Melanoma and the Greying Horse

A study of the grey horse melanoma, with special reference to prevalence, tumour structure and biology and associated pigment metabolism abnormalities.

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MELANOMA

and the

GREYING HORSE

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The grey horse melanoma is a widely recognized but poorly understood condition. Its importance lies in its high prevalence, as a cause of rejection of grey horses for slaughter at export abattoirs, and as a potential model for pigment cell disorders and malignancies in other species (including people).

Grey horses can be born any colour but with age there is a dilution of coat colour to grey and then white. Heavily pigmented tumours develop in many of these animals, apparently concurrent with the dilution in coat colour. The mechanism which underlies the progressive failure of hair follicles to produce pigment is unknown.

Although among the most common of equine tumours, the exact nature of the grey horse melanoma has been the subject of debate since the turn of the century. Some argue that it is indeed a true malignancy. Other scientists have suggested that it may be a storage disorder following an abnormality of pigment metabolism; as the hair follicles lose their ability to produce pigment or pass it on to the growing hair, pigment is stored away in slowly enlarging masses. Yet another argument is that it is a variant of a type of benign mole seen in people, the blue naevus. There is evidence to support and undermine each theory but, in truth, we have progressed little since Lord McFadyean’s description of the condition in 1933.

With recent advances in the study of mammalian pigmentation genetics, there may be new opportunities to understand the steps leading to the development of this condition. As a requisite first step, information needs to be gathered on the basic biology of the tumour in grey horses; are horses with different patterns of coat colour dilution at different levels of risk of contracting the disease, is there a sex predilection, are other anomalies of pigmentation such as vitiligo involved, what are the anatomical features of the tumour at both light and electron microscopic levels, and what ultrastructural changes can be determined in the hair follicles of horses as their coats lose their colour? These are the questions addressed in the present study.
MELANOMA AND THE GREYING HORSE

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Grey horses may be born any colour but with age their coats become grey and then white. Despite dramatic recent advances in the understanding of coat colour genetics, little is known about the mechanism/s causing greying in the horse. The pattern of coat colour dilution varies to an extent; horses may be either dappled or flea-bitten and their points may turn white with the rest of the body or remain dark. Although skin colour in general appears to be unaffected, some grey horses develop focal, patchy vitiligo - particularly around the eyes and anus.

Concurrent to the coat colour dilution, melanotic tumours frequently develop. About 80% of horses older than 15 years of age are said to possess pigmented lesions. Congenitally white or grey horses, and horses of other colours, have little risk of acquiring the disease. There is some debate as to whether there is a sex predilection for tumour development and whether certain patterns of coat colour dilution are associated with a higher prevalence of the condition.

Debate on the nature of the condition has been ongoing since the turn of the century. Some people consider it a true neoplasm, albeit not an aggressive one. Others consider it to be a storage disorder following an abnormality of pigment metabolism. It has also been likened to the human blue naevus (a benign dermal mole which occasionally undergoes malignant transformation). A further explanation is that there may be a widespread distribution of melanocytes which acquire neoplastic properties and form melanin deposits. Depending on which theory is accepted, internal tumours may thus be said to be metastases, or accumulations of pigment in macrophages as part of a storage disorder, or ectopic nests of pigment cells somehow stimulated to produce excess pigment in response to, or as part of, the process of coat colour dilution.

Until the true nature of the grey horse melanoma is elucidated, clinicians will have difficulty optimizing the management of affected horses. For example, if the condition is malignant and arises from a primary skin tumour, then surgical excision of skin lesions is warranted and chemotherapy may be of use in more advanced cases. If on the other hand, the condition reflects hyperplasia of a pre-existing network of melanocytes, then such intervention is unlikely to be warranted.

The export of horse meat from Australia for human consumption is a small but significant industry. Grey horses are not acceptable for this market because of the high risk that carcases will be spoiled by the heavily pigmented visceral lesions, many of which involve skeletal muscle. Also if the condition is a true neoplasia, then regulations state that affected carcases must be condemned. Because export abattoirs often purchase horses as mobs, the presence of grey animals within the mob represents sometimes significant loss to the company. The ability to predict the extent of any disease ante-mortem would help minimize these losses.
The objectives of this study included an attempt to detail the development and spread of the
tumour in grey horses and formulate a protocol whereby internal presence of the tumour could be
predicted by external markers. Histopathological, immunohistochemical and ultrastructural
examination of tumours was utilized to try and gain a better understanding of the nature of the
condition. Similar examinations of hair follicles from coloured, greying and white horses were
employed to try and document the morphological changes occurring in follicular melanocytes as
they lost the ability to produce pigment for inclusion in hair; the aim being to identify which of
the pigmentation genes were most likely to be involved in the process of greying in the horse.

An abattoir survey was conducted at a pet food abattoir in south-east Queensland. Ninety-seven
grey horses and sixteen coloured horses were examined. The age of each animal was estimated
and recorded, along with sex, pattern of coat colour dilution, presence or absence of vitiligo or
externally visible lesions. Pigmented tumours were recorded as either “melanosis” (flat foci of
pigmentation or a small focus of pigmentation around the base of a hair follicle) or “melanotic
tumour”. Animals were said to have generalized disease if lesions were recorded in 4 or more
sites.

Findings of the abattoir survey included;

- A significant difference between horses under 7 years of age and over 7 years of age in the
  presence of melanotic tumours. All 22 horses classified as very aged (≥15 years) had lesions.
- Generalized disease occurred in 25% of young horses (≤7 years) with lesions, 36.4% of aged
  horses (7-15 years) with lesions and in 72.3% of very aged horses.
- No differences could be demonstrated between the different patterns of coat colour dilution
  and prevalence of lesions. The dappled pattern was most obvious in young horses, but the
  flea-bitten pattern was more common in older animals. In many horses, no particular pattern
  was discernible.
- There was no sex predilection.
- The presence or absence of vitiligo had no affect on the prevalence of melanotic lesions.
- The presence of a lesion detectable on ante-mortem examination of a horse was a strong
  predictor of generalized disease. However, of the 53 horses for which no lesions were
  detectable ante-mortem, 10 were subsequently found to have generalized disease.

In other words, all grey horses appear likely to develop disease if they live long enough; tumour
development can be thought of as an inevitable consequence of coat colour dilution. There is no
sex predilection. There is no apparent link between the pattern of coat colour dilution or the
development of vitiligo and the development of melanotic tumours. Abattoirs are limited in their
ability to predict generalized disease by a simple visual inspection of horses ante-mortem, though
the presence of any obvious lesions strongly suggests the animal’s carcase is likely to be affected
by generalized disease.

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The distribution of lesions was also informative. Of those horses in which melanotic lesions were detected, 97.6% had involvement of the ventral tail, 48.1% had involvement of skeletal muscle groups, 46.3% involvement of the parotid salivary gland, 37.8% showed involvement of the perineum, 37.7% involvement of the lips, 32.9% involvement of the arterial walls, 30.5% involvement of abdominal fat, 30.2% involvement of the fascia of the face and head and 23.2% of affected animals showed involvement of the anorectal lymph nodes. Skeletal muscle involvement was especially obvious in regions close to the axial skeleton. Connective tissue structures (e.g. skeletal muscle fascia) were the main sites of involvement rather than organ parenchyma.

The pattern of lesion distribution often gave the impression of multicentric origins rather than metastases from a solitary primary tumour.

This is a somewhat surprising pattern of lesion distribution if the condition is in fact a true neoplasia; visceral organs with extensive capillary networks such as the liver and lung are frequently targeted in malignant disease. There is however some correlation between this pattern of lesion distribution and the likely paths of neural crest cells as they migrate towards the skin during embryological development.

The earliest change identifiable at a microscopic level was the accumulation of pigment around and below hair bulbs to form a “cup” of melanin pigment. This was seen in the permanent hairs of the ventral surface of the tail but not in association with the general cover hairs of the body or the other coarse permanent hairs of the mane and lateral tail. In sections which included both white and coloured hairs, the lesions were most prominent around follicles of white hairs. Pigment was first seen in the loose connective tissue outside the external hair sheath between the level of the follicular isthmus and the dermal papilla. In other species, melanocytic precursor cells have been identified around the dermal papilla and in the outer hair sheath of post-natal animals.

