Role of coat color genotypes in risk and severity of melanoma in gray Quarter Horses

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Dedication

This work is dedicated to my advisor Dr. Molly McCue for her support, guidance and teaching. I'm grateful for the time and effort she dedicates to help me succeed in several aspects of my career. I also want to dedicate this thesis to my committee member Dr. James R. Mickelson for his help and guidance.

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Abstract

Both graying and melanoma formation in horses have recently been linked to a duplication in the *syntaxin-17* (*STX17)* gene. This duplication, as well as a mutation in the agouti signaling protein (*ASIP*) gene that increases melanocortin-1-receptor (MC1R) pathway signaling, affect melanoma risk and severity in gray horses.

We hypothesized that melanoma susceptibility in gray Quarter Horses (QH) is lower than gray horses from other breeds, and that this might be due to decreased MC1R signaling resulting from a high incidence of the *MC1R* chestnut coat color allele in the QH population.

Blood or hair root samples were collected from 335 gray QH with and without dermal melanomas, for DNA extraction and genotyping for *STX17*, *ASIP* and *MC1R* genes. Age, gender and external melanoma presence and grade were recorded. The effect of age and genotype on melanoma presence and severity was evaluated by candidate gene association study.

The melanoma prevalence and grade in this QH cohort were lower than in other breeds. Age was significantly associated with melanoma prevalence and severity. No significant effect of *MC1R* genotype on melanoma prevalence or severity was identified. In contrast to prior reports, an effect of *ASIP* genotype on both melanoma prevalence and grade was not detected. Homozygosity of *STX17* was low and precluded evaluation of the gray allele effect on melanoma presence and severity.

Melanoma prevalence and severity appears to be lower in gray QH than in other breeds. This could be due to infrequent *STX17* homozygosity, a mitigating effect of the

MC1R mutation on *ASIP* potentiation of melanoma, other genes in the *MC1R* signaling pathway, or differences in breed genetic background.

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Chapter 1

Introduction and Literature Review

Melanomas are primary tumors of melanocytes and account for 15% of equine skin tumors[1]. Equine melanomas are classified into 4 groups according to clinical and histopathologic features: melanocytic nevi, anaplastic malignant melanoma, dermal melanoma and dermal melanomatosis [2]. Melanocytic nevi and anaplastic malignant melanoma affect horses of any age, breed or coat color [2]. In contrast, dermal melanoma and dermal melanomatosis occur primarily on the glabrous skin of the ventral tail, perineum, external genitalia, lips and eyelids of gray horses [3, 4]. Dermal melanoma and dermal melanomatosis are distinguished clinically by the number of masses and the presence of metastasis, but are not distinguishable histopathologically and may represent a clinical continuum [2, 3].

Up to 80% of gray horses older than 15 years develop melanomas [1,13] and 14% to 66% of dermal melanomas eventually metastasize [4,3]. All dermal melanomas should be considered potentially malignant since histopathologic changes and location cannot predict the risk of metastasis [4]. Melanoma therapy is more effective when instituted early in the course of disease. Therapeutic options include surgical excision, intralesional cisplatin injections, intralesional cisplatin beads, systemic cimetidine or autologous vaccine. When the tumors become enlarged, locally invasive or metastatic, therapy is usually not feasible [4,11,12,15]. Therefore, early identification of the animals that are at greater risk of developing melanomas is necessary. A better understanding of the risk factors involved in melanoma susceptibility, severity and the risk of metastasis may allow for better prediction of tumor behavior and allow early intervention in horses that are more likely to develop severe, life threatening consequences to dermal melanomas, before therapy options are limited.

STX17 duplication, gray coat color, and increased melanoma risk

Graying and melanoma have recently been linked to a 4.6 kilobase (kb) duplication in intron 6 of the *syntaxin-17* (*STX17)* gene[5]. This duplication is found in a non-coding segment of DNA; however this region harbors a regulatory element containing two microphthalmia-associated transcription factor (MITF) binding sites, and a NGFI-B-response element (NBRE). Both MITF and NRBE play a role in pigmentation; MITF enhances melanocyte production and survival and controls melanin production within melanocytes, and NRBE serves as a binding site for *NR4A3* which is a key component of the melanocortin-1-receptor signaling pathway (**Figure 1**). Studies in transgenic zebrafish have demonstrated that this regulatory element drives reporter gene expression in the *mitfa*-expressing neural crest cells that become melanoblasts, and eventually skin and hair follicle melanocytes [6]. Studies in transgenic zebrafish with an analogous duplication (i.e. similar to horses with the gray mutation) have demonstrated that this regulatory element drives a significantly higher level of reporter gene expression in melanoblasts and skin melanocytes than zebrafish with a single copy "wild-type" construct [6]. MITF is necessary for the expression of *STX17* [6], and both *STX17* and its neighboring gene *NR4A3*, are up-regulated in melanomas from gray horses [5]. Taken together these data suggest that the duplicated region is a melanocyte-specific, *cis*-acting regulatory element for both *STX17* and *NR4A3*.

It has been hypothesized that duplication of the regulatory elements in *STX17* results in up-regulation of melanin production and proliferation of hair follicle and dermal/epidermal melanocytes and is the link between the *STX17* duplication, gray coat

color and melanoma formation [5, 6]. The hair follicle melanocytes responsible for melanin production and hair pigmentation are recruited from melanocyte stem cells at the base of the follicle and undergo apoptosis at the end of each hair cycle. Up-regulation and/or increased recruitment of hair follicle melanocytes in horses with the *STX17* mutation leads to increased hair follicle melanin production early in life, resulting in a dark coat color [5]. With age, the melanocyte stem cell pool becomes depleted resulting in a loss of hair pigmentation and progressive graying. Horses homozygous (*GG*) for the *STX17* mutation show more rapid graying, more frequent vitiligo (loss of skin pigmentation, **Figure 2**), little to no speckling (**Figure 2**) and are more homogenously white in the final stage of the graying process than horses heterozygous (*Gg*) for the duplication [5].

In contrast to follicular melanocytes, the dermal and epidermal melanocytes in glabrous skin (skin without hair) do not undergo apoptosis at the end of the hair cycle [5]. *MITF* regulated proliferation of skin melanocytes, may predispose horses to melanoma formation; and *MITF* has been identified as a melanoma oncogene in humans[24]. Further, up-regulation of *NR4A3* has been associated with carcinogenesis in humans[25]. Horses which are homozygous gray, and thus have two copies of the duplication have a higher melanoma incidence and higher mean melanoma grade than horse that are heterozygous gray (one copy of the duplication)[5], consistent with increased *MITF* and *NR4A3* mediated regulation of melanocyte behavior. *NR4A3* mRNA levels, and the mRNA levels of its downstream target *CCND2* are both elevated in equine dermal melanoma, suggesting a key role for this pathway in melanoma development [5].

Melanocortin-1-receptor signaling, coat color mutations and melanoma

Signaling through the melanocortin-1-receptor (MC1R) is the primary pathway regulating melanin synthesis in melanocytes (**Figure 1**). Signaling through MC1R and its regulation by agouti signaling protein (ASIP) determines the amount of eumelanin (black pigment) and pheomelanin (red/orange pigment) produced by melanocytes, and the pattern of eumelanin distribution responsible for the basic coat colors. In horses, a mutation in the *MC1R* gene results in *decreased* MC1R signaling, and horses that are homozygous for the mutation (*MC1R* $^{e/e}$), have a switch from eumelanin to pheomelanin production resulting in production of red pigment and the chestnut coat color [8]. Horses carrying a wild-type *MC1R* gene (*MC1R* $^{E/e,\ E/E}$) produce eumelanin instead of pheomelanin, resulting in the bay/brown or black phenotype [7]. ASIP *normally antagonizes* MC1R and *decreases* signaling through the receptor. However, a mutation in the *ASIP* gene results in a loss of ASIP antagonistic function and *a relative increase* in MC1R signaling. In the presence of wild-type *MC1R* gene, horses homozygous for the *ASIP* mutation (*MC1R*$^{E-}$ ASIP $^{a/a}$), have an even distribution of eumelanin and black coat color versus the bay/brown phenotype (*MC1R*$^{E-}$ *ASIP* $^{A/a,\ A/A}$) [7]. In horses that are homozygous for the *MC1R* mutation (*MC1R* $^{e/e}$), *ASIP* genotype has no effect on coat color as eumelanin is not produced, thus chestnut coat color is epistatic over bay/black/brown [8].

