Optimisation of therapeutic approach in equine sarcoids

Katie Susan Offer BVMS(Hons) MRCVS

Submitted for the degree of MVM to the University of Glasgow, College of Medical, Veterinary and Life Sciences, School of Biodiversity, One Health, and Veterinary Medicine

September 2024

Table of Contents

List of Tables

List of Figures

List of Accompanying Material

Acknowledgements

The following thesis is submitted in an alternative format with 2 chapters comprised of published works, and 1 further chapter prepared for submission for publication. I would therefore like to acknowledge the co-authors of each paper for their contributions, as follows. Please also see the relevant Author Declaration documents accompanying this thesis for full details of each co-author's contribution to each paper.

Chapter 2: Offer K.S.[1] Dixon C.E.[2], Sutton D.G.M.[1] (2024). Treatment of equine sarcoids: A systematic review*. Equine Veterinary Journal,* 56(1):12-25. doi: 10.1111/evj.13935. Epub 2023 Mar 23. PMID: 36917551.

[1]School of Biodiversity, One Health and Veterinary Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

[2] Tufts University, Cummings School of Veterinary Medicine, Department of Clinical Sciences, Administration Bldg, N. Grafton, MA, 01536, USA

Chapter 3: Offer, K.S.[1] & Sutton, D.G.M.[1] (2024) Retrospective study: Laser excision versus combined laser, cryosurgery and intralesional 5-fluorouracil in the treatment of equine sarcoids. *Equine Veterinary Education*, 00, 1–10. : doi: 10.1111/eve.14031

[1]School of Biodiversity, One Health and Veterinary Medicine, College of Medical Veterinary and Life Sciences, University of Glasgow, Glasgow, UK

Chapter 4: In preparation for publication: Offer, K.S.[1], Venkateswaran, S.[1], Foote AK[2], Sutton, D.G.M.[1,] Immunohistochemical investigation of equine sarcoids: a pilot study.

[1]Glasgow Equine Hospital, University of Glasgow, 464 Bearsden Rd., Glasgow.

[2]Rossdales Equine Hospital and Diagnostic Centre, Suffolk, UK

I would also like to acknowledge the following individuals who also contributed to the production of this thesis:

- Alex Raftery MA VetMB DipECEIM MRCVS for her help and guidance in the production of the systematic review.
- Joana Devesa MRCVS and Amie McGarva BVMS MRCVS for their help in retrospective data gathering.
- Dr Vanessa Herder PhD, for her guidance in the use of QuPath and bioimage analysis.

Declarations

I declare that the thesis has been composed by myself and that the work has not been submitted for any other degree or professional qualification elsewhere. I confirm that the work submitted is my own, except where work which has formed part of jointly authored publications has been included. My contribution and those of the other authors to this work have been explicitly indicated both below and in the attached declarations of authorships. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

The work presented in Chapter 2 was previously published in the Equine Veterinary Journal as 'Treatment of equine sarcoids: A systematic review', by Offer, K.S. (student) , Dixon, C.E. (co-supervisor), Sutton, D.G.M. (supervisor). Full author contribution has been credited in the attached declaration of authorships in accordance with the principles of the Contributor Roles Taxonomy system (CRediT) (Brand *et al.,* 2015).

The work presented in Chapter 3 was previously published in the Equine Veterinary Education as 'Retrospective study: Laser excision versus combined laser, cryotherapy and intralesional 5-fluorouracil in the treatment of equine sarcoids' by Offer, K.S. (student) and Sutton, D.G.M. (supervisor). Full author contribution has been credited in the attached declaration of authorships in accordance with the principles of the Contributor Roles Taxonomy system (CRediT) (Brand *et al.,* 2015).

The work presented in Chapter 4 is in the process of preparation for submission to Veterinary Pathology as 'Immunohistochemical investigation of equine sarcoids: a pilot study' by : Offer, K.S. (student), Venkateswaran, S., Foote, A.K., and Sutton, D.G.M. (supervisor). Full author contribution has been credited in the attached declaration of authorships in accordance with the principles of the Contributor Roles Taxonomy system (CRediT) (Brand *et al.,* 2015).

Funding for the work in this thesis was obtained from the University of Glasgow Veterinary Fund Post Graduate Research Scheme, and ethical approval was obtained from the University of Glasgow School of Veterinary Medicine Research Ethics Committee (ref. EA-4221). Written informed consent was included in the standard hospital consent form and was obtained for all included horses at the time of treatment.

Chapter 1: Introduction

The equine sarcoid (ES) is the most common neoplasm affecting equids worldwide (Marti *et al.,* 1993). Prevalence in the equine population is estimated between 1% and 11.5% internationally and is reported as 5.8% in the United Kingdom (Studer *et al.,* 2007, Knottenbelt, 2005, Ireland *et al.,* 2013). Sarcoids represent between 24-46% of equine cutaneous biopsy samples submitted for histopathology (Knowles *et al.,* 2016, Schaffer *et al.,* 2013) and may affect both wild and domesticated equids (Knottenbelt, 2005, Marais and Page, 2011, Wenker *et al.,* 2021).

The sarcoid was first described in 1936 as 'a unique locally invasive, benign neoplastic like tumour of the skin' (Jackson, 1936). Since then, the sarcoid has been further defined as locally invasive biphasic spindle cell tumour, referring to the proliferation of both dermal (mesenchymal) fibroblasts and epidermal (epithelial) keratinocytes in these neoplasms (Wobeser, 2016, Knottenbelt *et al.,* 2015). The tumours are rarely metastatic but may cause significant welfare and financial implications in affected equids due to their highly invasive behaviour and resultant secondary complications. Even when first described, the authors noted the sarcoid's 'high propensity for recurrence' which continues to frustrate clinicians and horse owners worldwide (Knottenbelt, 2019).

1.1 Aetiology

A viral aetiology for ES was first suggested following the induction of sarcoid lesions by the autotransmission of tumour tissue in a mule in 1937 (Montpellier, 1937). Since then, the associated viruses have consistently been identified as bovine papillomaviruses (BPV) -1 and -2 (Nasir and Reid, 1999).

BPV are a group of icosahedral DNA viruses known to result in papillomatous disease in bovine species. BPV 1 and 2 are part of the δ papillomavirus genus and are further classified as fibropapilloma viruses due to their ability to infect both the dermis and epidermis of an affected animal (Nasir and Reid, 1999). In their natural bovine host, infection generally results in superficial,

benign and self-limiting skin warts (Campo, 2002), though, interestingly, persistent infection may also occur in this host species and may predispose certain bladder and urinary tract neoplasias (Campo *et al.,* 1992). The equine sarcoid is considered to be the only example of cross species papillomavirus infection in domesticated animals (Borzacchiello *et al.,* 2008).

The association between ES and BPV-1 and BPV 2 infection is well established, though a direct causative relationship has not been confirmed and the specific pathogenesis remains elusive (Nasir and Reid, 1999, Nasir and Campo, 2008). BPV DNA is consistently expressed within sarcoid tissue (Nasir and Reid, 1999, Carr *et al.,* 2001b), and has been demonstrated in both the epidermal keratinocytes and dermal tissues in ES lesions (Brandt *et al.*, 2011). However, BPV DNA may similarly be demonstrated in surrounding grossly normal skin (Carr *et al.,* 2001b), equine inflammatory skin diseases (Yuan *et al.,* 2007), other equine dermal neoplasms (Chambers *et al.,* 2003) and in horses grossly unaffected by ES (Bogaert *et al.,* 2005). BPV infection in ES is deemed to be non-productive since viral DNA remains episomal in equids and entire viral particles have not been demonstrated (Amtmann *et al.,* 1980, Nasir and Campo, 2008). More recently, a further BPV type, BPV-13, has been identified in ES in equids in Brazil suggesting other BPV strains may also be associated with sarcoid development (Lunardi *et al.,* 2013), though this requires further investigation and may be dependent upon geographical area.

1.2 The viral genome and oncogenesis

The BPV genome consists of 3 distinct regions: a long controlling region (LCR) and those encoding the early phase (E) and late phase (L) proteins. The LCR region contains the regulatory elements necessary for control of viral transcription and translation and is under the control of the highly conserved regulatory oncoproteins encoded by the E region. L region encoded late proteins L1 (major) and L2 (minor) together form the BPV viral capsid and as such are inconsistently expressed in the non-productive infection in ES (Nasir and Reid, 1999).

Three major oncoproteins are encoded by the E region of the genome: E5, E6 and E7. Of these, E5 is of the most significance in ES. The most significant actions of these are the inhibition of normal fibroblast cellular gap junction function, dysregulation of intercellular signalling, and inhibition of Golgi apparatus and endosome acidification (Schapiro *et al.,* 2000, Faccini *et al.,* 1996). The resultant cellular dysregulation and uncontrolled proliferation is then further promoted by the additional activation of the platelet derived growth factor (PDGF) pathway (Borzacchiello *et al.,* 2006). In ES, the E5 protein plays a crucial role in the viral immune evasion mechanism resulting in persistence of the BPV virus (Wilson *et al.,* 2013, Marchetti *et al.,* 2009). The pathway has been well described and involves the activation of the p38 mitogen-activated protein kinase (MAPK) pathway; resulting in an inhibition of the production of MHC I heavy chain (Marchetti *et al.,* 2009).

The E6 protein has been less explored in ES specifically, but in other species acts as a transcriptional activator and to inhibit the function of the tumour suppressor gene, p53 (Zimmermann *et al.,* 2000). The protein also has a role in the disruption of the normal cellular cytoskeleton function and resultant neoplastic anchorage-independent growth of the tumour (Tong *et al.,* 1998). The E7 protein has a synergistic role with E5 by acting to promote the anchorage independent proliferation of neoplastic cells (Bohl *et al.,* 2001).

Aside from the above understanding of oncogene expression, the exact mechanism by which BPV induces sarcoid development has not been described. ES derived fibroblasts display the increased plasticity typical of neoplastic cells- increased genomic instability, polyploidy events and telomere dysfunction have been demonstrated leading to a heterogenous cell population predisposed to immature senescence (Potocki *et al.,* 2012, Potocki *et al.,* 2014). Selective expression of proteins (e.g., Bcl-2-associated athanogene (BAG) 3) that allow for neoplastic cell avoidance of apoptosis are expressed in ES samples (Cotugno *et al.,* 2013), and differential expression of various microRNAs has similarly been demonstrated from ES neoplastic fibroblasts in vitro when compared with normal skin (Terron-Canedo *et al.,* 2014).

The virus also appears to have a distinct immune regulatory role, interfering with both the innate and adaptive immune responses (Marchetti *et al.,* 2009, Yuan *et al.,* 2008). Upregulation of several immune regulatory chemokines has been demonstrated, in addition to down regulation of Toll Like Receptor-4 (TLR-4) and MHC-1- molecules has been demonstrated in BPV-1 transformed fibroblasts (Yuan *et al.,* 2008). This down regulation of the activators of the innate immune system (TLR-4) has been shown in vitro to be induced by the exogenous expression of both BPV-1 E2 and E7 oncoproteins, and so this is suggested to be a crucial mechanism of BPV immune evasion and ES oncogenesis (Dapalma *et al.,* 2010).

A further key feature of the BPV viruses is the maintenance of viral latency in the epithelia of clinically normal individuals (Nasir and Campo, 2008). Viral reactivation is again partially understood, but appears to be promoted by local trauma with the resultant release of inflammatory cytokines stimulating cellular proliferation (Nasir and Campo, 2008). In cattle infected with BPVs, latency may also be maintained in circulating peripheral lymphocytes (Stocco Dos Santos *et al.,* 1998), but this has inconsistently been demonstrated in horses (Nasir *et al.,* 1997, Brandt *et al.,* 2008). This infection of the effector cells of the adaptive immune response has been suggested as a further mechanism of BPV- induced immunomodulation (Nasir and Campo, 2008).

1.3 Transmission

Although first definitively demonstrated approximately 90 years ago (Jackson, 1936), understanding of ES transmission between horses remains incomplete. Early suggestions that ES may be caused by the direct transmission of a neoplastic cell line have been disproven (Gobeil *et al.,* 2007) but the mechanism of BPV spread is not yet established. More recent work has identified a possible role of stable flies (*Stomoxys calcitrans*), though suggested that this was more likely after contact with bovine BPV positive lesions than equine. This paper also demonstrated that transmission by this method will only occur shortly after BPV exposure (Haspeslagh *et al.,* 2018), and BPV DNA has also

been detected in various other species of flies (Finlay *et al.,* 2009). However, it has not yet been demonstrated that these species may actually transmit the virus between equids *in vivo*, and as such bovine species remain the most significant reservoir of BPV for ES infection.

1.4 Risk factors for disease

Despite the progress made in understanding ES pathogenesis, it is clear that some equids may be infected with BPV-1/2 without the development of clinical signs. Viral latency may have a role to play, as above, but the circumstances and reasons for viral re-activation are generally unknown. Similarly, spontaneous regression has been not infrequently described, again with an unknown mechanism (Berruex *et al.,* 2016).

A genetic predisposition to the development of ES has been clearly demonstrated and is associated with the presence of certain MHC class I and II haplotypes in certain Swedish, Swiss, French and Irish Warmblood horses (Broström *et al.,* 1988). A further whole genome wide association study identified several chromosomal regions that are linked to ES predisposition, and has suggested that this predisposition is a polygenic trait (Jandova *et al.,* 2012).

Age appears to be a significant risk factor for ES. Equids are usually reported to first develop sarcoids at between 3-6 years of age, but this is not universal (Knottenbelt *et al.,* 2015). Conversely, spontaneous sarcoid regression appears to be more frequent in young horses. In a longitudinal study following 61 3-year-old horses over a 5-7 year period, spontaneous regression without treatment was observed in 48% of cases (Berruex *et al.,* 2016), a proportion far greater than reported in the literature as a whole (Knottenbelt *et al.,* 2015). However, this study population was of one breed which is uncommon worldwide, and so the confounding effects of genetic predisposition must be considered when interpreting this data.

Further suggested risk factors for disease include geographic location, proximity to the native hosts (i.e., cattle) and density of the proposed viral vectors, although these are yet to be fully explored in the literature (Valentine, 2006).

1.5 Biological behaviour

A phenotypic classification system has been suggested for ES and is in routine clinical use (Knottenbelt, 2005). These are as follows:

- Occult: These sarcoids are superficial, alopecic, and roughly circular lesions that are generally hyperkeratotic in appearance. They may contain 1/2 small nodules (<5mm diameter). They can be difficult to identify in the haired horse, but generally are accompanied by a characteristic change in hair coat colour and density.

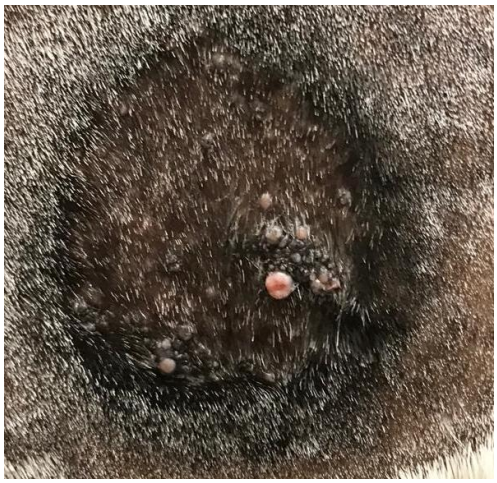


Figure 1: Photograph of an occult sarcoid.

- Verrucose: Rough, hyperkeratotic ('warty') sarcoids with epidermal scaling. They may be coalescing and extensive in nature and can be sessile (flattened) or pedunculated in nature. They are most commonly found on the face, axilla, and medial thigh/sheath regions.

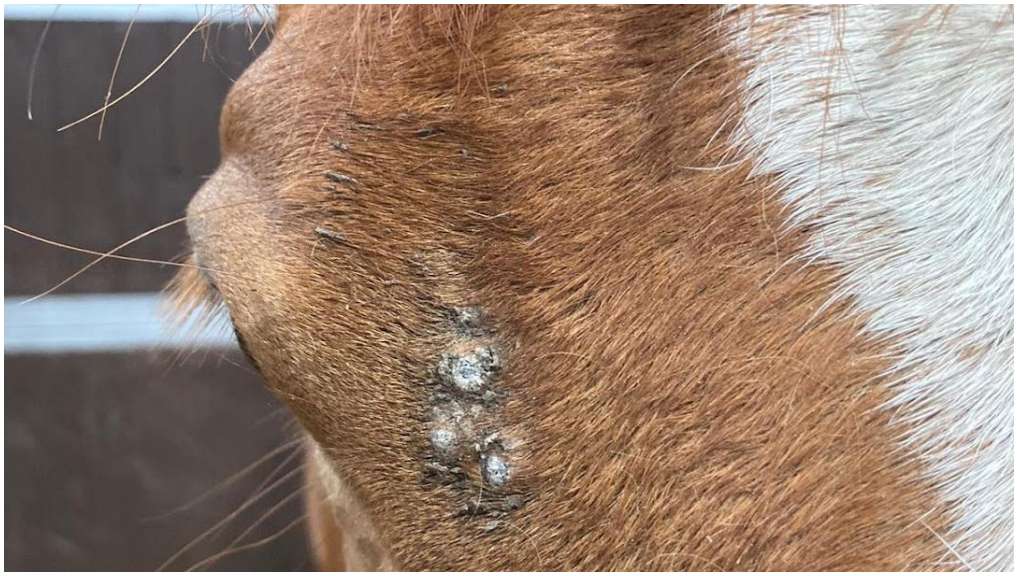


Figure 2: Photograph of a periocular verrucose sarcoid.

- Nodular: Spherical, well defined sarcoids of variable size and number. Occasionally, these may become ulcerated. These are further categorised into:
    - Type A: Overlying skin and underlying subcutaneous tissues are freely moveable over the mass.
    - Type B: Have a dermal component and so are firmly adhered to the overlying skin. Remain freely moveable over the subcutaneous tissues.

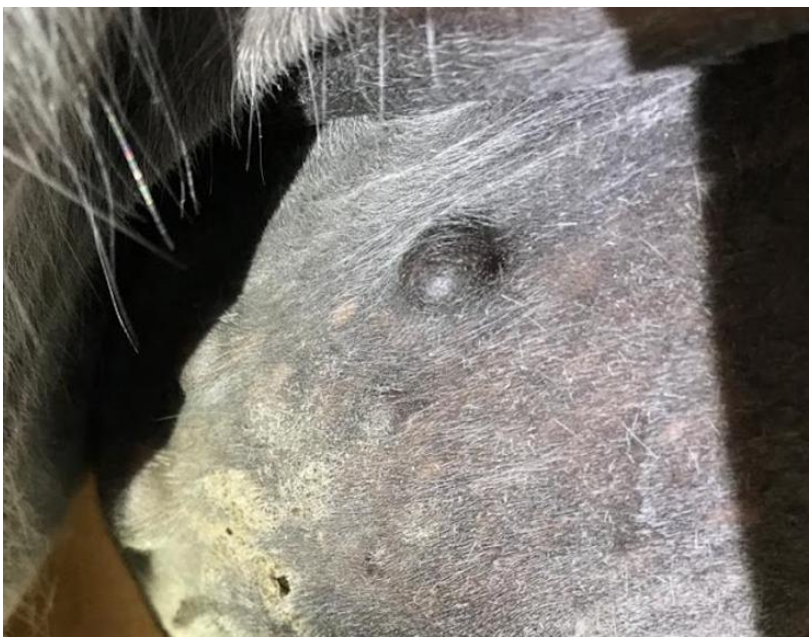


Figure 3: Photograph of a nodular sarcoid on a medial thigh.

- Fibroblastic: Proliferative sarcoids with an ulcerated appearance. These are further categorised as:
    - Type 1: Pedunculated. These sarcoids have a narrow ‘neck’ and poorly palpable root.
    - Type 2: Sessile. These sarcoids are flattened and are generally highly infiltrative.

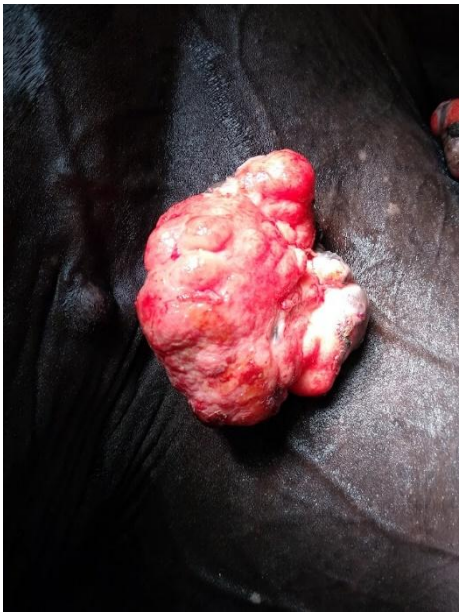


Figure 4: Photograph of a pedunculated fibroblastic sarcoid on the medial thigh.

- Mixed: Any combination of verrucose/occult/fibroblastic/ nodular sarcoids. The presentation and location of these sarcoids are highly variable.

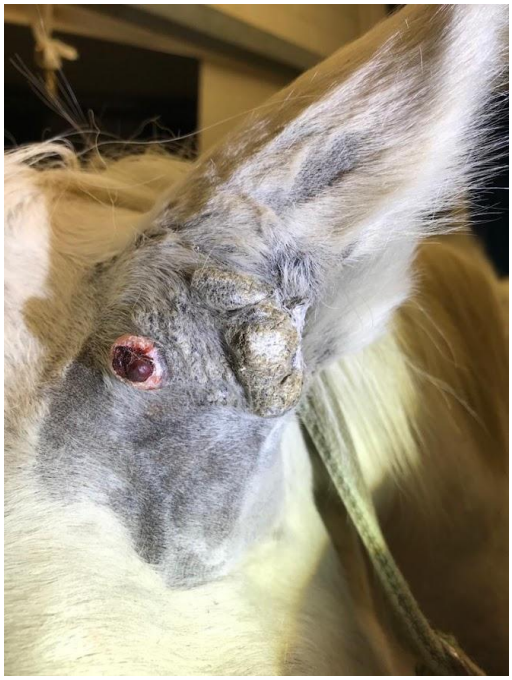


Figure 5: Photograph of a mixed nodular/verrucose/fibroblastic sarcoid at the base of a horse's ear.

- Malevolent/ Malignant: A highly infiltrative and aggressively proliferative form of sarcoid that rapidly invades the underlying lymphatic vessels. These are rarely described but are often associated with locations experiencing repeated trauma.

The reasons for these clinical manifestations of disease and the relationship between them are, as ever, poorly understood. There is no predictable linear disease progression between them (i.e., occult sarcoids do not necessarily progress into nodular etc.) and histopathologically they are difficult to differentiate without further diagnostics (Martens *et al.,* 2000, Knottenbelt, 2005). The utility of this classification comes in the selection of an appropriate treatment modality and not necessarily in the absolute prediction of their clinical behaviour (Knottenbelt, 2005, Martens *et al.,* 2001a). This gives rise to a key problem in the clinical management of ES; sarcoid behaviour is unpredictable and dynamic.

1.6 Differential diagnoses

Though clinical diagnosis of ES on the basis of gross appearance has been suggested to have reasonable sensitivity and specificity (83.3% and 79.6%, respectively (Koch *et al.,* 2018)), differential diagnoses for ES are numerous and clinical differentiation can be complicated (Knottenbelt *et al.,* 2015). These differential diagnoses are shown below in *Table 1.*

Neoplastic	**Non-Neoplastic**			
	Occult	**Verrucose**	**Nodular**	**Fibroblastic/ Malevolent**
Squamous cell carcinoma	Dermatophilosis	Dermatophytosis	Foreign body reaction	Exuberant granulation tissue
Fibroma	Alopecia areata	Dermatophilosis	Abscess	
Fibrosarcoma	Vitiligo	Trauma/rub marks	Scar tissue	
Papilloma	Trauma/ rub marks		Cystic structures	
Peripheral nerve sheath tumour			Granulomata: collagenolytic, bacterial, parasitic, fungal, foreign body	
Melanoma			Inflammatory polyp	
Carcinoma				
Histiocytoma				
Mast cell tumour				

Table 1: Differential diagnoses for ES (Knottenbelt et al., 2015).

1.7 Diagnosis

Given the above myriad of differentials and the vastly variable clinical behaviour of ES, an accurate diagnosis is crucial for treatment planning and prognostication. The basis of diagnostics in clinical practice remains histopathology, with typical features reported as a disorganised dermal proliferation of spindle-shaped neoplastic fibroblasts. Where present in the submitted samples, the epidermis is frequently hyperplastic or hyperkeratotic, forming rete peg projections extending into the dermis. Fibroblasts are frequently arranged perpendicular to the epidermal basement membrane (a 'picket fence' orientation) (Martens *et al.,* 2000). However, these features are inconsistently present (Martens *et al.,* 2000), and may be displayed by other spindle cell tumours (Wobeser, 2016).

A further complication in the use of histopathology for sarcoid diagnosis comes in obtaining the sample. Several authors have suggested that ES lesions should not be biopsied due to a risk of inducing accelerated growth and increasing local invasion secondary to the trauma of the procedure (Knottenbelt *et al.,* 1995). In a case series detailing the treatment of periocular sarcoids, all iatrogenically/ accidentally traumatised sarcoids were reported to show accelerated growth and transformed into fibroblastic tumours (Knottenbelt and Kelly, 2000). However, this same author later suggests in an editorial that biopsy may be appropriate when followed swiftly by appropriate treatment (Knottenbelt and Matthews, 2001). Indeed, the evidence that biopsy truly results in accelerated growth of a sarcoid, despite being a long-held belief by attending clinicians, is limited. A recent longitudinal study examining sarcoid growth dynamics after punch biopsy of 11 sarcoids suggests that this is not a consistent feature of sarcoids (Gysens *et al.,* 2024). Whilst some statistically significant changes in sarcoid dimensions were demonstrated in this small study, tumours showed both an improvement and deterioration following biopsy procedure. Further work is required in order to confirm this before the routine biopsy of ES lesions, but this suggests that biopsy may be an underutilised diagnostic procedure in many cases.

Further molecular techniques have been developed in an attempt to accurately diagnose clinical ES lesions. The identification of BPV DNA in excised lesions by PCR has been suggested in situations where the differentiation of sarcoid from non-neoplastic differentials (e.g., exuberant granulation tissue) is difficult (Carr *et al.,* 2001b). This technique is limited by the high incidence of BPV detection in grossly normal surrounding skin. In a study examining BPV incidence in sarcoids in the United States, 98% of sarcoid tissue samples showed BPV positivity on PCR, but 63% of normal skin samples from sarcoid affected horses were also BPV positive (Carr *et al.,* 2001b). A further study identified BPV DNA in 50% of healthy horses living in contact with sarcoid affected horses, 73% of horses living in contact with cattle, and 30% of control horses with no known in contact history with either (Bogaert *et al.,* 2005). This technique has the same limitations as the sampling required for histopathology, and so more recently it has been suggested that a fine needle aspirate may also be

appropriate for the obtaining of samples for BPV PCR (Gysens *et al.,* 2023). However, further validation of these techniques is required before they may be routinely employed in the diagnosis of ES.

1.8 Prognosis

As discussed above, the clinical behaviour of sarcoids is unpredictable and widely varied, and so the clinician faces a challenge in the prognostication of ES in order to inform the most appropriate treatment protocol. Several attempts have thus far been made to identify a reliable prognostic marker for ES.

1.8.1 Histopathology

The relationship between histopathological findings and sarcoid prognosis/ likelihood recurrence is inconsistent. Whilst histopathological findings are not associated with sarcoid type (Martens *et al.,* 2000), the presence of features indicative of superficial inflammation in ES samples has been associated with the likelihood of sarcoid recurrence (Curnow *et al.,* 2023). Clustering of data identified in the modelling in this paper suggests that this is not the sole determinant of sarcoid recurrence, and further factors require investigation.

1.8.2 Immunohistochemistry (IHC)

Several markers have been investigated as potential prognostic markers, in addition to attempts to further investigate the pathogenesis of ES. These are summarised below.

P53

Investigation of cell cycle regulatory proteins in the equine sarcoid have thus far focussed primarily on loss of the tumour suppressive action of protein p53. Loss of p53 function has been identified as an important factor in the pathogenesis of neoplasia in both human and veterinary species (Hollstein *et al.,* 1996, Teifke and Löhr, 1996).

Results in the literature regarding the role of p53 in ES are somewhat conflicting. Sequencing of the p53 gene in cases of ES failed to identify mutation in exons 5 to 9, suggesting the frequency of p53

mutation may be low (Bucher *et al.,* 1996, Nasir *et al.,* 1999). However, immunohistochemical investigation of p53 expression has demonstrated expression in up to 43% of sarcoid tumours (Finlay *et al.,* 2012). Rather than mutation of the p53 protein itself, current data suggest that the loss of p53 function is more likely related to abnormal cytoplasmic or perinuclear sequestration of wild type p53 protein (Finlay *et al.,* 2012, Kasperowicz *et al.,* 2006, Tura *et al.,* 2022, Martens *et al.,* 2000, Bogaert *et al.,* 2007). This has been reported in a range of tumours (Moll *et al.,* 1996, Gestl and Anne Böttger, 2012), and is in agreement with evidence supporting the loss of p53 function in ES (Nixon *et al.,* 2005). Furthermore, the BPV E6 protein has been shown to be carcinogenic in its own right and promotes clastogenesis in its target cells that lead to p53 downregulation and inactivation (Araldi *et al.,* 2015, Scheffner *et al.,* 1990).

A recent paper concluded that p53 expression was not correlated with response to treatment or occurrence of new sarcoids (Tura *et al.,* 2022). However, this study failed to identify p53 positivity on any immunohistochemical section studied. It is of note that this paper used the PAb- 240 monoclonal antibody rather than the DO-7 clone used more routinely in the literature and has shown to be the best available antibody to recognise equine p53 (Finlay *et al.,* 2012). The PAb-240 clone is able to bind only the mutant p53 protein, which is demonstrably absent in the equine sarcoid, rather than DO-7 that is able to recognise both the wild- type and mutated forms (Gannon *et al.,* 1990, Vojtěsek *et al.,* 1992). The prognostic implications of p53 positivity therefore warrant further investigation.

PCNA

PCNA is a histone- associated protein expressed in association with DNA replication, and so is expressed primarily in the S phase of the cell cycle (Bolton *et al.,* 1992). It has been widely used in the calculation of proliferation indices in both human and veterinary species (Madewell, 2001). Data regarding its behaviour in sarcoids is sparse- high PCNA expression was associated with local tumour recurrence after surgery only when intratumoral chemotherapy was delayed in one paper examining both ES and squamous cell carcinoma (Théon *et al.,* 1999). A further paper examining the

immunohistochemical markers of tumour cell proliferation in ES found that PCNA was not associated with sarcoid type, but expression in the fibroblast portion of the tumours only was associated with increased risk of recurrence (Kasperowicz *et al.,* 2006). The paper concludes that sarcoid classification on the basis of tumour cell proliferation markers may be useful in the determination of tumour behaviour and prognosis.