Histological, immunohistochemical and ultrastructural examination of tumours revealed little similarity between the grey horse melanomas and the classic malignant melanoma seen in other species. Skin tumours consisted of at least two populations of cells; rather small cells with oval to spindle shaped nuclei (which stained positively with S100, a marker of melanocytes and other cells) and larger polyhedral cells replete with melanin granules (which stained positive with macrophage markers). Inflammatory cells (other than macrophages) and epidermal involvement were not detected and the melanocytes generally showed uniform nuclei with heterogenous chromatin dispersal and very few mitotic figures or apoptotic bodies. This picture was consistent with a very slowly growing benign growth. There were many similarities in histological appearance between the equine condition and the human cellular blue naevus; a biphasic pattern is common in both and both are centred on the dermis. In addition, both may on occasion populate regional lymph nodes.
Some visceral lesions were less obviously benign. Melanocytes have been demonstrated ultrastructurally. Histologically, the melanocytes may show more pleomorphism and tumour cells may also push along areolar connective tissues or separate and disrupt collagen fibres. These lesions however, still had more features of a benign condition than an overtly malignant one.

Although these tumours do share histological and ultrastructural features with the human blue naevus, the pattern of lesion distribution is completely different; the human condition generally involves a single lesion. The comparison with the blue naevus is also not particularly informative as it is itself a very poorly understood condition.

The condition can not be said to represent a storage disease in the true sense of the word, as melanocytes are present in visceral lesions.

Histological features of malignancy are rare and the pattern of tumour distribution is also unusual for a malignant growth but this study has not been able to rule out the possibility that the grey horse melanoma represents a very slowly progressive neoplasia (perhaps arising from dermal melanocytes or melanocytes from the infundibular external hair sheath).

The other possibility supported by the current study is that of a pre-existing network of melanocyte precursor cells. Under this scenario, melanoblasts or other neural crest derived cells would normally be present at the tumour predilection sites due to drop-out during embryonic neural crest cell migration. Such cells may then be stimulated to differentiate into active pigment producing cells as a response to the process of coat colour dilution. The typical grey horse melanoma picture would thus represent hyperplasia of a pre-existing network of cells which were normally inapparent. It would not be surprising given the long course of this condition, and current knowledge of the step-wise progression of events leading up to cancer, if a neoplastic process was occasionally superimposed upon this hyperplastic stage.

Ultrastructural studies of dermal papillae from greying horses reveals a population of cells with many of the features of melanocytes but with only occasional or no pigment producing organelles (the melanosomes). This suggests that the melanocytes may persist for some time beyond when they cease producing melanin pigment; the original lesion does not seem to involve failure of melanocytes to survive in the follicular environment. Also the melanosomes identified were granular in structure, as seen in the phaeomelanosomes which produce yellow or red melanins. $\alpha$-MSH and the product of the agouti locus (agouti signal protein) interact in a complex manner to determine both the level of protein synthesis and the type of melanin produced. Phaeomelanins are produced when $\alpha$-MSH activity is comparatively low and low levels of this hormone have in fact been previously demonstrated in grey horses. Our study thus lends support to the suggestion that $\alpha$-MSH activity has a central role in the process of coat colour dilution in these horses. Further work might profitably focus on the genes encoding the melanocortin 1 receptor of melanocytes and the agouti signal protein. The mechanisms of coat
colour dilution and melanotic tumour development appear to be inextricably linked so a complete understanding of the pathogenesis of these tumours is likely to require an explanation of the process underlying greying of the hair.
Introduction

(i) Background

Grey horses may be born any colour but, with increasing age, their coats become grey and then white; they could more accurately be referred to as "greying" horses. Despite dramatic recent advances in the investigation of coat colour genetics, little is known about the mechanism/s causing greying in the horse. The pattern of coat colour dilution varies to an extent; horses may be either dappled (a network of darker areas imposed over lighter grey areas) or flea-bitten (small flecks of colour persisting throughout the coat) and their points may turn white with the rest of the body or remain dark (Sponenberg and Beaver, 1983). Rarely large patches of red hair may grow into the coat of a grey horse; these are referred to as blood marks and can apparently become progressively larger until a grey horse appears uniformly red (Sponenberg and Beaver, 1983). Although skin colour in general appears to be unaffected, some grey horses develop focal, patchy vitiligo - particularly around the eyes and anus (Lerner and Cage, 1973).

Concurrent to the dilution in coat colour, melanotic tumours frequently develop. Most modern texts quote (without acknowledgement) van Dorssen's 1903 study of 235 grey horses belonging to the Amsterdam Street railway; no lesions were seen in horses under the age of 6 years but 80% of horses older than 15 were found to possess pigmented lesions. Congenitally white or grey horses, and horses of other colours, have little risk of acquiring the disease (Levene, 1971).

No data has been published to indicate if all grey horses are equally at risk or if certain "types" have a higher prevalence; i.e. dappled or flea-bitten, those with vitiligo, those with white points etc. Most authors discount a sex predilection but there has been some debate on this question (Kerr and Alden, 1974; Levene, 1971).

(ii) Tumour Pathology

Lesions develop as one or more firm, raised, smooth nodules in regions of dark glabrous skin (i.e. perineum, ventral tail, external genitalia) and/or the parotid region (Levene, 1971; Yager and Scott, 1993). Ulceration of the epidermis overlying skin tumours is uncommon. On cut section, tumours are smooth and glistening black with, at most, only a touch of brown. Rarely, parts of an individual mass may contain varying degrees of pallor (McFadyean, 1933). Less commonly mentioned sites include the nuchal ligament at the base of the mane, the rhomboid muscles, the pinnae and periocular regions and the skin of the shoulder or lip or distal limbs (Levene, 1971).

Some authors have felt that there was no favoured primary site for tumours (Runnels and Benbrook, 1941) and, in fact, there is a certain confusion in the literature on the typical distribution of lesions; while many reviews do not mention some of the sites listed above, other papers claim frequent involvement of the small bowel, brain, spleen or various other soft tissues (Lerner and Cage, 1973; Mangrulkar, 1944). While skeletal muscle involvement is often overlooked, it is widely recognized by veterinarians working in abattoirs that slaughter horses (Furrow, Shalkop and Sturkie, 1977).
Tumour growth is slow but masses may attain a large size. This is reflected in the clinical course of the disease which is generally very long (several years); any problems seen reflect mechanical interference from the size and position of the masses; constipation, interference with mating, rubbing of girth straps etc. Occasionally a benign growth is said to assume features of malignancy and metastasize widely and rapidly, leading to the demise of the horse (Theilen and Madewell, 1987).

It is unknown if the condition arises as a single primary skin tumour which then metastasizes or if it is multicentric in origin.

Cellular details of tumours are frequently obscured by heavy pigmentation. Skin tumours consist of a mixture of benign-appearing spindle cells and larger, pigment-laden polyhedral cells (which often predominate). Most authors presume that these are melanocytes and macrophages respectively. There is no junctional activity and a sub-epidermal *Grenz* zone is invariably present. Malignant features such as mitotic activity and evidence of anaplasia are rare. It is not clear that visceral lesions do contain melanocytes or whether they consist solely of macrophages replete with melanin. (Yager and Scott, 1993; Levene, 1971)

No immunohistochemical studies of the condition have been reported. Such studies offer the prospect of verifying that the two cell populations seen in skin lesions do, in fact, represent melanocytes and macrophages. Also they will allow the determination as to whether visceral tumours contain melanocytes.

The ultrastructural features of the tumours have received little attention. Examination of skin lesions has verified that these masses consist of a mixture of melanocytes and macrophages but the authors noted the need to examine visceral lesions to ascertain if they also contain pigment cells (Ghadially, 1988). Ohmuro *et al* (1993) described the formation of compound melanosomes (the organelles responsible for melanin production) in cells from a skin lesion, but shed little light on the nature of the condition, neoplastic or otherwise.