Gray is epistatic to all other coat colors, so horses with the *STX17* duplication turn gray with age, regardless of their other coat color genotypes [26]. However, gray horses still have MC1R signaling in dermal and epidermal melanocytes even after the hair follicle

melanocytes have been depleted. Thus, genotypes at *MC1R* and *ASIP* still regulate MC1R signaling even though the effect on coat color is no longer phenotypically present. Transcription of both *NR4A3* and *MITF*, the link between *STX17* and melanoma, are mediated by signaling through MC1R; thus alterations in this signaling pathway have the potential to impact melanoma phenotype. The relative increase in MC1R signaling associated with the $ASIP^{a}$ allele has been linked to increased melanoma prevalence and melanoma grade in gray horses [5]. It is important to note however, that the effect of the $ASIP^{a}$ allele on melanoma grade is additive, whereas the effect of the $ASIP^{a}$ on coat color is recessive. Thus while the increase in MC1R signaling in $ASIP^{Aa}$ heterozygotes is not sufficient to alter coat color; it is enough to increase melanoma risk and severity.

A loss of function mutation in the *MC1R* gene results in the "chestnut" coat color in horses[8]. This mutation, by decreasing melanocyte cAMP levels, also has the potential to affect melanoma formation and severity in gray horses through the downstream effect of decreased *MITF* and *NR4A3* transcription (**Figure 1)**[9]. In humans, similar *MC1R* variants which alter MITF expression have been linked to better outcomes in melanoma patients[10].

The impact of altered MC1R signaling associated with the *MC1R* mutation on melanoma development and severity in horses has not been evaluated. In this study, we evaluate melanoma prevalence and severity in a population of greater than 300 gray Quarter Horses (QH), and the effects of genotypes at the *STX17, ASIP,* and *MC1R* loci on melanoma prevalence and severity. Since therapies for equine melanomas are most effective when they are initiated early, prior to extensive invasion of local tissue or

metastasis, early identification of the animals that are at greater risk of developing severe melanomas is necessary.

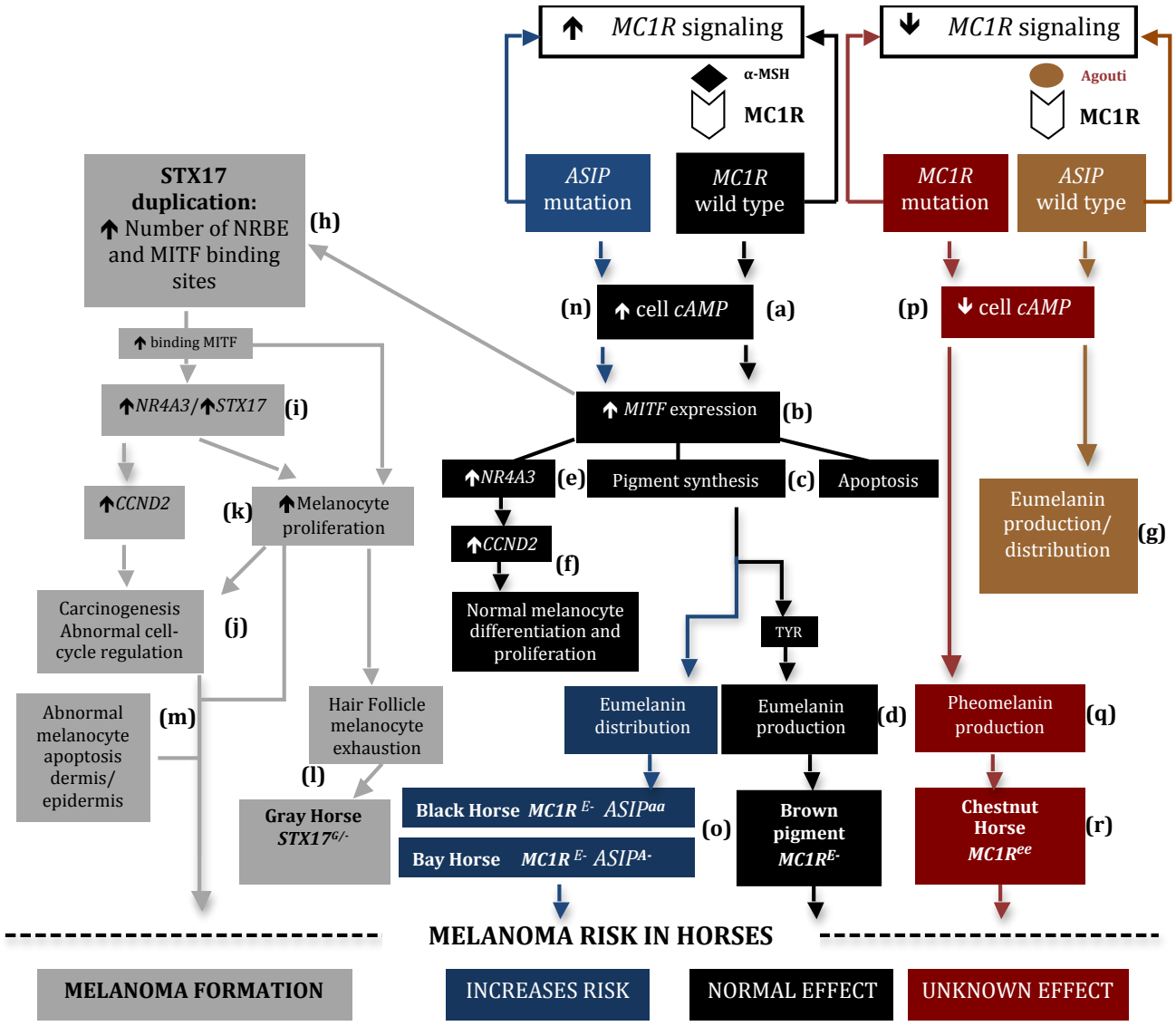
↑ MC1R signaling
↓ MC1R signaling
α-MSH
MC1R
Agouti
MC1R
ASIP mutation
MC1R wild type
MC1R mutation
ASIP wild type
STX17 duplication: ↑ Number of NRBE and MITF binding sites
(h)
(n)
↑ cell cAMP
(a)
(p)
↓ cell cAMP
↑ binding MITF
↑NR4A3/↑STX17
(i)
↑ MITF expression
(b)
↑NR4A3
(e)
Pigment synthesis
(c)
Apoptosis
↑CCND2
(k)
↑Melanocyte proliferation
↑CCND2
(f)
Eumelanin production/ distribution
(g)
Carcinogenesis Abnormal cell-cycle regulation
(j)
Normal melanocyte differentiation and proliferation
TYR
Abnormal melanocyte apoptosis dermis/ epidermis
(m)
Hair Follicle melanocyte exhaustion
Eumelanin distribution
Eumelanin production
(d)
Pheomelanin production
(q)
(l)
Gray Horse STX17G/-
Black Horse MC1R E- ASIPaa
Bay Horse MC1R E- ASIPA-
(o)
Brown pigment MC1RE-
Chestnut Horse MC1Ree
(r)
MELANOMA RISK IN HORSES
MELANOMA FORMATION
INCREASES RISK
NORMAL EFFECT
UNKNOWN EFFECT

Figure 1. Role of STX17 and MC1R signaling in melanoma formation.

Normal MC1R signaling (black boxes/arrows): MC1R signaling is activated by the binding of the agonists α –MSH or ACTH to the MC1R receptor. Activation of MC1R results in increased cellular cyclic-AMP (cAMP) levels (a) which results in increased *MITF* expression (b) leading to increased melanin synthesis (c); stimulation of tyrosinase leading to eumelanin (black/brown) pigment production (d); and increased *NR4A3* transcription (e) which mediates cell cycle and proliferation through the action of CCND2 (f). Normal ASIP (brown boxes/arrows) antagonizes the MC1R receptor resulting in a decrease in MC1R signaling and decreased eumelanin production (g) leading to the wild-type bay/brown coat color (in horses with at wild-type *MC1R*). Alterations in horses with *STX17* duplication (gray boxes/arrows): Horses with the duplication have additional binding sites for both MITF and NR4A3 (h), which are components of the normal MC1R signaling pathway. It is hypothesized that binding to these additional regulatory elements results in increased expression of *NR4A3* and *STX17* (i), which leads to altered regulation of the cell cycle and carcinogenesis (j). Increased binding of *MITF* also results in excessive melanocyte proliferation (k) which leads to exhaustion of hair follicle melanocytes and gray coat color (l) and abnormal proliferation (k) and/or regulation of apoptosis in dermal/epidermal melanocytes and melanoma (m). Altered MC1R signaling in horses with the ASIP deletion (blue boxes and arrows): In horses with the *ASIP* deletion, the antagonistic effect of ASIP is lost, resulting in a relative increase in MC1R signaling and increased cAMP levels compared to wild-type (n). Horses homozygous for this mutation ($ASIP^{a/a}$) produce more eumelanin, resulting in black coat color (o). In horses with the *STX17* duplication and the *ASIP* deletion, the relative increase in MC1R signaling from the *ASIP* deletion, results in higher levels of *MITF* and *NR4A3*, which exacerbates the abnormal cellular regulation with the gray genotype (h, i, j, k, l, m). In horses with the *MC1R* mutation (red boxes/arrows): MC1R signaling is altered and cellular cAMP levels are decreased (p). This alters pigment production causing a shift from eumelanin production to pheomelanin production (q). Pheomelanin is a red/orange pigment leading to the chestnut coat color in $MC1R^{e/e}$ horses (r). *The impact of decreased* MC1R *signaling and thus decreased MITF and NR4A3 expression on melanoma susceptibility and severity in gray horses is unknown*.

