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Pathology, coinfections and oncogenesis in South Australian koalas (*Phascolarctos cinereus*) and their association with koala retrovirus (KoRV)

by

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Abstract

Koalas are a vulnerable iconic species, for which disease is a significant threat to populations around Australia. Lymphoid neoplasia and immunomodulation in koalas have been associated with an important pathogen, koala retrovirus (KoRV). The prevalence and transmission of KoRV differs in northern koalas, from Queensland and New South Wales, compared to southern koalas, from Victoria and South Australia. Northern koalas inherit and horizontally transmit KoRV via endogenous and exogenous mechanisms, respectively with all koalas infected. Southern koalas are hypothesised to only have exogenous transmission, and within these populations the prevalence is less. Both proviral load (inserted viral genome into host DNA) and viral load (extracellular RNA viral genome) are significantly higher in northern koalas in comparison to southern koalas, representing active transcription in a greater number of cells. Mechanisms for KoRV-associated oncogenesis are still unknown, along with the prevalence of lymphoid neoplasia in the Mount Lofty Ranges population, South Australia. Increased susceptibility of disease has been inferred from studies exploring KoRV coinfection with *Chlamydia pecorum*, a bacterium which causes both ocular and urogenital disease in koalas. KoRV and *C. pecorum* are well-studied key pathogens of koalas, although the significance of another infectious agent Phascolarctid gammaherpesviruses, is unknown. Avian and human studies have shown increased incidence of neoplasia when coinfected with a retrovirus and gammaherpesvirus, but there is no current evidence for this in koalas.

Association of diseases is based on a confident diagnosis of infectious agents, for which koalas in South Australia have shown variations in proviral analysis creating complexity in the diagnosis of KoRV. The KoRV viral genome consists of three genes, *gag*, *pol* and *env*, flanked by long terminal repeats, LTRs. The *pol* gene qPCR has been a standard diagnostic

tool, but more investigation across multiple genome targets has shown variance in SA koala PCR and qPCR results. In this study, PCR and qPCR methods were used against two targets in the *gag* gene, one in the *pol* gene and two in the *env* gene. Koalas for which all proviral targets were positive were designated KoRV positive and koalas for which one *gag* target, *pol* target and *env* targets were negative were designated KoRV negative. There were 41.2% (89/216) KoRV positive, 56.9% (123/216) KoRV negative koalas and only 1.9% (4/216) deemed inconclusive. Viral gene expression analysed by qPCR was found to be present in 10/10 KoRV positive koalas, and absent in 5/5 KoRV negative koalas. RNAseq analysis revealed transcription of sequences homologous to terminal regions of the KoRV genome in all koalas, verified by the presence of one *gag* gene target in almost all (215/216) koalas tested by PCR. The presence of these regions in South Australian koalas without the presence of the full KoRV genome, suggests an endogenised retroviral element, potentially within the koala genome prior to KoRV.

Lymphomic koalas showed high expression of KoRV and higher proviral loads compared to KoRV positive koalas without lymphoma. Lymphoma was found in 1.2% (3/240) of koalas and these cases were collated with previous South Australian lymphoma cases for classification. All cases had abdominal involvement, were intermediate to large cell and of non-T cell origin. RNAseq data was compared from lymphomic lymph nodes, lymph nodes from KoRV positive and lymph nodes of KoRV negative koalas. High KoRV transcription was found in lymphomic tissue, 1207 genes showed differential expression between KoRV positive koalas diagnosed with lymphoma and KoRV negative koalas and 939 genes between KoRV positive koalas diagnosed with lymphoma and KoRV positive koalas. Oncogenes MYB, MYCL and FLT3 were significantly upregulated and possible candidates in the

incitement of oncogenesis. Dysregulation in IL10, and pathways associated with NF-kB also support the role of immunosuppression in lymphoma pathogenesis.

Based on theories of KoRV-associated immunosuppression leading to opportunistic infections and augmentation of disease with pathogens, coinfections and comorbidities were investigated in 247 necropsied koalas. KoRV was not found to be associated with *C. pecorum* or disease severity. However, *C. pecorum* was associated with another recently discovered infectious agent, Phascolarctid gammaherpesvirus (PhaHV), and in this cohort PhaHV was associated with the presence of paraovarian cysts, regardless of chlamydial status, similar to Victorian koalas. Also, KoRV and PhaHV-2 coinfection was associated with neoplasia and warrants further investigation. Disease and infectious agents were negatively correlated with victims of road traffic accidents showing the potential importance of this group of koalas.

Novel pathologies were found in this koala necropsy cohort. Pulmonary actinomycosis, a new presentation of respiratory disease of koalas, with a subset of these koalas presenting with secondary hypertrophic osteopathy, a second novel pathology in koalas. Fifteen koalas were found to have pyogranulomatous lobar pneumonia, predominantly affecting the left caudal lung lobe. Histological examination showed Splendore Hoeppli phenomenon with associated Gram-positive or Gram-variable, non-acid fast, filamentous bacteria. The pathogen was identified as a novel *Actinomyces* sp. and through 16S rRNA gene sequencing was closest to *A. timonensis*. Collaboration with other wildlife veterinarians found that two of the study koalas also presented with another undescribed pathology in koalas, hypertrophic osteopathy, found secondary to the pulmonary lesions. These discoveries highlight the necessity for ongoing necropsy studies in koalas to increase the knowledge of disease presentations in this iconic species.

Overall, this thesis aimed to examine the association of disease with several pathogens, with particular focus on the association between KoRV and lymphoma. Advancements were made in recommendations for KoRV diagnosis in South Australia, gene dysregulation in KoRV-associated lymphoma, interactions with other key pathogens; *C. pecorum* and PhaHV, and the report of novel disease presentations. The increased knowledge of infectious disease and interaction of disease in South Australian koalas can help management strategies protecting the health and welfare of these koalas. Also, identification of dysregulated genes has increased the knowledge of oncogenesis by koala retrovirus.

Contents

List of Abbreviations

Abl-MuLV	Abelson murine leukaemia virus
ALV	Avian leukosis virus
AMV	Avian myeloblastosis virus
BLV	Bovine leukaemia virus
BoHV	Bovine herpesvirus
DA	Dog attack/Canine attack
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EHV	Equine herpesvirus
EP	Eyre Peninsula
ERV	Endogenous retrovirus
FeLV	Feline leukaemia virus
FeSV	Feline sarcoma virus
FIV	Feline immunodeficiency virus
FMLV	Friend murine leukaemia virus
GALV	Gibbon ape leukaemia virus
HPF	High powered field
HIV	Human immunodeficiency virus
HTLV	Human T-cell leukaemia virus
IHC	Immunohistochemistry
ISD	Immunosuppressive domain
KI	Kangaroo Island
KoRV	Koala retrovirus
KSHV	Kaposi's sarcoma herpesvirus
LL	Lymphoid leukosis
LTR	Long terminal repeat
MD	Marek's Disease
MDHV	Marek's Disease herpesvirus
MelWMV	Melomys woolley monkey virus
MLR	Mount Lofty Ranges
MLV	Murine leukaemia virus
MMLV	Moloney murine leukaemia virus
NGS	Next Generation Sequencing
NSW	New South Wales
PCR	Polymerase chain reaction
PERV	Porcine endogenous retrovirus
PhaHV	Phascolarctid gammaherpesvirus
QLD	Queensland
RAV	Rous associated virus
RBD	Receptor binding domain
RIV	Riverland
RNA	Ribonucleic acid
RSV	Rous sarcoma virus
RTA	Road traffic accident
SA	South Australia
SESA	South-eastern South Australia
SIV	Simian immunodeficiency virus
SSV	Simian sarcoma virus

SU	Surface
TM	Transmembrane
qPCR	Quantitative polymerase chain reaction
VIC	Victoria
WA	Western Australia
WHO	World Health Organisation
WMSV	Woolley monkey sarcoma virus
WMV	Woolley monkey virus

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Chapter 1: Literature Review

Koalas (*Phascolarctos cinereus*) are a vulnerable species (Woinarski *et al.* 2016) that face

multiple threats, both environmental and biological. Environmental treats are through

deforestation and habitat fragmentation. Fragmented remnant populations are susceptible to

overbrowsing and movement through built-up areas can increase the incidence of road traffic

accidents and dog attacks (Waugh *et al.* 2017). Koalas are also vulnerable due to disease,

such as that caused by two key pathogens - koala retrovirus (KoRV) and *Chlamydia* spp.

(Woinarski *et al.* 2016; Quigley *et al.* 2020). In South Australia, koalas have a history of

local extinction and reintroduction, which the genetic bottleneck that occurs from these

events is important may have an implication in KoRV transmission. KoRV infections have

the ability to spread in two distinct ways, exogenously – which is horizontal (e.g. through

close contact, vector or fomite) or vertical (e.g. through milk or *in utero* infection), or

endogenously – for which the retroviral provirus has inserted into a germ cell line and been

passed from parental DNA to offspring in Mendelian dominant fashion (Tarlinton *et al.* 2005;

Stoye 2006). KoRV is currently hypothesised to be spreading through koala populations,

from Queensland southward, through New South Wales, Victoria and to South Australia

(Simmons *et al.* 2012). Northern koala populations, from Queensland and New South Wales,

have a KoRV prevalence of 100% and KoRV is endogenised, while southern populations,

from Victoria and South Australia, have a much lower prevalence, 51.5% (Simmons *et al.*

2012), and 49.2% (Fabijan *et al.* 2019b), respectively. Therefore, it is postulated that

generalised endogenisation is unlikely to have taken place in southern populations (Simmons

et al. 2012; Wedrowicz *et al.* 2016).