(iii) The Pathogenesis of Grey Horse Melanoma

While this condition is well recognized and very common, it remains rather engimatic and there is considerable disagreement as to what the underlying disease process actually is.

In view of the fact that the condition is only seen in greying horses, it seems likely that the processes of coat colour dilution and tumour development are linked. Greying is inherited as a character dominant to non-greying. Homozygotes are reputed to become white faster than heterozygotes but this is not certain; independent modifiers may also influence the rate of coat colour dilution (Sponenberg and Beaver, 1983). The responsible gene has been designated the *G* locus and is said to be epistatic to the basic coat colours such as bay, chestnut or black (Jones, 1982).
Visible pigmentation in the skin and hair is the culmination of a complex series of events; embryonic melanoblasts must develop in the neural crest and migrate to their final destinations, melanocytes must survive and proliferate once in place, melanocytes must function in response to environmental stimuli, and melanosomes must be distributed to neighbouring keratinocytes where they generally undergo subsequent processing (Furumura et al., 1996). Over the past decade there have been dramatic advances made in understanding the physiology and genetics of pigmentation using DNA technology and mutant strains of mice. Silvers, writing in the pre-molecular biology era (1979) was able to identify over 60 genes influencing mouse coat colour. Even more remarkable was the report by Furumura and co-workers (1996) that one quarter of these genes have now been cloned and characterized.

The mechanism of coat colour dilution in horses has received little attention. Examination of the morphological changes in the follicular melanocytes of greying horses is an important step as it will allow the search for candidate genes to be more finely focussed. An interesting observation was made by Altmeyer and co-workers (1984); plasma melanocyte stimulating hormone (α-MSH) levels decline as horses turn grey. α-MSH induces melanocyte proliferation and stimulates the production of pigment by melanocytes. Two of the most important coat colour genes, extension and agouti, code for α-MSH receptors and an α-MSH antagonist protein respectively; interactions between the two have an important influence on the level and type of melanin production (Furumura et al., 1996).

Much of the debate on the nature of the tumour itself has been reviewed by Levene (1971) who prefers the term "equine melanotic disease" to grey horse melanoma. Some consider it to be a true neoplasm, albeit not an aggressive one. Other people consider it to be a storage disorder following an abnormality of pigment metabolism (which is reflected in the dilution of coat colour). Levene likened it to the human blue naevus (a poorly understood type of mole which originates in the dermis and is not malignant in itself but which has the potential for malignant change). A further explanation is that there may be a widespread distribution of melanocytes which acquire neoplastic properties and form melanin deposits (which would explain a multicentric origin).

Internal masses may thus be metastases, or accumulations of pigment in melanophages as part of a storage disorder, or ectopic nests of pigment cells somehow stimulated to produce excess pigment.

(iv) Economic and Medical Aspects

Determining the sequence of events leading to the development of these melanotic tumours will answer important questions regarding the true nature of this condition. The principle benefit of the current project is to increase knowledge of a common but poorly understood disease of horses.

There are important economic consequences of this disorder for the Australian horse industry. Melanotic tumours, by virtue of their size and location, occasionally produce clinical disease in grey horses; veterinary journals are littered with reports of such cases. Undoubtedly these
tumours can also depress the monetary value of affected horses. Clinical management is problematic; surgery is unlikely to be curative and chemotherapy would appear unnecessary in view of the doubtful malignancy of the lesions (Goetz and Long, 1993). There have been conflicting reports of treatment with cimetidine (Goetz, Boulton and Ogilvie, 1989; Bowers, Huntington and Slocombe, 1994); the original rationale seemingly based on anti-tumour properties of this drug in some human malignant melanomas. It is apparent that, until the condition is properly characterized, practitioners will continue to have difficulty developing appropriate management plans for dealing with it.

Exports of horse meat from Australia for human consumption began in the late 1970s and developed rapidly before stabilizing at around 8000-9000 tonnes per annum. In 1991-92, 8850 tonnes of horse meat worth $23.8m were exported from Australia, equivalent to $2.69/kg (Ramsay, 1994). There are two export abattoirs processing horses in Australia; in Peterborough, South Australia, and in Caboolture, Queensland. Grey horses are frequently purchased as part of a mob and pose a dilemma for abattoir management and, in particular, for the meat inspectors. The regulations governing post-mortem decisions for export meat orders state;

"41.7 Malignant melanomata must result in condemnation of affected carcases and carcase parts.

41.8 Benign melanomata must be excised together with a margin of normal tissue and condemned, with the remainder of the carcase and its carcase parts being passed as fit for human consumption."

Meat inspectors in horse abattoirs are thus regularly placed in a position where they need to judge if this condition is truely malignant melanoma.

The likelihood of internal melanotic lesions obviously means that there is a high risk that the carcase of a grey horse will be condemned for human consumption; either due to spoilage from the heavy pigmentation in carcases with extensive involvement or because a few lesions cannot be definitively said to be benign. Grey horses are thus often sold on to pet food abattoirs and represent a loss of income to the export industry. Wholesale value of horse meat sold as pet food is around $1.10 - $2.00 per kilogram (Ramsay, 1994). Two issues arise; can the risk of visceral lesions be assessed by a visual examination of a horse ante-mortem and is this condition malignant melanoma?

A clearer understanding of the melanotic lesions of greying horses will also contribute to a more complete picture of melanocyte physiology and pathophysiology. This is useful at a time when the incidence of malignant melanoma continues to increase in the human population, afflicting large numbers of relatively young members of the community (Armstrong, 1994).
Objectives

1. To increase the acceptability of grey horses to export abattoirs by detailing the development and spread of the tumour and formulating a protocol whereby internal presence of the tumour can be predicted from external markers.

2. To determine the precise nature of the condition (e.g. neoplastic or storage disease) by post-mortem examination of affected animals and by the use of histopathological, ultrastructural and immunohistochemical techniques.

3. By detailing the nature of the condition, to allow for the development of better treatment regimes and to permit meaningful comparisons to be made between this condition and similar conditions in other species (e.g. the malignant melanomas of goats and humans, the blue naevi of humans and melanocytic tumours of dogs).

4. To provide preliminary information on which, if any, of the currently identified pigmentation genes are likely to be involved in greying of the horse by comparisons of the skin and coat melanocytes (pigment producing cells) of young and aged, grey and non-grey horses.

5. To provide background information to form the basis of future studies on the effects of α-MSH on pigmentation and tumour development in the horse (e.g. presence or absence of melanocytes in hair follicles and sub-cellular structural changes in the melanocytes may reflect α-MSH effects).
Methodology

(i) Abattoir Survey

The survey was conducted at a pet food abattoir at Rosewood in south-east Queensland. Because it deals in pet food, this facility almost exclusively handles grey horses (which are cheaper because the abattoirs exporting horse meat generally do not slaughter greys). Horses at this facility are bought at sales from a wide area of south-east Queensland and northern New South Wales. The export abattoir at nearby Caboolture frequently sells grey horses purchased in mobs of horses onto the various local pet food abattoirs. The horses slaughtered at Rosewood therefore originate from a huge "catchment area" which probably covers much of Queensland and New South Wales.

It was realized that slaughtered horses were likely to have a highly variable nutritional history which would be reflected in dental wear patterns. This along with the fact that most horses purchased by abattoirs were likely to be of mature age meant that achieving accurate age estimates was likely to be difficult. For these reasons, it was decided to simply separate horses into three age classes; young (7 years and younger), aged (from 7 years to 15 years) and very aged (over 15 years). Estimates of age were made according to the American Association of Equine Practitioners' Official Guide for Determining the Age of the Horse (AAEP, 1966).

