Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

SCIENZE VETERINARIE

Ciclo 34

Settore Concorsuale: 07/H2 – PATOLOGIA VETERINARIA E ISPEZIONE DEGLI ALIMENTI DI ORIGINE ANIMALE

Settore Scientifico Disciplinare: VET/03 – PATOLOGIA GENERALE E ANATOMIA PATOLOGICA VETERINARIA

MOLECULAR ANALYSIS OF PAPILLOMAVIRUS-INDUCED CUTANEOUS TUMORS IN EQUIDS

Presentata da: Giorgia Tura

Coordinatore Dottorato

Carolina Castagnetti

Supervisore

Barbara Bacci

Co-supervisore

Giuseppe Sarli

Esame finale anno 2022

Abstract

Papillomavirus associated tumors are well recognized entities in humans as well as in animals. Here we review the current understanding of human papillomavirus (HPV) associated cancers to better understand the oncogenic mechanisms of Equine papillomavirus (EcPV) and Bovine Papillomavirus (BPV) in horses.

In the first part of this study the interactions between Equine papillomavirus 2 (EcPV-2) and cell cycle proteins are discussed. EcPV-2 has been recognized as the cause of genital squamous cell carcinomas (SCCs) in horses, but the exact mechanism of carcinogenesis is not fully understood. The aim of the first part of study is to assess the expression of cell cycle proteins p53, p16, pRB and Cyclin D1 in a series of equine SCCs and papillomas.

Results of the study confirm the role of EcPV-2 in the pathogenesis of genital SCC, as previously reported. Moreover, in a small subset of ocular SCCs EcPV-2 was detected for the first time. By immunohistochemistry, p53 was mostly expressed in ocular SCCs and predominantly with a suprabasal localization. UV light exposure and *TP53* mutation may be involved in the pathogenesis of SCC in this location. However, this aspect was not reviewed in the present study. Regarding p16 expression, overexpression was associated with increased mitotic index but not with viral infection, as in humans. In addition to p16, investigation on pRB and Cyclin D1 proteins did not show significant correlation with other histological and molecular variables, suggesting that the p16-pRB-Cyclin D1 pathway is not involved in EcPV-2 induced carcinogenesis, but may be still dysregulated in a subset of equine SCC.

The second part of this study is focused on the carcinogenetic mechanisms of BPV in equine sarcoids. The association between BPV and equine sarcoids is well known, but several aspects of the disease remain unclear. The aim of the second part of the study was to characterize the typical histomorphological features of equine sarcoids, assess the expression of cell cycle proteins (pRB, Cyclin D1, p53, p16) and the Ki-67 proliferation index.

Our results confirm that the typical histological features of sarcoids cannot be used to correctly classify the clinical types of sarcoids. Moreover, in a subset of sarcoids low pRB-Cyclin D1 scores were associated with simultaneous increased p16 expression. This could suggest that BPV may act in a pRB-dependent pathway, but further studies are necessary to confirm this hypothesis. The analysis of the proliferative rate of sarcoids was carried out with calculation of Ki-67 index, confirming that sarcoids have a low proliferative activity except for tumors displaying a fascicular dermal pattern. Finally, a subset of sarcoids recurred after excision.

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Chapter 1 Cutaneous neoplasia in equids

In equids, cutaneous neoplasia has been reported to account for 50% of all neoplastic lesions.[175] So far, survey data on frequency and tumor types are very limited and often variable, depending on the characteristics of the studied population. The high variety may be due to age categories, environmental differences, and breeds. However, since 1977 sarcoids are reported to be the most frequently diagnosed tumor.[310] In fact, in the first study of survey of equine cutaneous neoplasia conduced on 236 horses, 43.6% of all the neoplastic diseases were diagnosed as sarcoids.[310] Similarly, the occurrence of sarcoids from 1935 to 1974 accounted 38.1% of the submissions in a 1983 study.[21] More recently, these data were confirmed by both Valentine *et al* and Shaffer *et al* in 2006 and 2013 respectively.[279,328] Moreover, in the past seven years, sarcoids were confirmed as the first most common cutaneous neoplasms, accounting 24% of all the submissions in United Kingdom.[175]

According to all the equine literature of survey on equine cutaneous tumors, squamous cell carcinoma (SCC) is the second most commonly diagnosed neoplasm.[19,21,175,279,310,328] Moreover, from recent literature, SCC has been also associated with breed and sex categories: mares seems to be at lower risk while Cob/Cob cross are at increased risk.[175]

However, it should be considered that the cause of the low odds in mares could be related with the higher incidence of penile and preputial SCCs in stallions.[324]

Regarding breed predisposition, the data described above disagree with the previous literature, in which the highest prevalence of developing SCCs was reported in ponies.[142,324] This discrepancy may be related to the older age of the pony population in the older studies compared with the population studied by Knowles *et al* in 2015.[175]

Other cutaneous tumors as melanomas, lymphomas, mast cell tumors and fibromas occur in variable proportions. In 1975 Baker and Leyland reported sarcoids as the third neoplasm, occurring in 12.90% of animals after SCCs (15.3%) and fibromas (35.5%).[19]

However, the highest incidence of fibromas and the relative low proportion of sarcoids may also be related with the confidence of clinicians to diagnose sarcoids without histological examination.[19] Two years later, melanomas were considered the less frequent tumors by Sundberg *et al*, which were reported to occur in 3.80% of cases.[310] However, recent data consider melanomas or lymphoma as the third most commonly diagnosed cutaneous neoplasms.[175,279] Even in melanomas, a connection between coat colour and high incidence has been recognized: an autosomal dominant mutation in syntaxin-17 has been proposed, but further studies are needed to evaluate the association between this genetic mutation and the onset of melanomas.[228] By contrast, cutaneous lymphomas are not associated with any risk factor and comprised 13.6% of all the skin neoplasms, moving down to the third place for Knowles *et al*.[175]

Lastly, mast cell tumors are reported to occur in less than 6%. Although Arabian and Arabian X seem to be predisposed, up to now no association between mast cell tumors and this breed has been demonstrated.[202]

Chapter 2 Overview of Papillomaviruses

2.1 Overview and Papillomavirus Taxonomy

Papillomaviruses (PVs) are small, non-enveloped viruses belonging to the *Papillomaviridae* family. According to the International Committee on Taxonomy of Viruses (ICTV), the family is divided in two subfamilies, the *Firstpapillomavirinae*, and *Secondpapillomavirinae*.[97]

The subfamily of *Firstpapillomavirinae* currently contains more than 50 genera and many species. Genera can include both human and animal PVs and are named using a Greek letter (e.g., Alpha-) while species are named using a numbering system.[336]

In 2010, the discovery of novel genera exhaust the Greek alphabet, therefore the classification was revised by adding the prefixes "dyo" and "treis" after the Omega-PVs to accommodate the growing list.[23,336] Exceptions are *Alphapapillomavirus*, *Betapillomavirus* and *Gammapapillomavirus*, whose PVs-genera infect humans and are not used in combinations with prefixes.[97]

By contrast, genera within the *Secondpapillomavirinae,* are named according to the Semitic abjads, a kind of alphabet composed by consonants. The only genus belonging to this subfamily is *Alefpapillomavirus.*[97]

Within the subfamilies, division into genera and species is based on the highly conserved L1 open reading frame (ORF).[336] In particular, different genera must show less than 60% similarity in their L1 ORF while PVs of different species less than 30%.[336]

Most PVs are highly species-specific and can infect either cutaneous or mucosal epithelium. An exception of natural cross-species infection in domestic animals is represented by Bovine delta-PVs. Within this group, Bovine Papillomavirus (BPV)-1, -2, and -13 have been associated with the development of sarcoids in horses.[198,247] Although BPV-1 and BPV-2 in cattle are the cause of fibropapillomas, in horses they are responsible for the malignant transformation of dermal fibroblasts with concomitant pathognomonic epithelial changes.[27] To date, 28 types of BPV have been characterized and the majority cluster into five genera: delta (BPV-1, -2, -13, -14), epsilon (BPV-5, -8,

-25), xi (BPV-3, -4, -6, -9, -10, -11, -12, -15, -17, -20, -23, -24, -26, -28), dyokappa (BPV-16, -18, -22) and dyoxipapillomavirus (BPV-7). The remaining are not yet classified.[349,360]

By contrast, only 9 types of Equine Papillomavirus (EcPV) are known and classified into three genera: zeta (EcPV1), dyoiota (EcPV2, EcPV4, EcPV5) and dyorhopapillomavirus (EcPV3, EcPV6, EcPV7). As for BPV, EcPV-8 and -9 are unclassified.[194,326]

2.2 Genome

All PVs, have very similar genome structure and organization. PVs are DNA viruses with a double-stranded, circular genome of 8kb. The genome is conventionally divided into three regions, separated by two polyadenylation sites: early region (E), late region (L) and long control region or non-coding (LCR).[132]

The early region is located from the 5' extremity onwards and contains up to 8 early genes that encode the E1, E2, E3, E4, E5, E6, E7 and E8 proteins.[356] In general, early proteins are non-structural proteins that regulate viral transcription, replication and adaptation within infected cells.[356] Comparative analysis of PVs of different genera has shown that not all the E genes can be necessary expressed or have the same function.[336] For example, the lack of E4 ORF as well as different functions of E6 and E7 ORF have been demonstrated for Avian PVs.[239] Moreover, the E8 ORF is only contained in BPV-1 [69,179]and Human Papillomavirus (HPV)-31 [258,305] and encode a E8^E2 fused protein which acts as a repressor of viral replication.[100]

The late region is located downstream the E region and consists of L1 and L2 ORF. These two genes encode for L1 (major) and L2 (minor) capsid proteins, two structural proteins that assemble in capsomers and contribute to the icosahedral capsid formation.[356] In addition to structural properties, L1 protein functions as portal entry into the host cell by binding to surface receptors.[294] Likewise, L2 as well as being the minor structural protein, also facilitates viral escape from late endosomes and promotes viral DNA transport to the nucleus by interacting with L1.[342]

The LCR, also called upstream regulatory region (URR), is the last part of the genome. It doesn't encode for proteins but acts as regulator of viral transcription.[112]

2.3 Life cycle and Replication

This chapter describes the mechanisms of infection, establishment, and replication of the well-known human PVs.

PVs are epitheliotropic viruses that can infect both cutaneous and mucocutaneous epithelia.[335] In haired skin the infection is thought to occur through microwounds that expose basal keratinocytes [71,118] or via hair follicles.[283] For mucocutaneous junctions, and in particular for cervical lesions, viral infection starts in the transition zone from columnar to stratified squamous epithelium.[140] Although the mechanisms of viral attachment to the host cell are still unknown, several studies established the role of heparan sulphate (HS) in viral internalization. Heparan sulphate (HS) is a linear polysaccharide present both in extracellular matrix and in the phospholipid bilayer of the plasma membrane.[125,154] PVs can attach to glycosaminoglycans chains of heparan sulphate (HSPG) of the plasma membrane or to HSPG ectodomains attached to laminin 332 of the extracellular matrix.[63,86,289] Nevertheless, in epithelial cells the most representative HS is syndecan-1 which function as a primary attachment receptor.[291] Viral interaction with HS cell-surface receptors, triggers conformational changes in viral capsid proteins, promoting L1 cleavage by kallikrein-8. Next, cyclophilin B exposes L2 N-terminus which is cleaved by furin[43] allowing viral transport to an entry receptor complex. This complex comprises different classes of proteins including CD151 and CD63, integrins α6 and β4, annexin A2 and an undefined secondary receptor.[4,105,113,280] Binding to the receptor complex induces actin polymerization and subsequent viral internalization through clathrin and caveolin independent pathway.[282,300] After internalization, the virus is delivered to an early endosome which than matures into late endosome. Here the host chaperone cyclophilin B (CyPB) and the lower PH, contribute to viral capsid proteins disassemble.[24] Thus, L2-viral DNA complex (L2/vDNA) is transported to the *trans* Golgi, where it remains during interphase.[17,262] In the late prophase, L2/vDNA is delivered to the internuclear space until nuclear envelope breakdown.[17,262] As the cell progresses to the metaphase, L2 associates to the host cell chromosomes establishing viral infection.[52,262]

PVs lifecycle can be divided into three steps which are strictly linked to epithelial cell replication and differentiation: establishment, maintenance, and amplification.[95] The first step is the establishment, where viral genome starts to express E1 and E2 proteins and is maintained as episomes in basal cells.[345] In the maintenance phase, as basal cells move up to the surface, viral genome starts to express E6 and E7 proteins which further delay terminal differentitation.[132] During this process, episomes replicate simultaneously to the host chromosomes to be maintained in a constant copy number.[115] The third step, called genome amplification, occurs in the upper intermediate layer of the epidermis and starts with the expression of E4 and E5 proteins.[110,253] Concurrently, E7 overexpression within the nucleus of the host cell triggers the expression of E1 and E2 proteins.[96] Once amplified, viral genome starts to synthetize L1 and L2 and viral particles are than packed into mature virions.[116] Thereafter, following the physiologic process of anoikis of fully keratinized cells, virions are released and a new infection can take place.

2.4 Cross-species infection

Most PVs are highly species-specific but an exception of natural cross-species infection in domestic animals is represented by BPV-1, -2, and -14. In fact, these PVs have been associated with sarcoids development in horses, donkeys, zebras and buffaloes.[65,249] In addition, there is strong evidence that BPV-1 can also infects cats, with development of sarcoid-like lesions.[60] In cats, BPV cross-infection produce characteristic proliferation of neoplastic fibroblasts, often with picket-fence arrangement.[286] The first report on BPV cross specie infection, described a proliferative papillomatous lesions characterized by variable cytopathic effect and hypergranulosis of the stratum spinosum in two Persian cats.[59] Because of many similarities between equine and feline BPV-induced tumors, the lesion in cats has been named “feline sarcoid”. In 2003, Teifke *et al*, detected BPV-1 and -2 DNA from mesenchymal cells of 4/14 feline sarcoids, with no evidence of viral detection in the overlying epidermis.[319] More recently, PCR from oral and mucosal sarcoids, allowed the diagnosis of a BPV-14 infection.[129,160] These

findings support the ability of BPV-1, -2 and -14 to infect other species other than cattle.

2.5 Diagnosis of Papillomavirus infection

The diagnostic approach to PVs infection is based on histopathology and molecular biology. Histological examination is useful to detect epithelial and/or mesenchymal changes induced by some PVs. However, in the majority of cases, additional diagnostic techniques are required to confirm the diagnosis. For example, immunohistochemistry (IHC) can be used to detect Papillomavirus antigen[109] or, in challenging cases, it is necessary for the distinction from other tumors. Moreover, PVs DNA can be detected by polymerase chain reaction (PCR) on formalin-fixed paraffin embedded (FFPE) samples or on fresh tissue specimens.[27,213] Despite being a highly specific and sensible technique, the main disadvantage is the risk of false negative results: viral DNA can be degraded and therefore undetectable. In addition, cross-contamination and non-specific annealing can occur and skew the results.

To date, the most sensitive and specific molecular method used to identify viral infections, is chromogenic in situ hybridization (CISH). This assay allows simultaneous mRNA detection and localization within a specific cell type.[146]

Chapter 3 Interaction of Papillomaviruses with Cell Cycle Proteins

Cell cycle proteins are responsible for maintenance and regulation of the physiologic cell cycle. These include Cyclins and their coupled enzymes cyclin-dependent kinases (CDKs) that regulate phosphorylation of target proteins during cell-cycle phases. In PVs infection, viral oncogenes interact with specific cell cycle proteins resulting in abrogation of cell-cycle checkpoint. The consequence is DNA damage, cell immortalization, and carcinogenesis.[136]

3.1 Overview of the cell cycle

Cell cycle consists of five phases (G0, G1, S, G2, M) that result in cell division. It is regulated by activators and inhibitors, which are responsible for the correct progression till the end of the process.

Cells in quiescent state are in G0, and to enter G1 must be stimulated by growth factors.[16] At this point, the progression through G1 is regulated by Cyclins D and E. The family of Cyclins D includes three isoforms (D1, D2, D3) which are otherwise expressed within different tissues.[70] Binding of Cyclins D to its associated enzymes (CDK4 and CDK6) is essential in the early G1, whereas Cyclin E/CDK2 binding occurs in the middle/late G1.[333]

G1/S transition is controlled by a special mechanism of surveillance called "checkpoint", which prevent the replication of damaged DNA. This step is regulated by the retinoblastoma (RB) gene family which include Rb/p105, p107, and Rb2/p130 collectively named as "pocket proteins". This term refers to the highly conserved binding region (pocket region) which is responsible for cellular factors and/or oncoproteins interactions.[104] The retinoblastoma protein (pRB) is regulated by phosphorylation: in resting cells, pRB is hypophosphorylated and tightly bound with E2F transcription factors, blocking it in an unactive state.[2]

When mitogenic signals induced by the formation of Cyclin D/CDK4-6 complex gather on cell cycle, pRB is inactivated by phosphorylation. Hyperphosphorylation of pRB

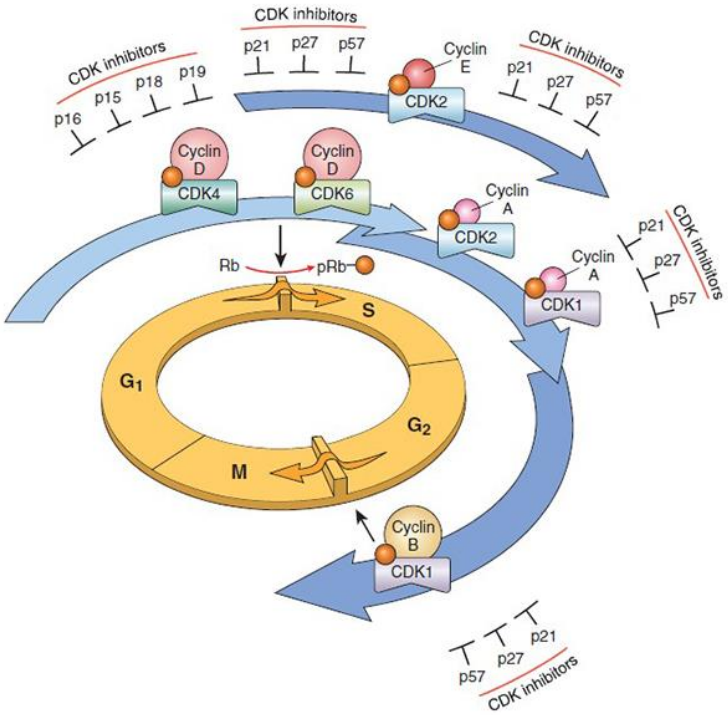
leads to E2F release and subsequent gene transcription for the S phase. Once E2F is released, the complex Cyclin E/CDK2 will form, to prepare the cell to enter the S phase. The maintenance of pRB in a hyperphosphorylated status till the end of the cell cycle is due to Cyclin A/CDK2 complex.[89]

The S phase is tightly controlled by Cyclin A/CDK2, which regulate the timing of mitosis and E2F phosphorylation.[26] Moreover, Cyclin A is responsible for the transition to G2 phase, in which binds to and activate CDK1, promoting entry in M phase.[333]

Another control mechanism acts on G2/M transition and avoids cells with damaged DNA to enter the M phase. This mechanism is regulated by Cyclin B/CDK1 complex whose activity is maintained upon mitosis. Specifically, chromosome condensation, centrosome separation and cell rounding, only occur if Cyclin B/CDK1 is activated.[332]

In M phase, cyclin A/CDK1 and Cyclin B/CDK1 promote the event of mitosis. Towards the end of M phase, the anaphase promoting complex (APC) causes ubiquitination and destruction of Cyclin A and Cyclin B, that leads to termination of M phase.[91] Figure 1 shows the main regulatory proteins of the cell cycle.

Figure 1. Cell cycle and the role of cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors (Robbins e Contran, 2020).



3.2 Negative regulators of the cell cycle

Cell cycle is further controlled by CDKs inhibitors, that block CDKs activity. To date, there are two families of CDKs inhibitors: the INK4 and Cip/Kip family, which act on specific cell cycle phases.

The first family, the INK4, includes $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$. They specifically block interaction and binding between CDK4-6 and Cyclin D at G1 phase.[332] The most studied CDKs inhibitor of this family is $p16^{INK4a}$. It negatively regulates the cell cycle by binding with CDK4 and CDK6 alone or with Cyclin D/CDK4-6 complex. The inhibition of Cyclin D/CDK4-6 prevents RB phosphorylation and therefore the cell arrests in G1.[334]

The Cip/Kip family includes p21 (Cip1), p27 (Cip2), p57 (Kip2). All these proteins can block the cell cycle by binding most of the CDKs. Of note, p21 inhibits CDK4, 6/Cyclin D and CDK2/Cyclin E, blocking the cell cycle at G1-S and G2-M respectively. Moreover, some studies demonstrate that p21 can also play an important role in cases of DNA damage.[333]

In conjunction with CDKs inhibitors, p53 ensures that only cells without DNA errors undergo replication. P53 is a tumor suppressor protein called “the guardian of the genome” referring to its role in preventing neoplasms and mutations. If a DNA damage occur, p53 is highly expressed and this triggers the expression of p21.[68]

3.3 Papillomavirus oncoproteins

3.3. Papillomavirus oncoproteins

3.3.1 E5

E5 is a small hydrophobic polypeptide involved in viral persistence and replication. At physiological levels it localizes in the endoplasmic reticulum and more rarely in the Golgi apparatus and early endosomes,[93] while when overexpressed, it also localizes in the nuclear envelope.[74]

The most studied PVs expressing E5 are HPV-16 and BPV-1. In HPV-16-cervical lesions, E5 is not the major oncogene but is essential in early phase of infection, enhancing E6

and E7 expression and tumor progression.[217] During early infection, E5 interferes with two signalling pathways: immune evasion and growth factors activation. In the first mechanism E5 binds to B cell receptor-associated protein 31 (BAP31) in the endoplasmic reticulum (ER) preventing the transport of the major histocompatibility complex (MHC) class I proteins to the cell surface.[15] From a functional view, MHC class I downregulation reduce cytotoxic T lymphocytes activity and leads to immune surveillance evasion.[55] The second mechanism is responsible for cellular proliferation and tumor progression. In HPV-16 infected cells, E5 binds to vacuolar ATPase on the endosome, interfering with endosomal acidification. Impaired acidification, induce epidermal growth factor receptor (EGFR) and endosome recycling, and a subsequent increase of the EGFR/EGF signal via the MAPK pathway.[147,227] However, as cervical cancer progresses toward malignancy, viral DNA integrates to the host genome while E2 and E5 extinguish.[137]

In contrast, BPV-1 and BPV-2 E5 is the most studied and powerful oncogene, interacting with many proteins, and signalling pathways. Of these, platelet derived growth factor receptor β (PDGRFβ) is one of the major target, interacting with E5 with a highly specific bound.[126] Hyperphosphorylation and subsequent activation of PDGRFβ trigger PI-3k-Akt pathway and recruitment of specific transforming factors.[36] In addition, E5 can also activate p38 MAPK to induce fibroblasts proliferation and tumor progression.[351]

Besides the association between HPV and genital cancers, recent works have emphasized the role of EcPV-2 in the development of equine genital squamous cell carcinomas (SCCs).[311] Nevertheless, the lack of E5 ORF within EcPV-2 genome, make the virus an unsuitable model for HPV-induced cancers.[278]

3.3.2 E6

E6 is a zinc-binding protein not expressed by all PVs, such as BPV-3, -4 and -6.[149] Along with E7, E6 is the best studied oncoprotein of high-risk HPV. It is responsible for abnormal cellular proliferation by inactivating the p53 pathway. Specifically, E6

inactivates p53 via different mechanisms: in the first, E6 binds to E3 ubiquitin ligase E6-associated protein (E6AP), a 100 kD enzyme. The E6-E6AP complex induce ubiquitination and degradation of p53 by the proteasome pathway.[281] The second mechanism involves p-300, CREB-binding protein (CBP) and ADA3, three histone acetyltransferases that inhibit p53 acetylation and p53-dependent gene activation.[178,322] P53 viral-mediated inactivation allow viral productive replication and contribute to cell cycle progression. Another function of E6 is the ability to activate the catalytic subunit of the telomerase reverse transcriptase (hTERT) via E6AP, promoting cellular immortalization and tumor progression.[124] Additional oncogenic activity of E6 is the ability to negatively regulate the intrinsic and extrinsic apoptotic pathways. The extrinsic pathway is blocked by E6 interaction with death receptors on the cell surface (e.g., tumor necrosis factor 1 (TNFR-1), Fas/CD95 and others). The net result is degradation of adapter molecules and caspase-8.[144] Inactivation of the intrinsic pathway occur through E6 binding to pro-apoptotic members of the BCL2 family Bax and Bak. The overall effect is the blocking of cytochrome c release in the cytosol and disruption of effector caspase cleavage.[327]

Conversely to high-risk HPV, BPV-1 E6 influence tumor progression in a p53-independent manner. Unlike high-risk HPV, BPV-1 E6 does not elicit p53 degradation by binding with E6AP, but it seems to induce its downregulation by interacting with CBP/p300.[359] The CBP/p300 family comprises the two transcriptional co-activator p300 and CBP which are involved in cellular growth and in p53 acetylation.[127] Recent studies demonstrate that interactions between BE6 and CBP/p300 interfere with p53 function and inhibit p53 transcription.[359] It is important to note that both high-risk HPV and BPV-1 can bind to paxillin, a focal adhesion molecule involved in focal adhesion and migration. Although the exact mechanism is unknown, paxillin binding to E6 prevents anoikis and allow cytoskeletal re-modelling, with a consequent anchorage-independent cellular growth.[339,340]

Regarding EcPV-2, little is known about host cell interactions and its oncogenic mechanisms. However, sequencing EcPV-2 genome, has revealed the presence of both

E6 and E7 genes, with E7 lacking the RB binding site.[278] Whereby E6 appear to be the major oncogene of EcPV-2, downregulating p53 in the same way of high-risk HPV.[266]

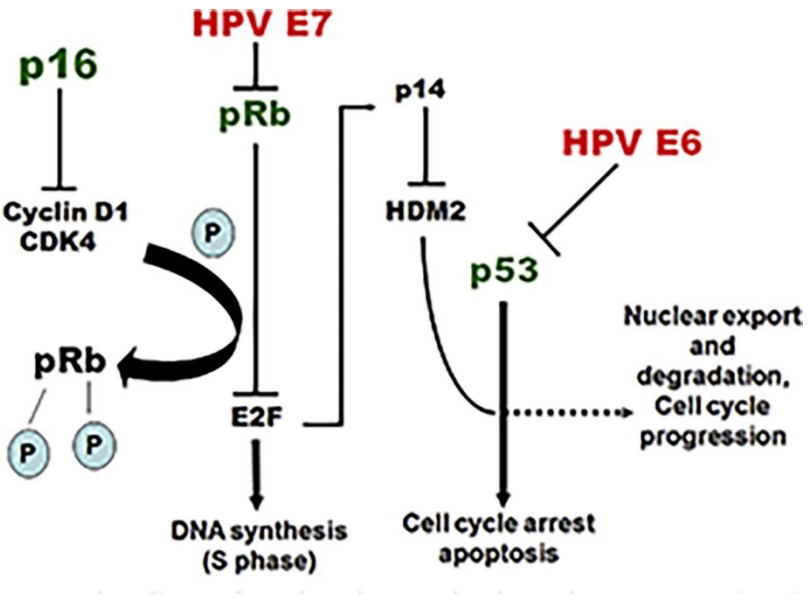
3.3.3 E7

E7 is a 100 amino acids polypeptide responsible for PVs proliferation.[219] In high-risk HPV, E7 contains a LXCXE motif that interact with the pRB "pocket" domain and induce E2F release. As the result, E2F become transcriptionally active and the cell progress through the S phase. Moreover, E7-pRB binding induce pRB degradation via the proteasome pathway therefore increasing p16 levels (Figure 2).[248] Also, E7 contribute to carcinogenesis by directly binding cyclin-dependent kinase inhibitors (CDKI) p21 and p27 further inducing cellular proliferation.[120,355] The combined action of CDKI inhibition and pRB inactivation also result in an increased levels of Cyclin E and Cyclin A which favour cell cycle progression and cellular division.[219]

By contrast, BPV-1 E7 lacks the LXCXE motif that mediates pRB ubiquitination, whereby its oncogenic activity is thought to rely on its ability to bind p600.[75] The E7-p600 interaction is essential for anoikis inhibition and therefore for anchorage-independent cell survival.[88] *In vivo* studies on equine sarcoids demonstrate the simultaneous co-localization of p600 and E7 in the cytoplasm of neoplastic fibroblast, reinforcing the role of E7 in natural occurring sarcoids.[75] In addition, cell invasion is further promoted by upregulation of matrix metalloproteinases (MMPs) by BPV-1 E7. Notably, cleavage of basal membrane by MMP-2 and MMP-9 modify the interstitial connective tissue and enhance tumor invasion.[209,352]

Although the entire EcPV-2 genome was sequenced in 2010 by *Scase et al*, the viral oncogenic mechanism is still unknown. However, the same work has showed that the E7 protein lacks the pRB-binding site, suggesting E6 as the major oncogene of EcPV-2.[278]

Figure 2. HPV E6 and E7 disrupting the p53 and pRB molecular pathways (Tanaka and Alavi, 2018).