There is a strong association of KoRV with cancer, especially lymphoma and leukaemia,

although the mechanism of oncogenesis in KoRV positive koalas remains unknown (Canfield

et al. 1990; Hanger 1999; Hanger *et al.* 2000; Tarlinton *et al.* 2005). South Australian koalas are hypothesised to be a key population in which to study KoRV-induced oncogenesis, due to the lack of KoRV endogenisation within this population. Lymphoid neoplasia has been highlighted as one of the most common neoplastic diseases of koalas, being described in 3-5% of the wild population and up to 55% in captive populations (Canfield *et al.* 1987; Hanger 1999). Lymphosarcoma, or lymphoma, has also recently been reported in SA in a female koala with overt chlamydiosis (Fabijan *et al.* 2017). In addition, KoRV is associated with increased susceptibility of disease, with associations found with chlamydiosis severity in several studies (Legione *et al.* 2017; Waugh *et al.* 2017; Maher *et al.* 2019; Fabijan *et al.* 2020). KoRV has also been associated with immunomodulation of key cytokines and immune genes, leading to a possible reduction in immune function (Maher *et al.* 2016; Maher *et al.* 2019). Gene expression data could be generated in the South Australian population to explore the role of KoRV in oncogenesis. Additionally, a greater understanding of the threats of disease within the SA population is needed, as disease is a significant threat to koalas in northern populations (QLD and NSW). Associations between the key pathogens; KoRV, *Chlamydia pecorum*, and the more recently discovered Phascolarctid gammaherpesviruses, could be more easily determined in populations where non-infected koalas exist, such as the South Australian koala population.

The following review outlines the threats to koalas, both environmental and biological, with a focus on pathogens of interest; KoRV, *Chlamydia pecorum* and Phascolarctid gammaherpesvirus (PhaHV). The role of retroviruses in formation of cancer will be discussed, with emphasis on mechanisms that could be extrapolated to KoRV and its role in lymphoid neoplasia.

1.1 Koalas distribution and status

The koala is an arboreal marsupial that inhabits eucalypt forests throughout eastern and south eastern Australia (Melzer *et al.* 2000), with its distribution divided into northern and southern populations. The northern population, which have been listed as vulnerable (Woinarski *et al.* 2016), spans the east coast and hinterlands of Queensland (QLD), New South Wales (NSW) inclusive of the Australian Capital Territory (ACT). The southern population is distributed throughout the majority of Victoria (VIC) and several divided populations in South Australia (SA), all of which are not considered as vulnerable (Woinarski *et al.* 2016). The most recent population modelling calculated a mean of 329,000 with a range of 144,000 – 605,000 for the total number of koalas in Australia (Adams-Hosking *et al.* 2016). Using the same principle, a decline of around 24% over three generations was calculated, with estimated losses of 53% for QLD, 26% for NSW, and 14% for VIC (Adams-Hosking *et al.* 2016). However, there remains uncertainty of exact koala population numbers, particularly following the devastating 2019-2020 bushfires. The 2019-2020 bushfire season was extremely ferocious, with 4,784,998ha, or an estimated 11% of koala habitat destroyed throughout Australia in this season alone (Ward *et al.* 2020).

In SA, the health and disease status of koalas has only recently begun to be described, with more KoRV investigation needed. There are currently five koala populations, fragmented by considerable breaks in desirable habitat. These populations are found in Adelaide and the Mount Lofty Ranges (MLR), Kangaroo Island (KI), South-East South Australia (SESA), the Riverland (RIV) and the Eyre Peninsula (EP). Recently the MLR population was estimated at 113,704 koalas (±2sd: 27,685 – 199,723), through citizen-science collected data (Sequeira *et al.* 2014). The KI population has been closely monitored over the past 20 years, with management through sterilisation and translocation. Following successful management, in

2010 the population was estimated at 14,270 (± 759) (Molsher 2017). Management was

reduced after 2010 and in 2015 the KI population was estimated at 48,506 (± 5,976), with

recent blue gum plantations accounting for 23,360 (± 3,330) koalas (Molsher 2017). In 2016,

the entire SA population was estimated to be approximately 33,000 (19,000-51,000), with

only 3% decline over three generations (Adams-Hosking *et al.* 2016). These studies clearly

show diverse estimates of the SA population, making assessment of their conservation status

difficult. More recently, the 2019/2020 mega-fire on KI significantly reduced the population,

which burnt more than 210,000 hectares and has been estimated to have reduced the island

population by approximately 80%. In 2019/2020 a bushfire in Cudlee Creek, MLR burnt

23,000 hectares and another in 2021 in Scott Creek National Park, burnt 2570 hectares, both

of which are densely populated by koalas. The combination of these fires will have

significantly reduced total koala population numbers in SA.

1.2 History of South Australian koalas

SA populations have a history of extinction and reintroduction. There are fossil records

which show the presence of modern koalas in SA in the Pliocene and Pleistocene ages (Black

et al. 2014), along with suitable habitat across the Nullarbor region and into Western

Australia (WA) up to approximately 74,000 – 130,000 years ago (Adams-Hosking *et al.*

2011; Black *et al.* 2014). Records of living modern koalas in the majority of SA prior to the

20th century are unknown with European settlers only finding the first koala 10 years after

first arriving in Australia and sightings only reported in the south-eastern region of SA (Black

et al. 2014; Sequeira *et al.* 2014). Indigenous Australians have been part of the Australian

country for over 65,000 years (Clarkson *et al.* 2017), but all modern-day faunal distribution

interpretations are records since European settlement. Modelling of habitat from the Last

Glacial Maximum, demonstrates possible suitable habitat in SA, focused on the MLR and the Fleurieu Peninsula (Black *et al.* 2014).

In the early part of the 20th century, it was presumed that koalas in SA were extinct due to hunting practices, baiting and possible disease events (Lindsay 1950; Robinson 1978). Conservationists set about the reintroduction of koalas as early as 1923 when six koalas from VIC were released into an enclosure in Flinders Chase National Park, KI. This founding population had a further addition of 12 koalas (six male, six female) from VIC in 1925 (Robinson 1978), as the population grew, koalas were released and began to populate the surrounding areas. On the mainland, Adelaide Koala Park acquired 10 koalas in 1950, of which seven were from QLD and three from Bangham Forest (SESA) and they also established a second breeding colony in MLR (Lindsay 1950). When the Adelaide Koala Park was closed, 10 koalas were translocated to Goat Island (RIV), and established another small breeding colony derived from their QLD and SA captive breeding program (Robinson 1978). Subsequent reintroductions from the ever-increasing KI population to three areas of SA occurred over the following 10 years with animals released around Waikerie, Berri and Renmark (RIV), South of Port Lincoln (EP) and to Ashbourne near Strathalbyn (MLR) (Robinson 1978). The koalas in the Adelaide Hills region (MLR) are also postulated to be derived from escaped animals from Cleland and Belair Native Fauna Parks and illegal translocations from NSW to Brownhill Creek in around 1935 (Robinson 1978). Hence the MLR koala population has had several contributions to its genetic diversity compared with KI, which may have ramifications for their genetic fitness and susceptibility to certain diseases.

1.3 Genetic diversity of South Australian koalas

Genetic diversity of the SA populations is important from a disease and disease susceptibility perspective. KI koala populations, founded from a small number of VIC koalas (Robinson *et al.* 1989), have shown low diversity based on their inherited mitochondrial DNA control region (Neaves *et al.* 2016). Microsatellite analysis of 29 KI koalas, 20 EP koalas, and 32 MLR koalas found greater diversity in the MLR koalas, but in comparison to Gippsland koalas (VIC), the genetic diversity was still low (Seymour *et al.* 2001). From mitochondrial DNA analyses, there was no evidence of QLD or NSW genetic contribution to koalas in KI, EP or MLR populations, contrary to historical accounts (Houlden *et al.* 2000; Seymour *et al.* 2001). Decreased genetic diversity has the potential to impact on the success of the koala in reproduction, adaption to disease and environmental change, like other animals that have suffered severe population bottlenecks (O'Brien *et al.* 1985; Houlden *et al.* 1999; Seymour *et al.* 2001). Indeed, certain pathologies, such as testicular abnormalities, have been associated with increased inbreeding coefficients in SA koalas (Seymour *et al.* 2001). Despite this, the MLR (Sequeira *et al.* 2014) and KI (Fabijan *et al.* 2019a) – pre-bushfires – populations are large and healthy population with high reproductive capability, indicating a currently reduced impact of the genetic bottleneck..

1.4 Threats facing koala populations

Koalas face a number environmental and biological threats; habitat loss and fragmentation, urbanisation, climate change and diseases such as *Chlamydia* and koala retrovirus (KoRV). Human impact on wildlife is extensive, in Australia the increase in human populations since the first European settlers arrived has influenced the use of land throughout koala habitats. Urbanisation and increased urban sprawl have reduced and fragmented Eucalyptus forests

and prime koala habitat (Melzer *et al.* 2000; McAlpine *et al.* 2006). A secondary effect has been the high proportion of koala deaths and veterinary euthanasia that are anthropogenic; through road traffic accidents (RTA) and dog attacks (DA) (Canfield 1991; Dique *et al.* 2003; McAlpine *et al.* 2015). Euthanasia from RTA made up 21.0% (109/519) and euthanasia from DA was 7.7% (40/519) of koalas in a recent study from south east QLD (Gonzalez-Astudillo *et al.* 2019). Another retrospective study of koala admissions and outcomes to Australia Zoo, QLD showed 36.4% (1307/3590) victims of RTAs and 13.8% (495/3590) of victims of DAs (Taylor-Brown *et al.* 2019). Collating 17 years of hospital admissions in southeast QLD, 41,606 aetiology incidences were found from 20,250 admissions, of which 15.5% (6432/41606) were RTA and 5.2% (2154/41606) were animal attacks (Gonzalez-Astudillo *et al.* 2017). Climatic changes, including increased ambient temperatures, decreased survival of eucalyptus species and changes in water, toxin and nitrogen content of the leaves are all changes that, for a specialist folivore such as the koala, could have detrimental effects (Reckless *et al.* 2017). The effect of recent climate change has been modelled for koala populations in QLD, showing that the increased frequency of high temperatures, drought and fire has contributed to the decline of numbers in this area (Lunney *et al.* 2014). This hypothesis can be expanded to most of the other populations in Australia and demonstrates overall decline (McAlpine *et al.* 2015).

