The association of *Felis catus* papillomavirus type 2 infection in feline cutaneous squamous cell carcinomas and its premalignant lesions (Bowenoid *in situ* carcinoma and actinic keratosis)

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Statement of Originality

This is to certify that to the best of my knowledge, the content of this thesis is my own work. This thesis has not been submitted for any degree or any purposes, other than for the aforementioned degree in the submission statement.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Alexander Teh

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Thesis Abstract/Summary

Background

Feline cutaneous squamous cell carcinomas (SCC) are the most common cutaneous malignant skin neoplasms of cats, comprising approximately 15% of feline skin neoplasms. Previous studies have demonstrated a potential causative role of infection with feline papillomaviruses, in particular *Felis catus* papillomavirus type-2 (FcaPV-2), in the development of cutaneous SCCs and its premalignant lesion Bowenoid *in situ* carcinoma (BISC). More recent studies in the human literature have also suggested a potential role of human papillomavirus (HPV) in the development of another premalignant skin lesion actinic keratosis (AK).

Study Aims

Firstly, to demonstrate the association between FcaPV-2 infection and cutaneous SCC oncogenesis in a population of Australian cats. Secondly, to explore whether there may be a possible association between FcaPV-2 infection and the development of AKs, similar to what has been recently reported in the human literature with HPV infection. Thirdly, to clarify whether p53, a key tumour suppressor protein, is altered as part of the feline papillomaviral infection pathogenesis.

Methodology

Immunohistochemistry (IHC) for p16 and p53 was performed on a variety of formalin-fixed paraffin embedded (FFPE) feline skin lesions including premalignant AKs and BISCs, and UV-exposed SCCs and UV-protected SCCs. IHC detection of p16 is often used as a proxy marker for papillomaviral infection in both human and veterinary pathology. Quantitative

polymerase chain reaction (qPCR) was also performed to detect viral DNA in the lesions. Correlation of the results of IHC and qPCR was then performed, combining the effect of viral infection (IHC) with the presence of viral DNA (qPCR), and the results were analysed using odds ratios, and Fisher's exact probability.

Major Findings

The IHC study demonstrated that feline BISCs exhibited positive p16 labelling at higher rates compared to AKs ($p = 0.0030$) but there was no statistically significant difference in p16 labelling between UV-exposed and UV-protected SCCs ($p = 0.0593$). IHC for p53 was unsuccessful. The qPCR study demonstrated that UV-protected SCCs were more likely to contain FcaPV-2 DNA compared to UV-exposed SCCs ($p = 0.0095$) but there were no statistically significant differences in viral DNA detection between BISCs and AKs ($p = 0.9512$). When IHC and qPCR results were combined together, no statistically significant difference was observed in FcaPV-2 aetiology (positive for both FcaPV-2 DNA by PCR and p16 labelling by IHC) between feline BISCs and AKs ($p = 0.1723$). However, there was a statistically significant difference observed in FcaPV-2 aetiology between UV-protected and UV-exposed cutaneous SCCs ($p = 0.0236$). Therefore, FcaPV-2 infection appears to be associated with the development of BISCs, AKs, and UV-protected SCCs but not UV-exposed SCCs. This is suggestive that FcaPV-2 infection may be involved in the development of more cutaneous lesions in felines than what is currently expected.

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Chapter 1. General Overview and Introduction

Lesions Involving Cutaneous Squamous Epithelial Proliferation – a Spectrum Ranging from Hyperplasia to Invasive Squamous Cell Carcinoma

The stratified squamous epithelium of the epidermis is subject to a wide range of lesions ranging from hyperplasia to dysplasia to premalignancy to invasive squamous cell carcinoma (SCC), and it is always best to view these lesions as different points on a spectrum rather than discrete lesions independent of each other (Hargis and Thomassen 1979; Meuten 2017). Epidermal hyperplasia is a very common and non-specific change involving orderly proliferation of epidermal cells, usually cells of the stratum spinosum, which can be accompanied by hyperkeratosis, resulting in a thickened epidermal layer (Meuten 2017). This can occur secondary to a wide variety of underlying causes such as inflammation, hypersensitivities, chronic irritation/trauma, and others (Bradley, Mauldin and Morris 2023). Viral papillomas and viral plaques are papillomaviral-associated lesions that evoke this response (Wilhelm et al. 2006).

Epidermal dysplasia refers to a disorderly development and proliferation of keratinocytes of the epidermis (Meuten 2017). Whilst dysplasia can also develop in response to a broad range of injurious stimuli such as inflammation, dysplasia is also a feature seen in premalignant lesions such as actinic keratoses (AKs) and Bowenoid *in situ* carcinomas (BISCs) (Favrot et al. 2009; Wilhelm et al. 2006). Histologically, dysplasia is characterised by the "jumbling" of cells of the epidermis resulting in disorganisation of the different layers (Wilhelm et al. 2006).

Cutaneous SCCs commonly progress from early premalignant lesions – AK and BISC (Favrot et al. 2009). Actinic keratoses are associated with chronic UV exposure, displaying many histological features that are related to solar damage such as epidermal hyperplasia, acute inflammation, spongiosis, and scattered necrosis of keratinocytes in the early stages; and mononuclear infiltration and dermal scarring in chronic stages. Solar elastosis is not usually a significant feature unlike human AKs (Mauldin and Peters-Kennedy 2016). In contrast, BISCs do not have an association with solar-induced damage, and the presence of feline papillomaviral DNA has been detected frequently in these lesions, creating speculation of their papillomaviral aetiology (Munday et al. 2007; Vascellari et al. 2019). Common histological features of BISCs include dysplasia and hyperplasia of both the epidermis and follicular outer root sheath adjacent the basement membrane (Mauldin and Peters-Kennedy 2016). Feline AKs usually exhibit less epidermal hyperplasia and lesser involvement of hair follicles compared to BISCs (Favrot et al., 2009). Nevertheless, it is often difficult or at times not possible to distinguish between AKs and BISCs histologically due to many overlapping features (Conceição et al., 2007; Favrot et al., 2009). Whilst feline and human AKs are accepted to be associated with UV-damage, multiple studies have demonstrated the presence of human papillomavirus (HPV) DNA within these premalignant lesions in people, suggesting that HPV infection may play an additive carcinogenic role to UV-damage in the development of AKs (Dianzani et al. 2017; Dianzani et al. 2008; Galati et al. 2020; Schneider et al. 2013; Struijk et al. 2006). Comparatively, the possible role of *Felis catus* papillomavirus (FcaPV) infection and feline AKs has not been thoroughly explored in the literature.

Cutaneous SCCs represent approximately 15% of all feline skin neoplasia and are the most common malignant cutaneous tumours of cats (Miller et al. 1991; Munday et al. 2009; Munday and Thomson 2021; Murphy 2013; O'Neill et al. 2011; Teh and Krockenberger 2021). SCCs are highly malignant neoplasms of stratified squamous epithelium that usually produce keratin (Fania et al. 2021; Gudenschwager-Basso et al. 2022). Due to their highly invasive behaviour, feline SCCs are a significant cause of morbidity and mortality in cats (Munday and Thomson 2021; Teh and Krockenberger 2021). Cutaneous SCCs commonly present as plaques or papillary masses usually associated with variable degrees of ulceration, erythema, alopecia, and crusting (Chandrashekaraiah et al. 2011). The most common SCC variants are well differentiated, moderately differentiated, and poorly differentiated (Nemec et al. 2012). Other uncommon SCC variants include clear cell, acantholytic, spindle cell, carcinoma arising in Bowen's disease, keratoacanthoma-like, verrucous, desmoplastic, and mucin-producing (Chandrashekaraiah et al. 2011; Nemec et al. 2012).

Cutaneous SCCs additionally represent a significant concern for humans, representing the second most common malignancy globally, and are common cancers in Australia (Corchado-Cobos et al. 2020; Fania et al. 2021; Parekh and Seykora 2017; Waldman and Schmults 2019; Wilson et al. 2022). Indeed, the incidence of cutaneous SCC in Australia is most likely significantly higher than what is currently reported, and the frequency in humans is increasing worldwide likely due to population aging and increased skin cancer screening (Corchado-Cobos et al. 2020; Fania et al. 2021; Wilson et al. 2022).

Cutaneous SCCs, across both human and veterinary medicine, exhibit relatively similar behavioural features. Most cutaneous SCCs are locally invasive within the dermis, which can often lead to regional destruction of the soft tissues, cartilage, and bone, causing devastating effects to the patient (Corchado-Cobos et al. 2020; Fania et al. 2021; Gudenschwager-Basso et al. 2022). Whilst metastasis is uncommon compared to oral SCCs, regional metastasis is more often observed in cases of poor differentiation and/or advanced stages (Fania et al. 2021; Gudenschwager-Basso et al. 2022). Surgical excision of the lesion is the usual first line treatment for most SCCs, however a number of them can recur and metastasise leading to death, especially because excision may prove difficult due to the locally invasive nature of the neoplasm (Fania et al. 2021).

There are a number of established risk factors for the development of SCC in animals which include chronic exposure to ultraviolet (UV) radiation, lack of epidermal pigmentation, and sparsely haired areas of skin (Munday and Thomson 2021; Murphy 2013). These are similar to risk factors observed in humans which include cumulative exposure to UV radiation, age, male sex, immunosuppression, and smoking (Corchado-Cobos et al. 2020; Fania et al. 2021). Indeed, the risk of cutaneous SCC development for cats with white fur is far greater than cats with other coat colours (Murphy 2013). The incidence of cutaneous SCC tends to increase with age, and the median age of affected cats is between 10 to 12 years (Murphy 2013).

Papillomaviruses

Papillomaviruses are non-enveloped double-stranded circular DNA viruses that infect almost all species of animals, often with multiple papillomaviral types (Geisseler et al. 2016; Harden and Munger 2017; Hoggard, Munday and Luff 2018; Munday and Thomson 2021; Sundberg et al. 2000). They are highly species and tissue-specific viruses that infect keratinocytes at cutaneous and mucosal sites and reproduce within keratinocyte nuclei (Geisseler et al. 2016; Hoggard, Munday and Luff 2018; Munday and Thomson 2021). The type and extent of disease induced by papillomaviral infection is variable, depending on the rate of epithelial replication that the virus can stimulate (Hoggard, Munday and Luff 2018; Munday and Thomson 2021). Most cases of papillomaviral infection involve slow viral replication thus resulting in mild epithelial replication which is not observable clinically (McBride 2017; Munday and Thomson 2021; Wierzbicka, San Giorgi and Dikkers 2023). This low level infection occurs as the virus attempts to establish itself in the basal cells of the epithelium, undergoing limited amplification and anchoring itself in the host nucleus by attaching to host chromatin (McBride 2017). Here, viral DNA is maintained at a low and constant copy number whilst the basilar epithelial cells do not differentiate into cells of the upper epithelial layers (McBride 2017). This asymptomatic infection is very common in both humans and domestic species (McBride 2017; Munday and Thomson 2021). However, a small number of papillomaviral types cause rapid viral and epithelial replication, commonly manifesting as hyperplastic viral papillomas or plaques (Hoggard, Munday and Luff 2018; Munday and Thomson 2021). This occurs as the infected basilar epithelial cells differentiate into cells of the upper layers, whereby the infected cells amplify the viral DNA to a high copy number, packaging them into progeny viral particles, finally permitting shedding of the virions from the sloughed epithelial squames (McBride 2017).

Due to papillomavirus interference with the regulation of cell replication, they can contribute to the development of neoplasia (Hoggard, Munday and Luff 2018; Munday and Thomson 2021; Teh and Krockenberger 2021). This is widely recognised in humans, and human papillomaviruses (HPV) are the most common viral causes of cancer and are the aetiological agent in most cervical SCC and many oral SCCs (Harden and Munger 2017). Around the world, it is estimated that 500,000 cases of cervical cancer are diagnosed each year resulting in over 250,000 deaths (Harden and Munger 2017). Neoplasia associated with papillomavirus infection is also recognised in domestic animal species such as dogs, cats, pigs, sheep, and cattle (Munday and Thomson 2021).

Cutaneous Squamous Cell Carcinomas – Interactions Between Host Genetics, Oncogenesis, and Papillomavirus Infection

1. Host Genetics and Oncogenesis

The pathogenesis of SCCs in animal species is not as well established as that of human SCC (Munday and Thomson 2021; Teh and Krockenberger 2021). This section will present the molecular pathogenesis of human SCC and laboratory animal models of oncogenesis, with comparisons to what is currently known about how feline papillomaviruses contribute to malignant transformation. The discussion on the molecular pathogenesis of SCC will focus on the interactions between host genetics and mutational/epigenetic changes that lead to oncogenesis.

