will develop EMM by age 15 and 66% of cases will progress to florid malignancy [13, 62]. The reliability of these statistics is questionable, as these estimations are based on limited published case reports and small-scale, breed-specific studies with short-term follow-up.

Approximately 10% of HMM cases are associated with a family history of the disease and heritable genetic mutations have been identified in 10-40% of families with a high rate of melanoma [4, 63]. Patterns of heritability and genetic mutations have also been identified in EMM, specifically associated with grey coat color [61]. EMM has been linked to the mutation responsible for grey coat color in horses, a 4.6-kb duplication in intron 6 of syntaxin-17 (STX17), and an overexpression of STX17 and a neighboring

gene, nuclear receptor subfamily 4, group A, member 3 (NR4A3) [61]. In both horses and humans, heritable traits such as hair color, speckling/freckling, and vitiligo have been associated with malignant melanoma [4, 61]. The most documented difference between EMM and HMM is that HMM has been associated with damage caused by UV-light, while EMM appears to be UV-independent. The most common sites for detection of EMM include the ventral surface of the tail and perianal skin[29]. As these sites are typically shaded from the sun, UV radiation is presumably not a significant risk factor for EMM. Recent studies have indicated that UV-independent, intrinsic factors may play an equally important etiologic role in HMM [64] and this may be highly relevant to the use of the horse as a translational model.

Among the specific genetic markers associated with HMM, activating mutations of the protooncogene *BRAF* have gained the most attention, as they have been documented in more than 50% of HMM tumors [65]. The V600E mutant, in particular, accounts for 90% of all *BRAF* mutations observed in HMM [65]. Patients with V600E *BRAF*-mutated metastatic melanoma have demonstrated marked tumor regression and improved survival times when treated with Vemurafenib, an inhibitor of mutated *BRAF*, further validating the significance of this mutation in melanoma maintenance [66]. B-Raf is one of 3 members of the Raf family of protein kinases found in mammalian cells. It serves as a central signaling molecule in the Ras/Raf/MAPK (mitogen-activated protein kinase)/ERK (extracellular-signal-regulated-kinase) pathway, which is a key component of signaling cascades that regulate cellular proliferation, differentiation, and survival. Expression of B-Raf is restricted in normal tissues [67].

Nestin has also recently been identified as a potential therapeutic target in HMM [68]. Nestin is a class VI intermediate filament protein that is expressed during embryonic development of the central nervous system. In adults, nestin expression is restricted to the subventricular zone (the site of neurogenesis) and endothelial cells of the brain. However, expression of nestin may also be induced during pathological circumstances, such as ischemic brain injuries and various solid neoplasms [69, 70]. Like *BRAF* mutations, nestin expression is seen in more than 50% of HMM tumor biopsies [71]. Akiyama *et al.* demonstrated decreased capacity for growth, migration, and invasion in HMM cells in which nestin expression was suppressed through the introduction of short hairpin RNA. Furthermore, increased nestin expression has been correlated with clinically advanced stages of HMM and metastasis [69, 72], making it an appealing therapeutic target for advanced stage HMM. Nestin expression may be a prognostic indicator for HMM, with nestin-positive cases demonstrating a significant decrease in 5-year survival rates [71]. Additionally, nestin has been identified as a cancer stem cell (CSC) marker for malignant melanoma [72].

Another CSC marker, Sox-10, has been identified as essential for activation of nestin in HMM [73]. Sox-10 is a protein that belongs to a family of transcription regulators. It is essential for neural crest development and vital to the development of neural crest-derived melanocytes [74]. Sox-10 expression persists in Schwann cells of the peripheral nerve sheaths, epidermal melanocytes, oligodendrocytes in the cerebral cortex, and myoepithelial cells of the parotid glands, bronchial submucosal glands, and mammary

glands [75]. It is expressed in an estimated 97% of HMM tumors and demonstrates more sensitivity and specificity for HMM than S100 protein, the current gold standard for diagnosis of melanocytic and peripheral nerve sheath tumors[75]. Additionally, Sox-10, along with another CSC marker, Pax-3, plays a critical role in the transcriptional control of the master regulatory gene for melanocytes, MITF (mirophthalmia-associated transcription factor) [76, 77]. MITF transactivates the gene for tyrosinase, a key enzyme for melanogenesis, and plays a vital role in melanocyte differentiation [78]. Pax-3 has also been specifically associated with UV-independent HMM tumors [79].

As HMM and EMM are very similar in that both have been strongly associated with certain heritable genetic factors related to hair and skin pigmentation, we hypothesized that they share similar genetic abnormalities and, by extension, aberrant protein expression patterns. Our understanding of the pathobiology of HMM vastly exceeds that of EMM, but it seems quite logical that an investigation of factors that have demonstrated diagnostic and therapeutic promise in human studies is a logical progression in the study of EMM. Consequently, we conducted an immunohistochemical study evaluating the expression of B-Raf, Nestin, Pax-3/7, and Sox-10 in biopsies of a clinically relevant population of horses either at risk for development of EMM or with clinically discernible EMM. This study was performed with the aims of further examining EMM as a translational animal model for HMM, investigating the potential role of CSCs in EMM, and identifying immunohistochemical markers with prognostic and therapeutic value for EMM.

## Materials and Methods

Grey Thoroughbred mares between the ages of 3 and 21 years with evidence of perianal EMM were identified on 10 farms in Lexington, Kentucky. Twenty mares, representing each clinical stage of EMM (7 Stage 0, 7 Stage 1-2, 6 Stage 3-4), were selected for inclusion in this study (**Table 1-1**) [80]. An additional 5 bay/brown, Thoroughbred mares, located at one of the studied farms, were included as controls. The sample population was restricted to individuals of a single breed and gender in an effort to reduce genetic variability and to allow for analysis of inheritance factors.

**Table 1-1: EMM Clinical Staging System**

| Stage | Tumor Burden | Individual Tumor Diameter | Dissemination / Metastasis | Growth Pattern |
|---|---|---|---|---|
| 0 | none | - | - | - |
| 1 | single | < 2 cm | absent | slow to quiescent; maintaining diameter of < 2 cm for months to years |
| 2 | multiple | < 2 cm | absent | slow to quiescent; maintaining diameter of < 2 cm for months to years |
| 3 | multiple | < 4 cm | present | slow; changes in size ≤ 25% over a course of months to years |
| 4 | multiple | > 4 cm | present | rapid; changes in size > 25% over a course of weeks to months |

Each mare was examined, photographed, and clinically staged for EMM using a clinical staging system we developed and published [80] (photographs, pedigrees, and brief clinical profiles for each study horse can be found in Appendix A). The mares were then sedated through intravenous administration of Xylazine and Butorphanol at 0.5-1 mg/kg and 0.01-0.05 mg/kg, respectively. Biopsy sites were selected, cleaned with betadine

scrub and water, and 1-2 ml of lidocaine was administered subcutaneously for localized pain management. Biopsy sites were then swabbed with alcohol and specimens were collected with sterile, 6mm biopsy punches. Biopsy sites were closed using 2-0 monofilament suture when necessary for hemostasis, but were primarily left to heal by second intention. A sample of normal, haired skin was collected from a site just lateral to the base of the tail and a sample of normal, glabrous skin was collected from the dorsal perineum of each mare to serve as autologous controls. Tumor samples were collected from mares with evidence of melanoma. In an effort to maintain anatomic consistency between tumor specimens, masses located in the glabrous skin found dorsal to the anus and ventral to the base of the tail were selected, whenever possible. Specimens were fixed in 10% neutral buffered formalin solution, trimmed, and embedded in paraffin polymer prior to being sectioned for staining.

All slides were processed by a Ventana BenchMark LT automated slide stainer (Ventana Medical Systems, Inc., Tucson, AZ, USA). H&E stained slides were produced from each sample for general histopathological evaluation and comparison. Additional tissue sections were processed using B-Raf (F-7), Nestin (H-85), Pax-3/7 (H-208), and Sox-10 (A-2) primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:50.Slides were warmed to 37°C and incubated withNestin, Pax-3/7, and B-Raf primary antibodies for 16 minutes and 32 minutes with Sox-10 primary antibodies. Samples were incubated with a biotinylated mouse and rabbit secondary antibody solution for 8 minutes. An indirect biotin streptavidin system for secondary antibody detection was employed through the use of Ventana Akaline Phosphatase Red

Detection Kits (Ventana Medical Systems, Inc., Tucson, AZ, USA). Slides were incubated with the streptavidin-alkaline phosphatase enzyme conjugate for 12 minutes, followed by incubation with an enhancer and naphthol for 4 minutes, Fast Red A for 8 minutes, and Fast Red B for 8 minutes. The B-Raf, nestin, and Pax-3/7 antibodies chosen were previously demonstrated to be cross-reactive in equine tissues. Reactivity for each antibody was confirmed through staining of known positive HMM tumor specimens. Cross-reactivity of the Sox-10 antibody with equine tissues was confirmed through staining of equine salivary tissue, as Sox-10 is normally expressed by the myoepithelial cells of salivary glands [75].

Slides were scored based on the intensity of chromagen staining using a scale ranging from negative to +++ by two, independent reviewers (**Table 1-2**). Patterns in the anatomic distribution of stain and neoplastic cells were also noted. The first reviewer was familiar with the history and clinical findings associated with each sample and had 5 years of experience evaluating EMM biopsy samples. The second reviewer was a veterinary pathologist with over 40 years of experience in the evaluation of surgical biopsy specimens and was blinded to the identification of the horses, their history/signalment, and the site of biopsy. This semi-quantitative reviewing system was developed in an effort to account for variability of interpretation, experience, and case familiarity.

**Table 1-2: Scoring System for Immunohistochemical Stain Analysis**

| Score | Description |
|---|---|
| - | No evidence of protein expression |
| +/- | Faint or inconsistent staining associated with protein expression |
| + | Mild staining associated with protein expression |
| ++ | Moderate staining associated with protein expression |
| +++ | Intense staining associated with protein expression |

## Results

Histopathologic review revealed abnormal melanocytes infiltrating the dermis and subcutis in 21 of the 51 samples collected from grey mares. Of these samples, 13 were from biopsies of grossly identified tumors. The remaining 8 samples included 4 biopsies of grossly normal, glabrous skin collected from Stage 0 mares, 3 biopsies of grossly normal, glabrous skin collected from Stage 1-2 mares, and 1 biopsy of glabrous skin collected from a Stage 3-4 mare. Evidence of melanocytic infiltration was absent in all 20 samples of grossly normal, haired skin collected from grey mares. As expected, melanocytic infiltration was not noted in any of the samples collected from the bay/brown control mares.

Based on observations from Stage 0-2 mares with melanocytic infiltrates, neoplastic melanocytes appear to originate in the dermis and subcutis with close proximity to sweat glands, not as foci surrounding the follicular bulb as originally reported by Levene [15]. Samples from the larger tumors of Stage 3-4 mares exhibited compression or obliteration of normal dermal structure, extensive melanocytic invasion encroaching upon the epidermis, epidermal thickening and keratinization, and increased numbers of pigmented melanocytes in the superficial epidermis. This differs from HMM, which

typically originates at the dermo-epidermal junction and infiltrates into the dermis during the vertical growth phase [81].

**Table 1-3: Concurrence of Reviewer Scoring of Immunohistochemical Stains**

| Sample Source | Overall Reviewer Scoring Agreement (%) | Pax 3/7 Reviewer Scoring Agreement (%) | B-Raf Reviewer Scoring Agreement (%) | Nestin Reviewer Scoring Agreement (%) | Sox-10 Reviewer Scoring Agreement (%) |
|---|---|---|---|---|---|
| Bay/Brown Mare Normal, Haired Skin | 70% | 100% | 80% | 60% | 40% |
| Bay/Brown Mare Normal, Glabrous Skin | 75% | 80% | 60% | 80% | 80% |
| Stage 0 Grey Mare Normal, Haired Skin | 75% | 86% | 71% | 86% | 57% |
| Stage 0 Grey Mare Glabrous Skin | 61% | 71% | 71% | 43% | 57% |
| Stage 1-2 Grey Mare Normal, Haired Skin | 57% | 71% | 57% | 71% | 29% |
| Stage 1-2 Grey Mare Glabrous Skin | 65% | 80% | 60% | 40% | 80% |
| Stage 1-2 Grey Mare EMM Tumor | 71% | 71% | 86% | 43% | 86% |
| Stage 3-4 Grey Mare Normal, Haired Skin | 83% | 100% | 50% | 100% | 83% |
| Stage 3-4 Grey Mare Glabrous Skin | 79% | 83% | 83% | 83% | 67% |
| Stage 3-4 Grey Mare EMM Tumor | 54% | 83% | 33% | 33% | 67% |

There was good agreement between expression score data of both reviewers (**Table 1-3)**, with 31% discord across all specimens. The reviewers' scores varied by only one

category in 65 of 76 (86%) instances of disagreement. Expression of Pax-3/7, B-Raf, Nestin, and Sox-10 was noted in all EMM tumor specimens, but was largely absent to mild in the control tissues from both grey and non-grey mares (**Figures 1-1, 1-2, 1-3**). Expression of all four proteins was primarily restricted to infiltrating melanocytic cells, but was also noted in 10-25% of sweat gland cells (**Figure 1-4, 1-5, 1-6)**, the occasional normal junctional melanocyte, and cells associated with hair follicles.

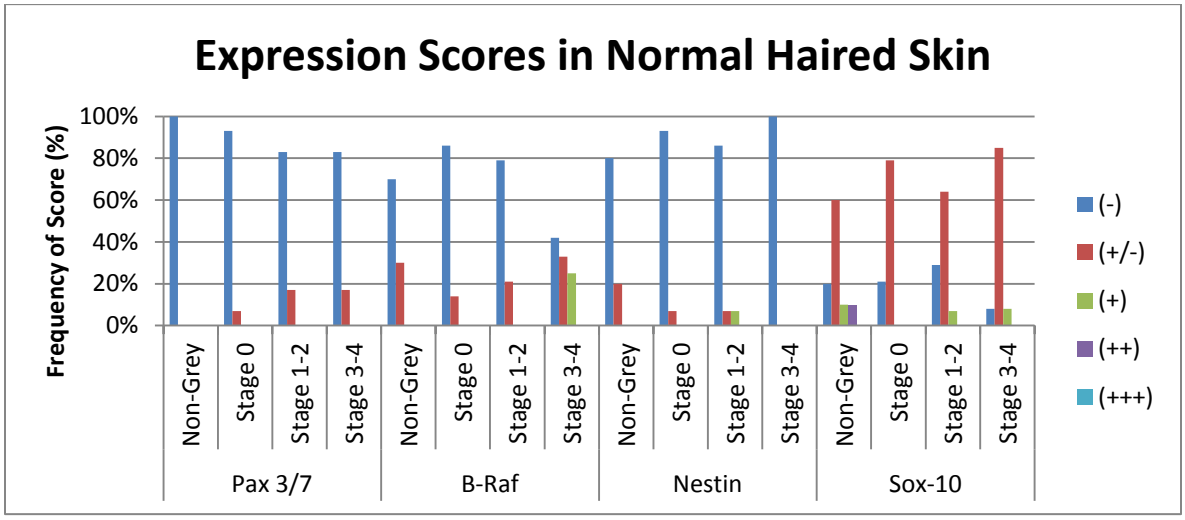


Figure 1-1: Immunohistochemical protein expression data from samples of grossly normal, haired skin. Scores from each reviewer were recorded as independent events.

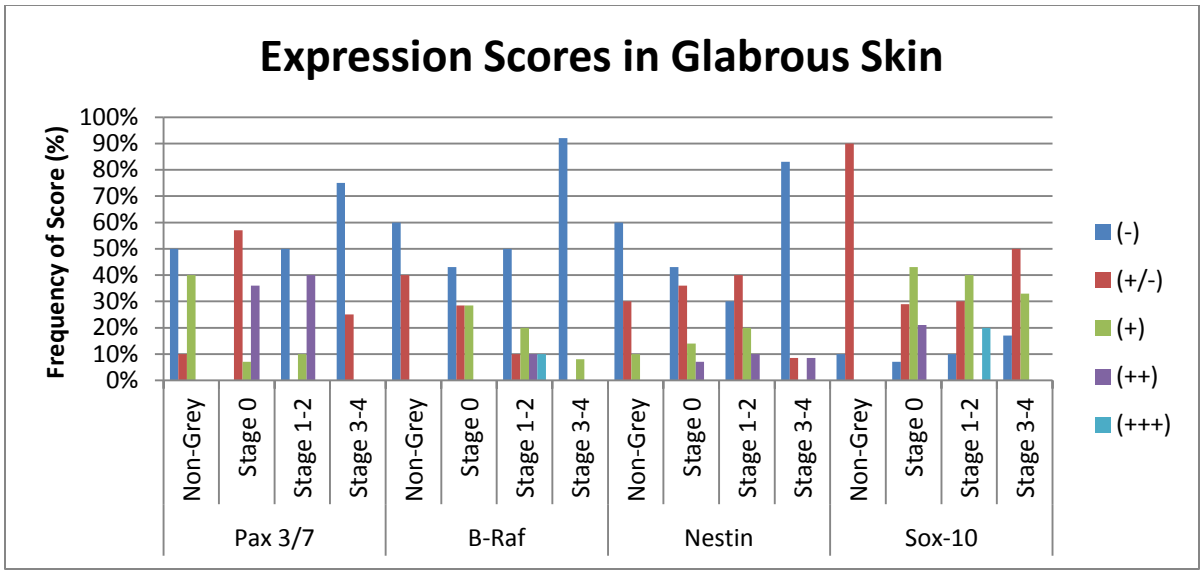


Figure 1-2: Immunohistochemical protein expression data from samples of glabrous skin. Scores from each reviewer were recorded as independent events. Data includes samples with evidence of melanocytic infiltration.

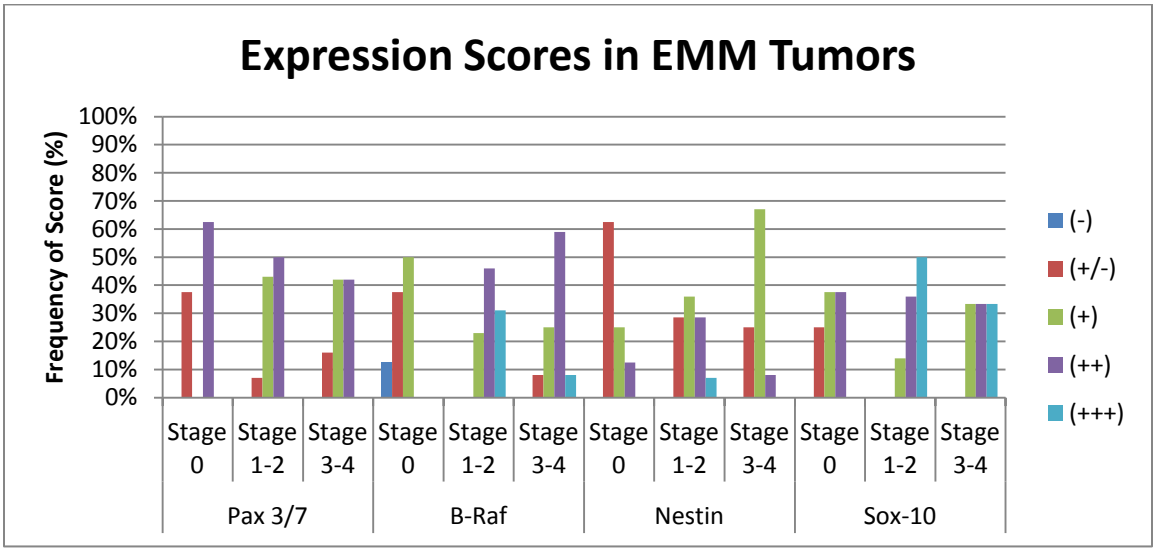


Figure 1-3: Immunohistochemical protein expression data from samples of EMM tumors. Scores from each reviewer were recorded as independent events. Data includes Stage 0 mares with evidence of neoplastic infiltration in samples from grossly normal, glabrous skin.

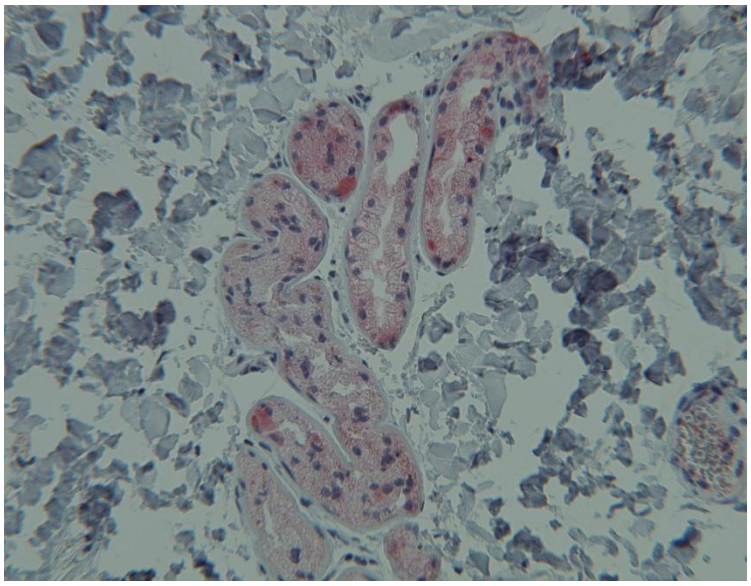

Figure 1-4: Photomicrograph demonstrating B-Raf protein expression (red stain) in the sweat glands of a glabrous skin sample from a Stage 0 grey mare with microscopic evidence of EMM (not shown). (250X Magnification)

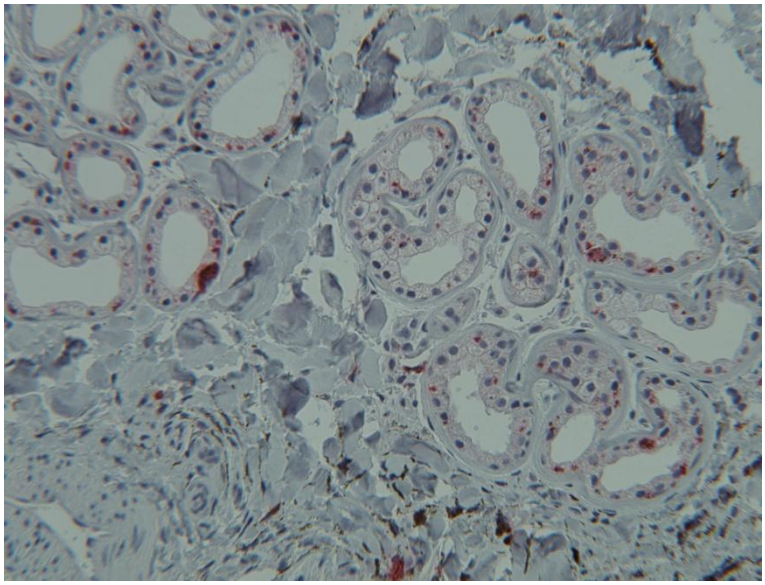

Figure 1-5: Photomicrograph demonstrating Nestin protein expression (red stain) in the sweat glands of a glabrous skin sample from a Stage 0 grey mare with microscopic evidence of EMM. (250X Magnification)

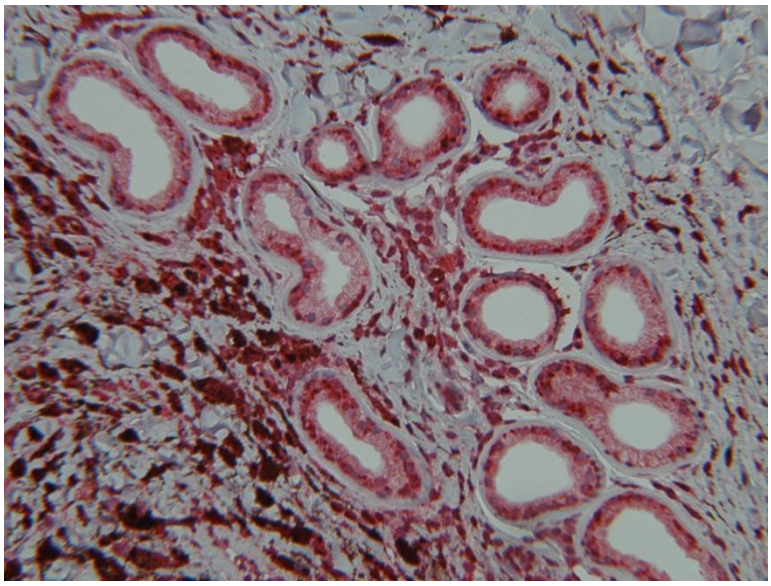

Figure 1-6: Photomicrograph demonstrating Sox-10 protein expression (red stain) in the sweat glands of a glabrous skin sample from a grey mare with Stage 1 EMM. (250X Magnification)

Pax-3/7 was expressed in cells closely associated with sweat glands in the glabrous skin specimens from at least 1 mare in each category, and the majority of glabrous skin specimens from the bay/brown mares and Stage 0 grey mares. Of the glabrous skin samples, 3 out 5 from bay/brown mares, 5 out of 7 from Stage 0 grey mares, 1 out of 7 from Stage 1-2 grey mares, and 1 out of 6 from Stage 3-4 grey mares exhibited Pax-3/7 expression associated with sweat glands. The highest expression levels of Pax-3/7 (**Figure 1-7**) were noted in the infiltrating melanocytes of grey mares with Stage 0 through Stage 2 EMM.

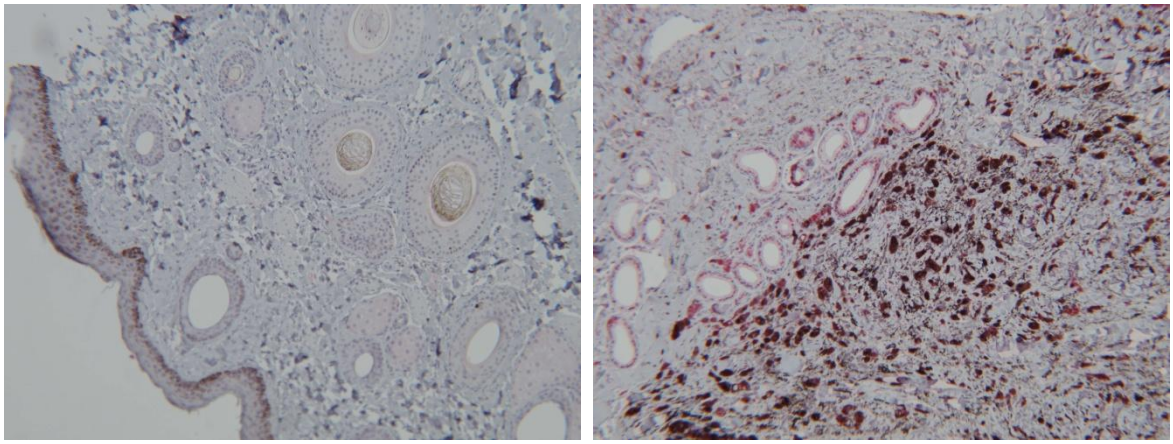

Figure 1-7: Photomicrographs demonstrating a Pax-3/7 (-) scored, normal, haired skin specimen from a Stage 0 grey mare (left) and (++) scored, glabrous skin specimen from a Stage 1 grey mare (right). Expression of Pax-3/7 is also noted in the sweat glands of the specimen from the grey mare with Stage 1 EMM. (100X Magnification)

B-Raf was expressed in close association with sweat glands of the glabrous skin in 2 out of 5 samples from bay/brown mares, 5 out of 7 samples from Stage 0 grey mares, 4 out of 5 samples from Stage 1-2 grey mares, and 1 out of 6 samples from Stage 3-4 grey mares. Sweat gland-associated B-Raf expression was noted to a lesser extent in tumor samples, which was somewhat attributable to the loss of dermal structure in advanced tumors. However, even in tumor samples with normal sweat gland structure, the expression of sweat gland- associated B-Raf was diminished in comparison to non-grey and Stage 0 grey mare specimens. Instead, B-Raf expression (**Figure 1-8)** was primarily noted in the infiltrating melanocytes of tumor specimens, with the most intense expression found in samples collected from Stage 1-2 grey mares and consistently mild-moderate expression noted in samples collected from Stage 3-4 grey mares.

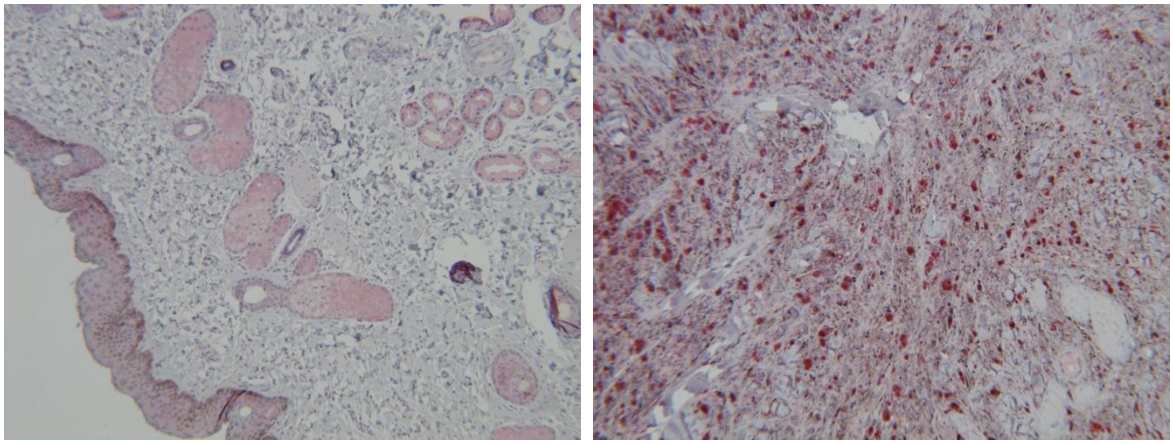

Figure 1-8: Photomicrographs demonstrating a B-Raf (-) scored, normal, glabrous skin specimen from a bay/brown mare (left) and (+++) scored, tumor specimen from a Stage 1 grey mare (right). Unstained sweat gland can also be observed within the tumor specimen from the grey mare with Stage 1 EMM. (100X Magnification)

Faint to mild expression of Nestin was noted in association with sweat glands in glabrous skin specimens from 3 out of 5 bay/brown mares, 6 out of 7 Stage 0 grey mares, 4 out of 5 Stage 1-2 grey mares, and 1 out of 5 Stage 3-4 grey mares. Nestin expression (**Figure 1-9**) of infiltrating melanocytes was absent to mild in specimens from Stage 0 mares, and faint to moderate in Stage 1-4 mares.