At slaughter, the sex and age class of each horse was recorded. Animals were then examined for externally visible lesions, presence or absence of vitiligo, and pattern of coat colour dilution (i.e. dappled or flea-bitten, dark or white points). As each animal was dissected, a record was kept of any pigmented lesions; these were recorded as melanosis (flat foci of pigmentation or a small focus of pigmentation around the base of a hair follicle) or a melanotic tumour. An animal was said to have generalized disease when lesions were recorded in 4 or more different anatomical sites.

Due to abattoir procedures, it was not always possible to follow carcases through to "boning out" but, of those carcases that were followed in this way, no horses were found that only had lesions detectable at boning out. The skull was retained from ten animals to allow examination of the brain.

Ninety-seven greying horses and sixteen coloured horses were examined.

(ii) Immunohistochemical and Histopathological Studies

Tissue samples were collected from slaughtered horses, formalin fixed and then paraffin embedded by standard procedures. From each horse, samples were taken from the parotid gland and skin of the ventral tail, perineum and ventral mid-line of the neck. Melanotic lesions were collected in a similar manner.
For routine histopathology, sections were stained with haematoxylin and eosin; bleaching was also performed on some tumour sections. Skin sections were examined and compared between young and aged horses, and between greying and coloured animals. Particular attention was paid to melanocytes around the dermal papillae of anagen phase hair follicles. When examining tumours, particular attention was paid to features that might reflect malignancy and visceral tumours were compared with skin lesions.

Representative specimens of tumours were further investigated using immunohistochemical techniques; the aims being to gather qualitative evidence regarding the cell type/s present and the level of proliferative activity within these tumours. Primary antibodies and negative controls were purchased from Dako corporation. Antibodies purchased detected the following antigens:

**S-100A** is a calcium binding protein normally expressed in a range of cell types, including glial cells, melanocytes and chondrocytes. It will also stain various tumours arising from these cell lines. Antibody used was a rabbit anti-bovine S-100A. Antibodies to S-100 protein are extensively used in medical diagnostic immunohistochemistry, generally as one of a "panel" of antibodies (Fleming and Bergfeld, 1990).

**HAM56** is expressed by tissue macrophages, monocytes and some endothelial cells. The mouse anti-human macrophage monoclonal antibody used is prepared by immunizing mice with human alveolar macrophages. It has been shown to reliably stain melanophages in human pigmented skin lesions (Fleming and Bergfeld, 1990). **CD68**, another macrophage marker, was used on some sections.

**HMB45** is found in premelanosomal vesicles (Schaumburg-Lever, Metzier and Kaiserling, 1991). It has been demonstrated in human foetal and neonatal melanocytes, junctional and blue naevus cells and some perivascular smooth muscle cell-derived tumours. Intradermal naevi, normal adult melanocytes and other cell types are not labelled by antibody to HMB45 in human tissues. It reacts with the majority of human melanomas and tumours showing melanoma/melanocytic differentiation. The antibody used in this study was a mouse anti-human melanoma monoclonal.

**PCNA**, or proliferating cell nuclear antigen, is an auxiliary protein for DNA polymerase δ which initiates leading-strand DNA synthesis. PCNA also plays a role in DNA repair so it is not necessarily only expressed during the S phase of the cell cycle. There has been some debate over the clinical usefulness of PCNA antibodies as a guide for prognosis in human cancer biopsies (Yu and Filipe, 1993). However it was considered useful in our investigations because it is known to be a highly conserved protein, the antibody is suitable for use on paraffin-embedded tissues, and we were interested in qualitative evidence of proliferation rather than trying to quantify exact levels. The mouse monoclonal antibody, PC10, was used.

**Controls** The same negative control reagent was used for HAM56, HMB45 and PCNA runs; foetal calf serum (supplied by DAKO). For S100A, the negative control used was nonimmune rabbit serum. S100A tested sections contained internal positive controls (epidermal melanocytes, nervous tissue). Similarly, liver sections were included in HAM56 tests (Kupffer cells positive)
and bone marrow from a healthy young cat was used as a positive control for PCNA. No human melanoma tissue was available as a positive control for HMB45; an anaplastic canine malignant melanoma was included as a positive control.

A commercial kit based on the labelled streptavidin-biotin method was used (L.V. DAKO LSAB®2, HRP). Sections were collected on to poly-L-lysine coated slides. All except those to which PCNA was to be applied were incubated initially in a 0.1% trypsin solution in Tris buffer (pH 7.6) containing 0.1% calcium chloride at room temperature for 5 minutes. Endogenous peroxidase was quenched by incubation in 3% hydrogen peroxide (5 minutes). Sections were then incubated in the primary antibody (10 minutes) followed by the biotinylated link antibody (10 minutes) and then peroxidase-labelled streptavidin. Slides were then incubated in the chromagen, DAB or 3,3’-diaminobenzidine tetrahydrochloride, for 5 minutes to produce an insoluble brown end-product. Counterstaining with haematoxylin, mounting and cover-slipping completed the procedure.

(iii) Ultrastructural Studies

Specimens examined include melanotic tumours (both dermal and visceral) recovered from freshly dead animals and plucked hairs from grey horses and coloured horses. Attention in the examination of the hairs has focused on the dermal papillae; do any melanocytes persist and, if so, do they show any sub-cellular abnormalities?

Specimens were fixed in cold 3% glutaraldehyde in 0.066M cacodylate buffer, pH 7.2, containing 2.5 mM CaCl₂. Samples were then post-fixed in 1% osmium tetroxide in 0.066M cacodylate buffer, then dehydrated through graded methanols (30% initial up to 100% final concentration) and embedded in hard Spurr's resin. The resin was infiltrated for one hour each at 25%, 50%, 75%, 100%, then at 100% overnight at 4°C for 3 nights before polymerisation at 60°C for 48 hours. Thin sections were cut on a Reichert Ultracut E and stained with 5% uranyl acetate in 50% methanol and Reynold's lead citrate before viewing in the transmission electron microscope.
Results

(i) Prevalence of Lesions - Effects of Age

Seventy greying horses were completely dissected. This included fifteen young horses of which 8 had melanotic lesions (53.3%), 33 aged horses of which 30 had lesions (90.9%) and 22 very aged horses which all had lesions. There is a significant difference ($P = 0.0005$ by Fisher exact test) between the young group of horses and those over the age of 7 years in the prevalence of melanotic lesions (see Table 1).

<table>
<thead>
<tr>
<th>Age</th>
<th>No Lesions</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young ($\leq$ 7 years)</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>Aged ($&gt;7$ years)</td>
<td>3</td>
<td>52</td>
</tr>
</tbody>
</table>

The observations in the different categories are significantly different than expected from random occurrence ($P = 0.0005$) using the Fisher exact test.

The extent of disease also varied with age. In most young horses (62.5%, or 5 out of 8) with lesions, they were confined to the tail. Of the aged horses with lesions, 23.3% (7 out of 33) showed tail involvement only whereas 13.6% of the very aged horses (3 out of 22) showed such a restricted distribution of lesions. Similarly, generalized disease occurred in 25% of young horses with lesions, 36.4% of affected aged horses and 72.3% of very aged horses (see Table 2).

<table>
<thead>
<tr>
<th>Age</th>
<th>No Lesions</th>
<th>Restricted Disease ($\leq 3$ lesions)</th>
<th>Generalized Disease ($\geq 4$ lesions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young ($\leq$ 7 yrs)</td>
<td>7</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Aged (7-15 yrs)</td>
<td>3</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Very Aged ($\geq$15 yrs)</td>
<td>0</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

There is an increased prevalence of melanotic lesions in grey horses as they age, and also an increased chance that such lesions will involve several regions of the body.

(ii) Prevalence of Lesions - Effects of Colour Dilution Pattern
Many horses were uniformly grey or white so that a pattern of coat colour dilution could not be ascertained. Flea bitten greys were seen much more frequently than dappled greys (20 flea bitten and only 6 dappled). Age appeared to be a significant confounding factor; all dappled greys were young horses but only 2 of the 20 flea bitten greys fell into this category. Similar problems were identified in comparing horses in which the hair colour of the points appeared to fade as part of the general coat colour dilution with those horses in which the points remained dark while the general coat colour had become diluted. These results are shown in tables 3 and 4; no statistically significant differences were able to be demonstrated between the general coat colour dilution patterns and the prevalence of pigmented lesions. A Fisher exact test comparing lesion prevalence between white points and dark points revealed no significant difference between the two patterns (P = 0.0999).