The pathogenesis of cutaneous SCCs involves a complex multistage process, primarily with mutations in genes involved in cell cycle control and epidermal homeostasis, with an additive effect of epigenetic changes often also contributing (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Further demonstrating how these cutaneous lesions exist on a continuous spectrum, AKs also share similarities with cutaneous SCCs at the genomic level (Wang, Z. et al. 2024). The most frequently mutated gene in cutaneous SCCs and AKs is TP53 which encodes p53, and a mutation in this gene occurs in approximately 54-95% of human cases (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022; Wang, Z. et al. 2024). The p53 protein is a crucial tumour suppressor protein which exerts its regulatory effects primarily in the cell cycle (Wang, H. et al. 2023). Upon the detection of cell stressors such as DNA damage or hypoxia, p53 is able to induce cell cycle arrest to permit DNA repair, or direct the cell towards senescence or apoptosis (Moulder et al. 2018; Wang, H. et al. 2023). A mutation in TP53 is

commonly caused by UV-induced damage involving the formation of pyrimidine dimers in DNA (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). TP53 mutations are usually early events in SCC carcinogenesis but are responsible for significant genomic instability, allowing keratinocytes to avoid apoptosis and promote unregulated clonal expansion of mutated cells (Corchado-Cobos et al. 2020; Fania et al. 2021; Wang, Z. et al. 2024). Another relatively commonly mutated gene is the cyclin-dependent kinase inhibitor 2A (CDK2NA) locus which has been observed in approximately 31% of metastatic and primary cutaneous SCCs in people (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). The CDK2NA locus gene encodes the tumour suppressor proteins p16INK4a (p16) and p14ARF (p14) which help to regulate the cell cycle by tampering the effects of cyclin dependent kinases (CDK), proteins which drive the events of the cell cycle (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). p16 is a CDK inhibitor regulating the G1/S checkpoint of the cell cycle by influencing pRb as previously elaborated, and p14 is produced in response to mitogenic stimulation whereby it inhibits HDM2 forming a stable complex (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). HDM2 degrades p53 and therefore p14-mediated inhibition of HDM2 permits p53 activity leading to cell cycle arrest or apoptosis (Corchado-Cobos et al. 2020; Fania et al. 2021). Thus, a loss-of-function mutation in the CDK2NA locus ultimately leads to dysfunction of the key tumour suppressor proteins p16 and p14 and ultimately uncontrolled cell growth as there is no longer appropriate p14-mediated inhibition of HDM2, allowing the cell cycle to progress in an unregulated manner (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022).

Mutations in other tumour genes such as NOTCH and the rat sarcoma (RAS) oncoproteins can occur which are proteins involved in various essential cell functions such as proliferation, differentiation, apoptosis, and maintenance (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022; Shah et al. 2020). Loss-of-function mutations in both NOTCH1 and NOTCH2 in particular have been observed in more than 75% of cutaneous SCCs in people (Corchado-Cobos et al. 2020; Fania et al. 2021). A loss-of-function of NOTCH1 may have several effects including disruption of keratinocyte differentiation, reduces transcription of the tumour suppressor protein IRF6, and upregulates the Wnt/beta-catenin pathway promoting tumour development (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Thus, dysfunction of the NOTCH signalling pathway has been associated with the progression of SCC and impaired cellular differentiation (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Mutations in the RAS family most commonly occur in the HRAS gene in humans which has been implicated in cutaneous SCC initiation and the malignant transformation of keratinocytes, possibly doing so by activating another pro-growth pathway – mitogen activated protein kinase (MAPK) (Campos et al. 2020; Corchado-Cobos et al. 2020; Fania et al. 2021).

Epidermal growth factor receptor (EGFR) overexpression can be observed frequently in cutaneous SCCs which can occur due to dysfunctions in the functions of the RAS-RAF-MEK-MAPK, PLC-gamma/PKC, PI3K-AKT-mTOR, STAT and NF-kB signalling pathways, all of which are frequently altered in cutaneous SCCs (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Ultimately, alterations in these pathways and EGFR expression result in cellular proliferation, survival, migration, avoidance of apoptosis, and

impaired differentiation (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022).

Epigenetic modifications refer to alterations in the mechanisms that regulate gene expression without changes to DNA (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Such modifications include DNA methylation and histone modifications such as ubiquitination, methylation, acetylation, chromatin remodelling, and phosphorylation (Corchado-Cobos et al. 2020; Cozma et al. 2023; Fania et al. 2021; Hedberg et al. 2022). Common examples of genes that often are affected by epigenetic changes include hypermethylation of p16, p14, CDH1, RB1, MGMT and RASSF1 (Corchado-Cobos et al. 2020; Fania et al. 2021). All of these genes are involved in DNA repair, signal transduction, regulation of the cell cycle, and epithelial adhesion (Corchado-Cobos et al. 2020; Fania et al. 2021). Epigenetic modifications can also occur in microRNAs and CpG islands (Corchado-Cobos et al. 2020; Fania et al. 2021).

2. Papillomaviruses and SCCs

In HPV infections, the viral protein E6 promotes: cell immortalisation through increasing the transcription of hTERT, and degradation and ubiquitination of p53 by mediating its binding to ubiquitin ligase E6AP, removing a key regulator of the cell cycle and ultimately contributes to uncontrolled cellular proliferation (Altamura et al. 2018; Graham 2017; Munday and Aberdein 2012; Patel et al. 1999; Zimmermann et al. 1999). Additionally, E6 can further interfere with p53 function through the binding of acetyltransferase CBP/p300, inhibiting p53 transcriptional activity, inducing conformational changes and sequestering the protein in the cytoplasm (Graham 2017; Patel et al. 1999; Zimmermann et al. 1999). As a

result of p53 degradation, ubiquitination, and functional interference by the oncoprotein E6, p53 immunoreactivity is not observed within HPV-induced SCCs (Munday and Aberdein 2012). However, the immunohistochemical expression of p53 is less straightforward in veterinary medicine (Munday and Thomson 2021; Teh and Krockenberger 2021). A previous study demonstrated that p53 levels remained unaffected in FcaPV-infected cutaneous SCC lesions via immunohistochemistry, and that there was no significant association between FcaPV-infected SCCs and the presence or absence of p53 immunostaining (Munday and Aberdein 2012). This suggests that FcaPV does not degrade p53 in the same manner that high risk HPVs do (Munday and Aberdein 2012). However, more recent *in vitro* molecular studies appear to conflict with this suggestion and have demonstrated that the E6 oncoprotein can possibly promote intracellular p53 degradation (Altamura, Corteggio and Borzacchiello 2016; Altamura et al. 2018). This is an area of aetiopathogenesis that is currently not yet fully clarified (Teh and Krockenberger 2021).

In the conceptual model of HPV oncogenesis, the E7 oncoprotein causes functional inactivation of the pRb protein by binding to the retinoblastoma susceptibility protein via cullin 2 ubiquitin ligase complex which results in the rapid degradation of pRb via ubiquitination (Chung et al. 2014). This simultaneously displaces the E2F transcription factor from the transcriptional repression complex with p16 (Graham 2017; Stephen et al. 2013). Displacement of E2F permits it to activate other cell cycle-related genes such as cyclins A and E which promote cell progression through the G1/S checkpoint (Stephen et al. 2013). Furthermore, E7 can interfere with E2F inhibitory transcription complex activity which leads to enhancement of promoter activity of growth control genes (Graham 2017). Since the function of p16 is also dependent on pRb, the functional loss of pRb results in a marked

accumulation of intracellular p16, thought to be due to a negative feedback loop (Chung et al. 2014; Stephen et al. 2013). This large intracellular p16 accumulation can be detected via immunohistochemistry and is commonly used as a surrogate/proxy marker for an active PV infection in both humans and cats (Stephen et al. 2013; Chung et al. 2014; Geibler et al. 2013; Munday and Thomson 2021). Additionally, when HPV integrates its viral DNA into the host genome during infection, the viral E2 gene, an inhibitor controlling the promoter of E7, is disrupted, causing increased expression of E7 (Chung et al. 2014).

The pathogenesis of papillomavirus infection and its link to oncogenesis is not as well understood in the veterinary literature compared to human medicine, and it is not known to what extent papillomavirus infection contributes to neoplastic transformation in animals (Munday and Thomson 2021; Teh and Krockenberger 2021). In humans, high-risk HPV strains (types 16 and 18) are implicated in the development of SCC of the cervix, head and neck, and the anogenital region (Graham 2017). In cancerous lesions associated with high-risk HPV infection, the viral genome is integrated into the host genome which subsequently results in loss of the E2 viral repressor protein and increased expression of the E6 and E7 genes (Graham 2017; Pal and Kundu 2019). It is the E6 and E7 genes that contribute to the oncogenic potential of the virus with the ultimate outcome of immortalising cells and markedly increasing cell proliferation (Graham 2017; Pal and Kundu 2019). The E6 protein binds to and degrades p53 - the tumour suppressor gene and key regulator of the cell cycle (Graham 2017; Pal and Kundu 2019). Simultaneously, E6 triggers the expression of telomerase reverse transcriptase (TERT) which contributes to cell immortalisation (Altamura, Corteggio and Borzacchiello 2016; Graham 2017; Pal and Kundu 2019). The E7 protein binds to the pRb and displaces the E2F transcription factors which help promote cell

progression through the cell cycle (Altamura et al. 2016; Graham 2017; Pal and Kundu 2019). Additionally, E7 inactivates the cyclin-dependent kinase (CDK) inhibitors p21 and p27 which results in increased CDK4/cyclin D complex formation which further contribute to progression through the cell cycle (Pal and Kundu 2019).

Most domestic cats are infected with feline papillomavirus, however only a small number develop disease (Geisseler et al. 2016; Munday and Thomson 2021; Teh and Krockenberger 2021). Immunosuppression is considered a likely risk factor that predisposes cats to the development of papillomaviral-induced disease (Mauldin and Peters-Kennedy 2016; Munday and Thomson 2021). Lesions where feline papillomavirus is either known or suspected to influence the onset of disease include viral papillomas, viral plaques, BISCs, feline sarcoids, and SCCs (Mauldin and Peters-Kennedy 2016). In viral-induced lesions, certain viral cytopathic effects may be observed histologically in keratinocytes within the epidermis (Munday and Thomson 2021; Wilhelm et al. 2006). These include the presence of enlarged cells with expanded grey-blue cytoplasm; cells with a perinuclear clearing and a shrunken hyperchromatic nucleus (koilocyte); enlarged, prominent, and/or irregular keratohyalin granules, and intranuclear inclusion bodies (Wilhelm et al. 2006). Histological observation of these viral cytopathic effects is suggestive of a papillomaviral aetiology (Wilhelm et al. 2006).

Biomarkers of SCC Neoplasia – p53 and p16

The p53 protein is a key tumour suppressor protein which is often mutated in various neoplastic diseases, especially those induced by UV damage (Munday and Aberdein 2012; Park et al. 2004). p53 regulates progression of cells through the cell cycle and maintains genetic integrity (Campos et al. 2020; de Bakker et al. 2022; Einspahr et al. 1997). Specifically, p53 is a regulator of the G1/S phase of the cell cycle which is a transition phase between cell growth and DNA replication (Campos et al. 2020; de Bakker et al. 2022; Einspahr et al. 1997). In response to DNA damage detected at the G1/S checkpoint, p53 can arrest the cell cycle, initiate DNA repair proteins such as GADD45, or induce apoptosis of cells in response to irreparable DNA damage (Benzerdjeb et al. 2021; Lu, El-Mofty and Wang 2003; Munday and Aberdein 2012). Mutations of the p53 gene disrupt normal function of the protein resulting in a stable protein which can be detected via immunohistochemistry (Finlay et al. 1988; Munday and Aberdein 2012; Ziegler et al. 1994).

The p16 protein is another key tumour suppressor protein that functions at the G1/S phase of the cell cycle similar to p53. p16 is a CDK inhibitor and slows down the cell cycle by inactivating the cyclin D/CDK4 complex (Geibler et al. 2013). Inactivation of the cyclin/CDK complexes by p16 prevents phosphorylation of the retinoblastoma protein (pRb) which in turn binds and inhibits E2F transcription factors which prevents progression of cells through the G1/S checkpoint (Geibler et al. 2013). Therefore, the p16 and pRb proteins function synergistically as another key regulatory pathway of the G1/S cell cycle transition phase (Benzerdjeb et al. 2021; Geibler et al. 2013). Previous studies in feline SCCs have largely demonstrated a significant association between FcaPV DNA and p16 immunostaining

suggesting similarities with the HPV model of oncogenesis with regards to the E7-pRb-p16 degradation pathway (Munday and Thomson 2021; Teh and Krockenberger 2021). In cats, the G175-405 clone has been demonstrated to cross-react with feline p16 and is therefore confidently utilised for the immunohistochemical detection of p16 overexpression (Munday, Gibson and French 2011; Munday and Thomson 2021). Similar to human papillomavirus associated disease pathogenesis, p16 labelling is also commonly utilised as a proxy marker of FcaPV aetiology in feline SCCs (Munday and Thomson 2021).

Feline-Specific Papillomavirus Studies

Whilst the role of HPV infection and cancers in humans (especially cervical cancer) is well established, the veterinary literature currently provides moderate strength of evidence to support the oncogenic potential of FcaPV in feline SCCs (Teh and Krockenberger 2021).