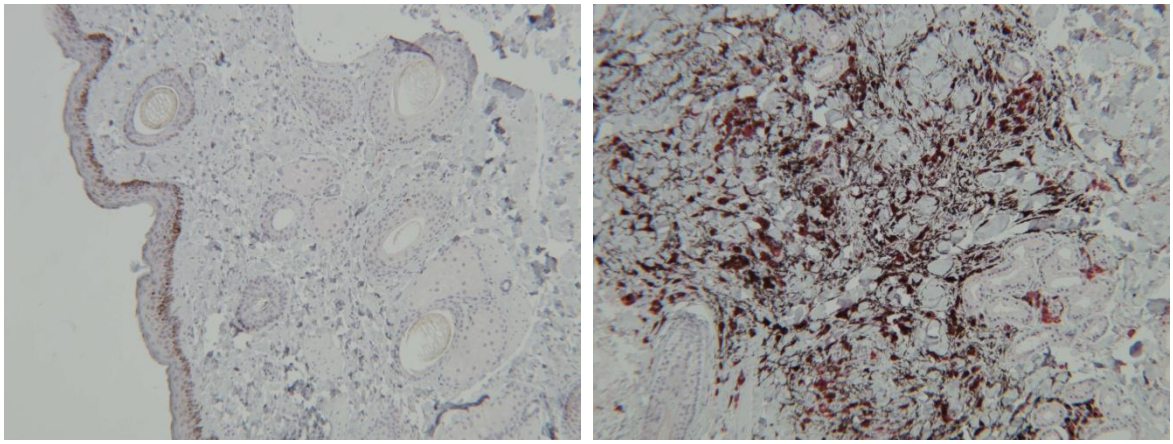

Figure 1-9: Photomicrographs demonstrating a Nestin (-) scored, normal, haired skin specimen from a Stage 0 grey mare (left) and (++) scored, tumor specimen from a Stage 1 grey mare (right). Unstained sweat gland can also be observed within the tumor specimen from the grey mare with Stage 1 EMM. (100X Magnification)

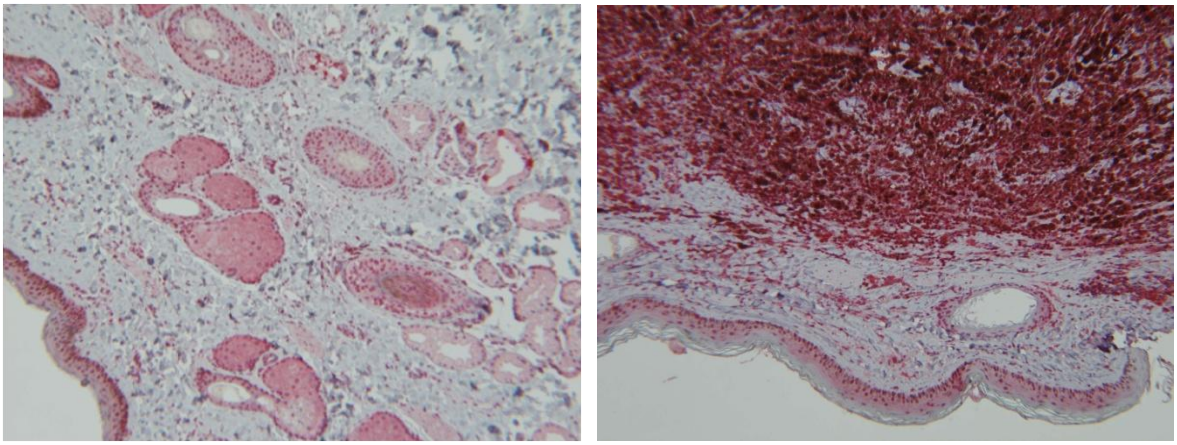

Figure 1-10: Photomicrographs demonstrating a Sox-10 (+/-) scored, normal, haired skin specimen from a Stage 0 grey mare (left) demonstrating faint, non-specific staining and mild evidence of Sox-10 expression in select sweat gland cells and a (+++) scored, tumor specimen from a Stage 4 grey mare (right) demonstrating obliteration of normal derma structure and encroachment on the dermo-epidermal junction. (100X Magnification)

Localization of faint to mild Sox-10 expression was non-specific in the majority of specimens without melanocytic invasion. Occasionally, intense staining of select cells was associated with sweat glands (**Figure 1-10**). Moderate to intense expression of Sox-10 was noted in infiltrating melanocytes in samples from all stages of EMM. Sox-10

expression was particularly intense in samples collected from Stage 2-4 grey mares (**Figure 1-10**).

The most intense expression of Pax-3/7 was associated with infiltrating melanocytes in samples from Stage 0-2 mares. B-Raf and Nestin expression was most intensely exhibited in the infiltrating melanocytes of Stage 1-2 mares. Sox-10 demonstrated the highest degree of baseline expression, but was consistently increased in the tumors of Stage 2-4 mares.

## Discussion

Far more is known about the histological and immunohistochemical signature of HMM than is known about EMM; few studies have been done to improve the diagnostic, prognostic, and therapeutic tools used for EMM. This study identifies four markers with diagnostic, prognostic, and therapeutic potential in EMM. Sox-10, in particular, showed consistently elevated expression patterns among EMM tumors and could be a useful diagnostic marker in unusual cases of EMM. B-Raf and Nestin are proteins with demonstrated therapeutic potential in HMM. Further studies confirming widespread prevalence and upregulation of B-Raf and Nestin expression in EMM would support further investigation of the therapeutic potential of these proteins in EMM. Evaluation of the significance of B-Raf in the pathogenesis of EMM could lead to use of Vemurafenib or a similar *BRAF* inhibitor in the treatment of EMM. Additional studies searching for genetic mutations responsible for the increased expression of these proteins and evaluating the effects of inhibition of these genes and protein products could lead to the development of targeted, novel therapies with the potential to benefit both horses and

humans with malignant melanoma. Whole genome sequencing of EMM tumors would be the most thorough means of identifying mutations associated with EMM. However, in order to produce statistically significant correlation between genomic mutations and EMM, the genomes of hundreds of horses would have to be analyzed. A multitude of mutations would likely be identified and further evaluation of individual mutations would need to be prioritized based on current knowledge. The financial and temporal factors associated with a study of this magnitude in horses with EMM precluded its inclusion within the scope of this work. Since EMM-associated mutations identified through whole genome sequencing would likely be prioritized based on knowledge gained from the study of HMM, it seems equally logical to pursue the study of genes known to be commonly mutated in HMM and whose protein products are known to be over-expressed in EMM. Protein expression patterns remained relatively consistent between samples from mares within each clinical classification, but substantial variability existed between specimens from mares with different clinical classifications. Early stages of EMM, specifically stages 0 and 1, showed the highest expression levels of Pax-3/7. Increased B-Raf expression was associated with EMM stages 1 and 2. EMM stages 2-4 were associated with the highest levels of Sox-10 expression. This data correlates with clinical staging of EMM and supports the EMM Staging System presented by Moore *et al*. [80]. Additionally, 57% of Stage 0 mares in this study had microscopic evidence of EMM. The protein expression profiles identified within the sampled sections of grossly normal, glabrous skin from Stage 0 mares were similar to those described in Stage 1-2 EMM tumor samples. These findings support continued identification and staging of grey horses without gross manifestation of EMM, as these horses likely have

microscopic lesions with potential for growth, invasion, and metastasis. Furthermore, these findings impact the validity of prior statistical studies of EMM prevalence, in which EMM was strictly diagnosed through evidence of grossly identifiable masses.

Elevated levels of Pax-3/7, Nestin, and Sox-10 in cells associated with hair follicles and in occasional junctional melanocytes are consistent with findings demonstrating melanocyte stem cells in these locations in humans [82]. Additionally, studies have associated premature graying with progressive loss and abnormal anatomic distribution of melanocyte stem cells in mouse models and human hair follicles [83]. These findings may account for the increased expression of melanocyte stem cell markers in cells associated with sweat glands in the dermis and subcutis of the grey mares in this study. Alternatively, previously unidentified nests of stem cells may normally exist in association with the sweat glands in the dermis and subcutis of equines. If normal melanocyte stem cell populations exist in close association with sweat glands, and we consider the embryological, structural, and functional similarities of sweat glands and salivary glands, it follows that melanocyte stem cells may also be present in salivary tissues. This hypothesis is supported by the common observation of pigmented regions in equine salivary tissues during necropsy and may account for the frequent occurrence of EMM in parotid salivary glands.

Stem cells normally exist in small numbers within specific anatomic locations, known as niches, of adult tissues and possess the capacity for infinite self-renewal and proliferation and for production of progenitor cells which then differentiate into a variety of cell types that constitute the tissue or origin. CSCs possess these same characteristics of normal, adult stem cells, but have also acquired mutations that

deregulate the normally homeostatic functions of stem cells and lead to the formation of tumors. CSCs were originally identified in hematopoietic neoplasia, and have since been discovered in a multitude of solid tumors, including HMM [72, 84]. Our findings suggest that melanocyte CSCs may play a role in the pathogenesis of EMM and support further investigation of melanocyte CSCs in EMM.

Melanocyte CSCs have been implicated in the pathogenesis of HMM [82] and abnormal, increased expression of B-Raf and the stem cell marker proteins, Pax-3, Nestin, and Sox-10 have been associated with HMM [65, 71, 75, 79]. This study shows that abnormal over-expression of these proteins also occurs in EMM tissues. These finding suggest that Pax-3, B-Raf, Nestin, and Sox-10, as well as melanocyte CSCs, may also be involved in the pathogenesis of EMM and provides further evidence that EMM is a valid translational model for HMM.

## Chapter 2: Frankincense Oil Induces Apoptosis in Human Malignant Melanoma Cells in Tissue Culture

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### Abstract

Malignant melanoma is an aggressive, infiltrative skin neoplasm that can be deadly, particularly in patients diagnosed at advanced stages of disease. Current therapies for stage III and IV malignant melanoma are limited and provide minimal improvements in overall survival (about 15-20% at 5 years for Stage IV patients). Frankincense oil, a distillate botanical oil from tree resin, has demonstrated cytotoxic effects in various neoplastic cell lines. Little work has been done to evaluate its efficacy in treating neoplasms of the skin. Our data demonstrates FO-mediated dose- and time-dependent cytotoxicity in SK-Mel-5 human malignant melanoma (HMM) cells, with the majority of cell deaths resulting from apoptosis. These findings support further investigation of FO as a treatment for malignant melanoma.

### Introduction

The number of patients with human malignant melanoma (HMM) has been increasing steadily for the past 30 years, primarily as a result of increased exposure to ultraviolet radiation [85]. While conventional therapies have some efficacy, HMM remains the leading cause of skin cancer-related deaths [4]. A variety of chemotherapeutic agents, immune-modulatory therapies, and vaccines have been used in the treatment of malignant melanoma [86], but as of 2008 the average 5-year survival rate for Stage III-IV HMM was still less than 50% [5]. HMM has been reported to demonstrate innate

and acquired resistance to commonly used chemotherapeutic agents [87]. The use of these drugs is often associated with significant side effects [86] as a result of their indiscriminate killing of both healthy and neoplastic cells. Selective, specific, and efficient targeting of cancer cells is a primary goal in the development of effective anti-cancer therapies for aggressive malignancies, such as HMM [88].

In a natural products screening study performed in the Department of Radiation Oncology at Wake Forest University School of Medicine, a variety of essential oils were tested for cytotoxic activity in the presence of human glioblastoma (GBM) cells and normal fibroblasts *in vitro*. Fifteen of the essential oils exhibited varying degrees of cytotoxicity. Frankincense oil (FO) was found to have the greatest cytotoxic effect in the GBM cells, while exhibiting the least effect on normal fibroblasts.

FO is a distillate of the gum resin collected from the trunks of trees of the genus *Boswellia*. There are multiple species in the genus and each produces a slightly different resin. *Boswellia* resin and its derivatives have been prized for their medicinal qualities for centuries. Research efforts have identified at least a dozen different pentacyclic triterpenic acids within *Boswellia* resin [89]. Among these, boswellic acids have been identified as the most biologically active and pharmacologically significant components; however, others have found the complete extract to be more efficacious than its purified fractions [90].Following the initial screening study, western immunoblotting was performed to probe for caspase-3 cleavage, an indicator of apoptosis, in GBM, C-6 rat glioma, and normal rat astrocyte cell lines at 8 and 16

hours post-treatment with FO at doses of 0.1, 0.15, and 0.2 µg/ml. Caspase-3 cleavage was noted in the 0.15 and 0.2 µg/ml treatment groups of the GBM cell line at 8 hours and in all treatment groups at 16 hours, exhibiting both a time- and dose-dependent effect. The C-6 rat glioma cell line exhibited caspase-3 cleavage at all concentrations at each time point, with an increase in caspase-3 cleavage noted between 8 and 16 hours. Cleavage of caspase-3 was not noted at either time point for any of the concentrations tested in the normal rat astrocyte cell line. [91]

A separate study evaluating the effects of frankincense oil derived from the resin of *Boswellia carteri* in a human urinary bladder transitional cell carcinoma (J82) cell line and an immortalized normal bladder urothelial (UROtsa) cell line also found it to selectively suppress neoplastic cell viability while allowing for continued growth of normal urinary cells in a dose-dependent manner [92]. Cell viability was determined through multiple methods, including an XTT cell proliferation and *in situ* cell death detection kit and Trypan blue dye exclusion. RNA was isolated from the J82 cells and gene expression data was collected using an Illumina microarray to determine the nature of cell death. FO was found to influence transcription factors and other gene products involved in cell cycle arrest and cellular proliferation, and in apoptosis, indicating that FO causes cell cycle arrest and apoptosis in J82 cells. A TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) analysis was also performed using an immunohistochemical-like staining procedure, and provided further evidence of FO-induced apoptosis in J82 cells. However, FO was not found to cause DNA fragmentation, a hallmark of apoptosis, in J82 cells within 6 hours of treatment.

The authors speculated that FO may induce apoptosis without DNA fragmentation or DNA fragmentation may have occurred after 6 hours post-treatment. The demonstrated selective activity of FO- killing tumor cells while sparing normal cells- is a highly desirable property for an anti-cancer therapeutic.

Clinically, anti-cancer therapeutics that induce cytotoxicity via apoptosis are preferred to those that result in necrosis, because apoptosis results in less tissue damage and inflammation. Cell culture studies provide a means for evaluation of the cytotoxic efficacy and mechanisms of potential therapeutics without risk to animal or human subjects. The cytotoxic and apoptotic attributes of FO and its components have been studied in numerous cell lines. Few studies have evaluated the efficacy of FO as a therapy for neoplastic skin cells, despite the fact that skin cancer is one of the most prevalent cancers in humans [93]. Only one published study to date has specifically explored the effects of boswellic acids derived from FO in metastatic melanoma cells [94]. This study demonstrated dose and time-dependent inhibition of cell proliferation and migration, as well as increases in cell differentiation, in B16F10 mouse melanoma cells in the presence of boswellic acid [94]. In the present study, we examined the cytotoxic activity of medicinal grade FO against the HMM cell lines WM-115 and SK-Mel-5 and studied the mechanism(s) of HMM cell death following exposure of SK-Mel-5 cells to FO using flow cytometry. This study represents the first step in evaluating the therapeutic potential of FO against malignant melanoma.

## Materials and Methods

### Cell Culture

The WM-115 human melanoma cell line was obtained from The American Type Culture Collection (ATCC CRL-6475; Manassas, VA, USA). WM-115 is an adherent cell line derived from the primary site of melanoma on the skin of a 58-year-old woman with metastatic malignant melanoma. The WM-115 cell line was selected for study because it was established from a primary site of metastatic malignant melanoma. For studies, WM-115 cells were maintained in Eagle's Minimum Essential Medium (Quality Biological, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Quality Biological, Gaithersburg, MD, USA) and 1% penicillin and streptomycin (Quality Biological, Gaithersburg, MD, USA) at 35°C in humidified air with 5% $CO_2$. Media was replaced every 2-3 days and cells were passaged when they reached about 80% confluence, using Trypsin 0.25% EDTA 0.02% (Quality Biological, Gaithersburg, MD, USA) to lift adherent cells. A growth curve analysis (**Figure 2-1**) averaging daily manual hemocytometer counts from each of 3 flasks of untreated WM-115 cells was performed over a period of 5 days to estimate the baseline growth rate and time required for cell adherence.

The SK-Mel-5 human melanoma cell line was obtained from The American Type Culture Collection (ATCC HTB-70; Manassas, VA, USA). SK-Mel-5 is an adherent cell line derived from a metastatic site of malignant melanoma in an axillary lymph node of a 24-year-old, Caucasian woman. The SK-Mel-5 cell line was selected for study

because it was established from a metastatic site of malignant melanoma. For studies, SK-Mel-5 cells were maintained in Eagle's Minimum Essential Medium (Quality Biological, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Quality Biological, Gaithersburg, MD, USA) and 1% penicillin and streptomycin (Quality Biological, Gaithersburg, MD, USA) at 37°C in humidified air with 5% $CO_2$. Media was replaced every 2-3 days and cells were passaged when they reached about 80% confluence, using Trypsin 0.25% EDTA 0.02% (Quality Biological, Gaithersburg, MD, USA) to lift adherent cells. A growth curve analysis (**Figure 2-2**) averaging daily manual hemocytometer counts from each of three flasks of untreated SK-Mel-5 cells was performed over a period of 5 days to estimate the baseline growth rate and time required for cell adherence

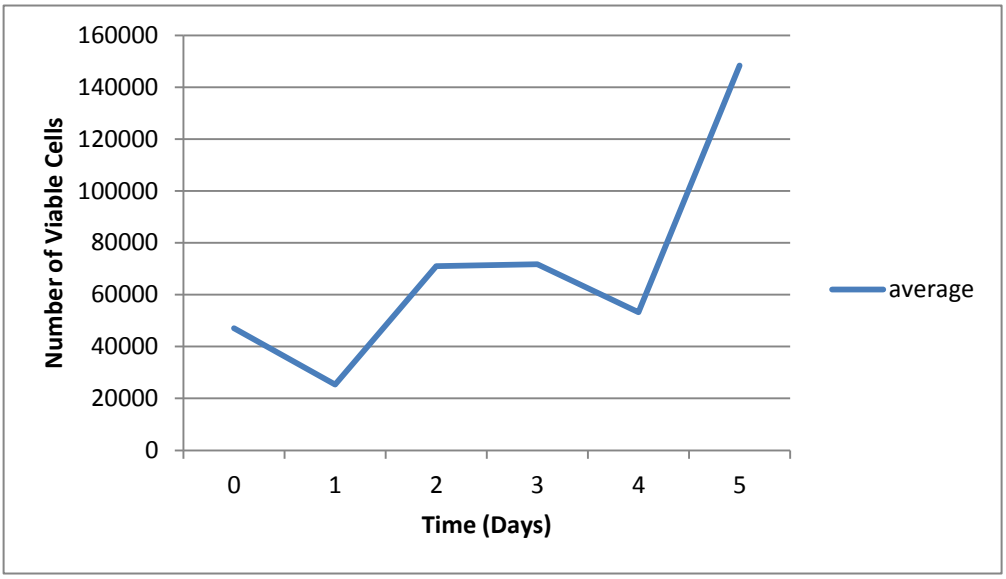


Figure 2-1: WM-115 Growth Curve. Mean cell viability data from a 5-day growth curve analysis of untreated WM-115 HMM cells.

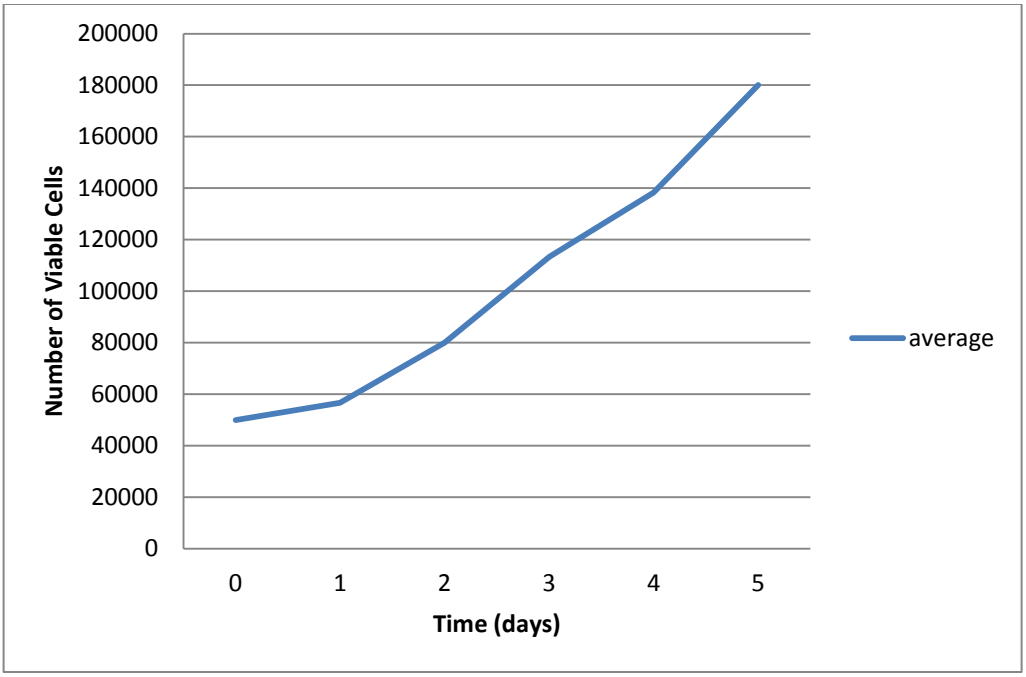


Figure 2-2: SK-Mel-5 Growth Curve. Mean cell viability data from a 5-day growth curve analysis of untreated SK-Mel-5 HMM cells.

**Preliminary cytotoxicity analysis**

In order to gauge the response of HMM cells to treatment with FO, a preliminary cytotoxicity study evaluating a wide range of concentrations (0.02%-0.24%) and an extended (48 hour) trial length was performed in the primary HMM cell line. Medicinal-grade FO, supplied as a hydrodistillate derived from the resin of *Boswellia carteri* (Boswellness, Burlington, Vermont, USA) was added to the growth medium of WM-115 cells at concentrations ranging from 0.02% to 0.24% by volume. Each treatment group was tested in duplicates for comparison and averaging of data. Two untreated flasks of WM-115 cells were included as negative controls. For assessment of cytotoxicity (positive control), 70% ethanol, diluted with growth medium to a concentration of 28%, was used in two flasks of WM-115 cells. Cell viability was determined using the Trypan

blue dye exclusion method and manual hemocytometer cell counts were performed at 24 and 48 hours post-treatment (**Figures 2-3 and 2-4**).

### Statistics

Comparisons of WM-115 cell survival following frankincense oil treatment were made using the one-way analysis of variance (ANOVA). $P \leq 0.05$ was considered significant.

### Flow cytometric analysis of apoptotic or necrotic cell death

After demonstrating that FO induced cell death in primary HMM (WM-115) cells, the cytotoxic effects of FO were tested in metastatic HMM (SK-Mel-5) cells, followed by flow cytometric analysis to determine the mode of cell death. The use of flow cytometry allowed for rapid analysis of each individual treated cell, thereby providing substantial replication and reliability of results. SK-Mel-5 cells were split into 25 $cm^2$ flasks with approximately 200,000 cells per flask. Following 12-24 hours of incubation for adherence, cells were exposed to concentrations of FO from *Boswellia frereana* (Boswellness, Burlington, VT, USA) at 0.02%, 0.1%, and 0.2% by volume. Untreated cells were included as negative controls and ethanol treated cells (as above) were included as positive controls. Additionally, a flask of SK-Mel-5 cells treated with 0.04% carboplatin by volume was included for comparison, since it is a chemotherapeutic with demonstrated efficacy in HMM clinical trials and it's analog, cisplatin, is a commonly used chemotherapeutic in EMM [56, 95]. At 1, 4, 8, and 12 hours post-treatment, cells were trypsinized and harvested for analysis. Harvested cells were washed with phosphate buffered saline (PBS) and centrifuged for 10 minutes. After centrifugation,

the supernatant was discarded and the pellet was washed again with PBS followed by 1X binding buffer (Annexin V Apoptosis Detection Kit FITC, eBioscience, San Diego, California, USA). The cells were then resuspended in 1X binding buffer at 1-5 x $10^6$ cells/ml and 4 100 µl aliquots of each cell suspension were placed in eppendorf tubes. Five µl of Annexin V FITC (Annexin V Apoptosis Detection Kit FITC, eBioscience, San Diego, California, USA) was added to 2 of the 4 100 µl cell suspensions and allowed to incubate at room temperature for 10-15 minutes. All of the cells were then washed with 1X binding buffer following 10 minutes of centrifugation and resuspended in 200 µl of 1X binding buffer. Five µl of PI staining solution (Annexin V Apoptosis Detection Kit FITC, eBioscience, San Diego, California, USA) was added to one of the Annexin V FITC treated cell suspensions and one of the untreated cell suspensions and stored at 2-8°C for up to 4 hours prior to analysis by flow cytometry. An unstained, Annexin V FITC stained, PI stained, and combined Annexin V FITC and PI stained sample from each treatment group was analyzed using a BD FACS Aria I (Becton Dickinson, San Jose, California, USA) and FlowJo software (Tree Star Inc., Ashland, Oregon, USA).

**Results**

FO from *Boswellia carteri* induced cell membrane damage in the WM-115 cell line in a dose-dependent manner, allowing for uptake of Trypan blue dye (**Figures 2-3 and 2-4**). The results shown in **Figures 2-3** and **2-4** demonstrate the individual data points, means, and confidence intervals for each treatment group at 24 and 48 hours. Cell viability was found to decrease significantly in the 0.12% and 0.24% FO treatment groups compared to untreated controls at both time points (p=0.05). Cell viability

decreased precipitously between the 0.02% and 0.12% FO treatment groups (**Figures 2-3 and 2-4**). WM-115 cells treated with FO from *Boswellia carteri* exhibited nearly identical mean cell viability at 24 and 48 hours post-treatment (**Figure 2-5**). Additionally, the total number of cells counted in each of the FO-treated flasks was less than 55% of the number of cells counted in the negative control flasks (**Figure 2-6**).

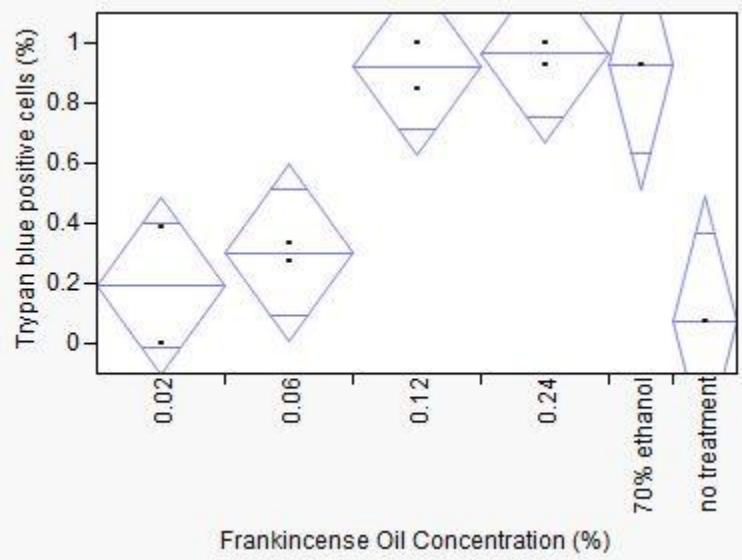


Figure 2-3: WM-115 Cell Viability 24 Hours Post-Treatment. Percentage of dead (trypan blue positive) WM-115 cells treated with varying concentrations of FO from *Boswellia carteri* at 24 hours after treatment.

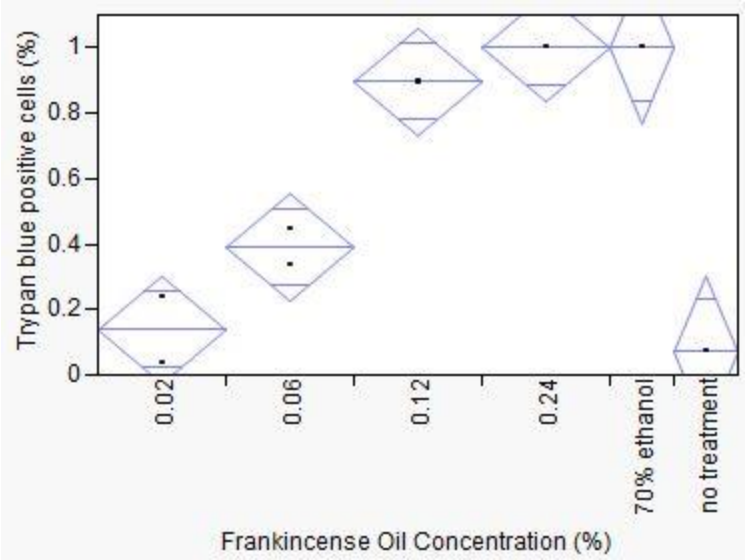


Figure 2-4: WM-115 Cell Viability 48 Hours Post-Treatment. Percentage of dead (trypan blue positive) WM-115 cells treated with varying concentrations of FO from *Boswellia carteri* at 48 hours after treatment.