1.5 Diseases in koalas

Necropsy studies to determine what diseases affect koalas date back to the 1970s (McKenzie 1981; Obendorf 1983; Canfield 1987; Speight *et al.* 2018; Gonzalez-Astudillo *et al.* 2019) and more recently, retrospective long-term admission studies to wildlife hospitals have also added to this knowledge base (Griffith *et al.* 2013; Gonzalez-Astudillo *et al.* 2017). Comparison of the findings of these studies shows distinct differences in disease

presentations based on geographical region. Table 1 shows a summary of findings from these broad-focused necropsy or long-term admission studies, excluding data regarding anthropogenic causes for euthanasia, death or admission such as RTA or DA. Chlamydiosis, the most extensively studied koala disease, is common in NSW and QLD koalas and appears to be increasing over time (McKenzie 1981; Canfield 1987; Griffith *et al.* 2013; Gonzalez-Astudillo *et al.* 2017; Gonzalez-Astudillo *et al.* 2019). Neoplasia, which in koalas is predominantly lymphoma or lymphoid leukaemia , is most prevalent in QLD and NSW and associated with KoRV infection (Canfield 1987; Griffith *et al.* 2013; Gonzalez-Astudillo *et al.* 2017; Gonzalez-Astudillo *et al.* 2019). By contrast, oxalate nephrosis, a renal disease that causes intratubular and interstitial inflammation, tubular degeneration, necrosis and loss, glomerular atrophy and cortical fibrosis, is much more common in SA populations (Speight *et al.* 2013; Speight *et al.* 2018). Differences in levels and types of reported parasitic infections can also be observed, with the tapeworm *Bertiella obesa* seen reported in NSW and QLD (McKenzie 1981; Canfield 1987), ticks predominating in NSW and QLD (McKenzie 1981; Griffith *et al.* 2013; Gonzalez-Astudillo *et al.* 2017) and mange, caused by *Sarcoptes scabiei* reported in VIC and SA (Obendorf 1983; Speight *et al.* 2017; Speight *et al.* 2018). Aetiologies of pneumonia have been extensively studied in the koala, predominantly associated with *Chlamydia* spp. (Brown *et al.* 1984; Mackie *et al.* 2016). *Cryptococcus gattii* can also cause an extensive fungal pneumonia which can disseminate to other tissues, including the central nervous system (Krockenberger *et al.* 2003). Cryptococcosis caused by *Cryptococcus neoformans* var. *gattii* and is found in has been reported in koalas in QLD (McKenzie *et al.* 1979) and NSW, (Canfield 1987; Krockenberger *et al.* 2003) but not in VIC or SA. *Bordetella bronchiseptica* has also been identified in outbreaks of pneumonia within captive koala colonies in QLD (McKenzie *et al.* 1979) and NSW (Canfield *et al.* 1986; Canfield 1987) either as a primary pathogen, or secondary to other diseases such as

cryptococcosis or chlamydiosis (Canfield *et al.* 1986; Mackie *et al.* 2016). Other likely

genetically associated disease have shown higher incidence in SA koalas and include

testicular abnormalities, in the form of atrophy (Speight *et al.* 2018) or aplasia (Seymour *et al.* 2001) and spinal deformities such as scoliosis or kyphosis (Fabijan *et al.* 2020).

Table 1: Summary of key findings from necropsy and long-term admission studies

Study	Mackenzie	Obendorf	Canfield	Griffith	Gonzalez-Astudillo	Speight	Gonzalez-Astudillo
Year	1975-1978	1975-1980	1980-1986	1975-2004	1997-2013	2012-2103	2013-2016
State	QLD	VIC	NSW	NSW	QLD	SA	QLD
Captive (C) and/or Wild (W)	11C, 15W	W	W	W	W	W	W
Number	26	44 necropsy 11 treatment	127	3,781 (admissions)	20,250 (41,606 aetiologies)	85	519
Chlamydiosis or *Chlamydia*-like signs	not recorded	not recorded	not recorded	20.40% (772/3781)	52.00% (21,619/41,606)	11.70% (10/85)	58.50% (304/519)
Urogenital disease*	11.50% (3/26)	29.10% (16/55)	33.80% (43/127)	11.20% (422/3781)	27.90% (11,629/41,606)	5.90% (5/85)	51.40% (267/519)
Conjunctivitis*	7.70% (2/26)	14.50% (8/55)	7.80% (10/127)	9.30% (350/3781)	17.20% (3,485/20,250)	10.60% (9/85)	16.90% (88/519)
Respiratory disease/ pneumonia	58.50% (8/26)	not recorded	5.50% (7/127)	not recorded	12.30% (2,493/20,250)	3.50% (3/85)	4.00% (21/519)
Renal disease	not recorded	"several"	11.80% (15/127)	not recorded	12.00% (2,432/20,250)	31.80% (27/85)	36.40% (189/519)
Oxalate nephrosis	not recorded	not recorded	not recorded	not recorded	not recorded	31.80% (27/85)	0.90% (5/519)
Gastrointestinal disease	15.40% (4/26)	not recorded	4.70% (6/127)	0.03% (1/3781)	not recorded	8.20% (7/85)	not recorded
Neoplasia	11.50% (3/26)	not recorded	4.70% (6/127)	0.03% (1/3781)	2.10% (420/20,250)	0.00% (0/85)	7.90% (41/519)
Lymphoma and/or leukaemia	7.70% (2/26)	not recorded	3.90% (5/127)	not recorded	not recorded	0.00% (0/85)	4.60% (24/519)
Parasitic	57.1% (4/7) *Bertiella obesa* 3.8% (1/26) *Ixodes holocyclus*	3.6% (2/55) *Sarcoptes scabiei*	"Common" *Bertiella obesa*	0.03% (1/3781) "ticks"	0.2% (49/20,250) "ticks"	8.2% (7/85) *Sarcoptes scabiei*	not recorded

1.6 Infectious pathogens of the koala

The most extensively studied and most important pathogen in koalas is *Chlamydia*. Koalas

can be affected by two species of *Chlamydia*; *C. pecorum* and *C. pneumoniae,* with the

former regarded as more pathogenic (Polkinghorne *et al.* 2013). Prevalence of *C. pecorum* has been found to be up to 100% in QLD and up to 88% in SA (Polkinghorne *et al.* 2013; Speight *et al.* 2016; Gonzalez-Astudillo *et al.* 2017; Gonzalez-Astudillo *et al.* 2019; Robbins *et al.* 2020), whilst koala populations on KI have been modelled to be free of *C. pecorum (Fabijan et al. 2019a). C. pecorum* is detected through PCR of DNA from ocular and urogenital swabs. Presentations can be further classified into apparent overt chlamydiosis, inapparent overt chlamydiosis and inapparent, undetectable disease. All are positive for *Chlamydia* by PCR with apparent overt chlamydiosis showing signs of disease without using veterinary techniques, such as wet bottom and/or keratoconjunctivitis. Inapparent overt chlamydiosis is diagnosed with the use of veterinary techniques, inclusive of ultrasound, urinalysis or postmortem examination. Asymptomatic koalas with undetectable chlamydiosis are classified as having inapparent disease (Wan *et al.* 2011; Polkinghorne *et al.* 2013). Chlamydiosis can present as ocular, urogenital and/or reproductive disease leading to morbidity, mortality and infertility (Weigler *et al.* 1988; Polkinghorne *et al.* 2013; Speight *et al.* 2016). *Chlamydia* spp. have also been associated with cases of rhinitis and/or pneumonia in QLD and NSW (Brown *et al.* 1984) with a recent report of pneumonia due to *Chlamydia pecorum* (Mackie et al. 2016). There is greater incidence and severity of chlamydiosis in koalas from QLD and NSW in comparison to koalas from SA (Polkinghorne *et al.* 2013; Speight *et al.* 2016; Fabijan *et al.* 2019a). A large retrospective study in QLD showed chlamydiosis was the most common presentation to wildlife hospitals; 52% (21,619/41,606) of aetiologies, where the number of total cases was 20,250 (Gonzalez-Astudillo *et al.* 2017). Chlamydiosis was further categorised and showed 26.8% had cystitis, 17.2% conjunctivitis, 13.5% bursitis, 12.3% pneumonia, 12% nephritis, 5% metritis and there were nine prostatitis and three orchitis cases (n = 21,619) (Gonzalez-Astudillo *et al.* 2017). Speight et al. (2016) described overt clinical disease (conjunctivitis or wet bottom) in 21% of koalas presented in

SA (n = 65), but this was only mild in severity. Moreover, histologically apparent but not clinically evident lesions consistent with *Chlamydia* were found in 42% (24/57) of koalas that were PCR positive for *C. pecorum* and 28% (16/57) were subclinically infected, highlighting lower levels of chlamydiosis within SA populations (Speight *et al.* 2016).

Chlamydia pecorum infection have also shown a strong correlation with the recently discovered *Phascolarctid gammaherpesvirus* (PhaHV) infection in Victorian koalas, for which the presence of reproductive disease was also associated (Vaz *et al.* 2019). It is hard to determine cause and effect with cross-sectional studies, but augmentation of reproductive Chlamydiosis by PhaHV warrants further investigations. Numbers of overtly affected koalas were small in this study, therefore it was more difficult to draw definitive conclusions as to the clinical impact (Vaz *et al.* 2019). There are two subtypes of PhaHV, type 1 (PhaHV-1) and type 2 (PhaHV-2) (Vaz *et al.* 2011; Vaz *et al.* 2012), and an association has been found between infection with PhaHV-2 and coinfection with PhaHV-1 (Vaz *et al.* 2019).