Initial studies that explored the role of FcaPV infection and cutaneous SCCs were important in identifying the early link between PV DNA within feline SCCs. Several of these early studies utilised PCR to demonstrate an association between feline SCCs and the presence of PV DNA, including FcaPV-2 (Munday et al. 2008; Munday et al. 2007; Nespeca et al. 2006; O'Neill et al. 2011). One of these studies also included AKs in the investigation in addition to SCCs (O'Neill et al. 2011). However, whilst PCR techniques were able to identify association between the presence of PV DNA and cutaneous SCCs, it was difficult to attribute the presence of PV DNA in feline SCCs to an active causative role, since FcaPV-2 can also be isolated from the skin of many clinically normal cats (Munday and Kiupel 2010; Munday, Sharp and Beatty 2019; Thomson, Munday and Dittmer 2016). This makes it difficult to conclude whether the presence of viral DNA in the SCC lesions is due to it being an active carcinogen, an innocent bystander, or a transient infection (Hoggard, Munday and Luff 2018). Regardless, these early studies were critical in identifying the association and allowed for further exploration into FcaPV's pathogenesis and oncogenic potential.

To provide stronger evidence of the oncogenic role of FcaPV in feline SCCs, later studies began to explore the effects of viral infection on host cellular proteins (Munday and Aberdein 2012; Munday et al. 2011; Munday, Gibson and French 2011). Specifically of interest were proteins involved in cell cycle regulation and tumour suppression – p16, p53,

and pRb; all of which are known to be affected by the PV oncoproteins E6 and E7 in the HPV model of oncogenesis (Munday and Aberdein 2012; Munday et al. 2011; Munday, Gibson and French 2011). Numerous IHC studies which looked at the expression of p16, p53, and pRb found that cutaneous SCCs containing FcaPV DNA also concurrently overexpressed p16 with a simultaneous reduction in pRb (Munday and Aberdein 2012; Munday et al. 2011; Munday, Gibson and French 2011). The detection of FcaPV-2 DNA in combination with p16 overexpression was also reportedly more common in UV-protected feline SCCs compared to UV-exposed SCCs, suggesting a likelihood of underlying FcaPV-2 aetiopathogenesis (Munday, Gibson and French 2011). Interestingly, one IHC study found that p53 levels appeared to be unaffected in SCCs with FcaPV DNA (Munday and Aberdein 2012). This was particularly interesting given that in the HPV model of oncogenesis, the E6 oncoprotein is known to bind to and inhibit p53, resulting in reduced p53 levels and concurrent overexpression of the CDK inhibitor p16 (Graham 2017; Isaacson Wechsler et al. 2012; Lu, El-Mofty and Wang 2003). Reduced expression of pRb however shares similarities with the pathogenesis of HPV in which the E7 oncoprotein binds to and inhibits the function of pRb (Graham 2017; Munday et al. 2011; Munday and Aberdein 2012). Another IHC study found that AKs overexpressed p53 and not papillomaviral antigen, and conversely BISCs did not tend to overexpress p53 but exhibited papillomaviral antigen immunoreactivity (Favrot et al. 2009). A limitation of this study was the use of IHC for papillomaviral L1 antigen. The L1 antigen is only produced in the late stages of viral replication, and therefore if viral replication is not present within a lesion (which is usually the case in PV-induced cancers), the L1 antigen cannot be detected (Munday and Thomson 2021). Furthermore, as there are no specific antibodies against feline PV types, it is possible that there is a lack of cross-reactivity between the antibody and PV antigen, and thus it can be difficult to definitively

conclude true negative results (Munday and Thomson 2021). Thus, these IHC studies were crucial in determining that the effects of FcaPV involved disruption of the pRb/p16 pathways similar to HPV, however the lack of p53 degradation was intriguing.

Additionally, other studies attempted to further explore the presence and effects of the FcaPV E6 and E7 oncogenes on the development of cutaneous SCC in cats (Altamura, Corteggio and Borzacchiello 2016; Altamura et al. 2016; Hoggard, Munday and Luff 2018; Thomson, Munday and Dittmer 2016). Collectively, these studies were able to demonstrate the presence of E6 and E7 transcriptionally active FcaPV-2 with simultaneous p16 overexpression, the presence and unregulated transcription of E2, E6, and E7, and the ability of the E6 and E7 oncoproteins to disrupt the p53 and pRb pathways *in vitro* (Altamura, Corteggio and Borzacchiello 2016; Altamura et al. 2016; Hoggard, Munday and Luff 2018; Thomson, Munday and Dittmer 2016). Another study highlighted a key finding which appears to differ from the HPV pathogenesis model (Altamura, Corteggio and Borzacchiello 2016). This study found that the FcaPV-2 E6 oncoprotein was able to upregulate both the RAF/MAPK and PI3K/Akt pathways independently of EGFR (Altamura, Corteggio and Borzacchiello 2016). This is a different mechanism to HPV in which the upregulation of these two signalling pathways depends on EGFR (Altamura, Corteggio and Borzacchiello 2016). Both the RAF/MAPK and PI3K/Akt signalling pathways are critical for the promotion of cell growth and survival, and inhibition of apoptosis (Ranieri et al. 2013).

A couple of previous studies with overlapping methodologies to the current research were identified (Mazzei et al. 2018; Thomson, Munday and Dittmer 2016). In one of these studies, a main limitation were small sample sizes of both the SCC and AK categories (four SCCs and

one AK) which restricted the strength of the conclusions that could be drawn from the findings (Mazzei et al. 2018). Furthermore, of the four SCCs included, they were all from UV-exposed areas, and so the results pertaining to the SCCs in this study are difficult to extrapolate to all feline SCCs which encompass both UV-exposed and protected areas (Mazzei et al. 2018). The other study did not investigate AKs, and the number of PV-induced premalignant lesions included in the sample size were limited (Thomson, Munday and Dittmer 2016). Nevertheless, these two studies were important in demonstrating the usefulness of qPCR and correlation of viral loads with p16 expression in a variety of feline skin lesions. A recent case report demonstrated an aetiological role of a novel papillomavirus (FcaPV-6) in an Australian cat through PCR and p16 immunostaining associated with a squamous cell carcinoma (Carrai et al. 2020), but no other study specifically examined the role of FcaPV infection in the development of skin lesions amongst an Australian feline population.

The current veterinary literature collectively suggests a possible or likely role of FcaPV infection in the oncogenesis of feline cutaneous SCCs. Whilst there may be some differences with the HPV model of oncogenesis, such as whether p53 is degraded by E6, and the upregulation of the pro-growth and survival pathways RAF-MAPK and PI3K/Akt with or without EGFR; these studies were able to demonstrate a number of important findings that support FcaPV's oncogenic potential. However, there are still multiple areas that require clarification/further investigation such as whether p53 is degraded or unaffected as part of the oncogenic process; whether FcaPV is involved in the development of the premalignant AK; and whether an aetiological link can be demonstrated in an Australian feline population.

These are knowledge gaps and/or areas for further investigation that are hoped to be addressed by this study.

Study Overview – Aims, Justification, Methodology, and Hypotheses

1. **Broad Study Aims and Justification**

The study aims to address the following points: 1) to confirm the association between FcaPV-2 infection and the development of cutaneous SCCs in a population of Australian cats; 2) to investigate whether there may be a possible association between FcaPV-2 infection and the development of AKs, similar to what has been recently reported in the human literature with HPV infection; and 3) to clarify whether p53, a key tumour suppressor protein, is altered as part of the pathogenesis of FcaPV-2 infection.

These are critical gaps to address given the high prevalence of both cutaneous SCCs and FcaPV-2 infection in felines, and the significant impact on feline morbidity and mortality associated with SCCs (Geisseler et al. 2016). Whilst it is known that papillomaviruses can be involved in the pathogenesis of SCCs in felines and other species including people, to what extent viral infection is truly involved particularly in different parts of the epithelial proliferation spectrum and in other premalignant lesions such as AKs is not fully understood. By identifying whether FcaPV-2 infection can act as a co-factor in addition to UV-damage in feline AKs, this may be suggestive that the virus has a significantly greater role in feline SCC carcinogenesis than what is currently known. Furthermore, with the exception of a recent case report identifying a novel papillomavirus associated with SCC in a cat, this would represent the first study exploring this potential aetiology within a population of Australian cats (Carrai et al. 2020).

2. **Specific Study Aims**

The aims of this study were to describe the expression of the proteins p16 and p53 by immunohistochemistry (IHC), and the presence of FcaPV-2 DNA by quantitative polymerase chain reaction (qPCR) in feline cutaneous squamous cell carcinomas (SCC) and its premalignant lesions: actinic keratosis (AK) and Bowenoid in situ carcinomas (BISC) in a population of Australian cats.

Due to the many overlapping histological features between feline BISCs and AKs, further elaboration on p53 overexpression in feline AKs may provide a useful avenue for the differentiation between the two tumours (Conceição et al. 2007; Favrot et al. 2009).

3. Methodology

The chosen methodology of anti-p16 and p53 IHC in combination with qPCR to amplify a portion of the FcaPV-2 L1 gene would provide evidence of both the effects of viral infection (detectable by IHC) and presence of viral DNA (detectable by qPCR). Immunohistochemistry and qPCR were performed on formalin-fixed paraffin-embedded (FFPE) tissues from feline cutaneous SCCs (both UV-exposed and UV-protected) and the premalignant lesions BISCs and AKs.

4. Hypotheses

It was hypothesised that positive p16 labelling and lack of p53 labelling would be detected in UV-protected SCCs and BISCs via IHC in conjunction with detectable viral DNA. Conversely, in AKs and UV-exposed SCCs, there would be negative p16 labelling, and variable p53 labelling depending on the presence of UV-damage in combination with low to undetectable viral DNA.

Chapter 2. Expression of p16 and p53 in Feline Cutaneous Squamous Cell Carcinomas, Bowenoid *in situ* Carcinomas, and Actinic Keratoses by Immunohistochemistry

Introduction

Feline squamous neoplasia are common skin diseases with complex aetiology, that includes agents of disease ranging from chronic ultraviolet (UV) radiation exposure to viral infections. Among these, actinic keratoses (AKs), Bowenoid *in situ* carcinomas (BISCs) and squamous cell carcinomas (SCCs) represent a spectrum of common skin lesions from pre-malignant to malignant epithelial proliferations. BISCs and UV-protected SCCs are thought to be associated with feline papillomavirus (FcaPV) infection, whereas AKs and UV-exposed SCCs are often linked to chronic UV exposure (Teh and Krockenberger 2021).

In cell cycle regulation, there are two key tumour suppressor proteins involved – p16 and p53. The p16 protein is encoded by the CDKN2A gene and is commonly overexpressed in lesions associated with FcaPV infection (Munday and Thomson 2021; Teh and Krockenberger 2021). This is due to the effect of the E7 viral oncoprotein which disrupts and degrades the retinoblastoma protein (pRb) resulting in a compensatory increase in p16 levels (Teh and Krockenberger 2021). Indeed, p16 overexpression is often used as a surrogate/proxy marker for FcaPV infection (Munday and Thomson 2021). In contrast, the p53 protein, which is encoded by the TP53 gene, is degraded by the E6 viral oncoprotein (Altamura et al. 2018). However, the current veterinary literature appears to have conflicting findings with initial IHC studies finding that FcaPV does not degrade p53, but later molecular based studies showing that the E6 oncoprotein does degrade it (Altamura,

Corteggio and Borzacchiello 2016; Altamura et al. 2018; Munday and Thomson 2021; Teh and Krockenberger 2021; Munday and Aberdein 2012).

The expression of p16 in relation to FcaPV infection has been previously explored in other feline studies globally (Mazzei et al. 2018; Munday and Thomson 2021; Thomson, Munday and Dittmer 2016). However, this study represents the first to specifically examine the FcaPV aetiology in a variety of pre-malignant and malignant skin lesions in a population of Australian cats. In doing so, this will determine whether findings in an Australian context align with other studies, or if there are inconsistencies, whether there are certain features amongst Australian felines accounting for potential differences. Assessing p53 expression patterns would also help to address conflicting findings in the current veterinary literature and clarify the pathogenesis of FcaPV associated disease. Understanding the association of FcaPV infection in the development of these common feline skin neoplasms may potentially open up therapeutic and/or preventative options in the future.

This study aims to investigate the expression of p16 and p53 in feline cutaneous SCCs, BISCs, and AKs, in order to explore the associations of FcaPV with the development of epidermal malignancy in the Australian cat.

Materials and Methods

Tissues

Formalin fixed paraffin embedded (FFPE) tissues from feline SCCs, BISCs, and AKs were identified from tissue archives at the Veterinary Pathology Diagnostic Services (VPDS), the University of Sydney. Feline SCCs were further subdivided into UV-protected (face, digit,

thigh, neck) or UV-exposed (nasal planum, pinnae, eyelids, third eyelid) sites as previously described (Munday, Gibson and French 2011).

Case Metadata

The following patient information was obtained from VPDS case accession records attached to each sample included in the study: histopathology diagnosis, case date, age, sex, breed, desexing status, and lesion site.

Histological Confirmation

Haematoxylin and eosin (HE) stained tissue sections of each sample were examined using standard light microscopy for histological confirmation of the diagnosed lesion. Lesions were considered to exhibit evidence of viral cytopathic effects if at least one of the following features were observed in neoplastic cells within the lesion: 1) cytoplasmic vacuolation with a perinuclear clearing and a shrunken hyperchromatic nucleus (koilocytosis), 2) cells with expanded blue-grey to amphophilic fibrillar cytoplasm, 3) eosinophilic intranuclear viral inclusion bodies, and/or 4) prominent, clumped, enlarged, coarse to irregular keratohyalin granule formation (Wilhelm et al. 2006).