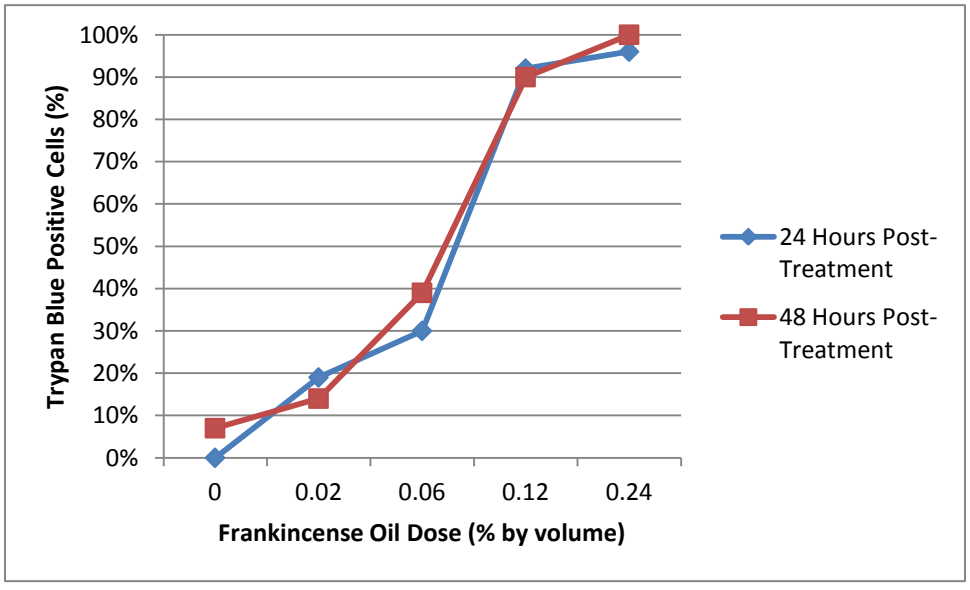


Figure 2-5: WM-115 Mean Cell Viability at 24 and 48 Hours Post-Treatment. Comparison of mean cell viability data from manual hemocytometer counts of WM-115 cells at 24 and 48 hours after treatment with varying doses of FO from *Boswellia carteri* demonstrating similar cell viability at both time points.

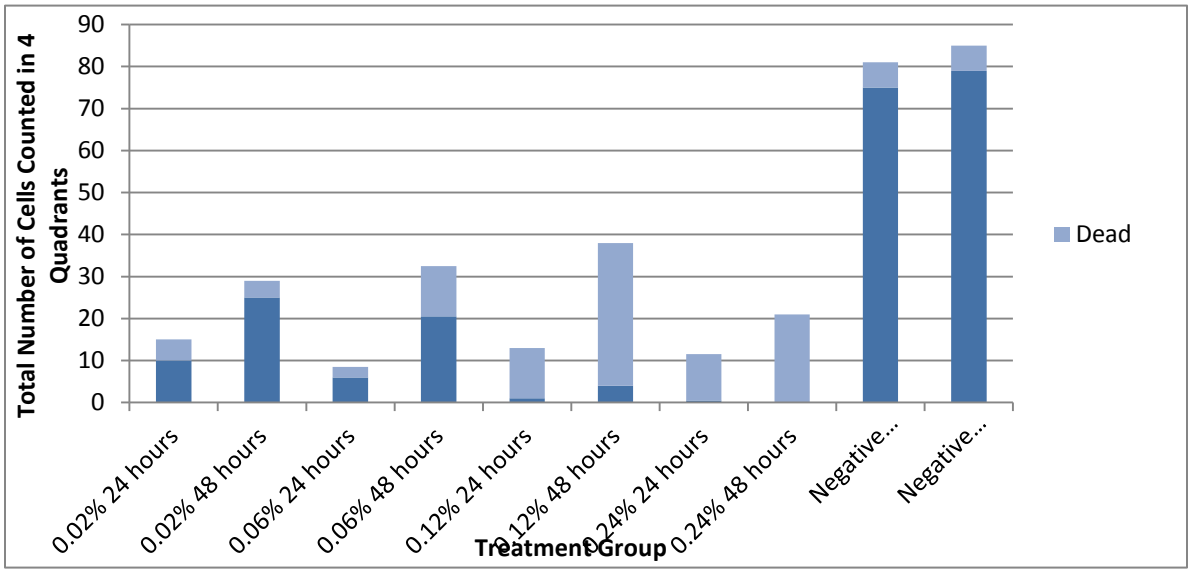


Figure 2-6: WM-115 Hemocytometer Counts. Data from manual hemocytometer counts of WM-115 cells at 24 and 48 hours after treatment with varying doses of FO from *Boswellia carteri*.

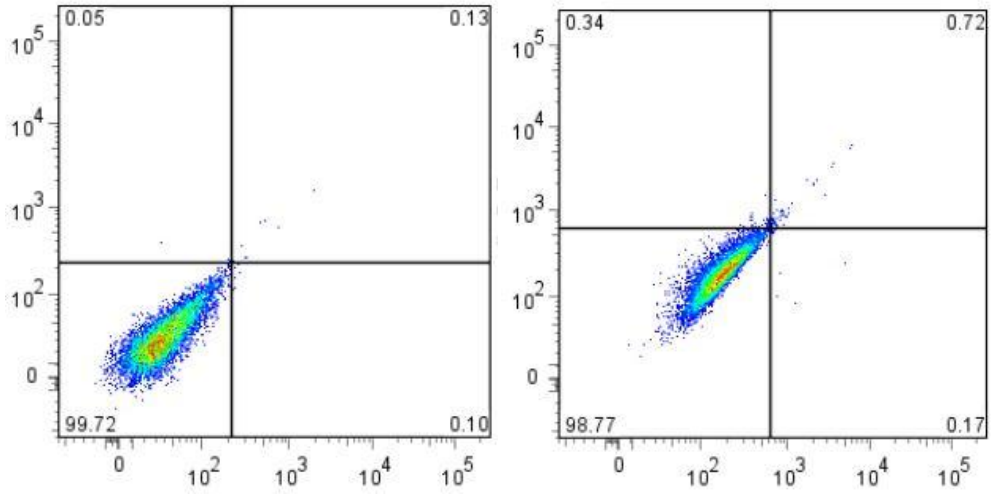


Figure 2-7: Example of fluorescence of SK-Mel-5 cells treated with FO from *Boswellia frereana.* Comparison of flow cytometry data from unstained, untreated, SK-Mel-5 cells (left) and unstained SK-Mel-5 cells treated with FO from *Boswellia frereana* at 0.2% FO by volume (right) at 8 hours post-treatment demonstrating fluorescence of FO from *Boswellia frereana*.

During preliminary flow cytometric analysis of FO-treated cells, FO from *Boswellia frereana* was determined to exhibit fluorescent properties (**Figure 2-7**). This was ascertained through comparison of treated and un-treated unstained, Annexin V FITC-only stained, and PI-only stained samples. Unstained samples from each treatment group were processed and compared to stained samples and the gate location was adjusted to account for the fluorescence contributed by FO. Apoptosis and necrosis were distinguished by combined staining of samples with Annexin V FITC and Propidium Iodide. Flow cytometric analysis using combined staining with annexin V and PI has recently been demonstrated to be useful for the separation of these two forms of cell death, with annexin $V^+/PI^-$ indicating cells undergoing apoptosis, annexin $V^+/PI^+$ indicating secondary necrosis, annexin $V^-/PI^+$ indicating necrosis, and annexin $V^-/PI^-$ indicating viable cells [96].

On average, 60% of SK-Mel-5 cells treated with medium alone were viable (FITC-/PI-), 28% were early apoptotic (FITC+/PI-), 3.5% were necrotic (FITC-/PI+), and 7% were late apoptotic/secondary necrotic (FITC+/PI+) across all time points (all data from flow cytometry, after corrections for FO fluorescence, is presented in Appendix B). Low cell viability in untreated groups was likely due to cellular damage incurred during trypsinization. Data summaries are presented by treatment group below:

**Table 2-1: Flow Cytometric Data for 0.02% FO Treated SK-Mel-5 Cells**

| | 1 Hour | 4 Hours | 8 Hours | 12 Hours |
|---|---|---|---|---|
| FITC-/PI- | 63% | 26% | 40% | 58% |
| FITC+/PI- | 21% | 7% | 46% | 24% |
| FITC+/PI+ | 10% | 63% | 11% | 15% |
| FITC-/PI+ | 6% | 4% | 3% | 4% |

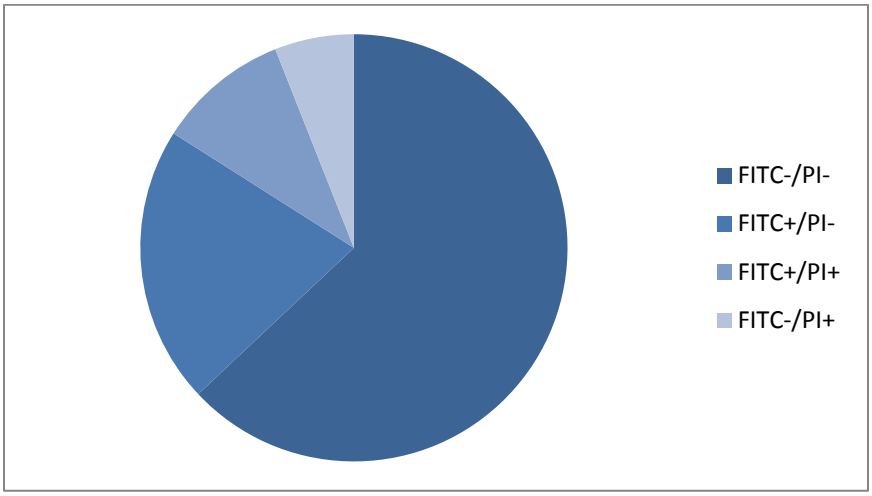


Figure 2-8: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 1 hour post-treatment with 0.02% FO

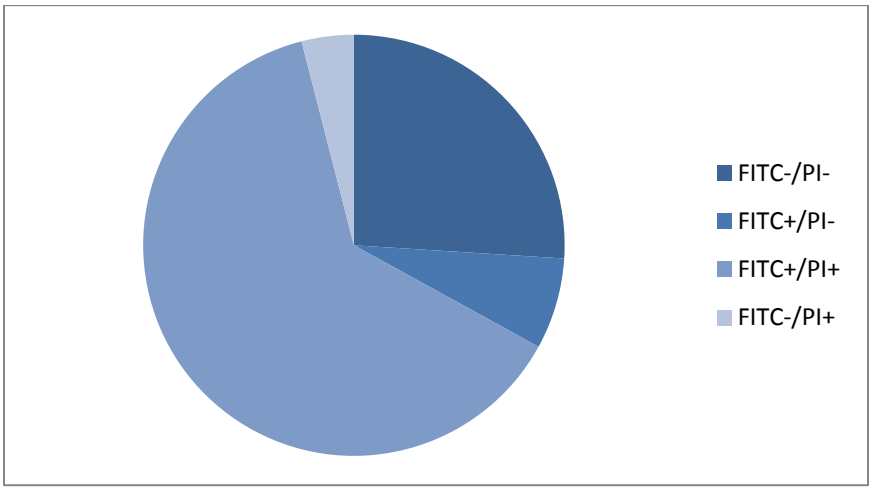


Figure 2-9: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 4 hours post-treatment with 0.02% FO

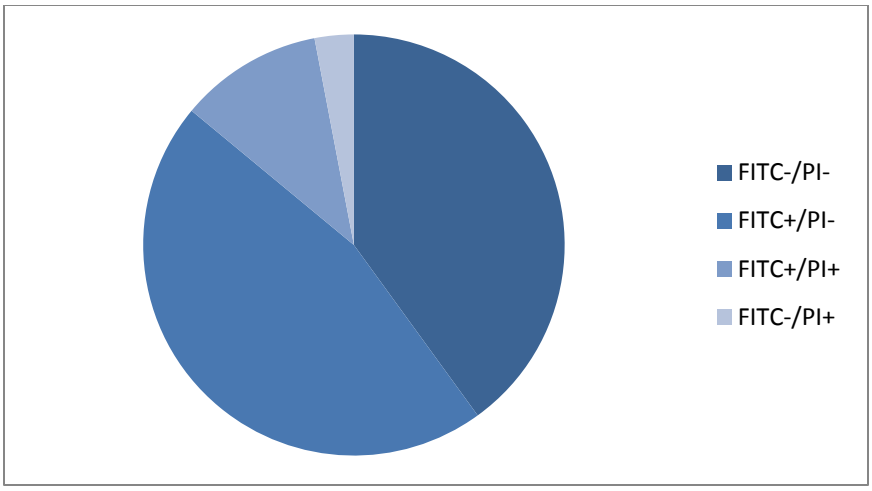


Figure 2-10: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 8 hours post-treatment with 0.02% FO

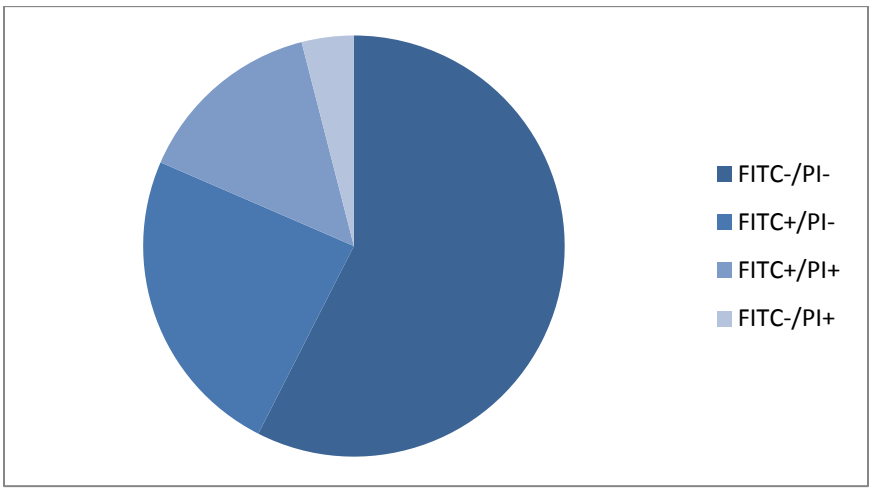


Figure 2-11: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 12 hours post-treatment with 0.02% FO

**Table 2-2: Flow Cytometric Data for 0.1% FO Treated SK-Mel-5 Cells**

| | 1 Hour | 4 Hours | 8 Hours | 12 Hours |
|---|---|---|---|---|
| FITC-/PI- | 55% | 12% | 0% | 2% |
| FITC+/PI- | 15% | 38% | 6% | 5% |
| FITC+/PI+ | 12% | 48% | 93% | 89% |
| FITC-/PI+ | 19% | 2% | 1% | 4% |

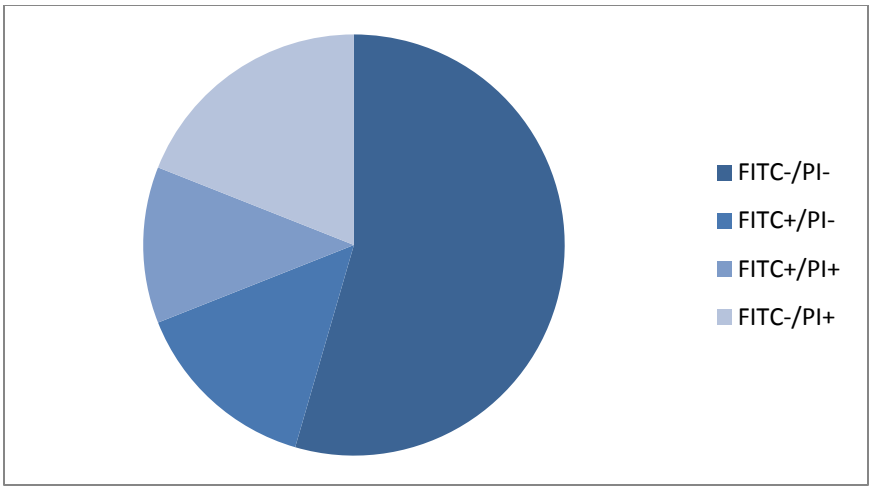


Figure 2-12: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 1 hour post-treatment with 0.1% FO

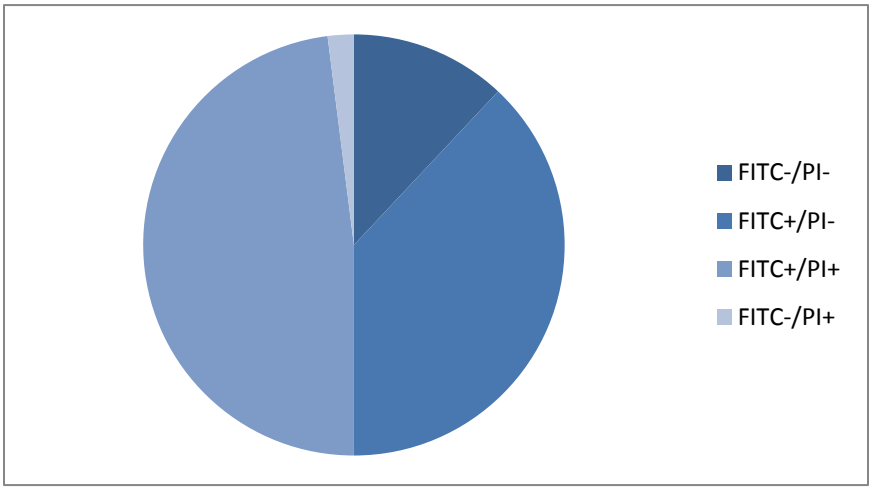


Figure 2-13: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 4 hours post-treatment with 0.1% FO

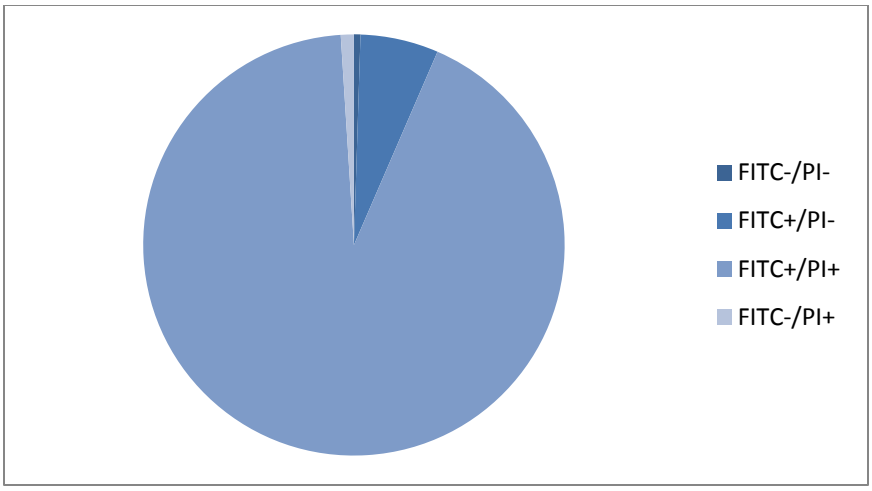


Figure 2-14: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 8 hours post-treatment with 0.1% FO

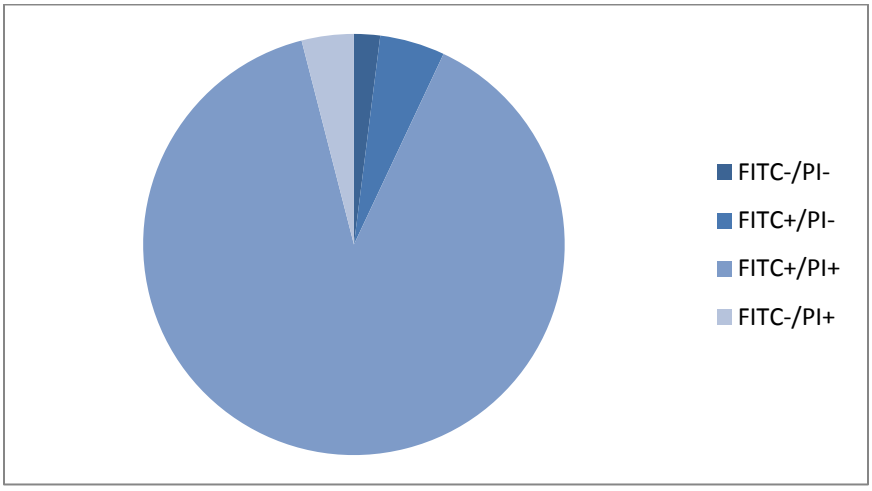


Figure 2-15: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 12 hours post-treatment with 0.1% FO

**Table 2-3: Flow Cytometric Data for 0.2% FO Treated SK-Mel-5 Cells**

| | 1 Hour | 4 Hours | 8 Hours | 12 Hours |
|---|---|---|---|---|
| FITC-/PI- | 20% | 20% | 13% | 4% |
| FITC+/PI- | 32% | 2% | 35% | 6% |
| FITC+/PI+ | 36% | 39% | 44% | 64% |
| FITC-/PI+ | 12% | 39% | 8% | 26% |

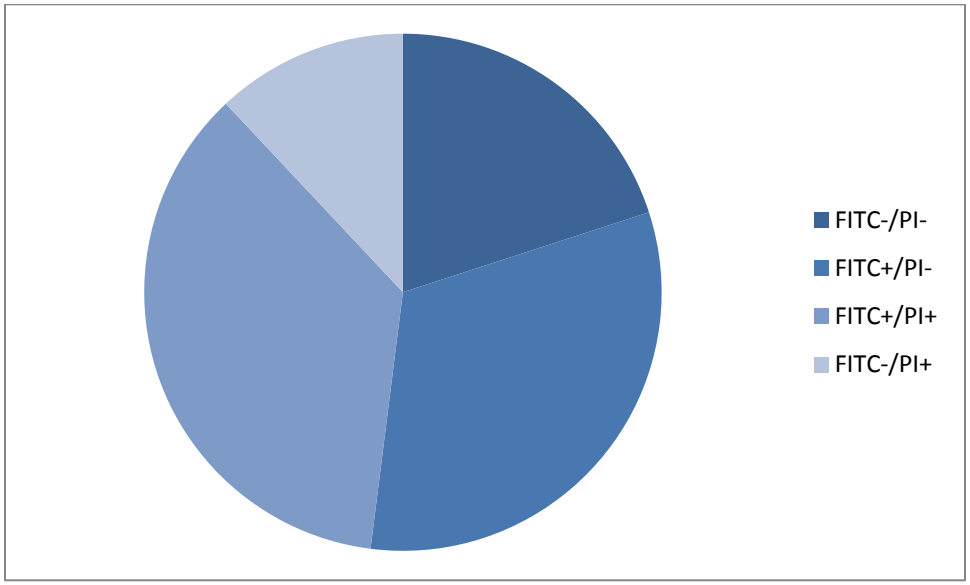


Figure 2-16: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 1 hour post-treatment with 0.2% FO

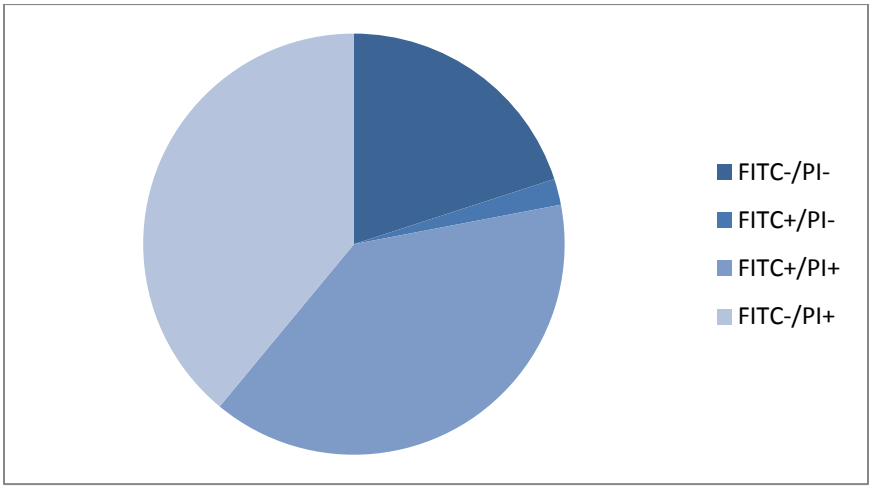


Figure 2-17: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 4 hours post-treatment with 0.2% FO

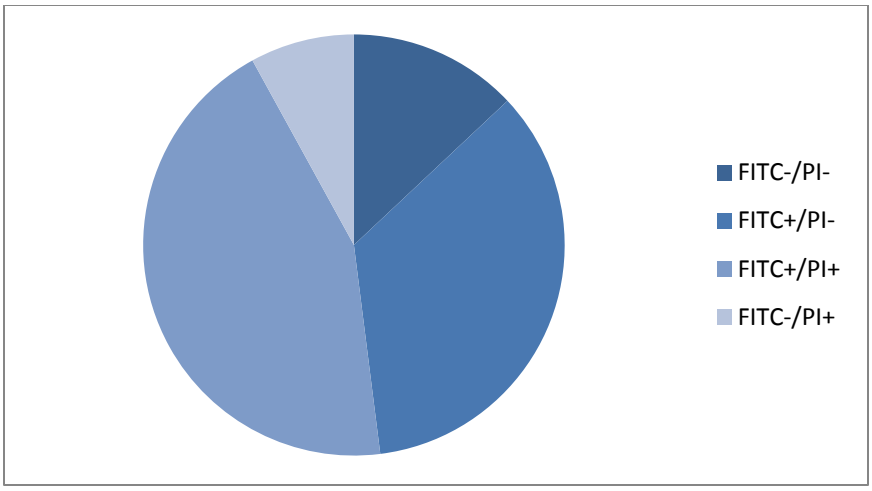


Figure 2-18: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 8 hours post-treatment with 0.2% FO

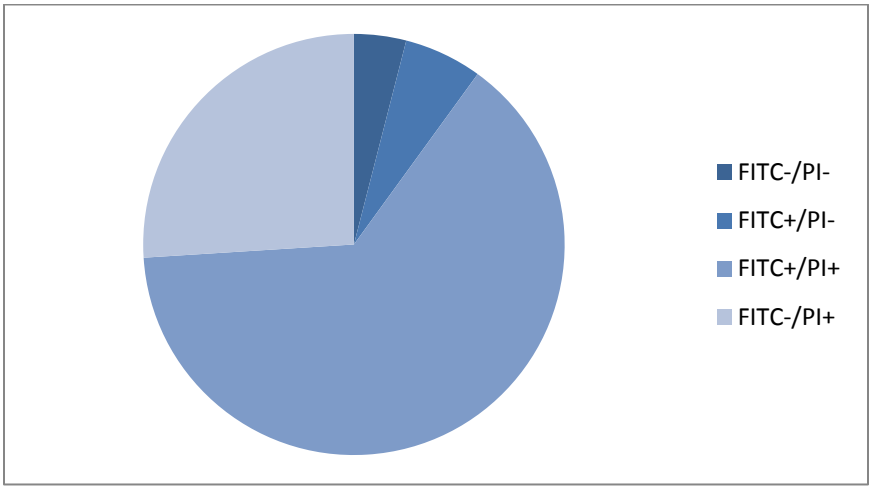


Figure 2-19: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 12 hours post-treatment with 0.2% FO

**Table 2-4: Flow Cytometric Data for Carboplatinum Treated SK-Mel-5 Cells**

| | 1 Hour | 4 Hours | 8 Hours | 12 Hours |
|---|---|---|---|---|
| FITC-/PI- | 58% | 68% | 42% | 40% |
| FITC+/PI- | 22% | 16% | 51% | 50% |
| FITC+/PI+ | 12% | 13% | 6% | 9% |
| FITC-/PI+ | 9% | 3% | 2% | 2% |

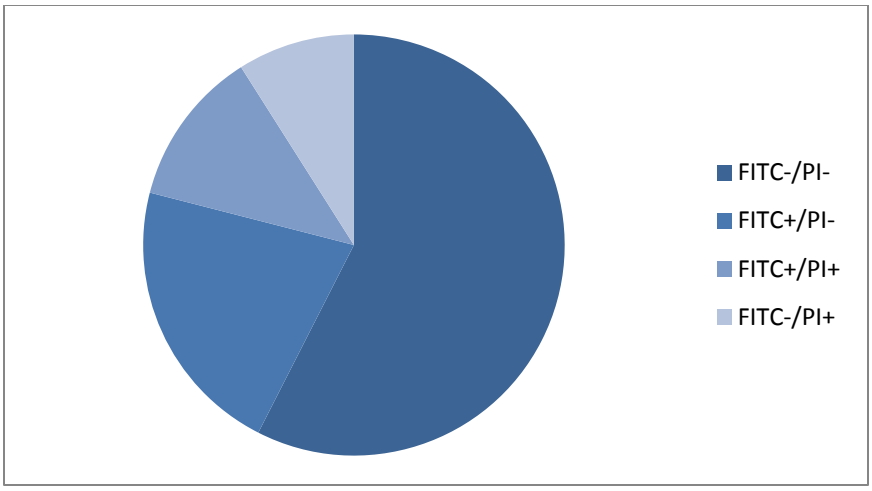


Figure 2-20: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 1 hour post-treatment with 0.04% Carboplatinum

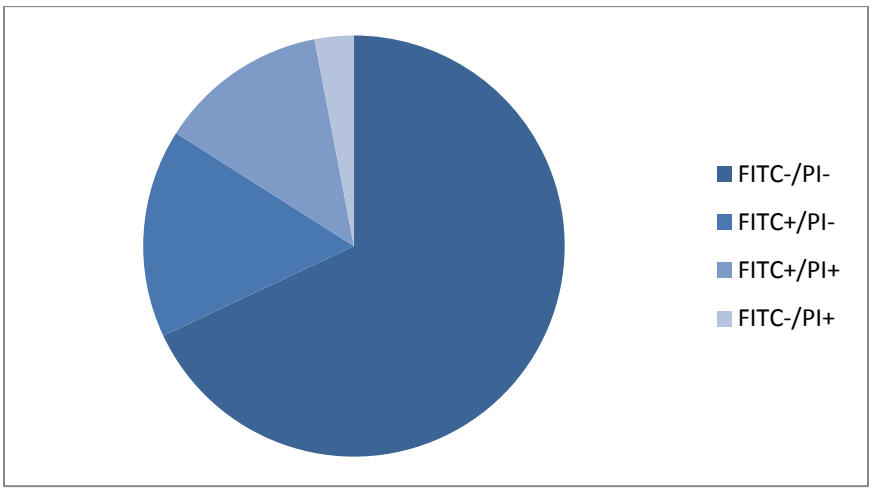


Figure 2-21: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 4 hours post-treatment with 0.04% Carboplatinum