Furthermore, associations have been found between *Chlamydia* and another significant pathogen of koalas; koala retrovirus (KoRV). KoRV is a simple RNA gammaretrovirus with a competent endogenous (inherited KoRV-A) and several exogenous (horizontal/vertical transmission) variants (KoRV-A – I) circulating throughout koala populations (Tarlinton *et al.* 2005; Tarlinton *et al.* 2006; Miyazawa *et al.* 2011; Simmons *et al.* 2012; Shojima *et al.* 2013; Xu *et al.* 2013; Chappell *et al.* 2017). In a 2005 study, KoRV plasma viral loads (transcribed virus) in koalas with overt chlamydiosis averaged 6.9×10^8 copies/mL, in comparison to those koalas without overt disease, 7.7×10^7 copies/mL. These results were not statistically significant, but showed a trend of higher viral load with increased severity of

chlamydiosis (Tarlinton *et al.* 2005). However, in a more recent study, koalas infected with exogenous forms of KoRV (KoRV-A or KoRV-B) have been associated with increased prevalence and severity of chlamydiosis (Xu *et al.* 2013; Legione *et al.* 2017; Waugh *et al.* 2017). Infection with KoRV-B has recently been shown to upregulate the production of key immune genes IL17A and IL10 and therefore dysregulation of the immune system (Maher *et al.* 2016), which could increase the susceptibility to infections such as *Chlamydia* (Maher et al. 2019). *Chlamydia* spp. undergo a subclinical or persistent state and transformation to chlamydiosis is likely multifactorial but could involve compromise of the host immune system (Weigler *et al.* 1988; Maher *et al.* 2019).

KoRV was first discovered after viral particles were seen in the leukaemic bone marrow of a koala (Canfield *et al.* 1988), but it wasn't until 1999 when Hanger identified it as koala retrovirus or KoRV (Hanger 1999; Hanger *et al.* 2000). Increased proviral load (reverse transcribed and integrated viral genomic DNA into the host DNA) and high plasma viral load (viraemia) have been shown to have an association with lymphoid neoplasia (Tarlinton *et al.* 2005). In this study of QLD koalas, all were viraemic, but those with lymphoid neoplasia had an average plasma value of 1.6×10^9 copies/mL in comparison to healthy koalas 7.7×10^7 copies/mL (Tarlinton *et al.* 2005).

1.6.1 Prevalence of KoRV

Prevalence of KoRV throughout various wild populations in Australia shows remarkable differences, suggesting a recent appearance and southward spread of this virus (Tarlinton *et al.* 2006; Simmons *et al.* 2012). In 2006 KoRV was found in every koala tested in QLD (n = 98), with less prevalence found in VIC (n = 22) and no infection found in SA (n=26) (Tarlinton *et al.* 2006), Figure 1. By 2012 NSW also had 100% prevalence (n = 100) and prevalence had increased for VIC (n = 169) and for SA (n = 162). Notably, no KoRV infection was found on Phillip Island (n = 11), (Simmons *et al.* 2012). In a review, Denner and Young (2013) suggested a further increase in KoRV prevalence on KI to 30-35% based on their own unpublished data (Denner *et al.* 2013). This was confirmed by a recent study in SA populations, finding KI KoRV prevalence at 42.4% (n = 175) (Fabijan *et al.* 2019b). The population in the MLR was also surveyed in this study and shown to have a KoRV prevalence of 65.3% (n = 75) (Fabijan *et al.* 2019b).

Greater complexity in retroviral status within SA koalas has been identified utilising a combination of multiple PCR targets and expression studies in MLR koalas (Sarker *et al.* 2019; Sarker *et al.* 2020). Results found 38-99% of MLR koalas positive over nine different proviral targets, and 3% - 51% positive for viral RNA targets (Sarker *et al.* 2020). It has also been shown that MLR KoRV *pol*-negative koalas produced transcripts that aligned to the KoRV genome at positions 1-1389 (5' LTR and partial *gag* gene) and 7124-8431 (partial *env* gene and 3'LTR), with a lack of transcription of other KoRV genes (Tarlinton *et al.* 2017). Additionally, KoRV *pol*-positive (recognised as KoRV positive) koalas had low transcription of genes between genome positions 1389 and 7124, representing the 5' end of the *gag* gene, the *pol* gene and the 3'end of the *env* gene, compared with QLD koalas, in which expression was high across the entire KoRV genome (Tarlinton *et al.* 2017). It is currently postulated that truncated and potentially defective virus could inhibit viral replication in MLR koalas

(Tarlinton *et al.* 2017; Sarker *et al.* 2019). In other studies, recombinant KoRV, or rec-KoRV, has been attributed to sequences where the terminal sequences from KoRV have recombined with and earlier or ancient retroviral elements, such as Phascolarctid endogenous retroelement (PhER) (Lober *et al.* 2018; Yu *et al.* 2019). RecKoRV and has one prominent subpopulation designated recKoRV-1 and has been shown to be present in the MLR population in both KoRV positive and KoRV negative koalas, but it has also not been found in the KI population (Lober *et al.* 2018). Hence, question remains as to the host integration, expression and detection of KoRV in SA koalas.

1.6.2 Structure of KoRV

Retroviruses are spherical, enveloped virions which carry two copies of positive sense, single-stranded RNA (ssRNA) that use viral reverse transcriptase to transcribe their genome prior to insertion into the hosts cellular DNA. KoRV is a simple gammaretroviruses which is approximately 8.4kb in length and compose of four protein-coding regions; 5'-*gag-pol-pro-env-3'*, flanked by long terminal repeats (LTRs) (King *et al.* 2012). Three genes are present; *gag*, *pol-pro* (this region is often referred to as *pol*) and *env* (Figure 1).
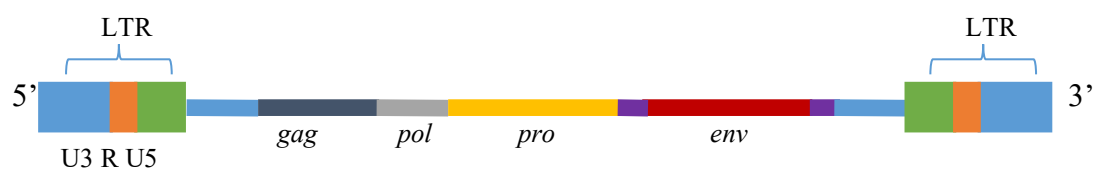
Figure 1: Schematic of simple retroviral genome adapted from (Vogt 1997; King *et al.* 2012)

The *gag* region codes for the matrix (MA), capsid (CA) and nucleocapsid (NC), the *pol* region codes for reverse transcriptase (RT) and integrase (IN), the *pro* region codes for a

protease (PR) and the *env* gene codes for two envelope proteins, surface (SU) and transmembrane (TM), (Vogt 1997; King *et al.* 2012) (Figure 2).
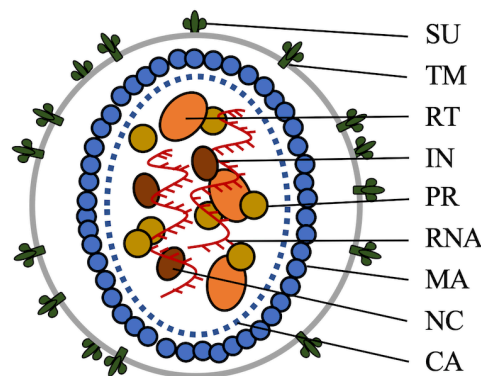
Figure 2: Schematic of the structure of a simple retrovirus, showing the following; surface (SU) and transmembrane (TM) – forming the receptor binding glycoprotein, reverse transcriptase (RT), integrase (IN) and protease (PR) – viral enzymes, viral RNA (RNA), nucleocapsid (NC), and matrix (MA) and capsid (CA) proteins forming inner and outer membranes (adapted from King *et al.* 2012).

The envelope proteins of retroviruses have been studied extensively because within the SU region there is coding for the receptor binding domain (RBD) which is highly variable and can differentiate strains within genera and antigenic gp70 peptide is also located within this region (Denner *et al.* 2013). The TM region of the *env* protein also hold several proteins and peptides of interest to immunological studies, FPPR – fusion peptide proximal region, isu – immunosuppressive domain, which includes p15E peptide, and MPER – membrane proximal external region (Denner *et al.* 2013). These antigenic regions have been specific targets for neutralizing antibodies for several retroviruses, including HIV and FeLV (Langhammer *et al.* 2011; Denner 2013) and are potential epitopes for immunohistochemical techniques within tissue sections (Lang *et al.* 1994).