One horse was identified with a large blood mark over its left shoulder region. It was an aged mare and showed melanosis of the ventral tail and lip, and melanotic tumours in skeletal muscle fascia.

### Table 3: Effects of Colour Dilution Pattern on Lesion Prevalence

<table>
<thead>
<tr>
<th>Colour Dilution Pattern</th>
<th>No Lesions</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flea bitten grey</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td>Dappled grey</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Uniform dilution</td>
<td>4</td>
<td>27</td>
</tr>
<tr>
<td>White Points</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Dark Points</td>
<td>5</td>
<td>8</td>
</tr>
</tbody>
</table>

Uniform patterns of coat colour dilution or the flea-bitten pattern were most common among grey horses examined; no meaningful analysis of the prevalence of melanotic lesions was possible between the different patterns. Horses in which the hair of the points faded with the body showed no difference in the prevalence of lesions than horses in which the hair of the points remained dark (P = 0.0999 using the Fisher exact test).

### Table 4: Interaction Between Age and Coat Colour Dilution Pattern

<table>
<thead>
<tr>
<th>Colour Dilution Pattern</th>
<th>Young (≤7 yrs)</th>
<th>Aged (7-15 yrs)</th>
<th>Very Aged (≥15 yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dappled</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Flea-bitten</td>
<td>2</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Uniform dilution</td>
<td>4</td>
<td>18</td>
<td>9</td>
</tr>
</tbody>
</table>
The patterns of coat colour dilution are not evenly distributed between the different age classes of horses. Horses classified as having a uniform dilution pattern were those in which neither a dappled nor a flea-bitten pattern could be identified.

(iii) Prevalence of Lesions - Effects of Sex

No sex predilection for pigmented lesions could be demonstrated with this survey; 85.2% of geldings and 86.0% of mares were found to have lesions. There was no significant difference between mares and geldings (P=1.0) using the Fisher exact test (see Table 5).

<table>
<thead>
<tr>
<th>Sex</th>
<th>No Lesions</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geldings</td>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>Mares</td>
<td>6</td>
<td>37</td>
</tr>
</tbody>
</table>

There is no significant difference between geldings and mares in the prevalence of melanotic lesions (P = 1.0000 by the Fisher exact test).

(iv) Prevalence of Lesions - Effects of Vitiligo

The proportion of horses with vitiligo on the muzzle, eyes and/or perineum, and which also had melanotic lesions, was not statistically different to the proportion of horses with no vitiligo which were afflicted (P = 1.0000 by the Fisher exact test), as illustrated in table 6.

<table>
<thead>
<tr>
<th>Vitiligo of Muzzle, Eyes &amp;/or Perineum</th>
<th>No Lesions</th>
<th>Lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>2</td>
<td>18</td>
</tr>
<tr>
<td>Absent</td>
<td>7</td>
<td>47</td>
</tr>
</tbody>
</table>
There is no statistical difference in the proportion of horses with melanotic lesions between those with vitiligo and those without vitiligo ($P = 1.0000$ by the Fisher exact test).

(v) Predictors of Generalized Disease

Table 7: Ante-Mortem Identification of Tumours as a Predictor of Generalized Disease

<table>
<thead>
<tr>
<th>Lesions Detectable Ante-Mortem</th>
<th>No Lesions or Lesions at ≤3 Sites</th>
<th>Generalized Disease (Lesions at ≥4 Sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesions Found</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>No Lesions Found</td>
<td>43</td>
<td>10</td>
</tr>
</tbody>
</table>

The presence of externally visible lesions on ante-mortem inspection of horses is a strong predictor of generalized disease ($P < 0.0001$ by the Fisher exact test). However, 10 out of 53 horses in which no lesions were detectable at ante-mortem inspection also had generalized disease.

As illustrated in table 2, the greater the age of an animal, the more likely it was to have melanotic lesions in four or more distinct anatomical sites (i.e. generalized disease for the purposes of this study).

Animals were examined ante-mortem, with particular attention being paid to the perineal regions and ventral surface of the tail. Identification of a tumour at this stage was a strong predictor of generalized disease (16 out of 17 horses with externally visible tumours had generalized disease). See Table 7.

However of the 53 horses in which no lesions were detected ante-mortem, 10 had generalized disease (18.9%), 33 had lesions in 3 sites or fewer (62.2%) and 10 had no lesions at all (18.9%). Fifteen of the 33 horses with lesions at 3 sites or fewer had lesions confined to the tail only.

(vi) Lesion Distribution

In total, 82 grey horses were found to have melanotic lesions. The distribution of lesions is illustrated in table 8; not every anatomic site was examined in each of the 82 horses. By far the most common site for lesions was the ventral surface of the tail, which was involved in all but two horses (97.6% of affected animals). Next in prevalence were skeletal muscles (48.1%) and the parotid salivary gland region (46.3%). Skeletal muscle lesions occurred in close proximity to the axial skeleton, especially involving the hypaxial groups but also muscles such as the serratus ventralis (region of insertion onto cervical vertebrae) and the rhomboideus thoracis (near the
origins of the muscle on the spinous processes of T1-T7). Both the skeletal muscle and the parotid gland region lesions appeared to involve the superficial fascia of these organs rather than organ parenchyma.

**Table 8: Distribution of “Grey Horse Melanoma” Lesions**

<table>
<thead>
<tr>
<th>Site</th>
<th>No. of Horses with Lesions at Site</th>
<th>No. of Horses with Lesions in which this Site was Examined</th>
<th>% of Affected Horses in which this Site was Involved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ventral tail</td>
<td>80</td>
<td>82</td>
<td>97.6</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>37</td>
<td>77</td>
<td>48.1</td>
</tr>
<tr>
<td>Parotid salivary gland</td>
<td>38</td>
<td>82</td>
<td>46.3</td>
</tr>
<tr>
<td>Perineum</td>
<td>31</td>
<td>82</td>
<td>37.8</td>
</tr>
<tr>
<td>Lips</td>
<td>20</td>
<td>53</td>
<td>37.7</td>
</tr>
<tr>
<td>Arterial walls</td>
<td>25</td>
<td>76</td>
<td>32.9</td>
</tr>
<tr>
<td>Abdominal fat</td>
<td>25</td>
<td>82</td>
<td>30.5</td>
</tr>
<tr>
<td>Face/fascia of head</td>
<td>16</td>
<td>53</td>
<td>30.2</td>
</tr>
<tr>
<td>Anorectal lymph nodes</td>
<td>19</td>
<td>82</td>
<td>23.2</td>
</tr>
<tr>
<td>Thoracic cavity</td>
<td>3</td>
<td>82</td>
<td>3.7</td>
</tr>
<tr>
<td>Kidney capsule</td>
<td>1</td>
<td>82</td>
<td>1.2</td>
</tr>
</tbody>
</table>

The tail is the major site for involvement of melanotic lesions; all but two horses with evidence of the condition exhibited tail involvement. The parotid salivary gland region and skeletal muscles close to the axial skeleton were involved in nearly half of all horses. The glabrous skinned perineum and lip each were involved in around 37% of affected horses. Fascia of the head, abdominal fat and arterial walls each were involved in around 30% of affected animals. The anorectal lymph nodes were involved in 23.2% of affected horses.

The perineum and lip followed with 37.8% and 37.7% respectively. The fascia of the face and base of the ears, the abdominal fat, and arterial walls (particularly the external iliac artery) each were found to have melanosis or small tumours in around 30% of those horses in which these sites were examined. The anorectal lymph nodes were involved in 23.2% of cases. Sporadic lesions were found in the kidney capsule (1.2%) and the thoracic cavity (3.7%). Lesions were not found involving the parenchyma of viscera in any animals. There was no brain involvement in any of the ten horses from which the skull was retrieved.