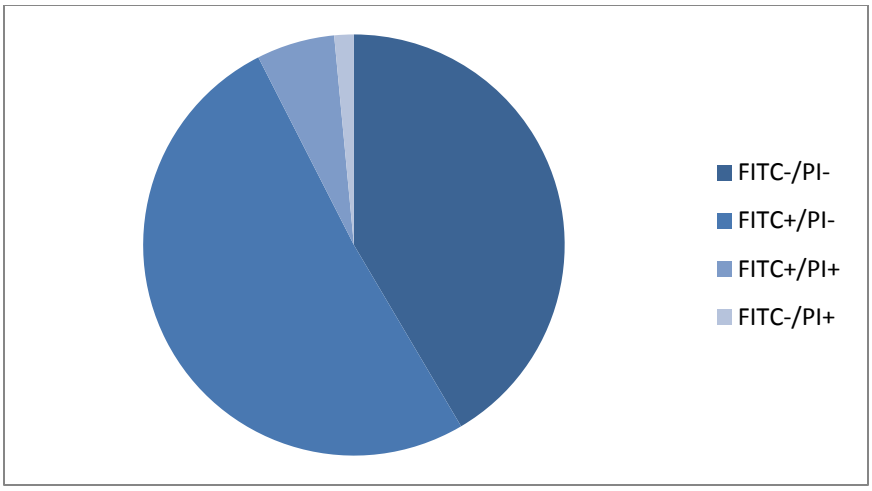


Figure 2-22: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 8 hours post-treatment with 0.04% Carboplatinum

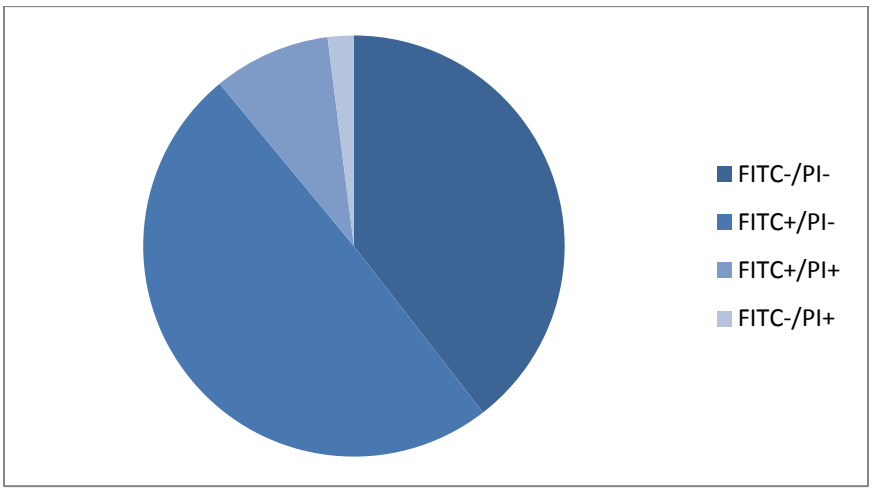


Figure 2-23: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 12 hours post-treatment with 0.04% Carboplatinum

**Table 2-5: Flow Cytometric Data for Ethanol Treated SK-Mel-5 Cells**

| | 1 Hour | 4 Hours | 8 Hours | 12 Hours |
|---|---|---|---|---|
| FITC-/PI- | 5% | 0% | 3% | 1% |
| FITC+/PI- | 3% | 5% | 7% | 2% |
| FITC+/PI+ | 48% | 95% | 86% | 94% |
| FITC-/PI+ | 44% | 0% | 5% | 4% |

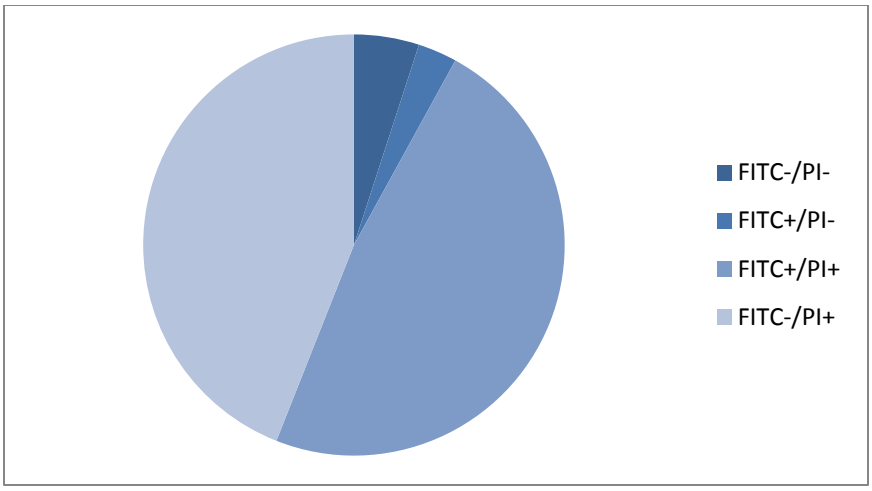


Figure 2-24: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 1 hour post-treatment with 28% Ethanol

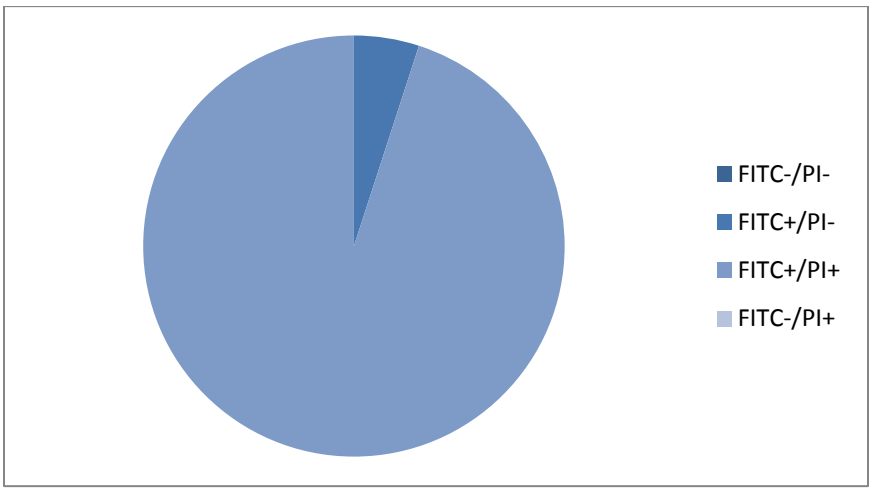


Figure 2-25: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 4 hours post-treatment with 28% Ethanol

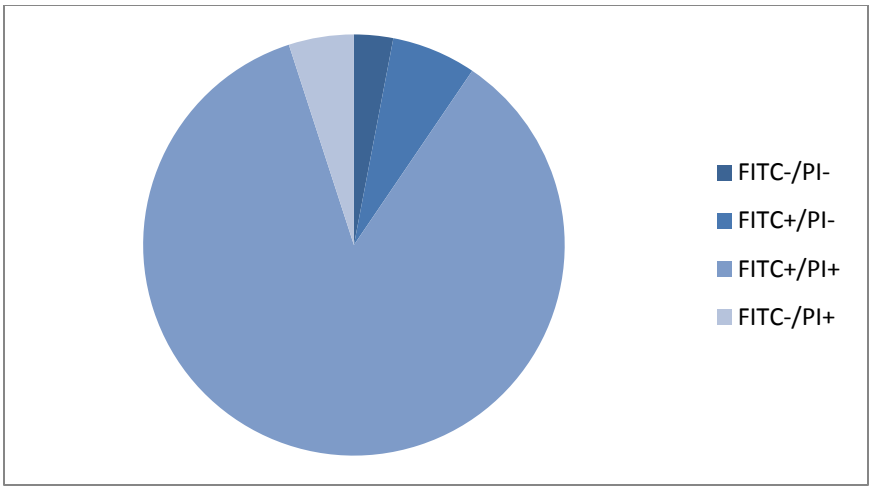


Figure 2-26: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 8 hours post-treatment with 28% Ethanol

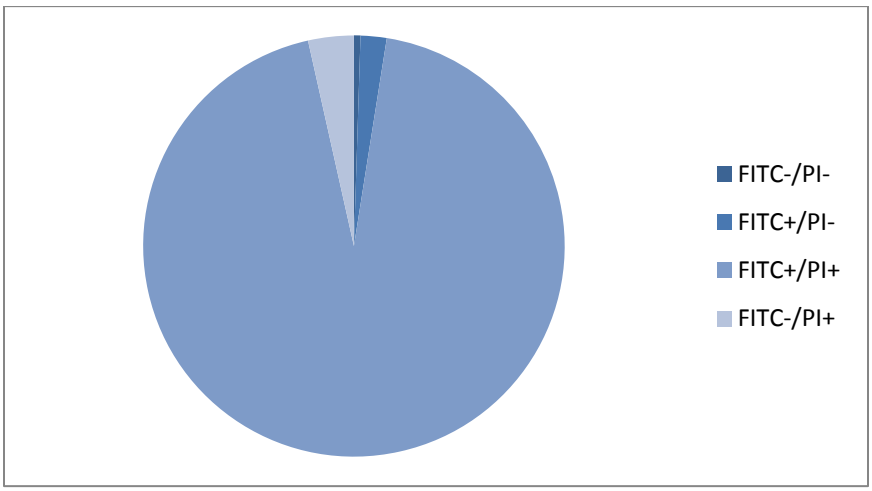


Figure 2-27: Flow cytometric data demonstrating percentage of viable, apoptotic, and necrotic SK-Mel-5 cells 12 hours post-treatment with 28% Ethanol

**Discussion**

FO from *Boswellia carteri* and *Boswellia frereana* induced cell death in a dose-dependent manner in HMM cells. The cytotoxic activity of FO from *Boswellia carteri* in WM-115 cells peaked within 24 hours of treatment and within 12 hours in SK-Mel-5 cells treated with FO from *Boswellia frereana*. Additionally, the total number of cells counted

in each of the FO-treated flasks was less than the number of cells counted in the negative control flasks, even in the flasks receiving the lowest doses of FO. These finding demonstrate that FO from *Boswellia carteri* and *Boswellia frereana* also inhibit cellular proliferation.

Apoptosis, or programmed cell death, is a normal physiological process of cellular self-destruction and degradation. Another means of cell death, necrosis, involves loss of cell membrane integrity resulting in a failure of homeostasis in response to cellular injury. For decades, apoptosis has been the preferred mechanism of cell death for chemotherapeutic activity due to end-stage phagocytic clearance,resulting in minimal surrounding tissue damage. In contrast, the leakage of intracellular components and enzymes during necrosis promotes an inflammatory response which may lead to substantial damage of healthy tissue. However, this proinflammatory response may also improve the efficacy of therapies through recruitment of immune cells. Furthermore, as neoplastic cells often have mutations altering critical apoptotic elements, induction of necrosis may be an essential component of effective cancer therapy [97]. To an extent, these two processes can be differentiated by morphological features, DNA fragmentation analysis, and flow cytometry. [98]

During the early stages of apoptosis, phosphatidylserine (PS) is translocated from its normal position on the inner surface of the cell membrane to the exterior. Annexin-V is a protein that preferentially binds with negatively charged phospholipids, like PS, while demonstrating minimal attraction to the phospholipids present on the external leaflet of

normal, healthy cells. When tagged with the green dye fluorescein isothiocyanate (FITC), Annexin V can be used to identify the loss of phospholipid asymmetry, indicating initiation of the apoptotic pathway through flow cytometry [96, 99]. Necrotic cells differ from apoptotic cells in the loss of cell membrane integrity and, consequently, degradation of associated phospholipids [98]. Consequently, DNA-binding dyes, like propidium iodide (PI), are excluded by early apoptotic cells while inducing red fluorescence in cells undergoing necrosis [96]. The combination of these markers allows for differentiation of early apoptotic and primary necrotic cells via flow cytometry.

In the current study, flow cytometric analysis demonstrated that FO, as a hydrodistillate from *Boswellia frereana* resin, induced cell death in SK-Mel-5 cells via apoptosis and, to a lesser extent, primary necrosis. The ethanol-treated control samples largely exhibited primary and secondary necrosis, with minimal evidence of induction of apoptosis. In contrast, all FO-treated groups and the carboplatin-treated cells exhibited a trend of early apoptosis followed by progression into the late apoptotic/secondary necrotic quadrant. These findings suggest that FO from *Boswellia frereana* induces apoptosis similar to carboplatin. FO from *Boswellia frereana* demonstrated cytotoxic effects on SK-Mel-5 cells in a dose and time-dependent manner. The highest concentration of FO from *Boswellia frereana* induced increasing levels of necrosis and secondary necrosis over time, suggesting that the lower concentrations are likely more appropriate for therapy. FO-induced apoptosis peaked at 8 and 4 hours post-treatment in the 0.02% FO and 0.1% FO treatment groups, respectively. In contrast, early apoptotic levels in the

carboplatin-treated cells gradually increased over the 12 hour trial, indicating a longer duration of cytotoxicity.

While apoptosis and necrosis are distinctly different forms of cell death, no stimulus, including toxicants, has been shown to produce death by purely apoptosis or necrosis. This is due to the influence of both the nature of the injury and the apoptotic/necrotic threshold of the cell on the mode of cell death [98]. Treatment of SK-Mel-5 cells with 0.2% FO from *Boswellia frereana* resulted in an increase in primary necrosis, approaching apoptotic levels. This data suggests that the boundary between the apoptotic and necrotic thresholds for SK-Mel-5 cells with respect to treatment with FO from *Boswellia frereana* is close to 0.2% by volume. These findings could help guide future dosing strategies in translational studies targeting malignant melanoma, since apoptosis is clinically preferable to necrosis.

The percentage of secondary necrotic cells in FO-treated samples increased over the 12 hours of this trial and, to an extent, with increased FO dose. Secondary necrosis is a terminal state noted most commonly in cell culture situations, as a result of the absence of phagocytic cells. In the current study, the patterns of fluorescence (see Appendix B) indicated progression of FO-treated cells from the apoptotic quadrant into the secondary necrotic quadrant, suggesting that the accumulation of secondary necrotic cells was a result of the lack of phagocytic clearance [98]. However, in the 0.1% and 0.2% treatment groups, the apoptotic and necrotic cells accumulated at a faster rate than the carboplatin-treated cells. *In vivo*, secondary necrosis following apoptosis can occur

when phagocytic clearance systems are overwhelmed [98]. Therefore, secondary necrosis may be a consequence in future *in vivo* translational studies evaluating FO as a therapy for malignant melanoma. Again, this could have an impact on FO dosing strategies used in translational research. Large doses of FO would not be advisable for *in vivo* studies, as the rapid accumulation of apoptotic and necrotic cells would likely lead to necrosis secondary to overwhelmed phagocytic cells. Since extensive apoptosis with failure of clearance can be mistaken for a primary necrotic response [98], evidence of necrosis in translational FO studies should be confirmed through immunohistochemistry and doses should be decreased to levels below the necrotic threshold.

In the present study, we examined the cytotoxic efficacy of FO against HMM cells in culture and the mode of cell death induced by FO. Our findings, derived from cell cultures studies, show that FO primarily induces cell death via apoptosis in a dose-dependent manner and has potential as an effective treatment for patients with melanoma.

## Chapter 3: Frankincense Oil as an Injectable and Topical Cytolytic Therapy for Reducing Tumor Bulk in Equine Malignant Melanoma

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(in preparation for publication in *Equine Veterinary Education*)

### Abstract

Frankincense oil (FO) has long been recognized as a compound with diverse medicinal properties. In more recent years, the anti-neoplastic properties of FO have gained increased attention and multiple studies have been performed evaluating the cytotoxic effects of FO in neoplastic cell lines. FO has been demonstrated to selectively induce apoptosis in neoplastic cells in culture, making it a promising chemotherapy. In this study, we evaluated the toxicity of topical and intratumoral injectable FO therapy in horses with equine malignant melanoma (EMM). FO was found to be well-tolerated and, through a series of follow-up case studies, useful for reducing tumor bulk in advanced cases of perianal EMM.

### Introduction

Equine malignant melanoma (EMM) is a spontaneously occurring neoplasm associated with inherited genetic factors and grey coat color [100]. It accounts for 6-15% of all equine skin tumors [101] and it is estimated that 80% of all grey horses will develop EMM by age 15, with 66% of cases progressing to florid malignancy [13, 23]. Early stages of EMM may be managed with surgical excision [102], but this becomes impractical at advanced stages of the disease (Stage III-IV in the clinical classification system proposed by [80]). Cimetidine, a selective histamine antagonist with suspected immunomodulatory properties, initially showed potential for reducing tumor volume in a

study of 3 horses with early stages of melanoma [44]; but, subsequent, larger trials have shown it to be ineffective against advanced stages of the disease [103]. Similarly, studies evaluating cisplatin therapy (administered as intratumoral injections or cis-platinum impregnated polymeric beads) found it to be less effective in larger masses [104]. Immunotherapies, including interleukin therapy and autologous vaccination, have also had limited success in reducing tumor volume [105, 106]. At this time, there are no reliable therapies for late stages of EMM.

In a screening study performed in the Department of Radiation Oncology at Wake Forest University School of Medicine, a variety of essential oils were tested for cytotoxic effects in the presence of human glioblastoma cells and normal mouse fibroblasts *in vitro*. Fifteen of the essential oils exhibited varying degrees of cytotoxicity; however, frankincense oil (FO) was found to have the greatest cytotoxic effect in the glioblastoma cells, while exhibiting the least effect on normal fibroblasts [91]. In our own follow-up study, human melanoma cells were tested for evidence of cell death mediated by apoptosis in response to exposure to FO. FO was found to reduce cellular proliferation and induce apoptosis in a dose- and time-dependent manner in both primary, WM-115, and metastatic, SK-Mel-5, malignant melanoma cell lines (chapter 2).

FO is a distillate of the lipophilic fraction of gum resin collected from the trunks of trees and shrubs of the genus *Boswellia*. There are at least twenty species in the genus and each produces a unique gum resin [107]. For centuries, the gum resin produced by *Boswellia* species has been prized for its medicinal properties. Civilizations across the

globe have long recognized its ability to combat inflammation and infection and to promote wound healing [107]. Research efforts have identified at least a dozen different pentacyclic triterpenic acids within *Boswellia* resin [89]. Among these, boswellic acids have been identified as the most biologically active and pharmacologically significant components. While considerable attention has been placed on identifying the component that confers *Boswellia*'s medicinal properties, the complete extract may be more efficacious than its purified fractions [90].

Early research primarily focused on elucidating the mechanisms behind the anti-inflammatory effects of *Boswellia* products. These efforts uncovered biological activities that produce anti-neoplastic effects, including inhibition of 5-lipoxygenase (5-LO), triggering further investigation into the anti-cancer properties of *Boswellia* [108]. Additional studies with boswellic acid have shown it to interfere with protein binding in prostate cancer cells [109], inhibit enzyme activity in a murine colon cancer model [110], induce apoptosis in human leukemia cells [111], block cell-to-cell signaling in human multiple myeloma cells [112], and specifically down regulate chemokine receptor 4 in pancreatic cancer cells [113]. Recent evidence suggests that boswellic acids may also achieve anti-cancer effects through modulation of DNA methylation resulting in activation of tumor suppressor genes [114].

The data gathered at Wake Forest University combined with our findings evaluating cytotoxicity in human malignant melanoma (HMM) cell lines supported further study of FO in an animal model of melanoma. As a common, spontaneous neoplasm of the skin,

EMM represents an appropriate translational model for continued study of the cytotoxic effects of FO. This chapter presents results from a Phase I-II study evaluating the toxicity and safety of intratumoral injection and topical application of FO in horses with EMM and evaluates the use of FO as a treatment for perianal EMM, through clinical observation of approximately 50 cases.

## Phase I-II Toxicity and Clinical Efficacy Study Arm of Trial

### Materials and Methods

Horses of either sex with multiple perianal cutaneous melanoma lesions were solicited for study from clients in the eastern United States (primarily Virginia, Maryland, Kentucky, and New Jersey). Equine patients were considered eligible for the study if they were not currently receiving therapy for the condition. Eligible horses were donated to the Virginia-Maryland Regional College of Veterinary Medicine for teaching and research or temporarily volunteered for enrollment in the study by their owners following client education and obtaining Informed Consent for Experimental Study. Prior to initiation of treatment, medical histories were obtained and thorough physical examinations were performed on each horse to ensure eligibility and overall health. A total of 6 horses with late stage EMM were enrolled in the study (**Table 3-1**).

**Table 3-1: Overview of Horses Enrolled in the Phase I-II Study**

| Treatment Group | Age (years) | Breed | Sex | EMM Stage |
|---|---|---|---|---|
| Topical | 19 | Anglo-Arabian | Gelding | 4 |
| Topical | 16 | Thoroughbred | Gelding | 3 |
| Topical | 16 | Connemara/ Thoroughbred | Mare | 4 |
| Topical | 22 | Thoroughbred | Gelding | 3 |
| Injectable | 10 | Thoroughbred | Gelding | 4 |
| Injectable | 15 | Thoroughbred | Gelding | 4 |

On each horse, pairs of comparably sized perianal tumors were selected, confirmed to be EMM via biopsy, and then allocated for treatment, with one mass identified as the control and treated with medicinal grade, non-therapeutic, sesame seed oil (SO) and the remaining mass treated with medicinal grade FO (*Boswellia carteri*) (Young Living Essential Oils, Lehi, Utah, USA). In addition, to assess the normal tissue effects of FO (and SO), each material was topically applied to normal skin or injected into normal skin/subcutaneous tissue in the region of the tuber ischii. These control sites were treated in a manner identical in frequency and duration to the tumor sites. Treatment consisted of either twice daily topical application of FO and SO or daily intratumoral injections of FO and SO for 14 days. In the event of symptomatic tumor progression, significant concurrent illness, unacceptable toxicities, wound infection with purulent exudation, or a general deterioration in the condition of the horse, treatment was discontinued. Four of the 6 horses received only topical application of oil, while the remaining 2 received intratumoral injections. Prior to treatment, masses were photographed, measured with calipers, and biopsied along with normal perianal skin for histopathological evaluation. The length, perpendicular width, and height of each mass

were recorded. Blood was collected for a complete blood count and serum chemistry profile. Measurements, photographs, and bloodwork were repeated on days 4, 10, and 14 of treatment. Additional biopsies were collected on days 4 and 14. Tumor biopsy samples were collected using a biopsy punch following cleansing of the tumor surface with surgical scrub and alcohol and local administration of 2% lidocaine. Biopsies were stored in 10% neutral buffered formalin.

The FO was formulated at a concentration of 1000 µl/ml. Pre-treatment measurements of the masses were used to calculate the dose for intratumoral injection of FO, dosed at 100 µl/$cm^3$ using an 18 gauge needle and 3 ml luer lock syringe. Tumors receiving topical therapy were treated with 1-1.5 ml FO applied with a cotton-tipped applicator that had been immersed in FO. Skin toxicity was graded using an adaptation of the revised National Cancer Institute (NCI) Common Toxicity Criteria (CTC) version 3.0 for Toxicity and Adverse Event Reporting (http://ctep.info.nih.gov). Toxicity was categorized as none, mild (erythema), moderate (desquamation), or severe (necrosis).

A positive effect of therapy was defined as a minimum of 20% reduction in the volume of a treated lesion following FO administration for 14 days.

**Results**

Horses reacted to the initial intratumoral injection of FO as they would to an insect bite. Most horses glanced at the injection site, rubbed or attempted to bite at the injection site, and paced in their stall for a brief period of time following injection. These effects

generally lasted for approximately 10 minutes. Subsequent injections rarely elicited a response. We suspected that the injections had mild analgesic and anesthetic properties, but did not investigate this further. It was also worth noting that FO rapidly corroded plastic syringes. So, it was drawn up within 60 seconds of injection.

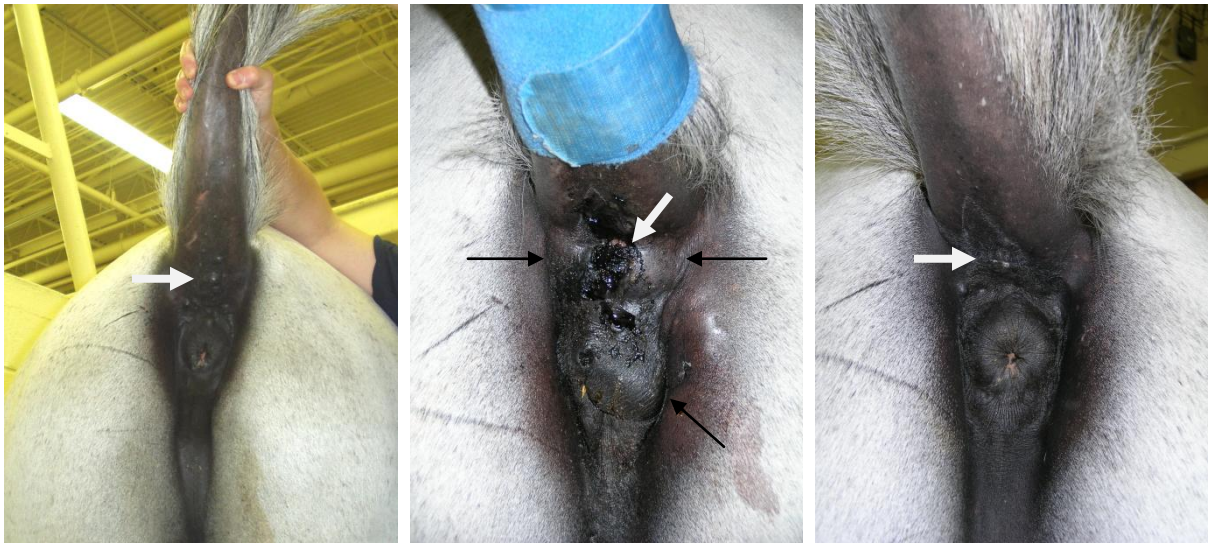

Figure 3-1: Photographs of the perineum of the 10-year-old, Thoroughbred gelding enrolled in the injectable FO study prior to treatment (left), 5 days after treatment (middle), and 28 days after treatment (right). White arrows indicate the treatment site and black arrows outline the extent of associated edema.

Both horses enrolled in the injectable study demonstrated mild to moderate, edematous swelling of the FO-treated tumors within 24-48 hours of FO injection. Palpation of the swollen treatment sites did not appear to elicit a pain response. At 72-96 hours after injection, the FO-treated tumors began discharging black, muco-purulent material and drainage persisted for several days after completion of the trial. The core of the FO-treated mass in the 10-year-old, Thoroughbred gelding sloughed one week after initiation of treatment (**Figure 3-1)**. A positive therapeutic effect, previously defined as a 20% decrease in tumor volume, was not observed within the 14 day trial period in either horse treated with intratumoral injections of FO (**Table 3-2**). However, observations of

tumors and skin sites of the 10-year-old Thoroughbred gelding were continued for 28 additional days after therapy was discontinued. Within that time, the discharging core of the FO-treated tumor closed, leaving a mildly depigmented focus, measuring 10 x 10 x 1 mm. Similarly, 6 months after completion of the trial period, the 19-year-old Anglo-Arabian gelding presented for euthanasia due to complications of metastatic EMM. At the time of necropsy, the FO-treatment site had healed, leaving a negligibly small lesion. These findings suggest that, given an extended trial period, injectable FO therapy would result in a positive therapeutic effect. Injection of normal skin sites with FO was discontinued in both horses after 7-10 days due to swelling and abscess formation. The complete blood counts of both horses demonstrated mild stress leukograms and one developed a mild, normocytic, normochromic anemia, but were otherwise within reference intervals. The serum chemistry profiles were unremarkable throughout the trial.

**Table 3-2: Tumor Dimensions of Horses Treated with Injectable FO**

| 10-year-old Thoroughbred Gelding | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| FO-Treated tumor | 20 x 17 x 6 | 2040 | 62 x 20 x 15 | 18600 | 55 x 34 x 27 | 50490 | 24.75x |
| | | | | | | | |
| Control tumor | 27 x 22 x 15 | 8910 | 43 x 25 x 20 | 21500 | 28 x 27 x 20 | 15120 | 1.7x |

| 15-year-old Thoroughbred Gelding | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| FO-Treated tumor | 21 x 16 x 8 | 2688 | 40 x 36 x 18 | 25920 | 52 x 28 x 16 | 23296 | 8.66x |
| | | | | | | | |
| Control tumor | 22 x 14 x 12 | 3696 | 22 x 23 x 11 | 5566 | 38 x 30 x 20 | 22800 | 6.16x |

In horses treated with topical FO, there was rarely any reaction to its application, even when applied to ulcerated skin/tumors. Responses to topical application of FO ranged from imperceptible to mild, transient skin irritation. One of the topically-treated horses was noted to rub the FO-treated area following treatment. Another was recorded as appearing ‘slightly agitated’ for approximately 30 minutes following FO-application. Evidence of skin toxicity was not noted at any of the treated tumor sites. A positive therapeutic effect was recorded in 3 of the 4 horses treated with topical FO; however, a positive therapeutic effect was also recorded in the SO-treated control tumor of 1 of these 3 horses (**Table 3-3**). The control tumors of the remaining 2 horses, in which a positive therapeutic effect was demonstrated, increased in size during the 14 day trial. Although the fourth horse did not demonstrate a positive therapeutic effect based on the preset definition for this study, the FO-treated tumor did demonstrate a lesser increase in size when compared to the control tumor. No significant changes were noted in the complete blood counts or serum chemistry profiles during the course of the trial.

Follow-up information was attained for 5 of the 6 horses involved in the toxicity study 5 years after its completion. All 5 of the horses had been euthanized due to complications associated with EMM.

