Coat Color Genotypes and Risk and Severity of Melanoma in Gray Quarter Horses


**Background:** Both graying and melanoma formation in horses have recently been linked to a duplication in the STX17 gene. This duplication, as well as a mutation in the ASIP gene that increases MC1R pathway signaling, affects melanoma risk and severity in gray horses.

**Objective:** To determine if melanoma susceptibility in gray Quarter Horses (QH) is lower than gray horses from other breeds because of increased MC1R signaling resulting from a high incidence of the MC1R chestnut coat color allele in the QH population.

**Methods:** Blood or hair root samples were collected from all horses for DNA extraction and genotyping for STX17, ASIP, and MC1R genotypes. Age, sex, 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.

**Results:** Melanoma prevalence (16%) and grade (0.35) in this QH cohort was lower than that reported in other breeds. Age was significantly associated with melanoma prevalence (P = 5.28 × 10⁻¹¹) and severity (P = 2.2 × 10⁻¹¹). No significant effect of MC1R genotype on melanoma prevalence or severity was identified. An effect of ASIP genotype on melanoma risk was not detected. Low STX17 homozygosity precluded evaluation of the gray allele effect.

**Conclusion and clinical importance:** Melanoma prevalence and severity is lower in this population of gray QH than what is reported in other breeds. This could be because of the infrequent STX17 homozygosity, a mitigating effect of the mutation on ASIP potentiation of melanoma, other genes in the MC1R signaling pathway, or differences in breeds.

**Key words:** ASIP; MC1R; Metastasis; STX17.

Melanomas, primary tumors of melanocytes, account for 15% of equine skin tumors and are classified into 4 groups according to clinical and histopathologic features. Melanocytic nevi and anaplastic malignant melanoma affect horses of any age, breed, or coat color, while dermal melanoma and dermal melanomatosis occur in gray horses. Dermal melanoma and melanomatosis are distinguished by the number of masses and the presence of metastasis, but are not distinguishable histopathologically. Up to 80% of gray horses older than 15 years develop melanomas and 14–66% of dermal melanomas eventually metastasize.

Graying and melanoma have been linked to a 4.6 kilobase duplication in intron 6 of the syntaxin-17 (STX17) gene. 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. The expression of both STX17 and its neighboring gene, NR4A3, is 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 (see Supplemental Material). Horses that are homozygous for the STX17 duplication have 2 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. NR4A3 and MITF transcription are mediated by signaling through the melanocortin-1-receptor (MC1R) pathway that results in increase in cellular cAMP concentration (Fig 1). MC1R signaling is antagonized by the agouti-signaling protein (ASIP) resulting in decreased cAMP, MITF, and NR4A3 expression (Fig 1). Horses carrying a deletion in exon 2 of the ASIP gene that results in a loss of ASIP antagonistic function, have increased signaling through the MC1R pathway and homozygotes are

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**Abbreviations:**
- ASIP: agouti-signaling protein
- MC1R: melanocortin-1-receptor
- MITF: microphthalmia-associated transcription factor
- QH: Quarter Horses
- STX17: Syntaxin-17

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black in color. The relative increase in MC1R signaling in horses with the ASIP mutation has been linked to increased melanoma severity.5 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 (Fig 1).9 In humans, similar MC1R variants that 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. We hypothesized that melanoma susceptibility is lower in gray QHs relative to gray horses from other breeds; and further, this lower susceptibility is because of decreased MC1R signaling resulting from a high incidence of the MC1R chestnut coat color allele in the QH population.

Materials and Methods

Study Population and Sample Collection

Age, sex, melanoma phenotype, and samples for isolation of genomic DNA (whole blood [EDTA] or hair root samples) 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 1/2 grade increments) by a single investigator (RT) according to the scale of Peilberg et al. (Supplemental Table S1).

**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 Quagen’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. 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. 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. Agouti genotypes were recorded as homozygous wild-type ASIP^A/A, heterozygous ASIP^A/e, or homozygous ASIP^e/e.

**Statistical Analysis**

Statistical analyses were performed using R statistical software. Descriptive statistics were calculated for age, sex, melanoma prevalence, and grade. 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 3 factor levels for genotype). Further explanation and coding for genotype in each analysis is in Supplemental Table S2. All horses in the study cohort were gray; therefore, the homozygous wild-type genotype STX17^G/G was not present in this data. 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 as a dichotomous variable (≥15 years versus <15 years). Sex was coded as gelding, stallion, or female and as male (stallion and gelding) and female.