1.6.3 Diversity of KoRV

Currently several studies have looked at the genetic diversity of KoRV and have revealed several variants or sub-types, A-I (Miyazawa *et al.* 2011; Shojima *et al.* 2013; Xu *et al.* 2013; Fiebig *et al.* 2015b; Xu *et al.* 2015; Chappell *et al.* 2017). The majority of the earlier viral work was carried out on the first recognised variant of KoRV, KoRV-A (Tarlinton *et al.* 2005; Tarlinton *et al.* 2006; Simmons *et al.* 2012). KoRV-A is the dominant sub-type and is highly prevalent in both captive and wild populations (Xu *et al.* 2013; Hobbs *et al.* 2014; Young 2014). Genomic studies of koalas from zoos around the world has shown the following sub-types; KoRV-B, isolated from the United States and Germany (Xu *et al.* 2013; Fiebig *et al.* 2015b), KoRV-B (which now includes previously identified KoRV-J), C and D from zoos in Japan (Miyazawa *et al.* 2011; Shojima *et al.* 2013), KoRV-E from the United States (Xu *et al.* 2015) and through sampling wild QLD koalas, variants A-I have been phylogenetically arranged (Chappell *et al.* 2017). This viral genetic diversity of KoRV is based on the hypervariable region of the receptor binding domain on the envelope protein and divergence was through deletions, point mutations, insertions and potential recombination events, which could occur when a koala was infected with multiple exogenous KoRV strains, as has been found in QLD (Chappell *et al.* 2017). KoRV is one of the few retroviruses that is being transmitted both endogenously and exogenously, and appears to be undergoing endogenistaion (Tarlinton *et al.* 2006; Chappell *et al.* 2017). From the sequencing of KoRV found within museum specimens and sequencing from endogenised KoRV-A in QLD koalas it has been shown that there was little sequence variability, suggesting stability of an endogenised variant (Avila-Arcos *et al.* 2013; Kinney *et al.* 2016). Endogenisation stablises the viral genome since it is then subject to cellular mechanism of DNA repair, therefore the mutation rate is considerably lower than that of an exogenous retrovirus (Higgins *et al.* 2014). Exogenous forms of retroviruses are not regulated by DNA repair mechanisms and their rate of mutation is considerably higher (Levy 2011; Higgins *et al.* 2014). Immune

regulating sequences from KoRV-A and KoRV-B have also been compared. These sequences have shown 100% homology within the immunosuppressive domain of p15E, which is also highly conserved throughout retroviruses, implying the ability to carry out the same mechanisms of immunomodulation between variants and potentially between retroviruses (Denner *et al.* 2013).

1.6.4 Retroviral replication and KoRV

Replication in retroviruses occurs through the following stages described below and shown numbers are shown in Figure 3. Viral entry into the cell is dependent on the variation of receptor binding domain in order to target specific cell types and receptors. The virus binds with a specific cellular receptor (1) and enters the cell through multistage viral fusion with the plasma membrane or endocytosis (2). Once inside the cell the virion uncoats (3) and using the retroviral reverse transcriptase, the genome is reverse transcribed into DNA or provirus (4). The provirus transferred to the nucleus and integrated into the host DNA, mediated by viral integrase (5). From there, host cellular mechanisms are used to transcribe the viral genes with enhancer regions in the LTRs help to promote the rate of transcription and increase the amount of virus. These transcripts are translated by cellular ribosomes (6a) and with viral genomic RNA (6b) they assemble near the plasma membrane (7). Budding then occurs to allow infectious complete virions out of the cell (8) (Levy 2011; King *et al.* 2012).

KoRV-A has been shown to use a sodium-dependent phosphate transporter PiT1 for viral entry into the cell, similar to that of GALV (Oliveira *et al.* 2006; Denner *et al.* 2013). KoRV-B has an altered receptor binding domain (RBD) and has been shown to utilise a thiamine

transporter protein (ThTR1) to gain cellular entry (Shojima *et al.* 2013; Xu *et al.* 2013). Other variant do not have identified mechanisms of cellular entry (Quigley *et al.* 2020), KoRV-E has been shown not to utilise PiT1, ThTR1, PiT2, riboflavin transporters SIC52A1 and sodium dependent neutral amino acid transport SIC1A5 (Xu *et al.* 2015). KoRV-F is closely related to KoRV-C, therefore it is assumed that KoRV-F would share the same transporters to gain entry into the cell, although the exact transporter is not known (Xu *et al.* 2015). A recent study demonstrated there were several koalas that were infected with 2 or more KoRV variants (Xu *et al.* 2015), co-infections can facilitate mutation and recombination events and evolution of retroviral strains (Rosenberg *et al.* 1997).
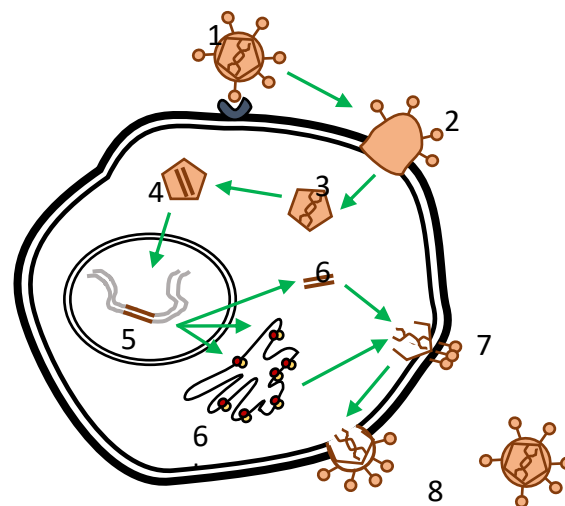
Figure 3: Retroviral replication adapted from Levy (2011), described in the first paragraph of *1.6.4*.

1.6.5 Retroviral transmission and KoRV

Transmission of retroviruses can occur through exogenous or endogenous pathways. Exogenous transmission occurs with competent infectious particles through close contact, most commonly sexual contact or insect vectors. The epidemiological study of human immunodeficiency virus (HIV) highlighted that promiscuity and intravenous drug use significantly increased the risk of becoming infected with HIV, therefore sexual intercourse

and blood-borne were determined to be the main transmission routes for this disease (Gallo 1988; Vogt 1997). Endogenous transmission, is where the provirus is found within the germ cell line and is passed directly to the offspring, through Mendelian dominant inheritance (Vogt 1997). This means that all the cells in the offspring have retroviral DNA within them. Endogenous retroviruses (ERVs) can be competent, silent or partial defective viral genomes and are subject to cellular surveillance (Vogt 1997).

KoRV appears to be the only retrovirus to be currently undergoing the process of endogenisation, whereby it is transmitted endogenously in some koala populations and exogenously in others (Oliveira *et al.* 2006; Tarlinton *et al.* 2006). Invasion of the germ cell lines was investigated in QLD koalas through *in-situ* hybridisation of sperm cells showing positive fluorescence with digoxygenin-labelled probe prepared from full length virus DNA and vertical transmission to offspring was shown (Tarlinton *et al.* 2006). For koalas, once endogenisation had occurred KoRV has remained competent and able to produce viral particles (Tarlinton *et al.* 2006). Proviral copy number has been correlated to infection status, endogenous infection showing higher copy number due to all cells being infected (in host genome), in comparison to exogenous infection which shows lower copy numbers as not all cells are infected, generally only target cells (lymphocytes). However, in theory, a koala with an exogenous marked viraemia could have a proviral load equivalent to that of an endogenous infection. In turn, this has the potential for a greater number of cell types to be infected (due to ubiquitous nature of viral entry receptors), including germ cells within that koala, contributing to the process of endogenisation throughout the koala populations. Proviral load in endogenously infected koalas can be measured in ear punch biopsies, whereas exogenous proviral load results has to be measured from peripheral blood supply as this contains a greater number of target cells (Simmons *et al.* 2012). These findings of KoRV

prevalence and proviral load variability support both exogenous and endogenous spread of KoRV, with northern populations showing greater proviral copy numbers in comparison to southern populations (Tarlinton *et al.* 2006). One study found that KoRV has been incorporated into the genome of northern koalas for greater than 100 years, with museum specimens testing positive for the virus (Avila-Arcos *et al.* 2013). Exact mechanisms for exogenous KoRV transmission are unknown. Mating, fighting, mother-joey interactions and biting arthropods are likely routes of transmission, with viral RNA isolated from both milk, sperm and experimentation demonstrating possible transmission through ticks (Simmons *et al.* 2011).

1.6.6 Phylogenetics of KoRV

There are two subfamilies of Retroviridae; *Orthoretrovirinae* and *Spumaretrovirinae*. Koala retrovirus has been established as a member of the *gammaretrovirus* genus, which belongs to the *Orthoretrovirinae* sub family (Hanger *et al.* 2000). Other members of this family include; *Gibbon Ape Leukaemia Virus* (GALV)*, Murine Leukaemia virus* (MuLV)*, Feline Leukaemia Virus* (FeLV) and *Woolly Monkey Sarcoma Virus* (WMSV) (King *et al.* 2012). As evident from the naming of many members of this genus, gammaretroviruses are associated with the development of neoplasia; five out of the six genera that belong to the subfamily *Orthoretrovirinae* have oncogenic viruses within the genus (Rosenberg *et al.* 1997).

Further to isolation of virus from koala tissues, Hanger et al. (2000) described a virus whose closest phylogenic species is GALV, sharing 78% of its viral DNA (Hanger *et al.* 2000). Due to the terrestrial separation of gibbons and koalas, it is postulated that at least one intermediate species would have been necessary for this cross-species transmission (Martin

1999), or even the possibility of iatrogenic cross species transmission with infected fomites (Hanger *et al.* 2000). An Australian rodent, *Melomys burtoni*, was a member of the 42 screened vertebrates and a novel retrovirus, Melomys burtoni retrovirus (MbRV) was shown to have 83% homology to KoRV and 93% homology to GALV (Simmons *et al.* 2014). It is suggested that this rodent is part of the cross-species transmission picture (Simmons *et al.* 2014). This is further supported by the discovery of GALV-like sequences in the genome of a subspecies of *Melomys burtoni* from the south eastern islands of Indonesia (Alfano *et al.* 2016). These GALV-like sequences were classified as Melomys Woolly Monkey Virus (MelWMV) due to its similarity with Woolly Monkey Virus (WMV), a likely GALV subspecies (Alfano *et al.* 2016). MelWMV was noted to be as ancient as the divergence of the *Melomys* subspecies from a common ancestor (Alfano *et al.* 2016). It is also plausible that these rodents are the geographical link due to the significant likelihood of rodent migration between Asia and Australia, similar to that of the black rat (*Rattus rattus*) or the house mouse (*Mus musculus*) through accidental human dispersal (Banks *et al.* 2012). More recently, retroviral examinations of flying foxes and microbats have also revealed closely related gammaretroviruses (Hayward *et al.* 2020). A reproduction competent, exogenous retrovirus was identified from the Australian black flying fox (*Pteropus alecto*) with homology to both KoRV and GALV. *In vitro* experimentation showed similar tissue tropisms and therefore is likely to be another candidate for cross-species viral spread (Hayward *et al.* 2020).