### Table 3-3: Tumor Dimensions of Horses Treated with Topical FO

| 19-year-old Anglo-Arabian Gelding | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| Treated tumor | 38 x 21 x 11 | 8778 | 48 x 28 x 14 | 18816 | 46 x 19 x 12 | 10488 | 1.19x |
| | | | | | | | |
| Control tumor | 26 x 17 x 11 | 4862 | 38 x 23 x 9 | 7866 | 38 x 30 x 11 | 12540 | 2.58x |

| 16-year-old Thoroughbred Gelding | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| Treated tumor | 30 x 21 x 8 | 5040 | 25 x 21 x 8 | 4200 | 24 x 21 x 6 | 3024 | 0.6x |
| | | | | | | | |
| Control tumor | 17 x 11 x 6 | 1122 | 19 x 16 x 7 | 2128 | 18 x 16 x 9 | 2592 | 2.3x |

| 16-year-old Connemara/ Thoroughbred Mare | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| Treated tumor | 38 x 33 x 13 | 16302 | 24 x 24 x 10 | 5760 | 19 x 20 x 8 | 3040 | 0.19x |
| | | | | | | | |
| Control tumor | 47 x 24 x 17 | 19176 | 64 x 18 x 10 | 11520 | 59 x 20 x 8 | 9440 | 0.49x |

| 22-year-old Thoroughbred Gelding | Day 0 Dimensions (mm) | Day 0 Volume (cu mm) | Day 4 Dimensions (mm) | Day 4 Volume (cu mm) | Day 14 Dimensions (mm) | Day 14 Volume (cu mm) | Change in volume Days 0-14 |
|---|---|---|---|---|---|---|---|
| Treated tumor | 13 x 13 x 6 | 1014 | 12 x 11 x 5 | 660 | 12 x 10 x 4 | 480 | 0.47x |
| | | | | | | | |
| Control tumor | 12 x 6 x 5 | 360 | 12 x 8 x 5 | 480 | 14 x 10 x 4 | 560 | 1.56x |

### Case Study Arm of Trial

In the 5 years following the completion of the Phase I-IIstudy, we have worked with veterinary colleagues and owners to guide the continued use of FO as a treatment for perianal EMM in an estimated 50 cases. Through these cases, additional information regarding therapeutic protocols and efficacy of FO as a treatment for EMM has been

gained. With regard to toxicity and side effects, approximately 4 cases exhibited transient clinical signs associated with colic directly following intratumoral injection of FO. This is in congruence with studies in humans evaluating oral administration of *Boswellia serrata* in which some subjects reported gastrointestinal discomfort [115]. All occurrences of FO-associated colic were successfully managed with basic medical treatment. FO-associated colic is presumed to be a result of inadvertent intravascular administration of FO; however, this has not been tested. Consequently, as a precaution, we recommend ensuring the needle is not in a blood vessel prior to administering FO intratumorally. Additionally, one pony experienced an episode of mild laminitis, in addition to colic, approximately 24 hours after intratumoral injection of 2.5 ml of FO. This is the only case in which laminitis developed following FO injection and may have been related to a variety of factors, including the pony's history of laminitis and the relatively high dose of FO administered.

Intratumoral administration of FO in a subauricular/parotid mass was only attempted in one case. Immediately following injection of 20 µl of FO, the horse developed bruxism and diaphoresis around the head and neck, pressed the mass to the ground, and appeared generally uncomfortable (pacing, pawing, and intermittently lying down). The scent of FO was detectable in the horse's breath. The mass was iced for 20-30 minutes without improvement. Intravenous flunixin meglumine was administered and improvement was seen within 30 minutes, but intermittent head tossing and bruxism was still noted. An hour later, symptoms worsened again and the horse was administered xylazine and butorphanol intravenously to control pain. Within 3 hours of

injection, his condition returned to baseline Forty-eight hours later, pitting edema was noted from a few inches cranial to the ramus of the mandible to several inches caudal on the neck and extending to the ventral midline and was approximated 10 cm in diameter(twice the size of the original mass). Further treatment of the mass with FO was discontinued.

The therapeutic protocol for intratumoral FO injections typically involved weekly or bi-weekly injections of a standard 1 ml dose, rather than the daily injections of smaller volumes administered during the toxicity trial. Topical therapy generally remained a twice daily application of 1-1.5 ml of FO. Three case examples are presented here, demonstrating the efficacy of FO as a therapy for EMM.

**Case 1**

Case 1 was a grey, Lipizzaner mare with stage IV EMM that presented for treatment in August of 2011 due to difficulty with defecation resulting from confluent perianal masses. Intratumoral injectable FO therapy was initiated using medicinal grade FO from *Boswellia frereana* Boswellness, Burlington, VT, USA). On day one of therapy, a 1 ml dose of FO was injected into the right side of the mass approximately 4 cm ventral to the base of the tail. A second 1 ml dose of FO was injected 2 cm ventral to the original injection on the following day and weekly injections were administered thereafter. After swelling was noted for approximately 3 weeks without evidence of rupture or drainage, the referring veterinarian drained purulent material from the area of injection using a needle and syringe. A week later, the referring veterinarian drained approximately 15 ml

of black liquid from the treated area. Rupture and natural drainage occurred approximately one week later. The mass was noted to be softer and the anus was less obstructed. Massages and hydrotherapy were regularly performed to promote further drainage. After 2 months of weekly injections, the mare was no longer experiencing difficulty with defecation. After 6 months of weekly injections, the mare began resisting treatment and injections ceased for approximately 3 weeks. Seven months after treatment was initiated, the mass was estimated to be approximately 50% of the original size (**Figure 3-2**).

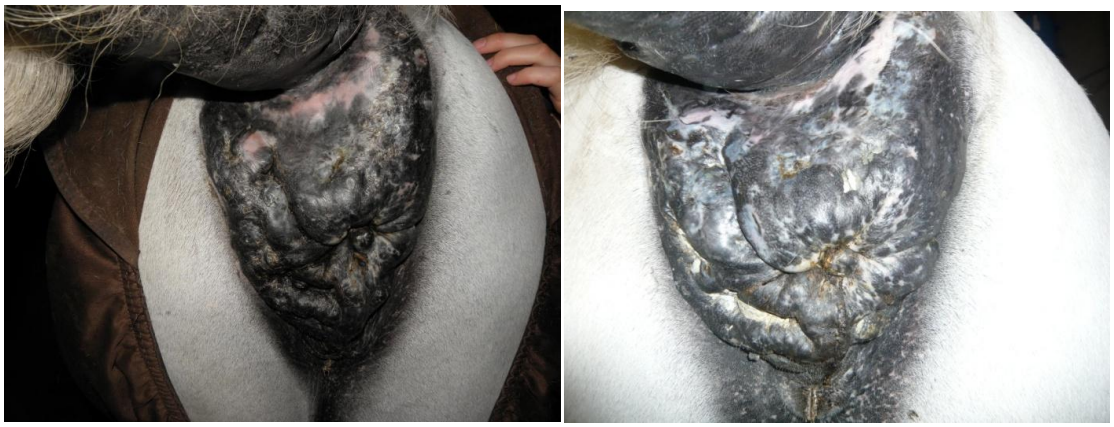

Figure 3-2: Images of the confluent perianal masses prior to treatment (left) and after 7 months of intratumoral FO therapy (right) from case 1. The region of the mass dorsal to the anus is estimated at 50% of the original size (note the change in size and location of the depigmented region).

**Case 2**

Case 2 was a 22 year old, grey, Arabian mare with stage 3 EMM that presented for treatment of a perianal mass in September of 2011. The mare had been receiving oral Cimetidine for one month prior and the perianal mass was estimated to decrease in size by approximately 30%. Cimetidine therapy was continued concurrently with intratumoral

FO therapy (Boswellness, Burlington, VT, USA). On day one of therapy, a 1 ml dose of FO was injected into the lateral aspect of the perianal mass. The injection was well-tolerated and the mare did not demonstrate any discomfort afterwards. Twenty-four hours later, extensive edematous swelling of the anus, and medial aspect of the left hind limb was noted. Within 5 days, the mass was noted to be draining sero-purulent material. Nine days after the injection, the core of the tumor (~1 inch in diameter, conical, black mass) was extruded, leaving primarily healthy, pink granulation tissue. The site was cleaned and irrigated twice daily and allowed to granulate in with healthy tissue (**Figure 3-3**).

Figure 3-3: Images of perianal masses prior to treatment (left) and after 1 week of intratumoral FO therapy (middle and right) from case 2.

**Case 3**

Case 3 was a 25 year old**,** grey, grade pony gelding with stage 4 EMM that presented for treatment in September of 2011 due to difficulty with defecation resulting from confluent perianal masses. Topical FO therapy was initiated using FO from *Boswellia carteri* (Purple Flame Aromatherapy,Warwickshire, UK). Within 7 days of twice daily application of FO, the treated mass became edematous, ruptured, and began draining thick, black exudate. Approximately one month after beginning topical FO therapy, concurrent oral Cimetidine therapy was added. Three weeks after introduction of oral Cimetidine therapy, the core of the tumor (3-4" long, 1" diameter, black mass) was extruded. The site was cleaned and irrigated twice daily and allowed to granulate in with healthy tissue (**Figure 3-4**). Since that time, he has not demonstrated difficulty with defecation or tumor recurrence at the treated site.

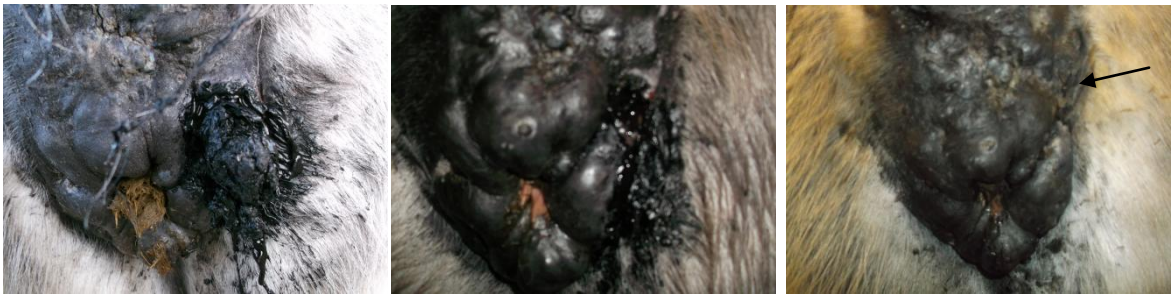

Figure 3-4: Images of draining perianal mass after 11 weeks of topical FO therapy (left), after extrusion of the core (middle), and after 3 weeks of healing (right) from case 3. The black arrow indicates the area of scar tissue formation following treatment.

**Discussion**

The combined results of the Phase I-II study and follow-up case studies demonstrate that FO is safe for topical and intratumoral medicinal use in cases of perianal EMM,

when properly administered. A positive therapeutic effect was noted in both horses involved in the injectable FO trial after a lengthened follow-up period, and in 3 out of 4 horses involved in the topical FO trial after 14 days. These findings are corroborated by the follow-up case study. Based on the data from cell culture studies, the lysis and drainage associated with FO treatment is presumed to be a result of secondary necrosis following apoptosis [98]. However, further histopathologic and immunohistochemical evaluation of samples collected from horses enrolled in the Phase I-II study would be necessary to acquire data to validate this assumption.

In our experience, the response to intratumoral injection of FO is typically much more rapid and dramatic than the response to topical application of FO. The information presented in Case 3 is atypical and represents the most dramatic response to topical FO therapy achieved to date. More commonly, owners report no change or subtle variations in tumor bulk following topical FO application. However, it is also worth noting that in Case 3, FO was applied to the tumor twice daily for nearly 3 months before the core of the tumor was expelled. While topical therapy has the benefit of being a therapy that can be applied by owners, this aspect also adds to the potential for decreased compliance. Furthermore, areas of the tumor surfaces of the horse in Case 3 were somewhat ulcerated and may have allowed for increased penetration of FO.

In both Case 2 and Case 3, FO therapy was administered concurrently with oral Cimetidine therapy. The success associated with both of these cases warrants further

investigation into the potential synergy between oral Cimetidine therapy and local FO therapy.

As demonstrated by the 3 cases presented, FO therapy is particularly beneficial in cases of advanced perianal EMM. Due to the confluence and proximity to important structures, such as the anal sphincter and associated nerves, surgical resection of these masses is often complicated. FO therapy can be used to successfully reduce tumor bulk in these cases and reduce discomfort and the risk of rectal impactions without compromising continence.

## Chapter 4: Dissertation Discussion and Conclusions

Understanding of the pathogenesis and availability of effective treatment options are limited with regard to malignant melanoma, especially malignant melanoma of horses. This doctoral dissertation presents new data demonstrating: 1) increased abnormal expression of Pax-3/7, B-Raf, Nestin, and Sox-10 proteins in EMM, 2) microscopic evidence of EMM in grey horses without clinical manifestation of the disease, 3) FO-induced cytotoxicity mediated by apoptosis in HMM cell lines, and 4) reduction of tumor bulk in cases of advanced (Stage 3-4) EMM treated with topical application and intratumoral injections of FO.

Our findings show that the melanoma tumors of grey horses with EMM demonstrate increased expression of B-Raf, Nestin, Pax-3/7, and Sox-10. The same proteins are over-expressed in HMM tumors, have been implicated in melanoma pathogenesis, and have been linked to genetic mutations. Demonstration of similar patterns of abnormal protein expression in EMM and HMM indicates that similar genetic abnormalities and cellular pathways are involved in the development of malignant melanoma in both species. Furthermore, the expression of Nestin, Pax-3/7 and Sox-10 in EMM and HMM tissues suggest involvement of melanocyte cancer stem cells in the pathogenesis of both EMM and HMM. In addition, the origins of multiple tumors on a single horse have not been confirmed. Studies comparing the histopathologic and immunohistochemical characteristics of multiple EMM masses from distant sites on the same horse and investigation of primary EMM cell cultures for evidence of stem cell populations could uncover key factors in EMM pathogenesis.

Grey horses commonly develop malignant melanoma and yet many survive into their teens and twenties, allowing for prolonged study of disease and extended follow-up periods for therapeutic trials. Most grey horses with EMM develop multiple masses, which permits autologous comparison of control substances in therapeutic trials. Unlike the Platyfish-Swordtail hybrid, EMM is naturally occurring and does not require induction of disease [10] and, unlike the Sinclair swine model, EMM has not been demonstrated to spontaneously regress [8]. Heritable risk factors related to skin and hair color have been established in both EMM and HMM and the data presented herein demonstrates similar abnormal protein expression in EMM and HMM. These characteristics accomplish the key features of a good translational animal model [11]. The translational potential of this model was tested and validated through demonstration of the clinical efficacy of FO (a medicinal oil with verified cytotoxicity in HMM cell lines) in EMM. One of the major limitations of EMM as a translational model is the expense of the facilities, staff, and general care required for horses. However, because EMM is a spontaneous condition with limited treatment options, many owners of afflicted horses are willing to enroll in experimental studies and will often help offset the cost of care. In areas, like Lexington Kentucky, where the horse population is extensive, large scale studies could be performed with greater ease. The other major limiting factor for the use of EMM as a translational model is the limited knowledge base regarding EMM pathogenesis. Although the evidence to date shows considerable similarity between EMM and HMM, further study of melanoma in both species is necessary to establish their inevitable differences. No translational model can perfectly replicate human disease. We found

that EMM appears to originate in the dermis, typically in close association with sweat glands, which is unlike most cases of HMM, where the condition originates in the epidermis or dermo-epidermal junction. Furthermore, the majority of HMM cases have been associated with damage due to UV radiation, which is not a readily apparent risk factor in EMM. Consequently, EMM would not be suitable for translational studies requiring a model demonstrating invasion of neoplastic melanocytes originating in the epidermis or demonstrating damage caused by UV radiation. However, EMM would be useful for studies requiring an animal model with spontaneous, inherited, and/or UV-independent melanoma development. The combined results from the three studies presented herein demonstrate the beneficial potential of translational malignant melanoma research, encourage use of EMM as a translational disease model for HMM, and promote further investigation of EMM pathogenesis.

The findings presented in this dissertation not only contribute data to the research community, but also contribute to the clinical diagnosis and treatment of EMM. Discovery of microscopic EMM in more than 50% of biopsied skin samples from Stage 0 mares from a small population of Thoroughbred mares further underscores the frequency with which this condition afflicts grey horses. This data suggests that actual EMM prevalence in grey horses may be even higher than previously reported statistics. Consequently, veterinarians should educate all owners of grey horses about EMM and both veterinarians and owners should be even more vigilant in monitoring common sites of EMM on Stage 0 grey horses for signs of disease progression.

The presented work also validates the use of FO for treatment of EMM and provides treatment options for veterinarians and horse-owners struggling to manage advanced cases of EMM. Based on my experience with FO therapy, I would advise use of intratumoral injectable therapy in advanced cases of EMM in which perianal masses are causing discomfort. A comparative study evaluating the margins for evidence of EMM infiltration following injectable FO therapy vs. surgical excision is warranted; however, given the current knowledge base, I would recommend surgical excision of small, distinct EMM nodules over injectable FO therapy, because of the follow-up care associated with FO injection. Additionally, for owners that are reluctant to pursue excision, topical FO therapy represents an improved alternative to the current "benign neglect". Investigation of the combined effects of oral Cimetidine and FO therapy could also prove promising for treatment of EMM.

Further investigation of FO therapy in both *in vivo* and *in vitro* models is indicated. Cytotoxicity of FO should be evaluated in equine cell lines and investigation of the specific pathways initiating FO-induced apoptosis should be examined in both EMM and HMM cells. Induction of cell cycle arrest following low dose treatment of EMM and HMM cells should also be evaluated. Further investigation of FO-related colic and laminitis would also be critical, and should include trials evaluating the effects of intravenous administration of FO in terminal subjects. Additionally, large scale studies evaluating the therapeutic and toxic dose thresholds for injectable FO are necessary for improved treatment protocols.

FO should be investigated as an alternative and adjunctive therapy for surgical resection of dermal HMM. The effects of systemic administration of FO are largely unknown. Consequently, at this time, it does not represent an option for treatment of metastatic HMM. However, further investigation evaluating the bioavailability and efficacy of oral *Boswellia* products could lead to its use as a systemic therapy. However, gaining approval from the FDA for use of medicinals derived from *Boswellia* in HMM patients would cost millions of dollars. In order for its use to be profitable and off-set the cost of FDA approval, a synthetic analogue would have to be developed and patented.

Heritable risk factors associated with pigmentation of the hair and skin are influential in the pathogenesis of both EMM and HMM. Studies evaluating hundreds of thousands of human subjects are performed annually to evaluate these risk factors and their impact on HMM incidence, pathogenesis, and prognosis. A link between grey coat color inheritance and melanomagenesis has long been established in EMM. Breed-specific pedigree information has been maintained for decades in horse breeds like Arabians and Thoroughbreds Still, studies correlating EMM incidence with familial inheritance of grey coat color are absent from the literature. In the study presented in Chapter 1, Thoroughbreds were selected as the sample population due to the availability of pedigree information through The Jockey Club. The Jockey Club has maintained the pedigree information of Thoroughbreds in the United States, Canada, and Puerto Rico since 1894. Using this information, a combined pedigree demonstrating inheritance of grey coat color, and presumably the associated risk factors for EMM, for all mares involved in the study was created (see Appendix A). This combined pedigree chart

demonstrates a common grey ancestor, Le Sancy, for all stage 1-4 mares involved in the study. Further study of pedigree information and expansion of data to include a larger population of grey Thoroughbreds could lead to a better understanding of EMM inheritance and, ultimately, prevention or reduction of disease severity through selective breeding. Additionally, the prognostic implications of EMM have not been statistically analyzed and few case reports provide enough follow-up data for estimation. An ongoing study following the development and progression of EMM linked with pedigree data would be very beneficial to the understanding of heritable risk factors and prognosis associated with EMM.

Little work has been done to investigate the pathogenesis of EMM, resulting in poor characterization of disease progression and limited treatment options. The findings presented in this dissertation represent substantial advancement in the histopathologic diagnosis, immunohistochemical characterization, and treatment of EMM. The data herein also supports the use of EMM as an animal model for translational study of malignant melanoma. However, as the study of EMM is still in its infancy, this work simply represents a starting point. Similarly, few studies have evaluated the effects of FO in melanoma cells and, to my knowledge, ours is the first translational study of FO in an animal model of melanoma. Continued investigation of the pathogenesis of EMM, comparative aspects of EMM and HMM, and the anti-neoplastic properties of FO are necessary. Far more work is required if the treatment and survival times for both horses and humans with malignant melanoma are to be improved and if the full potential of EMM as a translational model for malignant melanoma is to be realized.

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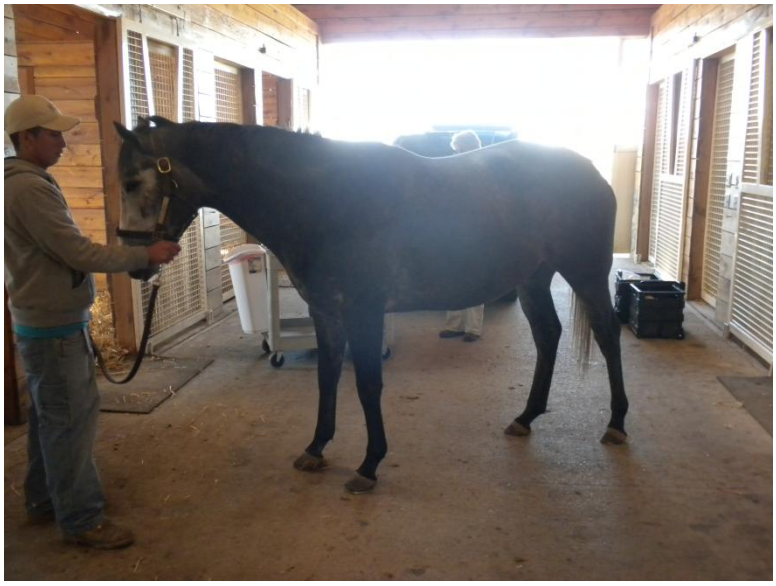
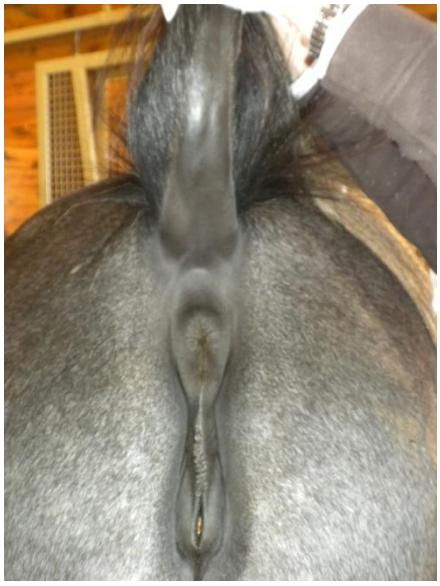
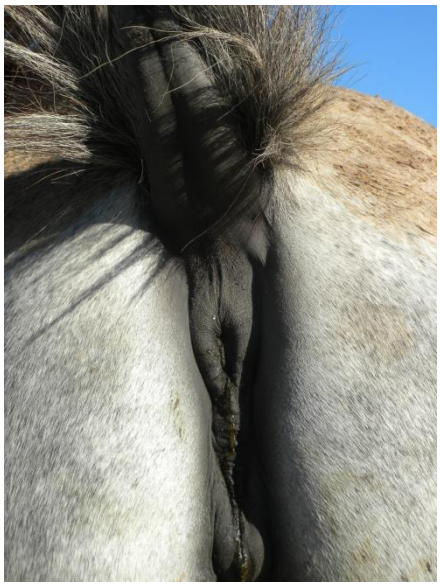
Appendix A: Photographs and Pedigrees of Mares in Chapter 1 Study

“Flatter Me” 2008 Thoroughbred Mare. Stage 0 EMM

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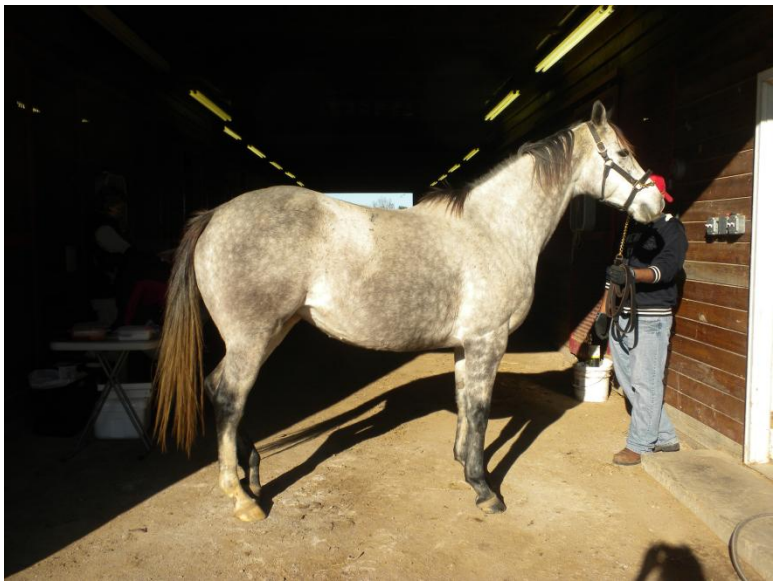
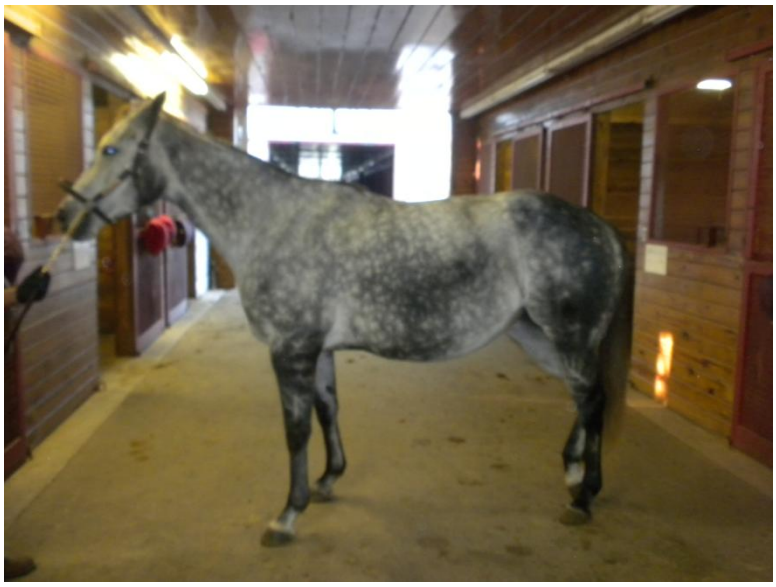
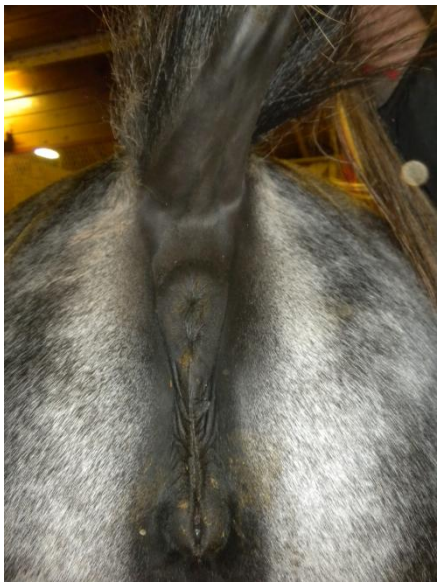
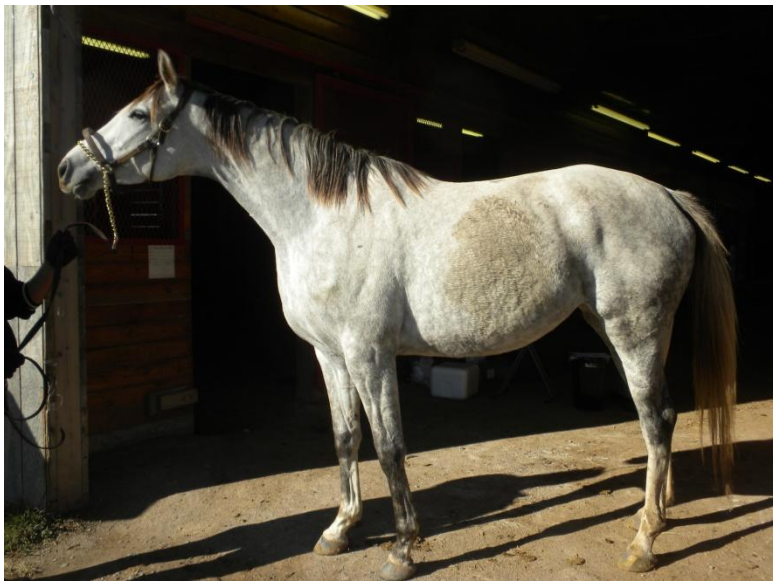


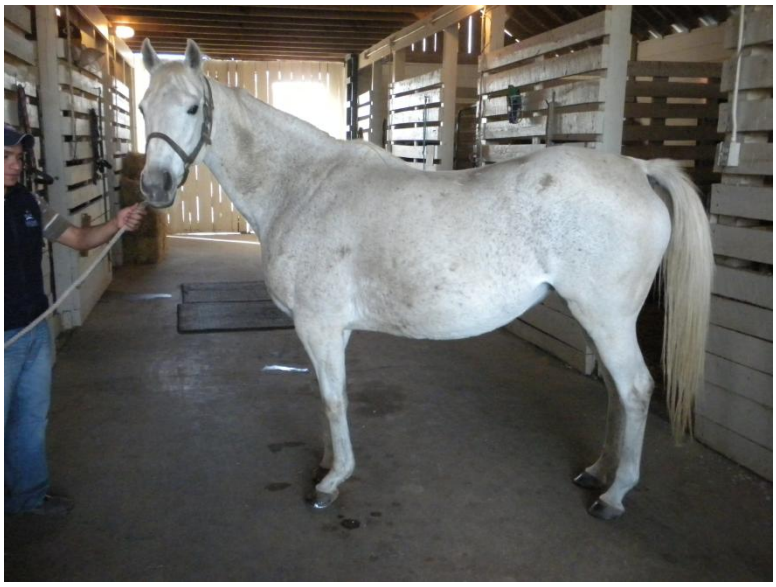
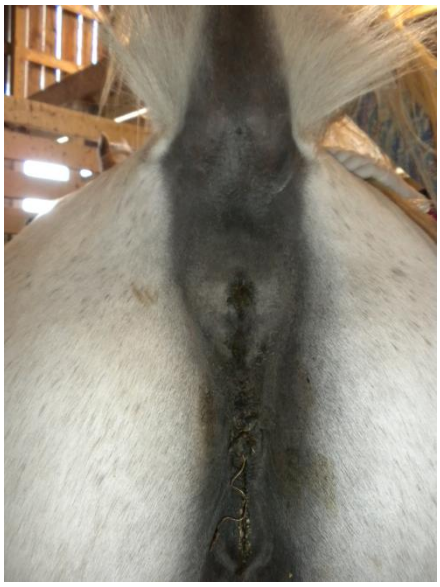

“Saturday Matinee” 2006 Thoroughbred Mare. Stage 0* EMM

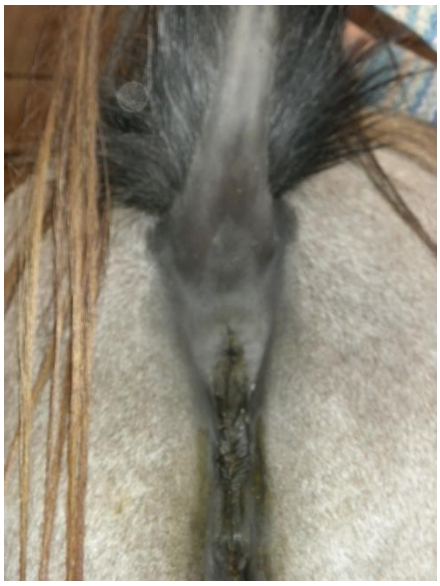
“Holy Moment” 2006 Thoroughbred Mare. Stage 0 EMM