Chi-square tests were performed to identify significant deviation of genotypes from Hardy-Weinberg expectations. 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 sexes. Multiple regression analysis was performed using age (cubic) and sex to further evaluate for an effect of sex on outcome.

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 Supplemental Methods and Supplemental Figure S2 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. 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 before the enrollment of horses.

**Results**

**Melanoma Prevalence and Grade**

Three hundred thirty-five gray QH, 227 females, 87 geldings and 20 stallions, and 1 of unknown sex were phenotyped for dermal melanoma. Age ranged from 1 to 33 years (mean 9.21). Age was not recorded in 3 horses. Fifty-six horses (17.7%) had visible dermal melanomas and were classified as cases. Of these 56 cases, 28 (50%) had a solitary mass, and 28 (50%) had 2 or more masses (mean number of masses per horse 3.2; range 2–8). Melanomas were most commonly under the tail (75%), followed by around the anus (18%), at the commissure of the lip (11%), surrounding the parotid salivary gland (9%), on the prepuce (9%) or external genitalia (7%), and on the neck (7%). Less common locations included the following: 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, 89.3% had masses under the tail and in a 2nd location; only 10.7% of horses with masses in multiple locations did not have melanomas under the tail. Melanoma grade distribution in the case cohort is presented in Supplemental Table S3. Mean melanoma grade across the case cohort was 2.15, with only 7% of the horses assigned a grade 4 with ulcerated masses (Fig 2). 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 sexes (Supplemental Table S4).

Melanoma cases ranged in age from 2 to 30 years, with clear increase in melanoma prevalence with age (Supplemental Table S5); prevalence was 52% in horses >15 years compared to 10% in horses <15 years. Age was significantly associated with the presence or absence of melanoma (logistic regression \( P = 5.28 \times 10^{-11} \)). 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) (Supplemental Table S5). Age was also significantly correlated with melanoma grade (linear [cubic] regression: Spearman’s correlation coefficient = 0.387, \( P = 2.2 \times 10^{-13} \)).

**Genotypes (MC1R, ASIP, STX17)**

Three hundred twenty-six 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 \textit{MC1R} and \textit{STX17} loci (allele frequencies: \textit{MC1R}^e 0.76, \textit{MC1R}^E 0.23; \textit{STX17}^G 0.53, \textit{STX17}^g 0.46). Genotype frequencies for \textit{ASIP} and \textit{MC1R} were consistent with Hardy-Weinberg expectations ($P = .294$ and $P = .244$, chi-square test for derivation from Hardy-Weinberg equilibrium; Table 1). Because of the sampling scheme that collected only gray horses, Hardy-Weinberg expectations could not be tested for \textit{STX17} genotype frequencies; however, \textit{STX17}^G/G homozygotes (7.07\%) were much less frequent than \textit{STX17}^G/g heterozygotes (92.93\%; Table 1).

\section*{Effect of Genotype on Melanoma Prevalence}

Melanoma prevalence by genotype at each locus is presented in Table 1. Melanoma prevalence was almost 2 times greater in \textit{STX17}^G/G homozygotes when compared to \textit{STX17}^G/g heterozygotes (27.27\% versus 15.91\%). Melanoma prevalence did not appear to follow a particular pattern in \textit{MC1R} (\textit{MC1R}^E/E 12.5\%, \textit{MC1R}^E/e 18.64\%, and \textit{MC1R}^e/e 15.59\%) or \textit{ASIP} (\textit{ASIP}^A/A 15.86\%, \textit{ASIP}^A/a 18.54\%, and \textit{ASIP}^a/a 10.0\%) genotypes. Because of the large effect of age on melanoma prevalence, logistic regression with age