Chapter 4 Papillomaviruses and Squamous cell carcinomas

Squamous cell carcinoma is one of the most common malignant neoplasm in veterinary medicine, affecting dogs, cats, cows and horses.[77,206,344] In horses, the periocular region and external genitalia are the most frequently affected sites, followed by ear pinnae, perianal region and extremities.[321] To date, the association between EcPV-2 and equine genital SCCs is well established, but the exact mechanisms of carcinogenesis are not fully understood.[130,182] Recent works on equine EcPV-2 induced SCCs showed many histological and molecular similarities with human HPV-induced SCCs: in both cases viral genome is reported to reside within infected cells as viral, episomal or integrated.[311] Moreover, genital tumor similarities between high-risk HPV and EcPV-2 suggest that the two viruses can have similar carcinogenic mechanisms and features. For this reason, high-risk HPV may represent a good model for the equine counterpart. In human medicine, oncogenic PVs are defined as "high-risk" and are implicated in a variety of cancers, including anogenital, head and neck and cervical SCCs.[49,90]

4.1 HPV and genital SCCs in humans

In human medicine, about 15 genotypes of HPV are considered oncogenic and are associated with different types of cancer.[251] Among these, HPV-16 is the most commonly observed in cervical and penile cancers (54.4%), followed by HPV-18 (16.5%), HPV-58 (5.1%), HPV-33 (4.7%) and HPV-45 (4.4%).[80,235] As the development of cervical neoplasia is strictly linked with HPV, it is important to consider the factors involved in the development of HPV-induced cervical SCCs.

Studies have shown that cigarette smoking is one of the major risks for progression from precancerous lesion towards malignancy.[12] In fact an epidemiological study on HPV status and smoking levels showed that the risk for developing cervical cancer is twofold increased with the number of cigarettes and the age at the start of smoking.[12] Moreover, tobacco carcinogens can either damage DNA and cause genomic instability,

and impair innate immunity response by reducing the number of T lymphocytes and Langhans cells in the cervix.[53,139,259]

Besides smoking, the long-term use of hormonal contraceptive increase the risk of developing cervical cancer in HPV positive women.[229,297] Repeated administration of oestradiol seems to enhance the expression of E6 and E7 ORF and promote viral proliferation.[121]

Furthermore, high parity and childbirth are associated with the development of HPV-induced SCCs.[150,237] During pregnancy, intense exposure to oestrogen and progesterone promote the maintenance of the transition zone within the cervix, facilitating HPV infection.[236] Similarly, concurrent epithelial damage and immunosuppression during pregnancy could favour HPV infection and subsequent development of SCC.[150]

Apart from lifestyle factors, also individual factors as immunosuppression and other infections have been demonstrated to increase the risk of developing cervical SCCs. Immune suppressed women due to infection with human immunodeficiency virus type 1 (HIV) or organ transplantation, are more prone to develop precursor lesions and then SCCs.[102] This, in HIV-positive women with lower CD4 count, is attributable to an impaired cell-mediated immunity.[271] At least, even opportunistic infections with common microorganisms as *Candida albicans* or *Herpes simplex virus* can alter cervical microflora. Disruption of the cervical microenvironment enhance local pro-inflammatory response which can damage cervical epithelium and promote HPV entry.[35,292]

Human genital squamous cell carcinomas are classified based on tumor location.

In males, penile SCCs are grouped in two categories according to the 2016 WHO classification: HPV-related SCCs and non-HPV related SCCs.[225] Within HPV-related SCCs, tumors are further classified based on histological appearance in: basaloid, warty (condilomatous),[82,83] warty-basaloid,[67] papillary-basaloid[84] and clear cell[275]. Besides the most common histological types, also rare variants as lymphoepithelioma-like[221] and medullary SCCs have been reported.[57] Moreover, for penile SCCs it is also

recommended to apply a three-tiered grading system, based on histological features of malignancy: well differentiated SCCs (grade 1) are characterized by large nests of neoplastic cells whereas poorly differentiated SCCs (grade 3) are composed of trabeculae or solid areas of pleomorphic cells. Moderately differentiated SCCs (grade 2) show intermediate histological features of grade 1 and 3.[225] However, if the histological diagnosis cannot be made certainly with routine hematoxylin and eosin (H&E) staining, it is further recommended to perform IHC for p16 which can be used as a surrogate marker for HPV-related neoplasm.[66,85]

In female HPV-induced cervical SCCs, tumors are classified using a two-tiered grading system which divides the lesions into high-grade squamous intraepithelial lesions (HSIL) and low-grade squamous intraepithelial lesions (LSIL).[87] LSIL are commonly self-limited and show cytopathic or metaplastic changes of the lower one third of the cervical epithelium. On the other hand, HSIL are characterized by full thickness cellular proliferation with loss of nuclear polarity and cellular atypia. Additionally, for equivocal lesions, the WHO guidelines recommend the use of p16 as immunohistochemical marker for better classifying the disease.[141]

4.2 EcPV associated diseases in horses

To date, nine types of EcPVs have been identified and the infection seems to be limited to horses.[184,185,192,194,254] Table 1 summarizes EcPV types and associated lesions. Currently, there are three different syndromes caused by EcPV: classical viral papillomatosis, genital papillomas and aural plaques.

Classical viral papillomatosis is a highly contagious disease caused by EcPV-1. Young horses (≤ 3 years old) are mainly affected and fortunately spontaneous regression occurs within 4 months.[169] The most commonly affected sites are the lips and muzzle although viral papillomatosis is also reported in the eyelids, limbs an genital region. The infection requires a breach in the skin and the transmission occurs through direct contact or via contaminated fomites.[199] The lesion begins as small papules (≃ 1mm in

diameter) which than develop into more large broad-based pedunculated papillomas.[288]

Genital papillomas are caused by EcPV-2. The most commonly affected sites are the glands, vulval lips, vestibulum and clitoris.[278,298] Unlike classical papillomatosis, the disease typically occurs in older horses and may evolve to SCC.[325]

Aural plaques are associated with EcPV-3, -4, -5 and -6 and occur as solitary or multiple lesions in the ear pinnae.[185,314] When fully developed, they are typically bilateral and symmetrical, raised, white, with thick hyperkeratotic surface. No breed or sex predilection has been reported and horses of any age can be affected.[326] Although histologically similar to papillomas, aural plaques have less finger-like projections and contain less melanin.[288]

Table 1. EcPV associated diseases in horses.

Disease	**Genera**	**EcPV-type**
Classical papillomatosis	Zeta	EcPV-1
Aural plaques	Dyoiota	EcPV-4, -5
	Dyorho	EcPV-3, -6
	Zeta	EcPV-1
Genital SCCs and precursors	Dyoiota	EcPV-2, -4, -9
	Dyorho	EcPV-6, -7
	Treiskappa	EcPV-8

4.2.1 Genital SCCs in horses

The genital region is considered one of the most important sites of occurrence of equine SCCs. In males, SCCs often develop in the free parts of the penis[204,325] while in females they develop in the vulva.[260,263,298] Geldings are reported to be more prone to developing SCCs than stallions.[324] Although accumulation of smegma or low hormone levels in castrated horses have been proposed as potential risk factors, their role in the pathogenesis of SCCs is still unconfirmed.[106,143] Older horses are more frequently affected, and an increased prevalence is reported in lightly pigmented breeds as Appaloosa, American Paint Horse, Clydesdale and Pinto.[169]

SCCs start as small, solitary lesions and in the majority of the cases go unnoticed or are mistaken for infected granulation tissue.[204,288] Histologically, SCCs are composed of trabeculae or cords of neoplastic epithelial cells that breach the basal membrane and infiltrate adjacent tissues. There is often keratin pearls formation and prominent intercellular bridges. Intratumoral lymphocytes, plasma cells or macrophages are common.[288]

The etiology of equine SCCs in not fully understood, but the presence of EcPV-2 seems to be associated with the development of the neoplasm.[33,164,183] Nevertheless, a recent study demonstrates the presence of viral DNA also in the skin of healthy horses.[131]

From a molecular point of view, little is known about the oncogenic mechanism of EcPV-2 and the development of equine SCCs. In human penile SCCs, the presence of high-risk HPV is considered a strong indicator of a favourable outcome.[196] In addition, p53 and Ki-67 expression are correlated to lymph node metastasis and thus, they can be considered as prognostic markers.[233,261] By contrast, in equine penile SCCs, neither p53 nor Ki-67 seem to be correlated with the degree of differentiation of the neoplasm or with the expression of viral genes hence they cannot be used as prognostic markers. However, statically significant correlation was found between p53 expression and poorly differentiated or invasive lesions (in situ carcinomas/SCCs) compared to the benign counterpart (hyperplasia/papillomas).[323] Table 2 summarizes EcPV-2 detection in SCCs-related lesions and normal tissues (2010-2020).

Table 2. EcPV-2 detection in normal tissues and SCCs-related lesions from previous studies.

Study by	Molecular technique	Matrix type	Localization	EcPV-2
Scase *et al* (2010)	PCR	SCCs, papillomas, CIS	Penis and vulva	26/35 (74.3%)
		Normal skin	ND	0/1 (0%)
		Sarcoid	ND	0/1 (0%)
Kinght *et al* (2011)	PCR	SCCs	Genital	9/20 (45%)
		Other lesions	Penis	1/20 (5%)
Bogaert *et al* (2012)	PCR	SCC	Penis, vulva, anus, oral cavity	32/34 (94.1%)
		Normal swab	Penile	4/39 (10.2%)
			Vaginal	0/20 (0%)
Vanderstraeten *et al* (2011)		SCCs, papillomas, PIN	Genital	36/36 (100%)
			Ocular	9/9 (100%)
		Normal mucosa	Genital	49/90 (54.4%)
		Normal mucosa	Ocular	32/56 (57%)
Sykora *et al* (2012)	PCR	SCCs, papillomas, CIS	Penis and vulva	4/4 (100%)
		Normal swabs	Ocular	1/30 (3.3%)
			Genital	4/94 (4.2%)
		Normal semen	Semen	0/54 (0%)
		Normal milk	Milk	0/15 (0%)
Lange *et al* (2013)	PCR	SCCs, papillomas, CIS	Penis	24/24 (100%)
		Other lesions	Penis	6/11 (54.5%)
Kinght *et al* (2013)	qPCR	SCCs, papillomas, CIS	Penis	16/20 (80%)
			Oropharynx	3/20 (15%)
		Other lesions	Penis	3/19 (15.8%)
		Normal mucosa	Penis	2/32 (6.2%)
			Vulva	1/40 (2.5%)
			Oral cavity	2/75 (2.7%)
			Ocular	0/75 (0%)
Newkirk *et al* (2014)	PCR	SCCs, papillomas, CIS	Penis	10/22 (45.4%)
			Ocular	0/42 (0%)
Fisher *et al* (2014)	PCR	Normal swab	Genital	9/50 (18%)
Van den Top *et al* (2015)	PCR	SCCs, papillomas, CIS	Penis/preputium	91/103 (88.3%)
Zhu *et al* (2015)	PCR, ISH	SCCs	Penis/preputium	6/13 (61.5%)
Ramsauer *et al* (2019)		SCCs, papillomas, CIS and benign hyperplasia	Penis	19/19 (100%)
		Normal skin	Penis	0/5 (0%)
Greenwood *et al* (2020 a, b)	PCR, ISH	SCCs	Penis, vulva	10/35 (28.6%)
			Eyelid	0/18 (0%)
		Normal skin	Genital	16/68 (23.5%)
			Eyelid	8/68 (11.8%)
			Retropharyngeal lymph node	2/64 (3.1%)
			muzzle	7/68 (10.3%)
Alloway *et al* (2020)	PCR, ISH	SCCs	Stomach	7/11 (63.6%)
		Normal mucosa	Stomach	0/4 (0%)

4.2.2. Equine ocular SCCs

In horses SCCs are the most common neoplasms of the eyes and ocular adnexa, accounting 72% of all mucocutaneous SCCs.[99,315] Preferential locations are the eyelid and canthus, followed by third eyelid, cornea, conjunctiva and the orbit.[155,186,203]

Interestingly, the growth of corneal SCCs seems to be limited to the corneal epithelium and/or the stroma, with no reports about Descemet's membrane invasion.[155,156] Ocular SCCs are slowly metastatic and the recurrence depends on the treatment of the neoplasm.[161,231] As for genital SCCs, the pathogenesis is unclear, but a strong prevalence is reported in grey-pigmented breeds.[187] UV light exposure seems to play a major role in the development of SCCs in this location.

4.2.3 Equine Pharyngeal and oral SCCs

Tumors of the oral cavity and pharynx rarely develop in horses, however, SCCs account about 5% of all the neoplasms in this locations.[98,285] Although there is no breed or sex predilection, the prevalence is higher in older horses.[153,285] They are reported to develop on the tongue, gingiva, pharynx and larynx. Clinical signs reflect the anatomic location and include dysphagia, ptyalism, dyspnea, cough and nasal discharge.[153,230,304]

Oral and pharyngeal SCCs are locally invasive and can metastasize to regional lymph nodes and lungs.[285]

Pre-neoplastic lesions are still not described because of the difficulty to examine these sites. For this reasons, the diagnosis is usually made late in the clinical course and the treatment is not attempted.[153]

4.2.4 Other Locations

Other locations of SCCs include haired skin and stomach. Cutaneous SCCs develops preferentially in the pinnae, perineal area, and face. As for other locations, a widely accepted predisposing factor for the development of SCCs, is the degree of pigmentation.[173] However, there are three reports of SCCs in sites of previous trauma, including an injection site, a laceration and a thermal burn.[18,111,287]

SCCs of the stomach are the most common neoplasm of the equine gastro-intestinal tract and are reported to occur more frequently in horses with more than 12 years old. Clinical signs include anorexia, weight loss, abdominal distension or regurgitation.[78,244,320] Histologically they appear as exophytic, cauliflower-like masses that develop from the squamous epithelium that lines the dorsal part of the stomach.[77] Metastasis to regional lymph nodes or invasion to adjacent tissues as distal esophagus, liver and great omentum is a common feature.[320]

Sinonasal SCCs can develop from the paranasal sinuses or, more frequently, from the caudal maxillary sinus.[38,138] Clinical signs are nasal discharge, dysphagia, facial swelling and dyspnea. In contrast to SCCs of other locations, they rarely metastasize. Nevertheless, sinonasal SCCs are locally invasive and usually diagnosed late in the disease, with no effective treatment options for horses.[138,200]

Recent publications have demonstrated the presence of EcPV-2 in a subset of gastric SCCs.[6]

4.3 Association between UV light exposure and SCC development

A wide range of risks factors other than PVs infection, have been proposed for the development of SCCs, including UV light exposure, geographical location (altitude and longitude), and the paucity of hair coat and the pigmentation. All these causes are briefly discussed in the following sections.

4.3.1 UV radiation in Horses

UV radiation is the most important environmental factors that contribute to the development of cutaneous neoplasia. In fact, the onset of SCCs in horses increases with the increase of annual solar radiation and high longitude and altitude.[103,288] Whereby genital and ocular regions are considered, predilection sites of occurrence both for the scarcity of hair coat and for being more exposed than other areas.[106] Moreover, the lack of melanin is believed not to protect the skin against UV light. This is demonstrated by the higher incidence of SCCs in Appaloosa and American Paint horses, that are characterized by absent or poor pigmentation in the genitalia.[50,287] Several works have also related the onset of ocular SCCs to the chestnut and gray coat colours, suggesting that the genes that control these phenotypes could explain the highest incidence in some breeds.[224,231,257] The chestnut colour result from a recessive mutation in the *melanocortin 1 receptor* (MC1R) gene, which regulates the switch from eumelanin to pheomelanin.[208] Conversely, the grey coat colour results from a duplication in *syntaxin-17* (STX17) gene, which results in a progressive pigment loss from melanocytes.[270]

Cutaneous SCCs frequently develop after pre-neoplastic lesions as actinic keratosis, which appear as hyperkeratotic and/or crusting plaques of 0.2-2 cm in diameter.[288] Generally, UV-induced neoplasia are characterized by C to T base substitution in TP53 gene.[358] Although there is not a clear relationship between SCCs development and UV exposure, some studies tried to investigate the role of p53 in the pathogenesis of SCCs. Interestingly, three different works on equine ocular SCCs demonstrate the expression of p53 in all the examined cases, proposing UV light as the leading cause.[5,296,318] These data are further reinforced by the work of Pazzi *et al*, in which point mutation of the p53 gene in neoplastic epithelial suggests that the amino acid substitution could be due to UV light exposure.[252]

By contrast, genital SCCs are characterized by moderate to high expression of p53, but the lack of knowledge of p53 status in this location, makes difficult to interpret the importance of p53 in UV-induced SCCs.[266,323]

4.3.2 UV radiation in Humans

Sunlight is the major carcinogen for human cutaneous SCCs. Epidemiological studies reveal an highest incidence of cutaneous SCCs in lightly-pigmented people living in regions with high UV exposure.[267] Human behaviour as the use of UV lamps for curing nails or tanning devices are associated with the risk of developing SCCs.[117,157] In addition to environmental and behavioural factors, SCCs is reported to develops also in UV-treated psoriasis patients.[158] Despite all the causes of cutaneous SCCs, all UV-induced neoplasms are able to induce DNA mutations. These are evident in p53 gene, whose product, the p53 protein, is dysfunctional and very stable at the same time and its accumulation can be detected by IHC in tissues.[9,42] TP53 mutations are called "hotspots" and are characterized by C to T substitution or CC vs TT double base changes in specific codons.[358]

4.4 Other risk factors for the development of SCCs in horses

In addition to the previously mentioned causes of SCCs, other factors have been proposed as possible source of neoplastic transformation in horses. In geldings smegma accumulation in the prepuce induce chronic inflammation possible leading to SCCs. Although smegma is mainly composed of exfoliated cells, it also contains lytic enzymes as lysozyme, cathepsin B and other compounds that were considered chronic irritants.[50] Nevertheless, this hypothesis has never been proven and the association between SCCs and smegma accumulation is still considered doubdful.[142,143]

Sites of previous injuries or irritations are also considered in the pathogenesis. There is also some evidence of ocular SCCs developed from sites of previous trauma or from delayed wound repair.[123] Even if the development of cutaneous SCCs from previously injured areas is reported in three works, their association is still unconfirmed.[18,111,287]

Experimental part

Chapter 5 Expression of cell cycle proteins in equine squamous cell carcinomas and carcinomas in situ

Squamous cell carcinoma is the second most common neoplasm of horses, occurring mostly in the periocular region and external genitalia. To date, the pathogenesis of this neoplasm is not fully understood, but the presence of EcPV-2 seems to be associated with the development of genital lesions.[130,183] An additional risk factor for SCC development is UV light exposure. In fact, an increased prevalence of SCCs was associated with an increase of altitude and longitude.[103]

In humans, the association between HPV and genital malignancy is well established, with cervical and penile cancers being related to HPV-16 and HPV-16, -18 respectively.[48,148] HPV integration into the host cell leads to E6 and E7 activation and interaction with many cell cycle proteins.[248] When HPV E6 integrates into the host genome, it induces p53 degradation. Its activity is further accomplished by HPV E7, which binds to pRB and induce its ubiquitination and E2F transcription factors release. The net result is CDKN2A overexpression. All these interactions with cell cycle proteins are responsible for DNA damage, cell cycle progression and tumorgenesis.[136,248]

CDKN2A is an important tumor suppressor and its overexpression in cervical high risk HPV is used to predict HPV status.[205] Another important cell cycle regulator, p53, is reported to be mutated in a variety of human cancer, being an indicator of tumor aggressiveness and poor overall survival.[220,306,307] Conversely to other neoplasms, in cervical intraepithelial lesions p53 overexpression is negatively correlated with histological grade and HPV positivity.[176,331]

In human medicine, the study of cell cycle proteins combined with the presence of HPV is considered a specific predictor for HPV-status and malignant transformation of the female genital neoplasia.[243]

To date, in veterinary literature, the study of cell cycle proteins and their interactions in equine SCCs are limited to p53. Although p53 cannot be considered as a prognostic marker, it can be useful in the distinction between benign and malignant lesions.[266,323] Moreover, EcPV-2 infection is known to be associated with equine SCCs, but in contrast to humans, EcPV-2 status is not considered a prognostic factor.[130]

Therefore, the aim of this study is to assess if EcPV-2 has any effect on cell cycle proteins pRB, Cyclin D1, p53 and CDKN2A in cutaneous and mucocutaneous SCCs, papillomas and carcinoma in situ (CIS).

5.1 Materials and Methods

5.1.1 Case selection and histology

Cases were retrieved from the Department of Anatomy, Physiology and Pathology archives at the University of Liverpool (UK). Cases were searched in the Department database using "squamous cell carcinoma" as keyword. A total of seventy-nine cases were identified. Clinical and signalment data were retrieved simultaneously, including signalment data and tumor location. Cases were then reviewed by histological examination of hematoxylin and eosin-stained slides and reclassified as papillomas, carcinoma in situ (CIS) and SCCs using previously described criteria.[266]. Briefly, papillomas were characterized by finger-like projections of hyperplastic epithelium supported by a fibrovascular core. Tumors were classified as CIS when neoplastic keratinocytes were confined to the epidermis without invasion into the dermis whereas a diagnosis of SCCs was made when trabeculae, islands or cords of neoplastic cells invade the dermis. For the purpose of the study, carcinomas (SCCs and CIS) and papillomas were analyzed separately.

SCCs were further divided in well differentiated or poorly differentiated using a modified grading system by Bryne *et al* (Table 3).[46] Tumor grading was calculated by adding each parameter and providing a total score from a minimum of 5 to a maximum of 20. Tumors with total scores ≤10 were classified as well differentiated whereas

tumors with total scores >10 were classified as poorly differentiated. For each tumor, the average number of mitotic cells per high power field (HPF) was assessed in 2.37 mm^2, avoiding necrotic areas.

Table 3. Modified grading system for equine squamous cell carcinomas (Bryne *et al*, 1992).