Immunohistochemistry

FFPE blocks for immunohistochemistry were cut at a thickness of 4 µm on positively charged adhesive slides, deparaffinised by placing in xylene for three minutes, then moved to a fresh pot of xylene for an additional three minutes. Sections were rinsed twice in 100% alcohol for two minutes, then twice in 95% alcohol for two minutes and then in 70% alcohol for two

minutes. This was then followed by additional rinsing with deionised water for five minutes and tap water for five minutes.

Following deparaffinisation and rehydration, antigen retrieval was achieved by heating sections in citrate buffer, pH 6.0 in a 1000W microwave for ten minutes, with a 45-minute cooldown. Blocking of endogenous peroxidases was achieved by incubating slides for ten minutes in 250 µL of 3% hydrogen peroxide at room temperature, then rinsed gently five times with 0.05M tris-buffer solution (TBS).

To detect p16, slides were incubated with a 1:200 dilution of purified mouse anti-human monoclonal antibody G175-405 (BD Biosciences, San Diego, United States) for 120 minutes at room temperature. Another p16 antibody (clone IHC116, GeneAb) was also trialled. For the positive control, a feline squamous cell carcinoma that has been previously demonstrated to overexpress p16 (G175-405 clone) was included (provided by Professor John Munday, Massey University). For the negative controls, the assay was run normally with the exclusion of the primary antibody for each block, and a section of normal feline skin was included in the assay following the current staining protocol. After incubation with the primary antibody, slides were rinsed gently with 0.05M TBS then placed in a fresh buffer bath.

To detect p53, slides were incubated with a mouse anti-human p53 clone pAb 240 antibody (BD Biosciences) at a dilution of 1:50 for 120 minutes at room temperature, then rinsed with 0.05M TBS and placed in a fresh buffer bath. For the positive control, a feline squamous cell carcinoma that has been previously demonstrated to overexpress p53 (pAb 240 clone) was

included (provided by Professor John Munday, Massey University). For the negative controls, the assay was run normally with the exclusion of the primary antibody for each block (negative antibody control), and a section of normal feline skin was included in the assay following the current staining protocol (negative tissue control).

After incubating slides with the primary antibodies, slides were incubated with Dako EnVision Dual Link System HRP for 60 minutes at room temperature then rinsed with 0.05M TBS, then incubated with ImmPact VIP HRP substrate (SK-4605, Vector Laboratories) chromogen solution for ten minutes. Specimens were counterstained by immersing in a bath of 0.5% methyl green for ten minutes and rinsed with distilled water. Slides were dehydrated in graded alcohol solutions and xylene, then coverslipped.

Assessment of immunostaining

Each labelled slide was initially examined microscopically using an Olympus CX43 light microscope to subjectively confirm positive/negative staining, appropriate tissue staining (whether the expected nuclear and/or cytoplasmic staining patterns for p16 and p53 precipitated), counter-staining, and quality (even staining distribution). Visual assessment of p16 positive labelling was adapted from previously described methodology (Supsavhad et al. 2016). For all examined lesions, lesions were considered to exhibit positive p16 labelling if greater than 50% of intralesional neoplastic cells contained cytoplasmic and nuclear immunostaining, evaluated through visual estimation in five different high power (400x magnification) fields within areas of most intense immunostaining. The expected staining

pattern for p16 labelling is cytoplasmic and nuclear, whereas the expected labelling pattern for p53 is nuclear.

Low magnification images (40x magnification) of each lesion labelled with p16 were then taken using an Olympus DP23 microscope camera, images subsequently analysed using the software program QuPath (Version 0.4.3) for positive cell detection (Table 1) (Bankhead et al. 2017). Prior to running the positive cell detection, the entire lesion was selected using the wand tool. A lesion was classified as p16-negative if no positive staining was detected at all using previously described visual confirmation and QuPath's positive cell detection (Figure 1). Conversely, a lesion was classified as p16-positive if positive staining was detected using both the previously described visual confirmation methodology and QuPath's positive cell detection.

p16-positive lesions were further subclassified as exhibiting weak or strong immunostaining based on the number of cells belonging to each detection threshold in QuPath (Table 1). Cells that were classified by QuPath as belonging to threshold 1 were considered weakly positive, and cells classified as belonging to threshold 2 were considered strong positive. All cells classified as threshold 3 were examined manually and determined most consistent with melanocytes due to their intense pigmentation and were excluded from the total cell count. The intensity threshold parameter for threshold 1 (weakly positive category) was determined by training the software thresholds with a previously assessed weak/faint p16 immunostaining case to ensure that greater than 50% of positively detected cells would fall into the category of threshold 1 (Figure 2). Similarly, the intensity threshold parameter for threshold 2 (strong positive category) was determined by training the software thresholds

with a previously assessed strong/intense p16 immunostaining case to ensure that greater than 50% of positively detected cells would fall into the threshold 2 category (Figure 3). The intensity threshold parameter for threshold 3 (melanin and melanocytes) was determined by taking a photomicrograph of a lesion exhibiting positive p16 labelling with the presence of prominent melanin in the background and adjusting the threshold parameter value such that all of the melanin and none of the p16-labelled neoplastic cells detected would fall into the threshold 3 category (Figure 4). A lesion was classified as a weak positive if more than 50% of neoplastic cells belonged to threshold 1 (Figure 2); whilst a lesion was classified as a strong positive if more than 50% of neoplastic cells belonged to threshold 2 (Figure 3), as adapted from elsewhere (Supsavhad et al. 2016). For lesions that exhibited very weak immunostaining which could not be detected by QuPath, p16 labelling was confirmed visually using the previously described visual assessment methodology and assigned a weak positive classification.

Table 1. Cell detection parameters for QuPath.

Setup Parameters	
Detection image	Optical density sum
Requested pixel size	0.5 µm
Nucleus Parameters	
Background radius	8 µm
Use opening by reconstruction	YES
Median filter radius	0 µm
Sigma	1.5 µm
Minimum area	10 µm^2
Maximum area	400 µm^2
Intensity Parameters	
Threshold	0.1
Max background intensity	2
Split by shape	YES
Exclude DAB (membrane staining)	NO
Cell Parameters	
Cell expansion	5 µm
Include cell nucleus	YES
General Parameters	
Smooth boundaries	YES
Make measurements	YES
Intensity threshold Parameters	
Score compartment	Cell: DAB OD max
Threshold 1+	0.09
Threshold 2+	0.12
Threshold 3+	0.6
Single threshold	NO

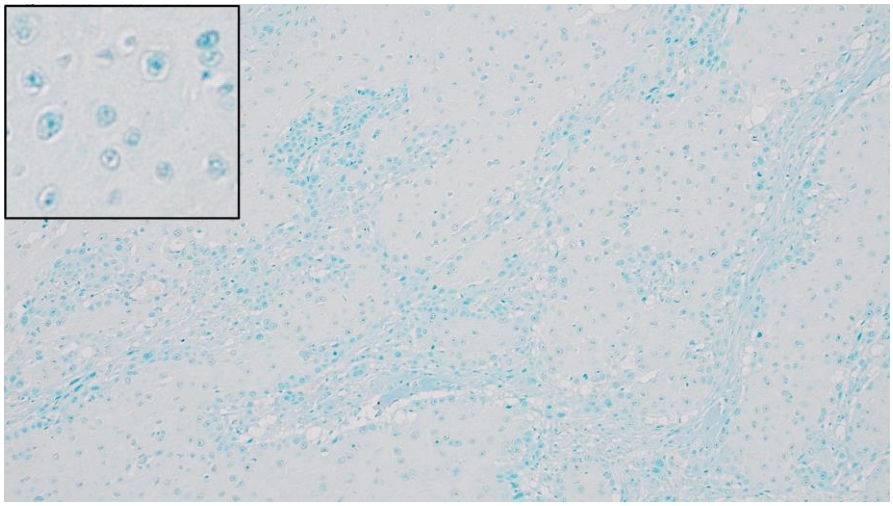

Figure 1. Photomicrograph of a feline cutaneous SCC negative control, with the assay run as normal but with the exclusion of the primary antibody (p16 G175-405, BD Biosciences) showing absent cytoplasmic and nuclear labelling. Inset shows higher magnification of the intralesional neoplastic cells with absent p16 labelling. ImmPact VIP HRP substrate (SK-4605, Vector Laboratories) chromogen solution, counterstained with 0.5% methyl green.

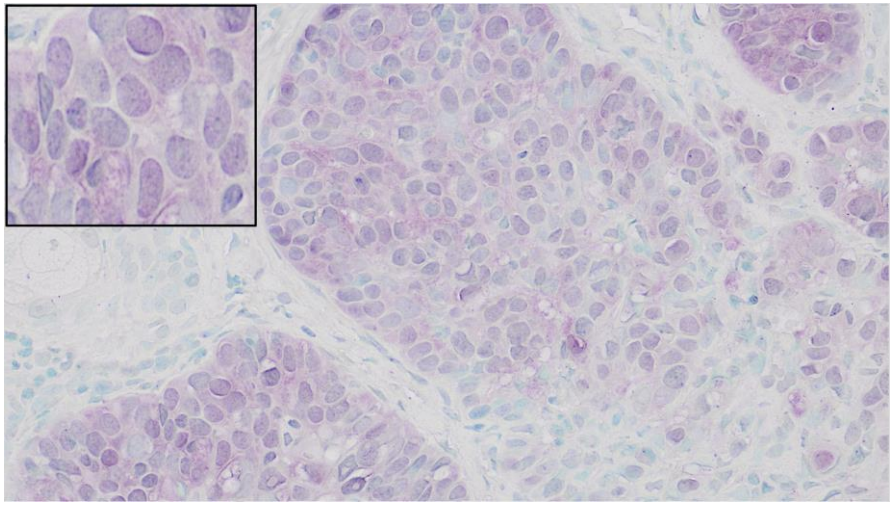

Figure 2. Photomicrograph of a feline cutaneous BISC labelled with p16 showing positive but weak cytoplasmic and nuclear staining. This lesion was classified as a weak positive (greater than 50% of positively detected cells belonging to threshold 1). Inset shows higher magnification of a cluster of p16 labelled neoplastic cells with nuclear and cytoplasmic

staining. ImmPact VIP HRP substrate (SK-4605, Vector Laboratories) chromogen solution, counterstained with 0.5% methyl green.

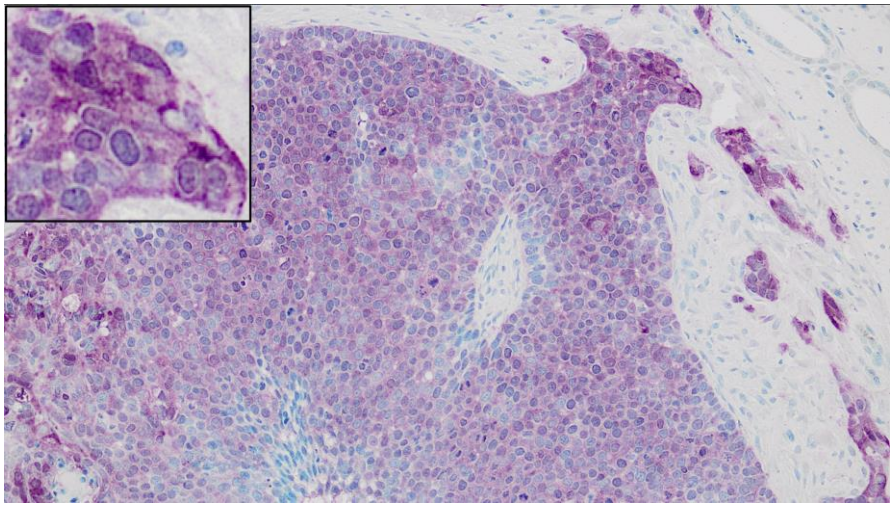

Figure 3. Photomicrograph of a feline cutaneous SCC labelled with p16 showing strongly positive cytoplasmic and nuclear staining. This lesion was classified as a strong positive (greater than 50% of positively detected cells belonging to threshold 2). Inset shows higher magnification of a cluster of p16 labelled neoplastic cells with nuclear and cytoplasmic staining. ImmPact VIP HRP substrate (SK-4605, Vector Laboratories) chromogen solution, counterstained with 0.5% methyl green.