E5

Expression of the major BPV-1 oncoprotein E5 is widely established in ES and is expected in both the epidermis and neoplastic fibroblasts of sarcoid tumours (Carr *et al.,* 2001a). It is found in a juxtanuclear position within the cytoplasm of positive cells (Marchetti *et al.,* 2009, Borzacchiello *et al.,* 2008), and has multiple oncogenic functions as discussed previously.

Limited information currently exists regarding the role of E5 in the prognostication of ES though, logically, quantification of the highly conserved E5 expression may act as a surrogate measure of BPV load due in the absence of other quantitative methods (Nasir and Reid, 1999). In human medicine, high human papillomavirus loads have been shown to predict cervical intraepithelial lesion progression independently of gross clinical appearance and/or histochemical grade (Ho *et al.,* 2006, Carcopino *et al.,* 2006). Current evidence in ES suggests that BPV mRNA expression (and accordingly, E5 expression) may be higher in nodular sarcoids compared with other morphological types (Bogaert *et al.,* 2007). This study also notes that small tumours and those with clinical signs of regression display lower expression of BPV related oncogenes. The authors suggest that this represents differences in relative cellularity and density of the sarcoid types. More recent work goes further and identified consistently increased viral load in rapidly growing and/or aggressive multiple sarcoids (Haralambus *et al.,* 2010). This study employed quantitative PCR of the E2, E5, L1 and L2 genes, and found the E5 gene to be among the most reliable indicators of disease severity. However, distribution of BPV expression within the tumour and surrounding tissues is not fully understood, and qPCR may have several practical disadvantages over immunohistochemistry, including availability and cost implications.

Ki67

Ki67 expression in sarcoid tissue is generally low and similar to those levels detected in normal skin (Martano *et al.,* 2018, Tura *et al.,* 2022, Martens *et al.,* 2000, Nixon *et al.,* 2005, Bogaert *et al.,* 2007). Its association with clinical sarcoid type and behaviour is unclear. No correlation between Ki67 index and sarcoid recurrence has been identified, though one study did find a significantly higher expression in fibroblastic and nodular sarcoids than other types. This paper also correlated Ki67 index with the other proliferative marker, Cyclin D (Tura *et al.,* 2022). In contrast, a further study did not find an association between Ki67 status and sarcoid type (Martens *et al.,* 2000). This inconsistency is presumably associated with current proliferative status of the tumour and makes this marker an inconsistent candidate as a reliable predictor of sarcoid behaviour.

HIF-1α

More recent work has demonstrated that the hypoxia-inducible factor -1 (HIF-1)/ vascular endothelial growth factor (VEGF) pathways are upregulated in ES (Martano *et al.,* 2020, Martano *et al.,* 2018). Specifically, HIF-1α is strongly expressed in ES fibroblasts, and is positively correlated with VEGF expression. HIF-1α appears to be abnormally accumulated in the cytoplasm of neoplastic sarcoid fibroblasts, in contrast to the nuclear expression previously reported (Depping *et al.,* 2015). The authors postulate that HIF-1α has a crucial role in the hypoxic environment of the equine sarcoid, leading to upregulation of the VEGF pathway and tumour angiogenesis, as is recognised with HPV (Nakamura *et al.,* 2009). HIF-1α may warrant further investigation in ES; increased expression is associated with aggressive tumour phenotypes and poor prognosis in both human and canine mammary cancers, and in human colorectal cancers (Yamamoto *et al.,* 2008, Baba *et al.,* 2010, Shin *et al.,* 2015).

Ag-NORs

One study investigated the association of silver staining nucleolar organiser regions (Ag- ORs) and sarcoid behaviour (Kasperowicz *et al.,* 2006). The authors demonstrated numerous granular staining regions in both the epidermal and cutaneous layers of the sarcoid, with the number of granular

regions greater in fibroblastic sarcoids when compared with occult or mixed types. However, there was no association with sarcoid behaviour or recurrence.

pRb and Cyclin D1

pRb and Cyclin D1 expression was examined in one study and were expressed in all included sarcoids (Tura *et al.,* 2022). For both markers, positivity was exclusively nuclear, and pRb and Cyclin D1 scores were positively correlated. This study found an overexpression of Cyclin D1 in 80% of sarcoid samples, with the highest expression present in nodular and fibroblastic sarcoids. Cyclin D1 expression was also positively associated with higher tumour Ki67 proliferation index. However, neither score was significantly associated with tumour recurrence rate, and so neither marker was prognostic.

1.8.3 Molecular techniques

PCR

Attempts to correlate sarcoid viral load (VL) on the basis of PCR and clinical behaviour of ES have been thus far conflicting. A study examining clinical sarcoid samples indicated that, whilst certain morphological types of sarcoid may be expected to have a higher BPV VL than others, this was not predictive of the likelihood of progression (Bogaert *et al.,* 2007). Conversely, a later study found a highly significant correlation between intralesional VL and disease severity (Haralambus *et al.,* 2010). The technique therefore may warrant further investigation as an ES prognostic marker.

MicroRNAs

Several attempts have been made to identify a microRNA (miRNA) marker profile that may predict sarcoid behaviour. The differential expression of several miRNAs has been identified both in BPV transformed fibroblasts *in vitro*, and clinical sarcoid samples. Aberrant expression of these post-transcriptional modifiers has been widely studied in Human Papillomavirus (HPV) associated cancers for their association with tumour pathogenesis, progression, metastasis and prognosis (Terron-Canedo *et al.,* 2014, Endale *et al.,* 2024). Early studies suggest that this may also be true for ES, with increased expression of a cluster of miRNAs on chromosome 24 in clinically mild versus aggressive

sarcoids (Bogedale *et al.,* 2019). Conversely, whole blood expression of these miRNAs was increased in horses with sarcoid progression, and was reduced in horses experiencing sarcoid regression in a further study (Unger *et al.,* 2019). The clinical significance of these biomarkers is therefore currently unclear, and further investigation of these serum miRNAs suggested they are of limited diagnostic sensitivity when used as a sole diagnostic marker for ES and provide little prognostic information (Unger *et al.,* 2021).

1.9 Treatment

The previous discussion indicates a rapidly evolving but highly conflicting body of evidence regarding the aetiology, pathogenesis, diagnosis, and prognosis of ES. Despite the wide range of articles on the subject, there is no profession-wide consensus on best practice. The clinician faces a problem first in the determination of an accurate diagnosis, and then in the formulation of the optimal treatment protocol.

The condition is clearly complex, and treatment relies also on sarcoid location, extent, chronicity and confounding horse and owner related factors (Knottenbelt, 2019). This, combined with the discussed difficulties in definitively determining an accurate diagnosis, is a key factor in limiting the quality of literature regarding the treatment of ES. Historically, clinicians have relied on small case series/ reports, anecdotal evidence and the experience of other clinicians, and the reluctance to biopsy lesions means that, in what literature is available, a definitive diagnosis for the treated lesions is often not recorded.

A brief literature search identified nearly 1500 papers on the subject, describing various treatments and combinations of treatments employed by clinicians worldwide. These vary in complexity from simple sharp excision (Martens *et al.,* 2001b), to more complex calcium electroporation (Frandsen *et al.,* 2020) and interstitial brachytherapy (Byam-Cook *et al.,* 2006). Immunotherapy is a rapidly emerging field of study, but thus far few reports of its clinical use exist (Jindra *et al.,* 2023). Of particular note is the general lack of prospective, randomised and/or placebo controlled trials in the

literature (Knottenbelt, 2019). A systematic approach to the review of the literature and objective assessment of the quality of literature regarding the treatment of equine sarcoids is therefore more appropriate than an extensive narrative review on the subject and so the latter is not included in this introduction. Publication of such a review would significantly aid in the evidence-based decision making of treating clinicians.

1.10 Aims

The aims of this thesis are to:

Chapter 2:

- Systematically review the literature regarding the treatment of ES and identify the effect(s) of currently available treatments on sarcoid resolution.
- Assess the quality of evidence regarding the treatment of ES.
- Develop guidelines for the treatment of sarcoids in equids and highlight gaps in the current evidence.

Chapter 3:

- Describe a previously unreported technique for the treatment of ES, a combination treatment protocol consisting of diode laser excision of the mass, followed by cryosurgical treatment and 5-fluorouracil chemotherapy.
- Compare the outcomes associated with this combination technique with laser excision alone.
- Investigate risk factors associated with sarcoid recurrence in a referral population of equids.

Chapter 4:

- Identify immunohistochemical markers expressed in clinical samples from this population of equine sarcoids for future investigation as to their associated with sarcoid behaviour and prognosis.
- An overall conclusion regarding the findings of this study and potential directions for future sarcoid research is given in the final chapter.

Chapter 2: Treatment of Equine Sarcoids: a systematic review

2.1 Summary

Background

The sarcoid is the most common equine cutaneous neoplasm. Evidence-based treatment of this condition is often lacking, and selection of treatment modality based on clinical experience or anecdotal evidence.

Objectives

Assess the quality of the currently available best evidence regarding the treatment of the equine sarcoid.

Study Design

Systematic review in compliance with PRISMA guidelines.

Methods

Literature searches were performed in PUBMED, Web of Science, CAB Abstracts, EMBASE (Ovid) and Scopus in April 2021. Included papers were required to describe an interventional study examining sarcoid treatment strategy, of level 4 evidence or greater. The case definition required confirmation of at least some included lesions on histopathology, and a minimum of 6 months of follow up was required on treated cases. Studies were assessed by two independent reviewers (KO, CD). Data extraction was performed manually, followed by risk of bias assessment. Methodological quality was assessed using the GRADE system.

Results

In total, ten studies were included in the review. Case definition was confirmed via histopathology in all included lesions in 60% of papers. Time to follow up was variably reported. Overall risk of bias ranged from 'some concerns', to 'critical'. Reported sarcoid regression rate ranged from 28-100% on an individual sarcoid level, and 9-100% on a whole horse level. Transient local inflammation was reported following most treatment strategies, with further adverse events reported infrequently.

Main limitations

Review methodology excluded a large proportion of available literature regarding the equine sarcoid. Significant heterogeneity between included studies prevented quantitative synthesis and most included papers were at significant risk of bias, indirectness, and imprecision.

Conclusions

There is insufficient evidence currently available to recommend one sarcoid treatment over another. There is an urgent need for sufficiently powered, randomised, placebo-controlled trials in order to allow more definitive comparison of the efficacy of different treatment strategies.

2.2 Introduction

The equine sarcoid is ubiquitous worldwide and is the most common equine cutaneous neoplasm, diagnosed in approximately 46% of neoplastic equine cutaneous biopsy samples (Schaffer *et al.,* 201µ). The condition has an owner reported prevalence in the United Kingdom of 5.8% and, although rarely metastatic, may be life limiting due to locally aggressive invasion and secondary ulceration and/or infection (Taylor and Haldorson, 2013, Ireland *et al.,* 2013). The condition therefore has a significant influence on the welfare and function of affected equids.

There is currently no uniformly effective therapy for the treatment of sarcoids. Reported success rates between studies are widely variable, and recurrence post treatment occurs frequently (Bogaert *et al.,* 2008). Multiple treatment protocols are reported, including sharp, or laser surgical excision (Knottenbelt and Kelly, 2000, Martens *et al.,* 2001b, Compston *et al.,* 2016, Mccauley *et al.,* 2002, Carstanjen *et al.,* 1997), cryosurgery (Martens *et al.,* 2001b, Lane, 1977), topical or intratumoral chemotherapy (Knottenbelt and Kelly, 2000, Knottenbelt *et al.,* 2020, Stewart *et al.,* 2006, Théon *et al.,* 2007, Nogueira *et al.,* 2006, Mcconaghy *et al.,* 1994), and immunotherapy (Knottenbelt and Kelly, 2000, Martens *et al.,* 2001b, Hodgson *et al.,* 1994, Klein *et al.,* 1986). Further techniques, such as interstitial brachytherapy or plesiotherapy and local electrochemotherapy, are commonly reported but may have limited practical availability (Hollis, 2020, Tamzali *et al.,* 2012, Byam-Cook *et al.,* 2006, Théon and Pascoe, 1995, Tozon *et al.,* 2016).

The range in treatment modalities is primarily due to widely variable lesion clinical behaviour. Traditionally, selection of treatment modality for this condition has often been based on clinical experience, or anecdotal evidence and case series. Evidence based treatment of this condition is currently lacking and is severely limited by the lack of prospective, double-blinded trials (Knottenbelt, 2019).

The question posed by this systematic review is therefore, ‘in equids with sarcoids (P) what effect do reported treatments (I) have on lesion resolution (O)? ‘.

The authors assess the quality of the currently available best evidence, in an attempt to develop guidelines for the treatment of sarcoids in equids and highlight gaps in the current evidence.

2.3 Methods

Eligibility criteria

Criteria for inclusion eligibility in this review was to be an interventional study examining a sarcoid treatment strategy. The study was required to be of Level 4 evidence and above, i.e., at least a case series or case-controlled study in the hierarchy of evidence (Howick *et al.,* 2011).

The case definition (i.e., of 'sarcoid') required confirmation on histopathology in at least some of the cases included, and a minimum of 6 months of follow up was required on treated cases. A publication date restriction of 1970 onwards was applied.

Exclusion criteria

Studies where the full text was not available, single case reports, or case series lacking a comparator group, non-systematic review articles, book chapters, newspaper articles and other documents not containing original data, and papers not available in the English language were excluded.

Search strategies

Literature searches were performed in April 2021 in the following electronic search databases; PUBMED, Web of Science, CAB Abstracts, EMBASE (Ovid), Scopus. Search strategies/ strings are available in Annex 1.

Selection process

All retrieved titles were deposited in EndNote reference manager (The Endnote Team, 2013). Duplicates were removed manually. They were then screened in an unblinded manner, first by title and then abstract, for relevance. Studies fulfilling the inclusion criteria, or in which fulfilment of the

criteria could not be established from the abstract, were retrieved as full texts. Two independent reviewers (KO, CD) then assessed the full contents of each study for inclusion in analysis.

Data collection process

A data extraction sheet was developed based on the Cochrane Consumers and Communication Review Group's data extraction template (Ryan *et al.,* 2016). Data were extracted manually from each report by the first author (KO), and then checked by the second (CD). Disagreement was resolved by a third party (DS). An example of the data extraction sheet is available in Annex 2.

Information was extracted regarding: study design, year of publication and source(s) of funding, the number of cases examined, sarcoid type and location, full details of the treatment, the number of repeat treatments and total treatment time, any adverse effects associated with treatment and the presence or absence of untreated/placebo treated control or, if not available, the treatment group used for comparison. The primary clinical outcome measure was the rate of complete regression, recorded both per horse and per lesion treated. This was defined as the percentage of sarcoids resolved or horses sarcoid free at the time of follow up, as specified by each individual study. Further secondary outcomes included the rate of tumour recurrence, and where available objective measures such as reduction in tumour volume or area.

Risk of bias assessment

The risk of bias for each included study was assessed using the Cochrane group's 'Risk Of Bias In Non-randomized Studies of Interventions (ROBINS-I)' tool for non-randomised trials, or the RoB 2.0 tool for Randomised controlled trials (Sterne *et al.,* 2016, Sterne *et al.,* 2019). The Rtool was used to illustrate this assessment (Mcguinness and Higgins, 2021).

Assessment of methodological quality

Methodological quality was then assessed using the GRADE system (Ryan and Hill, 2016). For outcomes explored by randomised controlled trials (RCTs), rating started at 'high', and non-RCTs started at 'low'. Studies were downgraded for risk of bias, inconsistency, indirectness, imprecision,

or publication bias. Quality of evidence was able to be upgraded where a large magnitude of effect of a treatment was present, a strong dose response to treatment was indicated, or where the effect of all plausible confounding factors would be to reduce the effect (where an effect is observed) or suggest a spurious effect (when no effect is observed).

Data Synthesis

Meta-analysis was not productive due to significant heterogeneity between studies. Data analysis was therefore descriptive. Where possible, results were combined utilising synthesis without meta-analysis (SWiM) guidelines (Campbell *et al.,* 2020). Studies were grouped by treatment protocol in order to compare clinical success rates.

2.4 Results

Study selection

In total, 1481 records were retrieved. Figure 6 describes the results of the search and selection process. The most common reasons for study exclusion included the lack of histopathological confirmation of diagnosis, review articles containing no original data, or case series lacking comparator groups. Ten papers met the criteria for eventual inclusion in this review.

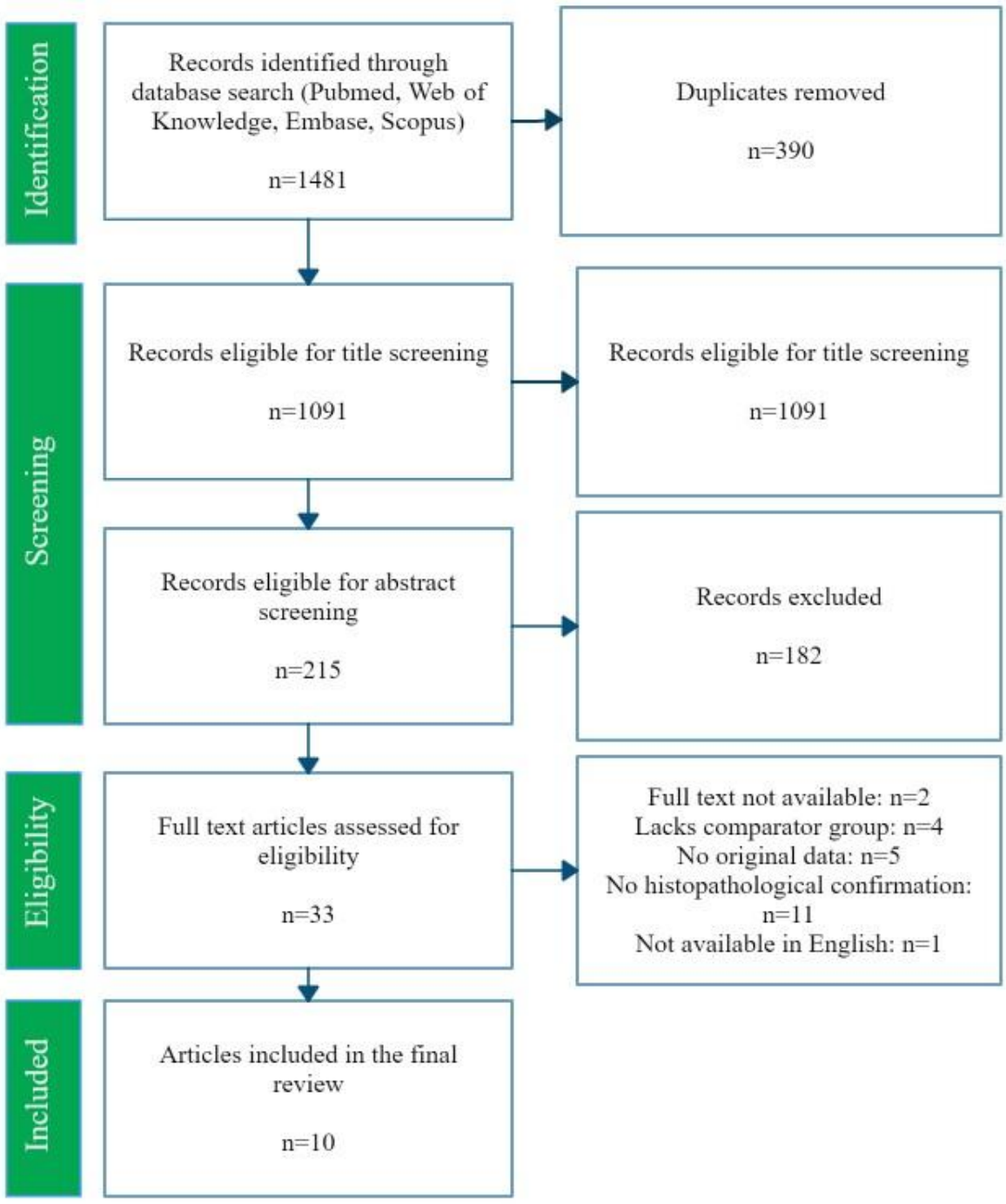


Figure 6: : PRISMA flow diagram of studies included and excluded from the review.

Study characteristics

Four randomised clinical trials were identified, in addition to three prospective, non- randomised clinical studies and three retrospective studies. Methodological characteristics of included studies, with sarcoid type, location, treatment strategies and outcome at follow-up, are described in Table 2.

Paper	Study design	Sarcoid types	Sarcoid locations	% Histo	Available for inclusion		Excluded		Lost to follow up		Time to follow up (months)	Treatment groups	Included	
					Horses	Sarcoids	Horses	Sarcoids	Horses	Sarcoids			Horses	Sarcoids
Christen - Clottu *et al.*, 2010	Prospective randomised blinded clinical trial	Occult-34% Verrucous- 50% Nodular- 3% Fibroblastic- 5% Mixed- 9%	Head- 12% Neck- 12% Prepuce- 6% Inner thigh- 6% Ventrum- 33% Axilla- 14% Thorax- 14%	79	53	163	11	34	17	48	12	Viscum album extract	23	72
												Placebo	13	43
Klein *et al.*, 1986	Prospective randomised clinical trial	Not stated	Head- 20% Leg- 26% Abdomen- 6% Breast- 20% Eye- 1% Anal- 9% Ear- 3% Groin- 14%	100	41	-	11	-	0	0	4-40	Live attenuated BCG vaccine	10	29
												BCG cell wall vaccine	10	16
												Cryosurgery	10	26
Knottenbelt and Kelly, 2000	Retrospective	Occult- 3% Verrucose- 29% Nodular- 36% Fibroblastic- 16% Mixed- 13% Malignant- 0.1%	Periorbital- 100%	12	445	-	-	-	*	*	Variably reported, ≤ 108	Benign neglect (Control)	42 [†]	-
												Surgical excision	28 [†]	-
												Cryosurgery	23 [†]	
												Radiofrequency hyperthermia	2 [†]	-
												BCG immunomodulation	309 [†]	-
												Radiotherapy (Ir^{192})	66 [†]	-
												Radiotherapy (Sr^{90})	3 [†]	-
												Intralesional cisplatin	18 [†]	-
												Topical AW4	146 [†]	-
												Topical 5% 5-fluorouracil	9 [†]	-
Martens *et al.*, 2001	Prospective clinical study	Occult- 5% Verrucose- 25% Nodular- 13% Fibroblastic- 24% Mixed- 34%	Head- 16% Trunk- 76% Extremities- 8%	- (39% of horses)	95	453	-	256	4	-	6 -60 [14]	Sharp excision	22	57
												CO_2 laser excision	28	81
												Cryosurgery	14	18
												Local BCG vaccination	27	30
McConaghy *et al.*, 1994	Retrospective	Not stated	Head- 33% Limbs- 59% Body- 8%	100	63	-	-	-	-	-	6 - 120	Sharp excision	-	18
												Cryotherapy	-	31
												BCG vaccination- Cell wall preparation	-	11
												BCG vaccination- Attenuated vaccine	-	5
												Radiotherapy (Au^{198})	-	1

Petterss on *et al.*, 2020	Prospective clinical study	Occult- 21% Verrucose- 4% Nodular- 20% Fibroblastic- 38% Mixed- 16%	Head- 15% Axilla- 10% Distal limb- 13% Trunk- 15% Genitalia- 15% Neck- 3%	20	25	164	-	-	-	6	3	Topical Imiquimod 5%		45
												Topical Sanguinaria canadensis + zinc		16
												Control (untreated)		107
Spoorm akers *et al.*, 2003	Prospective, randomised, clinical study	Occult- 4% Verrucose- 46% Fibroblastic- 35% Mixed- 15%	Girth/ventral abdomen/ genital- 17%. Pectoral/ neck- 20% Proximal limbs- 11% Distal limbs- 7% Head- 41% Back- 4%	100	36	-	-	-	-	-	12	5 days low dose intralesional IL-2	11	
												10 days low dose intralesional IL-2	10	
												Single high dose intralesional IL-2 + cisplatin	15	
Tamzali *et al.*, 2012	Retrospective	Occult- 8% Verrucose- 41% Nodular- 15% Fibroblastic- 18% Mixed- 18%	Head- 15% Neck- 5% Trunk- 30% limbs 30% genital/ paragenital 23%	100	48	194	14	-	*	*	48	Cisplatin ECT		110
												Cisplatin ECT plus surgical debulking		84
Théon *et al.*, 1999	Prospective, randomised, clinical trial.	Not stated	Periorbital- 58% Pinna- 6% Trunk/neck- 9% Limbs- 17% Genitals- 2%	100	70	89	-	25	-	-	20-69 [47]	Perioperative ITC	-	32
												Postoperative ITC	-	32
Théon *et al.*, 2006	Prospective clinical trial	Not stated	Periorbital- 58% Face and pinnae- 11% Trunk and neck- 10% Limbs- 17% Genitals- 4%	100	368	409	-	-	*	*	36	ITC alone	-	64
												Perioperative ITC, open wound, gross residual disease after Sx	-	47
												Perioperative ITC, closed wound, gross residual disease after Sx	-	99
												Postoperative ITC, open wound, gross residual disease after Sx	-	147
												Postoperative ITC, closed wound, microscopic residual disease after Sx	-	52

*Table 2: Methodological characteristics of included studies in the systematic review of equine sarcoids. – denotes 'no information' []= median, * indicates that only horses with available follow up were included in the study.*

[+] It is unclear from the original manuscript whether these numbers refer to individual sarcoids, or if horses received a combination of treatments and are included in multiple categories. They have been included as horses rather than individual sarcoids throughout this review. ECT= electrochemotherapy, ITC= intratumoural chemotherapy, BCG= Bacillus Calmette–Guérin vaccine, Sx= surgery, '% Histo' = the percentage of included horses/sarcoids with a diagnosis

confirmed via histopathology. 'Available for inclusion' denotes the total number of horses/ sarcoids initially presenting for inclusion in the study, 'Excluded' denotes the number of horses/sarcoids excluded on the basis of that study's inclusion criteria, 'Included' denotes the final number of horses/sarcoids included in the paper's analysis.

Case definition was confirmed via histopathology in all included lesions in 60% of papers. All but two papers lacked untreated or placebo controls, and in only one paper were those administering treatment blinded to the treatment protocol (Christen-Clottu *et al.,* 2010). Only one paper included a power calculation (Théon *et al.,* 1999). Time to follow up was variably reported but was up to 120 months in some cases (Mcconaghy *et al.,* 1994). Included sarcoid types varied between studies, but generally included all clinical morphological categories and, with the exception of Knottenbelt and Kelly (2000) on all regions of the body.

Risk of bias in studies

The risks of bias in individual studies are presented via the Robvis outputs below (Figure 7). Overall risk of bias ranged from 'some concerns' (Christen-Clottu *et al.,* 2010), to 'critical' (Théon *et al.,* 2007). In the randomised controlled trials, primary concerns arose regarding bias in the randomisation process and/ or lack of blinding. In the non- randomised studies, bias arose from confounding, particularly baseline confounding, and from lack of blinding in the assessment of outcomes. There was also concern regarding differences in co-interventions across groups, and regarding the selection of participants based on patient characteristics observed after the start of the study (e.g. exclusion of horses lost to follow up).

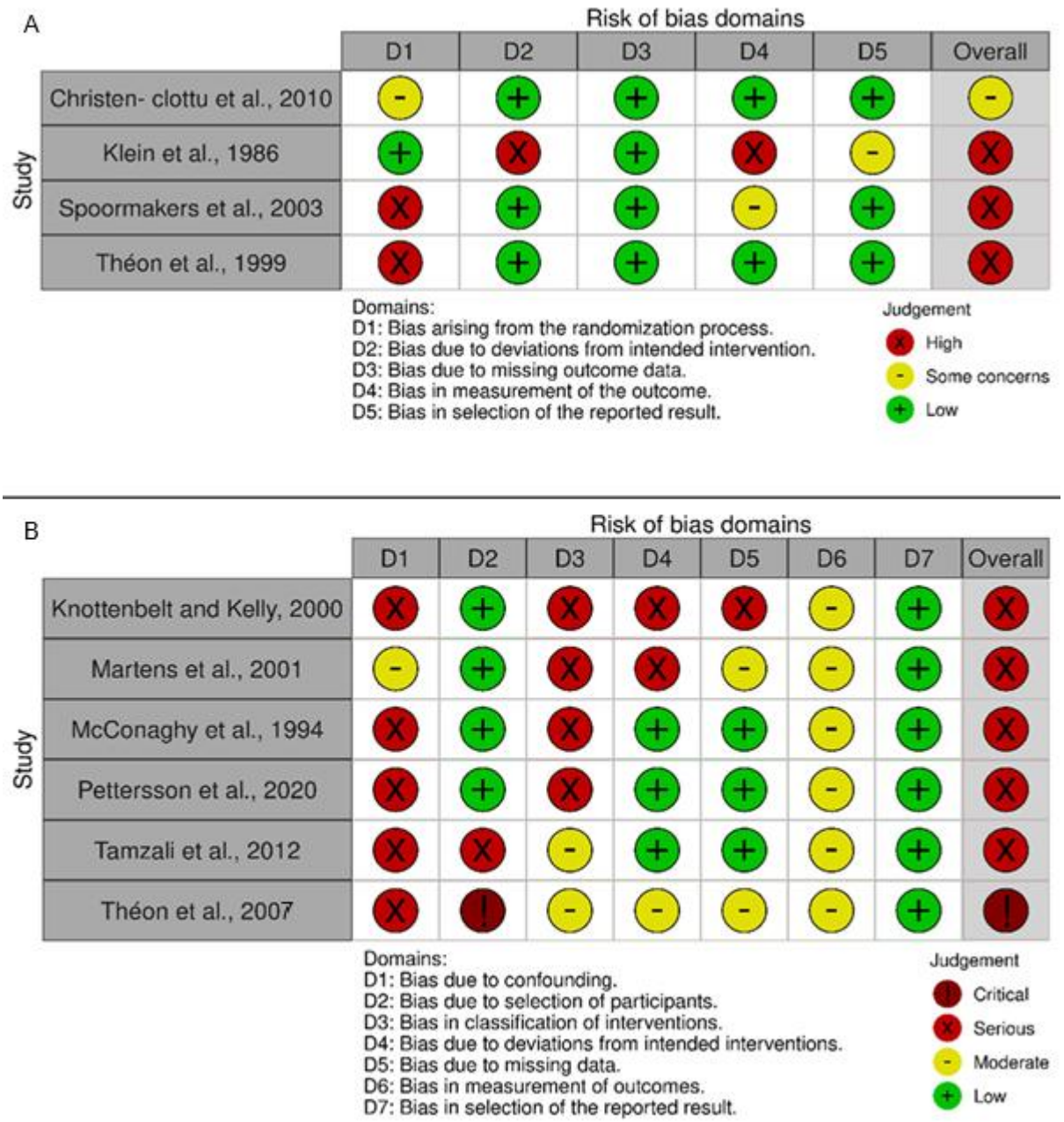
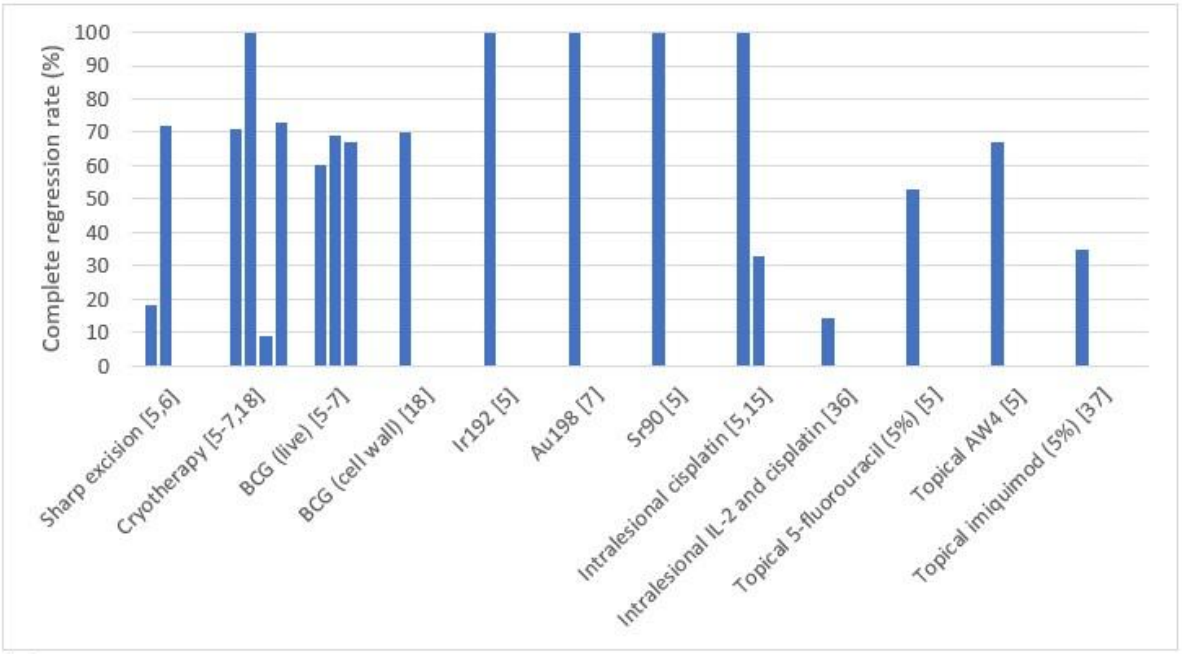


Figure 7: RoBvis diagrams of risk of bias in included (A) Randomised clinical trials and (B) Non-randomised studies of interventions regarding the treatment of equine sarcoids.