	Score			
Morphological feature	1	2	3	4
Degree of keratinization	Highly keratinized (>50% of cells)	Moderately keratinized (20-50% of cells)	Minimal keratinization (5-20% of cells)	No keratinization (0-5%)
Pattern of invasion	Pushing, well delineated infiltrating borders	Infiltrating, solid cords, bands and/or strands	Small groups or cords of infiltrating cells	Marked and widespread cellular dissociation in small groups and/or in single cells
Desmoplasia	None	Slight	Moderate	Marked
Nuclear pleomorphism	Little nuclear polymorphism (>75% mature cells)	Moderately abundant nuclear polymorphism (50-75% mature cells)	Abundant nuclear polymorphism (25-50% mature cells)	Extreme nuclear polymorphism (0-25% mature cells)
Mitotic index (mitosis in 2,37 mm^2)	0-1	2-3	4-5	>5

5.1.2 Tissue microarray construction

All cases were selected for tissue microarray technique (TMA). Firstly, representative areas containing neoplastic tissue without inflammation, hemorrhage, or non-neoplastic zones were selected from the H&E stained slides. Selected areas were marked on the slide and compared with the donor block to identify the sampling area, then were punched with a 6 mm diameter biopsy punch. TMAs were then constructed allocating the obtained tissue cores into new empty receiving paraffin blocks. One tissue core per tumor was used for the construction. A sector map was then created to recognize the position of each tumor core. Each TMA contained seven tumor cores, one equine lung core as a landmark. From each TMA, a H&E stained section was evaluated to confirm the presence of the selected tissue.

After validation of the TMA technique, TMA were used for EcPV-2 CISH.

CISH TMA validation will be evaluated by PCR in a secondary phase of the study with an estimation of the agreement level.

5.1.3 Western blot Analysis

To verify Cyclin D1 and pRB antibody specificity with equine tissues, Western Blot analysis was performed. The assay was carried out in collaboration with the Department of Experimental and Special Medicine (DIMES), at the University of Bologna. For protein extraction, fresh tissue from equine dermis and intestine was collected. From each, 25µg of tissue was homogenized with 500ml RIPA buffer (Thermofisher Scientific) and 100 µl of protease inhibitor (Halt Protease Inhibitor Cocktail EDTA-free, Thermofisher Scientific). Tubes were centrifuged at 12000 rpm for 30 minutes at 4°C and the supernatant was collected and stored at -80°C. Protein concentration was calculated using the DC Protein Assay Kit (Bio-Rad). Subsequently, 40 µg of proteins were denatured at 100°C for 10 minutes. Proteins were separated using a 8% gel and electrophoretically transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with milk 5% in TBS-T for 1h at RT. The blot was incubated at 4°C overnight with an antibody against pRB and Cyclin D1 respectively

(1:500, Abcam). Then, the membrane was incubated with anti-mouse peroxidase conjugated secondary antibody (1:200, Thermofisher) for both antibodies for 1 hour at room temperature. Reactive bands were visualized with a chemiluminescent detection kit (Westar nC 2.0 Cyanagen, XLS075,0020) using the Chemidoc instrument (Bio-Rad). B-Actin was used as the loading control and was detected using a β-actin-specific antibody (1:500 Santa Cruz, Biotechology).

5.1.4 Immunohistochemistry

Four-consecutive sections of each tumors were used for IHC, using the following antibodies: p53, CDKN2A, pRB and Cyclin D1. Data on the primary antibodies are summarised in Table 4.

Cross reactivity for Cyclin D1 and pRB with equine tissues was confirmed with western blot analysis, while for CDKN2A sequences were compared using an online sequence alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Equine CDKN2A sequences were compared to human sequences, showing 100% homology.

Three-micrometer-thick sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion in H_2O_2 3% in methanol for 30 minutes. For antigen retrieval, sections were immersed in 200 mL citrate buffer (PH 6.0) and heated in a microwave oven at 750W for two-10-minutes cycles (p53 and pRB) or for three-5-minutes cycles (CDKN2A and Cyclin D1). Slides were incubated with the primary antibody overnight at 4°C. The reaction was revealed using a commercial streptavidine-biotin-peroxidase technique (ABC kit elite, Vector, Burlingame, CA, USA) and visualized with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark). Slides were counterstained with Mayer's hematoxylin. Positive controls consisted of normal equine testis for pRB and Cyclin D1 whereas a SCC with known p53 expression served as a positive control for p53. Specific positive control for CDKN2A consisted of equine colon. As a negative control, the primary antibody was replaced with an isotype-matched non relevant antibody.

Immunoreactivity for p53 was considered significant when nuclear immunoreactivity was seen in at least 10% of neoplastic cells. If the percentage positive cells fell below 10% or was recorded as cytoplasmic then it was considered negative. Significant immunoreactivity was graded as basal (b) or non-basal (nb) as previously described.[266] CDKN2A immunostaining was evaluated as nuclear or cytoplasmic as previously described for human HPV-induced cervical lesions.[190] In addition, scattered positive keratinized cells were identified within the tumors and the positivity was further categorized as nuclear, cytoplasmic, or keratinized.

Immunolabelling for pRB and Cyclin D1 was scored along a 6-tiered scale ranging from 0 to 5: 0 (negative); 1 (1-25% positive cells); 2 (26-50% positive cells); 3 (51-75% positive cells); 4 (76-90% positive cells); 5 (91-100% positive cells). Cyclin D1 and pRB scores were added to obtain a combined score. A combined score ≤4 was considered low and a score >4 high.[101]

Table 4. Primary antibodies, resources, and dilutions.

Antibody	*Clone*	*Manufacturer*	*Dilution*
p53	PAb240	BD Pharmingen (Rodano (MI), Italy)	1:100
CDKN2A	Polyclonal	Elabscience Biotechnology (Texas, USA)	1:150
pRB	Sc-1	Santa Cruz Byotechnology (Segrate (MI), Italy)	1:100
Cyclin D1	A12	Santa Cruz Byotechnology (Segrate (MI), Italy)	1:150

5.1.5 EcPV-2 chromogenic in situ hybridization

Chromogenic *in situ* hybridization was performed using the RNA scope kit (Advanced Cell Diagnostics, Hayward, CA). The target genes and probe regions were designed to hybridize to messenger RNA (mRNA) and a 972-nucleotide portion of the E6/E7 region of EcPV2 (EU503122.1) as previously described.[357] Three-µm thick sections were deparaffinized in xylene, followed by dehydration in ethanol. Following endogenous peroxidase blocking, sections were incubated in 200ml 1X Target Retrieval solution (Advanced Cell Diagnostic, Hayward, CA) maintained at 100°C to 103°C using a water bath for 15 minutes, rinsed in deionized water and immediately treated with 4 drops of protease plus (Advanced Cell Diagnostic, Hayward, CA) at 40°C for 30 minutes in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA). Target probe was added to the slides and hybridized at 40°C for 2 hours in the HybEZ oven. Amplification steps were performed at first in HybEZ hybridization oven at 40°C for 30 minutes (from AMP1 to AMP4) than at ambient temperature for 30 and 15 minutes for AMP5 and AMP6, respectively. Chromogenic detection was obtained by adding Red-working solution (60 µl) for 10 minutes at room temperature. Slides were counterstained with Gill's hematoxylin and mounted with VectaMount (Vector Laboratories, Burlingame, CA). Ec-PPIB probe (*Equus caballus* peptidylprolyl isomerase B (cyclophilin B) (PPIB) mRNA) was used as positive control to assess gene housekeeping while *Bacillus subtilis* strain SMY methylglyoxal synthase dihydrodipicolinate reductase (dapB) gene was used as negative control.

5.2 Statistical Analysis

Normal distribution of continuous variables was assessed using the Shapiro-Wilk test of normality. Mean±standard deviation were indicated for normal continuous variables, whereas the median and range were calculated for non-normally distributed continuous variables. Distribution of categorical variables was assessed with the Chi square test. To assess the distribution of continuous variables, Kruskal-Wallis nonparametric test was used. Spearman coefficient was used to estimate the

correlations between continuous variables. For each test, differences were considered significant when $p \leq 0.05$. All statistical analysis was performed using IBM SPSS Statistics 26 (IBM Corporation, Chicago, IL, USA).

5.3 Results

5.3.1 Signalament and clinical data

Seventy-one (71) cutaneous, ocular, and genital SCCs and CIS from 69 horses were included in the study. Ages of horses were available for 52/69 equids and ranged from 5 to 33 years old with a mean of 17.44 years and a median of 18 years. Gender information was available for 58/69 and included 22 mares (37.9%) and 36 males (62.1%) of which 26 were geldings and 10 stallions. Information regarding breed was available for 52/69 submissions. Among horses, Cob (21.9%), Welsh (11%) and Thoroughbred (9.7%) were the most representative breeds. Table 5 summarises the clinical data and the histological diagnosis.

Table 5. Clinical data and histological diagnosis of squamous cell carcinomas.

Case ID	Breed	Age (years)	Sex	Tumor location	Histological diagnosis
15L-4937				Conjunctiva	Scc
15L-5601	Cob	15	MN	Eyelid	Scc
15L-5618 A			F	Third eyelid	CIS
16L-0457			MN	Penis	Scc
16L-0937 A, B		20	F	Skin	Scc
16L-1378 A	Welsh		MN	Penis	CIS
16L-1790	Cob	21	F	Vulva	Scc
16L-1929	Welsh	19	MN	Penis	Scc
16L-2103			F	Third eyelid	Scc
16L-2132	Haflinger	15	F	Third eyelid	Scc
16L-2921	Cob	22	F	Skin	Scc
16L-2974 B	Cob x	14	MN	Penis	Scc
16L-3066	Anglo Arabian	19	M	Conjunctiva	Scc
16L-3078 B			F	Vulva	Scc
16L-3484 A				Third eyelid	Scc
16L-3485 B				Third eyelid	Scc
16L-4612	Selle Francaise	5	F	Conjunctiva	Scc
16L-5059	Westphalian	10	MN	Third eyelid	Scc
16L-5245 A	Connemara x	18	MN	Penis	CIS
16L-5600 A	Cob	13	MN	Third eyelid	Scc
16L-5600 B	Cob			Cornea	Scc
16L-5915 B, C	Cob	19	F	Vulva	Scc
16L-6363			MN	Penis	Scc
17L-0634	Pony	31	MN	Conjunctiva	Scc
17L-0750	Irish cob		F	Third eyelid	Scc
17L-1957 A	Cob	10	MN	Third eyelid	Scc

17L-2178	Cob	20	M	Conjunctiva	Scc
17L-3182	Polo pony	16	F	Eyelid	Scc
17L-3343 A	Cob	17	MN	Penis	Scc
17L-3572 B		13	F	Third eyelid	CIS
17L-5007 B				Third eyelid	Scc
17L-5323 A	Trotter x	20	M	Penis	Scc
17L-5988	Sella Francaise			Conjunctiva	Scc
180 A	Thoroughbred	26	M	Penis	Scc
260 A	Welsh	8	M	Penis	Scc
640 A	Cob	22	M	Skin	Scc
783 C	Cob	13	F	Skin	Scc
1634			F	Cornea	Scc
1790 A	Shetland	12	F	Skin	Scc
2102 A	Cob x	14	MN	Eye globe	Scc
2151 B		25	M	Preputium	Scc
2525 A, B	Cob	10	MN	Third eyelid	Scc
3269 A	Falabella	22	F	Vulva	Scc
4352 A, B	Pony	19	F	Vulva	Scc
4356 A	Cob cross	14	MN	Eye globe	Scc
5117 A	Cob	17	M	Skin	Scc
5504 A, B	Appaloosa	33	MN	Skin	Scc
6425	Welsh A	11	F	Conjunctiva	Scc
7140 A	Connemara	25	F	Skin	CIS
7172 A	Welsh D	28	MN	Preputium	Scc
7300 A	Cob	9	F	Skin	Scc
8292 B	Cob	24	M	Penis	Scc
9598 A	Arabian x	20	MN	Preputium	Scc
9881 A	Quarter	17	MN	Eyelid	Scc
9934 B	Warmblood	19	MN	Skin	Scc
9950 A	Connemara	19		Third eyelid	Scc
10040 A	Welsh	11	MN	Third eyelid	Scc
10736 A	Standardbred	13		Third eyelid	Scc
11354 A				Eyelid	Scc
11356 A				Conjunctiva	Scc
12038 A			F	Third eyelid	Scc
12485 B	Clydesdale	18	MN	Conjunctiva	Scc
14224 A	Welsh A	20	MN	Penis	Scc
14447 A				Third eyelid	Scc
15156 C, D	Welsh A	22	MN	Penis	Scc
15727 A	Cross	12	F	Third eyelid	Scc
16735 A	Thoroughbred	7	F	Third eyelid	Scc
16948 A				Conjunctiva	Scc
17154 A	Thoroughbred	13	MN	Third eyelid	Scc
17296 A	Thoroughbred	14	M	Third eyelid	Scc
17547 A	Standardbred	15	MN	Penis	Scc

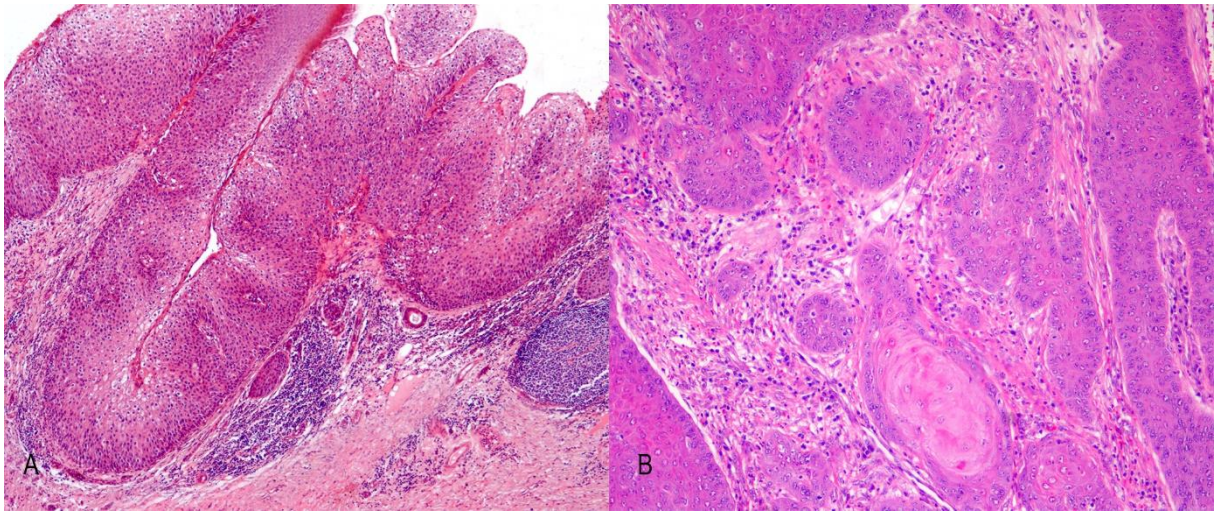
5.3.2 Histological diagnosis

Histological diagnosis and tumour location were evaluated in all the cases. Cases were histologically divided in CIS (5/71, 7%) and SCCs (66/71, 92.3%) according to previously published data (Figure 3).[266] Tumors were further divided based on tumor location in genital (22/71, 31%), ocular (39/71, 55%) and cutaneous (10/71, 14%).Within genital tumors, 17/22 (77.3%) were located in the penis/preputium whereas 5/22 (22.7%) in the vulva. Moreover, within ocular tumors, 14/39 (35.9%) were conjunctival/corneal and 25/39 (64.1%) in the eyelids.

In 45/71 (63.4%) cases, tumors were designated as well differentiated whereas 26/71 (36.6%) cases were graded as poorly differentiated. No statistically significant association between histological grading and anatomic site (p=0.26 *Chi-Square test)* was found.

Mitotic count values were not-normally distributed (Shapiro-Wilk test, p=0.001). The median mitotic count was 33.44 (range 1-124).

Figure 3. A. Eye, carcinoma in situ, 4x. **B**. Skin, squamous cell carcinoma, 20x.



5.3.3 Chromogenic in situ hybridization (CISH)

CISH was performed in all the cases. A positive hybridization signal was observed in 21/71 (2.6%) cases. Positivity appeared as strong nuclear or finely granular punctate dots in the cytoplasm of neoplastic epithelial cells (Figure 4). No signal was observed in peritumoral nor in non-neoplastic adjacent tissues. The presence of EcPV-2 was confirmed in 11/22 (50%) of genital SCCs and in 3/10 (30%) cutaneous SCCs. CISH signal was also detected in 7/39 (18%) ocular SCCs. EcPV-2 was significantly higher in genital tumors compared to ocular and cutaneous tumors (p=0.03 *Chi-Square test*) (Table 6). However, no statistically significant association between histological grading and EcPV-2 positivity (p=0.11 *Chi-Square test*) was found (Figure 5).

Table 6. Association between tumor location and EcPV-2.

	EcPV-2		
	Negative	**Positive**	Total
Genital	11	11	22
Ocular	32	7	39
Cutaneous	7	3	10

Figure 4. A, B. Strong hybridization signal within the nucleus of neoplastic cells. CISH, EcPV-2 probe, 20x and 40x. **C.** Strong hybridization signal in the cytoplasm of neoplastic cells. CISH, EcPV-2 probe 20x. **D.** Strong nuclear and cytoplasmic hybridization signal. CISH, EcPV-2 probe 20x.

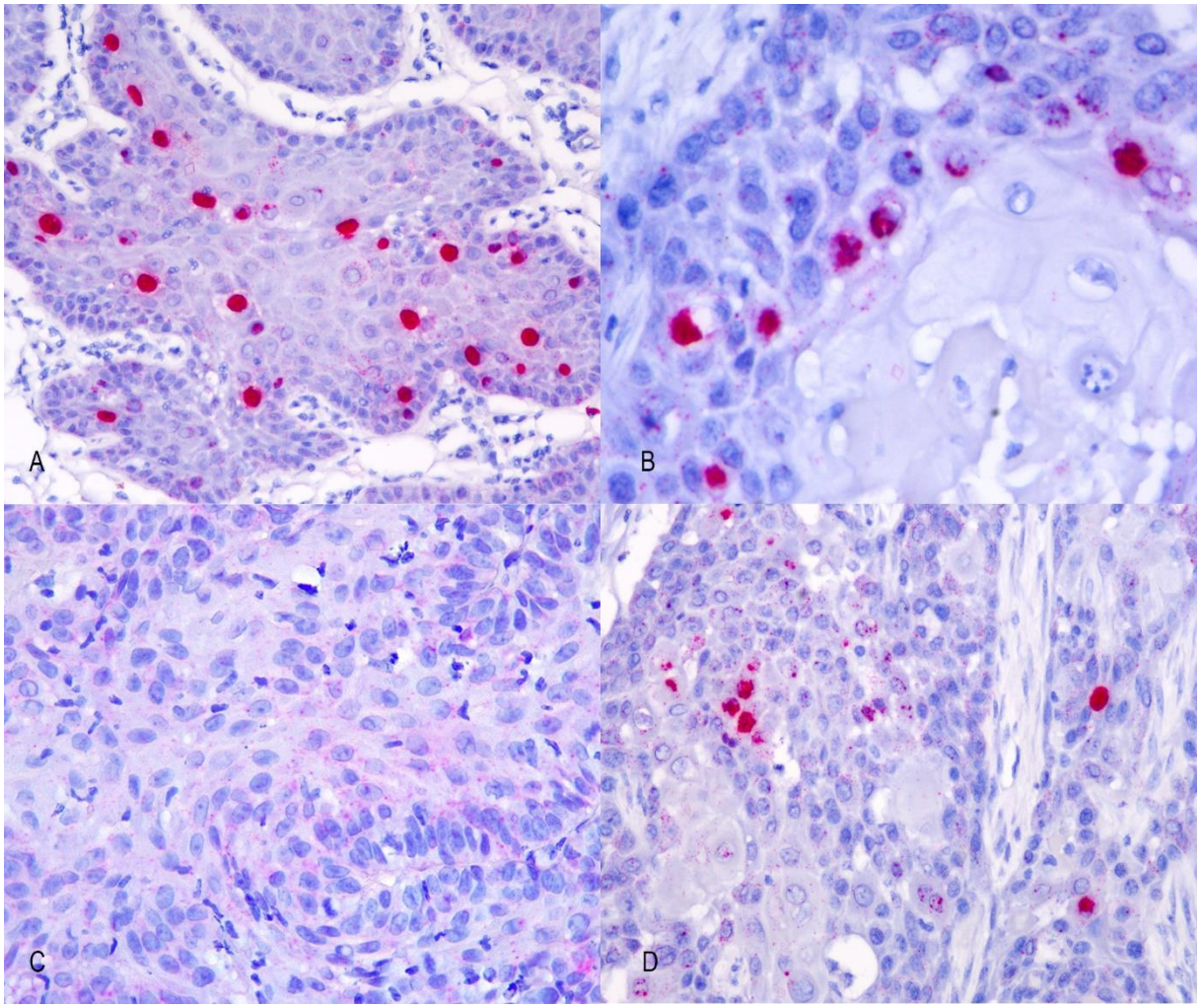
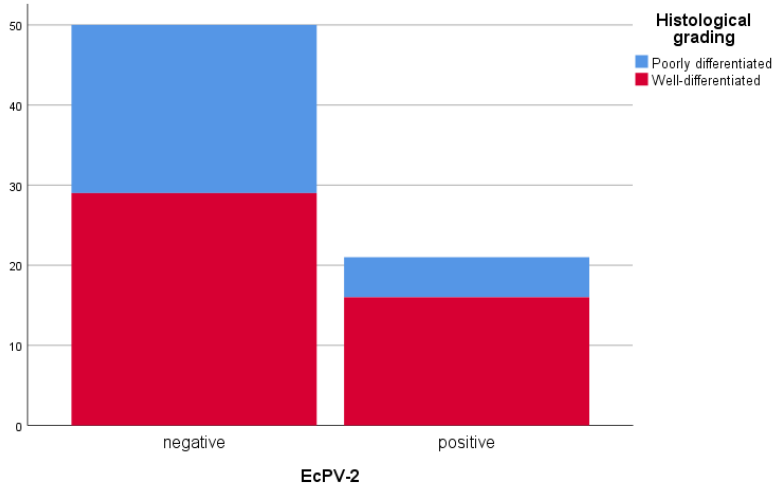


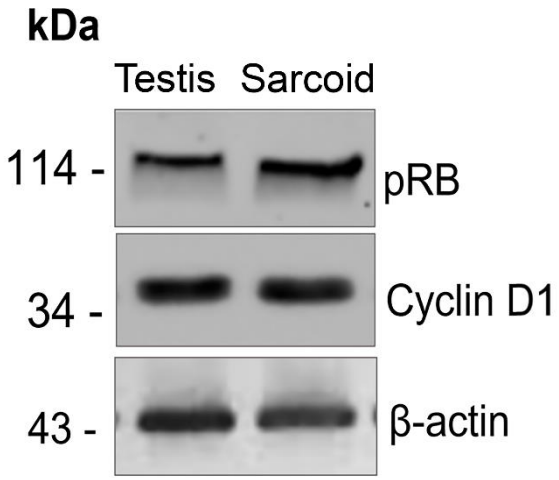
Figure 5. Distribution of EcPV-2 status and histological grading.



5.3.4 Immunohistochemistry (IHC)

Cross reactivity for Cyclin D1 and pRB were confirmed with Western blot analysis from equine testis and sarcoid (Figure 6). Cyclin D1 and pRB revealed a strong band at 34 kDa and 114 kDa protein respectively. An antibody to β-actin was used as a loading control.

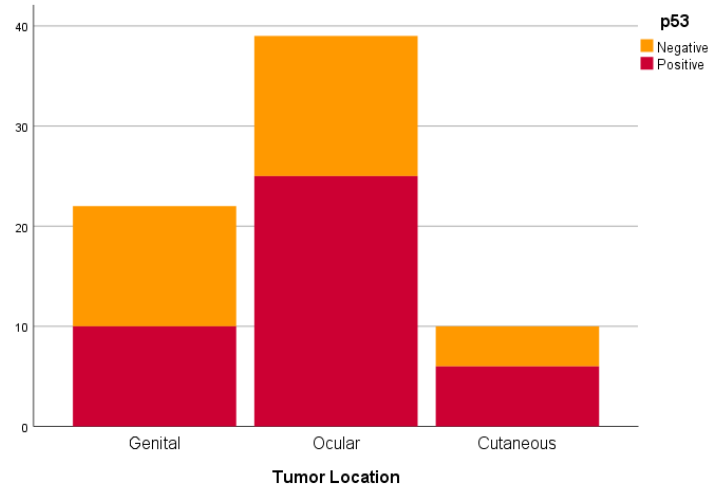
Figure 6. Western blot analysis of protein lysates from equine testis and sarcoid.



p53

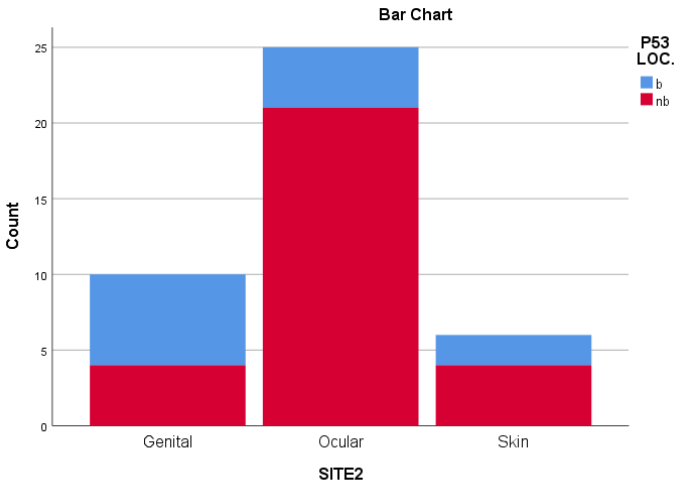
Positive nuclear p53 labelling, defined as >10% of positive cells, was observed in 41/71 (57.7%) of cases. Of these, 12 showed basal (b) positivity while 29 had a non-basal (nb) positivity distributed across all layers of epithelial cells (Figure 7). Differences of p53 labelling were detected based on anatomic sites: 25/39 (64.1%) ocular tumors positive labelled for p53 while 10/22 (45.4%) genital and 6/10 (60%) of cutaneous tumors showed positive p53 immunolabelling. Although not statistically significant, the majority of ocular tumors showed high p53 immunolabelling compared to the other sites (p=0.36, *Chi-Square test*) (Figure 8).