Figure 2a

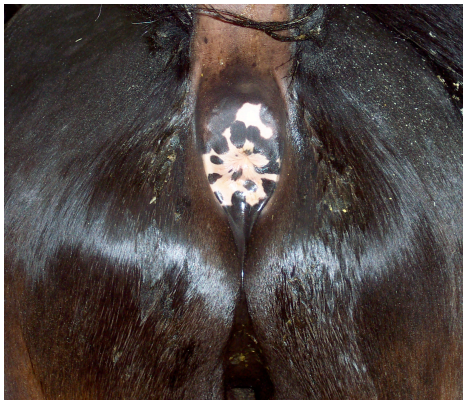

Figure 2b

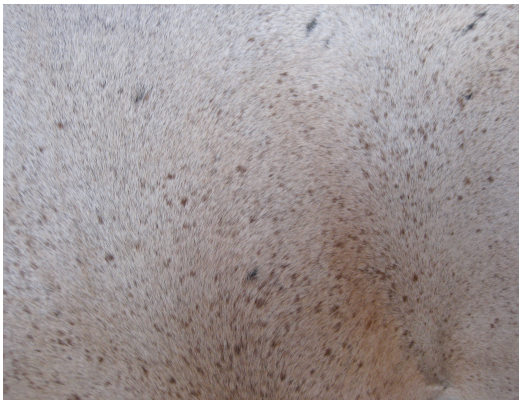

Figure 2. **Vitiligo and speckling**.

2a) Loss of skin pigmentation around the anus of a non-gray horse (vitiligo). 2b) Gray horse with red pigmented spots (speckling).

Rationale

There are no diagnostic tests available to predict tumor behavior and metastasis risk in gray horse melanoma. By better understanding the genetic risk factors involved in melanoma susceptibility, young gray horses can be tested and classified into risk categories (i.e. low and high risk for melanoma development and low or high risk of metastasis) which may allow early intervention in horses that are more likely to develop severe, life threatening consequences to dermal melanomas; when therapy options are limited to nonexistent.

Hypothesis for experiment 1

Up to 80% of gray horses older than 15 years develop melanomas [1,13], however,

preliminary results from a large family of 20 gray QH, varying in age from 4 to 22 years old (mean age of 11.3 years) indicated that melanoma prevalence was low in this breed . We hypothesized that melanoma susceptibility and severity is lower in the gray QH population, compared to gray horses from other breeds.

Aims for experiment 1

Determine the melanoma prevalence and grade in gray QHs and compare this prevalence and severity in gray horses of other breeds where melanoma prevalence and/or grade have been described.

Hypothesis for experiment 2

An increase in MC1R signaling has been linked to increased melanoma prevalence and grade in gray horses. We hypothesized that melanoma susceptibility in gray QHs is lower than gray horses from other breeds due to decreased MC1R signaling resulting from a high incidence of the *MC1R* chestnut coat color allele in the QH population.

Aims for experiment 2

Genotype over 300 gray QHs, with and without melanomas, for the *STX17*, *ASIP* and *MC1R* loci and determine the effect of decreased MC1R signaling on melanoma grade and severity, after accounting for the effect of age and the other two genotypes on

melanoma formation.

Power calculation: Prior to the study, a random population of 48 Quarter Horses were genotyped for the chestnut and black alleles. In this population the frequency of the chestnut $MC1R^{c}$ allele is 0.78 and the wild-type $MC1R^{C}$ allele is 0.22, thus the estimated genotypic frequencies for the chestnut genotypes from Hardy-Wienberg equilibrium are 0.05, 0.34 and 0.61 for $MC1R^{C/C}$, $MC1R^{C/c}$ and $MC1R^{c/c}$, respectively [$(MC1R^{C/C})^2$ + $2(MC1R^{C/c})$ + $(MC1R^{c/c})^2$ =1]. The frequencies of the black allele and wild type alleles in this population are $ASIP^{a}$ 0.32 and $ASIP^{A}$ 0.68, thus the estimated genotypic frequencies for the black locus are 0.46, 0.44 and 0.1 for $ASIP^{A/A}$, $ASIP^{A/a}$ and $ASIP^{a/a}$, respectively. These estimated population frequencies were used in power calculations to determine the ability to measure a possible effect of genotypes at these loci on melanoma incidence and severity. Assumptions used for power calculation are listed in **Table 1**.

Power calculations were performed for the ability to detect an effect of the chestnut locus on melanoma incidence and grade with α=0.05 and β=0.8, given the likely frequencies of the ASIP and MC1R genotypes in the QH population. The assumptions presented in **Table 1** about genotypic effect on melanoma grade for power calculations were made. Standard deviation estimation of 1.0 was used for the power calculations based on the average standard deviations from the regression analysis in 694 Lipizzaners by Pielberg et al [5] and the regression analysis of melanoma susceptibility in 296 Lipizzaners by Seletenhammer et al [17]. A reduction in R^2 of 0.3 was used in the

calculations to account for the 28% of total variability in melanoma grade due to age and stud farm reported by Seletenhammer et al [17]. Based on the above assumptions, power calculations demonstrate that a study cohort of 300 gray Quarter Horses is required to detect an effect of the chestnut allele on melanoma grade and susceptibility. Analyses done by Seletenhammer et al[17], and Pielberg[5] *et al* included horses 4 years or 6 years of age and older, respectively, and while our power calculations take into account the variability due to age and stud farm, we limited our analysis to horses 5 years of age and older to assure optimal power. Our goal was to identify a minimum of 300 additional gray QH (all ages) for inclusion in the study population to allow for accurate calculation of the impact of age on melanoma incidence, and assure that at least 300 horses aged 5 or older are available for analysis of the impact of *MC1R* genotype of melanoma grade.

Table 1. Genotypic effect on melanoma grade

risk factor	**mean (± SE) melanoma grade**	**frequency**
STX17$^{G/G}$	1.43 ± 0.04[a]	0.5[b]
STX17$^{G/g}$	0.67 ± 0.05 [a]	0.5[b]
ASIP$^{A/A}$	0.88 ± 0.06 [a]	0.46[d]
ASIP$^{A/a}$	1.06 ± 0.04 [a]	0.44[d]
ASIP$^{a/a}$	1.22 ± 0.07 [a]	0.1[d]
MC1R$^{C/C}$	1.18[c]	0.05[d]
MC1R$^{C/c}$	0.89[c]	0.34[d]
MC1R$^{c/c}$	0.6[c]	0.61[d]

[a] least squares mean of melanoma grade from polynomial regression analysis in a population of 694 gray Lipizzaner horses in Pielberg et al. [5]

[b] population frequencies of each gray genotype based on the data from Pielberg et al. [1]

[c] predicted effect of the MC1R locus genotypes estimated from the magnitude of effect of the ASIP genotypes in Pielberg et al. [5]

[d] estimated population frequencies of each genotype in Quarter Horses based on a population of 48 random Quarter Horses (preliminary data)

Chapter 2

Experiment 1

Melanoma Prevalence and Grade in Gray Quarter Horses

Introduction

Gray horses are born any color and turn gray with age. A strong correlation between the gray coat color and melanoma development has long been recognized with 80% of gray horses older than 15 years of age developing melanomas [1,13]. The genetic link between the gray coat color and melanoma development has recently been identified[5]. All dermal melanomas have the potential to metastasize and histopathology of the tumor cannot predict metastasis risk[4].

While the majority of gray horses older than 15 years of age will develop melanomas[1, 13], a large family of 20 gray horses ranging in age from 3 to 22 years old (mean age of 11.3 years) with no external melanoma was identified. Experiment 1 was designed to investigate if melanoma prevalence and grade is lower in gray QHs in general or if this lower susceptibility is present just in the family of gray horses identified.

The overall purpose of this study was to determine melanoma prevalence and grade in gray QH and compare it with gray horses from other breeds where those parameters have been described. Melanoma prevalence and grade have been studied in the Lipizzaner, Pura Raza Espanola and Camargue breeds.

Materials and Methods

Study population and sample collection. Age, gender and melanoma phenotype data were collected from 335 gray QH. Phenotype data included the presence, location, size and appearance of external melanomas recorded using a standardized form, and digital photography of melanomas (if present). Horses were assigned a melanoma grade 0-4 (in ½ grade increments) by a single investigator (RT) according to the scale of Peilberg et al[5] (**Table 2**).