1.6.7 Immune response to KoRV

Based on slow seroconversion when infected with *Chlamydia*, koalas have been labelled with a lazy immune response (Brown 1988). Contrary to these early studies serology studies from

recent *Chlamydia* and KoRV vaccination trials have shown strong and neutralizing humoral responses (Khan *et al.* 2014; Waugh *et al.* 2016b; Desclozeaux *et al.* 2017; Nyari *et al.* 2018; Olagoke *et al.* 2018; Olagoke *et al.* 2020). Immune tolerance to KoRV has been reported in wild koalas from QLD and captive koalas from Antwerp Zoo, Belgium and Duisberg Zoo, Germany (Fiebig *et al.* 2015b), suggesting that koalas may not mount an immune response to KoRV virus (Higgins *et al.* 2014). Endogenous retroviruses are subject to cellular regulation, DNA repair and continued cellular monitoring pathways, therefore QLD koalas harbouring endogenous KoRV could have accommodation by the koala's immune system (Higgins *et al.* 2014). Endogenous infections of retroviruses have also shown immunotolerance in pigs with porcine endogenous retrovirus (PERV) (Keller *et al.* 2014). The transmembrane (TM) segment of the *env* protein is generally antigenic in retroviruses. Hence, various studies on KoRV have investigated the *env* proteins, specifically the receptor binding domain (RBD), for its variability to identify different strains, and p15E and gp70 peptides for antigenic properties (Denner 2011; Kaulitz *et al.* 2011; Fiebig *et al.* 2015a; Ishida *et al.* 2015; Waugh *et al.* 2016a). The p15E protein is associated with the immunosuppressive domain (Denner *et al.* 2013), interaction of the immunosuppressive domain with immune cells reduces their response (Blinov *et al.* 2013). Blood samples from koalas positive for endogenous KoRV-A and exogenous KoRV-B infection were tested for their antibody response to viral lysates and KoRV-A peptides – p15E and gp70, of which 0/16 showed antibody production *in vitro* (Fiebig *et al.* 2015b). This was further investigated by Waugh et al (2016) with 7 koalas from different populations around Australia, QLD (n=4), NSW (n=1) and SA (n=2), finding that 6/7 koalas mounted an immune response to the transmembrane (TM) protein – KoRV-A p15E protein - and only 2/6 to the surface (SU) protein – KoRV-A gp70 protein (Waugh *et al.* 2016a). Both Feibig *et a*l. (2015) and Waugh *et al.* (2016) looked at the same envelope proteins and used western blot techniques, therefore differences seen may be due to the

individual koala's exposure to virus or laboratory differences (Fiebig *et al.* 2015b; Waugh *et al.* 2016a). After immunisation with TM and SU retroviral proteins, there was a measurable response in antibodies to the SU protein in the one koala that had previously not shown a response (Waugh *et al.* 2016a). Vaccination of MLR koalas that have been naturally infected with KoRV have shown humoral responses to the MPER peptide – part of the transmembrane *env* protein (Olagoke *et al.* 2018). Also, vaccination of koalas infected with endogenous KoRV in QLD not only showed the presence of neutralizing antibodies, but also reduction in their viral load (Olagoke *et al.* 2018). A study that immunised goats and rats with the KoRV p15E peptide have shown neutralising epitopes to the FFPR and MPER regions of the TM protein (Denner 2011). Discovery of immune-visible epitopes and assessment of efficacy of immune function are important in studies reflecting on immune-capabilities of koalas, for which both immune-tolerance from endogenous KoRV and potential immunosuppressive properties of KoRV are likely to play a role.

1.7 Lymphoid neoplasia

As noted in section 1.6, KoRV is associated with, and is the presumed aetiology for lymphoid neoplastic conditions in the koala. Currently the exact mechanism of molecular pathogenesis is unknown.

1.7.1 Neoplasia and lymphoid neoplasia overview

Neoplasia is the unregulated, uncontrolled proliferation of cells. A disruption to the normal cell cycle occurs, changing those processes responsible for cell growth, proliferation or cell

death. This makes the rate of division greater than that of apoptosis (Klopfleisch 2016). Malignancies occur when there have been one or more genetic disturbances to override the normal cell cycle, these mutations can be seen as deletions, insertions, translocations or substitutions. Survival of these neoplastic cells is multifactorial and tends to involve multiple and cumulative mutations (Klopfleisch 2016). Characteristic morphological changes occur in transformed or neoplastic cells, microscopically evident as changes in cell size and shape, cytoplasmic volume and colour, nucleus size and shape and changes in nucleolar appearance and number (Baba *et al.* 2007). Typically, nuclear size increases, resulting in an increase in the nucleus:cytoplasm ratio. Nucleoli become hypertrophied and segregated and may migrate towards the membrane (Baba *et al.* 2007). Division rate is increased in cancerous cells and histologically this is apparent through increased mitotic figures and the appearance of mitotic spindles within multiple cells (Baba *et al.* 2007). Cytoplasmic changes also occur with a general reduction in cytoplasm and, in some cell types, an increase in granularity and vacuoles (Baba *et al.* 2007).

Lymphoma, or lymphosarcoma, and lymphoid leukaemia are neoplastic diseases of lymphoid origin, disrupting the normal proliferation of immune cell lines (Vail *et al.* 2019). Lymphoma is a solid tumour made up of malignant lymphocytes and originate in lymphoid tissues. Lymphoid leukaemia originates from the bone marrow and more commonly consists of circulating malignant lymphocyte. Circulating malignant lymphocytes can also be found in end-stage lymphoma. Lymphoid neoplasms can derive from B-cell, T-cell or, more rarely, natural killer (NK) cells. B-cells are responsible for the humoral adaptive, specific immune response through production of antibodies, T-cells are also part of the adaptive immune response, underpinning cell mediated responses, inclusive of cytotoxic and regulatory roles. NK cells are part of the innate, non-specific immune response and like T-cells they have a

cell-mediated cytotoxic response (Snyder 2012). All of these lymphocytes are derived from the bone marrow and B-cells mature in lymphoid tissue such as lymph nodes or spleen, whereas T-cells mature and differentiate in the thymus (Snyder 2012). NK Cells have an important role in early viral and tumour responses (Snyder 2012). Identifying and differentiating the different lymphocytes is an important feature of classification of lymphoma and is carried out by immunohistochemical techniques on fixed tissue, each of the B-cell, T-cell and NK cell populations have differing surface proteins that can be visualised through antibody labelling. Examples of immunohistochemical antibodies for visualisation include; B cells: anti-human CD21, anti-human CD79a, anti-human CD79b and anti-human Pax5, T-cells: anti-human CD3, anti-human CD4 and anti-human CD8, NK cells: anti-human CD16 and anti-human CD56 (Spencer *et al.* 1996; Connolly *et al.* 1998; Valli *et al.* 2011; Snyder 2012; Day *et al.* 2014 ; Vail *et al.* 2019).

Classifications of neoplasms help to identify best practice with treatments and the prognosis of recovery. Human classification of lymphoid neoplasms is extensive, detailed and takes into account expanding molecular and genetic aspects of these disease (Swerdlow *et al.* 2016). The World Health Organisation (WHO) classification system has 101 named lymphoid neoplasms, each of which has staging through clinical signs, histopathology, molecular and genetic techniques, which is considerably more extensive than that of domestic animals and these techniques are not available for wildlife species like the koala (Hanger 1999). Classification for canine (Valli *et al.* 2011) and feline lymphoma (Chino *et al.* 2013) has more practical approaches for methodology that could be considered for a wildlife species. Classification of lymphoid neoplasms in the koala has been considerably simpler than that of human classifications. Available techniques include;

- Anatomical: tissues involved, site/s of involvement.

- Cytological: cell morphology, mitotic rates

- Cytological: Immunohistochemical techniques for B-cell, T-cell identification.

1.7.2 Lymphoid neoplasia in the koala

The first published pathological studies reporting lymphoid neoplasia in the koala (*Phascolarctos cinereus*) were in the early 1960s, both describing lymphoid leukaemia in koalas from the Taronga Zoo, NSW collections (Backhouse *et al.* 1960; Heuschele *et al.* 1961). More description of the pathology was reported on the case of acute lymphoid leukaemia, in which the koala had declined rapidly and died. Blood smears had shown a marked lymphocytosis with immature cells, these cells were also prominent in the sternal bone marrow, peripheral lymph nodes and lesions had infiltrated the stomach, kidneys, bladder and caecum (Heuschele *et al.* 1961). It was observed that koalas had a relatively high prevalence of lymphoid neoplasia, out of 344 koalas examined in the period of 1981-1985 from QLD and NSW, 3.8% had lymphoid neoplasms (Canfield *et al.* 1987). There was no sex predilection to lymphoid neoplasia noted and koalas over two years of age had a greater likelihood of disease (Canfield *et al.* 1987). Spontaneous proliferations were investigated by Canfield *et al.* (1990) on a wide range of marsupials, in which 26/154 koalas were found with neoplastic conditions. Lymphosarcoma was found in eight koalas, with a further three koalas highly suspicious of lymphosarcoma, but advanced autolysis did not allow for definite diagnoses (Canfield *et al.* 1990). Presentations to the University of Sydney of both free-living and captive koalas described 5.3% of koalas as having lymphoid neoplasms from 1982-1994 (Spencer *et al.* 1996). The majority of these cases were multicentric lymphomas, which