Figure 8. Distribution of p53 immunolabelling in different anatomical sites.



A statistically significant correlation between p53 localization and anatomic sites was found (p=0.034, *Chi-Square test*). Of 41 p53 positive cases, non-basal positivity was recorded in 4/11 (36.4%) genital, 21/25 (84%) ocular and 4/6 (66.6%) cutaneous tumors (Figure 9).

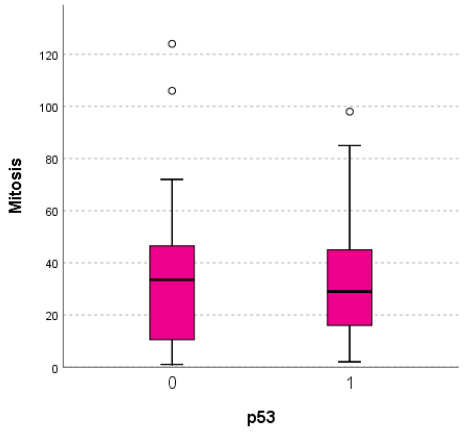
Figure 9. Distribution of p53 localization in different anatomical sites.



Regarding mitotic count, no correlation with p53 positivity (p=0.81, *Kruskal Wallis test*) nor with p53 localization (p=0.45, *Kruskal Wallis test*) was found (Figure 10).

When comparing histological grading with p53 immunolabelling, no significant association (*p*=0.1, *Chi-Square test*) was found. Likewise, histological grading and p53 distribution were not correlated (p=0.19, *Chi-Square test*).

Figure 10. Comparison between mitotic count and p53 immunolabelling. 0: negative cases; 1: positive cases.



P53 positive cases were not associated with EcPV-2 presence (*p*=0.42, *Chi-Square test*) (Table 7), similarly not statistically significant association between p53 localization and EcPV-2 was found. In 22/50 EcPV-2 negative cases, >90% of non-basal cells expressed p53 (*p*=0.81 *Chi-Square test*). Moreover, 7/50 EcPV-2 negative cases showed p53 basal distribution (Table 8).

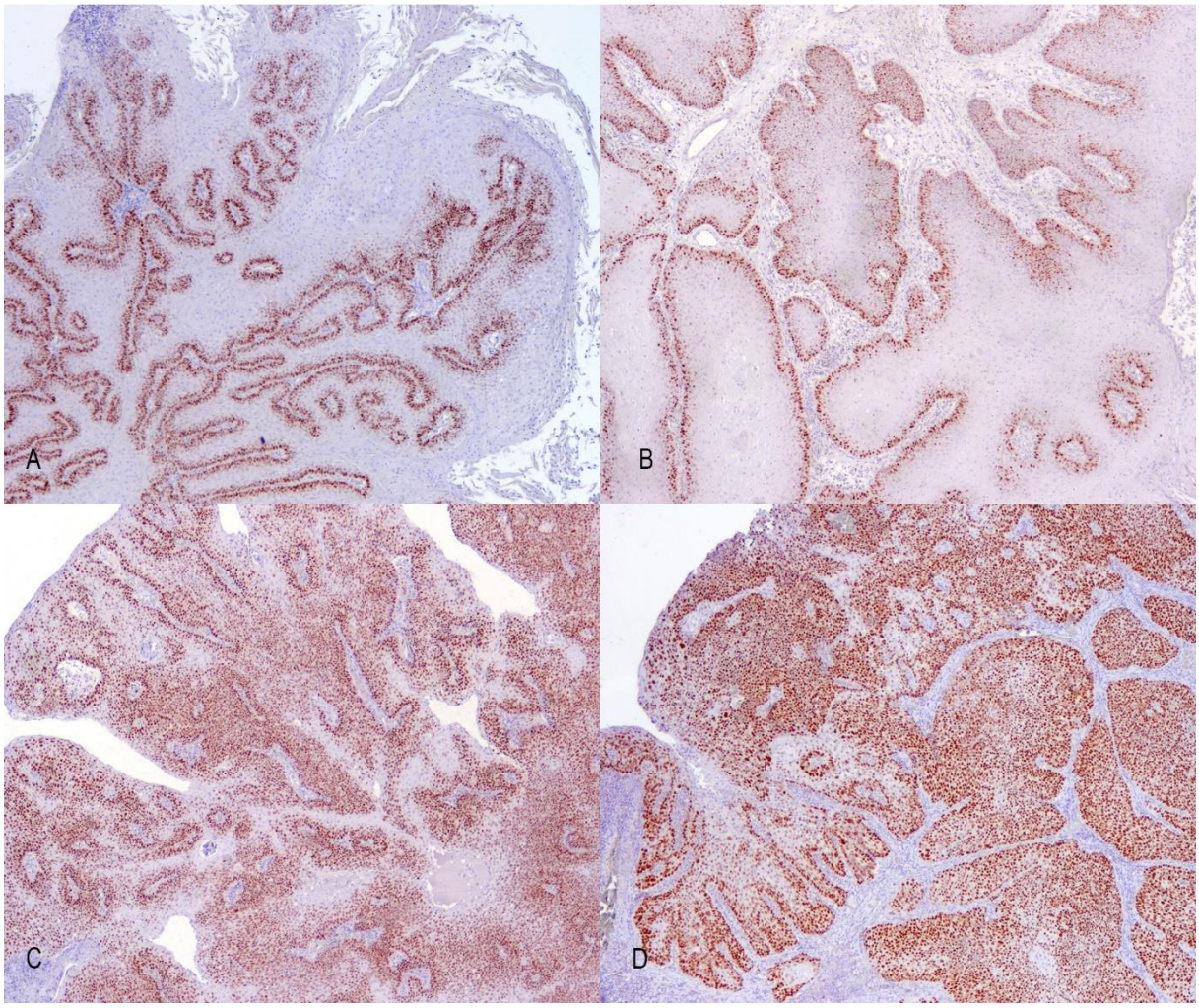
Table 7. Association between p53 immunolabelling and EcPV-2 status in equine squamous cell carcinomas and carcinomas in situ.

		EcPV-2		
		negative	positive	Total
p53				
	negative	22	8	30
	positive	28	13	41
Total		50	21	71

Table 8. Association between p53 localization and EcPV-2 presence.

		EcPV-2		
		negative	positive	Total
p53 localization		21	8	29
	basal	7	6	13
	non-basal	22	7	29
Total		50	21	71

Figure 8. A, B. Strong basal p53 immunolabelling, 4x. **C, D**. Strong non-basal p53 immunolabelling, 4x.



CDKN2A (p16)

CDKN2A immunolabelling was tested in 43/71 cases. Of these, 18/43 (17.5%) were negative, while 25/43 (24.3%) had either nuclear or cytoplasmic positivity. Moreover,

in 20/43 (46.5%) of cases, strong immunolabelling of keratinized cells was noted (Figure 11). The expression of CDKN2A, either nuclear only or nuclear and cytoplasmic, was not associated with EcPV-2 status. In fact, of the 25 CDKN2A-positive cases, 20 were EcPV-2 negative and 5 were EcPV-2 positive (p=0.55, *Chi-square test*). Although not statistically significant, the majority of CDKN2A-positive cases had a non-basal p53 distribution (14/25, >%) (p=0.062 *Chi-Square test).* Moreover, there was a statistically significant association between mitotic count and CDKN2A immunolabelling as CDKN2A positive cases had higher mitotic count compared to CDKN2A negative cases (*p*=0.009 *Kruskal-Wallis test*) (Figure 12).

In addition, no statistically significant association was observed with anatomic site (*p*=0.69 *Chi-Square test*), histological grading (*p*=0.08 *Chi-Square test*), and p53 positivity (*p*=0.20 *Chi-Square test*).

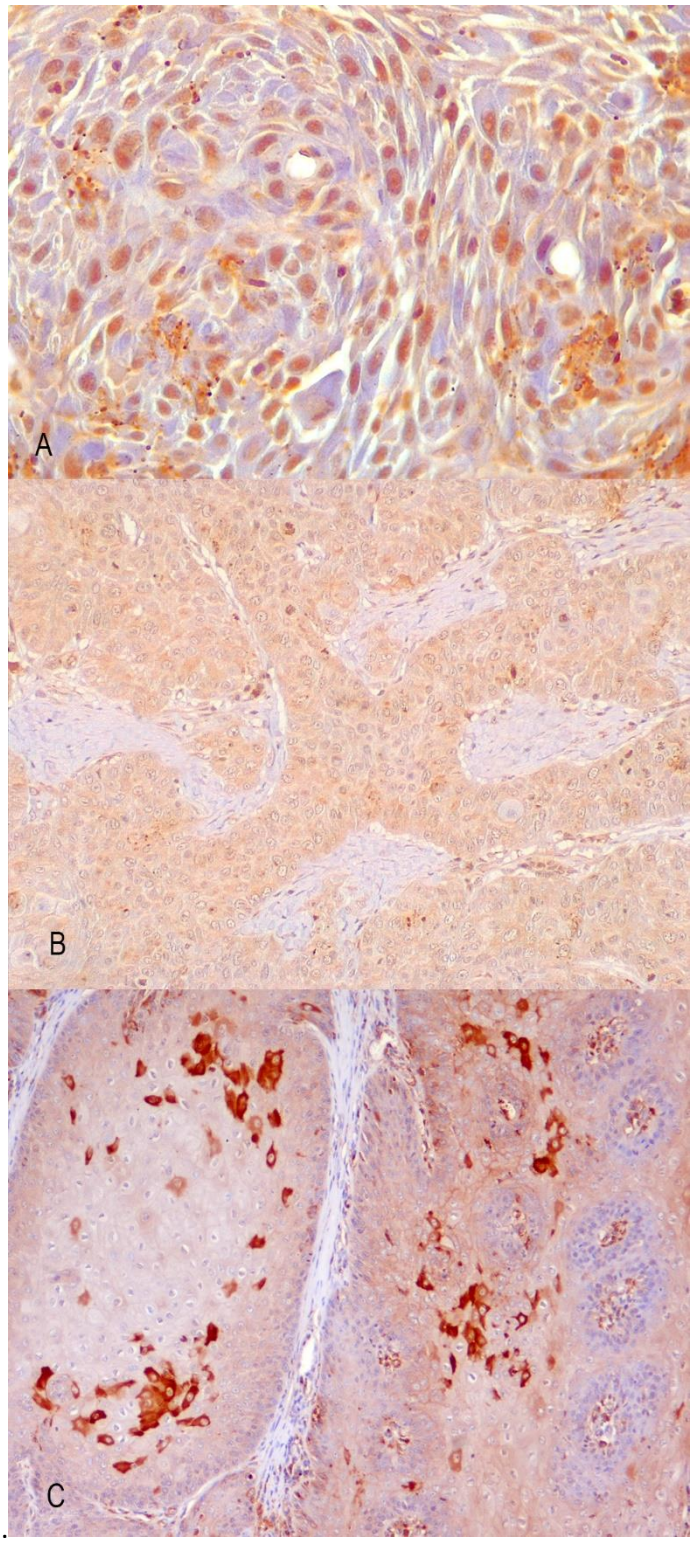
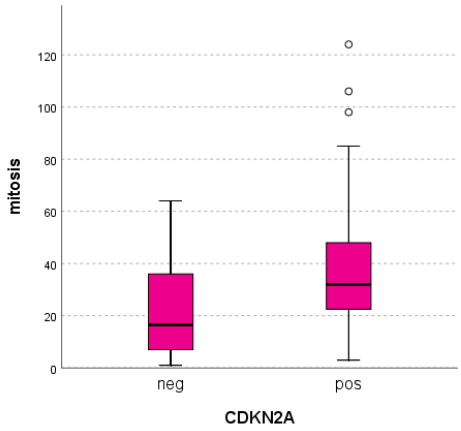


Figure 8. A. Intense nuclear labelling of CDKN2A neoplastic cells, 40x. **B.** Intense cytoplasmic labelling CDKN2A neoplastic cells, 20x. **C.** Intense CDKN2A labelling of keratinized neoplastic cells, 10x.

Figure 12. Comparison between CDKN2A immunolabelling and mitotic count.



pRB

For pRB, nuclear labelling was observed in 69/71 (97.2%) of cases (Figure 13). Two cases were excluded due to FFPE material unavailability. Regarding pRB score, 2 (2.8%), 2 (2.8%), 9 (13%), 22 (31.9%) and 34 (49.3%) cases showed expression scores of 1, 2, 3, 4 and 5 respectively (Table 9).

P53 positivity was significantly associated with high pRB scores (p=0.01, *Kruskal-Wallis test*). In addition, cases with high pRB score were associated with p53 non-basal positivity (p=0.002, *Kruskal-Wallis test*).

Furthermore, non-statistically significant association was found between pRB immunolabelling and other molecular variables (anatomical site, histological classification, mitotic count, EcPV-2 and CDKN2A positivity).

Moreover, pRB immunolabelling was not associated with histological grading (p=0.54, *Kruskal-Wallis test*). Although not statistically significant, pRB score was higher in ocular SCCs (p=0.05, *Kruskal-Wallis test*).

Figure 13. A, B. Strong nuclear pRB immunolabelling of neoplastic cells, 10x.

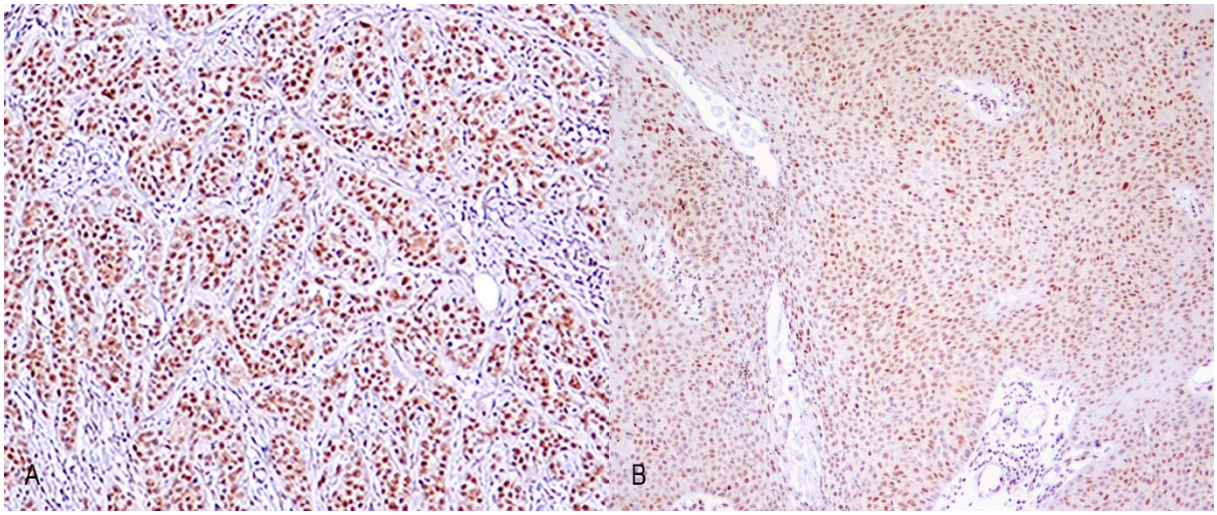


Table 9. Results of pRB score in squamous cell carcinomas and carcinomas in situ.

pRB score	Positve cases	Percentage (%)
0	0	0
1	2	2.8
2	2	2.8
3	9	13
4	22	31.9
5	34	49.3

Cyclin D1

Strong nuclear Cyclin D1 immunolabelling was observed in all the cases (Figure 14), with highly significant correlation between Cyclin D1 expression and p53 positivity (p=0.027 *Kruskal-Wallis test*). By contrast, no statistically significant association was found between Cyclin D1 and pRB expression (p=0.30 *Spearman Correlation Coefficient*) nor with mitotic count (p=0.31 *Spearman Correlation Coefficient*). Regarding Cyclin D1 score, 5 (7%), 18 (25.4%), 20 (28.2%), 11 (15.5%), 12 (17%) and 5 (7%) showed expression scores of 0, 1, 2, 3, 4 and 5, respectively (Table 10).

Although not statistically significant, Cyclin D1 score is higher in p53 non-basal positve cases (p=0.06 *Kruskal-Wallis test*). Similarly, not statistically significant correlation between Cyclin D1 score and histological grading (p=0.82, *Kruskal-Wallis test*) was

found. By contrast, high Cyclin D1 score was highly correlated with ocular location (*p*= 0.01 *Kruskal-Wallis test*) (Figure 15).

Figure 14. A, B. Strong nuclear Cyclin D1 immunolabelling of neoplastic cells, 10x.

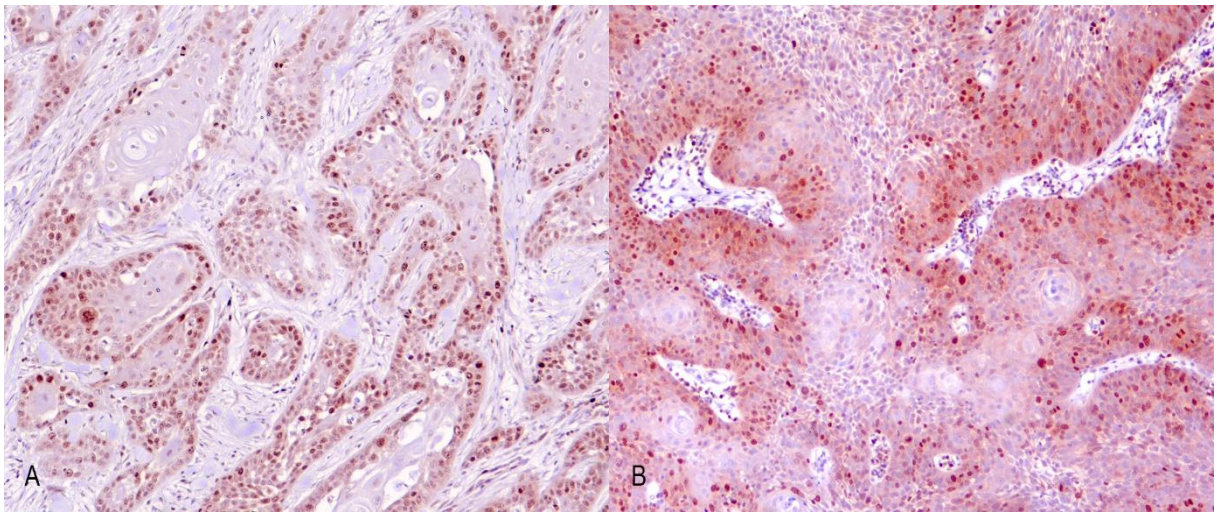
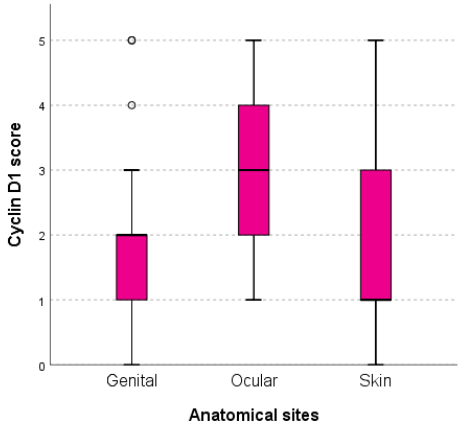


Table 10. Results of Cyclin D1 score in squamous cell carcinomas and carcinomas in situ

Cyclin D1 score	Positve cases	Percentage (%)
0	5	7
1	18	25.4
2	20	28.2
3	11	15.5
4	12	17
5	5	7

Figure 15. Distribution of Cyclin D1 scores and tumor location. High Cyclin D1 scores in ocular tumors.



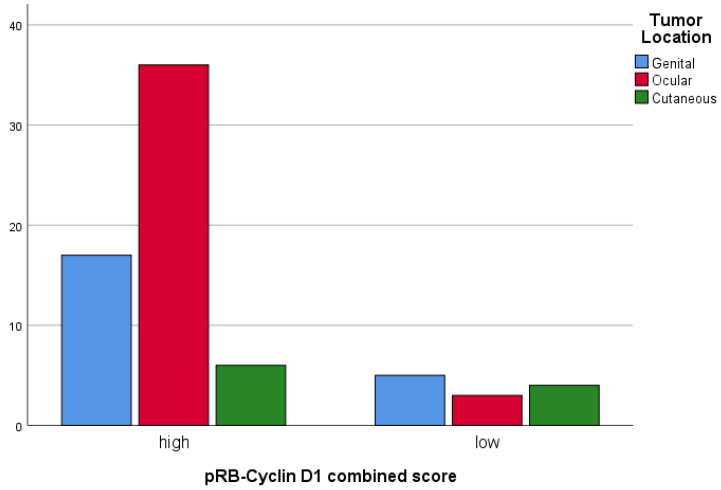
Cyclin D1-pRB combined score

When scores for pRB and cyclin D1 were combined, using 4 as cut-off, 59/71 (83%) samples showed high combined score and only 12/71 (17%) a low combined score.

Positve correlation between pRB-Cyclin D1 combined score and anatomical site (p=0.035 *Chi Square test*) was found (Figure 16). Similarly, a highly significant correlation between high pRB-Cyclin D1 combined score and p53 immunolabelling (p=0.002 *Chi Square test*) was found.

No statistically significant association was found between combined score and other variables: mitotic count (p=0.87 *Spearman Correlation Coefficient*), EcPV-2 positivity (p=0.50 *Chi Square test*), histological grading (p=0.28 *Chi Square test*) and CDKN2A expression (p=0.30 *Chi Square test*).

Figure 16. Distribution of pRB-Cyclin D1 combined score and tumor location. High pRB-Cyclin D1 combined scores in ocular tumors.



5.3.5 Papillomas

Of the seventy-nine cases selected initially, 8 were histologically classified as papillomas (Figure 17).

Papillomas were exclusively genital, either penile (6/8, 75%) or vulvar (2/8, 25%). Papillomas had a median mitotic count of 45.38. Moreover, a significant percentage (87.5%) of equine papillomas contained EcPV-2viral DNA.

P53 immunolabelling was observed in 5/8 (62.5%) cases with a basal positivity in all the cases. Expression of CDKN2A was recorded in 5/8 (62.5%) cases, displaying either nuclear (2/5, 40%) or keratinized pattern (3/5, 60%). No CDKN2A cytoplasmic immunolabelling was detected in equine papillomas.

Strong nuclear Cyclin D1 and pRB immunolabelling was observed in all the cases. Regarding the pRB-Cyclin D1 pathway, high combined score was observed in almost all (7/8, 87.5%) the cases, with only one papillomas displaying low combined score. Table 11 summarizes all the immunohistochemical and chromogenic *in situ* hybridization results.

Figure 17. A, B. Histological appearance of papillomas, 4x.

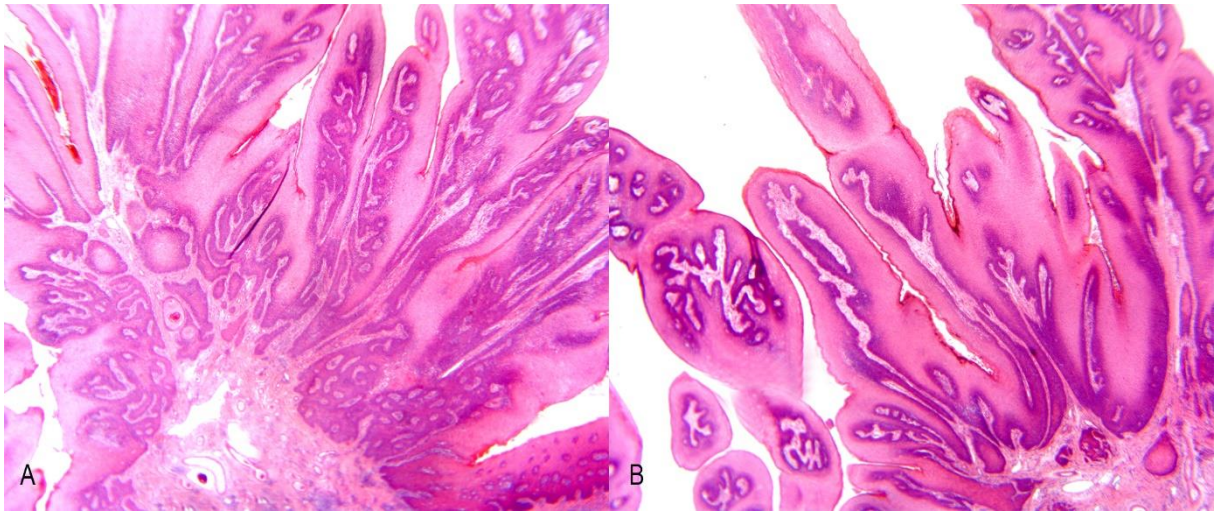


Table 11. Immunohistochemical and chromogenic in situ hybridization results in equine papillomas.

Case ID	p53	P53 pattern	CDKN2A	Cyclin D1 score	pRB score	pRB-Cyclin D1 score	EcPV-2
16L-0103	yes	B	yes	2	5	7	1
16L-1177	yes	B	yes	1	5	6	1
16L-2235	yes	B	yes	2	4	6	1
352 A	no		no	2	4	6	1
4581 A	no		yes	1	3	4	1
4756 A	no		no	2	4	6	1
12833 A	yes	B	yes	4	4	8	0
17345 A	yes	B	no	2	3	5	1

5.4 Discussion

Histological features of squamous cell carcinomas

Histological grading was previously applied to equine squamous cell carcinomas. Based on previous works, histological grade is not predictive of outcome and is not associated with EcPV-2 status.[13] Our results confirm that SCC histomorphological features are not correlated with the presence of the virus, nor with other molecular variables discussed later.

When comparing papillomas and SCC mitotic counts, we found that papillomas had a higher mitotic count compared to SCCs (median mitotic count of 45.38 and 35.2 respectively). Although malignant neoplasms are expected to have higher mitotic counts compared to their benign counterpart, in our study, results showed the opposite. The reason of this unexpected feature is not clear but may be related to the amount of mitosis in the basal cell layer, typically active in normal as well as in benign epithelia.