Table 2. Melanoma grading system (Rosengren et al, 2008)[1]

Grade	Description
0	No melanoma
1	Early stages of plaque-type or one solitary nodule of 0.5 cm or less
2	Several nodules of 0.5 cm or less or a single solitary nodule of 2 cm or more
3	One or several nodular melanomas 5 cm or more
4	Extensive confluent melanoma, necrosis or ulceration, or metastasis

Statistical analysis. Statistical analyses were performed using R statistical software. Descriptive statistics were calculated for age, gender, melanoma prevalence and grade. Age was evaluated as a continuous and categorical variable (1-4 years, 5-9 years, 10-14 years, 15-19 years and > 20 years or a dichotomous variable (≥15 years versus <15

years). Gender was coded as gelding, stallion or female and as male (stallion and gelding) and female. Univariate regression was used to test for the individual effect of age on melanoma case/control status (logistic regression) and melanoma grade (linear regression). Age was fit as a cubic term in the linear regression to better model the relationship between age and melanoma grade in the study cohort. Chi square test and regression models including age were performed to detect significant differences in melanoma prevalence between genders. Multiple regression analysis was performed using age (cubic) and gender to further evaluate for an effect of gender on outcome.

For all analyses, differences were accepted as significant when $p \leq 0.05$.

This protocol was approved by the University of Minnesota's Institutional Animal Care and Use Committee. Owner consent was obtained prior to the enrollment of horses.

Results

Melanoma prevalence and grade

Three hundred and thirty-five gray QH, 227 females, 87 geldings and 20 stallions, and 1 of unknown gender were phenotyped for dermal melanoma. Age ranged from 1 to 33 years (mean 9.21). Age was not recorded in four horses. Fifty-six horses (16.72%) had visible dermal melanomas and were classified as cases. Of these 56 cases, 28 (50%) had

a solitary mass, and 28 (50%) had two or more masses (mean number of masses per horse 3.2; range 2-8). Melanomas were most commonly under the tail (74.5%), followed by around the anus (18%), at the commissure of the lip (10.9%), surrounding the parotid salivary gland (9%), on the prepuce (9%) or external genitalia (7.2%) and on the neck (7.2%). Less common locations included: base of the ear, submandibular region, udder, medial aspect of the hind legs, forehead, shoulder, flank, and gluteal, abdominal or thoracic regions. In horses that had masses in 2 or more locations; 89.28% had masses under the tail and in a second location; only 10.71% of horses with masses in multiple locations did not have melanomas under the tail. Melanoma grade distribution in the case cohort is presented in **Table 3**. Mean melanoma grade across the case cohort was 2.15, with only 7% of the horses assigned a grade 4 with ulcerated masses (**Figure 3**). In no cases were the masses interfering with normal vital functions (defecation/urination). None of these horses had a history of weight loss or other complaints associated with melanoma. No significant difference in melanoma prevalence or mean melanoma grade was observed across genders (**Table 4**).

Melanoma cases ranged in age from 2 to 30 years, with clear increase in melanoma prevalence with age (**Table 5**); prevalence was 51.92% in horses >15 years compared to 10% in horses <15 years. Age was significantly associated with the presence or absence of melanoma (logistic regression $p=5.28x10^{-11}$). Similarly, melanoma grade increased with age, mean melanoma grade was lowest in cases between 1 and 4 years (1.63) and highest in cases ≥ 20 years (2.68) (**Table 5**). Age was also significantly correlated with melanoma grade (linear [cubic] regression: Spearman's correlation coefficient= 0.387,

$p=2.2x10^{-13}$).

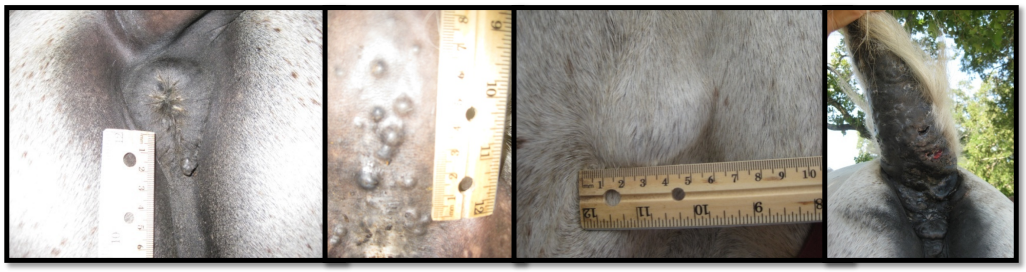

Figure 3. Example of melanoma grades 1-4.

a. Grade 1 melanoma around the anus: one solitary nodule of 0.5 cm or less. b. Grade 2 melanoma under the tail: several nodules of 0.5 cm or less. c. Grade 3 melanoma in the parotid gland region: one nodular melanoma 5 cm or more. d. Grade 4 melanoma under the tail: extensive confluent melanoma with the presence of necrosis, ulceration or metastasis.

Table 3. Distribution of melanoma grades in the case cohort

Melanoma grade	Count (%)
1	13 (21.82%)
1.5	9 (16.36%)
2	8 (14.55%)
2.5	12 (21.82%)
3	9 (16.36%)
3.5	1 (1.82%)
4	4 (7.27%)
Total	**56**

Table 4. Breakdown of melanoma prevalence and mean melanoma grade by gender

Gender	Total number of horses	Number of melanoma cases	Melanoma prevalence (%)	Mean melanoma grade (mean ± sd) entire cohort*	Mean melanoma grade (mean ± sd) cases only**
Female	227	35	15.42	0.36 ± 0.92	2.36 ± 0.85
Gelding	87	17	19.54	0.34 ± 0.80	1.76 ± 0.87
Stallion	20	4	20.00	0.40 ± 0.90	2.00 ± 0.91

Table 5. Breakdown of melanoma prevalence and mean melanoma grade by age

Age	Total number of horses	Number of melanoma cases	Melanoma prevalence (%)	Melanoma grade (mean ± sd) entire cohort[a]	Mean melanoma grade[b]
1-4	79	4	5.06	0.082 ±0.38	1.63
5-9	107	8	7.48	0.126 ±0.48	1.69
10-14	94	16	17.02	0.372 ±0.90	2.19
15-19	26	13	50.00	1.000 ±1.14	2.07
≥20	25	14	56.00	1.500 ±1.52	2.68
Total[c]	335	56	16.72	0.359 ±0.88	2.15
<15	280	28	10.00	0.196 ±0.64	1.96
≥15	51	27	51.92	1.245 ±1.35	2.35

[a]Mean melanoma grade calculated across the entire cohort

[b]Mean melanoma grade calculated only when melanoma was present (ie within the melanoma cases)

[c]The total number of horses is not equivalent to the summation of the horses in the different age categories because age is unknown in 4 animals (3 controls and 1 case)

Discussion

To date, studies of melanoma grade and prevalence have been limited to the Lipizzaner, Pura Raza Española (Spanish pure breed or Andalusians of Spanish origin) and Camargue breeds[13, 17,18].

The melanoma prevalence in our QH cohort was 16.72%, which is lower than previously described in the Lipizzaner (50%), Camargue (31.4%) and Pura Raza Española (89.6%). Our study confirmed the effect of age on melanoma prevalence in gray QH horses as previously described[1, 13,3]. However, the melanoma prevalence in QH older than 15 years old (51.92%) in our study was still much lower than the prevalence reported in Lipizzaner (75%) and Camargue (68%) horses older than 15 years[3, 13], or Pura Raza Española horses and crosses older than 10 years (100%)[18]. Further, the mean melanoma grade in this entire cohort was 0.35 (scale 0 to 4), and 2.15 in cases alone (horses with melanoma), which is again lower than melanoma grade reported in a population of 296 gray Lipizzaner horses, where mean melanoma grade across the entire study population was 1.19 and 2.40 in cases (grades 0-4) [17]. The same grading system was used between studies, so inter-investigator variability is unlikely based on the qualitative nature of the grading scale and the marked difference between grades.

The most common locations for melanoma development observed in the present study are consistent with previous reports. Also, no effect of gender was observed on melanoma prevalence and/or grade in the present study or previous studies.

In conclusion, melanoma prevalence and grade appears to be lower in gray QHs, compared with gray horses from other breeds.