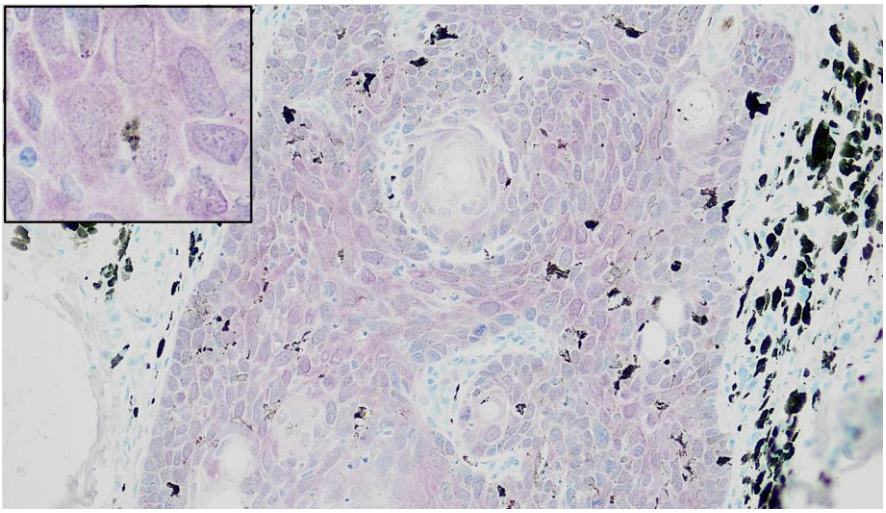

Figure 4. Photomicrograph of a feline cutaneous BISC labelled with p16 showing positive cytoplasmic and nuclear staining with large amounts of prominent melanin in the background. This lesion was used to determine the threshold parameter value for threshold 3 (melanin). Inset shows a cluster of p16 labelled neoplastic cells with nuclear and cytoplasmic staining. ImmPact VIP HRP substrate (SK-4605, Vector Laboratories) chromogen solution, counterstained with 0.5% methyl green.

Statistical Analyses

Statistical analyses were performed using MedCalc Software (version 23.2.6). Staining intensities (weak and strong) were analysed using an omnibus chi-squared test to look for statistically significant differences between staining intensities and the different lesion categories. To look for significant differences amongst p16 labelling according to the SCC's UV exposure site (exposed or protected), the premalignant lesions (BISC and AK), and whether p16 labelling was associated with the presence of histological viral cytopathic features – separate 2x2 contingency tables were created for the calculation of odds ratios and Fisher's exact probability tests.

Results

Sixty FFPE samples were included in this study, consisting of 22 BISCs, 13 AKs, and 25 SCCs. Of the 25 SCCs, 10 were from UV-protected sites, and 15 were from UV-exposed sites.

p16 immunostaining was successful with the G175-405 clone (BD Biosciences) but not the IHC116 clone (GeneAb). p53 immunostaining (pAb 240, BD Biosciences) was unsuccessful.

There was no statistically significant difference in p16 staining intensity across the examined lesions ($p = 0.6925$, $\chi^2 = 1.4558$) (Table 2). There was no statistically significant difference in p16 labelling between UV-exposed and UV-protected SCCs, although the p-value was marginally over the threshold ($p = 0.0593$) (Table 3). Feline BISCs exhibited positive p16 labelling at higher rates compared to AKs ($p = 0.0030$) (Table 4). Lesions that had at least one histological viral cytopathic effect feature exhibited positive p16 labelling more than lesions without detectable cytopathic effects ($p = 0.0004$) (Table 5).

Table 2. p16 labelling and staining intensity results across the different feline cutaneous skin lesions.

Diagnosis	p16 Staining	Staining Intensity	p-value	Chi-squared statistic (χ^2)
BISC	Positive 20 Negative 2	Strong 5 Weak 15	0.6925	1.4558
AK	Positive 5 Negative 8	Strong 2 Weak 3		
SCC (UV-Protected)	Positive 8 Negative 2	Strong 1 Weak 7		
SCC (UV-Exposed)	Positive 6 Negative 9	Strong 2 Weak 4		

BISC = Bowenoid *in situ* carcinoma, AK = actinic keratosis, SCC = squamous cell carcinoma

Table 3. p16 labelling amongst feline cutaneous SCCs according to whether the lesion developed in a UV-exposed or protected site.

Lesion	Positive	Negative
SCC (UV-Protected)	8	2
SCC (UV-Exposed)	6	9
OR = 6.0000, p = 0.0593 Fisher's exact probability = 0.0991		

SCC = squamous cell carcinoma, OR = Odds Ratio

Table 4. p16 labelling amongst feline cutaneous BISCs and AKs.

Lesion	p16 Positive	p16 Negative
BISCs	20	2
AKs	5	8
OR = 16.0000, p = 0.0030 Fisher's exact probability = 0.0017		

BISC = Bowenoid *in situ* carcinoma, AK = actinic keratosis, OR = Odds Ratio

Table 5. p16 labelling according to whether the feline cutaneous lesion contained histologically detectable viral cytopathic effects.

	p16 Positive	p16 Negative
Viral Features Present	29	5
Viral Features Absent	10	16
OR = 9.2800, p = 0.0004 Fisher's exact probability = 0.0003		

OR = Odds Ratio

Discussion

In this study, we investigated the expression of p16 in feline skin lesions from a population of Australian cats, including feline skin lesions which are thought to be associated with papillomavirus infection (BISCs and UV-protected SCCs), as well as lesions which are primarily related to UV exposure such as AKs and UV-exposed SCCs. Our findings demonstrate a significant presence of p16 (G175-405 clone) labelling in feline BISCs compared to AKs, but not in UV-protected SCCs compared to UV-exposed SCCs, in a population of Australian cats. The expression of p53 was unable to be observed and

assessed due to unsuccessful immunohistochemistry. The intensity of p16 immunolabelling did not have any statistically significant associations between the different lesions. To the author's knowledge, this study represents the first to thoroughly evaluate the expression of p16 in a variety of cutaneous neoplastic diseases in a population of Australian cats and in feline AKs.

The overexpression of p16 in feline BISCs aligns with observations in other feline studies globally, suggesting that the findings in Australian cats are consistent with overseas results (Mazzei et al., 2018; Munday & Thomson, 2021; Thomson et al., 2016). In feline and human medicine, the overexpression of p16 often serves as a surrogate or proxy marker for papillomaviral oncogenic activity (Graham 2017; Munday and Thomson 2021; Stephen et al. 2013). This is because the E7 oncoprotein expressed by PVs causes consistent degradation of pRb which results in overexpression of p16 within the cell (Munday and Thomson 2021; Teh and Krockenberger 2021). This is a key part of the pathogenesis of PVs that ultimately leads to unregulated cell cycle progression and tumorigenesis (Graham 2017; Munday and Thomson 2021). Thus, the detection of p16 overexpression via IHC permits visualisation of the effects of PV activity (Munday et al. 2011; Munday, Gibson and French 2011). Therefore, in this study, the significant overexpression of p16 in feline BISCs supports the hypothesis that these lesions are primarily driven by PV infection rather than UV damage. Thus, the findings support the tumorigenic potential of FcaPV in BISCs in a population of Australian cats, aligning Australian findings with similar global observations.

Conversely, AKs did not exhibit statistically significant p16 overexpression compared to the BISCs. This is consistent with the understanding that UV-induced skin carcinogenesis is less

associated with PV infection and more directly linked to DNA damage induced by chronic UV exposure. The findings from this study do not support a significant role of FcaPV infection in AKs, which is not consistent with recent observations in the human literature where certain types of HPV infections have been associated with human AKs (Dianzani et al. 2017; Dianzani et al. 2008; Galati et al. 2020; Schneider et al. 2013). Interestingly, there was also no statistically significant difference in p16 labelling between UV-exposed and UV-protected SCCs. Earlier studies suggested that FcaPV-2 infection was more commonly observed in UV-protected SCCs compared to UV-exposed ones (Munday, Gibson and French 2011). However, it is also important to note that other studies have observed p16 positive immunostaining in a proportion of UV-exposed SCCs on white-coated animals (Altamura et al. 2016). It was hypothesised in these lesions that PV infection may possibly have acted as a cofactor with chronic UV damage ultimately leading to carcinogenesis, which is similar to what occurs in people (Altamura et al. 2016). Therefore, whilst there was no statistically significant difference between p16 staining in UV-exposed and UV-protected SCC in this study, this may be indicative that FcaPV-2 infection may play a role in carcinogenesis across both lesion sites, acting as a primary aetiological contributor in UV-protected areas and a cofactor with chronic UV damage in UV-exposed areas (Altamura et al. 2016).

This study also provides further evidence to support the reliability of the p16 G175-405 clone in the investigation of FcaPV aetiologies in feline skin lesions. Another p16 clone (IHC 116) tested as part of this study was unsuccessful. The G175-405 clone reliably cross-reacts with the feline p16 protein (Munday and Thomson 2021).

There were some limitations to this IHC study. Whilst the detection of p16 overexpression is a well described diagnostic modality for the diagnosis of FcaPV-induced feline skin lesions, the sensitivity and specificity of p16 overexpression for FcaPV infection is currently unknown in cats (Munday and Thomson 2021). Whilst the relationship between PV infection and p16 overexpression is relatively strong, a spontaneous mutation of pRb independently of PV infection may also cause an increase in p16 expression (Munday and Thomson 2021). Conversely, a spontaneous mutation in p16 may reduce p16 levels within a cell even if it was associated with PV infection (Munday and Thomson 2021). Correlation of p16 expression with the detection of PV DNA through other mechanisms such as PCR and *in situ* hybridisation would help to strengthen the degree of confidence with ascribing a PV aetiology to a lesion. The retrospective design of the study itself presents another limitation given that it provides a moderate strength of evidence according to the hierarchy of evidence (Wallace et al. 2022). In the context of this study, specific limitations of the retrospective design included the inability to control for confounders and reliance on historical clinically submitted data originally intended for diagnostic rather than research purposes. The evaluation of histological viral cytopathic features was relatively subjective and was not verified by another blinded pathologist, nor was the assessor blinded. As such, the potential impact of unconscious and non-deliberate selection bias may have impacted and/or confounded the results evaluating histological viral cytopathic features and p16 positivity. Furthermore, not all BISCs are expected to show evidence of viral cytopathic effects, even though they are presumed to be related to a papillomaviral aetiology. Viral cytopathic effects are typically observed in the earlier stages of BISCs, becoming less prominent in more advanced cases (Meuten 2017). Whilst there was no statistically significant difference in the result of p16 labelling between UV-exposed SCCs and UV-

protected SCCs, the p value was marginally over the statistical significance threshold (p = 0.0593). It is possible that if more SCCs were included in the sample size, the result may have been different. Finally, the classification of a “UV-exposed” and a “UV-protected” lesion was based solely on the reported lesion site from the submitting clinician. Realistically, it is difficult to truly determine whether a lesion was UV-exposed or protected as animals vary widely in their pigmentation, hair coverage, and behaviours. It is possible that certain lesions classified as UV-exposed or protected based on the reported location may not have been. Furthermore, Australia receives some of the highest levels of UV radiation in the world owing to its geographical location, and main population centres in Australia such as Sydney, Brisbane and Perth receive higher UV doses compared to main population centres in New Zealand including Auckland, Wellington, and Christchurch (Gies et al. 2004; McKenzie 2017; Sharma et al. 2024). It is therefore plausible to speculate that due to higher UV exposure levels in Australia, anatomical sites that are considered UV-protected in other locales may actually be exposed in the current Australian cohort.

In conclusion, the overexpression of p16 in feline BISCs compared to AKs supports the role of FcaPV infection in the pathogenesis of certain feline skin lesions in an Australian population of cats. This aligns Australian findings with observations in other countries (Mazzei et al. 2018; Munday and Thomson 2021; Thomson, Munday and Dittmer 2016). Furthermore, the lack of significant p16 overexpression in feline AKs does not support a role of FcaPV infection in the aetiopathogenesis of this lesion, unlike in the human literature whereby HPV infections currently have a postulated association with AKs (Galati et al. 2020). The findings of this study therefore enhance our understanding of the role of FcaPV infection in feline cutaneous skin lesions, especially in an Australian context, however,

further research as to whether p53 levels are reduced as a result of degradation by the FcaPV E6 oncoprotein would be beneficial to clarify in detail the aetiopathogenesis of FcaPV.

Chapter 3. The Use of Quantitative Polymerase Chain Reaction (qPCR) for the Detection of *Felis Catus* Papillomavirus Type 2 (FcaPV-2) DNA in Feline Cutaneous Squamous Cell Carcinomas, Bowenoid *in situ* Carcinomas, and Actinic Keratoses, and Association with the Presence of Intralesional p16 Labelling

Introduction

Feline cutaneous squamous cell carcinomas (SCCs) are the most common malignant skin neoplasms of cats, representing approximately 15% of feline skin neoplasms (Munday and Thomson 2021). *Felis catus* papillomavirus type 2 (FcaPV-2) infection has been implicated in the development of various feline cutaneous lesions including SCCs and its premalignant counterpart Bowenoid *in situ* Carcinoma (BISC). Whilst previous studies have employed a combination of both polymerase chain reaction (PCR) and immunohistochemistry (IHC) to investigate a potential FcaPV-2 aetiology in feline cutaneous SCCs, this represents the first comparative study within an Australian feline population with a focus on examining the possible role in the development of actinic keratosis (AK).

The detection of p16 via IHC is used in both human and veterinary pathology as a surrogate marker for PV infection (Munday and Thomson 2021). However, reliance solely on p16 detection can be problematic as a spontaneous mutation in the retinoblastoma protein (pRb) may increase p16 levels independently of FcaPV-2 infection (Benzerdjeb et al. 2021; Chung et al. 2014; Geibler et al. 2013; Lu, El-Mofty and Wang 2003; Munday and Thomson 2021). Similarly, whilst PCR offers a sensitive method for identifying the presence of intralesional FcaPV-2 DNA, the results of PCR alone cannot differentiate between an active infection, transient infection, or a contaminant (Hoggard, Munday and Luff 2018). Given

that FcaPV-2 DNA has been detected in skin swabs from a large proportion of clinically healthy cats, it is difficult to definitively attribute the findings of standalone PCR results to an active FcaPV-2 infection (Geisseler et al. 2016; Munday and Thomson 2021; Munday and Witham 2010; Thomson et al. 2019).