A summary of sarcoid resolution rate expected with each treatment is provided in Table 3. Heterogeneity in study design and reporting meant that complete regression rate was not available by horse and sarcoid in every paper. It was also not possible to extract which individual sarcoids within each treatment or paper were histopathologically confirmed, and so all included lesions were

combined. Significant methodological differences existed between papers within each treatment category, for example, surgical debulking prior to cryotherapy, the frequency and number of cryotherapy treatments, or the inclusion of only superficial sarcoids within a treatment category (Table 2). Certainty in the evidence (GRADE scoring) for each treatment outcome is also presented in Table 3. Complete regression rates are displayed graphically in Figure 8.

(A)



(B)

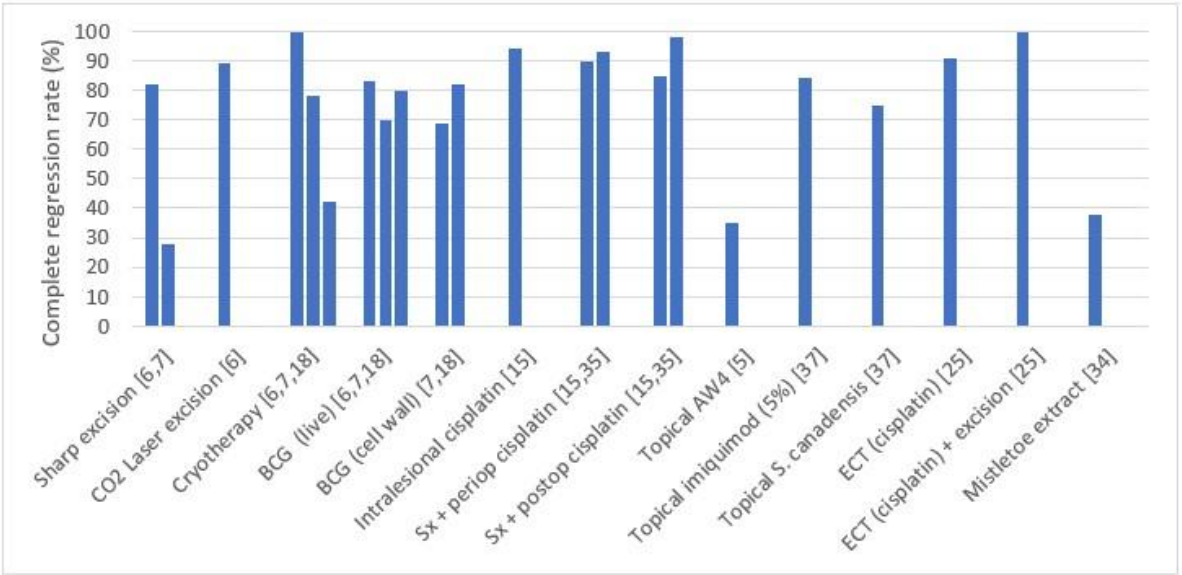


Figure 8:Complete sarcoid regression rates (A) per horse and (B) per tumour, reported with each treatment for equine sarcoids. Each bar represents an individual study (referenced in []) reporting that treatment). Due to the marked heterogeneity between studies review of the original manuscripts is recommended before using these figures solely for treatment selection. ECT= electrochemotherapy, BCG= Bacillus Calmette–Guérin vaccine, Sx= surgery

Treatment	Paper	Complete regression rate (%)		Timing of follow up (months)	Number of participants		Certainty in the evidence (GRADE)	Comments
		Per sarcoid	Per horse		Sarcoids	Horses		
Sharp excision	Knottenbelt and Kelly, 2000	-	18	≤ 108	-	28	Very Low	Periocular, superficial verrucose or Type A nodular sarcoids only.
	Martens *et al.,* 2001	82	72	6 -60 [14]	57	25		Surgical margins 8-16mm
	McConaghy *et al.,* 1994	28	-	6 - 120	18	-		Surgical margins 5-10mm. Base cauterised with electrosurgical unit.
CO_2 Laser excision	Martens *et al.,* 2001	89	71	6 -60 [14]	81	28	Very Low	Surgical margins 8-16mm
Cryotherapy	Klein *et al.,* 1986	100	100		26	10	Very Low	'Very large' tumours first frozen, then debulked, then 2 freeze- thaw cycles repeated at 2-3 weekly intervals between 1-5 times.
	Knottenbelt and Kelly, 2000	-	9	-	-	23		Periocular, <2cm^2 verrucose or occult lesions. 3 freeze- thaw cycles, once only.
	Martens *et al.,* 2001	78	73	6 -60 [14]	18	15		Debulked surgically prior to 2 freeze- thaw cycles, once only.
	McConaghy *et al.,* 1994	42	-	6 – 120	31	-		Debulked surgically prior to 3 freeze- thaw cycles, once only.
BCG immunotherapy (live attenuated vaccine)	Klein *et al.,* 1986	83	60	4-40	29	10	Very Low	0.25ml/cm^2, repeated after 12, 35 and 56 days.
	Knottenbelt and Kelly, 2000	-	69	-	-	300		Periocular only. Variable protocols reported.
	Martens *et al.,* 2001	70	67	6 -60 [14]	30	27		Ulcerated, fibroblastic sarcoids debulked to the level of the skin prior to treatment.
	McConaghy *et al.,* 1994	80	-	6 – 120	5	-		Surgically resected to skin level prior to treatment.
BCG immunotherapy (cell wall vaccine)	Klein *et al.,* 1986	69	70	4-40	16	10	Very Low	0.25ml/cm^2
	McConaghy *et al.,* 1994	82	-	6 – 120	11	-		Surgically resected to skin level prior to treatment. 5ml/3cm^2 tumour
Gamma radiotherapy-Ir^{192}	Knottenbelt and Kelly, 2000	-	100	12	-	66	Very Low	Periocular sarcoids only. Average dose 7000-9000 rads.
Gamma radiotherapy-Au^{198}	McConaghy *et al.,* 1994	-	100	6- 120	-	1	Very Low	Surgically debulked prior to treatment.
Beta radiotherapy-Sr^{90}	Knottenbelt and Kelly, 2000	-	100	12-48	-	3	Very Low	Periocular, single or few 'very small' verrucose/ occult sarcoids only. 10000 rads over 5 days.
Intralesional cisplatin	Knottenbelt and Kelly, 2000	-	33	-	-	18	Very Low	Periocular, fibroblastic, or extensive nodular lesions only. 1mg/cm^3 tumour
	Théon *et al.,* 2006	94	-	36	64	-		1mg/cm^{3-} tumour, four times at 2-week intervals.
Surgery + perioperative intralesional cisplatin	Théon *et al.,* 1999	90±6	-	20-69 [47]	32	-	Very Low	1mg/cm^3 four times at 2-week intervals, commencing at the time of surgery.
	Théon *et al.,* 2006	93	-	36	146	-		1mg/cm^3 four times at 2-week intervals, commencing at the time of surgery.
	Théon *et al.,* 1999	85±7	-	20-69 [47]	32	-	Very Low	1mg/cm^3 four times at 2-week intervals, commencing median 14 days postoperatively.

Surgery + postoperative intralesional cisplatin	Théon *et al.,* 2006	98	-	36	199	-		1mg/cm^3 four times at 2-week intervals, commencing 2-3 weeks postoperatively.
Intralesional IL-2	Spoormakers *et al.,* 2003	-	14	12	-	21	Low	200 000 IU IL-2 Daily for either 5 or 10 days
Intralesional IL-2 and cisplatin	Spoormakers *et al.,* 2003	-	53	12	-	15	Low	1mg/cm^2 cisplatin then daily 200 000 IU IL-2 treatment for 10 days
Topical 5-fluorouracil (5%) cream	Knottenbelt and Kelly, 2000	-	67	-	-	9	Very Low	Periocular, superficial occult or verrucose lesions away from the eyelid margins. Twice daily for 5 days, then once daily for 5 days.
Topical AW4	Knottenbelt and Kelly, 2000g	35	35	-	159	146	Very Low	Periocular, small, previously untreated, superficial verrucose lesions only.
Topical imiquimod (5%)	Pettersson *et al.,* 2020	84	-	3	45	-	Very Low	3 times weekly on non- consecutive days until remission or up to 45 weeks.
Topical Sanguinaria canadensis and zinc chloride	Pettersson *et al.,* 2020	75	-	3	16	-	Very Low	Facial tumours excluded. 6 days of daily treatment then every 4th day until remission or up to 45 weeks.
Electrochemotherapy (cisplatin)	Tamzali *et al.,* 2012	91	-	48	110	-	Very Low	Performed at 2-week intervals. Mean treatment number 2.6 ± 1.1
Electrochemotherapy (cisplatin) combined with sharp excision	Tamzali *et al.,* 2012	100	-	48	84	-	Very Low	ECT done either at the time of surgery, or 2 weeks following surgery, then at 2-week intervals. Mean ECT treatment number 2.9 ± 1.4
Radiofrequency hyperthermia	Knottenbelt and Kelly, 2000	-	0	-	-	2	Very Low	Periocular sarcoids only.
Mistletoe extract (Viscum album austriacus)	Christen- Clottu *et al.,* 2010	37.5	28	12	72	32	Moderate	3 subcutaneous injections of 1ml per week for 15 weeks.

Table 3: Complete regression rates by sarcoid and by horse for each included treatment, accompanied by the certainty in the evidence following GRADE assessment. – denotes 'no information' []= median. BCG= Bacillus Calmette–Guérin vaccine

Reported adverse events with each treatment strategy are available in Appendix 3. Transient local inflammation was experienced following nearly all reported treatments (Knottenbelt and Kelly, 2000, Mcconaghy *et al.,* 1994, Théon *et al.,* 2007, Klein *et al.,* 1986, Tamzali *et al.,* 2012, Christen-Clottu *et al.,* 2010, Théon *et al.,* 1999, Spoormakers *et al.,* 2003, Pettersson *et al.,* 2020). More significant adverse events were generally restricted to individual cases, but included cicatrisation of the upper eyelid following sharp excision (Knottenbelt and Kelly, 2000), septic arthritis of the tarsus following cryotherapy and sequestration of the underlying orbital bone following gamma radiotherapy of a periocular sarcoid (Knottenbelt and Kelly, 2000, Mcconaghy *et al.,* 1994). One case of anaphylaxis was reported following live attenuated BCG vaccine administration which resulted in collapse, but this horse survived with appropriate treatment (Knottenbelt and Kelly, 2000). Accelerated growth of fibroblastic sarcoids was observed in 91% of lesions treated with cryotherapy by Knottenbelt and Kelly, and resulted in the euthanasia of 11 horses, and in one case the treatment of a periocular sarcoid with topical AW4 cream resulted in the loss of the eye (Knottenbelt and Kelly, 2000).

2.5 Discussion

This is the first evidence synthesis study providing an objective assessment of the relevant literature to support equine practitioners in the important and common clinical problem of selection of treatment modality for equine sarcoid treatment. There are challenges in the interpretation and comparison between all described treatments for equine sarcoids due to the significant risk of bias, methodological differences, and underpowered studies. Clinical decisions must therefore continue to be made on a case-by-case basis.

The most effective treatment regimens based upon this study are radiotherapy, cryotherapy, intralesional cisplatin OR electrochemotherapy, with complete regression rates of >90% reported. We summarise the key considerations for these treatments.

Radiotherapy

Radiotherapy has long since been considered the gold standard treatment for sarcoids (Knottenbelt and Kelly, 2000, Hollis, 2019). Both the Ir^{192} and Au^{198} represent low- dose rate brachytherapy- a technique whereby radioactive wires or beads are inserted into the tumour and left in place until a total dose of 50-60Gy is administered (Byam-Cook *et al.,* 2006). There are a number of disadvantages to this approach; general anaesthesia is generally required for the implantation process, and the horse must be kept strictly isolated for several days. Accidental displacement of the implants represents a risk of exposure of personnel to high doses of radiation, and accidental ingestion of the implants by the horse may occur (Hollis, 2019). As such, this technique currently has very limited availability. Sarcoids treated by this approach were periocular (with the exception of one, where the location was not reported) or were surgically debulked prior to treatment (Mcconaghy *et al.,* 1994). This represents a major limitation of the technique- tumour response is inversely proportional to tumour volume and so the technique is best suited to small or superficial sarcoids only (Hollis, 2019).

There is one included report of strontium plesiotherapy included here by Knottenbelt and Kelly (2000). Limited further anecdotal reports exist in the literature (Hollis, 2019), and in one case series where treatment was not limited to the periocular region and all treated sarcoids resolved with variable time to follow up (Hollis, 2020). The advantage of this treatment is that the β radiation supplied by the strontium probe is poorly penetrating, and so significant side effects are less likely to occur (Knottenbelt and Kelly, 2000). This treatment is currently limited by availability, but it may represent an effective treatment for carefully selected lesions going forward.

Cryotherapy

Papers investigating cryotherapy as a treatment modality report success rates of up to 100% (Klein *et al.,* 1986). However, three of the four included papers citing 'cryotherapy' or 'cryosurgery' as a treatment protocol, do so after surgical debulking of the mass, and a significantly lower clinical regression rate of 9% is reported when cryotherapy was used as a sole therapy (Knottenbelt and Kelly, 2000). Case selection, anatomic site and sarcoid type likely contribute to this, and is variably

described (Knottenbelt and Kelly, 2000, Martens *et al.,* 2001b, Mcconaghy *et al.,* 1994). The number of freeze-thaw cycles applied to the tissue also varied between papers from 2 to 3 cycles per treatment, with variable repetition between 0 and 5 times at 2-3 weekly intervals (Mcconaghy *et al.,* 1994, Knottenbelt and Kelly, 2000, Klein *et al.,* 1986). The optimal number of freeze-thaw cycles in sarcoid treatment is unknown, however in human medicine it is accepted that repetitive freezing is crucial in the cryosurgical management of cancers, and that repetition of the freezing may increase the extent of the necrosis to up to 80% (Baust and Gage, 2005).

Cisplatin

Intralesional cisplatin demonstrated sarcoid regression rates of up to 98% when combined with surgical excision (Théon *et al.,* 2007). Success rates were comparable in this paper when used as a sole therapy (94%) but were as low as 33% in the Knottenbelt and Kelly paper (Théon *et al.,* 2007, Knottenbelt and Kelly, 2000). Direct comparison is perhaps not entirely useful- drug formulations (almond oil vs sesame oil emulsions) and concentrations (1mg/ml vs 3.3mg/ml) were different between papers, as was sarcoid type and anatomic location. Théon *et al.* found that larger tumour size and prior use of other treatments negatively affected treatment efficacy, possibly due to difficulty in achieving adequate drug concentrations throughout the tumour before its rapid metabolization (Théon *et al.,* 2007, Tamzali *et al.,* 2012). Cisplatin-containing biodegradable beads have been developed in order to address these limitations but as yet have not been compared with other treatment modalities (Hewes and Sullins, 2006).

Cisplatin electrochemotherapy, either as a sole treatment or in combination with surgical excision of the mass, gave complete regression rates of 91%-100% (Tamzali *et al.,* 2012). Electrochemotherapy has the advantage of increasing a cytotoxic drug's intracellular concentration and cytotoxicity when compared with intralesional injection alone (Heller *et al.,* 1995). Surgical debulking did not significantly influence sarcoid regression rate, but for medium and large-sized tumours significantly reduced the number of treatments needed (Tamzali *et al.,* 2012). Surgical debulking should therefore be considered prior to ECT in cases with large or invasive tumours. Though this study examined the

use of cisplatin with ECT, the most commonly used chemotherapeutic agent in other veterinary species receiving ECT is bleomycin (Spugnini and Baldi, 2019). A recent European Standard Operating Procedures of Electrochemotherapy study found no difference in cutaneous tumour response rate when comparing the use of cisplatin or bleomycin (Marty *et al.,* 2006), and so this may be considered in the future, given its low toxicity to non-tumour cells when compared with other chemotherapeutic agents (Knottenbelt *et al.,* 2020).

The above treatment modalities all have the disadvantage that they require, at the least, attendance to a veterinary facility. Given the ubiquitous nature of sarcoids in horses (Taylor and Haldorson, 2013), it is often more feasible to pursue topical treatment options, which may be employed on the yard. Though topical imiquimod appears to have the best complete regression rate at the individual sarcoid level, this is not true when considering treatment success within the whole horse. No significant difference in regression rate was found between sarcoids treated with either topical imiquimod or *Sanguinaria canadensis* and zinc chloride by Pettersson *et al.* (2020)*,* but small, fibroblastic tumours were more likely to respond favourably than larger sarcoids of other types (80% complete remission with either protocol) (Pettersson *et al.,* 2020). The use of AW4, a proprietary chemotherapeutic agent, for the treatment of small, periocular sarcoids was less successful (regression rate 34%), though direct comparison between topical treatments should be made with caution given the extreme heterogeneity between studies.

Throughout the included studies, employing a multimodal approach to the treatment of sarcoids appears to provide an advantage in complete regression rate over single treatment modalities, though the significance of this cannot be determined. For example, the addition of surgical debulking prior to cryotherapy or electrochemotherapy, or the addition of intralesional cisplatin to intralesional IL-2 protocols. Although not included in these studies, recent advances in the understanding of the molecular basis of cryotherapy also suggests that this will be true for the addition of cytotoxic agents to cryosurgical techniques (Baust and Gage, 2005). This has also been

suggested by previous authors as a method to improve clinical regression rate and prognosis (Knottenbelt, 2019).

Limitations of evidence and review process

This methodology of the review itself introduces a number of potential biases. The exclusion of grey literature, single case reports and studies lacking any histopathological confirmation of diagnosis removed the majority of the literature regarding equine sarcoids. However, in only 60% of included papers were all included sarcoids confirmed histopathologically. It was not possible to extract only those confirmed lesions from these studies, and so unconfirmed lesions unfortunately had to be included. This compromise was made in order to maximise the scope, whilst maintaining the validity, of the review, but clearly introduces a significant source of bias.

Heterogeneity between included papers also limited the available synthesis, and so only a basic narrative synthesis was appropriate. This heterogeneity introduced an important source of bias in this review, variation in sarcoid type. For example, fibroblastic sarcoids were over- represented in the paper by Pettersson *et al.* compared with other articles (Table 2), and, in contrast to the majority of other included studies, verrucose sarcoids the least frequently treated (Pettersson *et al.,* 2020). The influence of sarcoid morphological type on behaviour has not been defined in the literature, though anecdotally, fibroblastic lesions are likely to be perceived as increasingly aggressive and locally infiltrative than other classifications (Taylor and Haldorson, 2013). Anatomic location of the included sarcoid was also very variable (Table 2). Only periorbital sarcoids were discussed by Knottenbelt and Kelly (Knottenbelt and Kelly, 2000), and were over-represented in both papers by Théon *et al*. (Théon *et al.,* 1999, Théon *et al.,* 2007). It has been suggested that sarcoids of the face and upper forelimb display an increased frequency of malignancy and more aggressive local invasion (Knottenbelt, 2019). This cannot be confirmed by this review and may warrant further investigation, but likely influenced both treatment selection and tumour response in the included studies.

The most significant limitation in this review is the quality of available evidence. Included papers generally lacked power calculations or were underpowered (Théon *et al.,* 1999), and the persons administering the treatments were also unblinded to the treatment protocol in all but one of the included studies (Christen-Clottu *et al.,* 2010).

Sarcoid resolution rate was selected as the outcome of interest in this review as it was the most consistently available outcome available between studies. However, this outcome is also complicated by significant bias. In only one paper was there an untreated control or placebo group included therefore the use of this outcome is problematic (Christen-Clottu *et al.,* 2010). Spontaneous regression without treatment is reasonably frequently reported with sarcoids and may be expected in up to 48% of cases, particularly with young horses (Berruex *et al.,* 2016). As above, comparison of different sarcoid types and anatomic location is often not valid given their widely different clinical behaviours (Knottenbelt, 2019). The use of objective measures, e.g., measured reduction in tumour area or volume compared with a matched, untreated control would be more desirable, but was not available in the literature. The use of sarcoid recurrence rate would similarly be a more clinically significant outcome measure for this review, but available literature was widely variable in included follow up times, and so again this comparison was not useful.

The GRADE rating protocol for the included outcomes suggests that the quality of this evidence is generally 'very low', and most included papers were at significant risk of bias, indirectness, and imprecision (Atkins *et al.,* 2004). This contributed to the large variation in sarcoid regression rates between studies looking at the same or similar treatment protocols. The confidence in the effect estimates (Table 2) is therefore so low that any recommendation of one treatment strategy over another is speculative, and in the majority of studies no significant difference between treatment modality was demonstrated.

Whilst the traditional pyramid of evidence places systematic reviews at the top of the hierarchy for evidence based medicine, this may be too simplistic in this case on account of the significant

heterogeneity and risk of bias in the included papers (Vandenbroucke, 1998). More recently, a 'new evidence pyramid' has been suggested for medical evidence that views systematic reviews as a tool with which to examine and apply the available evidence, rather than evidence in their own right (Murad *et al.,* 2016). This may be more appropriate in this case, where there is significant uncertainly in the quality of the available evidence.

Implications for practice/policy/ future research

Given the above, it must be concluded in this review that there is insufficient evidence to routinely recommend one sarcoid treatment over another. We have identified an urgent clinical need for sufficiently powered, randomised, placebo- controlled trials to be performed. This would facilitate the adoption of standardised treatment protocols, for example regarding dose of chemotherapeutic agent/cm^3 tumour or frequency and repetition of cryotherapeutic freeze/thaw cycles. All decisions regarding the most appropriate treatment for any sarcoid are conditional on the sarcoid type, location, size and other patient and owner factors, and should be made at the discretion of the attending veterinary surgeon. If available, radiotherapy should be considered a good treatment option, or if not available then a multimodal approach should be considered. When a topical treatment is necessary, the greatest evidence of efficacy exists for the use of topical imiquimod (5%) or *Sanguinaria canadensis* (Pettersson *et al.,* 2020). Higher quality evidence is required to facilitate more definitive comparison of the efficacy of different treatment strategies for this common condition.

2.6 Appendices

2.6.1 Appendix 1: Full search strategy and results for included databases

Date	Source	Databases	Search strategy	Retrieved records
28.4.21	Pubmed	Pubmed	#1: “Equidae"[Mesh] OR horse*[tw] OR equi*[tw] OR donk*[tw] OR mule[tw] #2: ("Horse Diseases"[Mesh] AND "Skin Neoplasms"[Mesh]) OR sarcoid[tw] OR “cutaneous neopl*”[tw] OR “cutaneous mass” [tw] #3: treat*[tw] OR therapy[tw] OR management[tw] OR laser[tw] OR excision[tw] OR immun*[tw] OR cryoth*[tw] OR chemoth*[tw] OR brachyth*[tw] OR radioth*[tw] #4: #1 AND #2 AND #3 Year filter: 1970- present	270
28.4.21	Web of Knowledge	WOS, BCI, BIOSIS, CABI, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC	#1: TS=(horse OR equine OR donkey OR equid* OR mule) #2: TS=(sarcoid OR “cutaneous neopl*” OR “cutaneous mass”) #3: TS=(treat* OR therapy OR management OR laser OR excision OR immun* OR cryoth* OR chemoth* OR brachyth* OR radioth*) #4: #1 AND #2 AND #3 AND #4 Databases= WOS, BCI, BIOSIS, CABI, CCC, DRCI, DIIDW, KJD, MEDLINE, RSCI, SCIELO, ZOOREC Timespan: 1970-2021	512
28.4.21	Embase (OVID)	Embase	#1 (horse OR equi$ OR donkey OR mule).af #2 (sarcoid OR cutaneous neopl$ OR cutaneous mass).af #3 (treat$ OR therapy OR management OR laser OR excision OR immune$ OR cryoth$ OR chemoth$ OR brachyth$ OR radioth$).af #4 #1 AND #2 AND #3 Timespan: 1970-2021	219
28.4.21	Scopus	Scopus	TITLE-ABS-KEY (horse OR donkey OR equine OR equus OR equid*) AND (sarcoid OR cutaneous neopl* OR cutaneous mass) AND	480

			(treat* OR therapy OR management OR laser OR excision OR immun* OR cryoth* OR chemoth* OR brachyth* OR radioth*) Timespan: 1970- present	
			Total articles	1481
			Eliminate duplicates	1091
			After screening titles	215
			After screening abstracts	33
			After screening texts: KO	11
25.7.22			**After screening texts: CD**	9
27.7.22			**Agreed total after screening texts**	10

Appendix 1: Search strategies and search results from the systematic review (Chapter 2).

2.6.2 Appendix 2: Example of adapted data extraction template.

General review information

Review Title: Treatment of Equine Sarcoids: a systematic review

Name of review author completing this form:

Date form completed

Name of review author checking the data extracted to this form:

Other information and notes

Author contact details for study	
Further information required	
Correspondence with authors successful or not; what information was received and when	

Additional unpublished data?	
Notes	

Methods of the study

Aim of study	To evaluate the effects of a VAE (Iscador P, Viscum album extract) on clinically diagnosed equine sarcoid (CDES) of horses.
Study design	Prospective, randomised, blinded, clinical trial.
Number of treatment groups	Treatment group: VAE n=32 (horses) n=95 (sarcoids)

	Control/ comparator: placebo n=21 (horses) n=68 (sarcoids)
Funding source	Society for Cancer Research (Arlesheim) and Weleda AG (Arlesheim)
Ethical approval	*(Yes/No/Unclear)*

Study characteristics - Participants

Methods of recruitment of participants	Unclear			
Inclusion criteria	Clinically diagnosed equine sarcoid			
Exclusion criteria	Horses with other ES related therapies within 8 weeks of starting study or with signs of acute systemic diseases.			
Age range		VAE	Control	Total
	1-5 years	14 (44%)	8 (38%)	22 (42%)
	6-9 years	10 (31%)	10 (48%)	20 (38%)
	>9 years	8 (25%)	3 (14%)	11 (21%)
	Average	7.4±4.2	6.8±3.4	7.2± 3.9 3-17 Median 6 years
Breeds		VAE	Control	Total
	Freiberger	6 (19%)	5 (24%)	11(21%)
	Swiss Warmblood	15 (47%)	11 (52%)	26 (49%)
	Thoroughbred	4 (13%)	2 (10%)	6 (11%)

	Other	7 (22%)	3 (14%)	6 (11%)
Histopathological confirmation of sarcoid	Yes/ no/ unclear/ partial 79% n=42 horses/ 53, 129tumours/ 163			
Sarcoid type(s)	occult, verrucous, nodular, fibroblastic, mixed			
Sarcoid location(s)	head, neck, chest, axillary region, abdomen, prepuce and udder, inner hind thigh, and distal leg			

Study numbers	**Number**
Power calculation	Yes/**No**/ Unclear
Eligible for inclusion	53 horses 163 tumours
Excluded	11 horses 34 tumours No histological confirmation Horses with other ES related therapies within 8 weeks of starting study or with signs of acute systemic diseases.
Withdrawn (for each group; with reasons if relevant)	n/a

Lost to follow up (for each group; with reasons)	At 12 months: Intervention group (with reasons) • 9 horses/ 32 • 23 tumours/95
	Control group (with reasons) • 8 horses/ 21 • 25/68 tumours Owners decided to try other treatments
Included in the analysis (for each group, for each outcome)	Outcome 1: Outcome at end of trial on horse level Intervention 32 Control: 21
	Outcome 2: Outcome at end of trial on sarcoid level Intervention 95 Control: 68
	Outcome 3: Outcome at 12 months on horse level Intervention : 23 Control : 13

	Outcome 4: Outcome at 12 months on sarcoid level Intervention : 72 Control: 43

Study characteristics - Interventions

Item	Explanation, notes	Intervention	Control/ comparator
Intervention name		VAE= Viscum album extract	Placebo
Rationale	Describe any theory (with key references) or rationale relevant to the intervention. Describe any information on the quality of the intervention, assessed by study authors, others, or by you - such as the evidence base supporting the intervention.	Dose escalating scheme from manufacturer's recommendations and had been evaluated for tolerance previously in 7 healthy horses.	Blinded, 2:1 ratio
What was done?	<u>Materials:</u> Describe the content, format(s) or media, source of materials (if possible, where they can be accessed)	15 weeks treatment, 3 subcutaneous injections/ week of 1ml aqueous extract of fermented Viscum album austriacus (Iscador P). Variable concentrations 0.01-20mg/ml. SC in pectoral region. Clinical assessment performed prior.	15 weeks treatment, 3 subcutaneous injections/ week of 1ml of 0.9% sterile saline. SC in pectoral region. Clinical assessment prior.