Chapter 3

Experiment 2

Genetic Susceptibility of Melanoma in Gray Quarter Horses

Introduction

Graying and melanoma in horses have been linked to a 4.6 kilobase duplication in intron 6 of the *syntaxin-17* (*STX17)* gene[5]. This duplication harbors a regulatory element containing binding sites for microphthalmia-associated transcription factor (MITF) and *NR4A3*, key components in the regulation of melanocyte gene expression and cell function[6]. The expression of both *STX17* and its neighboring gene, *NR4A3*, are up-regulated in gray horse melanomas, suggesting that the duplicated region is a melanocyte-specific, *cis*-acting regulatory element. *MITF* and *NR4A3* mediated up-regulation of melanin production and proliferation of hair follicle and dermal/epidermal melanocytes is the hypothesized link between the *STX17* mutation, gray coat color and melanoma formation[5]. Horses that are homozygous for the *STX17* duplication have two additional copies of the regulatory element and have a higher melanoma incidence and higher mean melanoma grade than heterozygous gray horses, which have only a single extra copy of the regulatory element[6]. *NR4A3* and *MITF* transcription are mediated by signaling through the melanocortin-1-receptor (MC1R) pathway that results in increase in cellular cAMP concentration (**Figure 1**). MC1R signaling is antagonized by the agouti-signaling protein (*ASIP)* resulting in decreased cAMP, *MITF* and *NR4A3* expression (**Figure 1)**. Horses carrying a deletion in exon 2 of the *ASIP* gene that results in a loss of *ASIP* antagonistic function[7] have increased signaling through the MC1R pathway and homozygotes are black in color. The relative increase in MC1R signaling in horses with the *ASIP* mutation has been linked to increased melanoma severity[5].

The purpose of the present study was to determine the effect of decreased MC1R signaling associated with the *MC1R* chestnut allele on melanoma grade and severity in gray QHs. The final goal was to better understand the genetic basis of melanoma susceptibility in gray horses and develop a screening PCR test to be used in young gray horses, prior to melanoma formation, which would allow identification of animals at a greater risk of developing severe consequences of dermal melanomas. This would allow veterinarians and horse owners to make informed decisions prior to embarking on expensive therapy and would also allow selection of cases that are candidates for early intervention, prior to the development of metastasis.

Material and Methods

Study population and sample collection. Age, gender, melanoma phenotype and samples for isolation of genomic DNA (whole blood [EDTA] or hair root samples) were collected from 335 gray QH. Phenotypic data included the presence, location, size and appearance of external melanomas recorded using a standardized form, and digital photography of melanomas (if present). Horses were assigned a melanoma grade 0-4 (in ½ grade increments) by a single investigator (RT) according to the scale of Peilberg et al[5] (**Table 2**).

Sample recruitment and phenotyping. Samples were collected and phenotyped as described in Chapter 2.

DNA isolation. Genomic DNA was isolated from whole blood or hair roots with commercially available kits according to the manufacturers' protocols (Purogene Blood Kit C for whole blood and Qiagen's DNeasy Blood and Tissue Kit following the procedure for animal tissues for hair root isolations).

Genotyping. Horses were genotyped for gray (*STX17*), chestnut (*MC1R*) and agouti (*ASIP*) locus. Genotyping for gray was performed using the long range PCR method described by Peilberg et al.[5] and was recorded as heterozygous ($STX17^{G/g}$) or homozygous ($STX17^{G/G}$) gray. Chestnut was genotyped using the restriction fragment length polymorphism assay described by Marklund et al[8]; genotypes were recorded as homozygous wild-type $MC1R^{E/E}$, heterozygous $MC1R^{E/e}$, or homozygous $MC1R^{e/e}$ chestnut. Genotyping agouti was performed using PCR amplification and detection of the *ASIP* deletion by resolution of PCR products on a 4% agarose gel as described by Rieder et al[7]. Agouti genotypes were recorded as homozygous wild-type $ASIP^{A/A}$ (bay), heterozygous $ASIP^{A/a}$ (bay), or homozygous $ASIP^{a/a}$ (black).

Statistical analysis. Statistical analyses were performed using R statistical software. Genotypes were coded as additive (0, 1 or 2 copies of the risk allele), recessive effect (aa=1 and Aa or AA=0), where a and A are the risk alleles and wild-type respectively, dominant (aa or Aa=1 and AA=0), or genotypic (aa, Aa, and AA represent three factor levels for genotype) (**Table 6)**. Additive, recessive and dominant coding is relative to the

derived (mutant) allele. Both dominant and recessive coding has two levels of effect: 0 or 1. Additive coding allows each additional copy of the risk allele to result in a simple additive increase in risk, with three different levels of effect: 0, 1 or 2. Genotypic coding also allows for three different levels of effect (one for each genotype), however the effect differences do not have to be additive; in this circumstance the genotype coding is categorical. For example, genotypes AA, Aa, and aa, could have effects of 0, 2 and 6 respectively, or in a scenario where heterozygotes had the highest risk effects for AA, Aa, and aa could be 0, 6 and 2 respectively. The genotypic model treats all three genotypes as independent categories, without assigning an a priori guess as to the effect (ie does not have to be additive, dominant or recessive).

Table 6. Allele coding for each genetic model considered in regression analyses.

	Genotype coding for regression analyses			
Genotype	**Additive**	**Recessive**	**Dominant**	**Genotypic**
MC1R $^{E/E}$	0	0	0	E/E
MC1R$^{E/e}$	1	0	1	E/e
MC1R $^{e/e}$	2	1	1	e/e
ASIP $^{A/A}$	0	0	0	A/A
ASIP $^{A/a}$	1	0	1	A/a
ASIP $^{a/a}$	2	1	1	a/a
STX17 $^{G/g}$	1	0	1	G/g
STX17 $^{G/G}$	2	1	1	G/G

All horses in the study cohort were gray; therefore, the homozygous wild type genotype *STX17*$^{g/g}$ was not present in this data.

Chi-square tests were performed to identify significant deviation of genotypes from Hardy-Weinberg expectations. Multiple regression was performed using age (cubic), *STX17*, *MC1R* and *ASIP* on melanoma grade (linear regression), or case/control status (logistic regression) as the phenotypic response. Multiple regression models also included terms to model interaction between age and genotype and interactions between *MC1R* and *ASIP* genotype (see ***Equations*** **and Figure 3** for details of multiple modeling). Multiple regressions were performed in the entire cohort and repeated after limiting the analysis to horses > 5 years, or to melanoma cases.

Since we hypothesize that the high frequency of the *MC1R* chestnut allele ($MC1R^e$), and subsequent decrease in MC1R signaling, results in decreased melanoma prevalence and/or melanoma severity, we explored several multiple regression models to test this global hypothesis:

Equations:

1. $melanoma = \mu + age\ (cubic) + genotype + random\ error\ (\epsilon)$
2. $melanoma = \mu + age\ (cubic) + genotype + genotype*age + random\ error\ (\epsilon)$
3. $melanoma = \mu + age\ (cubic) + STX17\ genotype + MC1R\ genotype + ASIP\ genotype + random\ error\ (\epsilon)$
4. $melanoma = \mu + age\ (cubic) + MC1R\ genotype + ASIP\ genotype + random\ error\ (\epsilon)$
5. $melanoma = \mu + age\ (cubic) + MC1R\ genotype + ASIP\ genotype + MC1R\ genotype*ASIP\ genotype + random\ error\ (\epsilon)$
6. $melanoma = \mu + age\ (cubic) + STX17\ genotype + MC1R\ genotype + ASIP\ genotype$

*+ STX17 genotype*age + MC1R genotype*age + ASIP genotype*age + random error (ϵ)*

For all models, *melanoma* is either melanoma grade (linear regression), or case/control status (logistic regression), *age* is modeled as a cubic variable, *genotype* is coded as either additive, recessive, dominant or genotypic (**Table 6**), *genotype*age* indicates an interaction term between age and genotype, and *MC1R genotype* ASIP genotype* indicates an interaction term between the genotypes at both loci.

Equations 1 and 2 address the most straightforward questions, i.e., does the genotype at this locus affect melanoma (prevalence or grade) after accounting for the effect of age (**1**), and does the relationship between genotype and melanoma vary with age (**2**). *Equation* 3 is a multivariate regression equation simultaneously considering the effects of all 3 genotyped loci.

Equations 4 and 5 explore the relationship between *MC1R* and *ASIP*: *Equation* 4 tests for an effect of *MC1R* after accounting for *ASIP* (**Figure 1**); this hypothesis was also evaluated by testing for the effect of *MC1R* in only $ASIP^{AA}$ homozygotes. *Equation* 5 tests for interaction between the two loci, i.e., does the effect of either genotype depend on the genotype at the other locus. For this test, genotypes at *MC1R* were coded as recessive to test the hypothesis that in the absence of normal MC1R signaling (i.e., $MC1R^{ee}$), the effect of the *ASIP* mutation would be null (**Figure 4b**), and *MC1R* genotypes were coded as additive to test the hypothesis that the effect of the *ASIP* mutation is mitigated by the $MC1R^{e}$ allele (**Figure 4c**).