Recognising these limitations, the strength of evidence increases when both diagnostic modalities are considered in tandem. Concurrent detection of intralesional FcaPV-2 DNA by PCR alongside p16 labelling detected by IHC provides stronger evidence of an aetiological relationship by demonstrating the presence of viral DNA in combination with evidence of a key tumorigenic event – the PV E7 oncoprotein inhibiting the retinoblastoma protein resulting in overexpression of p16 detectable by IHC.

The aim of this chapter is to therefore detect the presence of FcaPV-2 DNA using quantitative PCR (qPCR) in feline cutaneous SCCs (UV-protected and UV-exposed), BISCs, and AKs; and to integrate findings from both qPCR and IHC analyses to assess whether FcaPV-2 infection constitutes an aetiological risk factor in the development of the aforementioned feline cutaneous lesions.

Materials and Methods

DNA Extraction

Additional unstained FFPE sections were prepared from the feline skin lesions previously described in Chapter 2 and mounted on glass slides. A sterile scalpel blade was used to

scrape the tissue into sterile clean Eppendorf tubes minimising the wax entering the DNA extraction process. DNA was then extracted from the FFPE sections using the Qiagen DNeasy® Blood and Tissue kit according to the manufacturer's protocol. Extracted DNA was stored frozen until qPCR analysis which was undertaken within 6 months of DNA extraction and storage. Formalin-fixed paraffin-embedded blocks with insufficient tissue, which made sectioning unfeasible, were excluded.

Primers and Positive Controls

Forward and reverse primers were used to amplify a portion of the FcaPV-2 L1 gene as previously described (Table 1) (Mazzei et al. 2018). For the papillomaviral positive control, a gBlock® Gene Fragment was designed with the nucleotide sequence "TTTTCTACACGCGGTACCAATTTCACCCTGTCTGTATCTACAGAAGCATCCCCTCAGGAATATGATG CCAGTAAATTTAACCAGTATCTGCGACATGTAGAGGAGTATGACCTGTC CTTTATATTCCAAGTGTGCGTGGTCACTCTTACCCCC" and synthesised by Integrated DNA Technologies, Coralville, United States. For the feline tissue positive control, primers for the *Felis catus* ribosomal 28s (Fel28S) housekeeping gene were used as previously described (Table 1) (Pinches et al. 2007).

Real-Time quantitative PCR

Real-time quantitative PCR (qPCR) was carried out using the Bio-Rad CFX Opus 96 Real-Time PCR system using SsoAdvanced™ Universal SYBR® Green Supermix reagent according to the manufacturer's instructions. The qPCR conditions were as follows: polymerase activation and DNA denaturation at 95.0°C for 3 minutes; denaturation at 95.0°C for 10 seconds; annealing/extension and plate read at 60.0°C for 20 seconds; 40 cycles; and melt curve

analysis at 65.0°C to 95.0°C with increments of 0.5°C every 5 seconds. The extracted DNA was tested for the presence of viral DNA (FcaPV-2 L1) and the feline reference gene Fel28S. A non-template control (NTC) consisting of all master mix reagents without the presence of template DNA; and the FcaPV-2 gene fragment were included in all runs serving as an internal negative and positive control respectively. A positive PCR result (for both viral and Fel28S DNA) was defined as a sample which yielded a cycle threshold (Ct) value, had a melt curve with a single sharp peak within an appropriate range, and displayed an appropriate sigmoidal amplification curve. Conversely, a negative PCR result was defined as a sample which lacked all of the aforementioned features. Samples which returned a negative result for Fel28S were tested again using the SYBR® assay and, if the negative result persisted, they were tested again using a TaqMan probe assay (SensiFAST™ probe, sequence: "TGGCTTGTGGCAGCCAAGTGT") (Pinches et al. 2007). The qPCR conditions for the TaqMan probe assay were as follows: polymerase activation at 95.0°C for 3 minutes; denaturation at 95.0°C for 10 seconds; annealing/extension and plate read at 58.0°C for 20 seconds; with 40 cycles. A positive result on the TaqMan qPCR assay was defined as a sample which yielded a Ct value and displayed an appropriate sigmoidal amplification curve. Samples which still returned a negative result for Fel28S using both the SYBR® and TaqMan probe assays were excluded from the analysis. All testing was performed in duplicate, including testing for FcaPV-2 and Fel28S DNA, except for the positive and non-template controls which were run in single replicates.

Table 1. Nucleotide sequences for the primers used to amplify the genes in the study. These included the viral DNA target (L1 of FcaPV-2) and the feline housekeeping gene used for quality control in the qPCR runs (Fel28S).

Gene	**Nucleotide Sequence (Forward Primer)**	**Nucleotide Sequence (Reverse Primer)**
FcaPV-2 L1	TACACGCGGTACCAATTTCA	AGAGTGACCACGCACACTTG
Fel28S	AGCAGGAGGTGTTGGAAGAG	AGGGAGAGCCTAAATCAAAGG

FcaPV-2 = *Felis catus* papillomavirus type-2

Statistical Analyses

Statistical analyses were performed using MedCalc Software (Version 23.2.6). To look for significant differences in qPCR positivity between general lesion UV exposure sites, BISCs and AKs, and UV-protected and UV-exposed SCCs, 2x2 contingency tables were created. Odds ratios and Fisher's exact probability tests were calculated using the created contingency tables.

Data from anti-p16 immunohistochemistry (described in Chapter 2) and PCR targeting FcaPV-2 were assessed, combined, and included in the analyses. Statistical analyses were undertaken using MedCalc Software (Version 23.2.6). A lesion was considered to be related to FcaPV-2 infection, and thus had a likely FcaPV-2 aetiology, if it had positive p16 labelling by IHC and FcaPV-2 PCR positive concurrently. A valid PCR result also needed to contain detectable Fel28S housekeeping gene DNA, whether it was considered a positive or negative result. 2x2 contingency tables were then created to compare the combined p16 and viral PCR positivity results amongst BISCs and AKs; UV-protected and UV-exposed cutaneous

SCCs; and with lesions that contained histological viral cytopathic features with subsequent calculation of odds ratios and Fisher's exact probability to detect statistical significances.

Results

Sixty FFPE samples were included in this study, consisting of 22 BISCs, 13 AKs, and 25 SCCs. Of the 25 SCCs, 10 were from UV-protected sites, and 15 were from UV-exposed sites. From this sample pool, 11 samples did not contain any detectable Fel28S DNA and were excluded from the sample size. These 11 excluded samples consisted of 7 BISCs, 2 AKs, and 2 SCCs. One SCC did not have sufficient tissue left in the FFPE block to permit further use for qPCR and was also excluded from the sample size. Therefore, the final sample size for qPCR comprised 15 BISCs, 11 AKs, and 22 SCCs (Table 2).

UV-protected SCCs were more likely to contain FcaPV-2 DNA compared to UV-exposed SCCs (Table 3, $p = 0.0095$). No statistically significant differences were detected in FcaPV-2 qPCR positivity between BISCs and AKs (Table 4, $p = 0.9512$).

No statistically significant difference was observed in FcaPV-2 aetiology (positive for both FcaPV-2 DNA by PCR and p16 labelling by IHC) between feline BISCs and AKs (Table 5, $p = 0.1723$). However, there was a statistically significant difference observed in FcaPV-2 aetiology between UV-protected and UV-exposed cutaneous SCCs (Table 6, $p = 0.0236$). Lesions that contained histological evidence of viral cytopathic features (at least one of: koilocytes, eosinophilic intranuclear inclusion bodies, prominent coarse to irregular

keratohyalin granules) were more likely to be related to a FcaPV-2 aetiology compared to lesions that did not contain cytopathic features (Table 7, p = 0.0294).

Table 2. Ct values of the samples tested by qPCR for both the Fel28S feline housekeeping gene and FcaPV-2 DNA. Samples which did not have a valid amplification of the Fel28S feline housekeeping gene were not included in the results.

Lesion	Ct (Fel28S)	Ct (FcaPV)
AK	37.13	31.45
AK	37.16	ND
AK	35.06	ND
AK	32.14	ND
AK	30.19	ND
AK	35.84	33.4
AK	31.38	ND
AK	35.23	35.36
AK	37.43	35.21
AK	39.23	33.11
AK	34.24	ND
BISC	37.39	ND
BISC	39.32	34.2
BISC	34.47	ND
BISC	29.41	ND
BISC	33.72	34.02
BISC	38.01	ND
BISC	34.16	27.38
BISC	35.73	31.52
BISC	39.32	ND
BISC	37.62	33.54
BISC	38.2	33.11
BISC	37.94	ND
BISC	33.41	ND
BISC	37.81	ND
BISC	31.92	28.58
SCC (UV-exposed)	39.15	ND
SCC (UV-exposed)	30.06	ND
SCC (UV-exposed)	35.71	ND
SCC (UV-exposed)	35.16	ND
SCC (UV-exposed)	34.45	ND
SCC (UV-exposed)	38.98	ND
SCC (UV-exposed)	33.02	ND
SCC (UV-exposed)	37.25	ND
SCC (UV-exposed)	31.15	ND
SCC (UV-exposed)	35.11	ND

SCC (UV-exposed)	32.45	ND
SCC (UV-exposed)	35.35	35.51
SCC (UV-protected)	36.74	33.31
SCC (UV-protected)	39.12	ND
SCC (UV-protected)	29.34	ND
SCC (UV-protected)	31.11	ND
SCC (UV-protected)	39.31	37.4
SCC (UV-protected)	36.04	29.38
SCC (UV-protected)	38.55	34.13
SCC (UV-protected)	34.11	26.27
SCC (UV-protected)	31.09	27.66
SCC (UV-protected)	34.12	25.18

AK = actinic keratosis, BISC = Bowenoid *in situ* carcinoma, SCC = squamous cell carcinoma, Ct = cycle threshold, FcaPV = *Felis catus* papillomavirus, ND = not detected

Table 3. PCR positivity for FcaPV-2 DNA by feline SCC UV exposure site.

Lesion	Positive	Negative
UV-protected	7	3
UV-exposed	1	11
OR = 25.6667, p = 0.0095 Fisher's exact probability = 0.0062		

PCR = Polymerase Chain Reaction, FcaPV = *Felis catus* papillomavirus, SCC = Squamous Cell Carcinoma, OR = Odds Ratio

Table 4. PCR positivity for FcaPV-2 DNA between feline BISCs and AKs.

Lesion	Positive	Negative
BISC	7	8
AK	5	6
OR = 1.0500, p = 0.9512 Fisher's exact probability = 1		

PCR = Polymerase Chain Reaction, FcaPV = *Felis catus* papillomavirus, BISC = Bowenoid *in situ* carcinoma, AK = actinic keratosis, OR = Odds Ratio

Table 5. Combined p16 and FcaPV-2 PCR positivity for feline BISCs and AKs. A lesion was considered to be related to an FcaPV-2 aetiology if it was positive for both p16 labelling and viral DNA by PCR concurrently. A lesion was considered to not be related to an FcaPV-2 aetiology if it was negative for both p16 labelling and viral DNA.

Lesion	p16/PCR Positive	p16/PCR Negative
BISC	7	2
AK	2	3
OR = 5.2500, p = 0.1723 Fisher's exact probability = 0.2657		

PCR = Polymerase Chain Reaction, BISC = Bowenoid *in situ* carcinoma, AK = actinic keratosis, OR = Odds Ratio

Table 6. Combined p16 and FcaPV-2 PCR positivity for feline cutaneous UV-exposed and protected SCCs. A lesion was considered to be related to an FcaPV-2 aetiology if it was positive for both p16 labelling and viral DNA by PCR concurrently. A lesion was considered to not be related to an FcaPV-2 aetiology if it was negative for both p16 labelling and viral DNA.

Lesion	p16/PCR Positive	p16/PCR Negative
SCC (UV-Protected)	7	2
SCC (UV-Exposed)	1	6
OR = 21.0000, p = 0.0236 Fisher's exact probability = 0.0406		

PCR = Polymerase Chain Reaction, SCC = Squamous Cell Carcinoma, OR = Odds Ratio

Table 7. Comparison between the presence of histological viral cytopathic features in lesions with p16 and viral PCR positivity. A lesion was considered to be related to an FcaPV-2 aetiology if it was positive for both p16 labelling and viral DNA by PCR concurrently. A lesion was considered to not be related to an FcaPV-2 aetiology if it was negative for both p16 labelling and viral DNA.

Viral Cytopathic Features	p16/PCR Positive	p16/PCR Negative
Present	11	3
Absent	6	10
OR = 6.1111, p = 0.0294 Fisher's exact probability = 0.0329		

PCR = Polymerase Chain Reaction, OR = Odds Ratio

Discussion

In this chapter, qPCR was used to detect the presence of FcaPV-2 DNA in feline cutaneous SCCs, both UV-exposed and UV-protected, and their premalignant lesions AKs and BISCs. Results from both the qPCR and p16 labelling were then combined and interpreted together. To the author's knowledge, this represents the first comparative study of FcaPV-2 within an Australian feline population, and the first study to specifically examine AKs.