affected several peripheral lymph nodes and infiltrated the liver and spleen (Spencer *et al.* 1996). Both multicentric and alimentary forms are common anatomical classifications in koalas from these studies (Canfield *et al.* 1987; Spencer *et al.* 1996). Sites of infiltration included; gastrointestinal tract, thymus, kidney, bladder, brain and bone marrow (Spencer *et al.* 1996). Hanger reported up to 55% of necropsies performed on captive koalas had a form of lymphoid neoplasia (Hanger 1999). From these studies it is evident that there is a high incidence of lymphoid cancers, but it should be noted the populations that are sampled were biased towards diseased cohorts and, as noted in Hanger (1999), these koalas were from populations with endogenised KoRV in their genome (Hanger 1999). Immunotyping of 51 lymphoma cases showed that T-cell lymphomas predominated (26/51, 51%) (Connolly *et al.* 1998). The antibodies that were utilised for this study were anti-human CD3 and CD5 to recognise T-Cells, anti-human CD79b to recognise B-cells and anti-koala IgG to recognise plasma cells (Connolly *et al.* 1998). Viral aetiology of these cancers was suspected since there seemed to be clustering of disease and spontaneous cases of leukaemia were similar to that seen in laboratory animals (Heuschele *et al.* 1961; Arundel *et al.* 1977; Canfield *et al.* 1987). This was confirmed with the use of electron microscopic visualisation of viral particles in association with lymphoid neoplasia (Canfield *et al.* 1988), followed by viral isolation and classification in 2000 (Hanger *et al.* 2000). This virus was named *Koala retrovirus* or KoRV, a member of *Retroviridae* family, *Orthoretrovirinae* subfamily and *Gammaretrovirus* genus (Hanger *et al.* 2000).

1.7.3 Classification of lymphoid neoplasia in the koala

Techniques for classifying koala neoplasia include gross pathology which describes the anatomical location of the lesions and the tissue/s involved and also histopathology, which describes the cell population and its morphology and current cross-reactive immunohistochemical techniques for B-cell, T-cell identification. Small populations of affected koalas have been used to describe and, in later years, to classify lymphoid neoplasms (Canfield *et al.* 1987; Spencer *et al.* 1996; Connolly *et al.* 1998). Using anatomical distribution, cell morphology and blood parameters Spencer *et al.* (1996) (n = 31) and Connolly *et al.* (1998) (n = 51) describe a classification for the koala; Table 2 (on next page) combines the classifications in these papers and taking pertinent information from WHO classification of lymphoma in the dog (Valli *et al.* 2011).

In wildlife species, historical timelines and all necessary samples for complete classification are often not acquired. Application of appropriate and consistent classifications are then needed, for which Tables 2 and 3 (on pages 46 and 47) collate appropriate data parameters, although often in wildlife studies time between euthanasia and postmortem investigation can inhibit complete evaluations and complementation with blood samples.

Table 2: Classification of lymphoma in the koala based on anatomical and cytological features (adapted from koala lymphoma (Spencer *et al.* 1996; Connolly *et al.* 1998) and Canine lymphoma (Valli *et al.* 2011; Vail *et al.* 2019))

Area	Class	Description	
Anatomical	Multicentric	Disseminated, bilateral involvement of lymph nodes with or without other organ involvement	
	Abdominal	Lymphoma withing the abdominal cavity with the following subgroups; Alimentary: Gastrointestinal tract, GALT and regional lymph node involvement Miscellaneous: involvement of other abdominal organs Combination: Combination of alimentary and miscellaneous subgroups	
	Cervicomediastinal	Involvement of lymph nodes of the lower neck, lymph nodes of the chest with or without thymic infiltration	
	Atypical	Other lymphomas that do not fit into another class, involvement of non-lymphoid organs such as CNS, conjunctiva, mammary gland	
	Mixed	Combination of two or more anatomical classifications	
	Primary Lymphoid leukaemia	Blood and bone marrow involvement	
Cytological	Cell population	Monoclonal	>75% same cell size – then divided into small-medium & large, dependent on nuclear size
		Mixed	<75% same cell size
		Immunoblastic	large cells, eccentric nucleus, prominent nucleoli and abundant cytoplasm
	Cell type (IHC)	B-cell, T-cell or Null type	B-cell markers: CD4, CD79a, CD79b[*], Pax5, CD20 T-cell markers: CD3[*], CD45, CD5 [*]routinely used in koalas, others used in other species
	Nuclear size	Small-medium	diameter up to width of 2 erythrocytes
		Large	diameter greater or equal to width of 3 erythrocytes
	Nuclear shape	Non-cleaved	Round nucleus
		Cleaved	Indented or folded nucleus
	Other	Nucleoli	present/absent
		Nuclear chromatin	pattern
		Cytoplasm	Amount and staining pattern
		Mitotic rate	Koala (Connolly *et al.* 1998): Low: 0 to 2, Medium: 3 to 5, High: 6+ (mitoses per HPF over 5 HPF)
			Canine/WHO (Valli *et al.* 2011): Low: 0 to 5, Medium: 6 to 10, High: 11+ (mitoses per HPF over 10 HPF)

Table 3: Staging of lymphoid neoplasia as described in Vail *et al.* (2019)

Staging	I	Involvement of single node, lymphoid tissue or organ (excluding bone marrow)
	II	Involvement of multiple regional lymph nodes
	III	Generalized lymph node involvement
	IV	Involvement of both the liver and spleen with or without generalized lymph node involvement
	V	Neoplastic cells in the blood, involvement of the bone marrow, involvement of other organs

1.8 Retroviruses and Cancer

The study of retroviruses has contributed significantly into the understanding of the development of cancer and oncogenes responsible in a wide range of species (Levy 2011; Beemon *et al.* 2012). Several mechanisms are utilised by oncogenic retroviruses to elicit malignant disease in their host, including insertional mutagenesis, activation of cellular microRNAs, expression of oncogenes, immunosuppression and through the effect of expression of accessory genes like *tax* and *HBZ* (Levy 2011; Beemon *et al.* 2012).

1.8.1 Insertional mutagenesis

Insertional mutagenesis is when the integration of the virus into the host DNA occurs within close vicinity to a cellular gene associated with cell proliferation, growth or cell death, altering its regulation of transcription (Levy 2011). In fact, the study of retroviral species that cause cancer through their insertion sites has led to many discoveries of genes involved in growth, differentiation and survival of cells (Levy 2011). The site of integration of the retrovirus will disrupt the DNA structure at that site, resulting for example, in separation of exons within cellular genes and production of proteins with altered function, reduction of mRNA stability through separation or regulatory elements and promoter enhancement from

viral LTR regions (Beemon *et al.* 2012). Viral LTR regions contain transcription promoters and depending on integration site can increase cellular gene transcription and disrupt regulation of protein production (Beemon *et al.* 2012). Avian leukosis virus (ALV) causes disease through insertional mutagenesis. In ALV induced bursal lymphoma there is an upregulation of *myc* gene and overexpression of this transcription factor that has a multitude of functions including driving cell proliferation (Kato *et al.* 1992).

Feline Leukaemia virus (FeLV) is also a member of the gammaretrovirus family and affects cats worldwide. It is associated with neoplastic, haematopoietic and immunosuppressive disease (Beatty 2014). There are three exogenous FeLV strains (FeLV-A, FeLV-B and FeLV-C), and an endogenous FeLV (enFeLV) (Roy-Burman 1996). FeLV-A has been found to be highly transmissible, but minimally pathogenic. Recombination between enFeLV and exogenous FeLV-A generates subgroups FeLV-B and FeLV-C (Dudley *et al.* 2011). FeLV-B is associated with malignant disease and FeLV-C with red cell aplasia (Fujino *et al.* 2008). Within the FeLV family six clonal sites of insertion (CISs) have been found; c-*myc*, *flvi*-1, *flvi*-2 (containing *bmi*-1), *fit*-1, *pim*-1 and *flit*-1, therefore it is likely that insertional mutagenesis plays a significant role in FeLV lymphanogenesis (Fujino *et al.* 2008). C-*myc* is a known proto-oncogene and *bmi*-1 and *pim*-1 are related to *myc*, a transcription factor involved in cell proliferation and transformation (Kato *et al.* 1992; Fujino *et al.* 2008). Lymphanogenesis has also been linked to dysregulation of *myc* proto-oncogene (Tsatsanis *et al.* 1994; Beatty 2014)

Duplication, triplication or other multiplications of the LTR regions can significantly increase transcription rates, leading to high virus expression in the target tissues and/or increasing expression of proto-oncogenes close to insertion sites, which has been found in retroviruses,

including FeLV, MuMLV and KoRV (Levy 2008). KoRV-B has been found to have an increased number of LTR repeats and has been associated with an increased incidence of lymphoid neoplasia (Xu *et al.* 2013). KoRV-F has been shown to have five repeats in the U3 region, but there has not been an inference to its pathogenicity (Xu *et al.* 2015). In other species infected with oncogenic retroviruses, LTR regions have been shown to incorporate enhancer regions (DesGroseillers *et al.* 1984; Chen *et al.* 1994). Triplication of LTR downstream of enhancer regions have shown increased transcriptional activity in FeLV affected splenic lymphosarcoma cells (Athas 1994). Also in FeLV, the LTRs from exogenous variants have shown more association with disease outcomes than those from endogenous variants (Ghosh *et al.* 2000). KoRV-A has been shown to only have a single copy of the LTR region, whereas KoRV-B has been shown to have four LTRs which encodes enhancer regions (Xu *et al.* 2013). The sample size was small in this study and all koalas had endogenised KoRV-A, therefore KoRV-A cannot be excluded from further studies of oncogenesis.