Genital papillomas and carcinomas are associated with EcPV-2 infection

Several studies have documented the presence of EcPV-2 in equine genital squamous cell carcinomas.[33,163,183] Consistent with previous works, the tropism of EcPV-2 for genital areas was confirmed in 50% of genital SCCs and 87.5% of genital papillomas.

The role of EcPV-2 in the development of SCCs was first discovered by Scase *et al* (2010) who found viral DNA in 74% of genital SCCs examined.[278] Subsequently, EcPV-2 was also found in papillomas in addition to SCCs, confirming the involvement of EcPV-2 in both benign and malignant epithelial genital lesions.[312] Since then, a number of other studies confirmed the presence of EcPV-2 in penile, vulvar, oral and gastric SCCs with percentages that range from 28.6% to 100%.[6,33,164,240,323]

In our study, the percentage of EcPV-2-positive genital malignant tumors (11/22, 50%) was similar to those reported in the literature. In addition, 87.5% of papillomas, which were all genital, either penile or vulvar, were associated with EcPV-2 infection. It is

possible that viral-induced papillomas progress into SCCs, but the proportion of tumors that undergo this progression is not known.

Although EcPV-2 was foud in a significant proportion of the ases examined, its detection does not necessarily prove that the virus influenced or caused tumor development. Detection of EcPV-2 in skin and mucosae from healthy horses in other studies may indicate that EcPV-2 can be an asymptomatic infection, as in humans, making it difficult to determine whether a neoplasm was truly caused by the virus. For this reason, we investigated the expression of cell cycle proteins, typically involved in HPV-related tumors in humans, which would be expected to differ in viral induced versus non-viral induced neoplasms.

However, it must be underlined that the accuracy of our method in detecting viral DNA has not been fully validated yet. For this reason, PCR for EcPV-2 is currently underway, so that CISH and PCR result concordance can be assessed.

A small percentage of ocular SCCs harbour EcPV-2 DNA

Currently, the pathogenesis of ocular SCCs is not fully understood, but it is thought that prolonged UV light exposure may play a primary role.[103] In humans, UV light exposure is often associated with other solar-induced lesions such as elastosis, actinic keratosis and epidermal plaques. In horses, these changes have been reported in association with SCCs of the conjunctiva and eyelid and are similar to those described in humans.[54] Most solar-induced equine SCCs develop in non-pigmented or lightly pigmented areas, which are more exposed to the sunlight. In a 2019 study, one case of solar elastosis associated with SCC of the eyelid was reported, highlighting the importance of considering UV light in the pathogenesis.[301] Mutations of the p53 gene are often related to UV light radiations and promote the development SCCs. Analysis of p53 expression in equine conjunctival SCCs, revealed higher p53 expression in moderately differentiated tumors versus well-differentiated. In the same work, a specific antibody for mutant p53 was used, suggesting the importance of considering UV-induced p53 gene mutation in the pathogenesis of ocular SCCs.[296]Previous studies have also

investigated the possible role of EcPV-2 in the pathogenesis of ocular SCCs. A number of studies have demonstrated the presence of EcPV-2 DNA in swabs from healthy ocular mucosa. The presence of viral DNA was also investigated in ocular SCC with highly contrasting results: while ocular SCCs were consistently negative in several studies (0/42 cases[240], 0/18 cases[130]) one report from 2011 demonstrated its presence by PCR in 9/9 (100%) cases examined.[329] According to our results where 6/40 (15%) ocular SCCs harboured viral DNA, we suggest that EcPV-2 may play a role in the development of a subset of ocular SCCs. It must be taken into account that the presence of intratumoral viral nucleic acid does not necessarily demonstrate its role in the development of the tumor. It is possible that the interaction of EcPV-2 and UV light radiation, contributes to the development of ocular SCCs. Recent studies of human cutaneous SCCs, demonstrate that beta HPV allow the accumulation of UV-induced DNA mutation.[269,299] The same mechanism may represent a possible model for the pathogenesis of equine ocular SCCs.

Ocular SCCs are associated with p53 dysregulation

Increased p53 expression has been widely demonstrated in equine SCCs.[252,296] In the present study, high nuclear p53 expression was observed in 25/39 (64.1%) ocular cases. These results are consistent with previous reports of p53 expression in equine ocular SCCs. In a study by Sironi *et al*, all the analysed equine ocular SCCs positively labelled for mutant p53 protein, while in normal tissue p53 expression was not detected.[296] In another study, mutant form of p53 was described in 6/6 (100%) equine and 26/41 (63.4%) bovine ocular SCCs. In the same work, p53 overexpression of both equine and bovine samples was higher in ocular areas compared with other tumor location (genital and cutaneous).[318] These findings are similar with those of this study, in which high p53 expression was detected in the majority of ocular SCCs.

It is known that high p53 expression levels may be due to mutations of the TP53 gene. Mutant p53 has greater half-life span and accumulates in the nucleus of neoplastic cells, thus it can be detected immunohistochemically.[9] Although p53 mutation has not

been investigated in the present study, the high p53 expression levels could be caused by UV exposure as in humans.[42,358]

In humans, two distinct pattern of p53 distribution, basal versus suprabasal, reflect the different response of the skin layers to UV damage.[81] In particular, when the skin is exposed to UVA, p53 expression is predominantly basal. This indicates the ability of UVA to penetrate in the basal layer of the epidermis and preserve the superficial keratinocytes layer. Basal cells are in fact less sensible to UVA-induced DNA damage and can efficiently stabilize p53. By contrast, when the skin is exposed to UVB, p53 expression is commonly diffuse. This specific pattern of p53 distribution is mainly attributed to loss of p53 tumor suppression function and subsequent TP53 gene mutation.[3] In our study the suprabasal positivity was significantly higher in ocular SCC, compared to the other anatomic sites; this may suggest a possible role of UV light in the pathogenesis of ocular SCCs.

Recent studies on equine SCCs and papillomas also demonstrated that p53 expression differs significantly in benign and malignant penile lesions, being confined to the basal layer in papillomas and extending to the basal layer in CIS and SCCs.[265] Our study further supports these findings, indicating that p53 can be used to more accurately distinguish between benign and malignant lesions.

Further studies will be aimed at investigating p53 mutations and correlating their presence with p53 immunohistochemical expression.

In addition to p53, the presence of EcPV-2 was also investigated in ocular SCCs. As reported above, a subset of ocular SCCs was positive for EcPV-2. Its presence did not show any correlation with p53 expression nor with its location, suggesting that p53 expression is not influenced by the presence of viral DNA, therefore that the virus probably does not interfere with p53 regulation.

Cell cycle proteins CDKN2A, pRB, and Cyclin D1 expression are not dysregulated in papillomavirus infection as it is in humans

In humans it is generally agreed that high-risk HPVs are the causative agents of precancerous and cancerous cervical lesions.[248] The malignant transformation of cervical epithelial cells is caused by HPV oncogenic proteins E6 and E7, with the latter targeting the pRB pathway.[159] When HPV E7 binds with pRB, the protein is dysregulated and CDKN2A (p16) is typically overexpressed. Simultaneously, HPV E6 degrades p53 via ubiquitin ligase E6AP.[354] In addition to CDKN2A overexpression, human HPV positive cases are strongly correlated with low pRB-Cyclin D1 combined scores. Therefore, high expression of pRB and Cyclin D1 as well as high pRB-Cyclin D1 combined scores, can be considered strong indicators of HPV negativity.[101] Similarly, Rb loss and high CDKN2A cytoplasmic expression were found in papillomavirus-associated feline plaques, Bowenoid in situ carcinomas and SCCs.[232] In contrast to human high-risk HPV, sequencing of EcPV-2 genome has allowed the identification of E6 as the most important oncogene. In fact, although EcPV-2 genome contains both E6 and E7 oncogenes, the latter lacks the pRB binding motif.[278] For this reason, there is no E2F activation and subsequent CDKN2A overexpression is not expected

As expected, in this study there was no correlation between CDKN2A expression (either nuclear or cytoplasmic) and EcPV-2 presence. Moreover, CDKN2A expression, regardless of its location, had no association with anatomic site, histological grading and p53 overexpression. However, the association between mitotic count and CDKN2A immunolabelling was highly significant: CDKN2A positive cases showed higher mitotic count compared to CDKN2A negative cases. CDKN2A is an important cyclin-dependent kinase inhibitor that functions as tumor suppressor that is also used as an indicator of malignancyr.[290] Based on this association we speculate that CDKN2A could be a possible marker of malignancy in equine SCCs, but further studies are needed to assess its potential prognostic role.

To verify any dysregulation of the pRB pathway in equine SCCs, pRB and Cyclin D1 were also tested. pRB was found to be upregulated in all tumors, confirming that EcPV-2 does not affect pRB activity; as CDKN2a is regulated by a pRB dependent mechanism, normal pRB expression further explains the absence of CDKN2A cytoplasmic

accumulation in these tumors. In summary, pRB and Cyclin D1 overexpression and lack of p16 accumulation indicate that EcPV-2 does not interfere with the pRB pathway as it does in humans and in cats.

In this study, high Cyclin D1 scores were significantly correlated with ocular location. This was also demonstrated in a previous study by Arthurs *et al* in which upregulation of Cyclin D1 was associated with EcPV-2 negativity, suggesting an upregulation of the Wnt pathway in EcPV-2 negative SCCs.[13]

pRB immunohistochemical results must also be interpreted considering the antibody used to perform these experiments. In fact, the antibody used in our study, detects both the hypo- and the hyperphosphorylated forms of the pRB protein, hence pRB expression is not related to its activation status.

5.5 Conclusions

To conclude, our results showed that the majority of genital SCCs were positive for EcPV-2, confirming the association between genital lesions and viral positivity. Moreover, a subset of ocular cases positively labelled for EcPV-2 by CISH, suggesting a possible role of EcPV-2 in the pathogenesis of equine ocular SCCs. In ocular SCC, p53 high positivity and suprabasal distribution suggest a possible role of UV light rather than EcPV-2 infection in its pathogenesis.

Cell cycle proteins CDKN2A pRB and Cyclin D1, typically dysregulated in HPV-induced SCC in humans, were tested for the first time in equine SCC and do not seem to predict EcPV2 status. This confirms the results of previous studies which demonstrated the absence of the pRB binding motif in EcPV-2 E7. However, the strong association between CDKN2A and mitotic count suggest that high CDKN2A expression may have a potential as a marker of tumor malignancy.

Future studies will aim at evaluating the involvement of other cell cycle proteins in EcPV-2 infection as well as the investigation of TP53 mutation status.

Chapter 6 Papillomavirus and equine sarcoids

6.1 Introduction

Sarcoids are the most common cutaneous spindle cell tumor affecting horses, mules, and donkeys, with an overall incidence of 90% of all cutaneous neoplasms.[64,170,207] Multiple lesions can occur in the same animal, mostly affecting limbs, groin/sheaths, face, and previously injured areas of the whole body.[170] Sarcoids are worldwide neoplasms with prevalence rates ranging from 0.5 to 2%.[128,216] This high variability is dependent on the geographical area: for example, in USA the Pacific north-west areas have a higher prevalence of sarcoids compared with north-eastern regions.[264,328] Interestingly, in a 1993 study, sarcoids were the most commonly diagnosed cutaneous tumor in Switzerland, accounting 90% of all cutaneous neoplasms.[216] Most reports suggest that geldings are at increased risk compared to mares and stallions.[174,347] However, retrospective studies demonstrate the lack of a sex predisposition for the occurrence of sarcoids. The suggested gender effect in previous works may be explained by the reference population and geographical areas.[343,347] In addition, breed predisposition has been reported, especially in horses carrying equine leukocytes antigen (ELA) haplotypes W3 and B1.[222] An epidemiological study on the association between sarcoids susceptibility and breed effects, showed that Quarter horses were at increased risk of developing sarcoids compared to Thoroughbred. In the same study, the frequency of sarcoids development in Standardbred was less than half that of Thorughbred.[11] Moroever, horses of all ages can be affected, but a higher incidence is reported in animals between 3 and 6 years old.[303,308]

Equine sarcoids are classified as non-metastatic and locally aggressive tumors, with a high tendency of recurrence after surgical treatment.[22,170]Currently, there are different modalities of treatment, which have been used separately or in combination, with variable efficacy. These methods include: cryotherapy, radiotherapy, chemotherapy and laser excision with local immune modulation.[51,214,302] Recently, surgical excision with wide surgical margins has been identified as one of the major factors that

influences the outcome: excision of small well defined sarcoids with margins of 16 mm from the tumor result in better prognosis and clinical outcome.[212] By contrast, the prognosis is significantly worse if one or more unsuccessful surgical treatment have been made previously.[166] Nevertheless, treatment efficacy is difficult to assess because there are no studies with relevant follow up analysis.

6.2 Association between sarcoids and Bovine Papillomavirus

Sarcoids are considered viral-induced tumors and their association with BPV-1, -2 and more rarely -13 is widely accepted.[198,247] The first evidence of a viral etiology was suggested in a 1951 study, in which horses inoculated with bovine wart material developed a sarcoma-like lesion with some features that resembled sarcoid.[245] Subsequently, many in situ hybridization studies demonstrated the presence of BPV-1 DNA in in equine sarcoids.[8,10,180,197] Furthermore, the presence of BPV-1 and -2 DNA in sarcoids samples was assessed by PCR in 62 Australian horses: of 76 examined sarcoids, 56/76 (73%) samples contained viral DNA.[25] Martens *et al* also detected BPV-1 and -2 DNA in 100% of sarcoid samples and 95% of adjacent normal skin at 4mm from the tumor borders.[212] A recent in situ hybridization study by Gaynor *et al* demonstrated the presence of BPV DNA in all the examined sarcoids and, to a lesser degree, in the adnexa and in the epidermis.[122] Although the presence of viral particles were detected in skin, In 2008, the presence of BPV in normal skin and peripheral blood of 4 groups of horses was evaluated. Viral DNA was detected in the skin of 24/42 (57%) healthy horses, in 73% of sarcoid affected horses and horses living in contact with cattle, and in 50% of horses living in contact with sarcoid-affected horses. However, only 58% of blood samples contained viral mRNA.[28] Although the percentage of viral mRNA was very low in the previous work, a same year report demonstrated that peripheral blood mononuclear cells (PBMCs) of sarcoid affected horses can represent a reservoir of viral DNA.[40]Besides sarcoids, evidence of BPV DNA has been reported in 59% of peripheral nerve sheath tumors (PNST), 37% of fibrosarcomas and 22% of other sarcomas

(myxosarcomas and fibromas), although the criteria used to classify these tumors were not specified by the authors.[107] Additionally, in a 2007 report, E1, E2 and E5 viral genes were also detected in cutaneous inflammatory conditions as eosinophilic granulomas, ulcerative dermatitis and others.[353] On the other hand, viral presence has not been described in cutaneous squamous cell carcinomas, papillomas and melanomas.[65,247]

6.3 Bovine Papillomavirus transmission and predisposing factors

Currently there is no clear evidence of a route of transmission, but direct contact with cattle, contaminated fomites, flies, and skin trauma are considered the most common routes. In the environment, BPV viral particles are extremely resistant, and could remain infectious for days even after dehydration.[268] However, the hypothesis of environmental contamination as a source of infection was excluded in a 2005 study in which samples from the surrounding habitat of hoses with and without sarcoids were not contaminated with BPV DNA.[30]

BPV DNA may also be transmitted by flies, which act as a vector moving between wound sites on different horses.[114,134] BPV-1 long control region (LCR) variant II was detected in biting and non-biting flies trapped in proximity of sarcoid bearing horses in the UK.[114] These findings were supported by a more recent work, in which BPV-negative stable flies (*Stomoxysis calcitrans*) harboured viral DNA after exposure to equine sarcoid and papilloma tissues. Although this route of transmission seems to be possible, it is thought that it can occur only for a short time after exposure.[134] In a 2000 study of periorbital sarcoids during the flies season, 69% of the lesions involved medial canthus, 26% the periocular skin and only 5% the lateral cantus.[167] The highest prevalence of sarcoids development at eyelid margins where flies assembles, support their role in the disease transmission.

However, it is also speculated that sarcoids develop from micro-trauma or wound sites. Limbs are particularly susceptible (distal to the stifle or elbow joints) due to the greater difficulty to heal.[168] Transformation of a wound into a sarcoid can occur by different

ways: the first is failure of a sarcoid removal, in which persistence of neoplastic fibroblasts is responsible for recurrence.[171] The second way is from normal skin of a horse bearing a sarcoid in other sites. It has been demonstrated that autograft transmission of neoplastic cells into the wound site may result in sarcoid transformation.[171] Moreover, horses without sarcoids but living in contact with affected equids can develop neoplastic lesions. In this case, viral particles are transferred to the unaffected horse via vectors or via reactivation of a latent BPV infection. Additionally, indirect transmission with BPV contaminated instruments as tack or needles can contribute to sarcoid development.[62] Lastly, even unaffected skin near an injured sarcoid can be susceptible to neoplastic transformation.[171]

Non-healing wounds are therefore responsible for sarcoids development, especially for fibroblastic and verrucous types.[41,189,309]

Genetic effects have an important role in sarcoids development. Although all breeds can be affected, Arabians, Quarter horses and Thoroughbreds seem to be more susceptible to sarcoids development.[22,64] In Arabian horses carrying a mutant DNA-PKcs allele there is a strong correlation between heterozygosis and sarcoids development.[92] Furthermore, horses with A3/B1 combination have an increased risk of sarcoid recurrence.[44,45] In a study of Swedish Halfbred horses, approximately 40% the A3 B1 haplotype was associated with sarcoids.[45] Additionally, major histocompatibility complex (MCH) class I A5 antigens have been associated with early onset of development.[44] Research has shown that equine leukocyte antigens (ELA) are also involved in human PV-induced cervical cancer, promoting the transition from low grade squamous intraepithelial lesions (LSIL) to high grade SIL (HSIL) and SCCs.[20,201] It is thought that PVs induce MHC class II downregulation with inadequate cytotoxicity of T killer lymphocytes.[64,188] Similarly, Thoroughbreds as well as Swiss, Irish and French horses carrying ELA haplotypes W3 and B1 associated with MHC class II, have a high incidence of sarcoids development.[222] If all these breeds are more vulnerable, Standardbred and Lipizzaners lack the ELA W13 allele and are therefore of lower risks.[22]

6.4 Oncogenic mechanisms

Numerous studies have suggested BPV-1 and -2 as the causative agents of equine sarcoids.[226,247,313] In cattle, infection with BPV-1 and -2 can cause cutaneous papillomas (warts), characterized by a predominant fibroblasts proliferation accompanied by epidermal changes.[56] Moreover, in fibropapillomas BPV-1 and -2 infect fibroblasts and keratinocytes, resulting a benign neoplasm that can even regress.[161]

In horses, the pathophysiology of BPV oncogenesis is based on cell culture experiments. In equine cell lines BPV-1 viral genes are capable to induce neoplastic transformation of fibroblasts.[61,350] The increased life span and cell proliferation of transformed fibroblasts is mediated by E5 and E6 oncoprotein, with E7 enhancing their actions.[145]

Once the infection is established, BPV E5 localizes in the endoplasmic reticulum and Golgi apparatus of the host cells and binds with platelet-derived growth factor-β receptor (PDGFβ-R).[255] The binding induce PDGFβ-R activation and recruitment of intracellular proliferative signals.[37] These findings were first described in a 2009 study, in which activation of PDGFβ -R was shown to trigger MAPK and P13K-AKT molecular pathways.[7,36] The MAPK and P13K-AKT pathways lie downstream the activation of PDGFβ -R and are necessary for cellular immortalization, apoptosis evasion, and cell-cycle progression.[58] Contrary to human medicine, in which D-type Cyclins are generally downregulated in the majority of cancers, in equine BPV-1 infections D-type Cyclins are upregulated in response to P13K-AKT activation.[36,293] However, it appears that in BPV-1 fibroblasts cell lines, activation of the p38 mitogen-activated protein kinase (MAPK) pathway is triggered by E6 rather than E5.[351]

The E6 protein is a zinc-binding proteins localized in membrane and nuclear fractions. As seen with high-risk HPV cervical lesions, it seems that in equines, BPV infections E6 induces p53 inactivation.[178,281,322] These findings are supported by detection of an abnormal p53 nuclear and cytoplasmic expression in equine sarcoids.[56,242] The hypothesis of abnormal p53 function was clarified by Nixon *et al*, which detected

cytoplasmic p53 expression in a subset of sarcoid cell lines. Therefore, it was shown that nuclear and cytoplasmic p53 colocalization were associated with its loss of function.[242] The obtained data provide further evidence of p53 compromission in equine sarcoids.

The BPV E7 protein cooperates with E5 and E6. When they are co-expressed, E7 is responsible for enhancing neoplastic transformation.[34] However, it is necessary to consider that BPV-1 E7 lack the retinoblastoma (RB) LXCXE binding motif. Therefore, in contrast to HPV E7, BPV-1 E7 is not able to binds with the pRB protein.[234] Nevertheless, recent works demonstrate the ability of E7 to complex with p600, allowing anchorage-independent cell growth.[34]

It should be noted, however, that in BPV infections, BPV-1 E2 also contribute to tumor development. E2 protein is a viral transcription regulator responsible for stable extrachromosomal maintenance of viral genome.[218,256] Moreover, it seems that E2 is involved in matrix metalloproteinases (MMPs) expression. The MMPs are zinc-dependent endopeptidases that break down matrix proteins and therefore play an important role in local invasion. The extracellular matrix remodelling in equine sarcoids was demonstrated by Martano *et al*, which reported an increased expression of MMP-2 and -9.[210]

6.5 Anatomical sites predilection

Sarcoids can occur anywhere in the body, however predilection sites are often associated with the clinical type. Interestingly, besides girth, limbs, head and neck and ventral body, sites with thinner skin covered by sparse coat including axillae, groin, penile sheath and scrotum have higher odds of sarcoids development.[181,317] Head lesions could develop anywhere, but periocular areas and ear pinnae are the most affected. However, mouth, coronary band skin, mammary gland and anal skin cannot be excluded from sarcoid development.[172] As mentioned before, previously injured areas seem to facilitate the development of the neoplasm.[31,210,316] Sarcoids can be

single or multiple, ranging from small wart-like lesions to large ulcerated exophytic masses.[288] Interestingly, it has been reported that horses in United Kingdom (UK) are more prone to develop multiple sarcoids than those in Australia, North America and Europe.[165]

6.6 Clinical presentation of equine sarcoids

Sarcoid size and gross appearance can be very different and clinically 6 variants can be recognized: occult, verrucous, nodular, fibroblastic, mixed, and malignant (malevolent).[170] Figure 1 shows the main clinical types.