The results from this chapter do not support a strict and separate aetiological relationship between FcaPV-2 infection and BISCs when compared to AKs, but there does appear to be a statistically significant association between FcaPV-2 infection and UV-protected SCCs compared to UV-exposed SCCs. A lesion was considered to be associated with FcaPV-2 infection if it contained viral DNA detectable by PCR with evidence of p16 labelling detectable by IHC. In doing so, this provided more convincing evidence that the presence of

FcaPV-2 was an active infection as visualisation of p16 labelling is often indicative of expression of the papillomaviral E7 oncoprotein which inhibits the retinoblastoma protein resulting in a consequential increase in retinoblastoma-dependent p16 levels (Chung et al. 2014; Geibler et al. 2013; Lu, El-Mofty and Wang 2003). Interpretation of p16 labelling and PCR positivity together was thus considered to be stronger than interpretation of the two results separately.

Interestingly, the results from this study do not completely align with findings in the veterinary literature. The findings conflict with current knowledge whereby it is thought that BISCs are due to a FcaPV aetiology whereas AKs are separately related to chronic UV damage (Mauldin and Peters-Kennedy 2016). This raises the suggestion that FcaPV-2 infection may play a greater role in the development of feline cutaneous premalignancies in general, possibly acting as co-factors in lesions such as AKs which are primarily due to UV damage (Altamura et al. 2016). This would be an understandable point given that unlike UV exposure, viral infection would not be restricted to only UV-protected or UV-exposed areas of the skin. This also prompts comparisons to similar findings in the human literature, whereby recent studies have found HPV DNA localised within or adjacent to AKs, hypothesising whether HPV can contribute to the development of these lesions in people as well (Dianzani et al. 2017; Dianzani et al. 2008; Galati et al. 2020). Speculatively, as this is the first study to specifically examine the role of FcaPV-2 in a cohort of Australian felines and their associated cutaneous lesions, another possible reason to explain the differences in the results with the conventional veterinary literature may be an underlying genetic component amongst Australian felines. However, there does appear to be a dichotomous aetiological relationship between FcaPV-2 infection and UV-protected SCCs compared to

UV-exposed SCCs which aligns with observations in other feline studies globally, suggesting that the findings in Australian cats with cutaneous SCCs (not the premalignant lesions) are consistent with results globally (Munday and Thomson 2021; Munday and Aberdein 2012; Munday, Gibson and French 2011). It is an interesting result that whilst there was no statistically significant difference amongst FcaPV-2 infection and the feline cutaneous premalignant lesions BISCs and AKs, there was a statistical significance between viral infection and UV-protected SCCs compared to UV-exposed SCCs. A possible explanation for this may relate to the earlier described theory whereby FcaPV-2 infection may be acting as a secondary co-factor synergistically with primary UV exposure potentializing the effects of UV damage in the development of premalignant AKs. However, given that FcaPV-2 may only be acting as a secondary co-factor in the primarily sun-induced skin lesions, the effects of viral infection are perhaps limited to the early premalignant stage of AKs and may have dissipated or has become undetectable using the study's methodology upon transformation into a malignant and invasive UV-exposed SCC.

The relationship between the observation of histological viral cytopathic features and FcaPV-2 infection does provide supportive evidence that the presence of these histological effects can correlate with a PV infection. This supports that identification of these histological features can be helpful in determining a PV aetiology.

However, there were several limitations to this study. Firstly, both the PCR and IHC studies were retrospective, and the sample size comprised entirely of archived FFPE tissues at The University of Sydney. Retrospective case-control studies provide a moderate strength of evidence compared to other study designs (Wallace et al. 2022). In the context of this study,

specific limitations related to the retrospective nature included the inability to control for confounders and reliance on historical clinically submitted data not originally intended for research purposes. Furthermore, as the samples were collected from one laboratory archive only, proximity bias may be a possible confounder and hinders the ability to extrapolate the results from this study to the wider Australian feline population. The University of Sydney Veterinary Pathology Diagnostic Services mostly receives tissue samples from metropolitan Sydney and the University Veterinary Teaching Hospitals in Camperdown and Camden, although it does receive samples outside of these regions to a lesser extent. Therefore, the sample size was unfortunately limited mostly to the aforementioned areas and excluded most of New South Wales and other interstate locations.

Whilst the classification of a lesion due to FcaPV-2 infection based on p16 labelling with PCR positivity together provides stronger evidence of an aetiological relationship rather than interpretation of the results separately, there may still be limitations with this assessment. Formalin fixation can damage and cross-link DNA leading to erroneous PCR results compared to testing a fresh, non-formalin fixed sample (Little et al. 2006). All of the samples included in this study were FFPE and thus susceptible to possible formalin-related DNA cross-linking. During the preparation of tissue samples for DNA extraction, pretreatment using xylene was not performed. Whilst excess wax entering the DNA extraction process was avoided, it is possible that some wax may still have been present in the extraction and therefore. This did not appear to be a significant inhibitor as qPCR product was still able to be detected appropriately, although may possibly have interfered with the Fel28S housekeeping gene amplification via the SYBR assay.

A limitation with the interpretation of PCR results in general is that whilst PCR is able to detect the presence of viral DNA within the lesions, it cannot differentiate between an active carcinogen/infection, a transient infection, or a contaminant (Hoggard, Munday and Luff 2018). Additionally, whilst there is strong evidence that p16 labelling is related to a PV infection in both human and veterinary medicine, spontaneous mutations in the retinoblastoma protein may also lead to a consequential overexpression in p16; or mutations in the p16 protein itself may lead to reduced levels (Munday and Thomson 2021). Both of these limitations regarding interpretation of either the qPCR or IHC data as standalone independent tests were meant to be negated by the interpretation of IHC and PCR results together, although it is also known that almost all clinically healthy cats may carry FcaPV DNA on their skin (Geisseler et al. 2016; Munday and Witham 2010; Thomson et al. 2019; Thomson, Dunowska and Munday 2015). Therefore, if a lesion was PCR positive due to a transient infection not related to tumorigenesis and had a concurrent spontaneous mutation in the retinoblastoma protein resulting in p16 overexpression, this may have been falsely classified as a positive result. Likewise, if a lesion was PCR positive and had a true FcaPV-2 aetiology but had a simultaneous mutation in the p16 protein leading to reduced/absent detectable levels, then this result may have been falsely classified as negative and not related to PV infection. Greater strength of evidence may be provided through more advanced methodology such as *in situ* hybridisation to localise the FcaPV DNA within the basal and suprabasilar layers of the lesion and/or the transcriptionally active E6 and E7 oncoproteins which play major roles in PV-related tumorigenesis. Another limitation with the study's qPCR methodology was the lack of quantitative normalisation of the results. Quantitative normalisation, through the calculation of ΔΔCt and/or ΔCt, would have helped to improve comparability between sample groups and possibly, with FcaPV-2-

positive samples, help discriminate between a bystander/latent infection and an active infection (Mazzei et al. 2018). However, the latter limitation was intended to be addressed through combination of the qPCR results with IHC results, providing evidence of both the presence of viral DNA with evidence of active viral infection.

This study focused on detecting and demonstrating an association with FcaPV-2 infection and the development of feline cutaneous SCCs and premalignant lesions. The reason for the study's focus on FcaPV-2 was that this viral genotype appears to be the most common cause of PV-induced disease in felines and the majority of the research has focused on this genotype, and thus currently it has the strongest experimental evidence supporting its potential oncogenic role (Munday and Thomson 2021; Teh and Krockenberger 2021). Other FcaPV genotypes (FcaPV-1, 3, 4, 5, and 6) have been variably associated with PV-induced disease as well (Carrai et al. 2020; Mazzei et al. 2018; Munday, Sharp and Beatty 2019; Munday and Thomson 2021; Medeiros-Fonseca et al. 2023) and, while less commonly observed than FcaPV-2, because other FcaPV genotypes were not tested, it is possible that other FcaPV genotypes may have been confounders in this study. The possible presence of other disease causing FcaPV genotypes may also explain the discrepancy between p16 labelling and FcaPV-2 DNA detection in those cases that displayed positive p16 labelling but FcaPV DNA was not detected.

Small sample sizes in this chapter were also a limitation, particularly once PCR and IHC data were combined. The classification system of a "UV-exposed" and a "UV-protected" lesion also carried a limitation. The categorisation of lesions as "UV-exposed" or "UV-protected" relied exclusively on the reported lesion site provided by the submitting clinician. However,

accurately assessing whether a lesion is truly UV-exposed or protected is challenging as there is considerable variability amongst felines with regards to their pigmentation, fur coverage, and behavioural patterns. Consequently, lesions which have been designated as "UV-exposed" or "UV-protected" based solely on their reported anatomical location may not accurately reflect their actual UV exposure status. Additionally, Australia receives some of the highest levels of UV radiation in the world, and its main population centres in Australia such as Sydney, Brisbane and Perth receive higher UV doses compared to main population centres in New Zealand including Auckland, Wellington, and Christchurch (Gies et al. 2004; McKenzie 2017; Sharma et al. 2024). It is therefore possible that anatomical sites which were classically considered to be UV-protected may actually have been UV-exposed owing to the harshness of the Australian sun. Lastly, whilst it appears that histological viral cytopathic effects have a significant correlation with FcaPV-2 aetiology, the histological assessment of the viral cytopathic features was not performed via a blinded methodology. Therefore, the results may be prone to unintentional and unconscious selection bias.

In conclusion, the study results are suggestive that FcaPV-2 may play a greater role in the development of feline cutaneous premalignancies including AKs, and UV-protected SCCs, but does not appear to be significantly associated with invasive cutaneous UV-exposed SCCs. Whilst this study carried several limitations, the results do prompt further investigation into the role of FcaPV and oncogenesis, especially lesions which were not previously thought to be due to FcaPV infection such as AKs. If FcaPV is indeed related to a wider variety of feline cutaneous skin lesions, then a preventative strategy may be a future consideration to lower the SCC burden in the Australian feline population (Thomson et al.

2019). Furthermore, exploration into how FcaPV-2 infection may potentialize UV damage in lesions such as AKs would be of interest.

Chapter 4. General Discussion

The following chapter will focus on presenting a general discussion of the overall study and relate the results of this study to its original aims. The broad aims of this study were to: 1) confirm the association of *Felis catus* papillomavirus type-2 (FcaPV-2) infection with the development of cutaneous squamous cell carcinomas (SCC) in Australian cats, and 2) explore the association of FcaPV-2 in the development of feline cutaneous premalignant lesions including actinic keratosis (AK). It was hypothesised that p16 labelling and detectable FcaPV-2 DNA would be related to UV-protected SCCs and Bowenoid *in situ* carcinomas (BISC), demonstrating not only presence of viral DNA but also the effect of viral infection whereby the E7 oncoprotein of FcaPV-2 inhibits the retinoblastoma protein (pRb) resulting in increased p16 levels (Munday and Aberdein 2012; Munday et al. 2011; Munday, Gibson and French 2011). Conversely, it was hypothesised that UV-exposed SCCs and AKs would not contain detectable FcaPV-2 DNA nor exhibit positive p16 labelling, ultimately demonstrating that the development of these lesions were not due to FcaPV-2 infection but rather UV-damage.

The study's methodology of correlating both the presence of FcaPV-2 DNA via qPCR and the detection of the viral effects via p16 IHC labelling was intended to strengthen the degree of evidence to support the aetiological/oncogenic relationship between feline cutaneous lesions and viral infection. Lesions that were positive for FcaPV-2 DNA in combination with positive p16 labelling were considered to be most likely related to a viral aetiology. The two positive results interpreted concurrently were indicative of the presence of intralesional viral DNA with simultaneous effects of active viral infection – accumulation of p16 as a

consequence of FcaPV-induced degradation of the pRb via expression of the E7 oncogene (Altamura et al. 2016; Munday and Aberdein 2012; Munday et al. 2011; Munday, Gibson and French 2011; Munday and Thomson 2021). Conversely, a lack of intralesional viral DNA detection and p16 labelling were suggestive that FcaPV-2 was not an important part of the pathogenesis of the lesion.