	<u>Procedures:</u> Describe each of the processes used in delivering the intervention <u>Mode of delivery:</u> Describe the mode of delivery of the intervention, such as whether it was delivered face-to-face (*e.g. in patient consultation, educational session, training*) or at a distance *(ego via phone, internet, mail);* and whether the delivery was to individuals or groups of participants. <u>Cointerventions:</u> Describe the delivery of any co-interventions	8 horses, selective surgery (sharp excision) 2 weeks before treatment. Selected by clinician Dose escalation protocol as per previous tolerance test (reported, not published data) 0.1 up to 20mg/ml VAE over the first 9 weeks (Table 2)	3 horses, selective surgery 2 weeks prior.
Who delivered the intervention?	Describe who was involved in delivery of each component of the intervention. Include description of any specific training given to providers to deliver the intervention, numbers of providers etc.	Horses were examined by 1^{o} author (O.C.)	Horses were examined by 1^{o} author (O.C.)

Where was the intervention provided?	Hospital vs yard	Field study- western Switzerland	Field study- western Switzerland
When and how often or how much of the intervention was provided?	Describe how the intervention was delivered, such as stages, timing, frequency, number of sessions, intensity, and duration of intervention delivery.	See above	See above
Was the intervention modified or adapted?	If the intervention was changed during the study, this should be described	No	No
How well was the intervention delivered?	Assessment of fidelity:	Treatment apparently well tolerated; courses completed	Treatment apparently well tolerated; courses completed

Risk of Bias assessment- summary from ROBINS-I

Risk of bias judgement- ROBINS-I **Risk of bias judgement- RoB**	Low / Moderate / Serious / Critical / NI Low/ **Some concerns**/ High
Optional: What is the overall predicted direction of bias for this outcome?	Favours experimental / Favours comparator / Towards null /Away from null / **Unpredictable**

Study characteristics - Outcomes and comparison groups

Primary outcomes			
Outcome	Method of assessing outcome measures *e.g., phone survey, questionnaire*	Method of follow-up for non-respondents	Timing of outcome assessment *(Including frequency, length of follow up)*
Tumour response	*% change in tumour volume*	*N/A*	*3,6,9,12 months*

Primary outcomes - adverse events (*e.g. complaints, levels of dissatisfaction, adverse incidents, side effects, increased inequities)*			
Adverse event	Method of assessment	Timing of assessment	Number
Mild oedema at injection site	*Clinical judgement*	*<3 days*	*16% treated horses (5)*

Data and results

Dichotomous outcomes

Outcome	Timing of outcome assessment (days/months)	Intervention group*		Control/ comparator group		Notes
		Observed (n)	Total (N)	Observed (n)	Total (N)	
Complete regression	3	95	6	64	3	
	6	91	14	68	6	
	9	91	16	54	9	
	12	72	27	43	9	

Partial regression	3	95	21	64	11	
	6	91	24	68	14	
	9	91	25	54	13	
	12	72	21	43	8	
Stabilisation	3	95	52	64	29	
	6	91	4	68	7	
	9	91	28	54	9	
	12	72	11	43	7	
CR + PR	3	95	27	64	14	
	6	91	38	68	20	
	9	91	41	54	22	
	12	72	48**	43	17**	Significant difference vs control
CR + PR + S	3	95	79	64	43	
	6	91	42	68	27	
	9	91	69	54	31	
	12	72	72**	43	43**	Significant difference vs control

**Note: add additional columns if there is more than one intervention group, e.g. Intervention Group A, Intervention Group B…*

Continuous outcomes

Outcome	Timing of outcome assessment (days/months)	Intervention group			Control/ comparator group			Notes
		*Mean / Mean change	Standard deviation	N	*Mean / Mean change	Standard deviation	N	
n/a								

Other results or data:

Logistic regression:

CR: better in verrucose (OR=2.38±1.03)

CR + PR: Less likely in previous biopsy (OR= 0.24±0.08)

CR+PR+S: Less likely in previous biopsy (OR=0.3±0.1)

Appendix 2: Example of the adapted data extraction form used in the systematic review of Chapter 2.

2.6.3 Appendix 3: Adverse events reported with respective treatments

Treatment	Event	Incidence (%)	Treated (N)	Paper
Sharp excision	Cicatrisation of upper eyelid	4	28	Knottenbelt and Kelly, 2000
CO_2 laser excision	Whitening of hair over treated region			Martens *et al., 2001*
Cryotherapy	Accelerated recurrence of fibroblastic sarcoid	91	23	Knottenbelt and Kelly, 2000
	Whitening of hair over treated region			Martens *et al., 2001*
	Septic arthritis of the tarsus	3	31	McConaghy *et al.,* 1994
BCG immunotherapy (live attenuated and cell wall)	Local swelling at injection site	100	31	Klein et al 1986
		85	309	Knottenbelt and Kelly, 2000
		12	16	McConaghy *et al.,* 1994
	Oedema away from injection site	19	31	"
	Pyrexia and anorexia	'common'	-	"
	Anaphylaxis	0.3	309	Knottenbelt and Kelly, 2000
	Discharging sinus tracts	15	309	"
Gamma radiotherapy (Ir^{192})	Transient corneal oedema	3	66	Knottenbelt and Kelly, 2000
	Sequestration of orbital bone	1.5	66	"
Intralesional cisplatin ± surgery	Local tissue reaction	-	32	Théon *et al.,* 1999
		100	368	Théon *et al.,* 2006
	Wound dehiscence	-	*32*	Théon *et al.,* 1999
	Seroma formation	-	*32*	"
	Infection	-	*32*	"
Intralesional IL-2	Localised swelling	-	-	Spoormakers *et al.,* 2003
Intralesional IL-2 and cisplatin	Localised inflammation	100	22	"
AW4	Scarring and secondary effects on eyelid function	4	146	Knottenbelt and Kelly, 2000
	Extensive necrosis of the upper eyelid and loss of eye	0.7	146	"
Imiquimod	Local inflammation- mild	84	45	Pettersson *et al.,* 2020
	Local inflammation- moderate	9	45	"
	Local inflammation- severe	7	45	"
	Scarring	4	45	"

	Alopecia	13	45	“
	Hypopigmentation	2	45	“
	Local pain	4	45	“
Sanguinaria canadensis and zinc chloride	Local inflammation- mild	69	16	“
	Local inflammation-moderate	19	16	“
	Local inflammation- severe	13	16	“
	Scarring	31	16	“
Electrochemotherapy	Acute local inflammation	51	99	Tamzali *et al.,* 2012
Mistletoe extract (Viscum album austriacus)	Mild oedema at injection site	16	32	Christen- Clottu *et al.,* 2010

Chapter 3: Retrospective study: Laser excision versus combined laser, cryosurgery and intralesional 5-fluorouracil in the treatment of equine sarcoids.

3.1 Preface

The preceding systematic review identified a significant lack of sufficiently powered comparative studies on the treatment of ES where clinical diagnosis is confirmed by the accepted gold standard, histopathology (Knottenbelt *et al.,* 2015). The review also indicated a possible benefit of employing a multimodal technique in the treatment of certain sarcoids.

At Glasgow Equine Hospital (GEH), an equine referral centre treating approximately 20 cases of ES annually, the standard treatment protocol employs a combination of laser excision of the grossly visible mass, followed by 3 freeze-thaw cycles of cryotherapeutic treatment of the resultant surgical site and instillation of approximately 1ml/cm^3 of 500mg/ml 5-fluorouracil solution. This protocol has, as yet not been described in the literature for the treatment of ES. The following article compares this combination treatment protocol, with the more commonly used simple laser excision of the mass (Compston *et al.,* 2016). The GEH cases represent an opportunity to examine the risk factors for sarcoid recurrence in a second opinion population where multiple and locally infiltrative sarcoids are common due to the referral nature of the work.

For the remainder of this chapter, 'cryotherapy' and 'cryosurgery' are used interchangeably, at the request of a reviewer of this article prior to publication.

3.2 Main Text

3.2.1 Summary

Background

Laser excision is used routinely in the treatment of sarcoids but may be ineffective in cases where complete excision cannot be achieved. A multimodal approach is warranted in these cases. 5- FU may improve the cytotoxic effect of cryosurgery as an adjunct to laser excision.

Objectives

To compare two treatment protocols for equine sarcoids, laser excision alone versus a combination protocol of laser excision, cryosurgery and 5- FU chemotherapy. Factors associated with sarcoid recurrence are also investigated.

Study Design

Retrospective case-controlled study.

Results

Eighty- four horses with 168 histologically confirmed sarcoids were included, with a median follow-up time of 39 months (IQR 21–62 months). Sarcoid recurrence at the treated site was reported in 38% of cases and in 23% of any individual sarcoid. No significant difference was demonstrated between treatment categories in either rate of sarcoid recurrence ($p = 0.45$ for any treated horse, $p = 0.63$ for individual sarcoid) or time to sarcoid recurrence ($p = 0.73$). Sarcoid recurrence was higher in horses with a greater number of sarcoids (OR 1.2 (1.0–1.5), $p = 0.03$); when treatment had been received prior to admission (OR 7.6 (2.0–33), $p = 0.004$). Horses with urogenital sarcoids or >1 mixed sarcoid experienced more rapid recurrence (HR 3.6 (1.3–10), $p = 0.02$ and HR 9.9 (3.3–30), pp < 0.001) and recurrence was less rapid following the treatment of a horse's first sarcoid (HR 0.3 (0.1–0.7), $p = 0.009$).

Main Limitations

Significant differences in case populations in each treatment category. Treatment selection was neither blinded nor randomised and missing data and recall bias limit the study's power. Sarcoid recurrence was owner reported.

Conclusions

When assessing the likelihood of sarcoid recurrence, characteristics of both the individual patient and sarcoid phenotype must be considered carefully when selecting a specific treatment protocol.

Keywords

Horse, sarcoid, recurrence, cryosurgery, chemotherapy

3.2.2 Introduction

The equine sarcoid (ES) is the most common equine cutaneous neoplasm, responsible for approximately 46% of neoplastic cutaneous biopsy samples (Schaffer *et al.,* 2013, Taylor and Haldorson, 2013). Although tumours are rarely metastatic, aggressive local invasion and secondary infection/ulceration may have a significant impact on equid welfare (Taylor and Haldorson, 2013, Ireland *et al.,* 2013). Currently no uniformly effective therapy for its treatment has been reported despite publication of numerous treatment protocols (Byam-Cook *et al.,* 2006, Carstanjen *et al.,* 1997, Compston *et al.,* 2016, Hollis, 2020, Hollis and Berlato, 2018, Klein *et al.,* 1986, Knottenbelt and Kelly, 2000, Knottenbelt *et al.,* 2020, Lane, 1977, Martens *et al.,* 2001b, Mccauley *et al.,* 2002, Mcconaghy *et al.,* 1994, Stadler *et al.,* 2011, Stewart *et al.,* 2006, Tamzali *et al.,* 2012, Théon *et al.,* 2007). Laser excision, either by CO_2 or diode laser, is widely used for the surgical removal of sarcoids (Carstanjen *et al.,* 1997, Compston *et al.,* 2016, Martens *et al.,* 2001b, Martens *et al.,* 2001a, Mccauley *et al.,* 2002). As with any surgical excision, one of the major limitations is that tumour- free margins must still be obtained and this may be difficult with equine sarcoids, depending on sarcoid type, size, number and anatomic location (Knottenbelt, 2019). Though thermal injury secondary to laser excision extends beyond the surgical field, this is variable and difficult to predict (Knottenbelt, 2019, Tate and Tate, 2019). A multimodal approach to sarcoid treatment has frequently been

suggested to reduce sarcoid recurrence rates (Klein *et al.,* 1986, Knottenbelt, 2019, Spoormakers *et al.,* 2003, Tamzali *et al.,* 2012), likely due to greater destruction of remaining neoplastic cells in situations where complete tumour excision has not been achieved.

Cryosurgery, the freezing of cells in this case by the application of liquid nitrogen, kills cells directly due to ice crystal formation and microcirculatory failure (Baust and Gage, 2005). Reported success rates as a sole therapy are variable (Klein *et al.,* 1986, Knottenbelt and Kelly, 2000), with several authors reporting this therapy is most useful when used as an adjunct to other therapies, particularly in the case of larger cutaneous masses (Klein *et al.,* 1986, Martens *et al.,* 2001a, Martens *et al.,* 2001b). Recent molecular research has focussed on tissues in the periphery of the cryosurgery treated zone where cell death is delayed and via apoptosis (Hollister, 1998). It has been suggested that further therapy promoting apoptosis in this region may be beneficial in improving the lethal effect of cryosurgery, and thereby improve outcome (Baust and Gage, 2005, Clarke *et al.,* 1999).

5- fluorouracil (5- FU) is a fluoropyrimidine antimetabolite used in the treatment of a range of cancers, particularly breast and colorectal cancers (Longley *et al.,* 2003). It exerts its effects via incorporation of the fluoronucleotides into cellular RNA and DNA, leading to subsequent cellular dysregulation and apoptosis (Longley *et al.,* 2003). In horses, its use has been reported in the treatment of ocular or peri- ocular squamous cell carcinoma (Offer *et al.,* 2022, Pucket and Gilmour, 2014), and as a topical agent for the treatment of peri- ocular sarcoids with variable success (Knottenbelt and Kelly, 2000). Intralesional 5- FU alone has a reported sarcoid resolution rate of 61.5%, though rates may be lower in large or 'resistant' tumours (Stewart *et al.,* 2006). 5- FU is ranked amongst the safest chemotherapeutic agents (Vodenkova *et al.,* 2020) and its use over more commonly reported platinum- based ES chemotherapy may be warranted given reduced evidence of any human carcinogenic activity (Cancer, 1987, Greene, 1992)

Its use as an adjunct to cryosurgery merits further investigation since it has been suggested that agents, such as 5- FU, may be beneficial in promoting the apoptotic cell death at the periphery of

cryotherapeutic sites (Baust and Gage, 2005, Clarke *et al.,* 2001). This may be particularly clinically useful in anatomic locations that limit the depth and/or extent of laser excision or cryosurgery in order to reduce the extent of damage to underlying normal tissues.

Objectives and hypotheses

This study aimed to compare two treatment protocols for the treatment of equine sarcoids: laser excision alone versus a combination protocol of laser excision, cryosurgery and 5- FU chemotherapy. As a secondary aim, factors associated with sarcoid recurrence were investigated. The authors hypothesise that the combination protocol will result in lower sarcoid recurrence rates than the laser excision protocol.

3.2.3 Materials and methods

Records were reviewed for horses referred to Glasgow Equine Hospital for the treatment of sarcoids between 2013 and 2022 (Figure 9). Criteria for case inclusion were horses with histologically confirmed sarcoids treated either by laser excision via diode laser or by the below combination therapy protocol. Cases were excluded if histopathological confirmation was not available, treatment was incomplete, or if a combination of laser excision/combination therapy/ any other combination treatment was employed.

Excision via diode laser consisted of use of the laser on continuous mode and a power setting of between 15 and 25 W, at the surgeon's discretion. Adherence to laser safety protocols was maintained. Where possible, the surgeon achieved a margin of at least 1 cm of grossly normal tissue around the sarcoid, although this was limited in cases where masses were very large and/or infiltrative, depending on their anatomic location. In this protocol, surgical sites were either left to heal by secondary intention or were closed using a 'smart' surgical technique, as previously described (Knottenbelt *et al.,* 2015). When using the combination protocol, laser excision sites were left open, and excision margins were then treated with three freeze–thaw cycles of cryosurgery followed by administration of 5- FU solution into the margins. Cryosurgery was performed using the CryoPro® cryosurgery unit (Cortex Technology, Denmark). The unit was held approximately 10 cm

from the desired site and liquid nitrogen was applied to spray freeze the epidermal margins and exposed dermis until firm. Care was taken to avoid excessive freezing of the underlying tissues. The tissues were allowed to thaw, before repeating a further two times. Thereafter, 50 mg/mL 5- FU (Medac) was injected into the resultant wheal to an estimated dose of 1 mL/cm3 target tissue. Appropriate personal protective equipment (two layers of chemotherapy- approved nitrile gloves, full length gown and face shield) was used during the application, and the horse handled only when wearing suitable nitrile gloves for 3 days thereafter. The horse was re- examined between 2 and 5 weeks thereafter, and the cryosurgery and 5- FU application re peated. This was repeated as required by clinical progress, for a median of three total treatments (IQR 2–3). The decision to repeat cryosurgery and chemotherapy after the three treatments was based on clinical assessment of the appearance of the granulation bed, with nodular growth a potential indicator of remaining sarcoid tissue.

Data obtained from medical records included: case history and signalment, details of the lesion(s) (sarcoid type, location, number, estimated tumour dimensions and volume), treatment protocol, as sociated adverse effects and duration, response to treatment and sarcoid regrowth/new lesion occurrence. The total number of suspected sarcoids, including unconfirmed and confirmed masses, was recorded for each case, but for all further analysis only histologically confirmed masses were included. For the purpose of recording sarcoid location, the body was split into seven anatomic regions: peri- ocular, head and neck, dorsum (above the level of the olecranon process), ventrum (below the level of the olecranon process), upper limb (above the level of the tarsocrural/radiocarpal joint), lower limb (below the level of the tarsocrural/radiocarpal joint) and urogenital (affecting the prepuce, penis, mammary glands or vulva). Sarcoid type was recorded using the currently accepted convention (Knottenbelt, 2005). Previous histopathological findings were recorded, including the presence or absence of confirmed surgical margins.

Following initial correspondence, owners were contacted via telephone between August and October 2022 to determine treatment outcome. An example of the telephone questionnaire is included in Appendix 4. Data were collected regarding the horse's history of sarcoid growth and treatment, outcome following sarcoid treatment, regrowth of treated sarcoids and growth of any new sarcoids.

Data Analysis

Data were analysed both by case, and then by individual sarcoid. Statistical analysis was performed in R studio (R version 4.2.0) (R Core Team, 2022). Normality was assessed for continuous variables using the Shapiro–Wilk test. Thereafter, as data were nonparametric, univariate analysis was performed by chi- squared test or Fisher's exact test for categorical variables, Wilcoxon's rank sum for continuous variables and Cochrane Armitage test (two sided) for trend, where appropriate (Appendix 5).

For variables of $p \leq 0.2$ in the univariate analysis, logistic regression was performed with sarcoid recurrence as the dependent variable (Appendix 6). Variables with $p \leq 0.2$ in the univariate analysis, $n > 10$ and low collinearity with other independent variables, were used to perform multiple logistic regression, again with recurrence as the control- dependent variable. Variables were removed from the model in a manual backwards elimination manner with the Akaike information criterion (AIC) used to assess the fit of the model at each step. Variables were retained in the final model despite not fulfilling $p \leq 0.2$ when their inclusion improved the AIC of the model. Variance inflation factors were used to assess final variables for multicollinearity, binned residual plots created to assess normality of the residuals and a receiver operating curve constructed in order to assess the adequacy of the model.

All variables with $p \leq 0.2$ in the univariate logistic regression were evaluated for inclusion in a Cox's proportional hazards model. Data were right censored at either time to recurrence of the treated sarcoid, time to follow- up telephone conversation or time to death or sale of the horse. Where

multicollinearity existed, the value with the lowest p- value was included. Numerical covariates were transformed to categorical due to violation of the linearity assumption of the Cox's proportional hazards model. The model was refined using a manual backwards elimination approach, with threshold for inclusion in the model at $p \leq 0.2$ whilst maximising concordance of the model. A plot of scaled Shoenfeld residuals was used to assess the proportional hazards assumptions of the model. Time to sarcoid recurrence was used to create a Kaplan–Meier plot, and differences between treatment protocols compared with a log- rank test. Statistical significance was considered at $p < 0.05$.

3.2.4 Results

One hundred and eighty equids were referred to Glasgow Equine Hospital for the treatment of sarcoids during this period, of which 84 met the inclusion criteria. Histological confirmation was available for 168 individual sarcoids in these 84 horses. Of these, 8 (5%) were categorised clinically as occult sarcoids, 26 (15%) as verrucose, 80 (48%) as nodular, 24 (14%) as fibroblastic and 26 (15%) as mixed. None were classed as 'malevolent', and sarcoid type was not recorded in four cases (2%). Seventeen different breeds were represented, of which 17 horses were crossbreeds (20%), 11 Irish Sport Horses (13%), 10 warmbloods (12%), six Thoroughbreds (7%) and five cobs (6%). Four donkeys were included in the study (5%), and the remaining horses were of various breeds. Thirty- two of included equids were mares (38%), 51 geldings (61%) and 1 stallion (1%). Ages ranged from 2 to 20 years (median 8 years (IQR 6–14 years)). Owner follow- up was available for 69/84 individuals, with regrowth ascertained from medical records in a further four individuals.

A flow chart describing included cases and sarcoids is presented in Figure 9. Of the 73 cases for which follow- up was available, 168 of the total of 320 suspected sarcoid masses were histologically confirmed (53%) and were included in further analysis. Specific follow- up regarding individual sarcoid regrowth was available for 118 histologically confirmed masses.

Horses/sarcoids for which follow- up was not available were excluded from further analysis regarding sarcoid regrowth. 62 horses had multiple sarcoids at the time of presentation, with a

median of 3 (IQR 2–7). 32 of these horses had ≥5 sarcoids, 15 ≥ 10 sarcoids and 3 horses had ≥20 sarcoids. All masses were removed at the time of initial presentation, with a maximum number 24 suspected sarcoids.

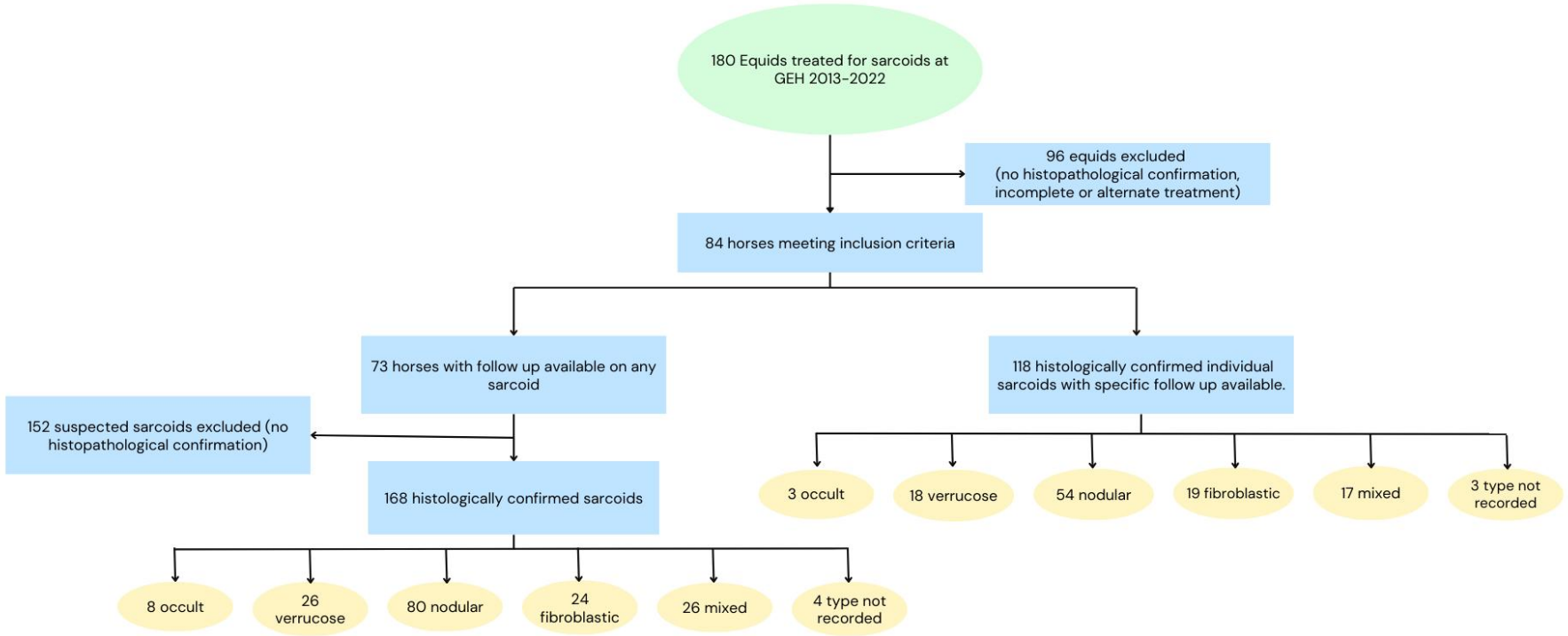


Figure 9 : Flow chart describing cases and sarcoids included in the retrospective analysis.

During the study period, the overall incidence of recurrence of any sarcoid on an equid at the same previously treated site was 38% (28/73) and time to follow- up ranged from 0.75 to 132 months (median 39 months (IQR 21–62 months)). For any individual treated sarcoid, total rate of recurrence was 23% (29/128) with a median length of follow- up of 48 months (IQR 24–61 months).

In total, 28 equids were treated by the combination treatment protocol (33%) and 56 by diode laser excision alone (67%). Table 4 displays pertinent differences between treatment groups. Equids in the combination category had larger sarcoids (median width 59 vs. 30 mm, $p < 0.001$) and were less likely to have any incision closed at the time of excision ($p < 0.001$). Length of follow- up was significantly longer in the laser category (52 months (39–72 months) vs. 20 months (14–25 months) $p < 0.001$).

		Laser	Combination	p	N
Age (years)		8 [6-10.75]	8.5 [7-10.8]	0.32	84
Site(s) affected	Periocular	12 (25%)	2 (7%)	0.13	14
	Head and neck	15 (27%)	7 (25%)	0.86	22
	Dorsum	2 (4%)	0 (0%)	0.55	2
	Ventrum	27 (48%)	19 (64%)	0.09	46
	Upper limb	33 (59%)	19 (64%)	0.43	52
	Lower Limb	2 (4%)	0 (0%)	0.55	2
	Urogenital	14 (25%)	4 (14%)	0.40	15
Chronicity at presentation	<3 months	16 (35%)	6 (26%)	0.69	22
	3-6 months	7 (15%)	4 (17%)		11
	6-12 months	6 (13%)	5 (22%)		11
	> 12 months	17 (37%)	8 (35%)		25
Total number of sarcoids		3 [1-7.25]	3 [2-6.5]	0.53	83
Max sarcoid width (mm)		30 [15-50]	59 [40-95]	<0.001*	64
Histological margins confirmed clear		10 (18%)	5 (18%)	0.75	84
≥1 Wound closed		27 (48%)	2 (7%)	<0.001*	84
Restraint	Standing sedation	28 (50%)	20 (71%)	0.06	48
	GA	28 (50%)	8 (29%)		36
Complications		5 (11%)	9 (36%)	0.03*	70
Length of follow up (months)		52 [39-72]	20 [14-25]	<0.001*	72

Table 4: Pertinent patient and sarcoid characteristics of cases assigned to either treatment category and results of univariate comparison. Continuous data are presented as median [IQR] and categorical data as count (% of total). 'Total number of sarcoids' denotes the total number of sarcoids recorded on each horse. Note: A total number of 84 cases were included in the study, but N is variable as full

*data were not available for every criterion assessed here and so the relevant field size is given in each case. *denotes statistical significance.*

Complications following treatment occurred in 14 horses included in the study (17%). These were more frequent in horses treated by combination protocol (9/25 (36%) vs. 5/45 (11%), p = 0.03) but were short lived and comprised: oedema surrounding the treated site (5/14, 36%), owner reported delayed healing (3/14, 21%) or infection (3/14, 21%) of the treated site, and individual cases of myiasis, facial nerve neuropraxia and excessive scarring (1/14, 7% each). Overall, 60/67 owners (90%) said they were happy with the final cosmetic result of the treatment. Of the 16 horses reported to have been euthanised prior to follow- up, four of these were for sarcoid- related reasons, resulting in a fatality rate of 4/69 (5.8%) over the duration of the follow- up period in this referral population.

Sarcoid recurrence at any treated site was reported in 20/47 (43%) of horses receiving laser excision alone and 8/26 (31%) treated by combination therapy for which follow- up was available. For any individual sarcoid, eventual recurrence at the same site occurred in 23/95 (24%) of those treated by laser excision and 6/33 (18%) receiving the combination protocol. These differences were not statistically significant in either case (p = 0.32 and p = 0.48 respectively).

Likelihood of Sarcoid Recurrence

A forest plot of variables significant in the univariate logistic regression on both a whole case and individual sarcoid basis is shown in Figure 10. Full results are available in Appendix 6.

Univariate logistic regression indicated that recurrence was more likely if the ventrum was affected (OR 3.9 (1.5–11) p < 0.001), and if sarcoids were of verrucose (OR 1.9 (1.2–3.4) p = 0.01) or mixed (OR 1.9 (1.3–3.5), p = 0.002) types. Nineteen horses and 20 individual sarcoids had received attempted sarcoid treatment prior to initial presentation, and this was significantly associated with subsequent sarcoid recurrence (13/19 (68%), OR 7.7 (2.5–27) p < 0.001 and 9/20 (45%), OR 4.9 (1.5–16) p = 0.004). Having a greater total number of sarcoids at presentation was also associated with

recurrence (OR 1.3 (1.1–1.5), p < 0.001) as was the surgical closure of any incision at the time of initial treatment (OR 4.0 (1.5–11.6), p = 0.007). Sarcoid chronicity was associated with recurrence both for affected animals (cases) and individual sarcoids (p = 0.03 and <0.001) and any individual sarcoid treated under general anaesthesia was more likely to recur (OR 6.3 (2.5–17) p < 0.001) than if treated standing under sedation. A significant association was also demonstrated between chronicity and sarcoid width (p = 0.02, OR (1.00–1.01)) and the likelihood of attaining histological margins(p = 0.01, OR = 0.68 (0.50–0.92)). Neither sarcoid site nor width was significantly associated with the confirmation of histological margins (p = 0.99 and p = 0.15, respectively).

Table 5 displays the multiple logistic regression model for variables predictive of sarcoid recurrence in any presenting case. The area under the ROC curve for this model as a predictor of sarcoid recurrence in this population was 0.86. A mixed effects logistic regression model was attempted with 'equid' included as the random effect variable. The random effect of horse- related factors was shown to be extremely large (between subject variance = 1065) and so this model was rejected due to clustering of data. Two horses were over- represented in the individual sarcoid data, these being 6- and 7- year- old Warmblood geldings with specific follow- up available on eight and seven sarcoids, respectively. Both geldings were treated by laser excision alone and only two sarcoids recurred.

The effect of treatment category on recurrence rate whilst accounting for significant population differences between treatment category (largest sarcoid width (mm), closure of any incision, occurrence of complications following treatment and length of case follow- up (days)) was examined. Treatment category remained non-significant in this model (OR 1.3 (0.14–13), p = 0.82).

Time to sarcoid recurrence

Data regarding time to sarcoid recurrence were available for 72 horses. Variables retained as significantly associated with time to sarcoid recurrence in the Cox's Proportional Hazards model are presented in Table 7, and the final Kaplan–Meier plot of sarcoid recurrence for each treatment

category is presented in Figure 11. The log- rank test comparing the survival curves again indicated no significant difference between treatment categories (p = 0.73).