**Histopathology**

The most consistently encountered lesion was the formation of a “cup” of melanin pigment around and below hair bulbs in the ventral skin of the tail. In those sections which included white and coloured hairs from the one animal, lesions were more prominent around follicles of white
hairs. The overlying epidermis was normal, but pigment granules were quite prominent in the infundibular external hair sheath cells. The most subtle lesions consisted of focal accumulations of pigment in the loose connective tissue outside the external hair sheath between the level of the follicular isthmus and the dermal papilla. In some areas the pigment appeared to be contained within elongate, spindle-shaped cells but in other areas strings of coarse melanin granules were seen; either enclosed within a dendritic cell process or simply melanin granules trapped between collagen fibres? Similar lesions were not seen in sections of ventral neck skin (typical cover hairs grow in this region) and were uncommon in glabrous skin (perineum and lip).

In more established lesions, the cellular nature of the process was clearer and consisted mostly of small cells with oval to spindle shaped nuclei. These cells were mostly arranged in a rather haphazard, swirling pattern but attempts at nest formation were also regularly seen. Mitotic figures, nuclear pleomorphism and other features of malignancy were not obvious. Larger polyhedral cells, replete with coarse melanin granules (presumably macrophages) occurred regularly; in smaller lesions they were more common in peripheral regions. In heavily pigmented regions, these cells sometimes coalesced to form heavily pigmented, multinucleate giant cells. Melanin granules were gathered together in the large polyhedral cells or occurred as "undulating lines" of pigment following the orientation of the adjacent collagen fibres. There was an impression of increased collagen density or desmoplasia within the lesions; whether this reflects crowding of the spaces between collagen bundles with melanin and cells, the production of new ground substance or laying down of new collagen fibres was unclear. In apparently more mature lesions, collagen fibres appeared to become disrupted, and then replaced. In such lesions, there was also often involvement of the stroma around apocrine sweat glands, blood vessels and nerves and in some sections the accumulations of pigment appeared to follow the contortions of the intermediate plexus. There was no attempt at encapsulation of lesions but they were generally well circumscribed. Apart from macrophages, inflammatory cells were not obvious.

All skin tumours were centred on the dermis; even with large perineal and tail lesions, there was a Grenz zone separating epidermis from tumour. Lesions in the perineum were similar to those described in the tail. The perineal epidermis was heavily pigmented and small amounts of pigment were sometimes scattered through the superficial dermis and around the adnexa; this melanosis rarely approached the extent of pigment accumulation in tail lesions. Lip lesions tended to be more cellular and unstructured (sebaceous glands may be eliminated from affected regions) but with essentially the same population of small bland cells. Although a Grenz zone was less identifiable in lip lesions, the overlying epidermis showed no obvious pathology.

In minimally affected lymph nodes, the only change was a scattering of large cells containing pigment in the subcapsular and trabecular sinuses. Occasional pigmented cells were also seen within lymphoid follicles. Eventually, lesions similar to those identified in the dermis develop (complete with desmoplasia). These appeared to be centred on similar areas. Finally there is development of typical melanotic tumours which obliterate normal lymph node; however, islands of essentially normal lymph node structure persist randomly throughout the lesion. There was little evidence of direct invasion of node structure. Tumours here, like other visceral tumours, were sometimes "phasic" in appearance (see below).

The most striking feature of visceral lesions was that they were centred on connective tissue supporting or surrounding organs and very rarely affected organ stroma itself. For instance, even large lesions (>3 cm diam) involving the parotid salivary gland were found histologically to
centre on connective tissue surrounding the gland and on inter-lobular septae but to spare the glandular epithelium. Similarly, lesions involving the skeletal muscle were in fact centred on the connective tissue fascia and support of these muscles. Collagen fibres were disrupted by pigment and the typical cell populations described in the skin lesions. Tumour cells appeared in some sections to push along the areolar connective tissue between bundles of collagen fibres or around small blood vessels. On the periphery of established lesions, smaller cells occasionally trickle off into and between adjacent, unaffected collagen fibres. Arterial lesions consisted of foci of pigmentation (generally contained within large polyhedral cells) scattered throughout the tunica adventitia. The arterial lesions were poorly circumscribed and appeared to trickle along between collagen fibres and into adipose and other areolar connective tissue.

Another feature of visceral lesions was a tendency towards greater variability in appearance of tumour cells. Desmoplasia was present to varying degrees. In some sections, the smaller cells showed a moderate degree of size variation with nuclei that occasionally had multiple nucleoli. There was often a clear zone surrounding the nucleus, with pigment present in strands peripheral to this zone. This variability within tumours may impart a phasic appearance to the lesion; some distinct regions were heavily pigmented with a cell population consisting mostly of replete, polyhedral cells but other regions of the same tumour could appear pale grossly and were heavily populated by the smaller size cells and contained less pigment and macrophages. The population of smaller cells were not prominent in arterial wall lesions.

Rarely, visceral lesions showed histological features more suggestive of malignancy. Nuclei of the smaller cells showed moderate pleomorphism but generally the chromatin remained homogenous. Mitotic figures, while not common, occurred regularly and occasional eosinophilic intra-nuclear inclusions imparted a “signet-ring” shape to some nuclei. Apoptotic bodies were rare. These lesions were intensely cellular, with little desmoplasia or obvious pattern of cell arrangement.

**(viii) Immunohistochemistry**

S-100A stained cells within the early lesions around the base of the hair follicles of the ventral tail. It also revealed a less apparent population of cells within the melanosis seen surrounding other adnexal structures in the ventral tail and perineum. Positivity was more difficult to interpret from within tumours due to the large amount of melanin present, in spite of various bleaching protocols being included in the study. Tumour cell reactivity appeared to be more variable but generally positive for the smaller cell population; the larger cells were difficult to read due to their heavy pigment content. After applying this stain, the nesting pattern of some of the tumour cells was more obvious. Positive controls stained strongly, negative controls did not stain.

Macrophage markers stained the large polyhedral cells found within tumours. Bleaching was necessary in order to demonstrate this. Positive and negative controls stained as expected.

HMB45 failed to stain any cells or any of the positive control samples.
PCNA stained a large proportion of the small tumour cells (up to half of all cells), basal keratinocytes and the positive control sample. Negative controls did not stain.

(ix) Tumour Ultrastructure

This work is still in progress. The presence of types II, III and IV eumelanosomes in some cells indicates the presence of melanocytic cells within the tumour. However granular melanosomes are also present. Most of the early melanosomes are seen as solitary organelles within cells. In other cells within tumours, melanosomes are collected together into compound melanosomes. Typical ultrastructural features of malignancy (nuclear changes etc) are not a feature.

(x) Histology and Ultrastructure of Coat Colour Dilution

This work is also still in progress. As hair colour fades, there is a decrease in the number of melanocytes detectable on routine sections. Melanocytes appear to disappear first from the tip of the dermal papilla (which supports fewer melanocytes normally) and remaining pigment cells in such follicles give an impression of being more centripetal than those in other follicles. Melanin accumulation around the hair follicle is generally more prominent in association with white hairs, but equally, white hairs may have no such change. Otherwise, there is little obvious change to signal the altered melanocytic environment.

Ultrastructural examination of the dermal papilla region of greying horses has to date only revealed granular melanosomes or phaeomelanosomes. The melanosomes are solitary rather than combined in compound melanosomes. Cells with many of the ultrastructural features of melanocytes are seen which have only a few or no melanosomes. The main criterion for identifying cells as melanocytic is the presence of melanosomes but other features of these cells include; no desmosomes or tight junctions, less electron dense cytoplasm than keratinocytes, no tonofilaments and well developed Golgi apparatus.
Discussion

The results of this study support the contention that all greying horses are likely to develop melanotic tumours provided they live long enough. There is no sex predilection. The observation that melanosis develops around the base of hair follicles as they progressively produce less pigment suggests that the process of coat colour dilution and tumour development are inextricably linked. Furthermore, the pathogenesis of the epidermal vitiligo that commonly accompanies coat colour dilution in the horse is apparently unrelated to tumour development.