The interpretation of discordant results was more challenging. Discordant results were observed between chapters 2 and 3, where there were significant statistical differences between p16 labelling in the AKs and BISCs and p16 labelling in the UV-exposed SCCs and UV-protected SCCs. However, once p16 labelling and qPCR results were combined and interpreted in tandem (chapter 3), there did not appear to be statistically significant differences in FcaPV-2 aetiology between BISCs and AKs, but a statistically significant difference emerged between UV-exposed and UV-protected SCCs. A possible reason to explain a lesion that exhibited positive p16 labelling but did not contain detectable FcaPV-2 DNA may be the presence of a genotype of FcaPV other than FcaPV (FcaPV-3, 4, 5, 6) causing the skin lesions. Whilst the other FcaPV genotypes do not appear to be commonly associated with PV-induced skin disease relative to FcaPV-2, disease has been reported in the literature (Carrai et al. 2020; Medeiros-Fonseca et al. 2023; Munday and Thomson 2021; Mazzei et al. 2018). As such, this study cannot exclude the possibility that other FcaPV genotypes were associated with the development of feline cutaneous lesions. Another possible reason for a lesion with positive p16 labelling but negative intralesional FcaPV-2 DNA may be a spontaneous mutation in pRb which would result in increased p16 levels independent of FcaPV-2 and its E7 oncogene (Munday and Thomson 2021). Conversely, a lesion that contained positive intralesional FcaPV-2 DNA but without overt p16 labelling

may represent a bystander/latent infection. It is known that the majority of felines are infected with FcaPV-2, however only a few develop disease (Geisseler et al. 2016; Munday and Thomson 2021; Teh and Krockenberger 2021; Thomson, Dunowska and Munday 2015). Indeed, one study from Switzerland reported a FcaPV-2 genoprevalence of 98% from a population of dermatologically healthy cats, however most did not mount an antibody response (Geisseler et al. 2016). This is suggestive that latent FcaPV-2 infection is widespread amongst felines and thus interpretation of a positive qPCR result alone is insufficient to draw significant associations between FcaPV-2 infection and the development of cutaneous lesions. A positive qPCR result without concurrent p16 labelling, or without appropriate quantitative normalisation, cannot be differentiated from a bystander/latent infection.

Discrimination between an active FcaPV-2 infection and a bystander/latent infection may also have been improved with quantitative normalisation of the qPCR results, through the calculation of ΔCt and/or ΔΔCt (Mazzei et al. 2018). Quantitative normalisation may have improved the qPCR sensitivity, allowing for the detection of subtle differences and the level of viral presence between sample groups, and helping to ascribe stronger association amongst FcaPV-2-positive lesions. This is a limitation with the study's qPCR methodology which reported binary categorical outcomes (positive/negative) reducing comparability amongst sample groups. Consequently, it is possible that there may have been differences in FcaPV-2 expression amongst the sample groups that were not detected, making it more difficult to discriminate between an active FcaPV-2 infection and a latent one.

There were two main reasons for the lack of quantitative normalisation. Firstly, whilst all of the FcaPV-2 qPCR testing was performed using the SYBR assay, some of the samples Fel28S qPCR SYBR assays failed and were re-tested using a TaqMan probe assay. The use of different assays precluded quantitative analysis of Fel28S and FcaPV-2 data, because of differing qPCR efficiency. Secondly, the methodology of ΔΔCt calculation involves the comparison of the ΔCt numbers from the experimental samples (in this study, a sample with active FcaPV-2 infection) with the ΔCt numbers from a reference/negative control group (a sample without active FcaPV-2 infection) (Livak and Schmittgen 2001). Each qPCR run consisted of a non-template control as the negative control group and a positive control consisting of a FcaPV-2 gene fragment. Because this study did not assume that AKs and UV-exposed SCCs were unrelated to FcaPV-2 infection, AKs were not judged to be a suitable FcaPV-2-negative control group. To the contrary, the study results suggest that AKs may have an association with FcaPV-2 infection.

Ultimately, whilst the lack of quantitative normalisation of the qPCR results was a limitation, reducing the assay's sensitivity and discriminative ability, the combination of the qPCR results in tandem with p16 labelling via IHC was intended to strengthen the ability to discern whether a FcaPV-2-positive lesion was an active infection or a bystander/latent infection. This is because the combination of qPCR with IHC results provided evidence of viral presence via qPCR, and evidence of the effects of viral infection via p16 labelling. In this study, a lesion was only considered to be appropriately associated with FcaPV-2 infection if it demonstrated positivity amongst both assays, thus strengthening the degree of association between FcaPV-2 infection and feline cutaneous lesions, providing a means to discriminate between an active and bystander/latent infection amongst qPCR FcaPV-2-

positive lesions.

The results of this study ultimately do not fully align with the hypotheses nor with the veterinary literature. It is currently thought that AKs are a UV-induced lesion whereas BISCs are related to FcaPV infection (Munday and Thomson 2021). In this study although there was a statistically significant relationship between FcaPV-2 infection and UV-protected SCCs compared to UV-exposed SCCs, a finding which is consistent with other feline studies (Munday, Gibson and French 2011), a statistically significant difference with FcaPV-2 infection and BISCs or AKs could not be demonstrated. Ultimately, this suggests that FcaPV-2 may be involved in the development of more feline cutaneous lesions, specifically AKs, than previously thought. It is possible that FcaPV-2 infection may be functioning as a secondary co-factor synergistically with primary UV damage in the development of AKs. These are similar observations to those currently reported in the human medical literature whereby it is now being postulated that human papillomavirus (HPV) may be involved in the development of AKs in people as well (Wang, Z. et al. 2024). Indeed, similar to this current study, HPV is being postulated as a possible cofactor along with chronic UV damage, immunosuppression, and genetic predispositions in the development of AKs and cutaneous SCCs (Wang, Z. et al. 2024). It is a plausible speculation that there may also still be a relationship between FcaPV-2 infection and UV-exposed cutaneous SCCs, but evidence of viral infection could not be detected using the study's methodology, or perhaps because viral infection may be a secondary co-factor potentializing primary UV damage, or because the effects of viral infection may have dissipated upon transformation to an invasive SCC. As this study also represents the first to investigate the role of FcaPV-2 infection in a

population of Australian felines, there may be a possible genetic component accounting for the difference in outcomes.

Pathways for future studies may include the utilisation of *in situ* hybridisation (ISH) rather than polymerase chain reaction (PCR) in the detection of FcaPV-2 DNA, examination of other protein biomarkers including p53, p16, pRb, and the E6 and E7 oncoproteins, including a larger sample size, and exploring how FcaPV-2 may be potentializing UV damage as a secondary co-factor particularly in AKs and UV-exposed cutaneous SCCs. Given that chronic UV exposure induces DNA damage and that papillomaviral oncogenes inactivate key tumour suppressor proteins such as p53 and pRb, detecting evidence of UV-induced DNA lesions in combination with FcaPV-2 infection via qPCR, ISH, and p16 IHC may help to support this theory of synergistic carcinogenesis. Specifically, evidence of UV-induced DNA damage has been demonstrated in studies involving people using IHC for anti-cyclobutane pyrimidine dimer (CPD/TDM-2) antibodies (Mabruk et al. 2009; El-Yazbi and Loppnow 2014). Other reported IHC antibodies used to detect UV-induced DNA damage in studies involving people include anti-8-hydroxydeoxyguanosine (8-OH-dG) to demonstrate the formation of DNA adducts; anti-4-hydroxy-2-nonenal (HNE) to demonstrate evidence of lipid peroxidation; and anti-advanced glycation end products (AGE) to show protein damage (Mamalis et al. 2014). The utilisation of ISH provides an advantage over PCR in that it is able to localise the presence of viral DNA within intralesional cells, which allows for the differentiation between an active infection from a contaminant or asymptomatic transient infection. Interpretation of the localisation of viral DNA with its effects on other protein biomarkers such as p53, p16 and pRb with correlation with E6 and E7 expression would help with understanding the mechanistic pathogenesis of viral infection. This would be beneficial

as this study focused primarily on identifying an association between viral infection and skin lesions rather than explore the mechanisms behind pathogenesis. Alternatively, qPCR with quantitative normalisation of the results by calculation of ΔCt and/or ΔΔCt would provide stronger evidence of an association with FcaPV-2 infection (Mazzei et al. 2018) and provide another means of discriminating between an active FcaPV-2 infection and a bystander/latent infection. One of the main limitations of this study was its relatively small sample size, particularly as the methodology evolved into combining the data from the PCR and IHC studies together. As such, the strength of evidence provided by the results in this study is not particularly strong and would be enhanced by the inclusion of more samples. Finally, it would be of great interest to explore in greater depth how FcaPV-2 may be acting as a secondary co-factor potentializing UV damage as the results from this study suggest it might be doing with AKs.

In summary, the original aims of the study – to confirm the association of FcaPV-2 infection in the development of cutaneous SCCs and its premalignant lesions BISCs and AKs in a population of Australian felines – have been answered. FcaPV-2 infection appears to be associated with BISCs, AKs, and UV-protected SCCs but not UV-exposed SCCs in a small sample population of Australian cats. This is suggestive that FcaPV-2 may be involved in the development of more cutaneous lesions than what is currently expected and prompts consideration of possible future preventative strategies with regards to viral infection to reduce the burden of cutaneous SCCs and premalignant skin lesions such as BISCs and AKs in felines.

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Appendix 1

Table 1. Case information for all samples used in both the immunohistochemistry and quantitative polymerase chain reaction (qPCR) studies.

Diagnosis	Age (years)	Sex	Breed	Desexed	p16 Positive	qPCR Fel28S Detection	qPCR FcaPV-2 Detection
AK	13	Female	Domestic Short Hair	Yes	No	Yes	Yes
AK	14	Female	Domestic Short Hair	Yes	No	No	N/A
AK	12	Male	Domestic Short Hair	Yes	Yes	Yes	No
AK	12	Male	Domestic Short Hair	Yes	No	Yes	No
AK	13	Male	Domestic Long Hair	Yes	No	No	N/A
AK	11	Male	Domestic Short Hair	Yes	No	Yes	No
AK	N/A	Female	Domestic Short Hair	Yes	No	Yes	No
AK	11	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
AK	3	Male	Domestic Short Hair	No	Yes	Yes	No
AK	N/A	Female	Domestic Short Hair	Yes	Yes	Yes	Yes
AK	17	Female	Domestic Short Hair	Yes	No	Yes	Yes
AK	8	Male	Domestic Short Hair	No	No	Yes	Yes
AK	14	Female	Domestic Short Hair	Yes	Yes	Yes	No
BISC	13	Male	Domestic Short Hair	Yes	Yes	Yes	No
BISC	6	Male	Domestic Long Hair	No	Yes	No	N/A
BISC	7	Male	Domestic Short Hair	Yes	Yes	No	N/A
BISC	12	Male	Domestic Long Hair	Yes	Yes	Yes	Yes
BISC	7	Male	Domestic Long Hair	No	Yes	No	N/A
BISC	12	Male	Domestic Short Hair	Yes	Yes	No	N/A
BISC	6	Male	Domestic Short Hair	Yes	Yes	No	N/A
BISC	13	Female	Devon Rex	Yes	Yes	Yes	No
BISC	17	Male	Russian Blue	Yes	Yes	No	N/A

BISC	16	Female	Domestic Short Hair	Yes	Yes	Yes	No
BISC	11	Female	Domestic Long Hair	Yes	Yes	Yes	Yes
BISC	15	Female	Domestic Short Hair	Yes	No	Yes	No
BISC	9	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
BISC	11	Female	Domestic Long Hair	Yes	Yes	Yes	Yes
BISC	14	Male	Domestic Short Hair	Yes	Yes	Yes	No
BISC	13	Female	Domestic Short Hair	Yes	Yes	No	N/A
BISC	13	Female	Domestic Short Hair	Yes	Yes	Yes	Yes
BISC	13	Female	Burmese	Yes	Yes	Yes	Yes
BISC	14	Male	Domestic Short Hair	Yes	Yes	Yes	No
BISC	10	Male	Domestic Short Hair	Yes	Yes	Yes	No
BISC	10	Male	Domestic Short Hair	Yes	No	Yes	No
BISC	9	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
SCC (UV-exposed)	6	Male	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-exposed)	6	Male	Domestic Short Hair	Yes	No	No	N/A
SCC (UV-exposed)	4	Female	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-exposed)	12	Male	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-exposed)	13	Male	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-exposed)	20	Female	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-exposed)	11	Female	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-exposed)	16	Male	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-exposed)	13	Male	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-exposed)	14	Female	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-exposed)	13	Male	Cornish Rex	Yes	No	No	N/A
SCC (UV-exposed)	15	Male	Domestic Short Hair	No	No	Yes	No
SCC (UV-exposed)	12	Female	Domestic Long Hair	Yes	No	N/A	N/A
SCC (UV-exposed)	11	Female	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-exposed)	11	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
SCC (UV-protected)	11	Male	Cornish Rex	Yes	Yes	Yes	Yes

SCC (UV-protected)	7	Male	Domestic Short Hair	Yes	Yes	Yes	No
SCC (UV-protected)	7	Male	Domestic Short Hair	Yes	No	Yes	No
SCC (UV-protected)	13	Female	Persian	Yes	No	Yes	No
SCC (UV-protected)	12	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
SCC (UV-protected)	15	Female	Domestic Short Hair	No	Yes	Yes	Yes
SCC (UV-protected)	15	Female	Domestic Short Hair	No	Yes	Yes	Yes
SCC (UV-protected)	17	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
SCC (UV-protected)	9	Male	Domestic Short Hair	Yes	Yes	Yes	Yes
SCC (UV-protected)	9	Male	Domestic Short Hair	Yes	Yes	Yes	Yes

AK = actinic keratosis, BISC = Bowenoid *in situ* carcinoma, SCC = squamous cell carcinoma, UV = ultraviolet, qPCR = quantitative polymerase chain reaction, FcaPV-2 = *Felis catus* papillomavirus type 2, N/A = not available.