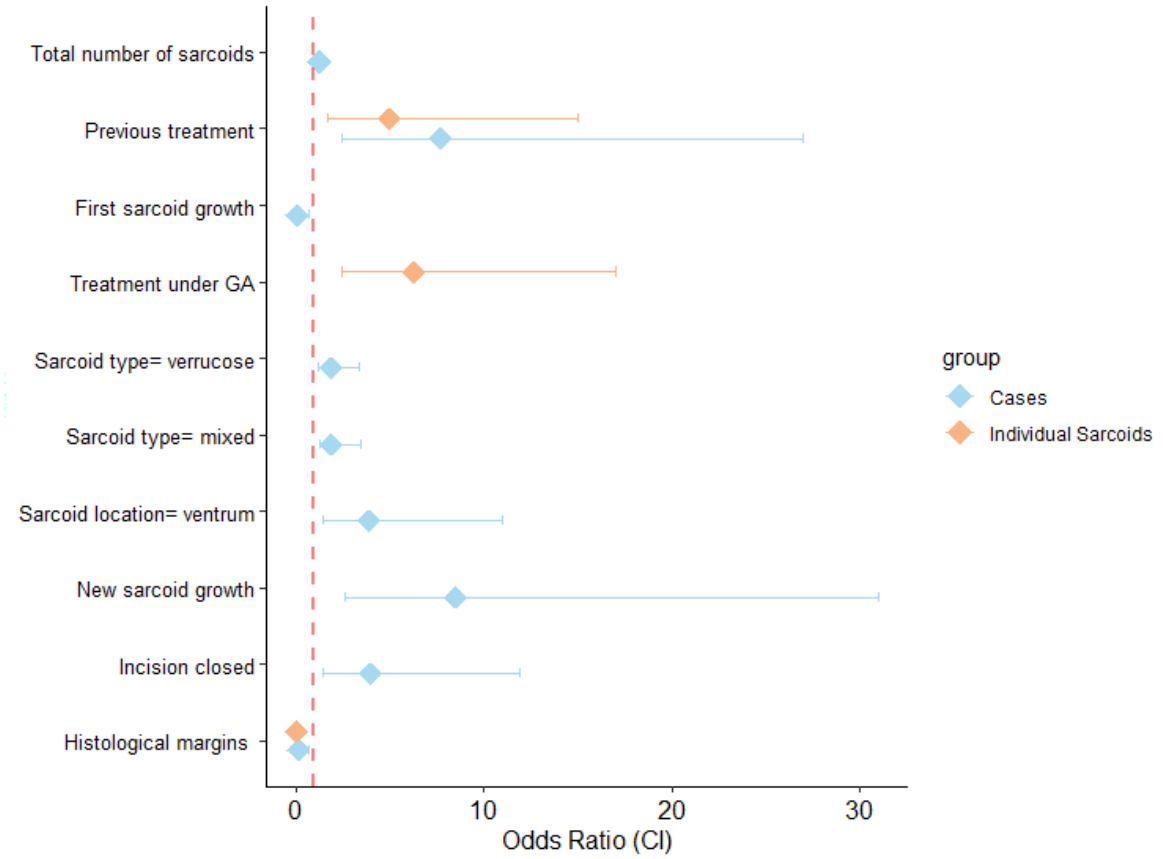


Figure 10: Forest plot showing odds ratios (OR) and 95% confidence intervals for variables significantly associated with likelihood of sarcoid recurrence within an individual case and for individual treated sarcoid lesion. The red dashed line indicates an OR of 1.

	Coefficient	**SE**	**Wald's test statistic**	**P**	**OR [CI]**	**AIC**
Intercept	-2.2	0.68	-3.3	0.001*	0.11 [0.02-0.36]	60
Total number sarcoids	0.19	0.09	2.1	0.03*	1.2 [1.0-1.5]	
Histological margins achieved	-0.90	0.95	-0.96	0.34	0.40 [0.05-2.3]	
Treatment prior to presentation	2.0	0.71	2.9	0.004*	7.6 [2.0-33]	

*Table 5: Multinominal logistic regression models for variables associated with sarcoid recurrence in 84 horses with histopathological confirmation of sarcoid(s). Note: *denotes statistical significance.*

		HR [CI]	p	Concordance
Lower limb		0.2 [0-1.6]	0.13	0.75
Urogenital		3.6 [1.3-10]	0.02*	
Number mixed sarcoids			<0.001*	
	0	Reference		
	1	3.5 [1.0-12]	0.046*	
	>1	9.9 [3.3-30]	<0.001*	
First sarcoid		0.30 [0.10-0.70]	0.009*	

*Table 6: Cox's proportional hazards model of variables associated with time to sarcoid recurrence showing hazard ratios (HR) and 95% confidence intervals [CI] for included variables. Note: *denotes statistical significance.*

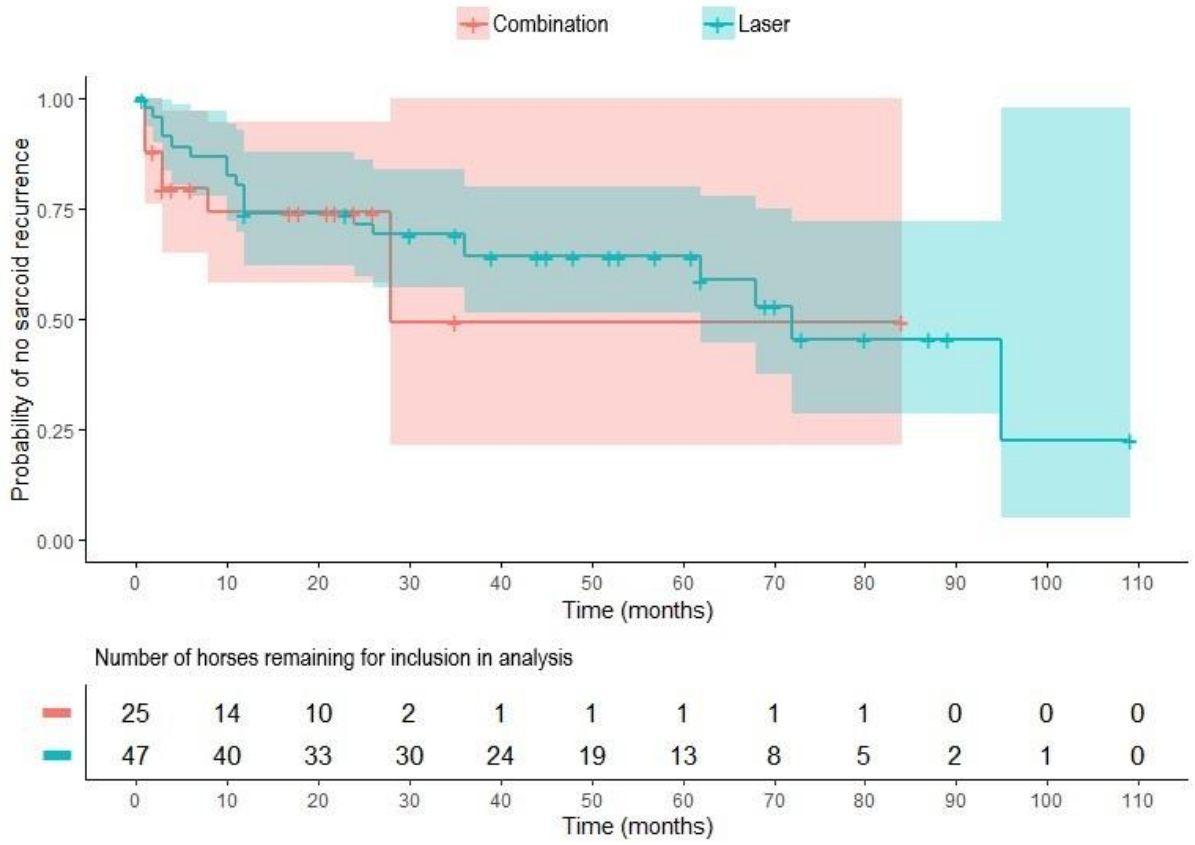


Figure 11: Kaplan–Meier plot showing time to sarcoid recurrence in both treatment categories. The table shows the number of included horses remaining with no sarcoid recurrence at each time point and in each treatment group, and therefore remaining for inclusion in analysis.

3.2.5 Discussion

Treatment protocols

The rate of recurrence of any sarcoid in this study was 23%, with a median length of follow- up of 48 months. There was no significant difference in recurrence between treatment category (laser 24% (23/95), combination 18% (6/33)). The lack of routinely accepted standardised outcome measure for the treatment of sarcoids makes comparison between studies very difficult (Offer *et al.,* 2024). Though these recurrence rates are marginally higher than those reported in the literature (sarcoid regression rates of 83%–89% with sole laser excision) (Compston *et al.,* 2016, Martens *et al.,* 2001a, Martens *et al.,* 2001b), this is highly likely to be influenced by numerous factors, including greater length of follow- up period in this study, and differences in case selection and treatment populations. These horses were all referred to the hospital for further evaluation and treatment, and by definition were positioned at the more severe end of sarcoid phenotype presentation.

Employing a multimodal approach to the treatment of sarcoids has been suggested by previous authors as a method to improve sarcoid regression rate (Knottenbelt, 2019) and was highlighted by this author's recent systematic review as likely to be advantageous over single treatment modalities (Offer *et al.,* 2024). This was not demonstrated in this study, though several confounding factors exist that may have prevented this. Firstly, there exist several significant differences in the case populations between treatment groups. Sarcoids treated by the combination protocol were significantly larger ($p < 0.001$) and were less likely to have been treated under general anaesthesia ($p = 0.04$). Both these factors may prevent attainment of an adequate surgical margin, depending on the location of the sarcoid and the temperament of the horse. As this study was not a randomised prospective trial, clinician preference is also likely to have resulted in selection of the combination protocol for sarcoid masses of greater clinical severity. Sarcoids in the combination treatment

category were also on significantly older horses. Whilst rare, spontaneous remission does occasionally occur in younger horses, and so this may have further confounded the results (Berruex *et al.,* 2016). Conversely, equids treated by laser therapy alone had a significantly longer time to follow- up than those treated by the combination protocol (median 52 vs. 20 months) due to the distribution of cases and historical clinician preference. Longer- term recurrence may therefore be underestimated in the combination protocol treatment category.

However, when a multivariable model was constructed to account for differences in case population between categories, treatment category remained nonsignificant in the model ($p = 0.82$). Since sarcoid recurrence is dependent on numerous variables (Knottenbelt, 2019), case selection is crucial in determining its likelihood. A true comparison of these treatment techniques would therefore require a fully randomised and blinded controlled trial and a recommendation between these two treatment protocols cannot be made on the basis of this study.

Cryosurgery and cryobiology are rapidly evolving fields of cancer therapy. Current recommendations for cryosurgical treatment of neoplasms include achieving a minimum temperature of −40°C for at least 5 min, and then allowing the thaw to be as slow as is practicable (Baust and Gage, 2005, Hoffmann and Bischof, 2002). Such a technique aims to produce a central zone of coagulative necrosis and direct cell death, as discussed previously, and requires the direct monitoring of freezing temperatures either by a needle mounted temperature probe or indirectly ultrasonographic changes. Freezing temperatures in this protocol were monitored only clinically, with the manual palpation of a direct zone of freezing. The use of multiple freeze–thaw cycles was designed to increase the cellular physiochemical changes occurring in neoplastic cells and is employed in several cancers as a technique to increase the lethal freezing temperature required and extend the zone of necrosis to closer to the tumour margins without endangering underlying tissues. Despite this, it is unlikely that the published target of −40°C was achieved, and indeed may have been undesirable given the expected damage to underlying tissues with such low temperatures.

In this clinical situation, the secondary inflammatory effects and delayed cellular apoptosis following cryosurgery become more significant, giving further weight to the use of an adjunctive pro-apoptotic agent such as 5- FU. Overcoming the immune evasive micro- environment of sarcoids is increasingly accepted to be crucial in the treatment of this disease (Jindra *et al.,* 2023), and so cryosurgery in this instance may represent a method of nonspecific immune stimulation and theoretically contribute to tumour resolution.

Risk factors for recurrence

The total number of sarcoids on the individual at the time of presentation was significant in both the univariate and multivariate logistic regression. This supports increased susceptibility of certain individuals to the development of sarcoids and was confirmed by the large inter- equid variance indicated in the attempted mixed effects logistic regression. This is well recorded in the literature—the presence of multiple sarcoids has been reported by several authors as predictive of an increased likelihood of sarcoid recurrence (Compston *et al.,* 2016, Lane, 1977), and it has been shown by numerous authors that the susceptibility to sarcoids has at least some genetic basis (Broström *et al.,* 1988, Christen *et al.,* 2014, Lazary *et al.,* 1994). The presence of equine leucocyte antigen (ELA) W13 allele has been correlated with susceptibility to sarcoid growth, although horses without the allele may also develop sarcoids (Goodrich *et al.,* 1998). Candidate genes within specific chromosomal regions also have been associated with increased susceptibility to sarcoid growth (Jandova *et al.,* 2012), and a polygenic mode of inheritance with 21% heritability has been demonstrated (Christen *et al.,* 2014). Presentation at the time of first sarcoid growth was similarly retained in the survival analysis as predictive of an increased time to subsequent sarcoid regrowth, that is, those individuals with increased susceptibility to sarcoid growth who present with multiple sarcoids spanning several years have a shorter time to regrowth than those with solitary sarcoids at first presentation.

Treatment of any sarcoid prior to presentation was associated with sarcoid recurrence and was retained in the multivariable model. The reason for this may be twofold; presentation following prior

treatment indicates that any previous intervention has failed to resolve the sarcoid. The sarcoid itself is therefore likely to be highly locally invasive and/or anatomically difficult to remove in its entirety. In addition, any traumatic intervention to a sarcoid may cause accelerated growth or increased malignancy, leading to subsequent difficulty in achieving full sarcoid resolution (Knottenbelt, 2019). Similarly, recurring sarcoids are likely to be more aggressive and infiltrative than those treated on first presentation (Taylor and Haldorson, 2013).

Further variables associated with sarcoid recurrence in this study may be associated with the ability to achieve surgical margins at initial laser excision. Sarcoid chronicity and size were significantly associated, and both likely to be linked to the ability to achieve complete tumour excision via laser. Complete excision of neoplastic cells logically is often stated as a significant factor contributing to sarcoid resolution (Carstanjen *et al.,* 1997, Knottenbelt, 2019), the desirable surgical margins for equine sarcoids have not been well defined. A margin of between 1 and 2 cm is often stated as desirable as BPV DNA has been demonstrated within a surgical margin of 16 mm in 33% of cases (Compston *et al.,* 2016, Knottenbelt, 2019, Martens *et al.,* 2001a, Martens *et al.,* 2001b) and histopathological evidence of sarcoid infiltration has been demonstrated at 2 cm from the sarcoid margin removed via laser excision (Mair and Fews, 2016). Coagulative necrosis extends beyond the surgical site when laser excision is employed but this may be insufficient to prevent sarcoid recurrence in cases where neoplastic cells extend beyond this region of necrosis (Compston *et al.,* 2016, Knottenbelt and Kelly, 2000). However, the presence of papillomaviral DNA within the surgical margin has inconsistently been associated with sarcoid recurrence, as has any association between width of surgical margin and subsequent recurrence rate (Martens *et al.,* 2001a, Martens *et al.,* 2001b).

Limitations

There are a number of further limitations to this study. Firstly, as with any retrospective study, missing data and loss of cases to follow- up limits its power. Owner recall bias may skew results,

though answers were corroborated with written clinical records where possible. The data described here apply only to a referral hospital with one case population in which most equids had more than one sarcoid and may have undergone previous treatment attempts. With the treatment of equine sarcoids, case selection plays a significant role in the likelihood of tumour recurrence. Though populations in each treatment group were compared, they were not identical and treatment assig nation was neither randomised nor blinded. Similarly, there was no untreated or placebo control population in this study, and so findings should be interpreted with caution. A significant limitation with the survival analysis was the differing follow- up times between treatment categories, although it should be noted that both exceeded the 12- month period used in many previous studies. This combination of factors may have prevented any demonstration of significant differences in time to sarcoid regrowth between groups.

Conclusion

No significant difference in sarcoid recurrence rate or time to sarcoid recurrence has been demonstrated with the use of a cryosurgery/chemotherapy protocol following laser excision versus laser excision alone, though this is limited by available time to follow- up and differences in case selection. The characteristics of the patient and sarcoid(s) were demonstrated to be more significant than the addition of adjunctive cryosurgery/chemotherapy in this population in relation to subsequent recurrence. Regardless of treatment protocol, approximately 25% sarcoid recurrence may be expected at 24 months following hospital discharge in a referral population of this type.

3.3 Appendices

3.3.1 Appendix 4: Owner telephone questionnaire template used to obtain case follow up.

Case number				
Owner name				
Horse name				
Contact number				
Date of telephone interview				
Date of initial presentation				
Age at presentation				
How long had the treated sarcoids been present by the time of presentation?	<3 months	3-6 months	6-12 months	>1 year
Was this the first sarcoid growth in this horse?	Yes		No	
How many sarcoids were present at the time of first treatment?	single	2-5	6-10	>10
Which body areas were affected?	Periocular	Head and neck	Dorsum	Ventrum	Upper limb	Lower limb	Urogenital
How many treatment (cycles) were given?	1	2	3	>3
Do you still have the horse?	Yes	No		
		Sold	Euthanised	Died
If no, date sold/ euthanised				
Reason for euthanasia/ death	Sarcoid related	Not sarcoid related		
Did you experience any complications following treatment of the sarcoids?	Yes	No		
If yes,	What?			
	Have these resolved?	Yes	No	
	How long did they take to resolve?			
If the horse is ridden, did it resume ridden exercise?	Yes	No	Not ridden	
Are you happy with the cosmetic outcome of the treatment?	Yes	No		
Comment				
Have any of the treated sarcoids regrown since last examination?	Yes	No		
If yes,	Which?			
	Time to recurrence?			

	Have they been treated?	Yes	No	
	With what?			
	Response to treatment:	Resolved	Improved but not resolved	No response
Have any other sarcoids occurred since last examination?		Yes	No	
If yes,	Where?			
	Describe lesion			
	When?			
	Have they been treated?	Yes	No	
	With what?			
	Response to treatment	Resolved	Improved but not resolved	No response

Appendix 3:Telephone questionnaire form used for data gathering in Chapter 3.

3.3.3 Appendix 5: Univariate analysis comparing populations between treatment groups.

		Cases				Individual sarcoids			
Variable		N	Laser	Combination	p	N	Laser	Combination	p
Age		84	8 [6-10.75]	8.5 [7-10.8]	0.32	168	8 [6-10]	11 [7.5-14]	0.04*
Sarcoid site	Periocular	14	12 (25%)	2 (7%)	0.13	4	4 (3%)	0 (0%)	0.55
	Head and neck	22	15 (27%)	7 (25%)	0.86	19	15 (12%)	4 (12%)	
	Dorsum	2	2 (4%)	0 (0%)	0.55	0	0 (0%)	0 (0%	
	Ventrum	46	27 (48%)	19 (64%)	0.09	56	46 (37%)	10 (32%)	
	Upper limb	52	33 (59%)	19 (64%)	0.43	58	43 (34%)	15 (48%)	
	Lower Limb	2	2 (4%)	0 (0%)	0.55	0	0 (14%)	0 (0%)	
	Urogenital	15	14 (25%)	4 (14%)	0.40	20	18 (90%)	2 (6%)	
Chronicity at presentation	<3 months	22	16 (35%)	6 (26%)	0.69	36	28 (32%)	8 (27%)	0.72
	3-6 months	11	7 (15%)	4 (17%)		20	15 (17%)	5 (17%)	
	6-12 months	11	6 (13%)	5 (22%)		9	5 (6%)	4 (13%)	
	> 12 months	25	17 (37%)	8 (35%)		52	39 (45%)	13 (43%)	
First sarcoid growth		52	35 (73%)	17 (68%)	0.90	-	-	-	-
Previous treatment		23	16 (32%)	7 (28%)	0.68	31	26 (36%)	5 (19%)	0.34
Total number of sarcoids		83	3 [1-7.25]	3 [2-6.5]	0.53	-	-	-	-
Sarcoid type	Occult	16	0 [0-0]	0 [0-0]	0.64	8	8 (6%)	0 (0%)	0.07
	Verrucose	25	0 [0-0]	0 [0-1]	0.64	26	24 (18%)	2 (6%)	
	Nodular	60	1 [1-2]	1.5 [0-4]	0.70	80	64 (48%)	16 (50%)	
	Fibroblastic	25	0 [0-0]	0 [0-1]	0.92	24	15 (11%)	9 (28%)	
	Mixed	29	0 [0-1]	0 [0-1]	0.39	26	21 (16%)	5 (16%)	
Sarcoid width (mm)		64	30 [15-50]	59 [40-95]	<0.001*	86	20 [10-30]	60 [30-100]	<0.001*
Histological margins		84	10 (18%)	5 (18%)	0.75	45	39 (52%)	6 (26%)	0.19
Initial sarcoid resolution		72	27 (59%)	19 (73%)	0.22	97	70 (74%)	27 (82%)	0.22
≥1 Wound closed		84	27 (48%)	2 (7%)	<0.001*	-	-	-	-
Restraint	Standing sedation	48	28 (50%)	20 (71%)	0.06	80	58 (44%)	22 (63%)	0.04*
	GA	36	28 (50%)	8 (29%)		88	75 (56%)	13 (37%)	

Complications		70	5 (11%)	9 (36%)	0.03*	13	5 (6%)	8 (26%)	0.004*
Repeat presentations		80	0 [0-0.25]	0 [0-1]	0.62	165	0 [0-0]	0 [0-0]	0.95
Owner happy with cosmetic outcome		67	39 (89%)	21 (91%)	1	-	-	-	-
New sarcoid growth since discharge		69	23 (51%)	5 (21%)	0.47	-	-	-	-
Horse still owned	Yes	45	27 (60%)	19 (79%)	0.19	-	-	-	-
	Sold	8	7 (18%)	1 (4%)					
	Euthanised	16	12 (27%)	4 (17%)					
Loss of horse sarcoid related		69	2 (11%)	2 (40%)	0.18	-	-	-	-
Length of follow up (months)		72	52 [39-72]	20 [14-25]	<0.001*	118	48 [44-68]	21 [14-26]	<0.001*
Regrowth of treated sarcoids		72	20 (43%)	8 (31%)	0.45	118	23 (24%)	6 (18%)	0.63

*Appendix 4: Univariate comparison of treatment group case populations. Categorical variables are presented as n(% of cases/sarcoids in that category), continuous variables are presented as median [IQR]. * Denotes statistical significance (p<0.05).*

3.3.4 Appendix 6: Logistic regression for variables where p<0.2 in univariate analysis.

Variable		Cases				Individual sarcoids			
		Coefficient	**OR [CI]**	**p**	**AIC**	**Coefficient**	**OR [CI]**	**p**	**AIC**
Age		-	-	-	-	0.06	1.1 [0.98-1.2]	0.13	139
Total number of sarcoids		0.22	1.3 [1.1-1.5]	<0.001*	81	-	-	-	-
Chronicity (months)	<3	Reference		0.03*	86	Reference		<0.001*	113
	3-6	-1.2	0.3 [0.01-2.2]	0.30			0.81 [0.1-4.6]	0.81	
	6-12	0.54	1.7 [0.33-8.6]	0.51			0.91 [0.04-7.3]	0.93	
	>12	1.2	3.23 [0.94-12]	0.07			3.0 [0.98-11]	0.07	
First sarcoid		-2.2	0.11 [0.03-0.75]	0.04*	82	-	-	-	-
Sarcoid type	Occult	-	-	-	-	Reference	$6e^{-8}$ [NA-$2e^{71}$]	0.99	138
	Verrucose	0.64	1.9 [1.2-3.4]	0.01*	89	16	$1e^{7}$ [$7e^{-72}$-NA]	0.99	
	Nodular	-	-	-	-	15	$3e^{6}$ [$1e^{-72}$-NA]	0.99	
	Fibroblastic	-	-	-	-	16	$7e^{6}$ [$7e^{-72}$-NA]	0.99	
	Mixed	0.64	1.9 [1.3-3.5]	0.01*	87	15	$5e^{6}$ [$6e^{-72}$-NA]	0.99	
Ventrum		1.35	3.9 [1.5-11]	<0.001*	81	-	-	-	-
Treatment prior to presentation		2.0	7.7 [2.5-27]	<0.001*	80	1.6	5.0 [1.7-15]	0.003*	107
Histological margins		-1.7	0.18 [0.03-0.75]	0.04*	82	-3.0	0.05 [0.003-0.24]	0.004*	118
Restraint	GA	-	-	-	-	1.8	6.3 [2.5-17]	<0.001*	124
≥1 incision closed		1.4	4.0 [1.5-11.6]	0.007*	94	-	-	-	-
Number of cryo/5fu treatments		-	-	-	-	-0.27	0.76 [0.45-1.2]	0.25	140
5fu dose (mg)		-	-	-	-	0.0009	1.0 [1.0-1.0]	0.22	34
Time between treatments (weeks)		-	-	-	-	1.9	6.6 [0.78-132]	0.10	15
Complications	Yes	-	-	-	-	1.3	3.5 [0.96-12]	0.05	111
Number of repeat presentations		2.8	16 [4.7-82]	<0.001*	81	2.2	9.0 [4.1-23]	<0.001*	104
Initial treatment success	Resolution	Reference			50	Reference			59
	Partial	4.4	81 [19-477]	<0.001*		5.3	196 [48-1140]	<0.001*	

	None	-	-	-	-	3.4	31 [1.1-944]	0.02*	
Further treatment required		4.9	128 [22-2508]	<0.001*	53	6.3	538 [85-11000]	<0.001*	45
Follow up length (months)		-	-	-	-	-0.002	1.0 [0.98-1.0]	0.84	114
New sarcoid growth		2.1	8.5 [2.6-31]	<0.001*	81	-	-	-	-

*Appendix 5: Logistic regression results for variables where p<0.2 in univariate analysis. Odds ratios are presented as median [IQR]. * Denotes statistical significance (p<0.05). AIC= Akaike information criterion.*

Chapter 4: Immunohistochemical investigation of equine sarcoids: a pilot study

4.1 Preface

The retrospective analysis of Chapter 3 identified several risk factors that contribute to the likelihood of sarcoid recurrence. However, significant horse level clustering was identified in the attempted multiple logistic regression model for each individual sarcoid. As discussed in the introduction, a significant genetic predisposition has been shown to exist to the development of ES. However, these traits have been demonstrated to be complex and polygenic in nature (Jandova *et al.,* 2012) and so are currently unsuitable for practical use as clinical prognostic markers.

Of crucial interest to the clinician is which specific characteristics of the equid and/or sarcoid can be readily identified in order to predict sarcoid behaviour. Immunohistochemistry is a widely used technique that may make use of already excised sarcoid tissue, and so may be readily applicable to clinical practice. In performing the retrospective study, a data set of clinical information was compiled regarding a large number of sarcoids treated at GEH. These cases have a long follow up time (median 39 months) far outweighing the majority of literature regarding ES treatments. For each of these cases, stored paraffin fixed tissue samples are available, representing a unique opportunity to investigate predictors of sarcoid recurrence in a robust data set.

The following article prepared for publication is a pilot study aiming to identify immunohistochemical markers in this case population that may be appropriate for investigation as to their relationship with the likelihood of sarcoid recurrence. Given the high prevalence of BPV infection in the surrounding skin of equids with sarcoids (65% (Carr *et al.,* 2001b)), IHC markers were compared between samples taken from the centre of confirmed sarcoid masses and the immediately adjacent grossly normal skin in order to confirm differential expression in the

neoplasm. The long-term aim is to identify a marker that may be used in practice in order to inform clinician and owner decision making regarding appropriate ES treatment.

4.2 Main Text

4.2.1 Abstract

Histopathological diagnosis of the Equine Sarcoid is complicated by inconsistent features and is poorly correlated with clinical prognosis. Sarcoid behaviour is unpredictable and immunohistochemical analysis of excised sarcoid tissue may represent an opportunity to identify prognostic markers. Samples were obtained from the centre of 14 equine sarcoids removed by diode laser, and from normal skin 1-2cm from the tumour margin. Expression of proliferating cell nuclear antigen (PCNA), p53, hypoxia inducible factor 1α (HIF-1α) and Bovine Papillomavirus (BPV) E2 protein were investigated quantitatively via bioimage analysis of immunohistochemical stained slides. BPV E2 (1.6% positive cells (0.16-3.6%) versus 0.09% (0.06-0.19%), p=0.01) and PCNA expression (24.9% positive cells (20-35%) versus 3% positive cells (1.4-9.3), p<0.001) were significantly greater in sarcoid tissue compared with peripheral skin. BPV staining and both HIF-1α (rho=0.88, p=0.004) and PCNA staining (rho= 0.77, p=0.02) were significantly correlated, and P53 and HIF-1α staining inversely correlated (rho= -0.85, p=0.008). This pilot study has identified the increased expression of BPV E2 protein and PCNA within sarcoid samples, which warrant further investigation as potential prognostic or diagnostic markers.

4.2.2 Introduction

The equine sarcoid (ES) is the most frequently diagnosed equine cutaneous neoplasm but has a variable and unpredictable clinical behaviour (Knottenbelt, 2019, Schaffer *et al.,* 2013). Histopathological diagnosis is often complicated, with the only consistent feature being the presence of an increased density of dermal fibroblasts. However, this is not pathognomonic and may be common to other spindle cell tumours. Other features, including epidermal hyperplasia and hyperkeratosis with deep projections into the neoplastic dermal tissue and 'picket fence' alignment of neoplastic cells along the dermal/epidermal border, are not consistently present (Martens *et al.,* 2000, Hewes and Sullins, 2009, Wobeser, 2016). Moreover, these histopathological features are poorly correlated to the clinical behaviour of the sarcoid (Martens *et al.,* 2000).

Several attempts have been made to identify both diagnostic and prognostic markers in ES, by the phenotypic categorization of sarcoid subtypes and various histochemical, immunohistochemical and biochemical markers (Knottenbelt, 2005, Bogaert *et al.,* 2011, Hamza *et al.,* 2023, Mählmann *et al.,* 2014, Kasperowicz *et al.,* 2006). Thus far these have either been unsuccessful or are not available for routine clinical use. A genetic basis for the predisposition for ES has been demonstrated, but similarly currently has limited clinical utility (Jandova *et al.,* 2012). Though incisional biopsy is not routinely recommended in suspected cases of ES due to the risk of provoking accelerated tumour growth (Taylor and Haldorson, 2013, Knottenbelt *et al.,* 1995), current treatments for ES often involve the initial surgical excision or debulking of the mass (Compston *et al.,* 2016, Martens *et al.,* 2001b, Haspeslagh *et al.,* 2016). There is therefore an opportunity to further examine sarcoid tissue at the initiation of treatment, and the identification of an IHC marker may represent an easily applicable aid to ES diagnosis and prognostication.