Equation 6 represents the full model of the data. These analyses were explored both across the entire cohort and within cases only.

For all analyses, differences were accepted as significant when $p \leq 0.05$.

This protocol was approved by the University of Minnesota's Institutional Animal Care and Use Committee. Owner consent was obtained prior to the enrollment of horses.

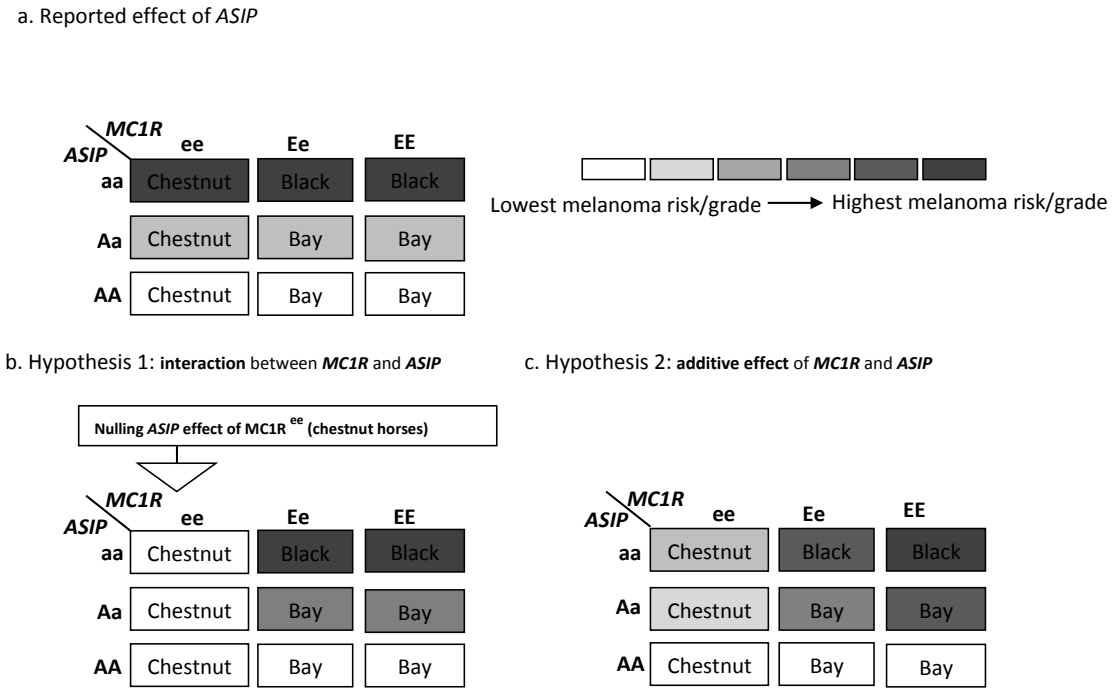

Figure 4. Previously reported effect of the *ASIP* mutation on melanoma grade (a)[5], and 2 hypotheses tested for the effect of *MC1R* on melanoma risk and grade in gray horses (*STX17*^{GG} *or STX17*^{Gg}) (b, c).

The horses initial coat color (prior to graying) based on *ASIP* and *MC1R* genotypes is indicated inside boxes. a) Effect of the *ASIP* mutation on increasing melanoma grade with increased copies of the mutated "a" allele as reported by Rosengren et al[5]. b) Hypothesis 1: Homozygosity for the chestnut allele ($MC1R^{ee}$) nulls the effect of the ASIP "a" allele; i.e. there is not an *ASIP* effect on $MC1R^{ee}$ horses. Statistically, this hypothesis was tested by coding the *MC1R* genotype as recessive and the *ASIP* genotype as additive, and testing for the interaction between the loci. c) Hypothesis 2: The *MC1R* mutation decreases melanoma risk and/or grade depending on the number of copies of the mutated allele "e", and the *ASIP* mutation increases melanoma risk with increased copies of the mutated allele "a". In this scenario, for statistical analysis, both genotypes were coded as an additive effect and the interaction between the *MC1R* and *ASIP* loci was tested.

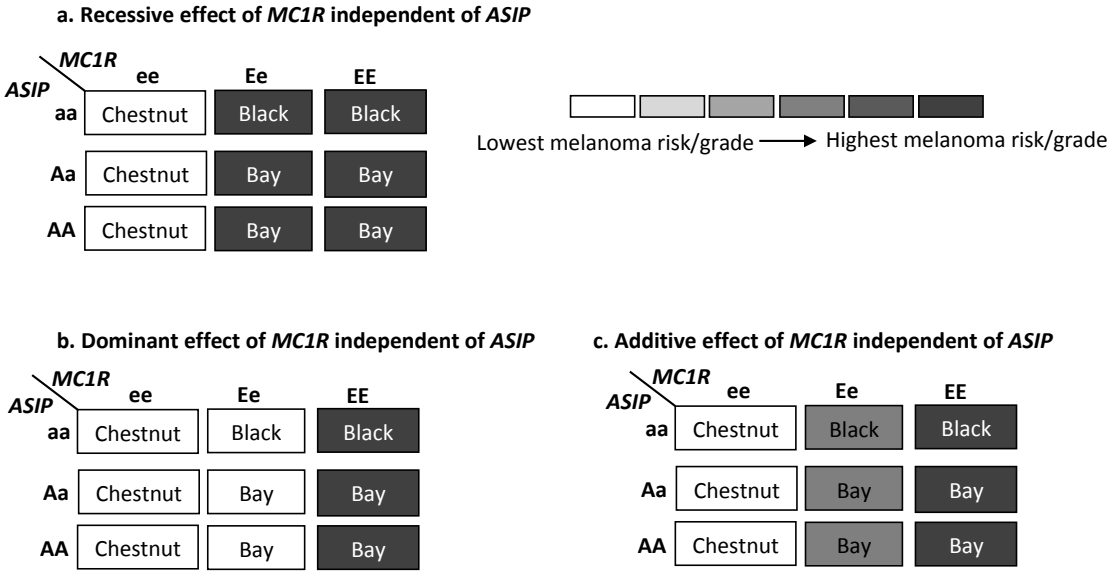

Figure 5. Testing for the effect of *MC1R* genotype.

The horses initial coat color (prior to graying) based on *ASIP* and *MC1R* genotypes is indicated inside boxes. The darker the box, the greater is the melanoma risk/grade. a) Recessive effect of *MC1R*, independent of *ASIP*. b) Dominant effect of *MC1R*, independent of *ASIP*. c) Additive effect of *MC1R*, independent of *ASIP*.

Results

Genotypes (MC1R, ASIP, STX17)

326 horses were successfully genotyped for *ASIP*, 320 horses for *MC1R* and 311 horses for *STX17*. In this cohort, the wild-type allele, $ASIP^{A}$, was the major allele at the agouti locus (allele frequencies $ASIP^{a}$ 0.32, $ASIP^{A}$ 0.67), and the mutant alleles were the major alleles at the *MC1R* and *STX17* loci (allele frequencies: $MC1R^{e}$ 0.76, $MC1R^{E}$ 0.23; $STX17^{G}$ 0.53, $STX17^{g}$ 0.46). Genotype frequencies for *ASIP* and *MC1R* were consistent

with Hardy-Weinberg expectations (p =0.294 and p =0.244, chi-square test for derivation from Hardy-Weinberg equilibrium) (**Table 7**). Due to the sampling scheme that collected only gray horses, Hardy-Weinberg expectations could not be tested for *STX17* genotype frequencies; however $STX17^{G/G}$ homozygotes (7.07%) were much less frequent than $STX17^{G/g}$ heterozygotes (92.93%) (**Table 7**).

Table 7.Genotype and allele frequencies for *ASIP*, *MC1R* and *STX17* and mean melanoma grade for each genotype.