6.6.1. Occult

Occult sarcoids are slow-growing and appear as alopecic or discoloured areas with a roughened, circular, often hyperkeratotic surface. In periocular regions it appears as more extensive, often with a scaly surface.[241] Occult sarcoids may evolve in verrucous or fibroblastic types but usually they do not progress. Although described as slow-growing and non-metastatic, if the surrounding area is not surgically excised, there will be a rapid recurrence.[165] Neck, face, sheath, medial thigh, shoulders are the most affected sites.[31,165]

6.6.2. Verrucous

Verrucous-type-sarcoids have a characteristic wart-like appearance, generally <6 cm in diameter. Moreover, they can also appear as hyperkeratotic cauliflowerlike lesions, broad-based or pedunculated. They are slow growing neoplasm, mostly affecting axillae, groin, periorbital areas and sheaths.[170]

6.6.3. Nodular

Nodular types appear as spherical, well-demarcated subcutaneous nodules that frequently coalesce. Two subtypes can be identified: movable masses with overlying intact skin (type A) or unmovable masses with variably sized areas of ulceration (type B). Type A is further classified as A1 (nodules with well-defined capsule and freerly mobile) and A2 (unmovable deepest nodules without skin involvement). Similarly, type B is subdivided in B1 and B2; B1 sarcoids are firmly attached to the overlying skin but can be moved to the deeper tissues.[172] Predilection sites are the groin, sheath, and eyelids.[170]

6.6.4 Fibroblastic

Appearance of fibroblastic sarcoids is extremely variable and often characterized by presence of an ulcerated or fleshy surface. Sometimes they can be also misdiagnosed with exuberant granulation tissue.[170] it is considered as the most aggressive type, composed of a large fibrous mass often associated with local infection or hemorrhage.[172] Fibroblastic sarcoids are divided in two types: type 1 and type 2. Type 1 is pedunculated and further subdivided in type 1a and 2a. Type 1a is pedunculated with no extension beyond the pedicle while type 1b has a dumbbell shape and extends beyond the pedicle. Type 2 fibroblastic sarcoids are sessile, invasive with a broad base.[170] Fibroblastic sarcoids are locally invasive and type 1b are generally complication of skin wound in the limbs.[165]

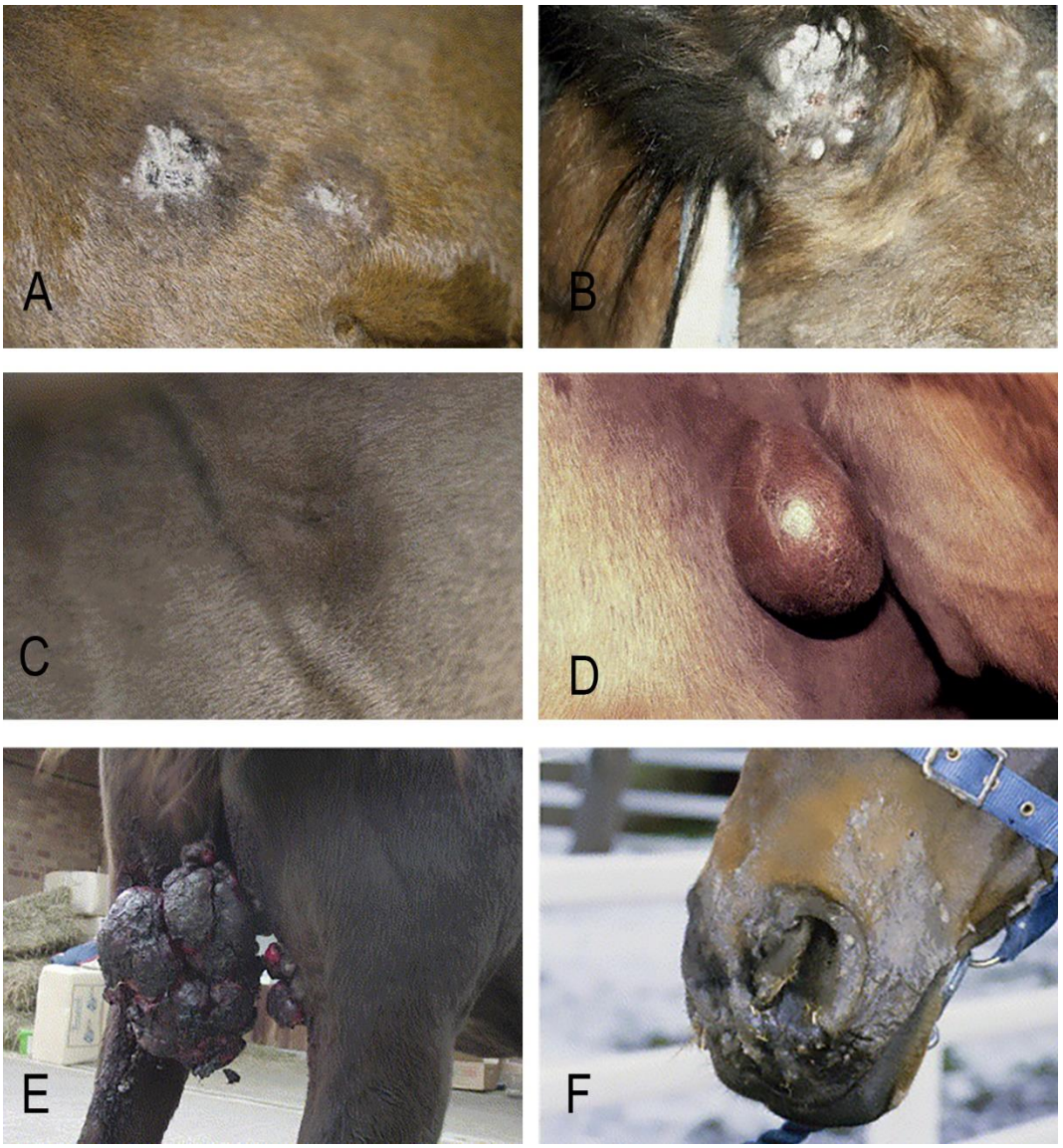
6.6.5 Mixed

Mixed type is a combination of any or all the aforementioned types. It is likely that mixed type represent a transition between verrucous and occult types and/or the fibroblastic and nodular types.[169]

6.6.6 Malevolent

Lastly, the malignant variant is the more aggressive form, characterized by multiple severe lesions, infiltrating lymphatics with palpable tumor cords.[170] Elbow, pre-femoral flank fold and cheek areas are the most frequently affected sites.[165,170]

Figure 1. A. Two mixed (occult-verrucous-nodular) sarcoids (Knottenbelt *et al*, 2005). **B.** Verrucous sarcoid (Knottenbelt *et al*, 2005). **C.** Occult sarcoid (Knottenbelt *et al*, 2005). **D.** Type A nodular sarcoid (Knottenbelt *et al*, 2005). **E.** Fibroblastic sarcoid developed from type A and type B nodule. **F.** Malevolent sarcoid in the muzzle (Knottenbelt *et al*, 2005).



6.7 Biological behaviour

Sarcoids are locally invasive cutaneous tumors that do not extend to internal organs. They remain static or grow slowly for years, depending on the clinical type. Horses bearing a single sarcoid usually have long-term stasis and are less susceptible to further development. By contrast, in multiple tumor bearing horses, sarcoids have a locally aggressive behaviour.[172] Spontaneous regression is a rare occurrence but cannot be excluded. A number of studies have suggested immune system upregulation as possible source of regression.[162,272,330] For example, injections of bacillus Calmette Guérin (BCG) showed complete regression in 36/61 (59%) and partial regression in 11/61 (18%).[330] More recently, vaccinated donkeys with chimeric virus-like particle composed of BPV-1 L1 and E7 proteins has shown enhanced sarcoid regression and reduced progression.[14]

6.8 Histological features

Histological features of sarcoids are well known and the most suggestive finding is the presence of interlacing streams and bundles of neoplastic fibroblasts supported by variably amount of collagen. Cells can vary from spindle/stellate to atypical fibroblasts with plumped nuclei and prominent nucleoli. Addition patterns can be seen as whirling, storiform, herringbone and haphazardly arranged fibroblasts, the latter most often occurring in occult sarcoids.[119] Mitotic index is typically low. As not always present, the perpendicular orientation of fibroblasts toward the dermo-epidermal junction called “picket-fence” is considered pathognomonic. Although being characteristic, the picket-fence feature is missing in up to 52% of sarcoids.[119] It is reported that, up to 46% of sarcoids lack epidermal hyperplasia and about 54% lack rete peg formation.[119] The epidermis can be variably hyperplastic and hyperkeratotic with long prominent branching (rete-peg formation), especially in the verrucous type. Moreover, notable epithelial changes including long exophytic digit-like projections with prominent orthokerathotic hyperkeratosis are typical features of verrucous type[31] whereas in

most nodular and occult types the epidermis can be normal to thinner.[288] It has been taken in account that occult sarcoids may be overlooked, especially at early stages: the mere histological characteristic is the presence of an increased fibroblasts density obscuring follicles and glands.[119] Moreover, in BPV DNA-positive occult sarcoids, epidermal changes may include swollen, pale keratinocytes with perinuclear halo and thickening of the basement membrane.[32] Conversely, nodular sarcoids are commonly ulcered.[31] In ulcered sarcoids, secondary infections with bacteria, fungi and parasites, including Habronema spp. are common. However, not all the aforementioned histological features can be present at the same time, making the diagnosis often challenging.

6.9 Differential diagnoses and immunohistochemical markers

There are several differential diagnoses when considering sarcoid-like neoplasms. Especially in biopsies, sarcoids can be misdiagnosed with other spindle-cell tumors such as fibromas, fibrosarcomas and nerve sheath tumors of Schwann cell origin.[27,107] Simultaneous presence of more densely areas of neoplastic cells (Antoni A) and loosely arranged neoplastic cells embedded in myxomatous matrix (Antoni B) or Verocay bodies are suggestive of Schwannomas and allow the differentiation with sarcoids.[284] Furthermore, fibrosarcomas are more cellular then sarcoids, with an high mitotic count and no association with the epidermis Moreover, fibromas are expansile, well-demarcated neoplasms composed of abundant collagenous stroma and mature fibroblasts.[288] Although most of these histological findings allow the distinction between sarcoids and other mesenchymal tumors, in some cases and especially in biopsies, IHC and PCR are required.[346] Currently there are no specific diagnostic markers but the combined S100 negativity and BPV DNA detection by PCR are considered the main diagnostic features.[27]

S100 is a calcium binding protein found in numerous cells of neuroectodermal origin. In veterinary oncology it is mostly used to differentiate Schwannomas, neurofibromas

and fibrosarcomas.[223] Although specific immunohistochemical markers for peripheral nerve sheath tumors and Schwannomas do not exist, previous works showed that most of these neoplasms positively labelled with S100.[284] In a 2011 study of 10 equine sarcoids histologically resembling peripheral nerve sheath tumors, all neoplastic cells positively labelled for vimentin but not for S100. Additionally, neoplastic cells positively labelled for laminin and collage IV, suggesting the presence of a basal lamina.[27] As previously mentioned, sarcoids and other mesenchymal tumors can share similar histological features. For this reason in 2011 Bogaert *et al*, defined the specific molecular features and markers for the diagnosis of sarcoids as the simultaneous S100 negativity and BPV DNA positivity.[27]

The role of p53 in equine sarcoids is still controversial. Whether in human HPV induced tumors E6 causes p53 degradation, in BPV infections E6 can transform cells in a p53 independent manner.[359] Some studies on p53 status, showed an abnormal cytoplasmic and perinuclear protein expression, speculating that the protein is not degraded within the cell.[215,242] Similar results were obtained by Bogaert *et al* in which perinuclear p53 expression was similar among different types of sarcoids, demonstrating that p53 expression is not associated with tumor behaviour.[29] Nevertheless, further works are necessary to understand the impact on p53 in equine sarcoids.

Ki67 is a marker of proliferation expressed in all cycling cells, with a peak in the M phase.[223] In equine sarcoids the proliferative fraction of neoplastic fibroblasts is typically low, reflecting the clinical observations that describe these tumors as slow-growing and non-metastatic.[29,215,242] However, the epidermis of verrucous and fibroblastic subtypes showed a higher proliferative fractions compared to the other subtypes.[31] Interestingly, the superficial dermis of fibroblastic and verrucous sarcoids have high Ki-67 index compared with the nodular and the occult types.[27] This finding agrees with in situ hybridization studies, in which most BPV infected cells are located in the dermo-epidermal junction.[197,317]

Platelet-derived growth factor β receptor (PDGFR-β) is a tyrosine kinase receptor involved in BPV-1 positive urinary bladder tumors and sarcoids.[36,76] In sarcoids its physiologic function is impaired by BPV-1 E5 protein, which binds to PDGFR-β and induce its phosphorylation.[255] PDGFR-β is subsequently activated and promote cell survival and growth. *In vitro* studies on equine sarcoid fibroblast cell lines demonstrate the transformative activity of BPV-1 E5 and its role in regulating cell proliferation by downstream activation of Ras-MAPK-ERK signalling.[7] Interestingly BPV-1 E5 and E6 can also trigger p38 mitogen-activated protein kinase (MAPK) pathway, which is crucial for cellular invasion.[351]

Vascular endothelial growth factor (VEGF) is a member of PDGF family involved in angiogenesis and vascular permeability.[177] The role of VEGF in sarcoids development is still not fully investigated. In a 2018 study, immunohistochemistry for VEGF revealed strong and finely granular cytoplasmic labelling in the majority of keratinocytes, neoplastic fibroblasts, and endothelial cells, suggesting a possible role of VEGF in sarcoids development. In the same study the authors conclude that VEGF overexpression maybe due to hypoxia, which further stimulates tumor growth via neoangiogenesis activation.[211]

Experimental part

Chapter 7 Expression of cell cycle proteins (CDKN2A, pRB and Cyclin D1), fibroblast associated protein (FAPα) and viral distribution in equine sarcoids

It is well known that in human papillomavirus (HPV) induced tumors, abrogation of cell cycle checkpoints is achieved by high-risk PVs rather than low risk. In HPV infections, E6 and E7 genes are responsible for cell cycle alteration by inactivating the two most important suppressor proteins: p53 and pRB.[151,338] The interaction of E7 with RB results in release of the transcription factor E2F from the RB-E2F complex. Moreover, as RB-E2F normally inhibits transcription of the p16 gene, expression of HPV E7 results in excessive p16 transcription and translation.[248] In a similar manner, the E6- p53 and -associated protein ligase (E6AP) binding trigger ubiquitination and subsequent degradation of p53.[273]

In addition to p53 and pRb, cyclin D1 and cyclin inhibitors such as p16 are key proteins in the study of the molecular alterations that characterize human cervical carcinoma.[73] As a consequence, p16 overexpression, in presence of high-risk HPV, is therefore used as a prognostic marker to predict HPV status.[66]

As well as pr3, pRB and p16, Ki-67 represent an efficient marker of proliferation, which is commonly used as prognostic marker of cervical cancer. Moreover, its increased expression is also an indication of increased cervical dysplasia.[277,295]

In literature, the study of cell cycle markers in equine sarcoids is limited to p53, Ki-67, Cyclin A, CDK2 and p27.[29,215,242] The contrasting role of p53 expression has been shown in several studies: Martens *et al* reported an aberrant p53 expression in 44% of cases[215], while in Johnston's work p53 immunolabelling was detected in 20% of cases.[152] On the other hand, Bogaert *et al*, demonstrated perinuclear p53 expression in 36.1% of the examined sarcoids, but its expression was not associated with any of the clinical types of the study.[29]

The proliferative rate of sarcoids, assessed by Ki-67 index, is reported to be low in all the studies, ranging from a minimum of 1.44% to 11%.[215,242]

By contrast, there is only one study on Cyclin A, CDK2 and p27 expression in equine sarcoids. Of a total of 13 cases, all were negative for the analyzed proteins.[242]
For these reasons, the study of cell cycle proteins in equine sarcoids can provide further insight in BPV oncogenic mechanisms.

7.1 Materials and Methods

7.1.1 Case selection and histology

Tissue samples from surgical excision or incisional biopsies with a histological diagnosis of sarcoid were retrieved from the archives of the Veterinary Pathology Centre at the University of Surrey (UK) and from the Pathology Service of the Department of Veterinary Medical Science of the University of Bologna (Italy), from 2010 to 2018. Clinical information, including signalment, tumor location and clinical subtypes were retrieved from the submission forms, when available. Samples were available as formalin-fixed, paraffin-embedded (FFPE) and H&E stained slides. Each case was reviewed to confirm the initial diagnosis of sarcoid based on H&E features.
To compare the histopathological features with clinical subtypes, each tumor was evaluated for six histological features: hyperkeratosis, hyperplasia of the epidermis, rete pegs, picket fencing, ulceration/crusting, and inflammation. Each feature was recorded as present or absent. Moreover, two growth patterns of fibroblasts in the dermis were identified and categorized into either interstitial (neoplastic fibroblasts arranged between and around collagen fibres) or fascicular (neoplastic cells arranged in interlacing streams and bundles), based on the prevalent pattern.
For cases that were sampled by surgical excision, the completeness of surgical margins was evaluated as complete or incomplete.

7.1.2 Tissue Microarray Technique

Firstly, representative areas containing neoplastic tissue without necrosis, inflammation, hemorrhage, or non-neoplastic tissue were selected from the H&E

stained sections. Selected areas were marked on the slide and compared with the donor block to identify the sampling area, then were punched with a 3 mm diameter biopsy punch. TMAs were then constructed allocating the obtained tissue cores into new empty receiving paraffin blocks. One tissue core per tumor was used for the construction. A sector map was then created to recognize the position of each tumor core. Each TMA contained eight tumor cores, one canine liver core as a landmark and one core of equine granulation tissue as positive control. From each TMA, a HE stained section was evaluated to confirm the presence of the selected tissue.

After validation of the TMA technique, IHC for FAPα was performed in each case; full sections were used for small tumors samples (<5mm) while large tumor samples were performed on TMA.

7.1.3 Protein Extraction and Western Blot analysis

To verify FAPα antibody specificity, Western Blot analysis was performed. The assay was carried out in collaboration with the Department of Experimental and Special Medicine (DIMES), at the University of Bologna. For protein extraction, fresh tissue from equine dermis, intestine and sarcoid was collected. From each, 25µg of tissue was homogenized with 500ml RIPA buffer (Thermofisher Scientific) and 100 µl of protease inhibitor (Halt Protease Inhibitor Cocktail EDTA-free, Thermofisher Scientific). Tubes were centrifuged at 12000 rpm for 30 minutes at 4°C and the supernatant was collected and stored at -80°C. Protein concentration was calculated using the DC Protein Assay Kit (Bio-Rad). Briefly, 40 µg of proteins were denatured at 100°C for 10 minutes. Proteins were separated using a 8% gel and electrophoretically transferred to a nitrocellulose membrane. Non-specific binding sites were blocked with milk 5% in TBS-T for 1h at RT. The blot was incubated at 4°C overnight with an antibody against FAPα (1:500, Abcam). Then, the membrane was incubated with anti-rabbit peroxidase conjugated secondary antibody (1:200, Thermofisher) for 1 hour at room temperature. Reactive bands were visualized with a chemiluminescent detection kit (Westar nC 2.0 Cyanagen, XLS075,0020) using the Chemidoc instrument (Bio-Rad). B-Actin was used

as the loading control and was detected using a β-actin-specific antibody (1:500 Santa Cruz, Biotechology).

7.1.4 Immunohistochemistry (IHC)

Immunohistochemistry for S100 and α-smooth muscle actin (αSMA) was performed to differentiate sarcoids from schwannoma and other nerve sheath tumors (NSTs) as previously described.[27,108] Additionally, immunohistochemistry for CDKN2A, pRB, Cyclin D1. p53 and Ki-67 was performed using full sections whereas for FAPα immunohistochemistry was performed using TMA blocks. Data on the primary antibodies are summarized in Table 1.

Ten sarcoid cases were selected for TMA validation as previously described.[238] From each case, both a full section and a punch section were used to perform immunohistochemistry (IHC) for fibroblast associated protein (FAP) α. IHC positivity was assessed blindly and the results of IHC on full section and TMA were compared to assess consistency.

Cross reactivity for Cyclin D1 and pRB with equine tissues was confirmed with western blot analysis in part 1 of this work.

Three-micrometer-thick sections were dewaxed and rehydrated. Endogenous peroxidase was blocked by immersion in H_2O_2 0.3% (for αSMA and Ki-67) or H_2O_2 3% (for S100, FAPα, CDKN2A, pRB, Cyclin D1 and p53) in methanol for 30 minutes. For antigen retrieval, sections were immersed in 200 mL citrate buffer (pH 6.0) or in tris-EDTA (pH=9.0) and heated in a microwave oven at 750 W for two 5-min cycles (αSMA, Ki-67 and pRB) or for three-5 min cycles (CDKN2A, Cyclin D1, p53 and FAPα) immunostaining respectively. Enzymatic antigen retrieval was performed in 37°C oven for 15 minutes with 0,05% trypsin for S-100. Slides were incubated with the primary antibody overnight at 4 °C. The reaction was developed using a commercial streptavidine-biotine-peroxidase technique (ABC kit elite, Vector, Burlingame, CA, USA) and visualized with 3-amino-9-ethylcarbazole (Dako, Glostrup, Denmark). Slides were counterstained with Harris hematoxylin. Positive controls consisted of normal

equine intestine for αSMA and Ki-67, equine melanoma for S100, and both a WB-positive equine sarcoid and an equine squamous cell carcinoma for FAPα and CDKN2A and p53 respectively. For pRB and Cyclin D1, a normal equine testis was used as positive control. As a negative control, the primary antibody was replaced with an isotype-matched non relevant antibody.

For FAPα, cells were evaluated positive when cytoplasmic labelling was observed, while any nuclear staining was considered unspecific.[1] Ki-67 proliferation index was assessed by manual image analysis based on the number of positive nuclei in 500 cells and expressed as a percentage. (Image J Software, National Institutes of Health, Bethesda, Maryland, USA). The number of positve nuclei in the epidermis was assessed separately in the epidermal and dermal component and the only the dermal percentage was used for statistical analysis. CDKN2A immunostaining was evaluated as nuclear or cytoplasmic[274]. Moreover, immunolabelling for CDKN2A, pRB and Cyclin D1 positve cells was scored along a three-tiered scale ranging from 1 to 3: 1 (<10%), 2 (10-50%) and 3 (>50%) as previously described.[246]

Table 1. Primary antibodies, resources, and dilutions.

Antibody	*Clone*	*Manufacturer*	*Dilution*
αSMA	1A4	Dako, Glostrup, Denmark	1:500
S-100	Polyclonal	Dako, Glostrup, Denmark	1:1000
FAPα	Polyclonal	abcam, Cambridge, UK	1:300
Ki-67	MIB-1	Dako, Glostrup, Denmark	1:600
p53	PAb240	BD Pharmingen (Rodano (MI), Italy)	1:100
CDKN2A	Polyclonal	Elabscience Biotechnology (Texas, USA)	1:150
pRB	Sc-1	Santa Cruz Byotechnology (Segrate (MI), Italy)	1:100
Cyclin D1	A12	Santa Cruz Byotechnology (Segrate (MI), Italy)	1:150

7.1.5 Polymerase chain reaction (PCR)

A PCR to detect BPV DNA (type 1, 2, 13) was developed. The primers BPV1213F 5'-CCACTACCTCCTGGAATGAAC-3' and BPV1213R 5' GGCAGACCTGTACAGGAGCA-3' were designed to amplify a 219 base pair region of the E2/E5 open reading frames. Tumor tissue for the PCR assays was obtained from FFPE blocks. Briefly, multiple 4µm sections were cut and purification was performed using NucleoSpin® DNA FFPE XS kit (Macherey Nagel, Milan, Italy) following manufacturer's instructions. To verify the DNA extraction and presence of BPV-1/-2-/13, all DNA extractions were subjected to qualitative β-actin and E2/E5 PCR, respectively.

7.1.6 Chromogenic In situ hybridization (CISH)

Chromogenic *in situ* hybridization was performed on full sections using the RNA scope kit (Advanced Cell Diagnostics, Hayward, CA). The target genes and probe regions were designed to hybridize to messenger RNA (mRNA) and viral genomic DNA of both BPV-1 and BPV-2 as previous reported.[122] Three-µm thick sections were deparaffinized in xylene, followed by dehydration in ethanol. Following endogenous peroxidase blocking, sections were incubated in 200 ml 1X Target Retrieval solution (Advanced Cell Diagnostic, Hayward, CA) maintained at 100°C to 103°C using a water bath for 15 minutes, rinsed in deionized water and immediately treated with 4 drops of protease plus (Advanced Cell Diagnostic, Hayward, CA) at 40°C for 30 minutes in a HybEZ hybridization oven (Advanced Cell Diagnostics, Hayward, CA). Target probe (4 drops) was added to the slides and hybridized at 40°C for 2 hours in the HybEZ oven. Amplification steps were performed at first in HybEZ hybridization oven at 40°C for 30 minutes (from AMP1 to AMP4) than at ambient temperature for 30 and 15 minutes for AMP5 and AMP6, respectively. Chromogenic detection was obtained by adding Red-working solution (60 µl) for 10 minutes at room temperature. Slides were counterstained with Gill's hematoxylin and mounted with VectaMount (Vector Laboratories, Burlingame, CA). Ec-PPIB probe (Equus caballus peptidylprolyl isomerase B (cyclophilin B) (PPIB) mRNA) was used as positive control to assess gene

housekeeping while *Bacillus subtilis* strain SMY methylglyoxal synthase dihydrodipicolinate reductase (dapB) gene was used as negative control. To analyze the probe signal a semi-quantitative score system was developed, utilizing the estimated number of punctate dots present in 10 high power fields (HPF) visible at 400x magnification (2,37mm^2). A score of 0 indicated absence of positive cells, a score of 1 indicated low numbers (20%–50%) of positive cells, a score of 2 identified an intermediate number (50%– 80%), and a score of 3 indicated that a high number (80% to 100%) of positive cells.[122] Moreover, the nuclear reaction pattern was classified as either diffuse or punctate: a diffuse signal throughout the nucleus was considered as diffuse pattern, while a dot-like signal within the cell nucleus was considered as punctate pattern.

7.1.7 Follow-up

Follow-up information was retrieved by telephone interview with the animal owners or referring veterinarians. Local recurrence (LR) and de novo occurrence (DNO) were defined as recurrence at the same site of previous sarcoid and occurrence of a distant sarcoid, respectively. These were recorded as present or absent. Time to recurrence (TR) was calculated as days elapsed from the histological diagnosis to LR/DNO. Cases in which no LR/DNO were recorded at the time of the study were censored.

7.2 Statistical analysis

Normality was evaluated with Shapiro-Wilk test. Mean and standard deviation (SD) were calculated for normally distributed data, while median and min-max were reported for non-normally distributed data. Chi-square test was performed for categorical variables; Kruskal-Wallis test was performed for continuous variables. Spearman correlation coefficient was calculated to compare continuous variables.

To evaluate LR/DNO Kaplan-Meier product-limit estimate were applied and compared by log-rank test. For each test, $p<.05$ was considered statistically significant.

7.3 Results

7.3.1 Signalament and clinical data

The study included 114 sarcoids from 88 equids (66 horses, 17 ponies and 5 donkeys). The age was known in 73/88 equids and ranged from 2 to 25 years old with a median age of 8 years. Sex was known in 72/88 cases of which 29 were female (40.3%), 13 (18%) were male and 30 (41.7%) neutered males. Among horses, Warmbloods (18.3%), Cobs (18.3%) and Thoroughbreds (12.7%) were the most represented breeds.

Of the 88 equids, 54 had one tissue sample included in the study and 14 had multiple tissue samples ranging from 2 to 6 per horse.

Four anatomical tumor locations were identified: head/neck region (29/114, 25.4%), genital area (24/114, 21.1%), abdomen/thorax (16/114, 14%) and limbs (13/114, 11.4%). In 32/114 (28.1%) cases location was unknown. Sarcoid clinical subtype was known in 74/114 cases and was categorized as follows: occult (4/114, 3.5%), verrucous (16/114, 14%), fibroblastic (9/114, 7.9%), nodular (35/114, 30.7%) and mixed (10/114, 8.8%).

Regarding the histological type and the anatomic location, all the cases were examined. Of the 114 sarcoids, the fibroblastic subtype was located mainly in the limbs (4/9, 44.4%) and the mixed subtype in the groin (6/10, 60%). Moreover, the nodular subtype was located mainly in head and neck region (12/35, 34.3%) and in the trunk (10/35, 28.6%) whereas the verrucous in the head and neck region (8/16, 50%) and in the groin (6/16, 37.5%).

Table 2 summarises the clinical data and histologic diagnosis.

Table 2. Clinical data and histological diagnosis of equine sarcoids.