While the findings presented here are not conclusive, it appears unlikely that different patterns of coat colour dilution carry different risks for tumour development. The fact that the dappled pattern was seen in younger horses, while the flea-bitten pattern was seen in animals of all age groups raises the possibility that some dappled horses may appear flea-bitten at a later stage of coat colour dilution. Of course, all animals ultimately become white (though the points may continue to be dark). It is probable that the basic mechanism of coat colour dilution remains the same whether horses become dappled or flea-bitten; other gene products may act as modifiers of the protein encoded at the G locus or, more likely, produce these patterns by acting at a different stage of the melanogenesis pathway.

Histological examination of dermal papillae from coloured, greying and white animals revealed an apparent elimination of the follicular melanocyte population as horses became white. Electron microscopy however revealed a population of cells which reflected many of the ultrastructural features of melanocytes but which had few or no melanosomes. This suggests that the melanocytes may persist for some time beyond when they cease producing melanin pigment; the original lesion does not seem to involve failure of melanocytes to survive in the follicular environment.

There are two types of melanosomes produced in normal melanocytes; eumelanosomes are ellipsoidal, with an organized internal array of parallel membranes and produce the black/brown eumelanin pigment whereas phaeomelanosomes are spherical and poorly organized and produce yellow/red phaeomelanin pigment (Furumura et al, 1996). Ultrastructural studies of follicular melanocytes have to date only revealed granular melanosomes akin to phaeomelanosomes. This is of interest in view of the report by Altmeyer et al (1984) that α-MSH levels are lower in grey horses; α-MSH is required for the synthesis of eumelanin and up-regulates the number and activity of melanocytes. It is not known if α-MSH acting at the hair follicle is systemically produced or produced locally and acting in a paracrine manner (Furumura et al, 1996). The hormone acts on melanocytes via a G protein-coupled receptor, melanocortin 1 receptor (MC1R). The action of α-MSH is countered by the activity of agouti signal protein which is produced by the most widely characterized of all the coat colour genes (the agouti gene). Thus, although this aspect of the project is not yet completed, it appears that further investigations of coat colour dilution in horses might usefully begin by establishing α-MSH levels in greying horses and identifying and characterizing the genes encoding the MC1R protein and the agouti signal protein in coloured and greying animals. MC1R sequences have previously been reported from the horse; a missense mutation is associated with chestnut coat colour (Marklund et al, 1996).
It is noteworthy that the accumulation of pigment around hair follicles was only seen in the ventral surface of the tail. These coarse hairs are said to be permanent whereas the general coat or cover hairs are shed periodically as part of the normal hair cycle (Talukdar, Calhoun and Stinson, 1972). The source of melanocytes for each new phase of hair growth remains unclear; unpigmented cells which may be melanocyte precursors have been identified around the dermal papilla and in the outer hair sheath of post-natal animals (Bennet, 1993). However other coarse hairs such as those of the mane and lateral tail were also unaffected; only those arising from the heavily pigmented, smooth skin of the ventral tail developed lesions.

Skin tumours of grey horses show little histological similarity to classic malignant melanoma. Epidermal involvement is not apparent, lesions are roughly symmetric and inflammatory infiltrates are rare; asymmetry and invasion by mononuclear cells occur commonly in malignant melanoma. Cytological features of tumour cells are also not suggestive of malignancy; nuclei are uniform in appearance with heterogenous chromatin dispersal. The rarity of mitotic figures and apoptotic bodies suggests a slow rate of tumour growth, although a significant number of cells stained positively with PCNA antibodies.

The skin lesions do however share many histological characteristics with the blue naevus of humans, particularly with the cellular blue naevus sub-group (Mooi and Krausz, 1992). Both have a dermal location and may extend into the subcutis. Ulceration and necrosis are rare but both the human and equine lesions will engulf cutaneous adnexae. Both may show a “biphasic pattern” of compact regions containing cells with little melanin alternating with heavily pigmented, dendritic cells which may be embedded in fibrotic tissue. There may be an abrupt change to a “pushing” growth pattern when either lesion extends into adipose tissue.

In a proportion of cases of cellular blue naevus, a similar population of cells will be seen in the regional lymph node, generally centred on the subcapsular sinus and lymph node parenchyma. The mechanism underlying this involvement is disputed (spread from skin via lymphatics or arrested melanoblast migration) but they do not adversely affect the prognosis (Mooi and Krausz, 1992). In the equine condition, the anorectal lymph node is regularly involved but other nodes are infrequently affected. This perhaps reflects the long-standing nature of the condition and the fact that the tail is generally the first region involved. Tumour cells are seen in both the subcapsular and trabecular sinuses in the horse. In equine lymph nodes, a large proportion of incoming lymph is immediately delivered to deep within the node via the trabecular sinuses so it is not surprising that lesions would commonly arise in both of these sites within the node (Nikles and Heath, 1992).

The pattern of lesion distribution is also of interest. The impression was that the condition first develops in the ventral skin of the tail. Later perineal skin and anorectal lymph nodes may become involved. In many animals though, lesions also develop in the lips, skeletal muscle and parotid salivary gland.

Some of these visceral lesions are less obviously benign. Mitotic figures may be more common, nucleoli are sometimes prominent and there may be more pleomorphism of the cells than what is seen in the dermal lesions. Lesions may push along areolar connective tissues but also can separate and disrupt collagen fibres. Ultrastructural features support the contention that both
melanocytes and macrophages are present, and this is further reinforced by the immunohistochemical demonstration of S-100 positive cells and cells which stain positive with macrophage markers. HMB45 was not useful as no animal tissues examined stained positively; as this is a monoclonal raised against human protein, it may be that the protein is not expressed in animal melanocytes. HMB45 normally stains “altered” human melanocytes including a large proportion of malignant melanoma and blue naevus cells.

The significance of the melanosomal structure in the visceral tumours is unclear. Type II to IV melanosomes are seen in blue naevi. Granular melanosomes have been reported in cases of human blue naevus but also in several other disorders, including malignant melanoma. None of the melanosomes so far identified could be described as bizarre with disordered internal structures as occur commonly in malignant melanoma. (Ghadially, 1985)

Melanotic lesions have a marked preference for connective tissue and very rarely involve thoracic or abdominal soft tissues. This is a most unusual pattern if the condition is thought of as a true neoplasm; metastatic lesions would be expected in areas such as the lungs, liver or even brain where slow flow of blood through capillaries is more conducive to malignant cells penetrating through the vascular basement membrane (Slauson and Cooper, 1990). In addition, the condition in the grey horse rarely has an obvious primary site; lesions in the ventral tail are invariably multifocal and it seems likely that, as the condition progresses, the various internal and dermal lesions develop concurrently.

The combination of lesion distribution and the presence of melanocytes within internal tumours argues against both the hypothesis that this condition is a pigment storage disorder and also the hypothesis that this lesion is a typical malignant melanoma as seen in other species. The analogy with the blue naevus of humans is also strained; both conditions have similarities in histology and both appear centred on the dermis but the blue naevus does not become as widely disseminated as the equine lesions unless it has undergone a histologically obvious malignant change (Levene, 1980; Mooi and Krausz, 1992).

The distribution of internal lesions shows some correlation with neural crest cell migration paths, at least in the avian embryo (Le Douarin, 1982; Serbedzija, Fraser and Bronner-Fraser, 1990). This study thus lends some support to the view that a pre-existing network of melanocytes (presumably arising along neural crest cell migration paths) become activated in greying horses and produce pigment. Although the subject of intense research, little is known of the mechanisms by which migrating neural crest cells stop in defined regions of the embryo; but interactions between cells and extra-cellular matrix components appear to be of central importance (Stemple and Anderson, 1993).