It is well established that the pathogenesis of ES involves the deltapapillomaviruses Bovine Papillomavirus (BPV) 1 and 2 (and the more recently identified BPV 13) (Otten *et al.,* 1993, Lunardi *et al.,* 2013, Campo *et al.,* 1992). Of these proteins encoded by their double stranded DNA genome, the transcriptional regulator, E2, is crucial for viral replication (Nasir and Campo, 2008, Hermonat *et al.,*

1988, Jackson and Campo, 1995). Both immunohistochemistry and in situ hybridization has demonstrated BPV expression/nucleic acid in up to 100% of sarcoids (Tura *et al.,* 2021, Gaynor *et al.,* 2016), and intralesional BPV DNA load by qPCR has been shown to reflect the severity of ES (Haralambus *et al.,* 2010). However, distribution of BPV expression within the tumour and surrounding tissues is not fully understood, and qPCR may have practical disadvantages over immunohistochemistry (IHC), including availability and cost implications.

Investigation of cell cycle regulatory proteins in the equine sarcoid has thus far focused primarily on loss of the tumour suppressive action of protein p53. Variable expression of p53 is reported in ES (Bucher *et al.,* 1996, Finlay *et al.,* 2012, Nasir *et al.,* 1999), and the BPV E6 protein has been shown to lead to p53 downregulation and inactivation in its own right (Araldi *et al.,* 2015, Scheffner *et al.,* 1990). The loss of p53 function has been identified as a principal factor in the pathogenesis of various neoplasia in both human and veterinary species (Hollstein *et al.,* 1996, Teifke and Löhr, 1996).

Proliferating cell nuclear antigen (PCNA) is a further potential IHC marker which has been widely used in the calculation of proliferation indices in both human and veterinary neoplasms (Madewell, 2001). The histone- associated protein is expressed in association with DNA replication (Bolton *et al.,* 1992), and several authors have suggested its potential utility in the determination of ES behaviour and prognosis (Théon *et al.,* 1999, Kasperowicz *et al.,* 2006). Recent work has demonstrated that the hypoxia-inducible factor-1α (HIF-1α) and associated vascular endothelial growth factor (VEGF) pathways are upregulated in ES and are involved in tumour angiogenesis (Martano *et al.,* 2020, Martano *et al.,* 2018). HIF-1α appears to be abnormally accumulated in the cytoplasm of neoplastic sarcoid fibroblast (Depping *et al.,* 2015), and increased expression is associated with aggressive tumour phenotypes and poor prognosis in both human and canine cancers (Yamamoto *et al.,* 2008, Baba *et al.,* 2010, Shin *et al.,* 2015). These proteins therefore warrant further investigation in clinical cases of ES.

This study aims to identify immunohistochemical markers that are expressed within equine sarcoids suitable for future investigation as to their association with sarcoid behaviour and prognosis. We aimed to quantitively compare the expression of PCNA, p53, HIF-1α and BPV E2 protein in central sarcoid samples versus grossly normal skin surrounding the sarcoid in clinical cases of ES. The authors hypothesized that these markers would each be overexpressed within the sarcoid samples relative to the peripheral samples.

4.2.3 Materials and methods

Samples were obtained prospectively from horses presented to Glasgow Equine Hospital for the treatment of ES. Written owner consent was obtained prior to treatment, and only sarcoids suitable for laser excision were included, at the clinician's discretion. Following complete diode laser excision of a suspected sarcoid, an 8mm punch biopsy sample was obtained from both the centre of the visible mass (hereafter termed 'central' sample (C)) and of the grossly normal skin between 1-2cm away from the apparent margin (hereafter termed 'peripheral' sample (P)). Samples were excluded from the study if the lesion was too small to obtain the relevant samples, or if insufficient margins were able to be obtained at the time of surgery to allow sampling of grossly normal peripheral tissue. Samples were formalin fixed prior to paraffin embedding.

Five sections at 3µm thickness were obtained from each paraffin fixed sample. Two sections were mounted on each slide in a randomized order, and identifiable only by a pseudonymized reference number. The researchers were blinded to the source (central or peripheral) of each sample.

H&E sections were examined by a registered veterinary pathologist (AF) to confirm histological diagnosis. Diagnosis was confirmed where the central section displayed characteristic histochemical characteristics of ES; namely proliferation of dermal fibroblasts with epidermal hyperplasia, hyperkeratosis and rete peg-like extensions into the tumour mass (Wobeser, 2016). Slides were excluded where diagnosis could not be confirmed in the central samples. Peripheral samples where evidence of neoplastic infiltration was suspected were retained in the analysis as were determined

to be representative of the normal clinical situation following mass excision where distinct surgical margins may not always be clear or feasible.

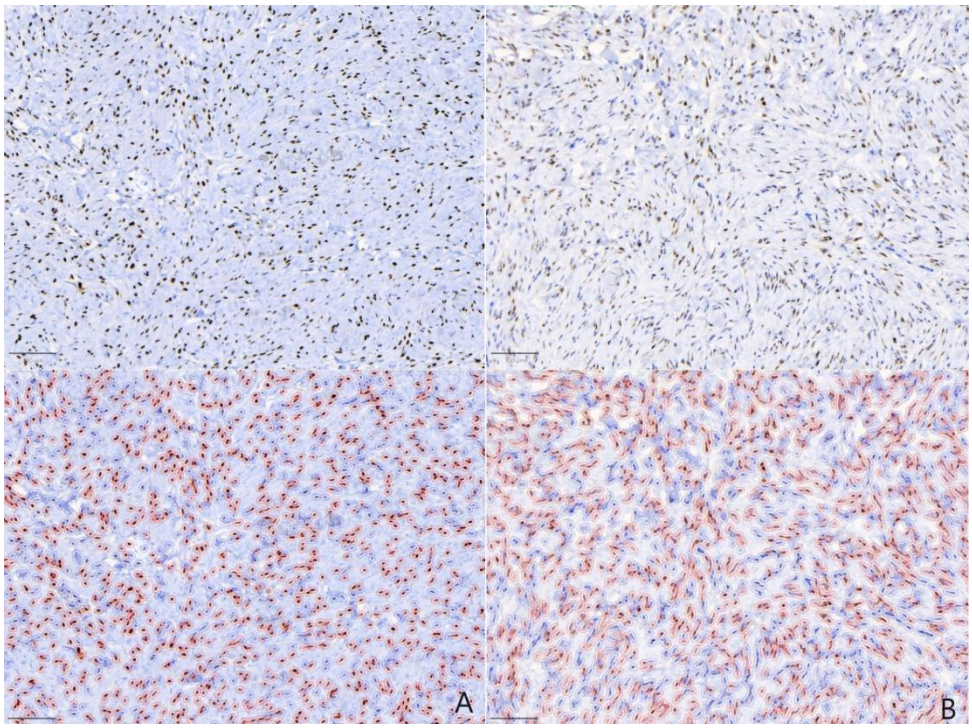
IHC staining was performed on the samples using the DAKO Universal staining system (DAKO LV-1 Universal Autostainer®, Agilent technologies, Inc.). Protocols were optimised on stored histologically diagnosed equine sarcoid tissue prior to application to study samples. Antigen retrieval was via Heat Induced Epitope Retrieval (HIER). Buffers used included citrate (pH6) or EDTA (pH9) as appropriate. Endogenous peroxidase activity was blocked using DAKO Real™ Peroxidase Blocking Solution (DAKO; Agilent Technologies, Inc.). Table 6 shows the relevant methodological details for each targeted protein. The samples were rinsed twice with buffer solution for 5 minutes between application of antibodies. Secondary antibodies were HRP conjugated rabbit/mouse antibodies, as appropriate, and samples were then counterstained with Gill's Haematoxylin. Negative controls were performed for each targeted protein using the same technique minus the primary antibody.

Antibody (Clone)	**Source (Catalogue number)**	**Antigen retrieval**	**Antibody dilution**	**Secondary Antibody (catalogue number)**
PCNA (PC10)	Bio-Rad Laboratories Ltd. (Hertfordshire, UK) (MCA1558)	HIER, sodium citrate buffer, pH6	1:3200	Secondary Dako Envision+ System-HRP Labelled polymer Anti-Mouse/Cat (K4001)
P53 (DO1)	Santa Cruz Biotechnology (Texas, USA) (sc-126)	HIER, EDTA buffer, pH9	1:50	Secondary Dako Envision+ System-HRP Labelled polymer Anti-Mouse/Cat (K4001)
HIF-1α (polyclonal)	Abcam (Cambridge, UK) (AB114977)	HIER, sodium citrate buffer, pH6	1:100	Secondary Dako Envision+ System-HRP Labelled polymer Anti-Rabbit/Cat (K4003)
BPV E2 (1E4)	Abcam (Cambridge, UK) (AB980)	HIER, sodium citrate buffer, EDTA pH9	1:50	Secondary Dako Envision+ System-HRP Labelled polymer Anti-Mouse/Cat (K4001)

Table 7: IHC methodological details for each target maker.

Following IHC staining, slides were digitized using the Motic EasyScan 60 (Motic Digital Pathology, California, USA) at x20 magnification. Each slide was examined by two independent observers (KO,

SV) in a blinded fashion. Bioimage analysis was performed using QuPath (Bankhead *et al.,* 2017). Artefacts larger than 500µm in diameter, skin associated glandular tissue and hair follicles were excluded. Due to inconsistent artefactual stain update, the epidermis was also excluded. Positive cell detection was used for the nuclear/perinuclear staining p53, PCNA and BPV slides. For the cytoplasmic staining HIF-1α, colour deconvolution was used to detect the positively staining pixels (hereafter denoted DAB (diaminobenzidine) staining pixels), with the threshold for detection set at 0.08 (*Fig 12*). Cell detection parameters are available in Appendix 7.



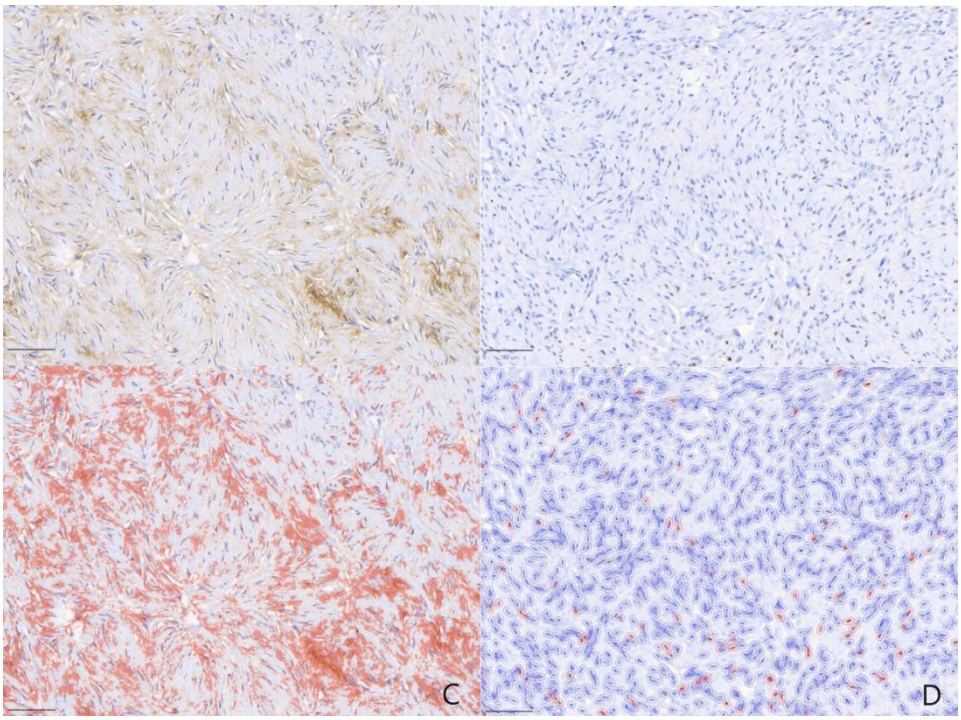


Figure 12: Images of IHC stained slides for PCNA (A), p53 (B), HIF-1α (C) and BPV E2 (D). The upper panels represent IHC stained slides, with corresponding bioimage analysis on the lower panels. Positive cell detection/ positive pixel detection is represented as a red overlay, as appropriate x40 magnification, bar represents 100µm

Data analysis

Positive cell % and positive cells/mm^2 were recorded for p53, PCNA and BPV slides. For HIF-1α, the percentage of DAB positive pixels were recorded, as was the HIF-1α positive pixels/µm^2. Data analysis was performed in R (R Core Team, 2022) For each stain, data were assessed for normality using the Shapiro Wilk test and were confirmed to be non-parametric. Results for peripheral and central samples were then compared using a Wilcoxon signed rank test. Correlation matrixes were formed to investigate any associations between stains within either central or peripheral samples and Spearman Rank Correlation coefficients calculated.

4.2.4 Results

In total, 28 slides were examined for each marker (14 central, 14 peripheral). Of the peripheral samples, five (36%) contained evidence of neoplastic fibroblast proliferation.. A median of 1 mitotic

figure/hpf was visible within the central samples (IQR 0-1/hpf). The outcomes for each of the stains are displayed in Table 8.

Target	Peripheral	Central	P
PCNA			
% positive cells (%)	3.0 (1.4-9.3)	24.9 (20-35)	<0.001*
Positive cells/mm^2	113 (64-310)	918 (513-1197)	<0.001*
P53			
% positive cells (%)	3.0 (1.2-8.2)	3.7 (1.9-26)	0.32
Positive cells/mm^2	59 (23-138)	55 (42-328)	0.45
HIF-1α			
% positive pixels (%)	3.3 (2.8-4.6)	6.6 (0.4-8.8)	0.12
Positive pixels/µm^2	0.03 (0.03-0.05)	0.07 (0.03-0.09)	0.11
BPV			
% positive cells (%)	0.09 (0.06-0.19)	1.6 (0.16-3.6)	0.01*
Positive cells/mm^2	0.80 (0.34-1.2)	12 (1.1-44)	0.003*

*Table 8: Median (IQR) results for quantitative IHC analysis for each marker. * Denotes statistical significance.*

Of the four markers investigated, a significant difference between positive cell/pixel % was demonstrated between peripheral and central samples for BPV (p=0.01) and PCNA stains (p <0.001) (*Fig 12*). The p53 and HIF-1α groups did not show a significant difference between peripheral and central samples. Results were similar for total number of positive cells/mm^2 (or positive pixels/µm^2 for HIF-1α), where BPV (p=0.003) and PCNA (p<0.001) remained significant (*Fig 13).*

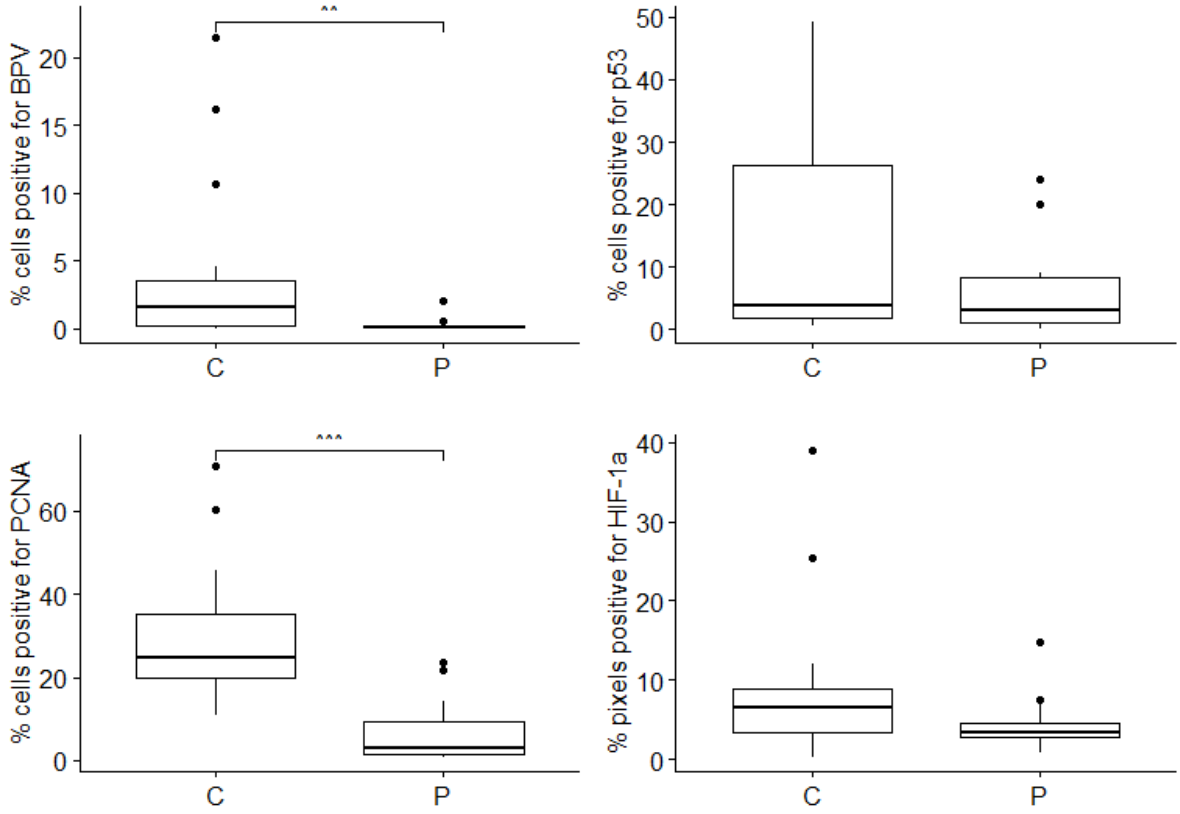


Figure 13: Box plot displaying the median and interquartile ranges of % positive cells in BPV, PCNA and p53 sections and % positive pixels in the HIF-1α sections in central (C) and peripheral (P) sarcoid samples. ^^ denotes p≤0.01, ^^^ denotes p≤0.001. Outliers are displayed as individual points.

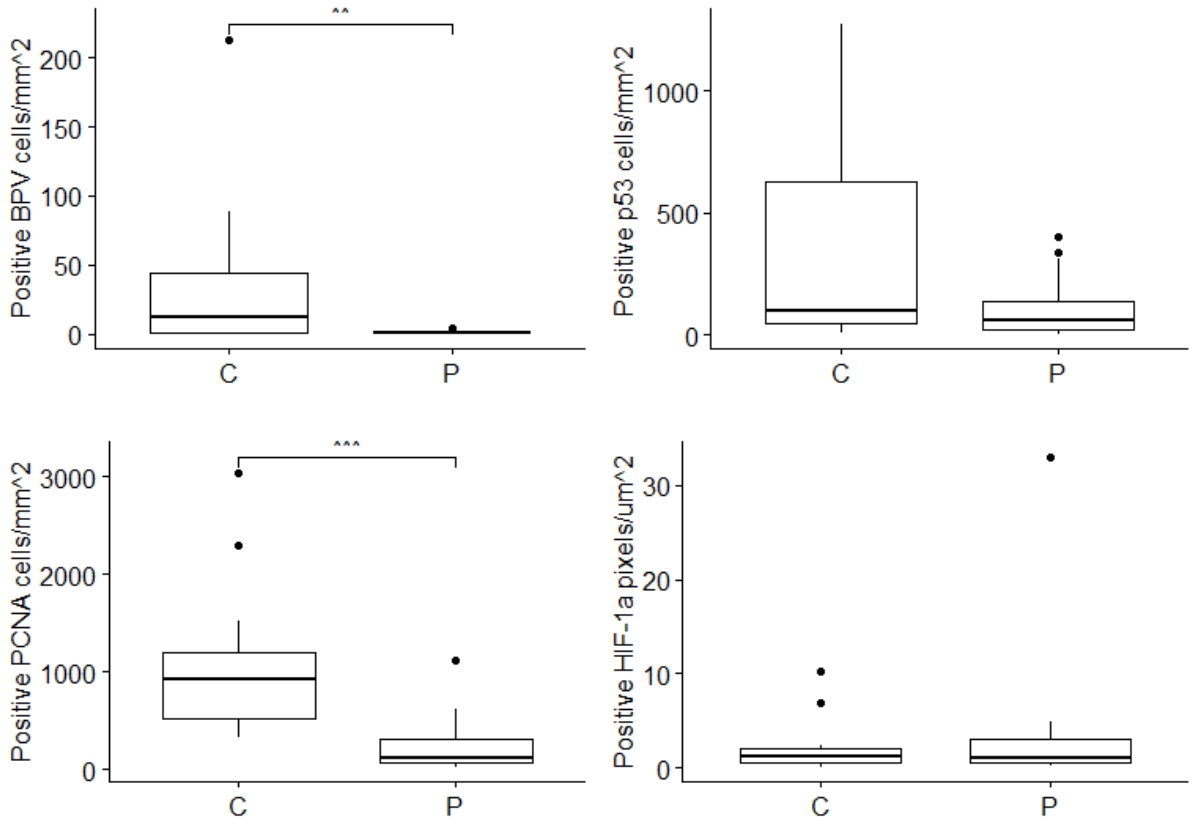


Figure 14: Box plot displaying the median and interquartile ranges of positive cells/mm2 in BPV, PCNA and p53 sections and positive pixels/µm2 in the HIF-1α sections in central (C) and peripheral (P) sarcoid samples. ^^ denotes p≤0.01, ^^^ denotes p≤0.001. Outliers are displayed as individual points.

A significant correlation was identified between BPV staining and both HIF-1α staining (rho=0.88, p=0.004) and PCNA staining (rho= 0.77, p=0.02). P53 staining was inversely correlated with HIF-1α staining (rho= -0.85, p=0.008) (*Fig 15*).

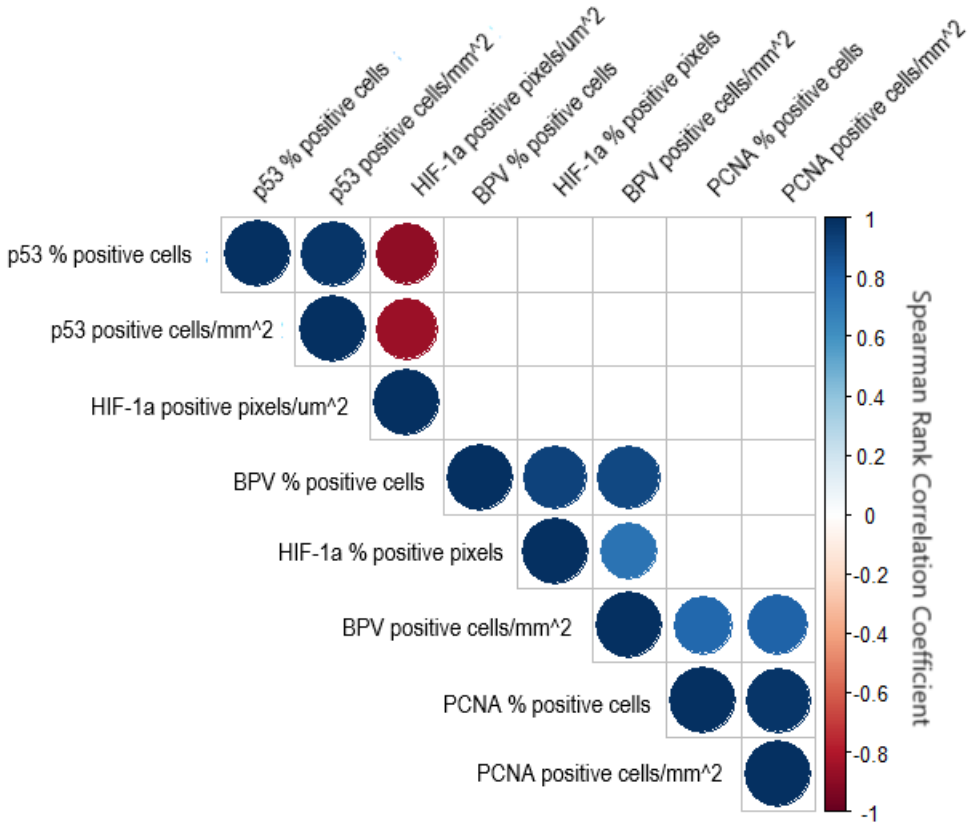


Figure 15: Correlation matrix displaying spearman rank correlation coefficients of variables significantly correlated ($p<0.05$). The colour of the circle denotes the direction and magnitude of the Spearman rank correlation coefficient.

4.2.5 Discussion

This study has demonstrated a significantly increased expression of both papillomaviral oncoprotein E2 and the DNA polymerase processivity factor PCNA in the equine sarcoid compared with surrounding skin.

Significant correlations were also demonstrated between BPV E2 expression and both HIF-1α and PCNA staining, and an inverse association between HIF-1α and p53 expression. These positive correlations may be understood by an appreciation of of the complex molecular interactions in sarcoid oncogenesis. An upregulation of the VEGF pathway, mediated primarily by the hypoxia induced expression of HIF-1α, is well described in equine sarcoids (Martano *et al.,* 2020, Martano *et al.,* 2018), though direct induction by BPV oncoproteins cannot be inferred by this study. In human

papillomavirus infection, increased HIF-1α expression is expected rather by the stabilisation of the HIF-1α secondary to inhibition of proteosomal degradation (Nakamura *et al.,* 2009). As oncogenic structure and function is apparently well conserved between papillomaviral types (Araldi *et al.*, 2017), it may be reasonable to suspect that a similar mechanism may be in place in BPV induced sarcoid tumourigenesis. An upregulation of PCNA expression may also be expected in actively dividing sarcoid tissue, given its participation in DNA synthesis and widely accepted role as a marker of cellular proliferation (Bolton *et al.,* 1992, Moldovan *et al.*, 2007).

P53 expression was inversely correlated with HIF-1α in this pilot study, though insufficient statistical power was attained to demonstrate further significant associations with other markers. The reported perinuclear sequestration of p53 within neoplastic fibroblasts was similarly not consistently observed (Bogaert *et al.,* 2007, Finlay *et al.,* 2012). However, its negative correlation with HIF-1α suggests a possible role of the loss of p53's tumour suppressive activities in sarcoid oncogenesis. Loss of DNA damage-induced G1 phase cell cycle arrest – p53s primary cellular function (Kastan et al., 1991)- is logically associated with an increase in cellular proliferation and thus cellular hypoxia and HIF-1α induction. These associations should be explored further on a larger data set to ensure repeatability, given the inconsistent expression of p53 described in the literature.

BPV expression was identified in the peripheral samples of all sarcoids in this pilot study. BPV expression has been demonstrated by PCR in both the normal skin surrounding sarcoids and in the skin of unaffected equids (Bogaert *et al.,* 2005, Bogaert *et al.,* 2008,). However, the recent use of chromogenic in situ hybridization failed to demonstrate BPV expression in the dermis or adnexa surrounding equine sarcoids (Tura *et al.,* 2021). To the authors' knowledge, this is the first report of BPV expression in the normal surrounding dermis demonstrated by immunohistochemistry- although the peripheral samples included apparent neoplastic fibroblasts in five of the included samples in this study, a further 9 demonstrated no histopathological evidence of neoplasm on H&E.

A useful addition to the study would have been the addition of distant normal skin samples from included horses, though this was prevented by experimental design in this case.

Though the detection of BPV by PCR at these surgical margins may have been more sensitive than detection by immunohistochemistry, its association with risk of sarcoid recurrence is unclear (Martens *et al.,* 2001a, Taylor *et al.,* 2014). Epithelial viral contamination as detectable by PCR on a surface swab is distinct from true viral latency (Bogaert *et al.,* 2008). BPV latency has been demonstrated in equine sarcoids both in epithelial tissue, as is recognized in human HPV infections, but also in peripheral blood mononuclear cells (Bogaert *et al.,* 2008, Brandt *et al.,* 2008, Maglennon *et al.,* 2011, Jones, 2022). Several mechanisms have been proposed, including E7 oncoprotein mediated inhibition of anoikis (a form of apoptosis induced by cellular detachment) and ability to maintain long term episomal replication within infected cells (Demasi *et al.,* 2007, Tu *et al.,* 2021). E5 mediated inhibition of surface MHC I receptors has also been demonstrated, with clear implications for host immunosurveillance (Ashrafi *et al.,* 2002, Nasir and Campo, 2008). Whilst the mechanisms surrounding viral reactivation are poorly understood, the persistence of immunopositivity to BPV in the peripheral tissues has implications for sarcoid regrowth or recurrence and warrants further investigation.

Quantifying BPV expression within sarcoids also warrants investigation as a prognostic marker, though no inferences may be made from this pilot study. Studies on the topic in ES are conflicting and vary with sampling technique. A recent paper from Gysens *et al.*¸ demonstrated that fine needle aspiration of a suspected sarcoid is a valid method for quantifying BPV viral DNA and varied with clinical sarcoid type, and the authors suggested that this technique may have prognostic potential (Gysens *et al.,* 2023). In contrast, a study utilizing quantitative, real time PCR of sarcoid tissue samples failed to demonstrate any association between clinical behaviour and viral load/expression (Bogaert *et al.,* 2007). In the human literature, the association between HPV viral load and tumour behaviour is similarly conflicting (Deng *et al.,* 2015, Flores *et al.,* 2006), though the persistence of

HPV DNA at the cervix following radiotherapy was demonstrated in a large scale longitudinal study to be associated with an increased risk of local tumour recurrence (Song *et al.,* 2010).

PCNA expression was shown to be upregulated in central sarcoid samples compared with peripheral samples in this sample. It is constitutively expressed in mammalian cells and is upregulated in neoplastic cells, regardless of their origin (González-Magaña and Blanco, 2020), and overexpression has demonstrated to be prognostic in both humans and veterinary species (González-Magaña and Blanco, 2020, Carvalho *et al.,* 2016, Miyamoto *et al.,* 2006, Mestrinho *et al.,* 2017). Initial work has suggested it may also be prognostic in the equine sarcoid (Kasperowicz *et al.,* 2006), and its role as a therapeutic target should also be evaluated given its inhibition reduces tumour cell growth both *in vitro* and *in vivo* in human prostate cancers (Zhao *et al.,* 2011, Zongqing *et al.,* 2012).

The expression of HIF-1α has been previously demonstrated in the equine sarcoid (Martano *et al.,* 2020). In this previous study, fibroblast immunohistochemical staining for HIF-1α was demonstrated in all 35 equine sarcoid samples, though a semiquantitative scoring system ranked only 80% of these as 'strong'. This previous study noted a complete lack of stain uptake within the fibroblasts of control skin samples, compared with a median 3.3% positivity in peripheral samples in this study. The difference between these findings is twofold. Control samples from the Martano *et al.* study were obtained from non-sarcoid affected equids postmortem and were confirmed to be BPV negative on PCR analysis. The peripheral samples in this study are more representative of the clinical scenario faced when excising sarcoid tissue. Despite attempts to achieve sufficient surgical margins, peripheral samples were confirmed to contain both neoplastic fibroblasts on routine H&E (36%) and BPV E2 expression on immunohistochemistry (0.09% positive cells detected in peripheral samples). Peripheral sample HIF-1α expression is therefore at least partially explicable by neoplastic fibroblast expression and should be considered clinically when selecting surgical margins during excision.