Genotype	Total number of horses	Number of melanoma cases	Genotype frequency (%)	Melanoma prevalence (%)	Melanoma grade (mean ± sd) entire cohort [a]	Melanoma grade (mean ± sd) Cases only [b]
$ASIP^{A/A}$	145	23	44.47	15.86	0.317 ±0.82	2.000 ±0.94
$ASIP^{A/a}$	151	28	46.31	18.54	0.407 ±0.92	2.196 ±0.83
$ASIP^{a/a}$	30	3	9.20	10.00	0.283 ±0.86	2.833 ±0.28
$MC1R^{E/E}$	16	2	5.00	12.50	0.156 ±0.43	1.250 ±0.35
$MC1R^{E/e}$	118	22	36.87	18.64	0.419 ±0.95	2.250 ±0.89
$MC1R^{e/e}$	186	29	58.12	15.59	0.327 ±0.83	2.103 ±0.85
$STX17^{G/g}$	289	46	92.92	15.91	0.346 ±0.87	2.173 ±0.89
$STX17^{G/G}$	22	6	7.07	27.27	0.522 ±0.96	1.916 ±0.86

[a]Mean melanoma grade calculated across the entire cohort

[b]Mean melanoma grade calculated only when melanoma was present (ie within the melanoma cases)

Effect of genotype on melanoma prevalence

Melanoma prevalence by genotype at each locus is presented in **Table 7**. Melanoma prevalence was almost two times greater in $STX17^{G/G}$ homozygotes when compared to $STX17^{G/g}$ heterozygotes (27.27% versus 15.91%). Melanoma prevalence did not appear to follow a particular pattern in *MC1R* ($MC1R^{E/E}$ 12.5%, $MC1R^{E/e}$ 18.64%, and $MC1R^{e/e}$ 15.59%) or *ASIP* ($ASIP^{A/A}$ 15.86%, $ASIP^{A/a}$ 18.54%, and $ASIP^{a/a}$ 10.0%) genotypes. Due to the large effect of age on melanoma prevalence, logistic regression with age as covariate was used to determine the effect of genotype on melanoma prevalence. Even after accounting for age in this manner, no significant differences in melanoma prevalence due to genotype were identified for *STX17, MC1R* or *ASIP* in any of the genetic models considered (i.e. additive, dominant, recessive or genotypic). Further, no significant differences in melanoma prevalence were detected when *ASIP*, *MC1R* or *STX17* were considered together in a multiple regression, or when age by genotype interactions were included in the models.

Effect of genotype on melanoma grade

The mean melanoma grade (grades 1-4) for each genotype across the entire cohort is presented in **Table 7.** The mean melanoma grade was higher in $STX17^{G/G}$ homozygotes when compared to $STX17^{G/g}$ heterozygotes (**Table 7**). When mean melanoma grade was calculated using only the case cohort however, $STX17^{G/G}$ homozygotes had a lower mean melanoma grade than $STX17^{G/g}$ heterozygotes. Further, melanoma grade calculated in only the case cohort followed a pattern consistent with an additive effect of the $ASIP^{a}$

allele on melanoma grade. Linear regressions with age as a covariate were fit to test for an effect of genotype on melanoma grade after accounting for age. Due to the dramatic effect of age on melanoma grade, the estimated effect of each genotype after accounting for age (least squares mean) and the estimated effect of genotype at 20 years of age are shown in **Tables 8** and **9**. After accounting for age, no statistically significant effects of genotype were identified. To test for varying effects of genotype with age, age by interaction terms were included in the model (see ***Equations***), no age by genotype interactions were detected.

Table 8.Least squares means and fit at age 20 across the entire cohort of samples.

Genotype	genotype + age^3		genotype + age^3 + interaction	
	least squares means (±SE)[a]	fit at 20 years of age (±SE)	least squares means (±SE)[a]	fit at 20 years of age (±SE)
$ASIP^{A/A}$	0.15 (±0.08)	1.18 (±0.13)	0.15 (±0.08)	1.09 (±0.15)
$ASIP^{A/a}$	0.26 (±0.07)	1.29 (±0.12)	0.26 (±0.07)	1.31 (±0.14)
$ASIP^{a/a}$	0.21 (±0.15)	1.23 (±0.18)	0.25 (±0.15)	1.59 (±0.35)
$MC1R^{E/E}$	0.11 (±0.20)	1.11 (±0.23)	0.08 (±0.20)	0.50 (±0.59)
$MC1R^{E/e}$	0.26 (±0.08)	1.25 (±0.13)	0.26 (±0.08)	1.42 (±0.16)
$MC1R^{e/e}$	0.20 (±0.07)	1.19 (±0.12)	0.21 (±0.07)	1.08 (±0.14)
$STX17^{G/g}$	0.21 (±0.06)	1.20 (±0.12)	0.20 (±0.06)	1.22 (±0.13)
$STX17^{G/G}$	0.40 (±0.17)	1.40 (±0.20)	0.40 (±0.17)	1.32 (±0.40)

[a]Mean age for *ASIP*= 9.21, mean age *MC1R*= 9.21, mean age *STX17*= 9.24

Table 9.Least squares means and fit at age 20 within the case cohort.

Genotype	genotype + age^3		genotype + age^3 +interaction	
	least squares means (±SE)[a]	fit 20 years of age (±SE)	least squares means (±SE) [a]	fit 20 years of age (±SE)
ASIP$^{A/A}$	1.89 (±0.22)	2.11 (±0.26)	1.92 (±0.23)	2.24 (±0.30)
ASIP$^{A/a}$	2.17 (±0.18)	2.39 (±0.22)	2.18 (±0.19)	2.35 (±0.24)
ASIP$^{a/a}$	2.76 (±0.49)	2.98 (±0.50)	2.79 (±0.51)	2.94 (±0.62)
MC1R$^{E/E}$	1.40 (±0.66)	1.62 (±0.69)	1.84 (±1.27)	2.38 (±1.95)
MC1R$^{E/e}$	2.16 (±0.20)	2.38 (±0.23)	2.16 (±0.21)	2.38 (±0.24)
MC1R$^{e/e}$	2.08 (±0.21)	2.30 (±0.26)	2.06 (±0.21)	2.31 (±0.29)
STX17$^{G/g}$	2.13 (±0.17)	2.37 (±0.22)	2.18 (±0.17)	2.43 (±0.22)
STX17$^{G/G}$	1.95 (±0.37)	2.19 (±0.39)	1.81 (±0.38)	1.66 (±0.54)

[a]Mean age for *ASIP*= 15.49, mean age *MC1R*= 15.27, mean age *STX17*= 15.48

Interactions between MC1R and ASIP

The hypothesized effects of *ASIP, MC1R* and *STX17* are all mediated through the MC1R signaling pathway, thus genotype by genotype interaction terms were modeled. In particular, the potential interaction between *ASIP* and *MC1R* were explored as the *MC1R^{ee}* genotype is epistatic over *ASIP* in relation to coat color (**Figure 1, Figures 4 and 5**). Across the entire sample cohort, no significant effects of either *ASIP* or *MC1R* were detected after accounting for genotype at the other locus (*equation 4*). Further, no interaction was detected between the two loci when the effect of *MC1R* was coded as additive or recessive (*equation 4*). When the effect of *ASIP* and *MC1R* on grade was considered in melanoma cases, there was a suggested additive effect of *ASIP* (p=0.077).

Similar results in all analyses were obtained when the cohort was restricted to horses greater than 5 years of age (data not shown).

Discussion

Studies of melanoma grade and prevalence have been limited to the Lipizzaner, Pura Raza Española (Spanish pure breed or Andalusians of Spanish origin) and Camargue breeds[13,17,18]. In these breeds gray coat color is predominant or breed-defining, the *ASIP* mutation is relatively common, and the *MC1R* chestnut allele is absent or segregating at extremely low frequency[5]. Thus the potential impact of altered MC1R signaling associated with the *MC1R* chestnut mutation on melanoma development and grade cannot be evaluated in any of these three breeds. The high chestnut allele frequency and resulting high homozygous chestnut genotype frequency in our gray QH cohort made it possible to determine the effect of diminished MC1R signaling resulting from this mutation on melanoma risk.

In a population of 694 Lipizzaner horses, the number of *STX17* duplications appeared to impact melanoma risk, as *STX17* homozygotes had greater melanoma prevalence than *STX17* heterozygotes, and the estimated mean melanoma grade (least-squares mean) for heterozygotes was 0.67 compared to 1.43 for homozygotes[5]. In our study cohort, melanoma prevalence and mean melanoma grade were higher in horses homozygous for the gray mutation (27.27% and 0.52, respectively), compared to heterozygous gray horses (15.91% and 0.34, respectively), although this difference was not statistically significant. There were very few homozygous gray horses in the study cohort (n=22) and only 6 of these horses had melanoma, resulting in poor statistical power to demonstrate the effect of the additional copies of the *STX17* mutation on melanoma prevalence and/or grade. The low frequency of the *SXT17* homozygosity in

our population is not surprising, as QH are not primarily bred for the gray coat color.

Work in the Lipizzaner breed also demonstrated an effect of the *ASIP* genotype on melanoma grade after accounting for the gray genotype. The effect of the *ASIP* deletion in that study was estimated as 1.06 (least-squares mean) for heterozygotes ($ASIP^{A/a}$) and 1.22 for homozygotes ($ASIP^{a/a}$) [5]. We did not detect a statistically significant effect of the *ASIP* mutation on melanoma grade in our study cohort. There are several possible explanations for this result. First, although no effect of the *ASIP* deletion was observed, a trend of increasing mean melanoma grade with increased copy of the mutate allele ($ASIP^{a}$) was observed in melanoma cases (**Table 7**); however after accounting for age (**Table 8**), these differences were not statistically significant. The confidence intervals around the estimated additive effect of the *ASIP* deletion on melanoma grade in our study cohort (estimate: 0.07; 95% CI -0.065 to 0.204) overlap with the additive effect reported by Pielberg et al (0.16-0.18)[5].