“Lou Ann’s Kitten” 2007 Thoroughbred Mare. Stage 0* EMM

“Moon Queen” 1998 Thoroughbred Mare. Stage 0* EMM

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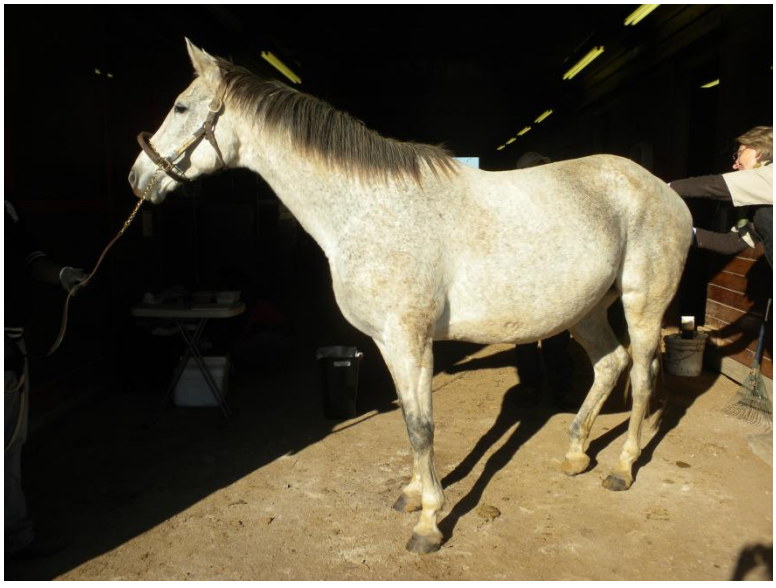
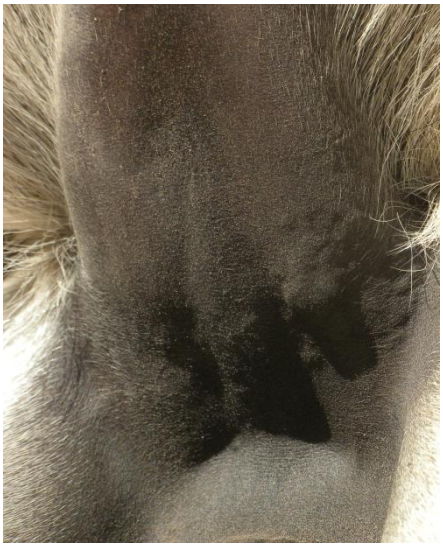
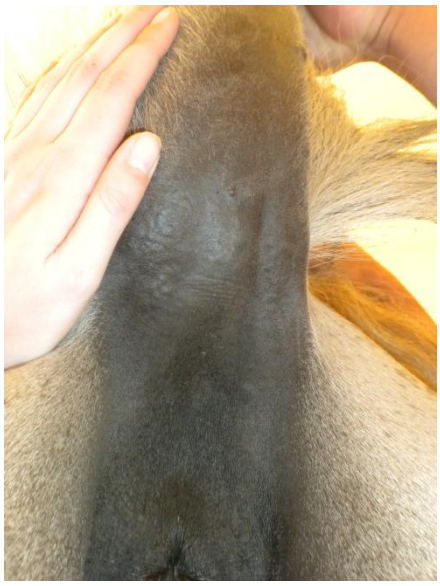



“Mortgage the House” 2002 Thoroughbred Mare. Stage 1 EMM

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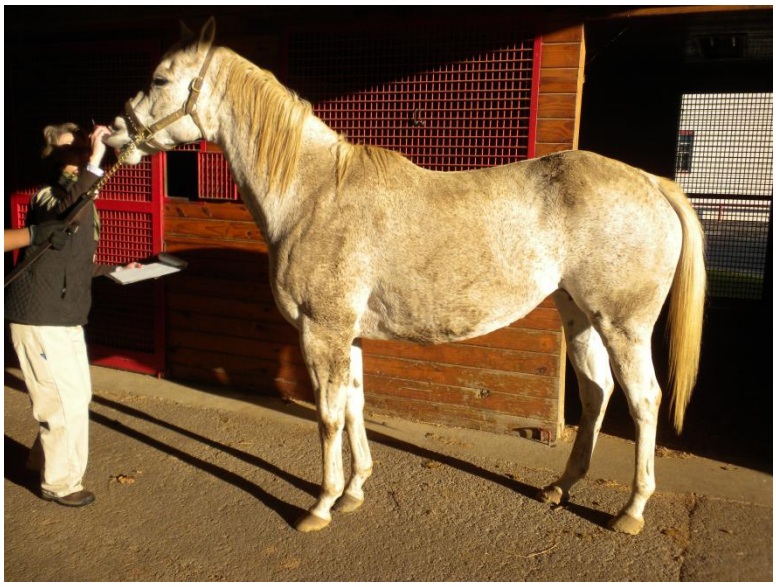
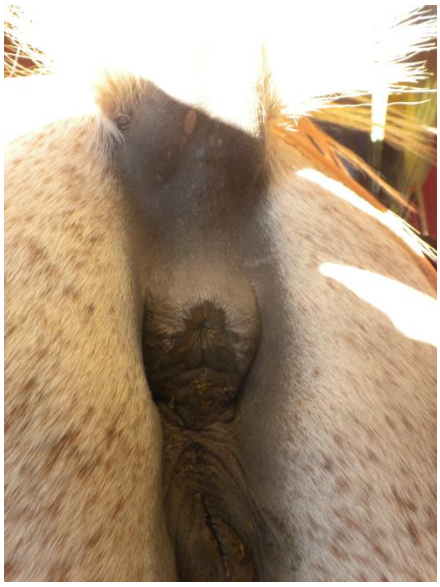

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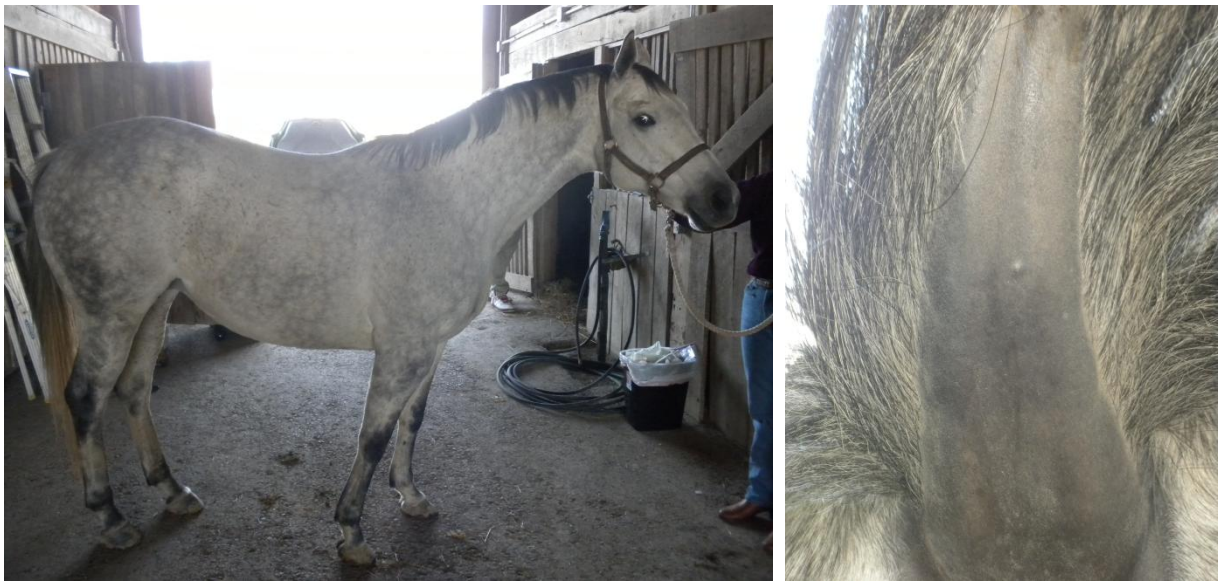
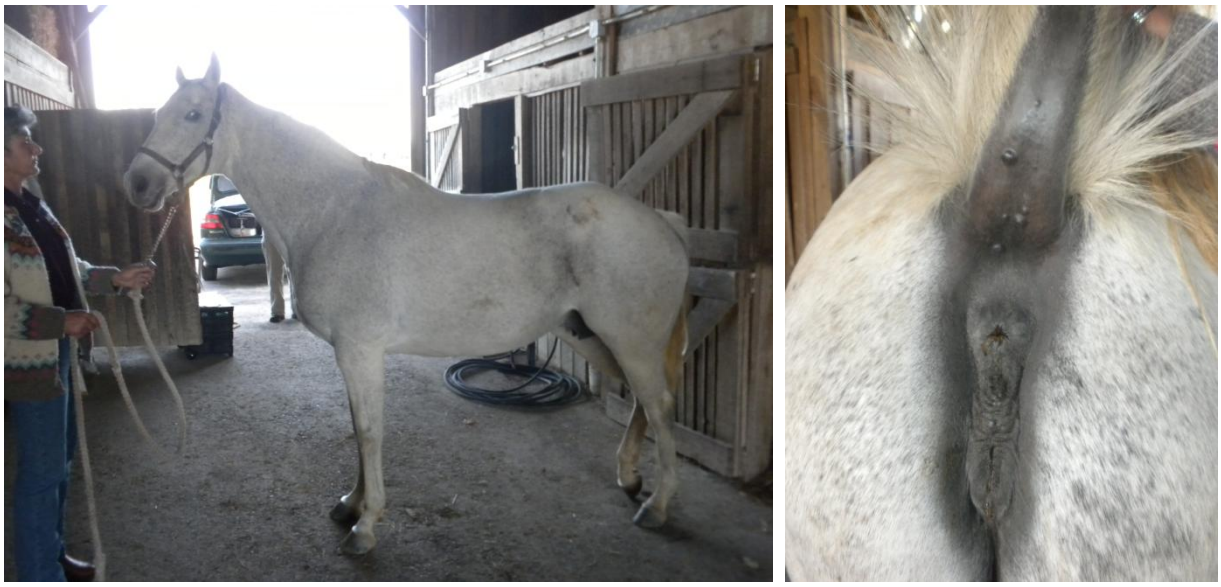


“Urbana Fizz” 2003 Thoroughbred Mare. Stage 1 EMM



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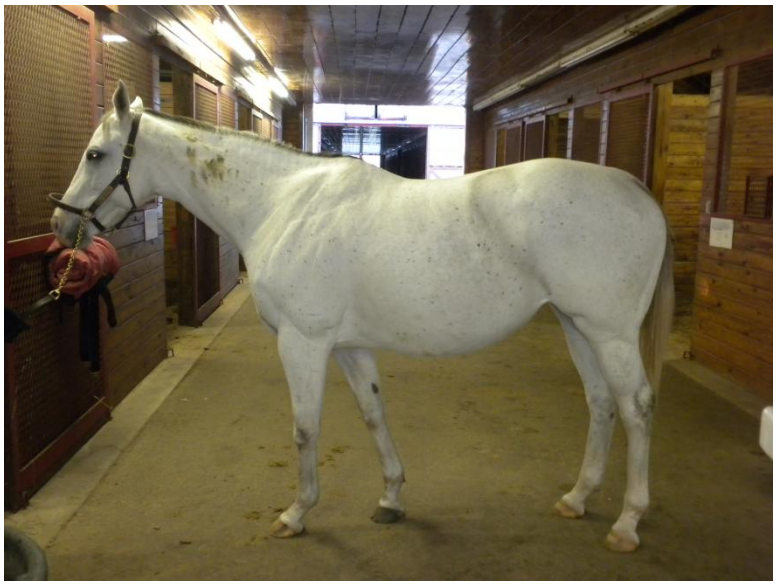
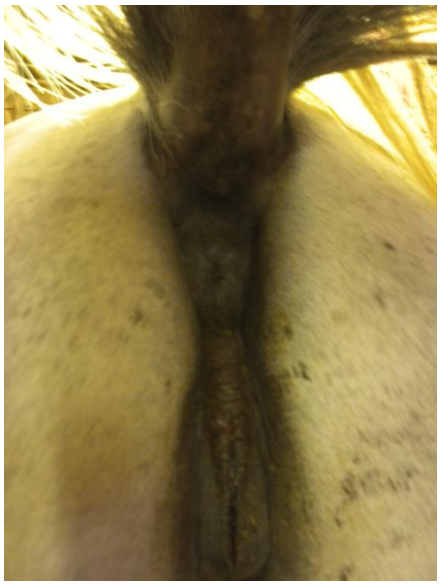
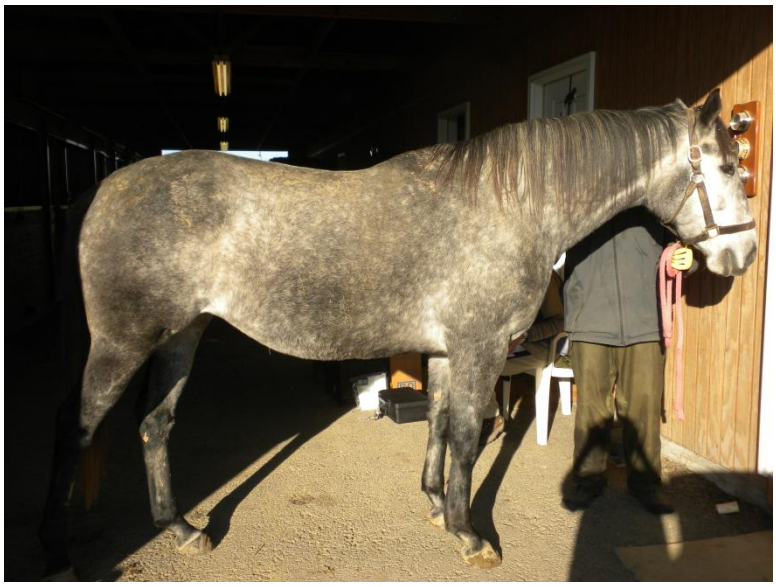
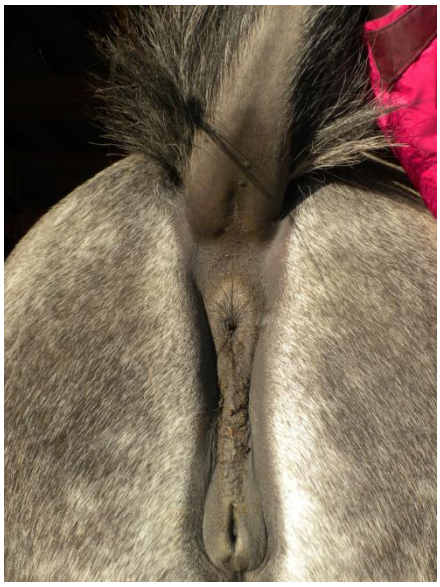
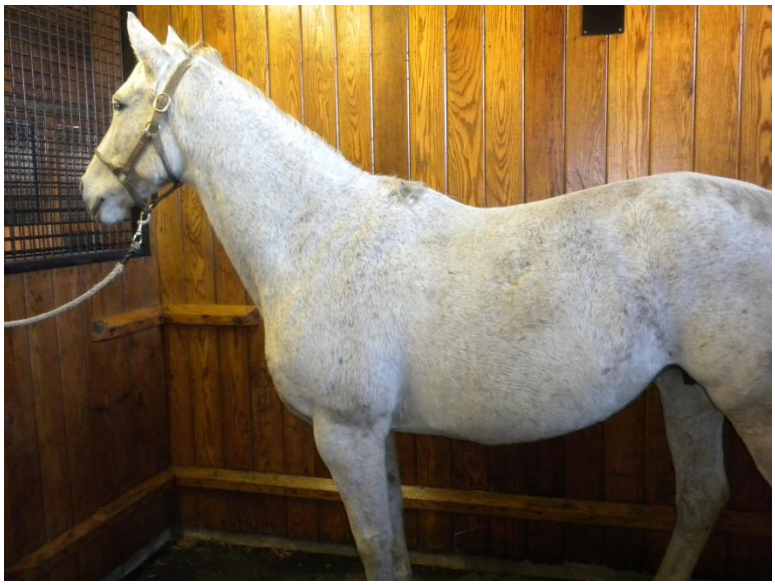
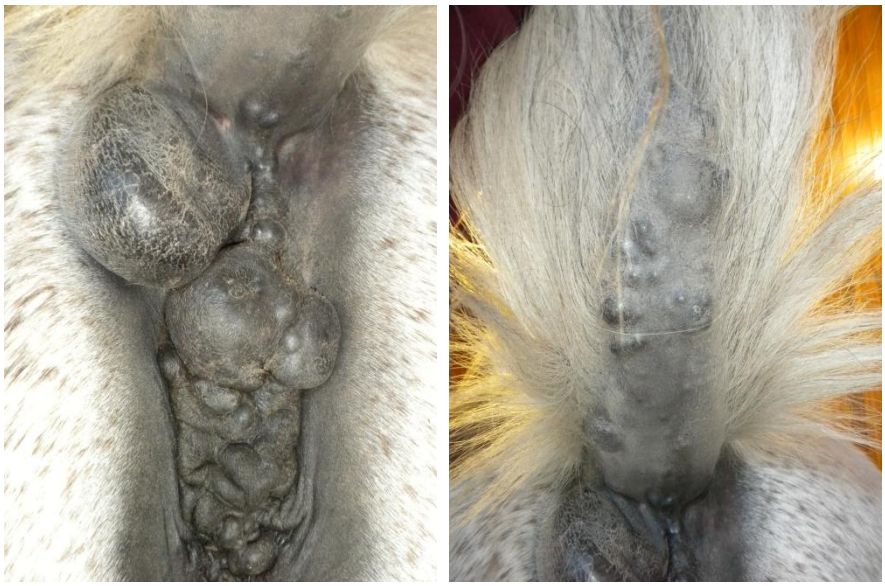



“Arazi Exchange” 2004 Thoroughbred Mare. Stage 2 EMM

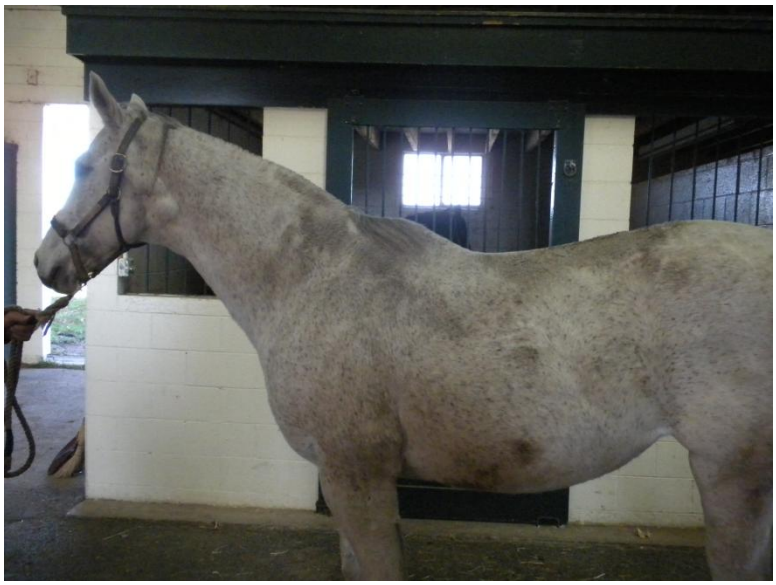
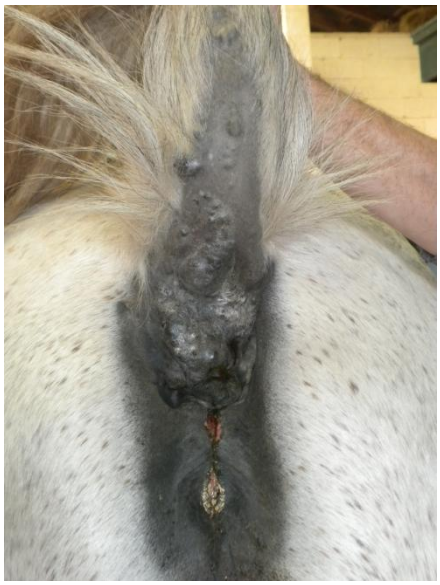
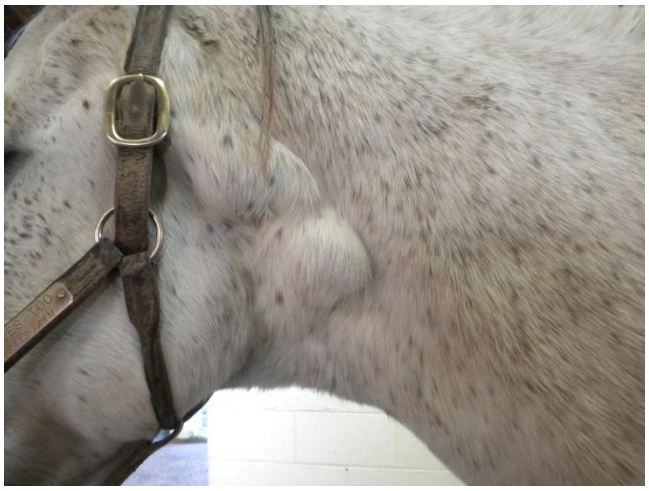
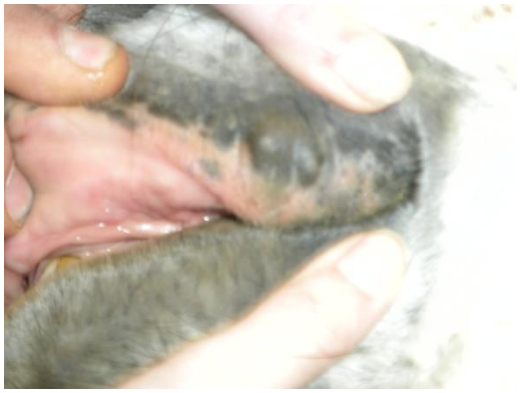



“Miss Mary Louise” 2005 Thoroughbred Mare. Stage 2 EMM

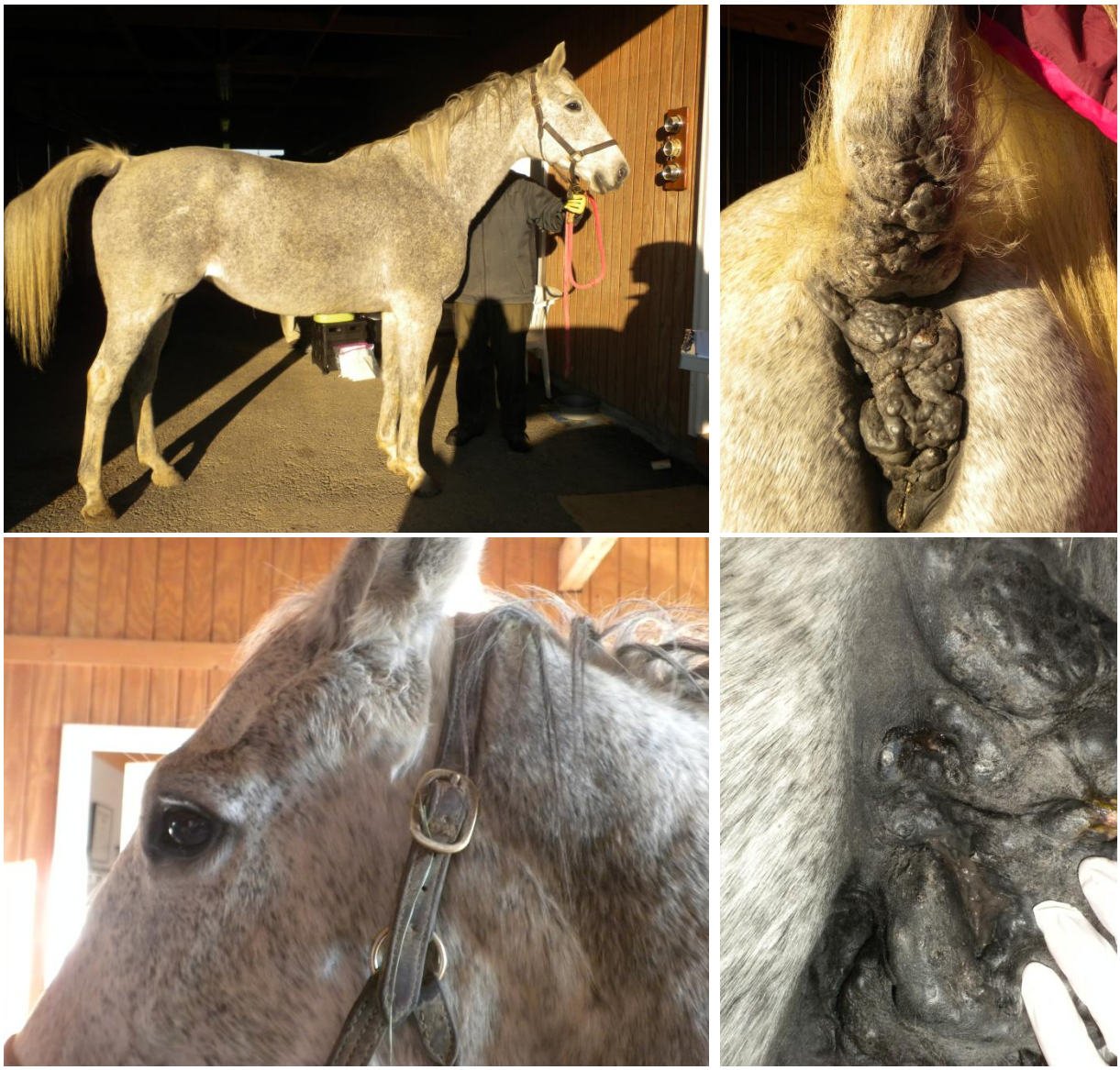




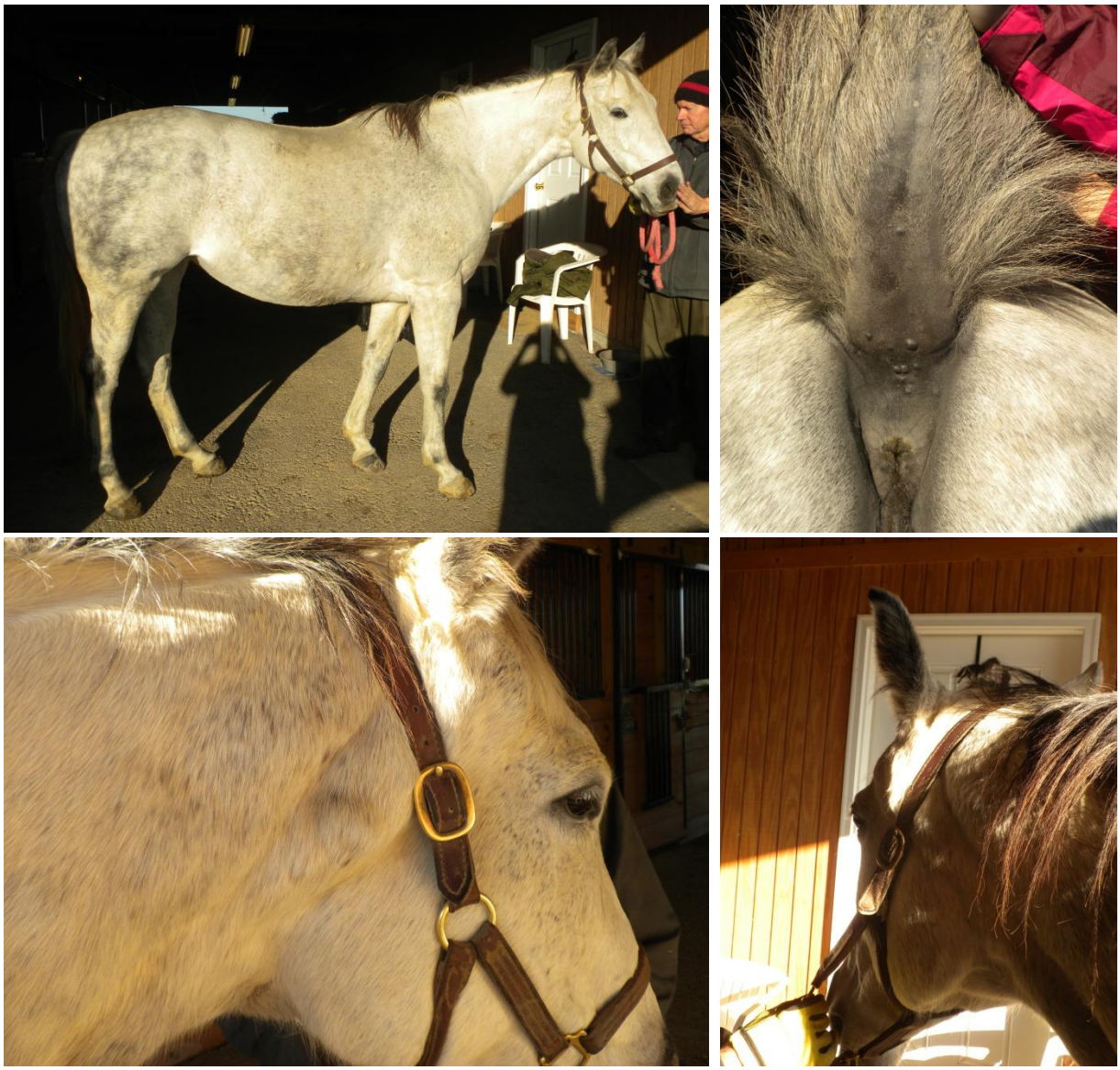
“Alexine” 1996 Thoroughbred Mare. Stage 4 EMM