Case ID	Breed	Age (years)	Sex	Tumor location	Histological type
170071			M	Lip	Verrucous
170105	Warmblood	7	F	Groin	Mixed
170122A	Sella Francais	6	F	Neck	Verrucous
170122B					Mixed
170135	Welsh D	16	MN	Ear	Mixed
170160	Warmblood	8	MN	Ear	Verrucous
170164	French WB	6	F	Abdomen	Mixed
170247	ND	2		Head	Mixed
170248	Pony	12	M	Eye	Mixed
170253	Fresian	12	M	Eye	Verrucous
170269A	Thoroughbred	10	MN	Chest	Nodular
170269 B, C				Limb	Mixed
170269 D-G				Limb	Mixed
170292 A	Warmblood	3	MN	Nose	Verrucous
170292 B				Eye	Mixed
170297 A	Dutch Warmblood	8	MN	Sheath/penis	Mixed
170297 C-E				Eye	Mixed
170376 C	Piebald Cob	14	MN	Abdomen	Mixed
170376 E				Sheath/penis	Mixed
170376 H				sheath/penis	Nodular
170390	Irish sport horse	10	MN	Neck	Mixed
170456	Warmblood	2	F		Nodular
170476				Thigh	Mixed
170511	Irish Cob	14	F	Limb	Verrucous
170535	Irish sport horse	15	MN	Sternum	Nodular
170567	Welsh D		MN	Ear	Verrucous
170570	Cob	7	F	Eye	Nodular
170591 A	Welsh D	2	F	Sheath/penis	Nodular
170591 C				Eye	Nodular
170603	Donkey	8	F	Nose	Mixed
170604	Sport Horse	11	M	Sheath/penis	Nodular
170628	Erisky	9	M	Sheath/penis	Mixed
170633	Welsh A	6	F	Abdomen	Nodular
170634	Irish sport horse	8	F		Nodular
170641	Donkey	8	F	Nose	Nodular
170661	Irish Sports	8	MN	Sheath/penis	Mixed
170813 A	Warmblood	4	M	Sheath/penis	Mixed
170813 C					Verrucous
170841	Thoroughbred	9	MN	Eye	Verrucous
170853				Thigh	Verrucous
170945	Andalusian	8	M	Thigh	Verrucous
170963	Cob	8	MN	Sheath/penis	Mixed
170969	Warmblood	5		Girth	Mixed
170971	Fell pony	4	F	Eye	Mixed
170997	Pony	18	F		Verrucous
171001	Warmblood	22	MN		Mixed
171008	Irish sport horse	17	F		Mixed

171009	Warmblood	25	F		Nodular
171011	Warmblood	16	MN		Verrucous
171017	Pony Shetland	23	F		Mixed
171037	Warmblood	7	MN		Nodular
171054	Welsh	5	F	Abdomen	Mixed
171056	Irish sport horse	3	M	Ear	Mixed
171141				Eye	Mixed
171167	Donkey	4	MN	Sheath/penis	Mixed
171221	Warmblood X	10	F	Teat	Nodular
171222	Thoroughbred	11	F	Abdomen	Mixed
171239		3	M	Sheath/penis	Mixed
171348	Andalusian	14	MN	Sheath/penis	Mixed
180103	Irish sport horse	8	MN	Thigh	Mixed
180117 A	Irish draught	4	MN	Sheath/penis	Mixed
180117 B					Mixed
180117 C				Sheath/penis	Nodular
180127	Welsh X	6	F		Nodular
180140	Clydesdale	7			Nodular
180142				Groin	Mixed
180143	Connemara				Nodular
180158	Pony	8		Head	Mixed
180199		3	M	Eye	Verrucous
180212 A-C	Donkey	6	MN	Scrotum	Nodular
180212 D-F				Scrotum	Nodular
180221	Thoroughbred X	12	F	Eye	Nodular
180225	Thoroughbred	17	MN		Nodular
180226	Quarter horse	14	MN	Sheath/penis	Mixed
180229 A	Cob	6	MN	Chest	Mixed
180229 D				Sheath/penis	Mixed
180229 E				Sheath/penis	Mixed
180229 F, G				Sheath/penis	Mixed
180265 A	Irish sport horse	6	MN	Ear	Verrucous
180265 C					Verrucous
180265 E					Verrucous
180295	Warmblood	9	F		Verrucous
180322	Warmblood	5	M	Sheath/penis	Verrucous
180369	Cob X	2	MN	Limb	Mixed
180443	Welsh	5	F	Abdomen	Verrucous
180446		12	MN	Axilla	Mixed
180455	Highland	3	MN	Sheath/penis	Mixed
180456					Nodular
180457					Mixed
180458 A					Nodular
180458 B					Mixed
180458 C					Nodular
180458 D					Nodular
180458 E					Mixed
180458 F					Nodular
180464 A					Mixed
180464 C					Mixed
180468 A					Mixed
180468 B					Nodular

180468 G					Verrucous
180502		17	MN	Head	Verrucous
180524	Warmblood	10	MN	Limb	Mixed
180536	Irish sport horse	8	F	Axilla	Nodular
180540	Warmblood	9	MN		Verrucous
180557	Highland	7	F	Abdomen	Nodular
180564	Highland	11	M	Eye	Verrucous
180565 A	Pony Shetland	14	F	Sheath/penis	Verrucous
180565 B				Sheath/penis	Mixed
180571	British	10	F	Eye	Mixed
180612	Hannoverian	18	F	Limb	Nodular
180646	Warmblood	4	F	Limb	Mixed
180654	Thoroughbred	10	F	Thigh	Nodular
AP20245	French Saddle Pony	17	F	Thigh	Mixed
AP20622	Sardinian donkey	6	M	Head	Mixed

7.3.2 Histological features

The predominant histological features in occult sarcoids were the presence of epidermal hyperplasia and interstitial fibroblast pattern which were both present in 4/4 cases (100%). Verrucous sarcoids were predominantly characterized by interstitial fibroblast pattern (15/16, 93.7%), hyperkeratosis and epidermal hyperplasia with rete pegs formation (14/16, 87.5%). The most prominent feature of nodular sarcoids was the presence of epidermal thinning (22/35, 62.8%) often with a neat separation between the dermis and the epidermis, while all the other features were detected in variable proportions. In fibroblastic sarcoids, the most prominent feature was the presence of epidermal ulceration (100%), while the other epidermal and dermal features were present in variable proportions. Similarly, mixed sarcoids showed a variable proportion of dermal pattern and epidermal features. Figure 2 shows the four histological types of sarcoids while Figure 3 the two different dermal pattern.

Surgical margins were complete in 50 cases, while in 49 cases they were incomplete. In 15 samples margins were not evaluated due to either partial sampling (incisional biopsy) or inadequacy of the sample.

Figure 2. A. Verrucous sarcoid, 4x. **B.** Nodular sarcoid, 4x. **C.** Occult sarcoid, 4x. **D.** Fibroblastic sarcoid, 4x. **E.** Mixed sarcoid, 10x.

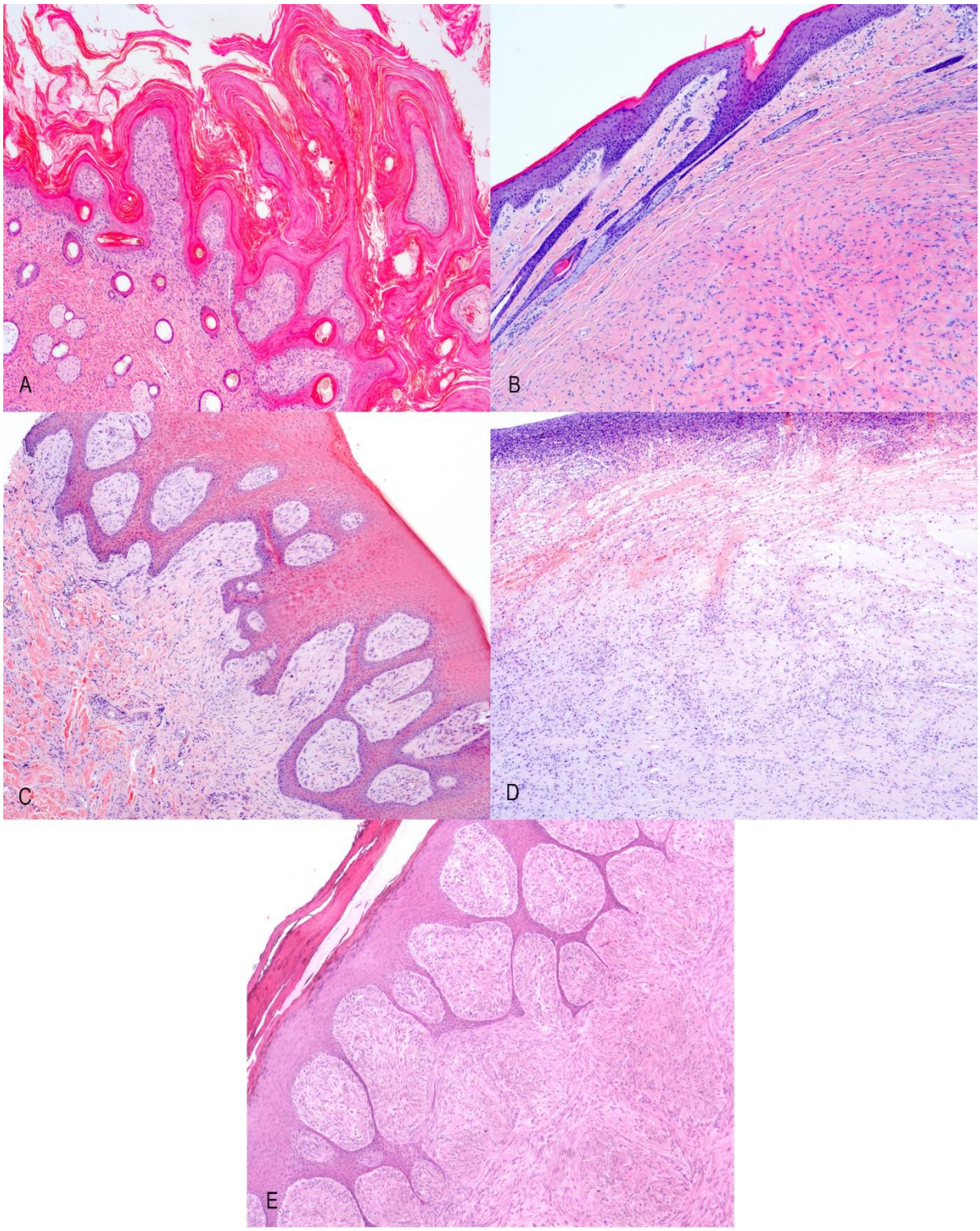
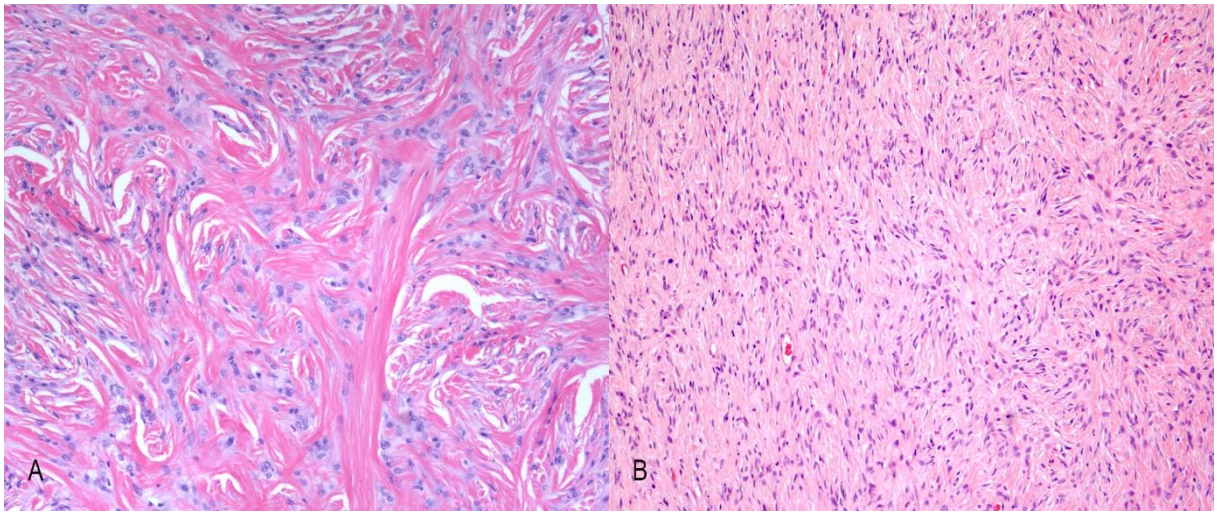


Fig 3. A. Interstitial pattern of fibroblasts, 20x. **B.** Fascicular pattern of fibroblasts, 4x.



Correlation between histological features and clinical subtype

To verify the presence of distinctive histological features in each clinical subtype, the frequency of each feature was evaluated according to the clinical aspect. Epidermal hyperplasia was significantly associated with occult and verrucous types (p=0.000 *Chi Square test*);picket fencing was significantly associated with epidermal hyperplasia (p=0.000 *Chi Square test*). By contrast, nodular type was significantly associated with decreased epidermal thickness (p=0.000 *Chi Square test*) (Figure 4). Regarding fibroblasts architecture, the interstitial pattern was significantly associated with both the verrucous (p=0.0003 *Chi Square test*) and occult subtypes (p=0.0014 *Chi Square test*). Figure 5 summarizes the association between dermal pattern and clinical types. No other histological features demonstrated a statistically significant association with clinical subtypes.

Figure 4. Association between clinical type and epidermal thickness.

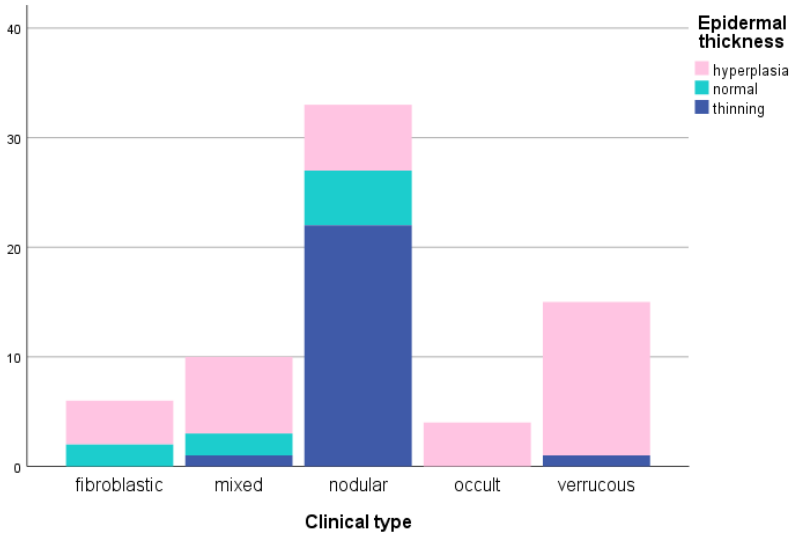
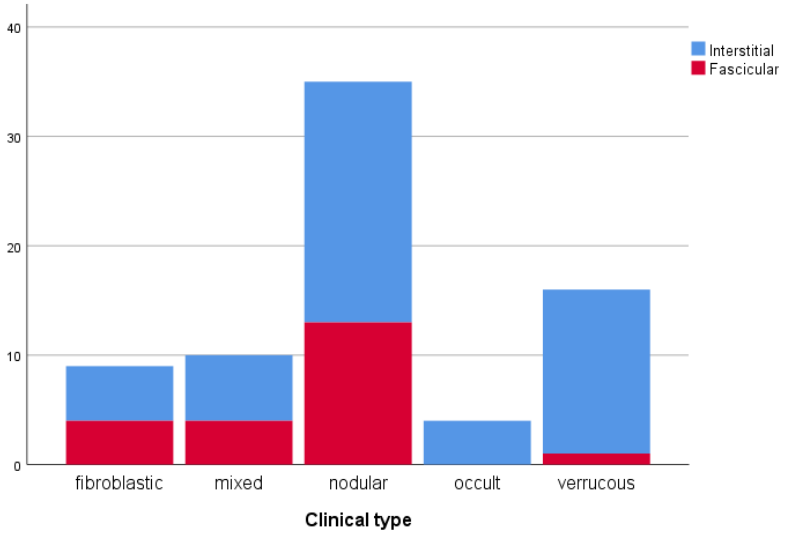


Figure 5. Association between clinical type and dermal pattern.



7.3.3 Immunohistochemistry

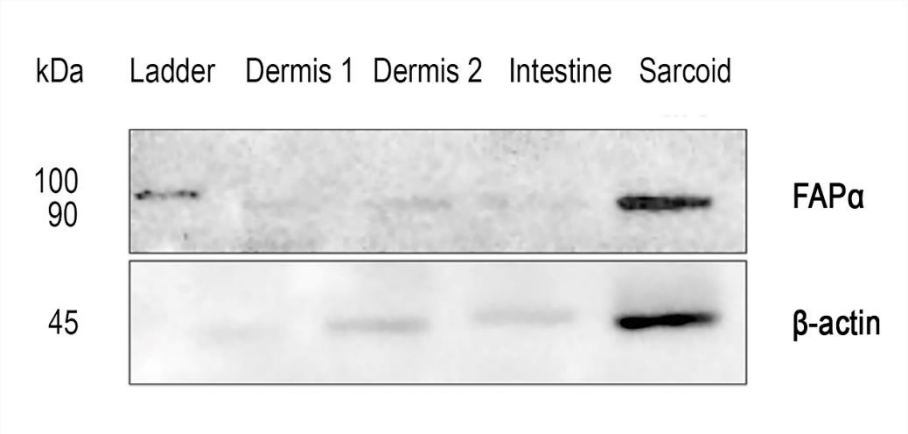
FAPα

Cross reactivity for FAPα was confirmed with Western blot analysis, which revealed a 90kD protein band that corresponded to the molecular weight of FAPα. A strongly

positive band was detected in the sarcoid sample, while in the dermis and intestine a weaker signal was present (Figure 6).

All 114 sarcoid cases labelled with FAPα, demonstrating the fibroblastic and/or myofibroblastic origin of neoplastic cells. FAPα protein was strongly expressed in the cytoplasm of neoplastic cells (Figure 7).

Figure 6. Western blot analysis of protein lysates from equine dermis (lanes 2 and 3), intestine (lane4) and sarcoid (lane 5). Marker proteins are shown in lane 1.

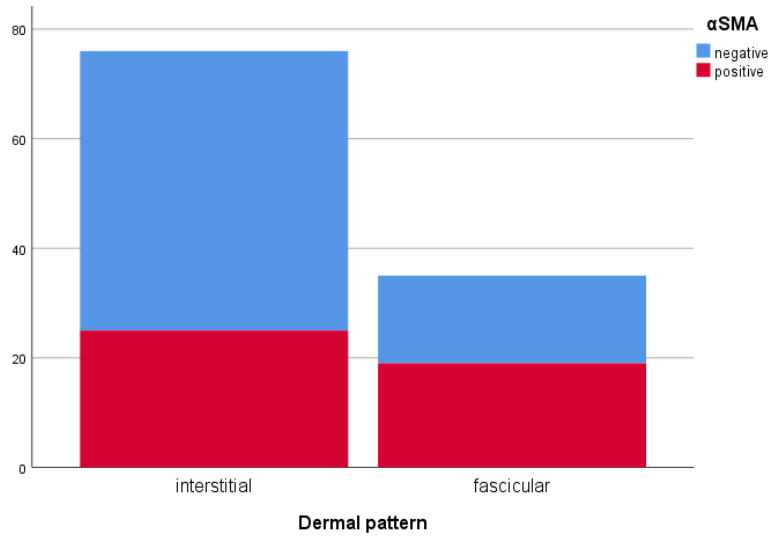


S100 and αSMA

All 114 equine sarcoids were negative for S100 and 45/114 (39.4%) of tumors were positive for αSMA, displaying variably intense cytoplasmic positivity (Figure 7). Three different tumor patterns were observed, diffuse intratumoral, peritumoral and subepithelial. According to the histologic subtypes, αSMA positivity was recorded as follows: 1/4 occult (25%), 4/16 verrucous (25%), 11/35 nodular (31.4%), 4/9 fibroblastic (44.4%) and 5/10 mixed (50%). Regarding αSMA immunolabelling, 24/ 44 (21.1%) cases had intratumoral pattern, 9/44 (7.9%) peritumoral pattern and 13/44 (11.4%) subepithelial pattern. Comparing αSMA positivity with dermal pattern, a statistically significant association was found between αSMA and the presence of streams and bundles (p=0.046 *Chi Square test*) (Figure 8). By contrast, no significant association was observed between either positivity (*p*=0.75 *Chi Square test*) or

distribution (*p*=0.46 *Chi Square test*) of αSMA labelling and clinical subtype respectively.

Figure 8. Association between dermal pattern and αSMA expression.



Cell cycle proteins p53, pRB, Cyclin D1 and CDK2NA

pRB immunohistochemistry was performed in 70cases. All cases were positive with intense nuclear labelling (Figure 9). Regarding pRB score, 32 (45.7%), 23 (32.8%) and 15 (21.4%) cases showed expression scores of 1, 2 and 3 respectively.

Cyclin D1 immunohistochemistry was performed in 73 cases. Strong nuclear Cyclin D1 immunolabelling was observed in all cases (Figure 9). Regarding Cyclin D1 score, 34/73 (46.5%) cases had score 2. The other positive cases had score 3 (27/73, 13.2%) or score 1 (12/72 (16.4%). Forty-one (for Cyclin D1) and forty (for pRB) cases were excluded from IHC due to insufficient tissue. Table 3 summarizes Cyclin D1 and pRB scores.

Table 3. Summary on pRB and Cyclin D1 scores in equine sarcoids, performed on 70 and 73 cases respectively.

		pRB	Cyclin D1
Score	1	32 (45.7%)	12 (16.4%)
	2	23 (32.8%)	34 (46.5%)
	3	15 (21.4%)	27 (36.9%)
Total		70	73

Immunohistochemistry for CDKN2A was performed in 56 cases. Positive immunolabelling was found in 45/56 (90.4%) cases whereas 11/56 (9.6%) were negative. In 27/45 (60%) cases CDKN2A immunolabelling was exclusively nuclear whereas 18/45 (40%) cases displayed both nuclear and cytoplasmic immunolabelling (Figure 9).

Regarding CDKN2A score, 19/56 (16.7%) cases had score 1. The other positive cases had either score 2 (14/56, 12.3%) or score 3 (12/56, 10.5%).

Cyclin D1 and pRB scores were positively correlated with each other (p=0.044, *Spearman correlation test*); moreover, inverse correlation between Cyclin D1 and pRB scores and p16 positivity was found (p=0.01, *Spearman correlation test*).

Moreover, p53 was negative in all the cases.

Ki-67 index

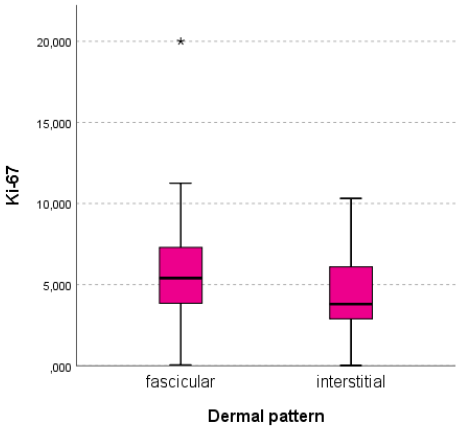
Immunohistochemistry for Ki-67 was performed in 109 cases. The median of Ki-67 positve cells was 4.73% (range 0.016%-20%). Statistical analysis revealed a strong correlation between Ki-67 index and the histological type: low Ki-67 index was prevalent in occult subtype compared to the mixed (*p*=0.15 *Kruskal-Wallis test*), nodular (*p*=0.01 *Kruskal-Wallis test*) and fibroblastic (*p*=0.01 *Kruskal-Wallis test*) subtypes. Similarly, verrucous sarcoids had low Ki-67 index compared to mixed (*p*=0.07 *Kruskal-Wallis test*), nodular (*p*=0.01 *Kruskal-Wallis test*) and fibroblastic (*p*=0.01 *Kruskal-Wallis test*) subtypes.

In equids with multiple sarcoids, Ki-67 index was not associated with the presence of multiple tumors (*p*=0.15 *Kruskal-Wallis test*).

Regarding fibroblasts dermal pattern, a statistically significant correlation between high Ki-67 index and fascicular pattern (p=0.03 *Kruskal-Wallis test*) was found (Figure 10). By contrast, no significant association was found between Ki-67 index and CDKN2A positivity (p=0.92 *Kruskal-Wallis test*) nor with αSMA immunolabelling (p=0.94 *Kruskal-Wallis test*).

Taking the median as cut-off value, cases were further divided in low and high proliferation. Based on this variable, 42/88 (47.7%) had high proliferation rate and 42/88 (47.7%) low proliferation.

Figure 10. Distribution of Ki-67 index and fibroblasts pattern.



7.3.4 Polymerase Chain Reaction (PCR)

Eighty-seven (87) sarcoids from 85 horses were tested by PCR for BPV1/2/13. In equids with multiple sarcoids, only one tumor was tested by PCR except in 2 cases. The presence of BPV-DNA was confirmed in 82/87 samples. Of the 5 samples that tested negative, 2 were also negative for β-actin, indicating unsuccessful DNA extraction. In the remaining 3 cases, 2 equids had multiple tumors of which at least one tested BPV-positive by PCR. Furthermore, sequencing of six cases suggested the presence of viral DNA referable to BPV-1.