Differing sampling methods also likely influence differences in HIF-1α expression between studies (sharp excision versus diode laser). Though the HIF-1α pathway has been shown to play a pivotal role in promoting tumour angiogenesis and pathogenesis of several neoplasms (Semenza, 2008, Chen *et al.,* 2022, Wang *et al.,* 2014, Yang *et al.,* 2022), it is also an adaptive cellular pathway crucial for mediating cellular responses to hypoxic conditions (Wang *et al.,* 1995, Jiang *et al.,* 1997). Thermal damage of surrounding tissues secondary to laser excision results in cellular hypoxia which may result in induction of HIF-1α in these peripheral tissues (Du *et al.,* 2011, Semenza, 2008), although the extent to which this may occur with laser excision is unknown. This is a limitation of the study and may have prevented the demonstration of a statistically significantly increased HIF-1α expression within the central sarcoid samples. However, the pathway warrants more consideration as a potential prognostic marker and a therapeutic target. Several i*n vitro* publications have demonstrated that inhibition of the HIF-1α pathway reduces the VEGF dependent angiogenesis necessary for tumour growth, and this pathway is the target of several human anti-cancer agents (Melillo, 2007, Ravi *et al.,* 2000, Stoeltzing *et al.,* 2004). If the above pathways could be elucidated for ES, similar agents may prove useful in the treatment of ES.

This study contributes to the conflicting reports of the role of p53 in ES. The P53 expression in this study was not significantly different between central sarcoid and peripheral skin samples and p53 expression was significantly negatively correlated with HIF-1α expression. Previous investigation of immunohistochemical p53 expression has demonstrated positivity in up to 43% of sarcoid tumours, but sequencing of the p53 gene in cases of ES failed to identify mutation in exons 5 to 9, suggesting the frequency of p53 mutation may in fact be low (Bucher *et al.,* 1996, Finlay *et al.,* 2012, Nasir *et al.,* 1999). Rather than mutation of the p53 protein itself, current data suggest that the loss of p53 function is more likely related to abnormal cytoplasmic or perinuclear sequestration of wild type p53 protein (Martens *et al.,* 2000, Bogaert *et al.,* 2007, Finlay *et al.,* 2012, Tura *et al.,* 2022, Kasperowicz *et al.,* 2006). This has been reported in a range of tumours (Moll *et al.,* 1996, Gestl and Anne Böttger,

2012), and is in agreement with evidence supporting the loss of p53 function in ES (Nixon *et al.,* 2005). However, this identified inconsistent expression of p53 means that it is unlikely to have further use as a prognostic marker or therapeutic target unless this pathway can be better understood in the equine sarcoid (Tura *et al.,* 2022).

This study has several clear limitations. Firstly, the small sample size limits the application of results to all cases of ES. As previously discussed, the samples used were obtained from clinical cases, and so were limited in the extent of their surgical margins and obtained only following diode laser excision. However, they have an advantage in that they represent the clinical scenario and provide information regarding what may be expected after routine sarcoid treatment. For this reason, peripheral samples that contained evidence of neoplastic fibroblast infiltration were retained within the study.

In conclusion, this pilot study has identified the increased expression of BPV E2 protein and PCNA within sarcoid samples as compared to peripheral skin. Further work in this region should aim to identify associations with these markers and tumour behaviour or prognosis to develop a readily available prognostic marker for ES.

4.3 Appendix 7: Cell detection parameters used in QuPath.

	PCNA	P53	BPV
Setup parameters			
Detection image	Optical density sum	Optical density sum	Optical density sum
Requested Pixel size (µm)	0.5	0.5	0.5
Nucleus parameters			
Background radius (µm)	8	9	8
Use opening by reconstruction	Yes	Yes	Yes
Median filter radius (µm)	0	0	0
Sigma (µm)	1.7	2	1.5
Minimum area (µm^2)	10	10	10
Maximum area (µm^2)	400	400	400
Intensity parameters			
Threshold	0.2	0.1	0.1
Max background intensity	2	0.2	2
Split by shape	Yes	No	Yes
Exclude DAB	No	No	No
Cell parameters			
Cell expansion (µm)	5	5	5
Include cell nucleus	Yes	Yes	Yes
General parameters			
Smooth boundaries	Yes	Yes	Yes
Make measurements	Yes	Yes	Yes
Intensity threshold parameters			
Score compartment	Nucleus: DAB OD mean	Nucleus: DAB OD mean	Nucleus: DAB OD mean
Single threshold	Yes	Yes	Yes
Threshold 1+	0.2	0.03	0.005

Appendix 6: Cell detection parameters used for QuPath analysis (Bankhead et al., 2017).

Chapter 5: Conclusion

This body of work contributes to the evidence base regarding the treatment and prognostication of ES.

Chapter 2 reviewed the available evidence regarding the treatment of equine sarcoids and attempted to formulate practical recommendations for veterinarians treating cases of ES. It must be emphasised that the quality of evidence was 'very low' on the GRADE rating scale (Atkins *et al.,* 2004) and so any such recommendations are at a significant risk of bias. The conclusion was therefore that there was insufficient evidence to formulate such recommendations, in the absence of further sufficiently powered, placebo-controlled trials. Collaboration should be encouraged within the veterinary community in order to standardise treatment protocols and allow comparisons between larger numbers of more diverse ES cases.

The literature suffered from poor documentation of accurate ES diagnosis, presumably due to the previously discussed concern regarding the biopsy of these lesions (Knottenbelt *et al.,* 1995). In addition, there is pressure on the clinician from the owner of the equid to keep costs to a minimum, further discouraging the submission of multiple samples for histopathology. The further validation of PCR as a reliable diagnostic modality, either on whole tissue samples (Carr *et al.,* 2001b) or via fine needle aspirate (Gysens *et al.,* 2023), in addition to recent literature suggesting the safety of biopsy of ES (Gysens *et al.,* 2024), will only encourage clinicians to obtain a precise diagnosis and improve the quality of literature in the future.

One of the tentative conclusions from Chapter 2 suggested that a multimodal approach may be beneficial in the treatment of some sarcoids. This was further investigated in Chapter 3, though no statistically significant benefit of the combination treatment protocol over laser excision alone was demonstrated. As discussed in this chapter, this may have been confounded by various factors, for example the intrinsic differences demonstrated between sarcoids and equids in either treatment

group. The study was retrospective rather than prospective and multimodal approach tended to be used on equids presenting in an advanced stage with multiple sarcoids rather than on early presented individuals with single sarcoid masses. Future work on this topic should therefore include a prospective, randomised, blinded and placebo-controlled study in order to better understand the effect of this combination treatment. The preliminary report included here has at least initially demonstrated the safety and plausibility of the technique, which will allow future researchers to further investigate and develop the protocol.

In the majority of papers examined in Chapter 2, little or no significant difference in outcome was demonstrated between treatment categories(s) or the control protocol. This was mirrored by the main findings of Chapter 3- no statistically significant difference was demonstrated between rate of sarcoid recurrence between the laser excision protocol and the combination treatment protocol. This suggests that a variable other than treatment protocol may be more significant in determining lesion outcome in cases of ES. This is given further evidence by the high degree of clustering identified in the mixed effects logistic regression model in Chapter 3; the random effect introduced by the individual equid introduced such a high degree of inter-equid variation so as to make the model largely otherwise meaningless. The reasons for this are not well understood. As previously discussed, a genetic predisposition to ES has been well demonstrated in the literature as a complex, polygenic trait (Jandova *et al.,* 2012). However, the full effects of this predisposition have not been investigated, nor is it understood the mechanism by which certain individuals experience spontaneous sarcoid regression whereas others suffer from multiple, infiltrative sarcoids (Berruex *et al.,* 2016, Knottenbelt *et al.,* 2015).

A current area of considerable research, which may explain some of these differences in susceptibility, is the role of immune dysregulation and the tumour microenvironment on ES. Recent evidence suggests that sarcoids induce an immune tolerant tumour microenvironment that allows persistence of the neoplastic fibroblasts and continued sarcoid proliferation (Wilson and Hicks, 2016,

Geisshüsler *et al.,* 2016). Whilst this requires further investigation, the implications of this suggests that the ability to induce sarcoid growth following implantation of sarcoid explants into a new individual may represent failure of allogenic tissue rejection secondary to this immunotolerant cytokine environment, rather than true de novo BPV infection (Wilson and Hicks, 2016). The tumour microenvironment is a topic of great interest in many human cancers, that is, the complex interaction between neoplastic cells and the non-neoplastic resident host cells, extracellular matrix and secreted factors that promotes tumour development (Anderson and Simon, 2020). This has led to the revolutionary development of immune checkpoint regulators as cancer therapeutics, for example the inhibitors of cytotoxic T-lymphocyte associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1) (Rotte, 2019). These are in use in several human neoplasms and have achieved good results in the treatment of many solid tumours (Xiang *et al.,* 2022). Future work should aim to better understand the tumour microenvironment in ES in order to better understand this individual variability in disease progression and identify similar specific therapeutic targets.

Chapter 3 provided practical clinical information regarding the risk factors influencing likelihood of recurrence and time to sarcoid recurrence. In this studied population, these were more significant in determining treatment outcome than the selection of treatment protocol itself. However, it should be emphasized that these are only applicable to the referral population studied, and analysis was limited by case numbers and missing data. A large scale, multicentred study would be required to better understand these risk factors and inform clinical decision making more generally. This approach may also allow the further untangling of the question of an individual's susceptibility to ES and the development of a more powerful prognostic model. Guidelines have been published in the veterinary literature in order to guide and standardise these future studies, and these should be adhered to in order to allow for future evidence synthesis and meta-analyses (Webster *et al.,* 2010).

Chapter 4 demonstrated increased expression of BPV E2 protein and PCNA within sarcoid samples compared with the immediately adjacent peripheral skin. Whilst HIF-1α staining did not reach

statistical significance between groups, induction of expression was suspected secondary to the thermal injury induced by diode laser excision. A future aim is to apply these findings to a larger set of 168 stored paraffin fixed samples, for which clinical follow-up was obtained as part of the data collection in Chapter 3 (median 39 months). The association between these IHC targets described in Chapter 4 and sarcoid behaviour will be investigated with the aim of identifying marker(s) that may be used for ES prognostication. This will ultimately improve the clinical management of cases of ES by working towards the development of tailored treatment protocols and will further understanding of the pathogenesis of ES and the tumour microenvironment.

In conclusion, this thesis has demonstrated via systematic review a severe lack of quality evidence regarding the treatment of ES and contributed to this literature with the addition of a retrospective case-control study. Clinical variables were investigated that were associated both with the likelihood and time to sarcoid recurrence, allowing clinicians to better understand an individual's susceptibility to ES and aid clinical case management. This was then further progressed to a pilot study investigating IHC markers differentially expressed in clinical sarcoid samples that are suitable for future analysis as to their association with sarcoid behaviour and prognosis.

References

- Amtmann, E., Müller, H. & Sauer, G. 1980. Equine connective tissue tumors contain unintegrated bovine papilloma virus DNA. *J Virol,* 35, 962-4.
- Anderson, N. M. & Simon, M. C. 2020. The tumor microenvironment. *Curr Biol,* 30, R921-r925.
- Araldi, R. P., Mazzuchelli-De-Souza, J., Modolo, D. G., De Souza, E. B., De Melo, T. C., Spadacci-Morena, D. D., Magnelli, R. F., De Carvalho, M. A., De Sá Júnior, P. L., De Carvalho, R. F., Beçak, W. & Stocco Rde, C. 2015. Mutagenic Potential of Bos taurus Papillomavirus Type 1 E6 Recombinant Protein: First Description. *Biomed Res Int,* 2015, 806361.
- Araldi, R.P., Assaf, S.M.R., Carvalho, R.F., Carvalho, M.A.C.R., Souza, J.M., Magnelli, R.F., Módolo, D.G., Roperto, F.P., Stocco, R.C., Beçak, W. 2017. Papillomaviruses: A Systematic Review. *Genet Mol Biol,* 40, 1–21.
- Ashrafi, G. H., Tsirimonaki, E., Marchetti, B., O'brien, P. M., Sibbet, G. J., Andrew, L. & Campo, M. S. 2002. Down-regulation of MHC class I by bovine papillomavirus E5 oncoproteins. *Oncogene,* 21, 248-59.
- Atkins, D., Best, D., Briss, P. A., Eccles, M., Falck-Ytter, Y., Flottorp, S., Guyatt, G. H., Harbour, R. T., Haugh, M. C., Henry, D., Hill, S., Jaeschke, R., Leng, G., Liberati, A., Magrini, N., Mason, J., Middleton, P., Mrukowicz, J., O'connell, D., Oxman, A. D., Phillips, B., Schünemann, H. J., Edejer, T., Varonen, H., Vist, G. E., Williams, J. W., Jr. & Zaza, S. 2004. Grading quality of evidence and strength of recommendations. *BMJ,* 328, 1490.
- Baba, Y., Nosho, K., Shima, K., Irahara, N., Chan, A. T., Meyerhardt, J. A., Chung, D. C., Giovannucci, E. L., Fuchs, C. S. & Ogino, S. 2010. HIF1A overexpression is associated with poor prognosis in a cohort of 731 colorectal cancers. *Am J Pathol,* 176, 2292-301.
- Bankhead, P., Loughrey, M. B., Fernández, J. A., Dombrowski, Y., Mcart, D. G., Dunne, P. D., Mcquaid, S., Gray, R. T., Murray, L. J., Coleman, H. G., James, J. A., Salto-Tellez, M. &

Hamilton, P. W. 2017. QuPath: Open source software for digital pathology image analysis. *Sci Rep,* 7, 16878.

- Baust, J. G. & Gage, A. A. 2005. The molecular basis of cryosurgery. *BJU Int,* 95, 1187-91.
- Berruex, F., Gerber, V., Wohlfender, F. D., Burger, D. & Koch, C. 2016. Clinical course of sarcoids in 61 Franches-Montagnes horses over a 5–7 year period. *Vet Qy,* 36, 189-196.
- Bogaert, L., Heerden, M. V., Cock, H. E., Martens, A. & Chiers, K. 2011. Molecular and immunohistochemical distinction of equine sarcoid from schwannoma. *Vet Pathol,* 48, 737-41.
- Bogaert, L., Martens, A., De Baere, C. & Gasthuys, F. 2005. Detection of bovine papillomavirus DNA on the normal skin and in the habitual surroundings of horses with and without equine sarcoids. *Res Vet Sci,* 79, 253-258.
- Bogaert, L., Martens, A., Van Poucke, M., Ducatelle, R., De Cock, H., Dewulf, J., De Baere, C., Peelman, L. & Gasthuys, F. 2008. High prevalence of bovine papillomaviral DNA in the normal skin of equine sarcoid-affected and healthy horses. *Vet microbiol,* 129, 58-68.
- Bogaert, L., Van Poucke, M., De Baere, C., Dewulf, J., Peelman, L., Ducatelle, R., Gasthuys, F. & Martens, A. 2007. Bovine papillomavirus load and mRNA expression, cell proliferation and p53 expression in four clinical types of equine sarcoid. *J Gen Virol,* 88(8), 2155-2161.
- Bogedale, K., Jagannathan, V., Gerber, V. & Unger, L. 2019. Differentially expressed microRNAs, including a large microRNA cluster on chromosome 24, are associated with equine sarcoid and squamous cell carcinoma. *Vet Comp Oncol,* 17, 155-164.
- Bohl, J., Hull, B. & Vande Pol Scott, B. 2001. Cooperative Transformation and Coexpression of Bovine Papillomavirus Type 1 E5 and E7 Proteins. *J Virol,* 75, 513-521.
- Bolton, W. E., Mikulka, W. R., Healy, C. G., Schmittling, R. J. & Kenyon, N. S. 1992. Expression of proliferation associated antigens in the cell cycle of synchronized mammalian cells. *Cytometry,* 13, 117-26.

- Borzacchiello, G., Russo, V., Della Salda, L., Roperto, S. & Roperto, F. 2008. Expression of Platelet-derived Growth Factor-β Receptor and Bovine Papillomavirus E5 and E7 Oncoproteins in Equine Sarcoid. *J Comp Pathol,* Elsevier Ltd.
- Borzacchiello, G., Russo, V., Gentile, F., Roperto, F., Venuti, A., Nitsch, L., Campo, M. S. & Roperto, S. 2006. Bovine papillomavirus E5 oncoprotein binds to the activated form of the platelet-derived growth factor β receptor in naturally occurring bovine urinary bladder tumours. *Oncogene,* 25, 1251-1260.
- Brand, A., Allen, L., Altman, M., Hlava, M. & Scott, J. 2015. Beyond authorship: attribution, contribution, collaboration, and credit. *Learned Publishing,* 28, 151-155.
- Brandt, S., Haralambus, R., Schoster, A., Kirnbauer, R. & Stanek, C. 2008. Peripheral Blood Mononuclear Cells Represent a Reservoir of Bovine Papillomavirus DNA in Sarcoid-Affected Equines. *J Gen Virol*, 89(6), 1390-1395.
- Brandt, S., Tober, R., Corteggio, A., Burger, S., Sabitzer, S., Walter, I., Kainzbauer, C., Steinborn, R., Nasir, L. and Borzacchiello, G. (2011) BPV-1 infection is not confined to the dermis but also involves the epidermis of equine sarcoids. *Vet Microbiol,* 150(1-2),35-40.
- Broström, H., Fahlbrink, E., Dubath, M. L. & Lazary, S. 1988. Association between equine leucocyte antigens (ELA) and equine sarcoid tumors in the population of Swedish halfbreds and some of their families. *Vet Immunol Immunopathol,* 19, 215-23.
- Bucher, K., Szalai, G., Marti, E., Griot-Wenk, M. E., Lazary, S. & Pauli, U. 1996. Tumour suppressor gene p53 in the horse: identification, cloning, sequencing and a possible role in the pathogenesis of equine sarcoid. *Res Vet Sci,* 61, 114-9.
- Byam-Cook, K. L., Henson, F. M. & Slater, J. D. 2006. Treatment of periocular and non-ocular sarcoids in 18 horses by interstitial brachytherapy with iridium-192. *Vet Rec,* 159, 337-41.
- Campbell, M., Mckenzie, J. E., Sowden, A., Katikireddi, S. V., Brennan, S. E., Ellis, S., Hartmann-Boyce, J., Ryan, R., Shepperd, S., Thomas, J., Welch, V. & Thomson, H. 2020.

Synthesis without meta-analysis (SWiM) in systematic reviews: Reporting guideline. *BMJ,*16,368, l6890

- Campo, M. S. 2002. Animal models of papillomavirus pathogenesis. *Virus Res,* 89, 249-261.
- Campo, M. S., Jarrett, W. F., Barron, R., O'neil, B. W. & Smith, K. T. 1992. Association of bovine papillomavirus type 2 and bracken fern with bladder cancer in cattle. *Cancer Res,* 52, 6898-904.
- Cancer, I. A. F. R. O. 1987. 5-FLUOROURACIL (Group 3). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.* Lyon (France).
- Carcopino, X., Henry, M., Benmoura, D., Fallabregues, A. S., Richet, H., Boubli, L. & Tamalet, C. 2006. Determination of HPV type 16 and 18 viral load in cervical smears of women referred to colposcopy. *J Med Virol,* 78, 1131-40.
- Carr, E. A., Théon, A. P., Madewell, B. R., Hitchcock, M. E., Schlegel, R. & Schiller, J. T. 2001a. Expression of a transforming gene (E5) of bovine papillomavirus in sarcoids obtained from horses. *Am J Vet Res,* 62, 1212-1217.
- Carr, E. A., Théon, A. P., Madewell, B. R., Griffey, S. M. & Hitchcock, M. E. 2001b. Bovine papillomavirus DNA in neoplastic and nonneoplastic tissues obtained from horses with and without sarcoids in the western United States. *Am J Vet Res,* 62(5), 741-4.
- Carstanjen, B., Jordan, P. & Lepage, O. M. 1997. Carbon dioxide laser as a surgical instrument for sarcoid therapy - A retrospective study on 60 cases. *Can Vet J,* 38(12), 773-6.
- Carvalho, M. I., Pires, I., Prada, J., Lobo, L. & Queiroga, F. L. 2016. Ki-67 and PCNA Expression in Canine Mammary Tumors and Adjacent Nonneoplastic Mammary Glands: Prognostic Impact by a Multivariate Survival Analysis. *Vet pathol,* 53, 1138-1146.
- Chambers, G., Ellsmore, V. A., O'brien, P. M., Reid, S. W. J., Love, S., Campo, M. S. & Nasir, L. 2003. Sequence variants of bovine papillomavirus E5 detected in equine sarcoids. *Virus Res,* 96, 141-145.

- Chen, H., Nio, K., Tang, H., Yamashita, T., Okada, H., Li, Y., Doan, P. T. B., Li, R., Lv, J., Sakai, Y., Yamashita, T., Mizukoshi, E., Honda, M. & Kaneko, S. 2022. BMP9-ID1 Signaling Activates HIF-1α and VEGFA Expression to Promote Tumor Angiogenesis in Hepatocellular Carcinoma. *Int J Mol Sci,* 23.
- Christen, G., Gerber, V., Dolf, G., Burger, D. & Koch, C. 2014. Inheritance of equine sarcoid disease in Franches-Montagnes horses. *Vet J,* 199, 68-71.
- Christen-Clottu, O., Klocke, P., Burger, D., Straub, R. & Gerber, V. 2010. Treatment of Clinically Diagnosed Equine Sarcoid with a Mistletoe Extract (Viscum album austriacus). *J Vet Intern Med,* 24, 1483-1489.
- Clarke, D. M., Baust, J. M., Van Buskirk, R. G. & Baust, J. G. 2001. Chemo-cryo combination therapy: an adjunctive model for the treatment of prostate cancer. *Cryobiology,* 42, 274-85.
- Clarke, D. M., Hollister, W. R., Baust, J. G. & Van Buskirk, R. G. 1999. Cryosurgical Modeling: Sequence of Freezing and Cytotoxic Agent Application Affects Cell Death. *Mol Urol,* 3, 25-31.
- Compston, P. C., Turner, T., Wylie, C. E. & Payne, R. J. 2016. Laser surgery as a treatment for histologically confirmed sarcoids in the horse. *Equine Vet J,* 48(4), 451-6.
- Cotugno, R., Gallotta, D., D'avenia, M., Corteggio, A., Altamura, G., Roperto, F., Belisario, M. A. & Borzacchiello, G. 2013. BAG3 protects bovine papillomavirus type 1-transformed equine fibroblasts against pro-death signals. *Vet Res,* 44, 61.
- Curnow, B., Rich, A. F., Ireland, J., Correa, D. C., Dunn, J., Jenkins, D., Carslake, H. & Ressel, L. 2023. Histological evidence of superficial inflammation is associated with lower recurrence of equine sarcoids following surgical removal: A follow-up study of 106 tumours in 64 horses. *Vet J,* 292, 105953.
- Dapalma, T., Doonan, B. P., Trager, N. M. & Kasman, L. M. 2010. A systematic approach to virus-virus interactions. *Virus Res,* 149, 1-9.
- Demasi, J., Chao, M. C., Kumar, A. S. & Howley, P. M. 2007. Bovine Papillomavirus E7 Oncoprotein Inhibits Anoikis. *J Virol,* 81, 9419-9425.

- Deng, T., Feng, Y., Zheng, J., Huang, Q. & Liu, J. 2015. Low Initial Human Papillomavirus Viral Load May Indicate Worse Prognosis in Patients With Cervical Carcinoma Treated With Surgery. *J Gynecol Oncol,* 26, 111-117.
- Depping, R., Jelkmann, W. & Kosyna, F. K. 2015. Nuclear-cytoplasmatic shuttling of proteins in control of cellular oxygen sensing. *J Mol Med (Berl),* 93, 599-608.
- Du, J., Xu, R., Hu, Z., Tian, Y., Zhu, Y., Gu, L. & Zhou, L. 2011. PI3K and ERK-Induced Rac1 Activation Mediates Hypoxia-Induced HIF-1α Expression in MCF-7 Breast Cancer Cells. *PLoS ONE,* 6, e25213.
- Endale, H. T., Mariye, Y. F., Negash, H. K., Hassen, F. S., Asrat, W. B., Mengstie, T. A. & Tesfaye, W. 2024. MiRNA in cervical cancer: Diagnosis to therapy: Systematic review. *Heliyon,* 10, e24398.
- Faccini, A. M., Cairney, M., Ashrafi, G. H., Finbow, M. E., Campo, M. S. & Pitts, J. D. 1996. The bovine papillomavirus type 4 E8 protein binds to ductin and causes loss of gap junctional intercellular communication in primary fibroblasts. *J Virol,* 70, 9041-5.
- Finlay, M., Yuan, Z., Burden, F., Trawford, A., Morgan, I. M., Campo, M. S. & Nasir, L. 2009. The detection of Bovine Papillomavirus type 1 DNA in flies. *Virus Res,* 144, 315-7.
- Finlay, M., Yuan, Z., Morgan, I. M., Campo, M. S. & Nasir, L. 2012. Equine sarcoids: Bovine Papillomavirus type 1 transformed fibroblasts are sensitive to cisplatin and UVB induced apoptosis and show aberrant expression of p53. *Vet Res,* 43, 81.
- Flores, R., Papenfuss, M., Klimecki, W. T. & Giuliano, A. R. 2006. Cross-sectional analysis of oncogenic HPV viral load and cervical intraepithelial neoplasia. *Int J Cancer,* 118, 1187-1193.
- Frandsen, S. K., Gehl, J., Tramm, T. & Thoefner, M. S. 2020. Calcium Electroporation of Equine Sarcoids. *Animals (Basel),* 10.
- Gannon, J. V., Greaves, R., Iggo, R. & Lane, D. P. 1990. Activating mutations in p53 produce a common conformational effect. A monoclonal antibody specific for the mutant form. *Embo J,* 9, 1595-602.

- Gaynor, A. M., Zhu, K. W., Cruz, F. N. D., Affolter, V. K. & Pesavento, P. A. 2016. Localization of Bovine Papillomavirus Nucleic Acid in Equine Sarcoids. *Vet Pathol,* 53, 567-573.
- Geisshüsler, H., Marti, E., Stoffel, M. H., Kühni, K., Stojiljkovic, A., Von Tscharner, C., Vidondo, B., Gerber, V. & Koch, C. 2016. Quantitative analysis of infiltrating immune cells and bovine papillomavirus type 1 E2-positive cells in equine sarcoids. *Vet J,* 216, 45-52.
- Gestl, E. E. & Anne Böttger, S. 2012. Cytoplasmic sequestration of the tumor suppressor p53 by a heat shock protein 70 family member, mortalin, in human colorectal adenocarcinoma cell lines. *Biochem Biophys Res Commun,* 423, 411-6.
- Gobeil, P., Gault, E. A., Campo, M. S., Gow, J., Morgan, I. M. & Nasir, L. 2007. Equine sarcoids are not induced by an infectious cell line. *Equine Vet J,* 39, 189-91.
- González-Magaña, A. & Blanco, F. J. 2020. Human PCNA Structure, Function and Interactions. *Biomolecules,* 10, 570.
- Goodrich, L., Gerber, H., Marti, E. & Antczak, D. F. 1998. Equine sarcoids. *Vet Clin North Am Equine Pract,* 14, 607-23.
- Greene, M. H. 1992. Is Cisplatin a Human Carcinogen? *JNCI,* 84, 306-312.
- Gysens, L., Martens, A. & Haspeslagh, M. 2023. Performance of fine-needle aspirate testing compared with superficial swab testing for quantification of BPV-1/−2 viral load in equine sarcoids. *Res in Vet Sci,* 159, 101-105.
- Gysens, L., Martens, A. & Haspeslagh, M. 2024. Longitudinal pilot study examining the effect of punch biopsy on equine sarcoid growth dynamics. *Vet derm,* 35, 148-155.
- Hamza, E., Cosandey, J., Gerber, V., Koch, C. & Unger, L. 2023. The potential of three whole blood microRNAs to predict outcome and monitor treatment response in sarcoid-bearing equids. *Vet. Res. Commun,* 47, 87-98.
- Haralambus, R., Burgstaller, J., Klukowska-Rötzler, J., Steinborn, R., Buchinger, S., Gerber, V. & Brandt, S. 2010. Intralesional bovine papillomavirus DNA loads reflect severity of equine sarcoid disease. *Equine Vet J,* 42, 327-31.

- Haspeslagh, M., Vlaminck, L. & Martens, A. 2016. Treatment of sarcoids in equids - 230 cases 2008-2013. *Vet Clin North Am Equine Pract*, 249(3), 311-8.
- Haspeslagh, M., Vlaminck, L. & Martens, A. 2018. The possible role of Stomoxys calcitrans in equine sarcoid transmission. *Vet Journ,* 231, 8-12.
- Heller, R., Jaroszeski, M. J., Leo-Messina, J., Perrot, R., Voorhis, N. V., Reintgen, D. S. & Gilbert, R. A. 1995. Treatment of B16 mouse melanoma with the combination of electropermeabilization and chemotherapy. *Bioelectrochem*, 36(1), 83-87.
- Hermonat, P. L., Spalholz, B. A. & Howley, P. M. 1988. The bovine papillomavirus P2443 promoter is E2 trans-responsive: evidence for E2 autoregulation. *Embo J,* 7, 2815-22.
- Hewes, C. A. & Sullins, K. E. 2006. Use of cisplatin-containing biodegradable beads for treatment of cutaneous neoplasia in equidae: 59 cases (2000-2004). *J Am Vet Med Assoc,* 229, 1617-22.
- Hewes, C. A. & Sullins, K. E. 2009 Review of the treatment of equine cutaneous neoplasia. *Proceedings AAEP Annual Convention - Las Vegas, USA*. 55:386-393.
- Ho, C. M., Chien, T. Y., Huang, S. H., Lee, B. H. & Chang, S. F. 2006. Integrated human papillomavirus types 52 and 58 are infrequently found in cervical cancer, and high viral loads predict risk of cervical cancer. *Gynecol Oncol,* 102, 54-60.
- Hodgson, D. R., Mcconaghy, F. F., Davis, R. E., Reppas, G. P., Rawlinson, R. J., Mcclintock, S. A., Hutchins, D. R. & Mcclintock, S. A. 1994. Management of equine sarcoids: 1975–93. *N Z Vet J*, 42(5), 180-4.
- Hoffmann, N. E. & Bischof, J. C. 2002. The cryobiology of cryosurgical injury. *Urology,* 60, 40-9.
- Hollis, A. R. 2019. Radiotherapy for the treatment of periocular tumours in the horse. *Equine Vet Educ,* 31, 647-652.
- Hollis, A. R. 2020. Strontium plesiotherapy for the treatment of sarcoids in the horse. *Equine Vet Educ*, 32, 7-11.