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
Genotype & Total Number of Horses & Number of Melanoma Cases & Genotype Frequency (\%) & Melanoma Prevalence (\%) & Mean Melanoma Grade (mean ± SD) Entire Cohort$^a$ & Mean Melanoma Grade (mean ± sd) Cases Only$^b$ \\
\hline
\textit{ASIP}^{A/A} & 145 & 23 & 44.47 & 15.86 & 0.317 ± 0.82 & 2.000 ± 0.94 \\
\textit{ASIP}^{A/a} & 151 & 28 & 46.31 & 18.54 & 0.407 ± 0.92 & 2.196 ± 0.83 \\
\textit{ASIP}^{a/a} & 30 & 3 & 9.20 & 10.00 & 0.283 ± 0.86 & 2.833 ± 0.28 \\
\textit{MC1R}^{E/E} & 16 & 2 & 5.00 & 12.50 & 0.156 ± 0.43 & 1.250 ± 0.35 \\
\textit{MC1R}^{E/e} & 118 & 22 & 36.87 & 18.64 & 0.419 ± 0.95 & 2.250 ± 0.89 \\
\textit{MC1R}^{e/e} & 186 & 29 & 58.12 & 15.59 & 0.327 ± 0.83 & 2.103 ± 0.85 \\
\textit{STX17}^{G/G} & 289 & 46 & 92.92 & 15.91 & 0.346 ± 0.87 & 2.173 ± 0.89 \\
\textit{STX17}^{G/g} & 22 & 6 & 7.07 & 27.27 & 0.522 ± 0.96 & 1.916 ± 0.86 \\
\hline
\end{tabular}
\caption{Genotype and allele frequencies for \textit{ASIP}, \textit{MC1R}, and \textit{STX17} and mean melanoma grade for each genotype.}
\end{table}

$^a$Mean melanoma grade calculated across the entire cohort.
$^b$Mean melanoma grade calculated only when melanoma was present (ie, within the melanoma cases).
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 because of genotype were identified for STX17, MC1R, or ASIP in any of the genetic models considered (ie, 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 1. The mean melanoma grade was higher in STX17<sup>G/G</sup> homozygotes when compared to STX17<sup>G/R</sup> heterozygotes (Table 1). When mean melanoma grade was calculated using only the case cohort, however, STX17<sup>G/G</sup> homozygotes had a lower mean melanoma grade than STX17<sup>G/R</sup> heterozygotes. Further, melanoma grade calculated in only the case cohort followed a pattern consistent with an additive effect of the ASIP<sup>a</sup> 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. Because of 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 2 and 3. 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 (Supplemental Material), no age by genotype interactions were detected.

**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 interactions between ASIP and MC1R were explored as the MC1Re<sup>e</sup> genotype is epistatic over ASIP<sup>a</sup> in relation to coat color (Fig 1, Supplemental Material, Supplemental Figures S1 and S2). 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, Supplemental Text). Further, no interaction was detected between the 2 loci when the effect of MC1R was coded as additive or recessive (equation 4, Supplemental Text). When the effect of ASIP and MC1R on grade was considered in melanoma cases, there was a suggested additive effect of ASIP<sup>a</sup> (<i>P = 0.077</i>).

**Table 2.** Least-squares means and fit at age 20 across the entire cohort of samples.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype + Age&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Genotype + Age&lt;sup&gt;3&lt;/sup&gt; + Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least-squares means (±SE)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fit at 20 years of age (±SE)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;1:A&lt;/sup&gt;</td>
<td>0.15 (±0.08)</td>
<td>1.18 (±0.13)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;1:a&lt;/sup&gt;</td>
<td>0.26 (±0.07)</td>
<td>1.29 (±0.12)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;a:a&lt;/sup&gt;</td>
<td>0.21 (±0.15)</td>
<td>1.23 (±0.18)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;E:E&lt;/sup&gt;</td>
<td>0.11 (±0.20)</td>
<td>1.11 (±0.23)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;E:e&lt;/sup&gt;</td>
<td>0.26 (±0.08)</td>
<td>1.25 (±0.13)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;e:e&lt;/sup&gt;</td>
<td>0.20 (±0.07)</td>
<td>1.19 (±0.12)</td>
</tr>
<tr>
<td>STX17&lt;sup&gt;G:G&lt;/sup&gt;</td>
<td>0.21 (±0.06)</td>
<td>1.20 (±0.12)</td>
</tr>
<tr>
<td>STX17&lt;sup&gt;G:G&lt;/sup&gt;</td>
<td>0.40 (±0.17)</td>
<td>1.40 (±0.20)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean age for ASIP = 9.21, mean age MC1R = 9.21, mean age STX17 = 9.24.