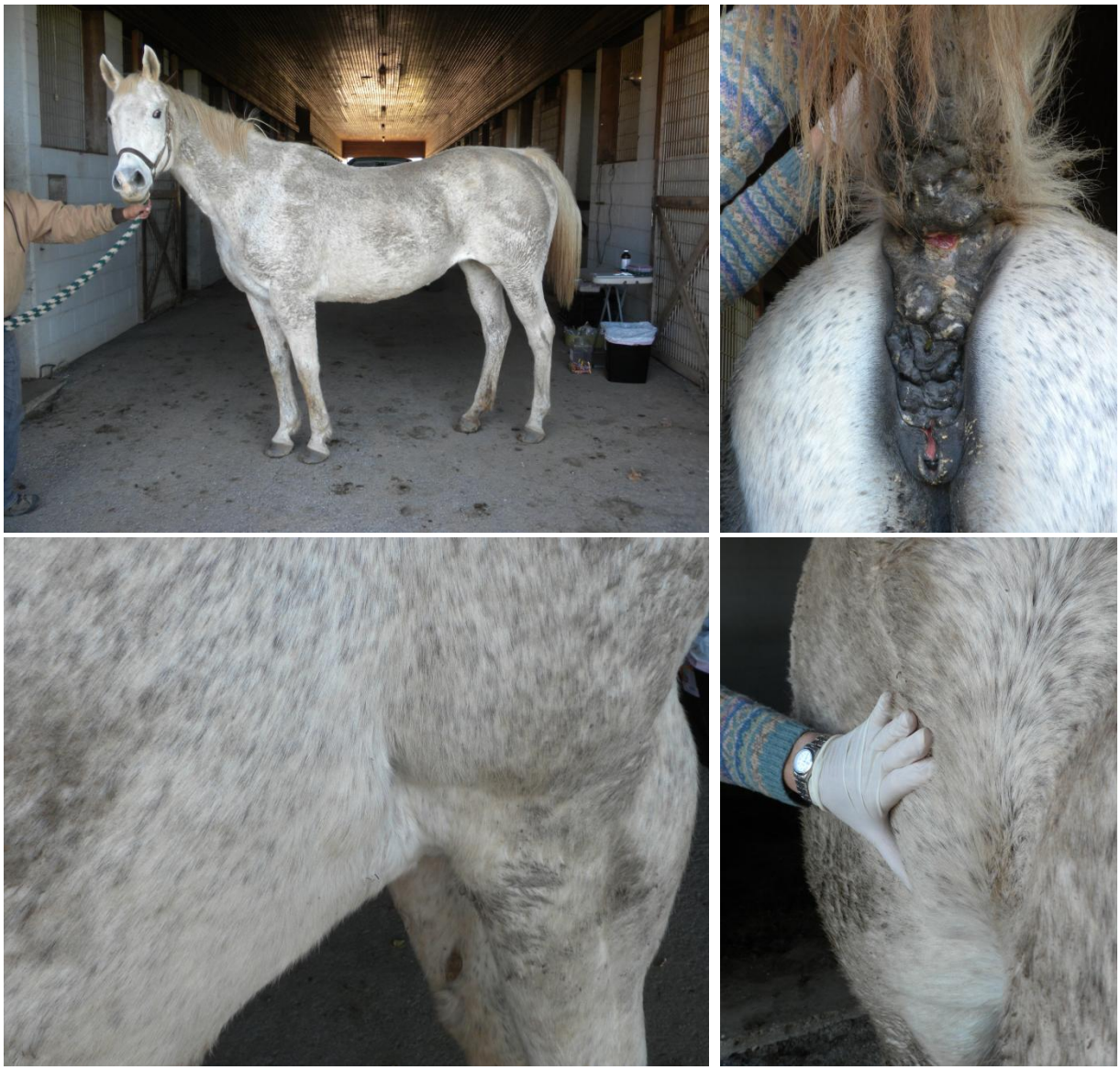
“Mercedes Too” 1996 Thoroughbred Mare. Stage 4 EMM



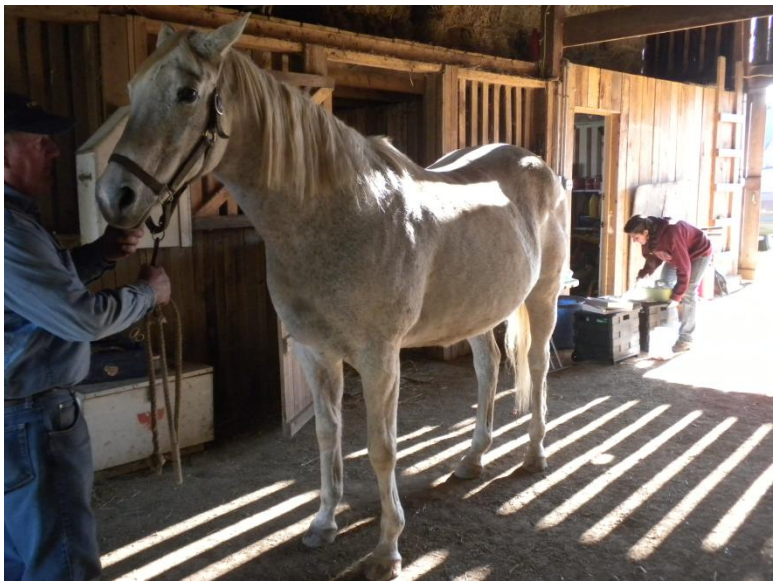
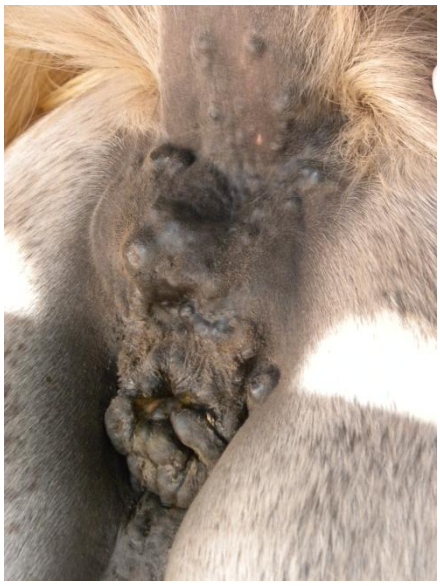
“Over N Back” 1994 Thoroughbred Mare. Stage 4 EMM



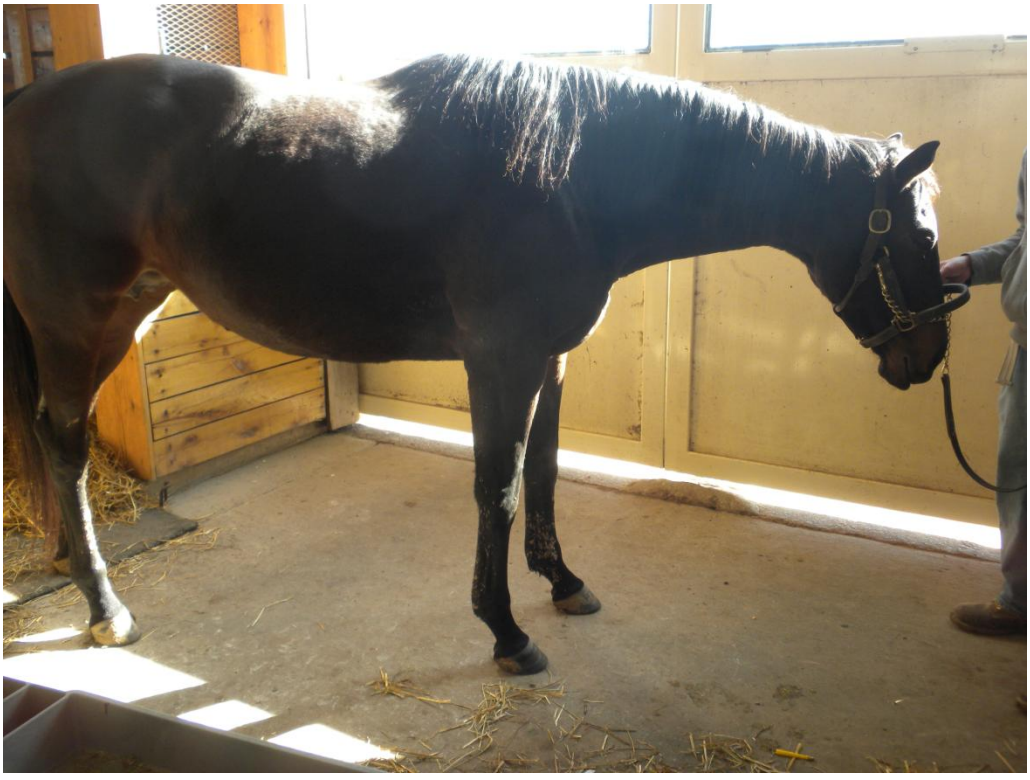
“Robyn Sweet Robyn” 2002 Thoroughbred Mare. Stage 4 EMM



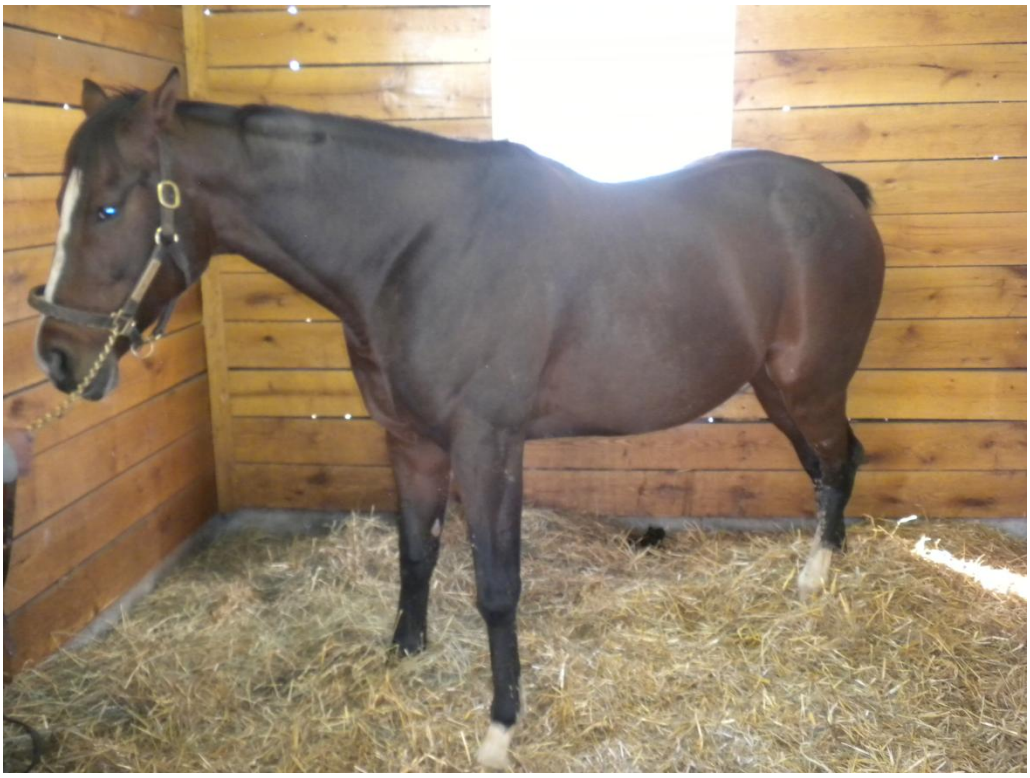
“Lady Aloma” 1990 Thoroughbred Mare. Stage 4 EMM

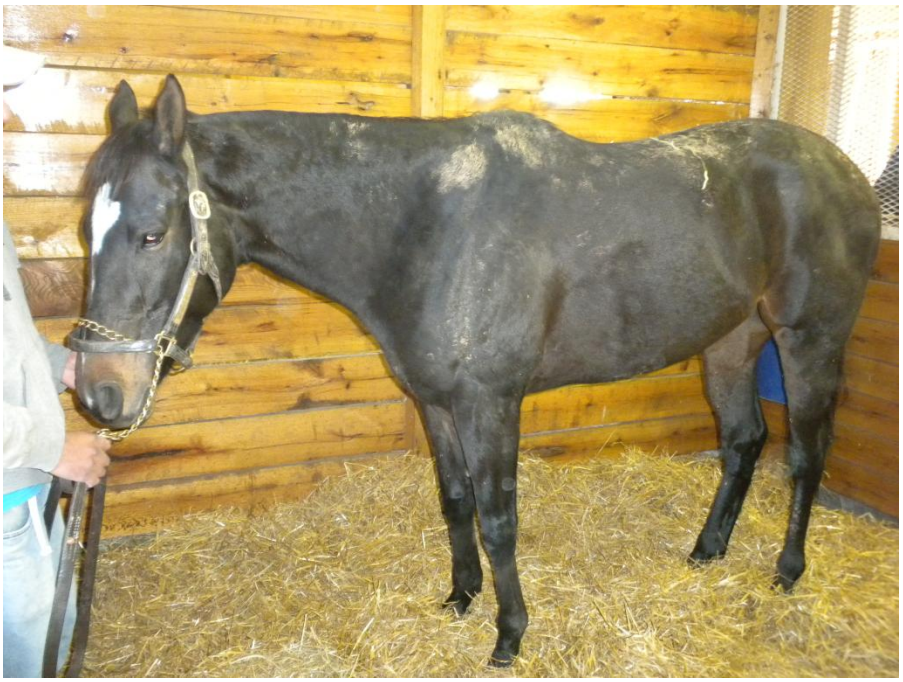
“Prancing Tess” 1991 Thoroughbred Mare. Stage 4 EMM



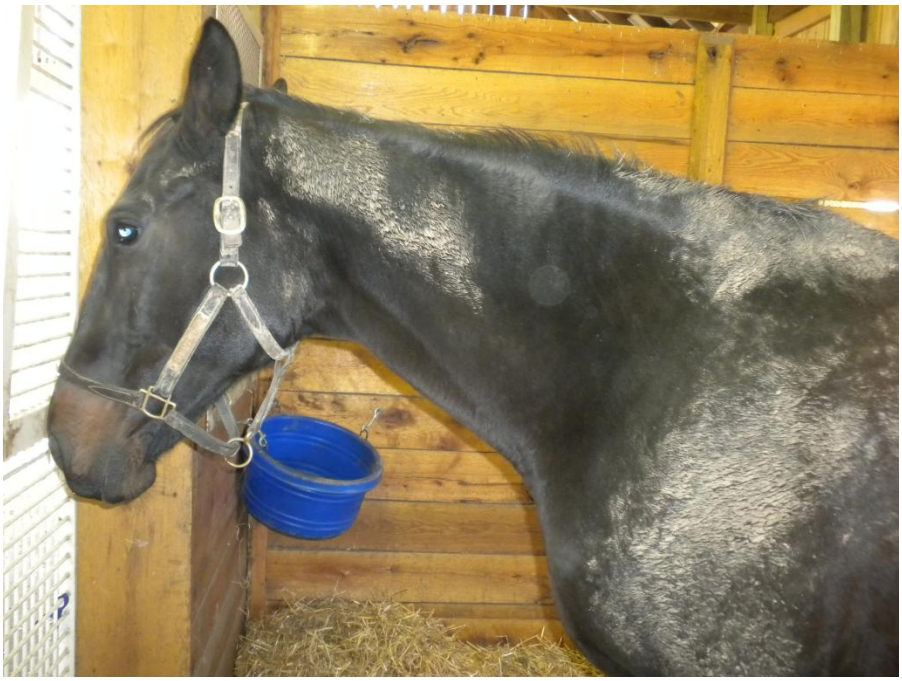
“Distinctively” 2006 Bay/Brown Thoroughbred Mare



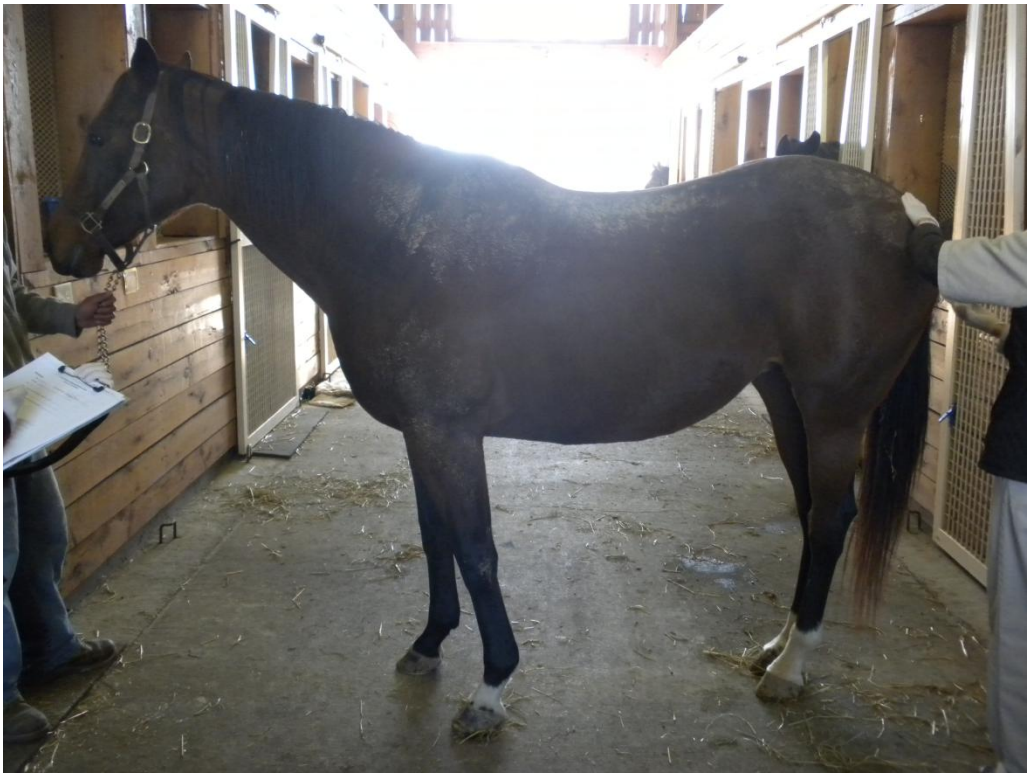
“Tiztimetokinect” 2003 Bay/Brown Thoroughbred Mare



“Miss Red Delicious” 2005 Bay/Brown Thoroughbred Mare



“Formal Affair” 1998 Bay/Brown Thoroughbred Mare



“SongTrack” 2001 Bay/Brown Thoroughbred Mare

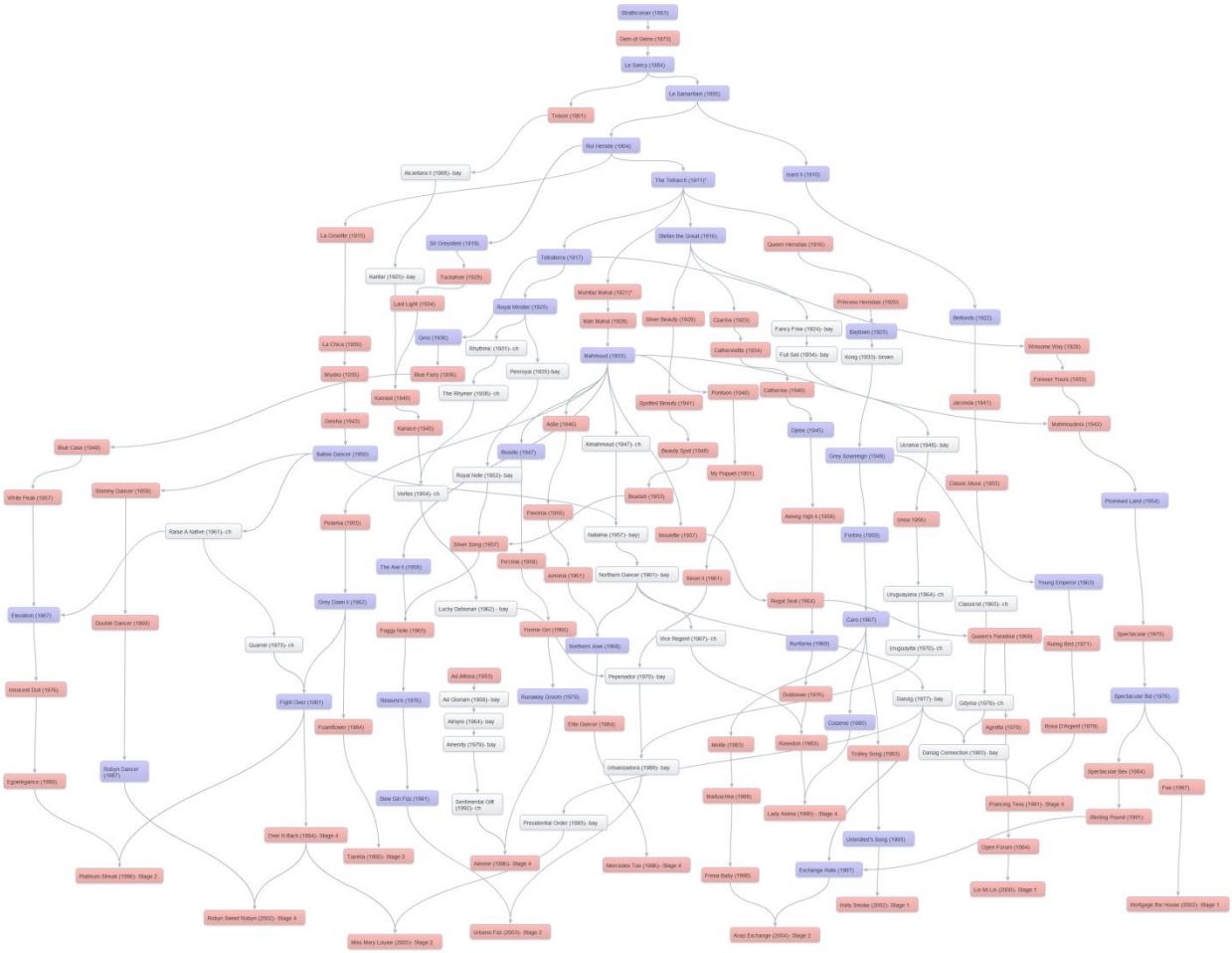
Inheritance of Grey Coat Color in Stage 0 EMM Mares


Pink = Mares

Blue = Stallions

White = Unknown/Non-Grey

Inheritance of Grey Coat Color in Stage 1-4 EMM Mares



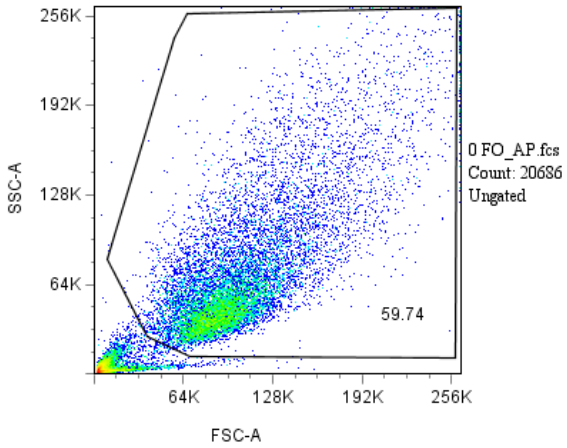
Pink = Mares

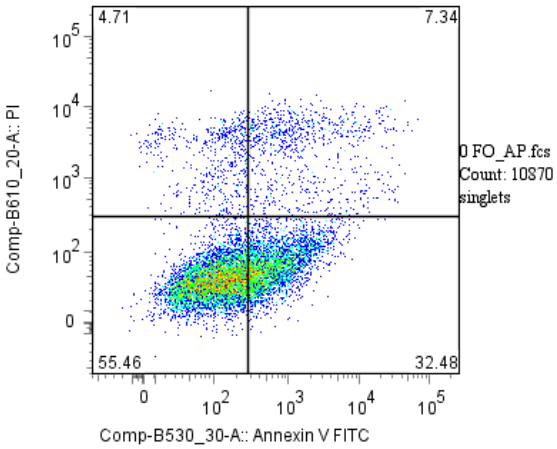
Blue = Stallions

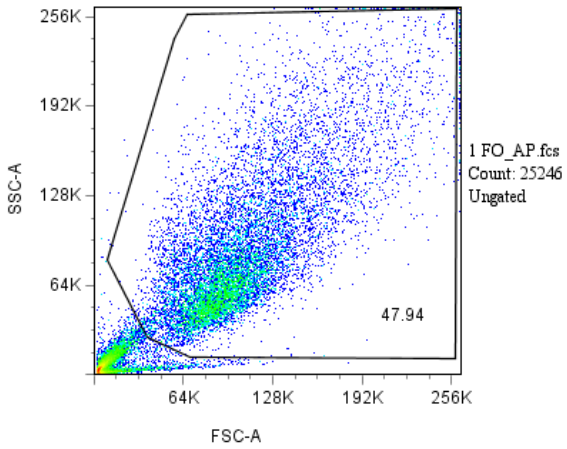
White = Unknown/Non-Grey

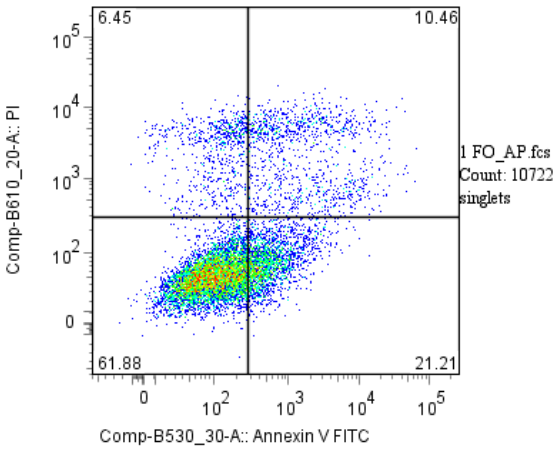
Appendix B: Flow Cytometry Data from Chapter 2 Study

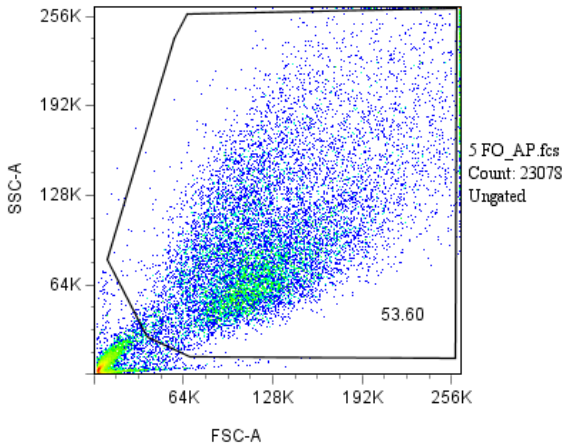
## 1 Hour Post-FO Data:

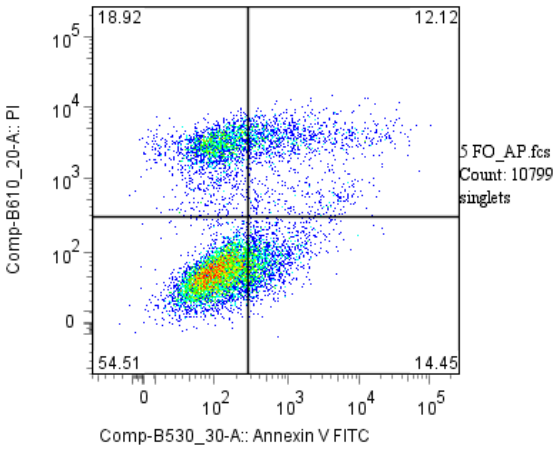


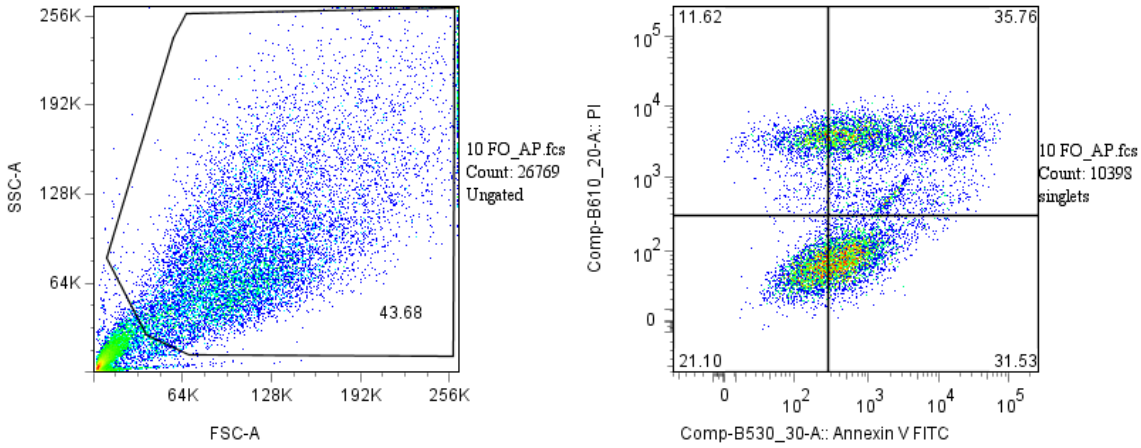

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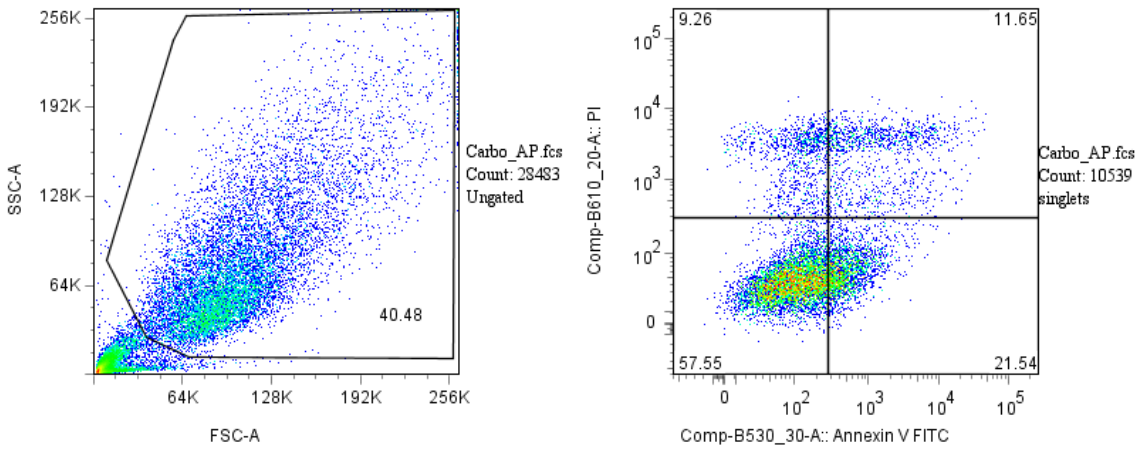
64K
128K
192K
256K
FSC-A
10 FO_AP.fcs
Count: 26769
Ungated
11.62
35.76
21.10
31.53
Comp-B610_20-A:: PI
Comp-B530_30-A:: Annexin V FITC
10 FO_AP.fcs
Count: 10398
singlets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FSC-A
Carbo_AP.fcs
Count: 28483
Ungated
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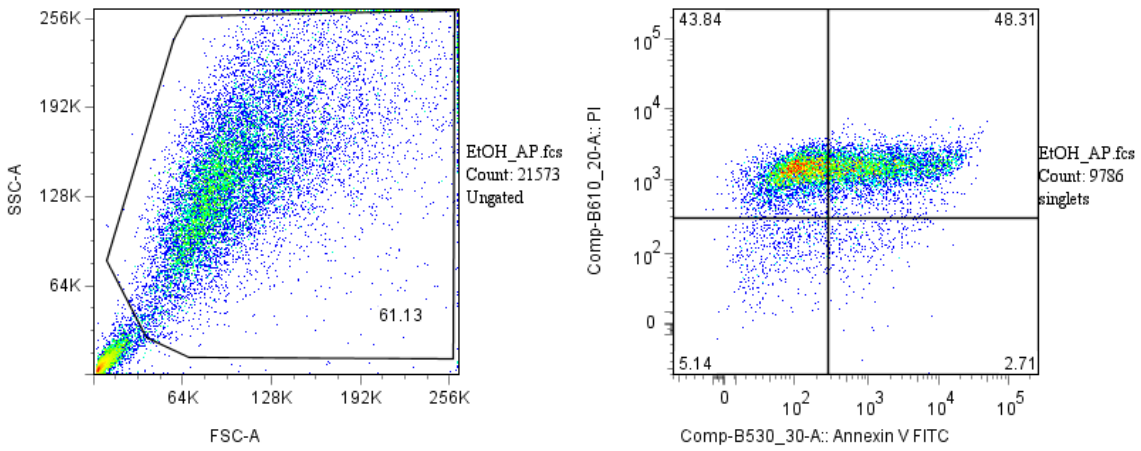
21.54
Comp-B610_20-A:: PI
Comp-B530_30-A:: Annexin V FITC
Carbo_AP.fcs
Count: 10539
singlets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FSC-A
EtOH_AP.fcs
Count: 21573
Ungated
43.84
48.31
5.14
2.71
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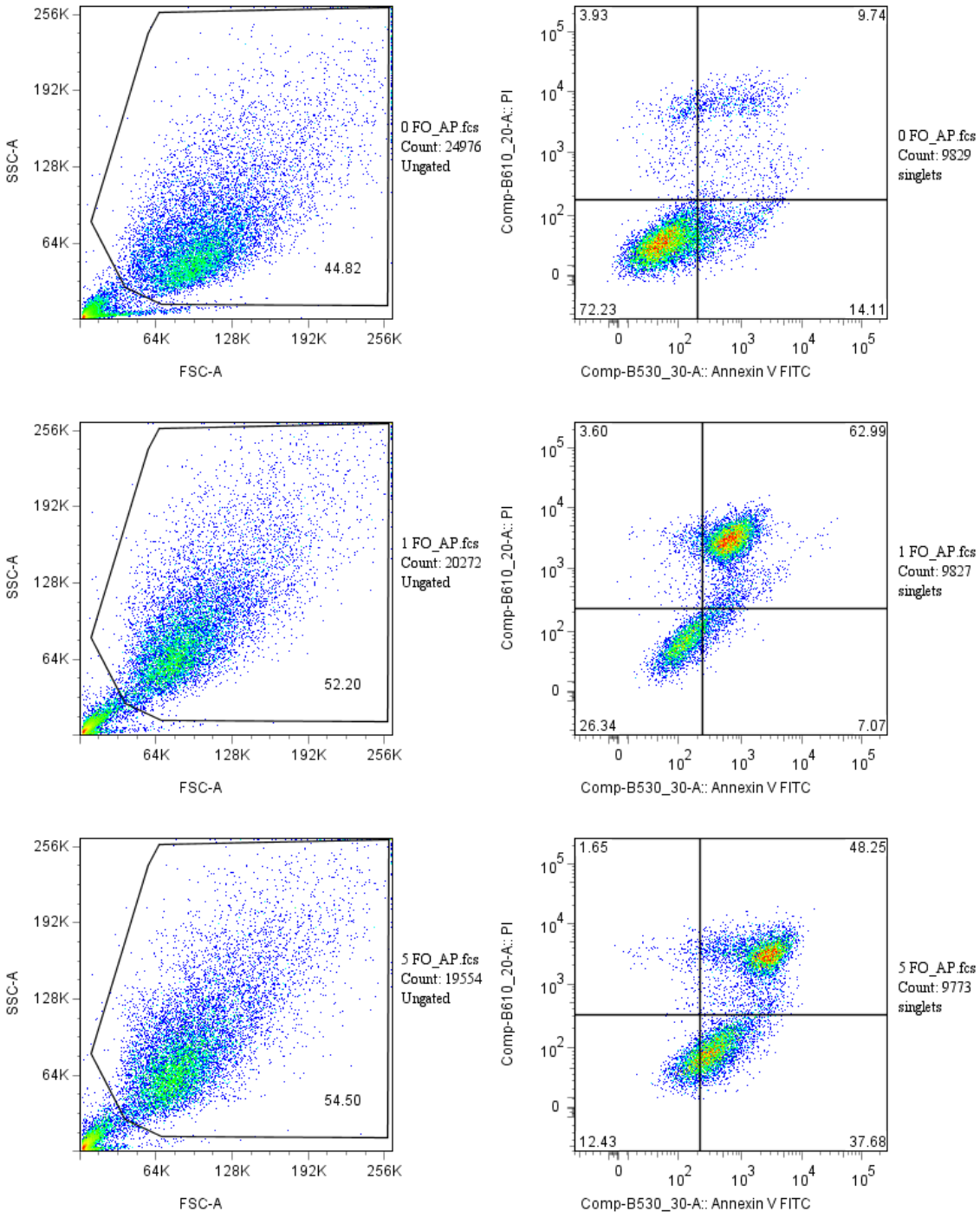
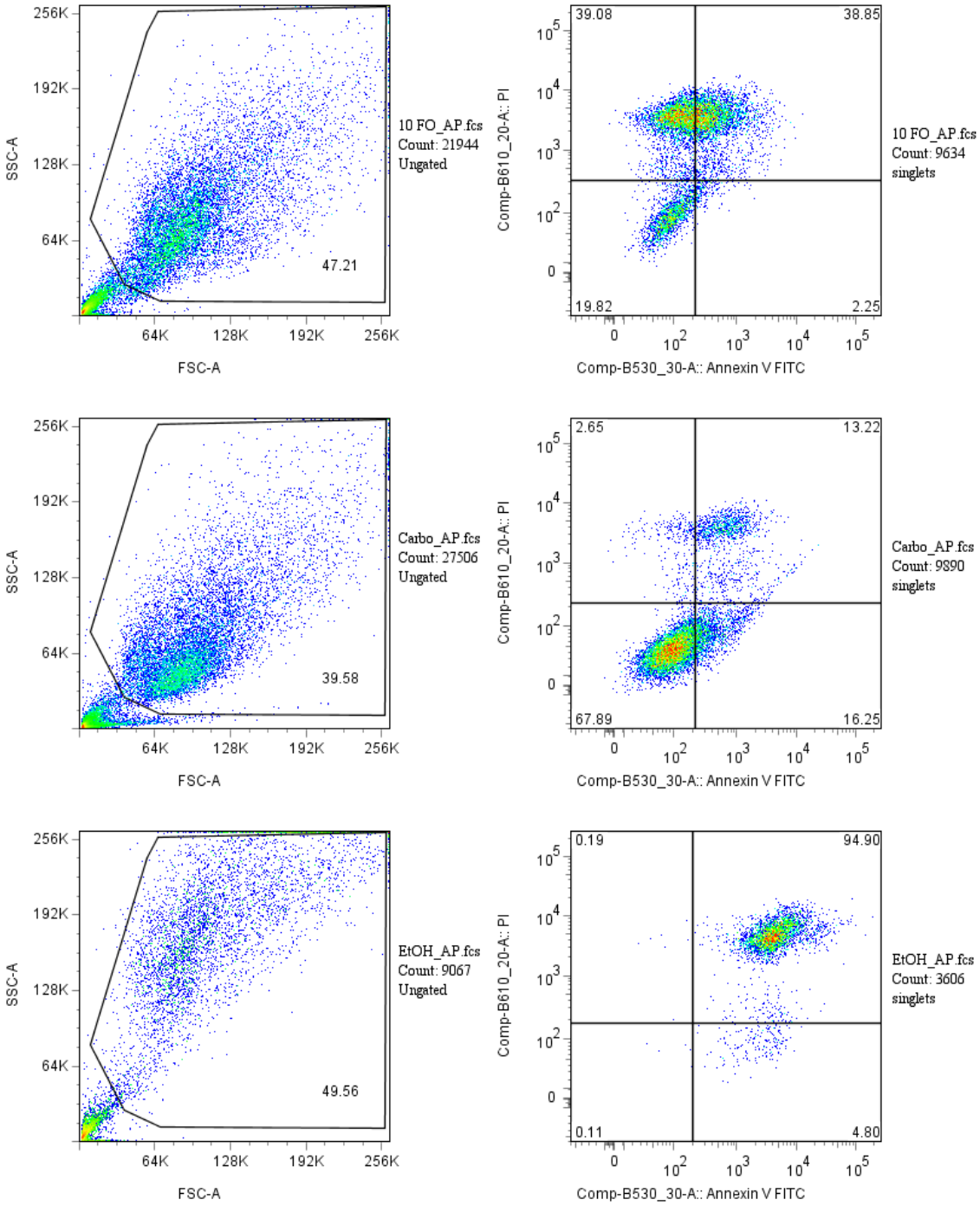
Comp-B530_30-A:: Annexin V FITC
EtOH_AP.fcs
Count: 9786
singlets

4 Hours Post-FO Data:

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FSC-A
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Count: 21944
Ungated
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38.85
19.82
2.25
Comp-B610_20-A:: PI
Comp-B530_30-A:: Annexin V FITC
10 FO_AP.fcs
Count: 9634
singlets
39.58
Carbo_AP.fcs
Count: 27506
Ungated
2.65
13.22
67.89
16.25
Carbo_AP.fcs
Count: 9890
singlets
49.56
EtOH_AP.fcs
Count: 9067
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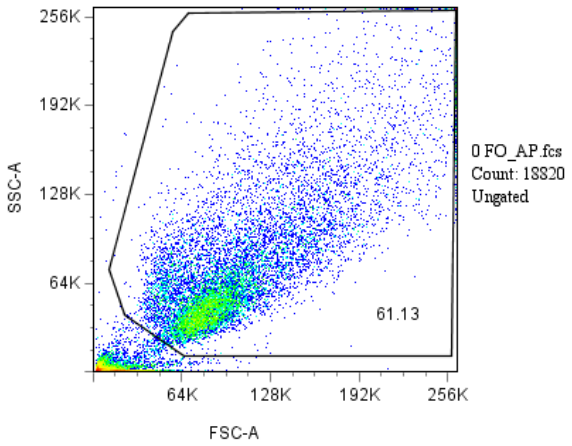
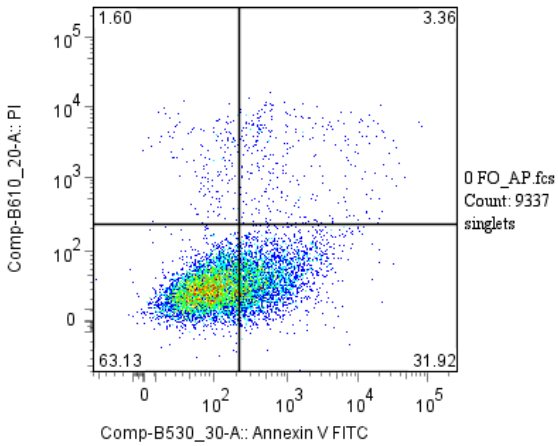
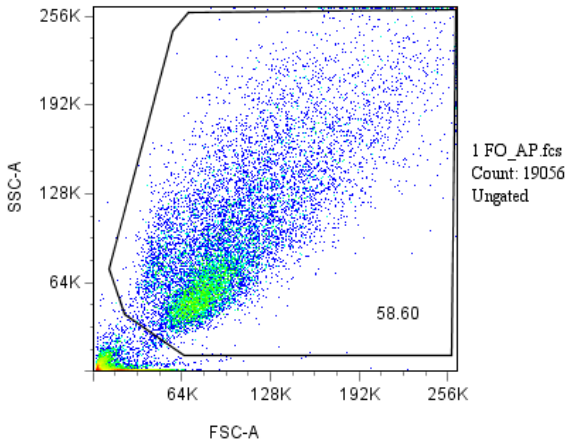
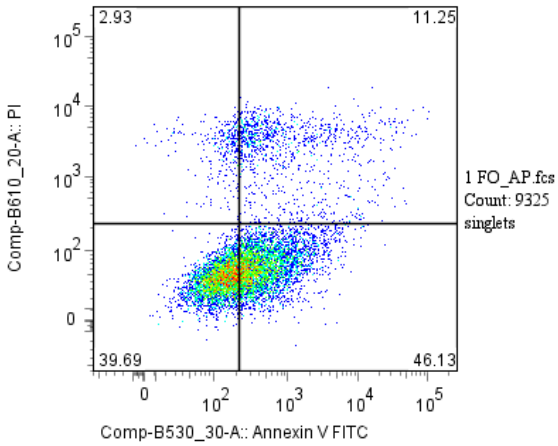
94.90
0.11
4.80
EtOH_AP.fcs
Count: 3606
singlets

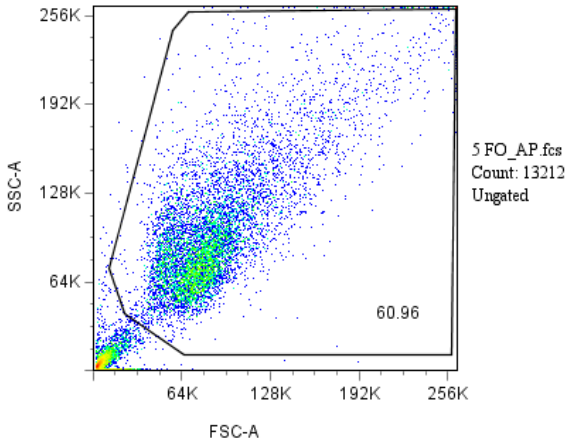
8 Hours Post-FO Data:

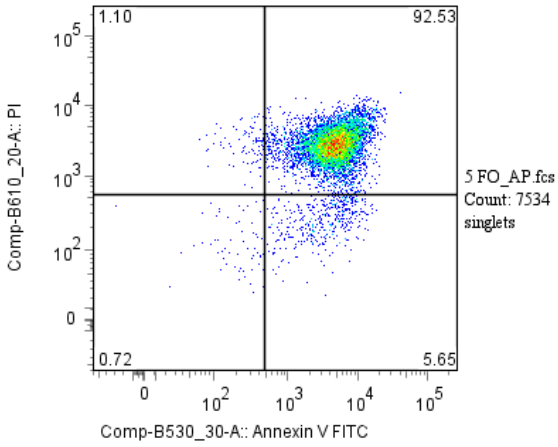


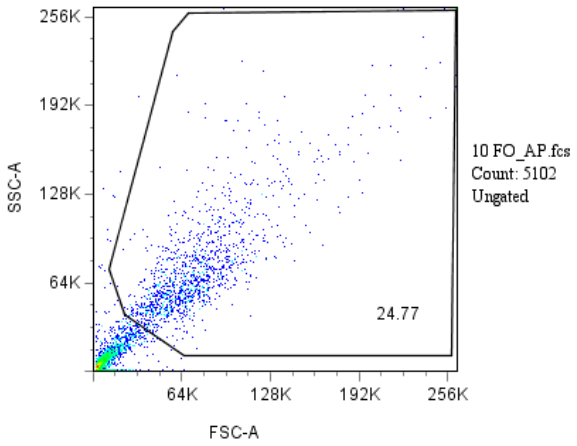

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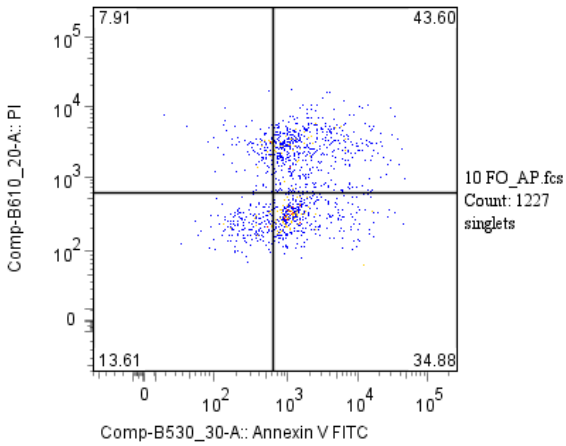
7.91
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13.61
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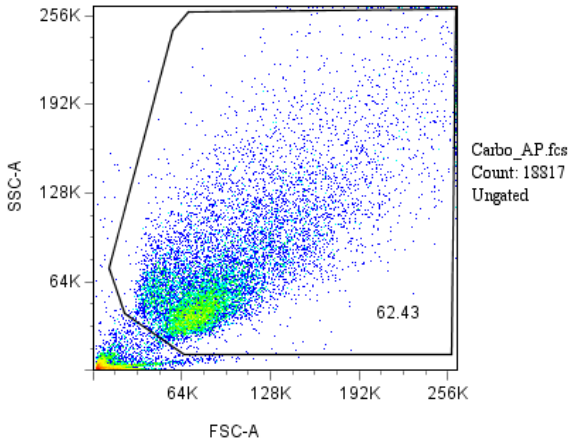
Comp-B530_30-A:: Annexin V FITC
10 FO_AP.fcs
Count: 1227
singlets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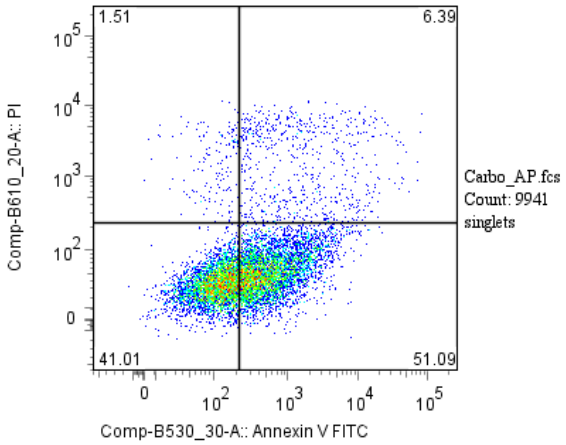
Ungated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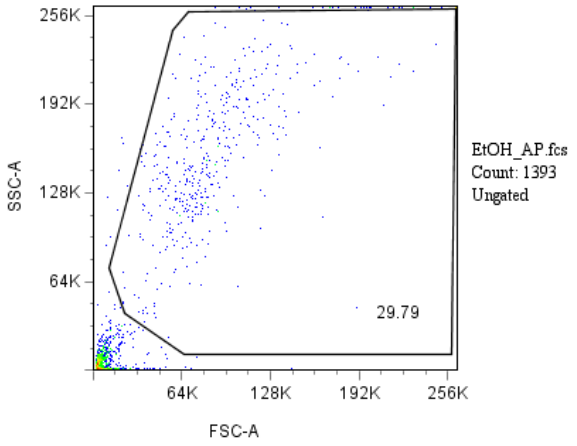
Count: 9941
singlets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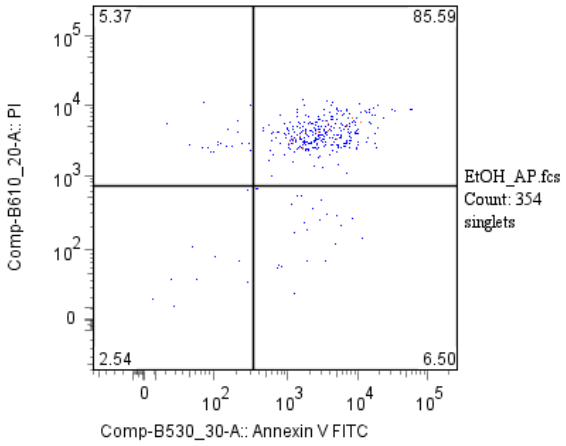
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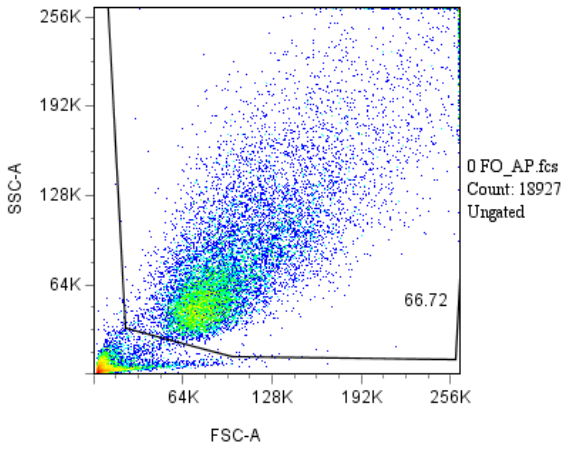
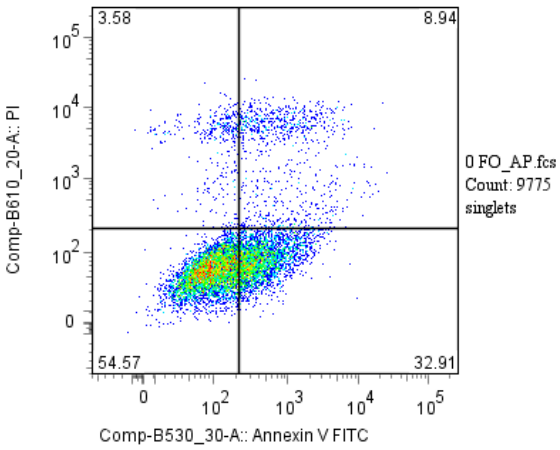
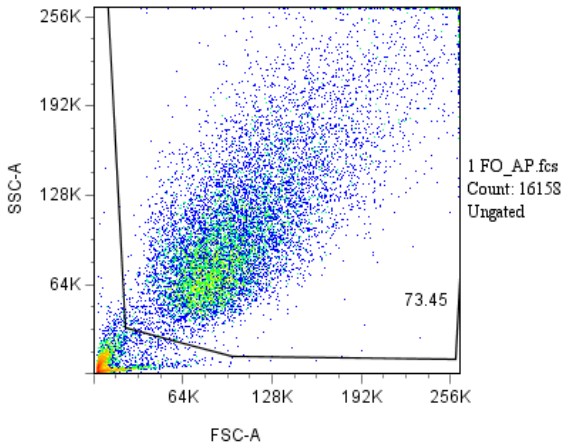
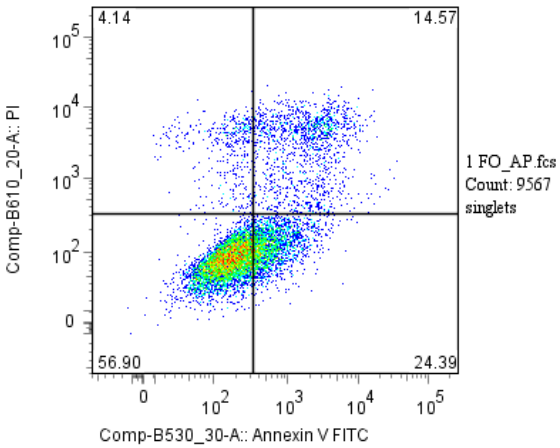
EtOH_AP.fcs
Count: 354
singlets

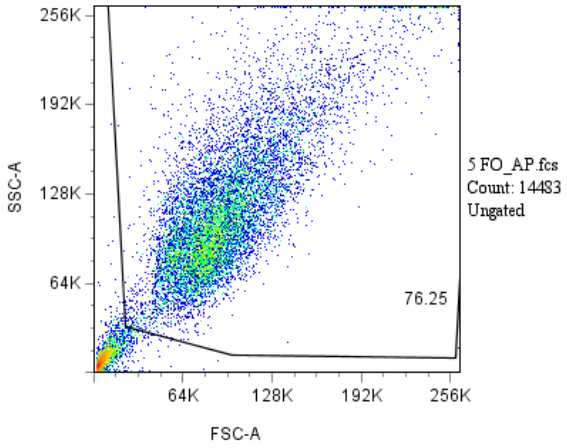
12 Hours Post-FO Data:

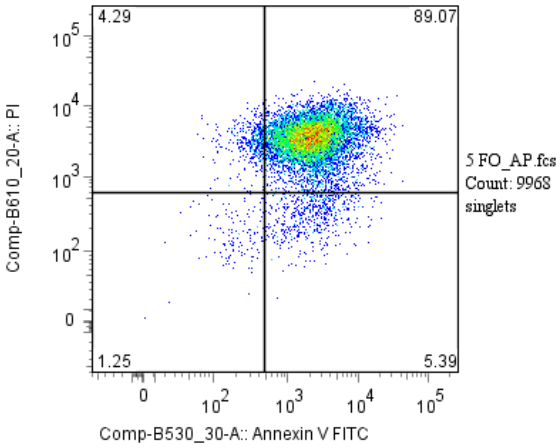


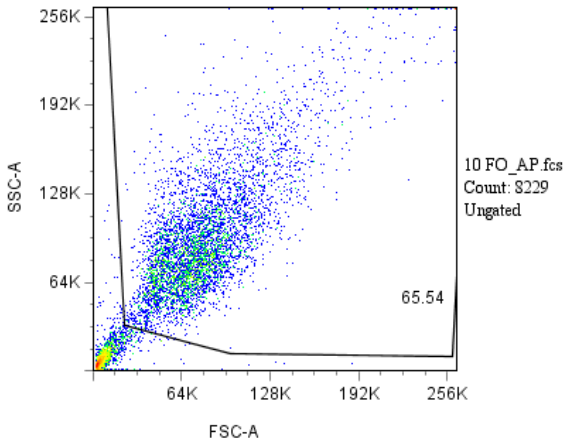

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FSC-A
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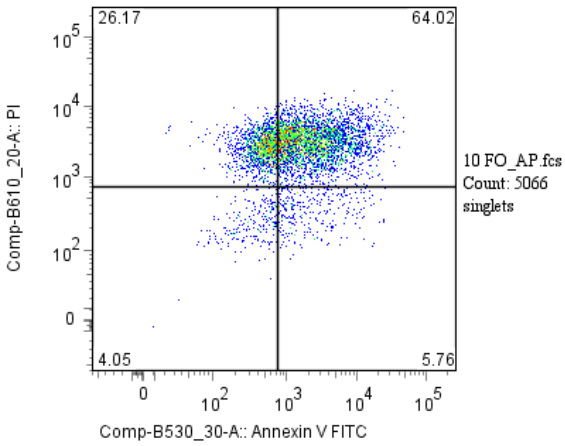
Count: 8229
Ungated


26.17
64.02
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Comp-B610_20-A:: PI
Comp-B530_30-A:: Annexin V FITC
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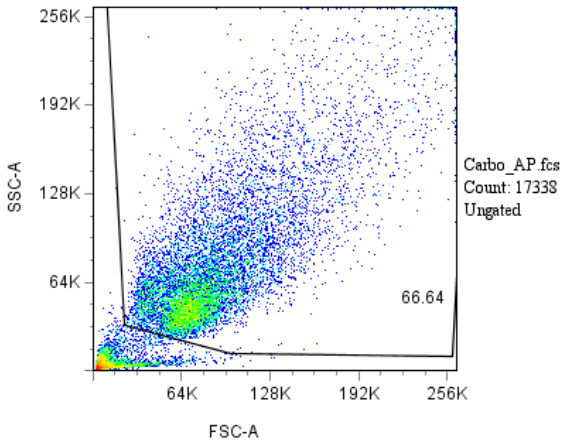
Count: 5066
singlets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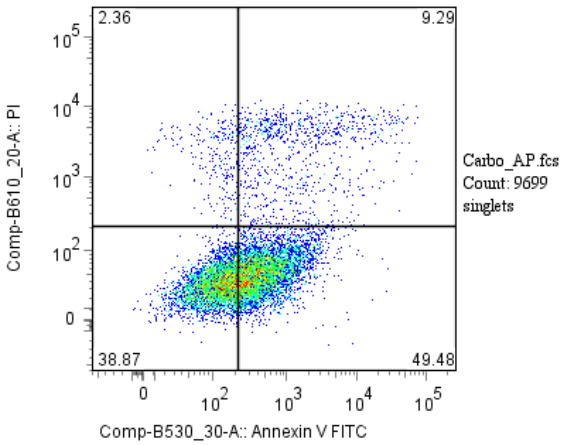
Carbo_AP.fcs
Count: 17338
Ungated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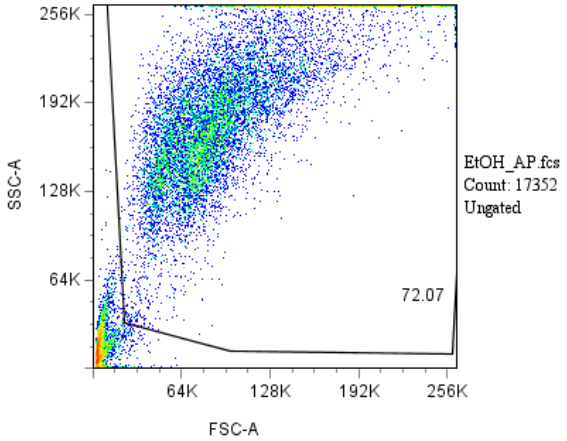
Count: 9699
singlets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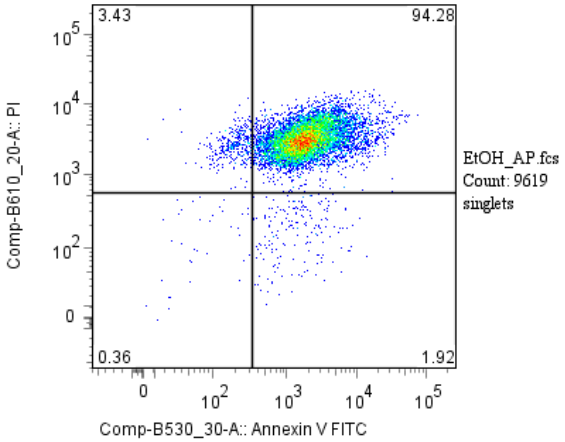
Count: 17352
Ungated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0.36
1.92
Comp-B610_20-A:: PI
Comp-B530_30-A:: Annexin V FITC
EtOH_AP.fcs
Count: 9619
singlets