It is also possible that the presence of the *MC1R* mutation mitigates an effect of the *ASIP* mutation on melanoma grade. Although we did not find a significant effect of the *MC1R* mutation itself, it is conceivable that the altered signaling in *MC1R* heterozygotes and homozygotes, specifically decreased levels of cAMP, balance the up-regulation of signaling in horses with the *ASIP* mutation (**Figure 1**). A high frequency of the *MC1R* mutation and the $MC1R^{e/e}$ and $MC1R^{E/e}$ genotypes were observed in this cohort. Only 5% of the horses were homozygous wild-type for the chestnut *MC1R* gene ($MC1R^{E/E}$), thus the vast majority of horses in this study had decreased MC1R signaling relative to wild-type. Although, we did not identify a statistically significant interaction between these

two genotypes, the low frequency of the *MC1R*$^{E/E}$ genotype resulted in poor statistical power for identification of this interaction. It is possible that the *ASIP* mutation has little to no effect on melanoma grade in the presence of the *MC1R* mutation due to decreased signaling through that pathway.

Another possible explanation for the lack of association between the *ASIP* mutation and melanoma grade is that the *ASIP* mutation might not be the functional allele underlying increased melanoma risk in Lipizzaners. In humans, *ASIP* variants have been shown to directly alter pigmentation phenotypes; and large genome wide association studies have implicated haplotypes containing *ASIP* in melanoma risk[19,20]. Yet, associations between specific variants in the *ASIP* gene and melanoma have not been demonstrated[21,22], leading to speculation that in humans, the increase risk for melanoma is attributed to other genes within the associated haplotype[19,20]. Thus, it is possible that the genetic variant responsible for increased risk in Lipizzaner horses is simply in linkage disequilibrium with the *ASIP* mutation, and is not the *ASIP* mutation itself. Due to differences in haplotype length between those two breeds, the two alleles might not be in linkage disequilibrium in QH, preventing detection of a positive association.

Although genetic variants in *MC1R* pathway and/or MITF regulated genes frequently underlie melanoma risk in humans, the pathophysiology of melanoma appears to be very distinct from horses. An *increase* in MC1R signaling associated with *increased* melanoma risk in horses[5] is in contrast with *decreased* MC1R signaling that is associated with red hair, fair skin, freckles, poor UVR-induced tanning response and *increased* melanoma risk in people[23]. Gray horses maintain dark pigmentation in the skin through

the process of graying and commonly develop melanomas in areas that are not exposed to the sunlight, thus UV exposure is unlikely an important environmental component in gray horse melanoma. Although *MC1R* variants are associated with increased melanoma risk in people, it was recently demonstrated that melanoma patients carrying *MC1R* variants had a better outcome than melanoma cases with black/brown hair, suggesting that *MC1R* mutations have a protective effect on survival. Signaling through MC1R regulates expression of the MITF transcription factor (**Figure 1**) that has many target genes in addition to pigment biosynthesis enzymes, including genes that regulate DNA repair, the cell cycle, apoptosis and invasion. The hypothesis is that melanoma cells carrying *MC1R* variants have less resistance to apoptosis, less sustained proliferation and poorer DNA repair, leading to better patient survival[10]. Although we found no effect of the *MC1R* mutation on melanoma prevalence/grade in gray QH, it is possible that this genotype is also associated with better survival in horses.

Chapter 4

Conclusions and Future Directions

Conclusions

All dermal melanomas should be considered potentially malignant since histopathologic changes and location cannot predict the risk of metastasis[4]. Therapy for equine melanoma is most effective prior to extensive invasion of local tissue or metastasis. When surgical removal is not feasible due to the size or location of masses, intralesional injection of cisplatin[11] or cisplatin beads[12] may be palliative for masses interfering with essential functions such as urination and defecation[13,3,11,14.] When metastasis has occurred, systemic cimetidine or autologous vaccine are the only therapeutic options[4,15], however both have relatively low efficacy unless initiated prior to the development of complications[15]. A better understanding of the risk factors involved in melanoma susceptibility, severity, and the risk of metastasis may allow for better prediction of tumor behavior and allow early intervention in horses that are more likely to develop severe, life threatening consequences to dermal melanomas. Classification of melanoma patients into risk categories (high and low risk groups) based on genetic predispositions would allow veterinarians to identify patients that are candidates for early intervention, before therapy options are limited, enabling owners and veterinarians to make informed decisions prior to embarking in expensive or prolonged treatment regimens. In humans, genetic testing is routinely used to identify individuals at high-risk for melanoma formation[16].

Our findings suggest a lower prevalence and severity of melanoma in gray QH compared to other breeds in which melanoma prevalence and grade have been

investigated, but could not conclusively tie this decrease in prevalence and severity to altered MC1R signaling. Further work is needed to determine if the decreased prevalence and severity of melanoma in gray QH is simply due to infrequent *STX17* homozygosity, a potential mitigating effect of the *MC1R* mutation on *ASIP* mediated enhancement of melanoma progression, or other genes in the MC1R pathway. Addressing these possibilities would require an expanded cohort of melanoma cases and controls and/or investigation of other loci. It is also possible, as in humans, that carrying *MC1R* variants do not prevent horses from getting melanomas, but rather provide better survival rates. A prolonged follow up of this cohort would be necessary to prove this hypothesis.

Future Directions

The low melanoma prevalence and grade observed in gray QH reinforces the need for future research in the area. While increasing the sample size would improve our power to detect an effect of the studied genotypes on melanoma prevalence/grade, inclusion of hundreds of gray QH is this study might not be feasible. Since gray horse melanoma is a complex genetic disorder, other approaches (apart from candidate gene studies) would make it possible to identify additional loci involved on melanoma susceptibility. Genome wide association studies and next generation sequencing technology are possible approaches to answer those questions.

I. Genome wide association studies - GWAS

Melanoma development in horses appears to be a complex disease, where multiple genes with different effects are responsible for the phenotype. While the *STX17* mutation appears to be a gene of major effect, other genes are involved in the pathway[5]. To further elucidate genomic regions involved in melanoma susceptibility in gray QHs, genome wide association analysis (GWAS) could be performed. GWAS aims to identify associations between SNPs (single nucleotide polymorphism) and a trait. GWAS is less hypothesis driven and involves characterization of a much larger number of SNPs. The goal is to identify regions of the genome, highly associated with the phenotype in question (SNPs that vary systematically between cases and controls) [31, 32, 33]. Those regions are further evaluated to identify possible candidate genes for further genotyping and validation in cases and controls. GWAS analysis relies on the concept of linkage disequilibrium (LD), which is the non-random association between markers and the causal allele. There is a low chance of recombination between two loci that are closely together within a chromosome segment, making it possible to find markers that are associated with disease in case-control GWAS analysis [31, 32, 33].

GWAS for case-control studies have the issue of optimal selection of cases and controls[31]. Since gray horse melanoma is commonly observed in older horses[1,13], controls should include horses older than 15 years or likely older, with no melanomas (grade zero), to avoid misclassification bias. Case criteria should include the horses with greater melanoma grade followed by age; the younger animals with more severe melanomas would likely represent the most aggressive type of the disease. Another concern when

designing those studies is sample size[31]. A large number of horses are needed to achieve enough power and detect genotypic markers associated with the trait of interest. DNA samples are available from 56 gray QH without visible melanomas, from which 22 are older than 15 years old.

Although GWAS is a powerful technology to identify genotypic markers associated with complex disease, a greater number of gray QHs are needed to achieve enough power. Another concern when performing GWAS is the presence of population stratification that can lead to spurious association, but several statistical tools exist to detect and correct for population stratification[31].

In conclusion, GWAS is a powerful tool to detect regions of the genome associated with the trait of interest, but a larger cohort of melanoma cases and controls is needed to achieve enough power.

II. Next Generation Sequencing, pooled approach

An alternative to GWAS is the use of genomic DNA pooling strategy for next generation sequencing. This technique is cost effective on estimating difference in allele frequency between groups, with a reduced cost. By pooling whole genome samples in two different groups (cases and controls) and sequencing them at a high depth of coverage, the difference in allele frequency between the pools can be estimated, in attempt to identify variants associated with the phenotype of interest. This alternative approach pools genomic DNA (non-barcoded) from several individuals and sequences

the pool sample. After sequencing, the first objective is to identify all polymorphic sites followed by difference in allele frequency between pools. For pooled sequencing, the frequency of a variant allele is a function of the population allele frequency and the size of the pool [27,28,29,30].

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