**Table 3.** Least-squares means and fit at age 20 within the case cohort.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Genotype + Age&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Genotype + Age&lt;sup&gt;3&lt;/sup&gt; + Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Least-squares means (±SE)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Fit 20 years of age (±SE)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;1:A&lt;/sup&gt;</td>
<td>1.89 (±0.22)</td>
<td>2.11 (±0.26)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;1:a&lt;/sup&gt;</td>
<td>2.17 (±0.18)</td>
<td>2.39 (±0.22)</td>
</tr>
<tr>
<td>ASIP&lt;sup&gt;a:a&lt;/sup&gt;</td>
<td>2.76 (±0.49)</td>
<td>2.98 (±0.50)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;E:E&lt;/sup&gt;</td>
<td>1.40 (±0.66)</td>
<td>1.62 (±0.69)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;E:e&lt;/sup&gt;</td>
<td>2.16 (±0.20)</td>
<td>2.38 (±0.23)</td>
</tr>
<tr>
<td>MC1R&lt;sup&gt;e:e&lt;/sup&gt;</td>
<td>2.08 (±0.21)</td>
<td>2.30 (±0.26)</td>
</tr>
<tr>
<td>STX17&lt;sup&gt;G:G&lt;/sup&gt;</td>
<td>2.13 (±0.17)</td>
<td>2.37 (±0.22)</td>
</tr>
<tr>
<td>STX17&lt;sup&gt;G:G&lt;/sup&gt;</td>
<td>1.95 (±0.37)</td>
<td>2.19 (±0.39)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean age for ASIP = 15.49, mean age MC1R = 15.27, mean age STX17 = 15.48.
Similar results in all analyses were obtained when the cohort was restricted to horses greater than 5 years of age (data not shown).

**Discussion**

The melanoma prevalence in our QH cohort was 16%, 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.1,3,11 However, the melanoma prevalence in QH older than 15 years old (52%) in our study was still much lower than the prevalence reported in Lipizzaner (75%) and Camargue (68%) horses older than 15 years.6,11 or Pura Raza Española horses and crosses older than 10 years (100%).12 Further, the mean melanoma grade in this entire cohort was 0.35 (scale 0–4), 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).13

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.11–14 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 3 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, grade, or both. The low frequency of the *SXT17* homozygosity in our population is not surprising, as QH are not primarily bred for the gray coat color.

Study 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) 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*) was observed in melanoa cases (Table 1); however, after accounting for age (Table 2), 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 (Fig 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 2 genotypes, the low frequency of the *MC1R*E/E genotype resulted in poor statistical power for the 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 because of 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.15,16 Yet, associations between specific variants in the *ASIP* gene and melanoma have not been demonstrated.17,18 leading to speculation that in humans, the increased risk for melanoma is attributed to other genes within the associated haplotype.15,16 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. Because of the differences in haplotype length between those 2 breeds, the 2 alleles might not be in linkage disequilibrium in QH, preventing detection of a positive association.

Although genetic variants in *MC1R* pathway, MITF regulated genes, or both frequently underlie melanoma risk in humans, the pathophysiology of melanoma...
MC1R is that melanoma cells carrying enzymes, including genes that regulate DNA repair, many target genes in addition to pigment biosynthesis of the MITF transcription factor (Fig 1) that has melanoma predisposition that is associated with red hair, fair skin, freckles, poor UVR-induced tanning response, and increased melanoma risk in people. 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 (Fig 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 hypotheses is that melanoma cells carrying MC1R variants would have less resistance to apoptosis, less sustained proliferation and poorer DNA repair, leading to better patient survival. 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.

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 treatment options are limited, enabling owners and veterinarians to make informed decisions before embarking in expensive or prolonged treatment regimens. In humans, genetic testing is routinely used to identify individuals at high risk for melanoma formation.

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 because of 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, investigation of other loci, or both. 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.

Acknowledgments

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Conflicts of Interest. Authors disclose no conflict of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Mechanism of graying, MC1R signaling, coat color and melanoma.

**Data S2.** Methods.

**Figure S1.** Testing for the effect of MC1R genotype.

**Figure S2.** Previously reported effect of the ASIP mutation on melanoma grade (a)1, and 2 hypotheses tested for the effect of MC1R on melanoma risk and grade in gray horses (STX17GG or STX17Gg) (b, c).

**Table S1.** Melanoma grading system (Rosengren et al, 2008).

**Table S2.** Allele coding for each genetic model was considered in regression analyses. Additive, recessive and dominant coding is relative to the derived (mutant) allele. Both dominant and recessive coding have 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 S3.** Distribution of melanoma grades in the case cohort.

**Table S4.** Breakdown of melanoma prevalence and mean melanoma grade by gender.

**Table S5.** Breakdown of melanoma prevalence and mean melanoma grade by age.