- Hollis, A. R. & Berlato, D. 2018. Initial experience with high dose rate brachytherapy of periorbital sarcoids in the horse. *Equine Vet Educ,* 30, 444-449.
- Hollister, W. 1998. Effects of freezing on cell viability and mechanisms of cell death in a human prostate cell line. *Mol Urol,* 2, 3-18.
- Hollstein, M., Shomer, B., Greenblatt, M., Soussi, T., Hovig, E., Montesano, R. & Harris, C. C. 1996. Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Res,* 24, 141-6.
- Howick, J, Chalmers, I, Glasziou, P, Greenhalgh, T, Heneghan, C, Liberati, A, Moschetti, I, Phillips, B and Thornton, H. "The 2011 Oxford CEBM Evidence Levels of Evidence (Introductory Document)". Oxford Centre for Evidence-Based Medicine. http://www.cebm.net/index.aspx?o=5653
- Ireland, J. L., Wylie, C. E., Collins, S. N., Verheyen, K. L. & Newton, J. R. 2013. Preventive health care and owner-reported disease prevalence of horses and ponies in Great Britain. *Res Vet Sci,* 95, 418-24.
- Jackson, C. D. T., P.J. 1936. The incidence and pathology of tumours of domesticated animals in South Africa : a study of the Onderstepoort collection of neoplasms with special reference to their histopathology. *Onderstepoort J Vet Res,* 6, 3-460.
- Jackson, M. E. & Campo, M. S. 1995. Both viral E2 protein and the cellular factor PEBP2 regulate transcription via E2 consensus sites within the bovine papillomavirus type 4 long control region. *J Virol,* 69, 6038-46.
- Jandova, V., Klukowska-Rötzler, J., Dolf, G., Janda, J., Roosje, P., Marti, E., Koch, C., Gerber, V. & Swinburne, J. 2012. Whole genome scan identifies several chromosomal regions linked to equine sarcoids. *Schweizer Archiv für Tierheilkunde,* 154, 19.
- Jiang, B. H., Zheng, J. Z., Leung, S. W., Roe, R. & Semenza, G. L. 1997. Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem,* 272, 19253-60.

- Jindra, C., Hainisch, E. K. & Brandt, S. 2023. Immunotherapy of Equine Sarcoids-From Early Approaches to Innovative Vaccines. *Vaccines (Basel),* 11, 769.
- Jones, S. E. 2022. Papillomaviruses in equids: A decade of discovery and more to come? *Equine Vet Educ,* 34, 236-240.
- Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. 1991. Participation of p53 protein in the cellular response to DNA damage. *Cancer Res*, 51, 6304–6311.
- Kasperowicz, B., Rotkiewicz, T. & Otrocka-Domagala, I. 2006. Pathomorphological and immunohistochemical study of selected markers of tumour cell proliferation in equine sarcoids. *Pol J Vet Sci,* 9, 109-119.
- Klein, W. R., Bras, G. E., Misdorp, W., Steerenberg, P. A., De Jong, W. H., Tiesjema, R. H., Kersjes, A. W. & Ruitenberg, E. J. 1986. Equine sarcoid: BCG immunotherapy compared to cryosurgery in a prospective randomised clinical trial. *Cancer Immunol Immunother,* 21, 133-40.
- Knottenbelt, D. C. 2005. A suggested clinical classification for the equine sarcoid. *Clin. Tech. Equine Pract,* 4(4), 278-295.
- Knottenbelt, D. C. 2019. The Equine Sarcoid: Why Are There so Many Treatment Options? *Vet Clin North Am Equine Pract,* 35(2), 243-262.
- Knottenbelt, D. C., Edwards, S. & Daniel, E. 1995. Diagnosis and treatment of the equine sarcoid. *In Practice,* 17, 123 - 129.
- Knottenbelt, D. C. & Kelly, D. F. 2000. The diagnosis and treatment of periorbital sarcoid in the horse: 445 cases from 1974 to 1999. *Vet Ophthalmol,* 3, 169-191.
- Knottenbelt, D. C. & Matthews, J. B. 2001. A positive step forwards in the diagnosis of equine sarcoid. *Vet J,* 161, 224-6.
- Knottenbelt, D. C., Patterson-Kane, J. C. & Snalune, K. L. 2015. 11 - Sarcoids. *In:* Knottenbelt, D. C., Patterson-Kane, J. C. & Snalune, K. L. (eds.) *Clinical Equine Oncology.* Elsevier, 203-219.

- Knottenbelt, D. C., Watson, A. H., Hotchkiss, J. W., Chopra, S. & Higgins, A. J. 2020. A pilot study on the use of ultra-deformable liposomes containing bleomycin in the treatment of equine sarcoid. *Equine Vet Educ,,* 32, 258-263.
- Knowles, E. J., Tremaine, W. H., Pearson, G. R. & Mair, T. S. 2016. A database survey of equine tumours in the United Kingdom. *Equine Vet J,* 48(3), 280-4.
- Koch C, Martens A, Hainisch EK, Schüpbach G, Gerber V, Haspeslagh M. 2018. The clinical diagnosis of equine sarcoids - Part 1: Assessment of sensitivity and specificity using a multicentre case-based online examination. *Vet J,* 242:77-82.
- Lane, J. G. 1977. The treatment of equine sarcoids by cryosurgery. *Equine Vet. J,* 9, 127-33.
- Lazary, S., Marti, E., Szalai, G., Gaillard, C. & Gerber, H. 1994. Studies on the frequency and associations of equine leucocyte antigens in sarcoid and summer dermatitis. *Anim Genet,* 25 (Suppl 1), 75-80.
- Longley, D. B., Harkin, D. P. & Johnston, P. G. 2003. 5-fluorouracil: mechanisms of action and clinical strategies. *Nat Rev Cancer,* 3, 330-8.
- Lunardi, M., De Alcântara, B. K., Otonel, R. A., Rodrigues, W. B., Alfieri, A. F. & Alfieri, A. A. 2013. Bovine papillomavirus type 13 DNA in equine sarcoids. *J Clin Microbiol,* 51, 2167-71.
- Madewell, B. R. 2001. Cellular Proliferation in Tumors: A Review of Methods, Interpretation, and Clinical Applications. *J Vet Intern Med,* 15, 334-340.
- Maglennon, G. A., Mcintosh, P. & Doorbar, J. 2011. Persistence of viral DNA in the epithelial basal layer suggests a model for papillomavirus latency following immune regression. *Virology,* 414, 153-163.
- Mair, T. S. & Fews, D. 2016. Detection of Sarcoid Tissue at 2 cm Resection Margins Following CO 2 Laser Excision. *Equine Vet J,* 48, 20.
- Marais, H. J. & Page, P. C. 2011. Treatment of equine sarcoid in seven Cape mountain zebra (Equus zebra zebra). *J Wildl Dis,* 47, 917-24.

- Marchetti, B., Gault, E. A., Cortese, M. S., Yuan, Z., Ellis, S. A., Nasir, L. & Campo, M. S. 2009. Bovine papillomavirus type 1 oncoprotein E5 inhibits equine MHC class I and interacts with equine MHC I heavy chain, *J Gen Virol,* 90, 2865-2870.
- Martano, M., Altamura, G., Power, K., Restucci, B., Carella, F., Borzacchiello, G. & Maiolino, P. 2020. Evaluation of Hypoxia-Inducible Factor-1 Alpha (HIF-1 alpha) in Equine Sarcoid: An Immunohistochemical and Biochemical Study. *Pathogens (Basel),* 9, 58.
- Martano, M., Power, K., Restucci, B., Pagano, I., Altamura, G., Borzacchiello, G. & Maiolino, P. 2018. Expression of vascular endothelial growth factor (VEGF) in equine sarcoid. *BMC Vet Res,* 14, 266-266.
- Martens, A., De Moor, A., Demeulemeester, J. & Ducatelle, R. 2000. Histopathological characteristics of five clinical types of equine sarcoid. *Res Vet Sci,* 69(3), 295-300.
- Martens, A., De Moor, A., Demeulemeester, J. & Peelman, L. 2001a. Polymerase chain reaction analysis of the surgical margins of equine sarcoids for bovine papilloma virus DNA. *Vet Surg,* 30, 460-7.
- Martens, A., De Moor, A., Vlaminck, L., Pille, F. & Steenhaut, M. 2001b. Evaluation of excision, cryosurgery and local BCG vaccination for the treatment of equine sarcoids. *Vet Rec,* 149, 665-669.
- Marti, E., Lazary, S., Antczak, D. F. & Gerber, H. 1993. Report of the first international workshop on equine sarcoid. *Equine Vet J,* 25, 397-407.
- Marty, M., Sersa, G., Garbay, J. R., Gehl, J., Collins, C. G., Snoj, M., Billard, V., Geertsen, P. F., Larkin, J. O., Miklavcic, D., Pavlovic, I., Paulin-Kosir, S. M., Cemazar, M., Morsli, N., Soden, D. M., Rudolf, Z., Robert, C., O'sullivan, G. C. & Mir, L. M. 2006. Electrochemotherapy – An easy, highly effective and safe treatment of cutaneous and subcutaneous metastases: Results of ESOPE (European Standard Operating Procedures of Electrochemotherapy) study. *EJC Supplements,* 4, 3-13.

- Mccauley, C. T., Hawkins, J. F., Adams, S. B. & Fessler, J. F. 2002. Use of a carbon dioxide laser for surgical management of cutaneous masses in horses: 32 cases (1993-2000) *J Am Vet Med Assoc,* 220(8), 1192-1197.
- Mcconaghy, F. F., Davis, R. E., Reppas, G. P., Rawlinson, R. J., Mcclintock, S. A., Hutchins, D. R. & Mcclintock, S. A. 1994. Management of equine sarcoids: 1975–93. *N Z Vet J,* 42(5), 180-184.
- Mcguinness, L. A. & Higgins, J. P. T. 2021. Risk-of-bias VISualization (robvis): An R package and Shiny web app for visualizing risk-of-bias assessments. *Res Synth Methods.* 12: 55–61.
- Melillo, G. 2007. Targeting hypoxia cell signaling for cancer therapy. *Cancer Metastasis Rev,* 26, 341-352.
- Mestrinho, L. A., Faísca, P., Peleteiro, M. C. & Niza, M. M. R. E. 2017. PCNA and grade in 13 canine oral squamous cell carcinomas: association with prognosis. *Vet Comp Oncol,* 15, 18-24.
- Miyamoto, S., Ito, K., Kurokawa, K., Suzuki, K., Suzuki, K. & Yamanaka, H. 2006. Clinical validity of proliferating cell nuclear antigen as an objective marker for evaluating biologic features in patients with untreated prostate cancer. *Int J Urol,* 13, 767-72.
- Moldovan, G. L., Pfander, B., Jentsch, S. 2007. PCNA, the maestro of the replication fork. *Cell*, 129, 665-679.
- Moll, U. M., Ostermeyer, A. G., Haladay, R., Winkfield, B., Frazier, M. & Zambetti, G. 1996. Cytoplasmic sequestration of wild-type p53 protein impairs the G1 checkpoint after DNA damage. *Mol Cell Biol,* 16, 1126-37.
- Montpellier J, B. P., Dieuzeide R. 1937. Tumeurs schannienne cutanees du mulet. *Rev Med Vet,* 86, 216.
- Murad, M. H., Asi, N., Alsawas, M. & Alahdab, F. 2016. New evidence pyramid. *Evid Based Med.* 21(4), 125-7.

- Mählmann, K., Hamza, E., Marti, E., Dolf, G., Klukowska, J., Gerber, V. & Koch, C. 2014. Increased FOXP3 expression in tumour-associated tissues of horses affected with equine sarcoid disease. *Vet J,* 202, 516-21.
- Nakamura, M., Bodily, J. M., Beglin, M., Kyo, S., Inoue, M. & Laimins, L. A. 2009. Hypoxia-specific stabilization of HIF-1alpha by human papillomaviruses. *Virology,* 387, 442-8.
- Nasir, L. & Campo, M. S. 2008. Bovine papillomaviruses: Their role in the aetiology of cutaneous tumours of bovids and equids. *Vet Dermatol*, 19(5), 243-54.
- Nasir, L., Mcfarlane, S. T. & Reid, S. W. 1999. Mutational status of the tumour suppressor gene (p53) in donkey sarcoid tumours. *Vet J,* 157, 99-101.
- Nasir, L., Mcfarlane, S. T., Torrontegui, B. O. & Reid, S. W. 1997. Screening for bovine papillomavirus in peripheral blood cells of donkeys with and without sarcoids. *Res Vet Sci,* 63, 289-90.
- Nasir, L. & Reid, S. W. J. 1999. Bovine papillomaviral gene expression in equine sarcoid tumours. *Virus research,* 61, 171-175.
- Nixon, C., Chambers, G., Ellsmore, V., Campo, M. S., Burr, P., Argyle, D. J., Reid, S. W. J. & Nasir, L. 2005. Expression of cell cycle associated proteins cyclin A, CDK-2, p27 kip1 and p53 in equine sarcoids. *Cancer Lett,* 221(2), 237-45.
- Nogueira, S. A. F., Torres, S. M. F., Malone, E. D., Diaz, S. F., Jessen, C. & Gilbert, S. 2006. Efficacy of imiquimod 5% cream in the treatment of equine sarcoids: a pilot study. *Vet Dermatol,* 17, 259-265.
- Offer, K. S., Dixon, C. E. & Sutton, D. G. M. 2023. Treatment of equine sarcoids: A systematic review. *Equine Vet J*, 56(1), 12-25.
- Offer, K. S., Marchesi, F. & Sutton, D. G. M. 2022. Topical 5-fluorouracil as an adjunct treatment in equine corneolimbal squamous cell carcinoma. *Equine Veterinary Education,* 34, e363-e368.

- Otten, N., Von Tscharner, C., Lazary, S., Antczak, D. F. & Gerber, H. 1993. DNA of bovine papillomavirus type 1 and 2 in equine sarcoids: PCR detection and direct sequencing. *Arch Virol,* 132, 121-31.
- Pettersson, C. M., Broström, H., Humblot, P. & Bergvall, K. E. 2020. Topical treatment of equine sarcoids with imiquimod 5% cream or Sanguinaria canadensis and zinc chloride – an open prospective study. *Vet Dermatol,* 31, 471-e126.
- Potocki, L., Lewinska, A., Klukowska-Rötzler, J., Bielak-Zmijewska, A., Grabowska, W., Rzeszutek, I., Kaminska, P., Roga, E., Bugno-Poniewierska, M., Slota, E., Mählmann, K., Koch, C. & Wnuk, M. 2014. Sarcoid-derived fibroblasts: links between genomic instability, energy metabolism and senescence. *Biochimie,* 97, 163-72.
- Potocki, L., Lewinska, A., Klukowska-Rötzler, J., Bugno-Poniewierska, M., Koch, C., Mählmann, K., Janda, J. & Wnuk, M. 2012. DNA hypomethylation and oxidative stress-mediated increase in genomic instability in equine sarcoid-derived fibroblasts. *Biochimie,* 94, 2013-2024.
- Pucket, J. D. & Gilmour, M. A. 2014. Intralesional 5-fluorouracil (5-FU) for the treatment of eyelid squamous cell carcinoma in 5 horses. *Equine Vet Educ,* 26, 331-335.
- R Core Team 2022. R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.
- Ravi, R., Mookerjee, B., Bhujwalla, Z. M., Sutter, C. H., Artemov, D., Zeng, Q., Dillehay, L. E., Madan, A., Semenza, G. L. & Bedi, A. 2000. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. *Genes Dev,* 14, 34-44.
- Rotte, A. 2019. Combination of CTLA-4 and PD-1 blockers for treatment of cancer. *J Exp Clin Cancer Res,* 38, 255.
- Ryan, R. & Hill, S. 2016. How to GRADE the quality of the evidence. *Cochrane Consumers and Communication Group,* available at http://cccrg.cochrane.org/author-resources. Version 3.0 December 2016.

- Ryan R, Synnot A, Prictor M, Hill S. Data extraction template for included studies. *Cochrane Consumers and Communication Group*. London: The Cochrane Collaboration; 2016. p. 1-5. https://training.cochrane.org/sites/training.cochrane.org
- Schaffer, P. A., Wobeser, B., Martin, L. E. R., Dennis, M. M. & Duncan, C. G. 2013. Cutaneous neoplastic lesions of equids in the central United States and Canada: 3,351 biopsy specimens from 3,272 equids (2000-2010). *J Am Vet Med Assoc,* 242(1), 99-104.
- Schapiro, F., Sparkowski, J., Adduci, A., Suprynowicz, F., Schlegel, R. & Grinstein, S. 2000. Golgi alkalinization by the papillomavirus E5 oncoprotein. *J Cell Biol,* 148, 305-15.
- Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J. & Howley, P. M. 1990. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell,* 63, 1129-36.
- Semenza, G. L. 2008. Hypoxia-inducible factor 1 and cancer pathogenesis. *IUBMB Life,* 60, 591-597.
- Shin, J. I., Lim, H. Y., Kim, H. W., Seung, B. J. & Sur, J. H. 2015. Analysis of Hypoxia-Inducible Factor-1α Expression Relative to Other Key Factors in Malignant Canine Mammary Tumours. *J Comp Pathol,* 153, 101-110.
- Song, Y. J., Kim, J.-Y., Lee, S.-K., Lim, H., Lim, M. C., Seo, S. W., Kang, S., Lee, D. O. & Park, S.-Y. 2010. Persistent Human Papillomavirus DNA Is Associated With Local Recurrence After Radiotherapy of Uterine Cervical Cancer. *Int J Cancer*, 129, 896-902.
- Spoormakers, T. J. P., Klein, W. R., Jacobs, J. J. L., Van Den Ingh Th, S. G. A. M., Koten, J. W. & Den Otter, W. 2003. Comparison of the efficacy of local treatment of equine sarcoids with IL-2 or cisplatin/IL-2. *Cancer Immunol. Immunother,* 52, 179-184.
- Spugnini, E. P. & Baldi, A. 2019. Electrochemotherapy in Veterinary Oncology: State-of-the-Art and Perspectives. *Vet Clin North Am Small Anim Pract,* 49, 967-979.

- Stadler, S., Kainzbauer, C., Haralambus, R., Brehm, W., Hainisch, E. & Brandt, S. 2011. Successful treatment of equine sarcoids by topical aciclovir application. *Vet Rec,* 168, 187-187.
- Sterne, J., Hernán, M., Reeves, B., Savović, J., Berkman, N., Viswanathan, M., Henry, D., Altman, D., Ansari, M., Boutron, I., Carpenter, J., Chan, A., Churchill, R., Deeks, J., Hróbjartsson, A., Kirkham, J. J., Jüni, P., Loke, Y., Pigott, T., Ramsay, C., Regidor, D., Rothstein, H., Sandhu, L., Santaguida, P., Schünemann, H., Shea, B., Shrier, I., Tugwell, P., Turner, L., Valentine, J., Waddington, H., Waters, E., Wells, G., Whiting, P. & Higgins, J. 2016. ROBINS-I: a tool for assessing risk of bias in non-randomized studies of interventions. *BMJ*, 12, 355. i4919.
- Sterne, J. A. C., Savović, J., Page, M. J., Elbers, R. G., Blencowe, N. S., Boutron, I., Cates, C. J., Cheng, H.-Y., Corbett, M. S., Eldridge, S. M., Emberson, J. R., Hernán, M. A., Hopewell, S., Hróbjartsson, A., Junqueira, D. R., Jüni, P., Kirkham, J. J., Lasserson, T., Li, T., Mcaleenan, A., Reeves, B. C., Shepperd, S., Shrier, I., L.A., S., K., T., I.R., W., P.F., W. & Higgins, J. P. T. 2019. RoB 2: a revised tool for assessing risk of bias in randomised trials. *BMJ*, 28, 366, l4898.
- Stewart, A. A., Rush, B. & Davis, E. 2006. The efficacy of intratumoural 5-fluorouracil for the treatment of equine sarcoids*. Aust Vet J,* 84(3), 101-6.
- Stocco Dos Santos, R. C., Lindsey, C. J., Ferraz, O. P., Pinto, J. R., Mirandola, R. S., Benesi, F. J., Birgel, E. H., Pereira, C. A. & Beçak, W. 1998. Bovine papillomavirus transmission and chromosomal aberrations: an experimental model. *J Gen Virol,* 79(9), 2127-35.
- Stoeltzing, O., Mccarty, M. F., Wey, J. S., Fan, F., Liu, W., Belcheva, A., Bucana, C. D., Semenza, G. L. & Ellis, L. M. 2004. Role of hypoxia-inducible factor 1alpha in gastric cancer cell growth, angiogenesis, and vessel maturation. *J Natl Cancer Inst,* 96, 946-56.
- Studer, S., Gerber, V., Straub, R., Brehm, W., Gaillard, C., Lüth, A. & Burger, D. 2007. [Prevalence of hereditary diseases in three-year-old Swiss Warmblood horses]. *Schweiz Arch Tierheilkd,* 149, 161-71.

- Tamzali, Y., Borde, L., Rols, M. P., Golzio, M., Lyazrhi, F. & Teissie, J. 2012. Successful treatment of equine sarcoids with cisplatin electrochemotherapy: A retrospective study of 48 cases. *Equine Vet J,* 44, 214-220.
- Tate, L. P. & Tate, K. B. 2019. Equine Laser Surgery Procedures. *Laser Surgery in Veterinary Medicine,* 246-263.
- Taylor, S. & Haldorson, G. 2013. A review of equine sarcoid. *Equine Vet Educ,* 25, 210-216.
- Taylor, S. D., Toth, B., Baseler, L. J., Charney, V. A. & Miller, M. A. 2014. Lack of Correlation Between Papillomaviral DNA in Surgical Margins and Recurrence of Equine Sarcoids. *J Vet Sci,* 34, 722-725.
- Teifke, J. P. & Löhr, C. V. 1996. Immunohistochemical detection of P53 overexpression in paraffin wax-embedded squamous cell carcinomas of cattle, horses, cats and dogs. *J Comp Pathol,* 114, 205-10.
- Terron-Canedo, N., Britton, C., Nicolson, L. & Nasir, L. 2014. Differential Expression of microRNAs in Equine Sarcoids: Preliminary Results from an In Vitro Model. *Equine Vet J,* 46, 15-15.
- The Endnote Team, E. 2013. EndNote. 64 bit ed. Philadelphia, PA: Clarivate.
- Théon AP, Wilson WD, Magdesian KG, Pusterla N, Snyder JR, Galuppo LD. 2007. Long-term outcome associated with intratumoral chemotherapy with cisplatin for cutaneous tumors in equidae: 573 cases (1995-2004). *J Am Vet Med Assoc,* 15, 230(10), 1506-13.
- Théon, A. P. & Pascoe, J. R. 1995. Iridium-192 interstitial brachytherapy for equine periocular tumours: treatment results and prognostic factors in 115 horses. *Equine Vet J,* 27, 117-121.
- Théon, A. P., Pascoe, J. R., Galuppo, L. D., Fisher, P. E., Griffey, S. M. & Madigan, J. E. 1999. Comparison of perioperative versus postoperative intratumoral administration of cisplatin for treatment of cutaneous sarcoids and squamous cell carcinomas in horses. *J Am Vet Med Assoc,* 215, 1655-60.

- Tong, X., Boll, W., Kirchhausen, T. & Howley Peter, M. 1998. Interaction of the Bovine Papillomavirus E6 Protein with the Clathrin Adaptor Complex AP-1. *J Virol,* 72, 476-482.
- Tozon, N., Kramaric, P., Kos Kadunc, V., Sersa, G. & Cemazar, M. 2016. Electrochemotherapy as a single or adjuvant treatment to surgery of cutaneous sarcoid tumours in horses: a 31-case retrospective study. *Vet Rec,* 179, 627.
- Tu, Q., Feng, W., Chen, Z., Li, Q., Zhao, Y., Chen, J., Jiang, P., Xue, X., Zhang, L. & Zhao, K.-N. 2021. Characterization of Episomal Replication of Bovine Papillomavirus Type 1 DNA in Long-Term Virion-Infected Saccharomyces Cerevisiae Culture. *Virol,* 36, 1492-1502.
- Tura, G., Brunetti, B., Brigandì, E., Rinnovati, R., Sarli, G., Avallone, G., Muscatello, L. V., La Ragione, R. M., Durham, A. E. & Bacci, B. 2022. Expression of Cell-Cycle Regulatory Proteins pRb, Cyclin D1, and p53 Is Not Associated with Recurrence Rates of Equine Sarcoids. *Vet Sci,* 9, 474.
- Tura, G., Savini, F., Gallina, L., La Ragione, R. M., Durham, A. E., Mazzeschi, M., Lauriola, M., Avallone, G., Sarli, G., Brunetti, B., Muscatello, L. V., Girone, C. & Bacci, B. 2021. Fibroblast-associated protein-α expression and BPV nucleic acid distribution in equine sarcoids. *Vet Pathol,* 9(9), 474.
- Unger, L., Abril, C., Gerber, V., Jagannathan, V., Koch, C. & Hamza, E. 2021. Diagnostic potential of three serum microRNAs as biomarkers for equine sarcoid disease in horses and donkeys. *J Vet Intern Med*, 35(1), 610-619.
- Unger, L., Gerber, V., Pacholewska, A., Leeb, T. & Jagannathan, V. 2019. MicroRNA fingerprints in serum and whole blood of sarcoid-affected horses as potential non-invasive diagnostic biomarkers. *Vet Comp Oncol*, 17(1), 107-117.
- Valentine, B. A. 2006. Survey of equine cutaneous neoplasia in the Pacific Northwest. *J Vet Diagn Invest,* 18, 123-6.
- Vandenbroucke, J. P. 1998. Observational research and evidence-based medicine: What should we teach young physicians? *J Clin Epidemiol,* 51, 467-72.

- Vodenkova, S., Buchler, T., Cervena, K., Veskrnova, V., Vodicka, P. & Vymetalkova, V. 2020. 5-fluorouracil and other fluoropyrimidines in colorectal cancer: Past, present and future. *Pharmacol Ther,* 206, 107447.
- Vojtěsek, B., Bártek, J., Midgley, C. A. & Lane, D. P. 1992. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. *J Immunol Methods,* 151, 237-44.
- Wang, B., Ding, Y. M., Fan, P., Wang, B., Xu, J. H. & Wang, W. X. 2014. Expression and significance of MMP2 and HIF-1α in hepatocellular carcinoma. *Oncol Lett,* 8, 539-546.
- Wang, G. L., Jiang, B. H., Rue, E. A. & Semenza, G. L. 1995. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. *Proc Natl Acad Sci U S A,* 92, 5510-4.
- Webster, J. D., Dennis, M. M., Dervisis, N., Heller, J., Bacon, N. J., Bergman, P. J., Bienzle, D., Cassali, G., Castagnaro, M., Cullen, J., Esplin, D. G., Peña, L., Goldschmidt, M. H., Hahn, K. A., Henry, C. J., Hellmén, E., Kamstock, D., Kirpensteijn, J., Kitchell, B. E., Amorim, R. L., Lenz, S. D., Lipscomb, T. P., Mcentee, M., Mcgill, L. D., Mcknight, C. A., Mcmanus, P. M., Moore, A. S., Moore, P. F., Moroff, S. D., Nakayama, H., Northrup, N. C., Sarli, G., Scase, T., Sorenmo, K., Schulman, F. Y., Shoieb, A. M., Smedley, R. C., Spangler, W. L., Teske, E., Thamm, D. H., Valli, V. E., Vernau, W., Euler, H. V., Withrow, S. J., Weisbrode, S. E., Yager, J. & Kiupel, M. 2010. Recommended Guidelines for the Conduct and Evaluation of Prognostic Studies in Veterinary Oncology. *Vet Pathol,* 48, 7-18.
- Wenker, C., Hoby, S., Steck, B. L., Ramsauer, A. S., Blatter, S. & Tobler, K. 2021. Equine Sarcoids in Captive Wilde Equids: Diagnostic and Clinical Management of 16 Cases- A Possible Predisposition of the European Cohort of Somali Wild Ass (Equus Africanus Somaliensis)? *J Zoo Wildl Med,* 52, 28-37.

- Wilson, A. D., Armstrong, E. L. R., Gofton, R. G., Mason, J., De Toit, N. & Day, M. J. 2013. Characterisation of early and late bovine papillomavirus protein expression in equine sarcoids. *Vet Microbiol,* 162(2–4), 369-380.
- Wilson, A. D. & Hicks, C. 2016. Both tumour cells and infiltrating T-cells in equine sarcoids express FOXP3 associated with an immune-supressed cytokine microenvironment. *Vet Res,* 47, 55.
- Wobeser, B. K. 2016. Making the Diagnosis: Equine Sarcoid. *Vet. Pathol.* 54, 9-10.
- Xiang, Z., Li, J., Zhang, Z., Cen, C., Chen, W., Jiang, B., Meng, Y., Wang, Y., Berglund, B., Zhai, G. & Wu, J. 2022. Comprehensive Evaluation of Anti-PD-1, Anti-PD-L1, Anti-CTLA-4 and Their Combined Immunotherapy in Clinical Trials: A Systematic Review and Meta-analysis. *Front Pharmacol,* 25 (13), 883655.
- Yamamoto, Y., Ibusuki, M., Okumura, Y., Kawasoe, T., Kai, K., Iyama, K. & Iwase, H. 2008. Hypoxia-inducible factor 1alpha is closely linked to an aggressive phenotype in breast cancer. *Breast Cancer Res Treat,* 110, 465-75.
- Yang, H., Geng, Y.-H., Wang, P., Zhang, H.-Q., Fang, W.-G. & Tian, X.-X. 2022. Extracellular ATP promotes breast cancer chemoresistance via HIF-1α signaling. *Cell Death Dis,* 13, 199.
- Yuan, Z., Philbey, A. W., Gault, E. A., Campo, M. S. & Nasir, L. 2007. Detection of bovine papillomavirus type 1 genomes and viral gene expression in equine inflammatory skin conditions. *Virus Res,* 124, 245-9.
- Yuan, Z. Q., Nicolson, L., Marchetti, B., Gault, E. A., Campo, M. S. & Nasir, L. 2008. Transcriptional changes induced by bovine papillomavirus type 1 in equine fibroblasts. *J Virol,* 82, 6481-91.
- Zhao, H., Lo, Y. H., Ma, L., Waltz, S. E., Gray, J. K., Hung, M. C. & Wang, S. C. 2011. Targeting Tyrosine Phosphorylation of PCNA Inhibits Prostate Cancer Growth. *Mol Cancer Ther,* 10(1), 29-36.

- Zimmermann, H., Koh, C. H., Degenkolbe, R., O'connor, M. J., Müller, A., Steger, G., Chen, J. J., Lui, Y., Androphy, E. & Bernard, H. U. 2000. Interaction with CBP/p300 enables the bovine papillomavirus type 1 E6 oncoprotein to downregulate CBP/p300-mediated transactivation by p53. *J Gen Virol,* 81, 2617-2623.
- Zongqing, T., Matthew, W., Kelsey, L. D., William, L. S., Chris, R. E., Shanna, J. S., Linda, H. M., Yi, Z., Shan, L. & Zhongyun, D. 2012. Small-Molecule Targeting of Proliferating Cell Nuclear Antigen Chromatin Association Inhibits Tumor Cell Growth. *Mol Pharmacol,* 81, 811.