From the point of view of abattoirs trying to identify horses which are likely to have extensive carcase involvement ante-mortem, the two most reliable features are age and the presence of externally visible lesions. Ageing horses at abattoirs ante-mortem can be expected to pose problems as it requires a certain level of restraint of animals that may be unused to handling. Degree and pattern of coat colour dilution can serve as a rough guide only (in this study, all of the dappled grey horses were under 7 years of age). This means that effectively the options of the abattoir are limited to inspecting animals for lesions before slaughter; the presence of such
lesions is strongly indicative of extensive carcase involvement. However, in this study 10 out of 26 horses with generalized disease were found to have no lesions detectable ante-mortem. The typical pattern of lesion distribution is also of interest to abattoirs; skeletal muscle was the second most commonly involved site.

A recent review of equine melanocytic tumours by Valentine (1995) described four distinct syndromes; melanocytic naevus (primarily affecting young horses), dermal melanoma (1 or 2 discrete dermal masses mostly affecting mature but not aged grey horses), dermal melanomatosis (the typical syndrome examined in the present study) and anaplastic malignant melanoma (histologically similar to malignant melanoma and reported in coloured horses). It is apparent that horses are afflicted with the same types of benign and malignant melanocytic neoplasms as other species, even though frankly malignant melanoma is quite rare. The two syndromes described by Valentine as dermal melanoma and dermal melanomatosis are likely to be variations on the one theme but there was a suggestion that surgical excision of one or two discrete lesions may be curative. Further work needs to be done to verify this finding as it would strongly suggest that the condition arose from a primary tumour and thus support the neoplasia hypothesis. However several of the horses classified as “dermal melanomas” in this study had a somewhat atypical presentation in that the skin tumour was found on the carpus, or fetlock or withers which is quite different to the pattern seen in this study. Metastases were recorded for animals from both the dermal melanoma and the dermal melanomatosis groups but details were not provided.

In summary, all grey horses are likely to develop melanotic tumours if they live long enough. While the ventral skin of the tail is generally the first affected site, skeletal muscle, parotid salivary gland, perineal skin and lips are often also involved. Tumours often appear multicentric in origin. Lesions share histological and ultrastructural features with the human blue naevus which is itself poorly understood. Histological features of malignancy are rare and the pattern of tumour distribution is unusual for a malignant growth but this study has not been able to rule out the possibility that this condition represents a very slowly progressive neoplasia (perhaps arising from dermal melanocytes or melanocytes from the infundibular external hair sheath). The condition cannot be said to represent a storage disease in the true sense of the word, as melanocytes are present in visceral lesions. It is possible that melanocyte precursor cells are normally present at predilection sites as a result of drop-out during embryonic neural crest cell migration (i.e. peri-follicular, dermis, arterial walls, face, fascia of skeletal muscle groups close to the axial skeleton). These precursor cells may be stimulated to differentiate into active pigment producing cells as a response to the process of coat colour dilution. The normal “grey horse melanoma” picture would thus represent hyperplasia of a pre-existing network of cells which were normally inapparent. It would not be surprising given the long course of this condition, and current knowledge of the step-wise progression of events leading up to cancer, if a neoplastic process was occasionally superimposed upon this hyperplastic stage.
Implications

The current project has significantly added to the level of understanding of a condition that affects a large percentage of the Australian horse population; all grey horses of either sex are likely to develop melanotic tumours given time. The nature of grey horse melanoma has been the subject of speculation since the turn of the century. By detailing the development and anatomical pathology of these tumours, the present study will allow a more informed appraisal of the options available for clinical management of the condition.

The grey horse melanoma is likely to represent either a very slowly progressive neoplasia or an activation and hyperplasia of a pre-existing network of melanocyte precursor cells, from which malignant transformation may occasionally arise. The fact that the processes of coat colour dilution and development of melanotic lesions appear so closely intertwined can be explained by the latter hypothesis if the activation of melanocyte precursor cells occurs as a consequence of the process of coat colour dilution. This study ruled out the possibility that this is a storage disease. The analogy with the human blue naevus would appear to be valid on several counts, but is not informative due to the limited understanding of the human disease.

From the point of view of abattoirs exporting horse meat for human consumption two issues arise. Firstly any grey horse with an externally visible lesion is likely to have generalized disease affecting the carcase. Age is another significant predictor of generalized disease but patterns of coat colour dilution are unhelpful in this regard. Secondly a neoplastic basis for this disease cannot be ruled out after consideration of this study’s findings; this needs to be kept in mind when making decisions on carcase disposal on the abattoir floor.

The implications for therapy of working or companion horses relate to the likely pathogenesis behind the condition. If the condition is a true neoplasia, then surgical excision of early solitary lesions may be curative, as reported by Valentine (1995). However the results presented here strongly suggest that, in the majority of grey horses, this condition has multicentric origins. If the condition is in fact neoplastic, it is only very slowly progressive. The fact that only a small proportion of cells are within the proliferative stages at any one time means that most attempts at anti-neoplastic chemotherapy are likely to be unrewarding.

Similarities in pathology between the grey horse melanoma and the blue naevus of people have been confirmed by this project. The ultrastructural features of greying hair follicles demonstrated here allow for more fruitful examination of the genetic basis of coat colour dilution in the horse; such work is likely to add significantly to the body of knowledge regarding the genetics of mammalian pigmentation. Further studies on the equine condition are thus likely to result in an improved understanding of melanocytic lesions such as the blue naevus in people and to our understanding of the basic biology of the melanocyte.
Recommendations and Communications Strategy

- Further ultrastructural examination of dermal papillae from hairs of white, greying and coloured horses. This has proven technically demanding. Results to date have been encouraging and informative but a greater number of hair follicles need to be looked at.

- Examine predilection sites of the tumour for melanocyte precursor cells. This could be done using DOPA staining of frozen sections or immunohistochemically (if an antibody could be demonstrated to be effective on equine tissues). A comparison of young and aged, grey and bay horses would be useful if such cells were able to be demonstrated.

- Confirm the report of lower $\alpha$-MSH plasma levels in greying horses when compared with coloured horses. This may be an important indicator for the mechanism of coat colour dilution.

- Design primers for the PCR amplification of the MC1R and agouti gene sequences from grey horse DNA and compare these sequences with those from bay horses. Results from further ultrastructural studies of hair follicles may refine the number of genes to be targeted in search of the mechanism of greying in the horse.

- Conduct a clinical trial to determine if surgical excision of solitary lesions is curative. This has been suggested by one author but would appear unlikely in view of the results of this project. The answer is of significance because, if surgical excision is curative, it is indicative of a neoplastic process. However the protracted course of the disease may entail quite a long-term approach to such a study.

- Disseminate the results of the report by publication in relevant journals (e.g. *Australian Veterinary Journal*) and presentations at conferences. Early results have already been reported at the Australian Society for Veterinary Pathology annual conference (1994), the New Zealand Society of Veterinary and Comparative Pathology annual conference (1992) and the International Melanoma Conference (*Melanoma: the way forward*) held in Brisbane, (1994).
References


Fleming MG and Bergfeld WF (1990) A simple immunohistochemical technique for distinguishing melanocytes and melanophages in paraffin-embedded tissue. *Journal of Cutaneous Pathology* **17**: 77-81


Marklund L, Johansson Moller M, Sandberg K and Andersson L (1996) A missense mutation in the gene for melanocyte-stimulating hormone receptor (MC1R) is associated with the chestnut coat colour in horses. *Mammalian Genome* 7: 895-899

McFadyean J (1933) Equine melanomatosis. *Journal of Comparative Pathology and Therapeutics* 46: 186-204


Runnels RA and Benbrook EA (1941) Malignant melanomas of horses and mules. *American Journal of Veterinary Research* **2**: 340-344


Sponenberg DP and Beaver BV (1983) *Horse Color*. Texan A&M University Press College Station


Theilen GH and Madewell BR (1987) *Veterinary Cancer Medicine, 2nd Edn.* Lea and Febiger Philadelphia


van Dorssen J (1903) Ueber die genese der melanome in der haut bei Schimmelpferden. *Inaugural dissertation*. Amsterdam