7.3.5 Chromogenic In situ hybridization (CISH)

CISH was performed in 106 cases in which sufficient FFPE material was available. A positive hybridization signal was observed in 101/106 (95.2%) cases. Positivity appeared as strong nuclear and occasionally finely granular punctate dots in the cytoplasm of neoplastic fibroblasts (Figure 7). Labelling was categorized into four grades as previously reported:[122] 5/106 cases (4.7%) were negative, 15/106 cases (14.2%) were scored 1, 17/106 cases (16,1%) were scored 2, and 69/106 were scored 3 (65.1%) (Table 4). Of the 106 samples that were tested by CISH, the clinical subtype was known in 70 cases; to assess whether the different clinical subtypes had different viral DNA distribution, the distribution of CISH positivity was evaluated within the tumor: 61 (87.1%) had a diffuse, uniform BPV DNA distribution throughout the tissue (Figure 7) while the remaining 9 (12.9%) cases showed a predominantly subepithelial CISH positivity (Figure 7) No association was observed between BPV DNA distribution and clinical subtype, although the 9 cases that had a predominantly subepithelial distribution all displayed picket fencing. Of these, 5 cases were verrucous, 1 was occult and 3 were mixed. However, no significant association was observed between the histological variables and nucleic acid distribution. In addition, no signal was observed in the epidermis overlying the neoplasm nor in the adnexa and/or dermis surrounding the neoplasm.

Of the 106 cases that were tested by CISH, 83 were also tested with PCR. 82/87 had concordant results, with 80 cases being positive for both CISH and PCR, 2 cases negative for both, 3 cases CISH+/PCR- and 2 cases CISH-/PCR+ (Table 5).

Table 4. *In situ* hybridization signal score of 106 tested sarcoids.

Score	No. of cases	%
0 (negative)	5	4.7
1	15	14.2
2	17	16.1
3	69	65.1
Total	106	100

Table 5. Comparison of PCR and CISH results for the detection of BPV-DNA in 83 cases

	CISH+	CISH-	Total
PCR+	80	2	82
PCR-	3	2*	5
Total	83	4	87

*β-actin negative

Figure 6. **A.** αSMA cytoplasmic immunoreactivity of myofibroblasts, 10x **B**. FAPα nuclear and cytoplasmic immunoreactivity of interlacing streams and bundles of neoplastic fibroblasts, 10x. C. Nodular sarcoid showing diffuse BPV distribution within neoplastic fibroblasts. Chromogenic *in situ* hybridization (CISH), BPV 1/2 probes, 4x. **D**. Mixed type sarcoid. Strong hybridization signal in the dermo-epidermal junction. CISH, BPV 1/2 probes, 4x. **E**. Strong hybridization signal within the nucleus of neoplastic fibroblasts. CISH, BPV 1/2 probes, 100x. **F**. Large pleomorphic fibroblasts with strong cytoplasmic hybridization signal. CISH, BPV 1/2 probes, 20x

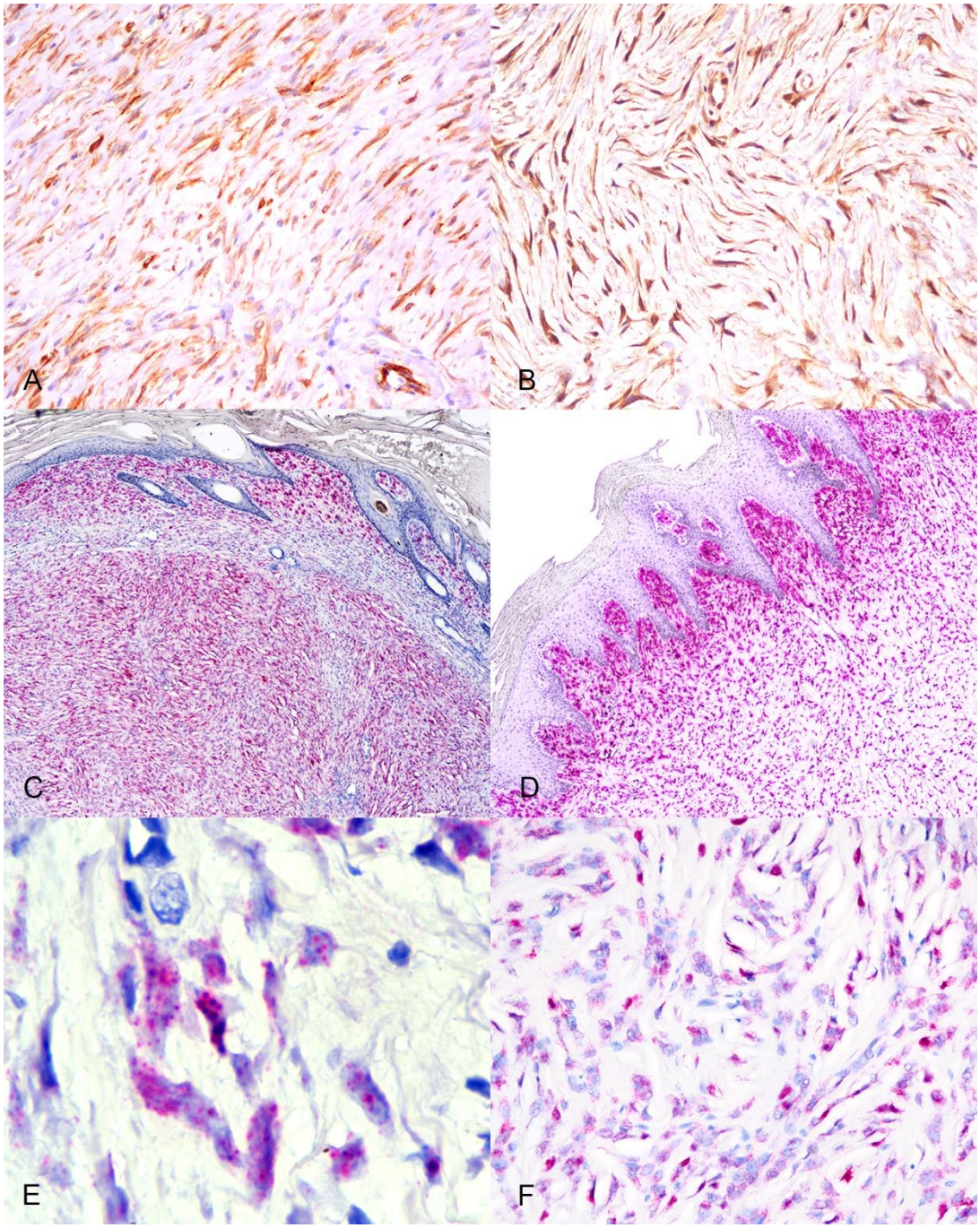
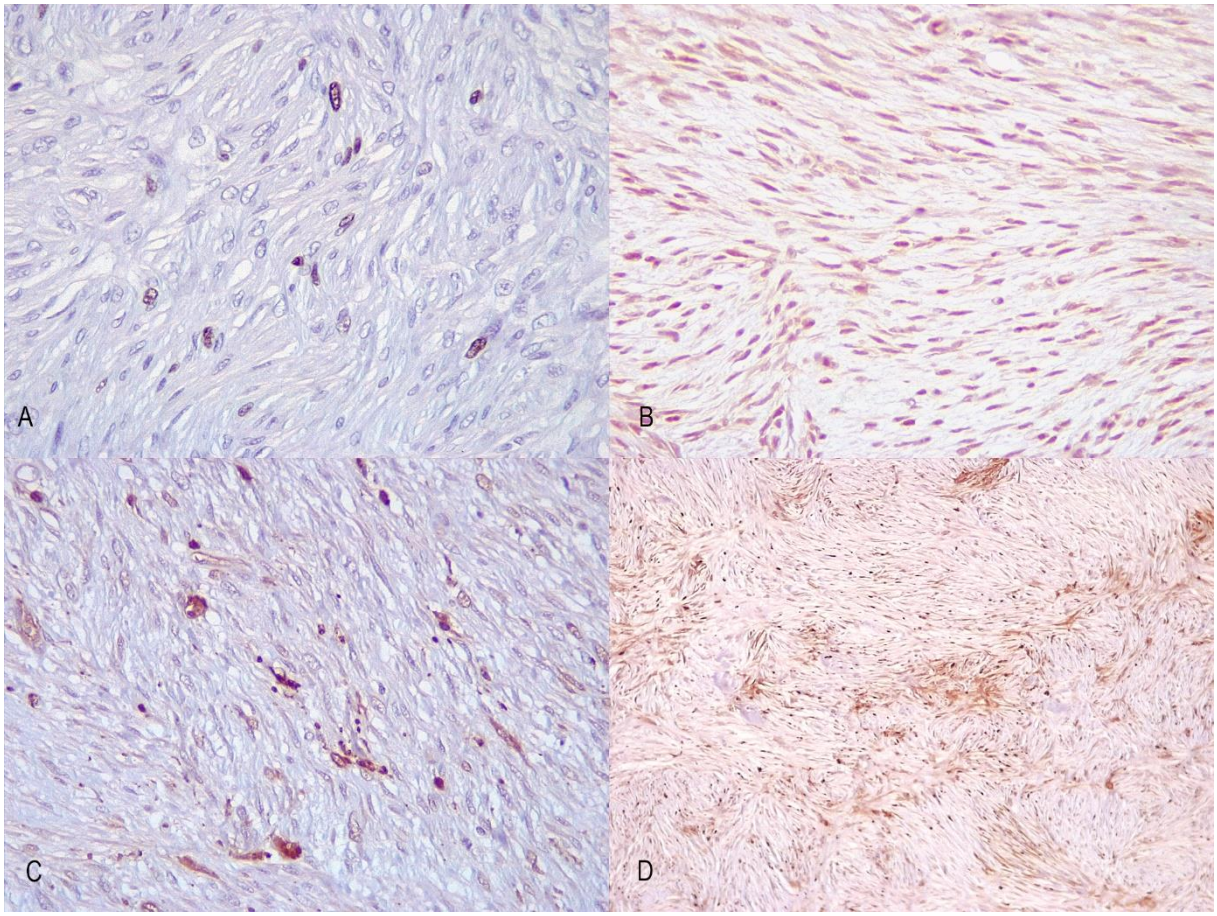


Figure 8. **A.** Strong pRB nuclear immunoreactivity of neoplastic fibroblasts, 20x. **B.** Cyclin D1 nuclear immunoreactivity of neoplastic fibroblasts, 10x. **C.** Strong CDKN2A nuclear immunoreactivity of neoplastic fibroblasts, 20x. **D.** CDKN2A nuclear and cytoplasmic immunoreactivity of neoplastic fibroblasts, 10x.



7.3.6 Analysis of follow-up

Follow-up information regarding recurrence was obtained in 29/114 cases. Among them, recurrence was confirmed by clinical examination or histology in 14 cases. Of these, 9 recurred on the same site, while in 5 horses new sarcoids were observed in distant sites (de-novo occurrence). Recurrence sites were as follows: groin (4/15, 26.7%), head and neck (3/15, 20%), limbs (3/15, 20%) and thorax (3/15, 20%). Regarding clinical classification, recurrence was recorded in 7/13 (53.8%) fibroblastic, 2/13 (15.4%) mixed and 4/13 (30.8%) verrucous sarcoids. Following resection, median LR/DNO time were 92 days for fibroblastic sarcoids, 65 days for mixed and 95 days for verrucous.

Comparing clinical type with tumor LR/DNO, statistical analysis revealed high tendency of recurrence to de-novo occurrence in verrucous sarcoids (5/6, 83.3%), and to recur locally for fibroblastic and mixed sarcoids (7/7, 100% and2/2, 100% respectively). By contrast, no recurrence was observed in nodular (12/12, 100%) and occult (2/2, 100%) types.

For equids with multiple sarcoids, recurrence was recorded in 6/29 (20.7%) cases while in 23/29 (79.3%) cases recurrence was not recorded. Although a higher proportion of equids with multiple sarcoids experienced tumor recurrence, the association was not statistically significant (p=0.54 *Chi square test*) (Table 6).

Moreover, LR/DNO rates were evaluated based on the completeness of excision margins: statistical analysis revealed that complete margins were not associated with local tumor recurrence (Chi-square test, p=0.33). In detail, of the 9 cases that recurred locally, 6 had incomplete surgical margins; similarly, of the 15 cases that did not recur, 7 had incomplete margins.

Although not statistically significant, higher Ki-67 index was identified in without recurrence (8/29, 27.6% and 10/29, 34.5% respectively) (p=0.59 *Chi square test*). The only histological variable correlated with higher LR/DNO rates was increased epidermal thickness (p=0.46 *Log-rank test*). Moreover, recurrence was not associated with immunohistochemical variables CDKN2A, pRB, Cyclin D1 and αSMA.

Table 6. Summary on recurrence in five clinical types of sarcoids.

	Local Recurrence (LR)	**De-novo occurrence**	**No recurrence**
Occult	0/2	0/2	2/2
Verrucous	0/5	5/5	1/6
Nodular	0/12	0/12	12/12
Fibroblastic	7/7	0/7	0/7
Mixed	2/2	0/2	0/2
Total	9/29	5/29	15/29

7.4 Discussion

Clinical types of sarcoids do not differ histologically

Although the histological features of equine sarcoids are well characterized, there is no well documented association between the clinical types and their histological features. The identification of clinical types of sarcoids by histopathology would be important to correctly classify the tumor types and verify any association with behaviour and/or response to therapy.

However, as previously reported, histopathologic features of sarcoids can be highly variable: epidermal hyperplasia with rete peg and picket fence formation are often present in the verrucous histological type;[31] epidermal ulceration and adjacent hyperplasia are commonly reported in fibroblastic sarcoids while epidermal thinning is usually described for the nodular type.[215] Moreover, in occult sarcoids, increased dermal density obscuring follicular units and adnexa seems to be the only distinctive feature. Lastly, the mixed type, the most variable type of sarcoid, can display all the previously reported histological characteristics.[31,215] The presence of an increased fibroblastic density remains the only common feature among the different types.[215]

Although in the present study we identified a number of features that were predominantly expressed in one clinical type compared to others, no single histological feature was exclusive to a single subtype. In particular, in our study interstitial pattern of neoplastic fibroblasts was observed in 100% of occult and 93.7% of verrucous clinical types. Moreover, increased epidermal thickness was recorded in 100% and 87.5% occult and verrucous types, respectively.

The inability to correctly identify tumor subtypes histologically was already documented by Bogaert *et al* and is further confirmed here in a larger number of cases. However, the presence of interstitial fibroblast arrangement and epidermal hyperplasia supports a diagnosis of verrucous and occult sarcoid; in addition, fibroblasts arranged in streams and bundles are more supportive of a diagnosis of fibroblastic, nodular or mixed subtype.

In addition, none of the cases included in the study had features of malignant (malevolent) sarcoid, confirming that this subtype is extremely rare.

αSMA, S100 and FAPα for the diagnosis of sarcoids

Several studies in the literature have highlighted the importance of finding reliable markers for the diagnosis of sarcoids. Earlier studies stated that BPV DNA can be found in other spindle cell tumors of horses[107], and similarly that the presence of BPV DNA does not guarantee a diagnosis of sarcoid without histological examination.

Since the publication on a series of equine schwannomas[284], much attention has been given in finding reliable markers to distinguish between schwannomas and sarcoid. In this respect, the combined S100 negativity and PCR positivity for BPV-DNA has been deemed as sufficient to solve this issue.[27] Indeed, in our study all cases were negative to S100, excluding Schwann cell origin of tumors. Regarding the positivity of other types of spindle cell tumors to BPV, the authors of the article did not specify the criteria used to classify the tumors;[107] it is reasonable to think that all BPV-positive spindle cell tumors should be diagnosed as sarcoids until proven otherwise.

In the attempt to finding additional and more reliable immunohistochemical markers to support the diagnosis of sarcoids, we further investigated FAPα expression in all our samples.[94] FAPα is a serine integral membrane proteinase encoded by FAP gene.[250] It is selectively expressed in reactive fibroblasts of epithelial tumors and malignant cells of bone and soft tissue sarcomas with no expression in normal fibroblasts or in other stromal tissues.[276] Recent studies also demonstrate the oncogenic role of FAP-expressing cells, enhancing tumor proliferation and tissue invasion.[79,195] In all the analysed cases, high FAP expression was recorded in neoplastic fibroblasts compared to normal skin fibroblasts. This could include FAP as a potential marker for sarcoid diagnosis. However, the inability to test other mesenchymal tumors in the same species, does not allow to confirm its usefulness in the differential diagnosis with other spindle cell neoplasms.

Sarcoids are also known to express αSMA.[27,210] In a 2011 study, 8/10 (80%) sarcoids positively labelled for αSMA, demonstrating a possible smooth muscle cells or pericyte origin of neoplastic fibroblasts.[27] More recently, Martano *et al* confirmed the expression of αSMA in 5/25 (20%) equine sarcoids. In our study, αSMA positivity was detected in 44/114 (38.6%) sarcoids. Positivity to αSMA appeared to be uniformly distributed across clinical subtypes, hence it cannot be used to differentiate clinical subtypes. However, its expression was associated with the presence of dermal streams and bundles, suggesting that this pattern may reflect an enhanced myofibroblastic differentiation of neoplastic cells.

Cell cycle proteins pRB, Cyclin D1 and CDKN2A (p16) expression are altered in a subset of sarcoids

In human papillomavirus (HPV) infections, the study of cell cycle proteins is used to predict the prognosis, especially for head and neck and cervical squamous cell carcinomas.[191,341,348] In HPV-induced tumors, pRB is degraded by HPV E7 resulting in CDKN2A (p16) overexpression. Moreover, when CDKN2A is overexpressed, Cyclin D1 is downregulated.[101] Therefore, in HPV-induced tumors, Cyclin D1 and pRB levels are typically low.[101] In this study, Cyclin D1 and pRB were positive in all cases with different scores. As expected, pRB and Cyclin D1 were positively correlated to each other, and their decrease was significantly associated with p16 positivity, either at nuclear or cytoplasmic level. Fan *et al* indicated that BPV-1 E7-induced cell proliferation could occur in the absence of pRB, although it could not completely rule out the possibility that some of the proliferative activity could involve Rb inactivation. In our study, the inverse correlation between CDKN2A and pRB-Cyclin D1 may indicate that pRB inactivation may occur and might be associated with CDKN2A accumulation. However, it must be noted that contrarily to what is observed in HPV-related cancer, accumulation of CDKN2A was observed both in the nucleus and cytoplasm, and if only cytoplasmic accumulation was considered, statistical significance was lost. Based on these findings, we speculate that a pRB-mediated pathway of tumorigenesis is not

excluded in BPV-induced sarcoids. In the same study, Fan *et al*, found a Cyclin D1 and Cyclin D-associated kinase downregulation, which would support our findings, at least in a subset of cases. Further studies are required to evaluate additional cell-cycle associated molecules, to verify their role in tumorigenesis and behaviour.

For completeness, p53 immunohistochemistry was also performed in all cases included in the study. In contrast with previous investigations, in which p53 positivity was observed in 22/50 (4.4%) cases, our results indicate that p53-mediated mechanisms of tumorigenesis do not occur in sarcoids. Our results are also supported by the lack of p53 mutation reported from Bucher et al. (1996).[47]

Ki-67 proliferation index is not useful as diagnostic or prognostic marker

In human and veterinary medicine the Ki-67 index is a widely used prognostic indicator of malignancy.[193] In horses only few studies reported the use of Ki-67 index to assess the degree of tumor proliferation.[29,215,242] Ki67 proliferation index through was analysed in 50 sarcoids, in which there was high variability of Ki-67 staining among the cases. All the cases had total mean proliferative fraction of 1.44%, slightly lower to our proliferative index results of 4,7% across all cases.[215] Similarly, in a 2004 study, Ki-67 expression ranged from 5 to 11%.[242] Our results confirm data reported in literature that in general, sarcoids have low proliferate rates. This finding parallels the clinical behavior of this tumor, typically characterized by a slow growth.

Ki67 expression was compared with other clinical pathological variables, but no associations were found, except for a higher Ki67 index in sarcoids displaying a fascicular dermal pattern. The reason of this finding is unclear as no increased recurrence rates were found associated with this feature. Overall, Ki67 does not seem to be useful in the diagnostic process and does not seem to predict clinical behaviour.

BPV distribution does not influence sarcoid histomorphological features

In the present study we analysed the link between viral distribution pattern and histological appearance of the tumor, to prove if BPV distribution has the ability to

influence the development of different morphological types of sarcoids. Of the 106 analyzed cases, 87.1% showed diffuse strong positivity for BPV by CISH and in only 12.9% the positivity was confined to the subepithelial areas, independently of the clinical type. In addition, no difference was found between signal distribution pattern and clinical types. Furthermore, to verify whether epidermal hyperplasia could be induced by the presence of the virus within the epidermis, CISH signal was searched in this location. In contrast to other studies, no hybridization signal was detected in epidermis, nor in the adnexa within and at the periphery of the tumor.[32,122] Older studies have used different laboratory techniques to localize BPV-DNA within the lesions, leading to contrasting results. In a previous study, laser-microdissection was used to separate the mesenchymal and epithelial components of sarcoids to better investigate the localization of BPV. After real-time PCR, 40% of keratinocytes of early stage sarcoids contained BPV-DNA, but in lower copy number than fibroblasts.[32] Moreover, immunohistochemistry for E5 and E7 oncoprotein positively labelled neoplastic fibroblasts and epidermal cells. The presence of viral oncoproteins in epithelial cells, could partially explain the pathogenetic mechanism of sarcoids characterized by a productive phase in the epidermis followed by release of virions by keratinocytes.[39] In another study, the presence of BPV-DNA in equine sarcoids was further investigated using CISH. In all the analyzed cases viral nucleic acid was detected in epidermis, sebaceous glands, and hair follicles.[122] However, in our study, no hybridization signal was detected in the epidermis nor in the adnexa, in any case. Hence, based on our results, we speculate that the epidermal hyperplasia of sarcoids may merely depend on the presence of neoplastic fibroblasts in close contact with the epidermis rather than being induced by the virus within keratinocytes. This also supports the hypothesis that the development of different clinical types may not depend on viral distribution.

In our study we also did not detect any viral nucleic acid in the tissues surrounding the tumors. This would suggest that local recurrence of sarcoids may not depend on the persistence of the virus in the tissue surrounding the lesion.

This is in contrast with other studies where the presence of virus was detected in the tissue adjacent to the tumor by PCR. In one study, surgical margins were evaluated in 19 horses of which 529 of margins were positive for BPV DNA: of these, the tumor margin was positive at 4, 8, 12, and 16 mm in, respectively, 95%, 73%, 39%, and 33% of the examined sarcoids. Local recurrence was observed in 3 sarcoids on 3 different horses in the same study.[212]

The reason of this discrepancy on margin positivity might be due to the different sensitivity of the techniques used to detect viral nucleic acid.

Finally, in our series of cases the vast majority of sarcoids had high nucleic acid content, that was visible in the majority of neoplastic fibroblasts, either in the nucleus or in the cytoplasm. Viral load was previously correlated with disease severity; in a study involving 38 horses, those affected by quiescent, slowly growing single tumors or multiple mild-type lesions had low viral load, while those that had rapidly growing and/or multiple aggressive sarcoids had a significantly higher viral content. In our study though, CISH score was not found to correlate with the other clinical or molecular features.[133]

Clinical types have different tumor recurrence features

Equine sarcoids are non-metastatic yet locally aggressive tumors, showing a high tendency of recurrence after surgical treatment.[170] So far, few reports have been published that correlate tumor recurrence with clinical types and molecular variables. Our results indicate that both local recurrence and occurrence in other sites are potential outcomes of sarcoids. In our case series, of the 29 horses with follow up data, nearly half recurred either locally or at distant sites.

In the literature, recurrence rates of sarcoids are highly variable. A study conducted by Compston *et al*, investigated recurrence of histologically confirmed sarcoids after laser surgery. Of 235 cases analyzed, they recorded that 82% did not recur locally and 72% did not occur in other sites.[72] Additionally, in a 2016 study, of 230 equids with 614 sarcoids treated with electrosurgical excision, 460/614 (74.9%) had no tumor

recurrence.[135] Vingerhoets *et al*, reported that 81% of 59 horses treated by CO_2 laser surgery were free from recurrence after 12 months.[337]

In our study we also observed that different clinical types appeared to have different outcomes. Specifically, nodular sarcoids did not tend to recur after surgical excision; fibroblastic and mixed sarcoids tended to recur locally, while in the majority of verrucous and occult sarcoids, de novo occurrences were recorded. This is in contrast with Compston *et al* study where verrucous and head and neck sarcoids were at increased risk of local recurrence.[72] In a 2019 study, Knottenbelt reported that with surgical excision the rate of recurrence was as high as 70%, often with development of a more aggressive type (e.g. fibroblastic).[174] In agreement with our study, verrucous and occult sarcoids did not tend to recur locally.[172]

We also investigated whether recurrence and *de-novo* occurrence were more frequent in horses already bearing multiple sarcoids, but we found no association between these two features. Other histological and molecular variables were compared with recurrence/*de-novo* occurrence data, but no statistically significant associations were found. Regarding time to recurrence, these did not differ significantly and had an overall median time of 92 days. In an epidemiological study, of 192 treated sarcoids, recurrence was recorded in 3 cases after 1 year.[321]

In addition, we evaluated whether surgical margins were predictive of local recurrence, but we observed that tumors tended to recur locally irrespective of the completeness of surgical margins.

It must be underlined that there are several limitations to the analysis of the follow-up data in our study which may have affected the quality of the results: firstly, only in a small proportion of cases we were able to retrieve follow up data; moreover, the excision technique was not specified, and we were not able to verify the clinical and pathological features of the recurred sarcoids. Hence, further follow-up data and analysis of recurred tumors will eb necessary to confirm our findings.

7.5 Conclusions

To conclude, our results indicate that there are no histological features that can confidently be used to classify the clinical types of sarcoids. In parallel, viral distribution and content does not seem to influence the histopathological features of the tumors.

A subset of sarcoids is associated with low pRB and Cyclin D1 scores and parallel increased CDKN2A expression. This could suggest that at least a proportion of sarcoids may be associated with pRB-dependent oncogenic mechanisms, but further studies are necessary to confirm this hypothesis. Finally, a subset of sarcoids recurred after excision. Of these, the fibroblastic and mixed subtypes had the highest local recurrence rates, the occult and verrucous were associated with development of other sarcoids at distant sites, while none of the nodular sarcoids recurred.

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