MicroRNAs (miRNAs) are associated with post transcriptional regulation of gene expression and RNA silencing and are thought to be critical for cellular processes such as growth, differentiation and apoptosis (Landais *et al.* 2007). Retroviruses can integrate into these loci and can cause overexpression of the miRNA. Murine retroviral integration and upregulation of *Kis2*, with overexpression of MiR-106-363 cluster miRNAs has been described in transformed tumour cells with possible disruption of tumour suppression pathways (Landais *et al.* 2007). MiRNAs have also been implicated in the progression of chronic lymphocytic leukaemia in humans and expression profiles of these miRNAs can distinguish between normal and malignant B-cells in this disease (Calin *et al.* 2004).

1.8.2 Viral oncogenes

Viral oncogenes (*v-onc*) are incorporated into the viral genome from the capture of cellular genes (Levy 2011). Integration of the provirus is generally upstream of the proto-oncogene, and with bypass of the stop codons, the oncogene is transcribed with the provirus (Swain *et al.* 1992). The incorporation of oncogenes into the viral genome is heterozygous and the maintenance of wild-type virus is key for the ability to infect and replicate within the host cells (Levy 2011). Oncogenic retroviruses that have incorporated *v-onc* have short latency periods prior to overt disease and generally transform cells in tissue culture (Levy 2011; Beemon *et al.* 2012). Viruses such as Rous sarcoma virus (RSV), Woolly Monkey Sarcoma Virus (WMSV) also known as Simian Sarcoma Virus (SSV) and Murine Sarcoma Virus (MuSV) have been shown to have *v-onc* incorporated into the viral genome and induce sarcomas, malignancies of mesenchymal origin (Rosenberg *et al.* 1997) (Table 4). Examples from Table 4 are expanded to show what the gene actions are and how they disrupt the cell cycle to initiate or augment oncogenesis:

- The v-*sis* viral gene is homologous to the cellular gene PDGF-B which is a strong mitogen for cells of mesenchymal origin (Kujoth *et al.* 1997).
- The v-*abl* viral gene is homologous to the cellular gene c-*abl* which codes a non-receptor tyrosine kinase with influences on pathways that regulate cell growth, survival and morphogenesis (Sirvent *et al.* 2008).
- The v-*cbl* viral gene is homologous to the cellular gene c-*cbl*, which negatively regulates tyrosine kinase pathways though E3 ligase. The dysregulation of tyrosine kinase pathways, an element of cytokine response, is linked to myeloproliferative disorders (Sanada *et al.* 2009).

Table 4: Examples of viral oncogenes in gammaretroviruses (adapted from Rosenberg, 2011)

Function	Representative Gene(s)	Representative virus(es)	Host	Disease
Growth factors	v-*sis*	SSV	woolly monkey	Sarcoma
	v-*sis*	PI-FeSV	cats	Sarcoma
Growth factor receptors	v-*fms*	SM-FeSV	cats	Sarcoma
	v-*kit*	HZ4-FeSV	cats	Sarcoma
Non-receptor tyrosine kinases	v-*abl*	Abelson MuLV	mice	lymphoma
	v-*abl*	HZ-2-FeSV	cats	Sarcoma
	v-*fgr*	GR-FeSV	chickens	Sarcoma
E3 ligase	v-*cbl*	Cas NS-1	mice	lymphoma
Serine/threonine kinases	v-*mos*	Mo-MSV	mice	Sarcoma
	v-*raf*	3611-MSV	mice	Sarcoma
G-proteins	v-*ras*^{H}	Ha-MSV	rats	Erythroleukaemia, Sarcoma
Transcription factors	v-*fos*	FBJ-MSV	mice	osteosarcoma

Abbreviations: SSV: Simian Sarcoma virus, FeSV: Feline sarcoma virus, MuLV: Murine leukaemia virus, Cas NS1: Murine sarcoma virus, MSV: Murine sarcoma virus

Gibbon Ape leukaemia virus (GALV), a closely related gammaretrovirus to KoRV, was isolated from a gibbon with lymphocytic leukaemia with very strong evidence for viral aetiology (Gallo *et al.* 1978). GALV has been identified as initiating both lymphocytic leukaemia and myelogenous leukaemia (Sun *et al.* 1980). Several strains of GALV are described, of which two contain *MYE* and *LYM* RNA sequences that show a correlation to leukaemic gibbons, suggesting that the expression of these are involved in oncogenesis (Sun *et al.* 1981). *LYM* and *MYE* fractions make up approximately 25% of the viral genome (Sun *et al.* 1980). GALV-1L strains contain *LYM* RNA which is lymphocytic leukaemia specific and GALV-3M strains contain *MYE* RNA, which is myelogenous leukaemia specific and it is hypothesised that these oncogenic RNAs are v-*oncs* and originate from proto-oncogenes within the gibbon genome (Sun *et al.* 1981).

The cell cycle has highly complex mechanisms and from the study of retroviruses and the cell cycle, the cancer genome project has suggested that there are between 120-140 driver genes that regulate oncogenesis, 70 of which are tumour suppressor genes and 50 are proto-

oncogenes (Vogelstein *et al.* 2013; Klopfleisch 2016). Tumour suppressor genes are those which reduce the likelihood of carcinogenesis and proto-oncogenes are those genes responsible for initiating carcinogenesis (Vogelstein *et al.* 2013). An important feature in the study of cancers is that each population of tumour cells stem from a single origin, making them monoclonal in nature. However, some cancers, including breast cancer in humans, can be of singular origin or have heterogenicity in the populations of cells in the tumours, called intra-tumour genetic heterogeneity (Klopfleisch 2016).

1.8.3 Accessory Genes

An example of a retrovirus that causes cellular dysregulation through viral accessory genes is Bovine Leukaemia Virus (BLV). BLV is a member of the *Deltaretrovirus* genus and similar to Human T-lymphotropic Virus type 1 (HTLV-1), therefore has been avidly studied for insights into oncogenesis. Deltaretroviruses have *Tax* and *Rex* viral genes within its genome and these have implications in their oncogenic mechanisms (King *et al.* 2012). Sheep have been used as a model for oncogenesis in BLV since the majority of sheep develop overt clinical signs within six months to four years of infection (Merimi *et al.* 2007). Cattle, in comparison, have a considerably lower B-cell malignancy rate (Klener *et al.* 2006). Mechanisms of oncogenesis in BLV are hypothesised to involve *Tax*, a viral transactivator. Through mechanisms of deregulation *Tax* modifies the cellular pathways to favour DNA damage and aneuploidy (Klener *et al.* 2006; Merimi *et al.* 2007). Through micro-array techniques Tax_{BLV} Has been shown to disrupt a broad spectrum of cellular pathway genes, including those concerned with DNA repair, apoptosis and proto-oncogenes (Klener *et al.* 2006) leading to a greater chance of mutations and malignancy. It is also hypothesised that acute leukaemia and progression to overt lymphoid neoplasia in these sheep models is

initiated through the silencing of viral oncoprotein *Tax* and evasion of the immune system through non-productive provirus within transformed cells (Merimi *et al.* 2007).

1.8.4 Immunosuppression

Immunosuppression has been associated with retroviruses since the HIV and AIDS epidemic in the 1980s and since then immunosuppression has been found with multiple retroviral infections (Denner 1998). Identification of the immunosuppressive domain (isu) within the transmembrane protein, a highly conserved region across multiple genera of retroviruses, such as FeLV, PERV, MuLV and KoRV, lead to more insights on the immunomodulating effects of retroviruses (Denner 1998; Fiebig *et al.* 2006). *In vitro* experiments found that the peptides corresponding to this region inhibited several lymphocytic reactions, decreased lymphocyte proliferation and altered cytokine activity (Denner 1998). Retroviral association with immunosuppression has also been linked to increased risk of malignant disease. A common complication of HIV induced AIDS is the formation of cancers and it is suggested that these cancers may arise as a consequence of inadequate immune surveillance, altered immune response or reactivation of oncogenic virus (Levy 2011). In koalas, higher levels of viral load would be likely to go hand in hand with increased mutagenesis and recombination, with possible induction of oncogenesis through evasion of the immune system or immunosuppression (Denner *et al.* 2013).

1.8.5 Herpesviral oncogenesis and association with retroviruses

Retroviruses are not the only viral aetiology of cancer. There are two major viral-associated neoplastic conditions that occur in chickens; Marek's Disease (MD), a neural and epithelial

neoplastic disease caused by Marek's disease herpesvirus (MDHV), and Lymphoid Leukosis (LL) an Avian Leukosis Virus (ALV) induced B-Cell lymphoma (Peters *et al.* 1973; Bacon *et al.* 1988; Mays *et al.* 2019). MDHV is an alphaherpesvirus and ALV is an *Alpharetrovirus* with subtypes, of which Rous-associated Viruses are in subgroup E. It has been hypothesised that interactions between these viruses increase the prevalence of LL (Peters *et al.* 1973; Bacon *et al.* 1988) and LL-like bursal lymphoma (Mays *et al.* 2019). Peters et al (1973) inoculated pathogen-free chickens with MDHV, Rous associated virus-2 (RAV-2), and both MDHV and RAV-2 (Peters *et al.* 1973). Within the first 60 days, none of the birds in the individual, either MDHV or RAV-2, inoculations succumbed, whereas there was a mortality rate of 81% and a tumour rate of 100% in the birds from the combined MDHV and RAV-2 inoculation group (Peters *et al.* 1973). Bacon et al. (1989) demonstrated an increased incidence of LL in serotype-2 MD vaccinated chickens when exposed to avian leukosis virus (ALV) and postulated this was the cause of B-cell hyperplasia in MD vaccinated birds (Bacon *et al.* 1988). Two novel strains of RAV were found in commercial flocks with a high incidence of LL, these strains were the used to infect birds, in combination with or without the MDHV vaccine (SB-1). It was found that those vaccinated had significantly increased incidence of LL-like bursal lymphomas (Mays *et al.* 2019).

Discovered in 2011, there are two known Gammaherpesviruses in koalas, *Phascolarctid gammaherpesvirus 1* (PhaHV-1) and *Phascolarctid gammaherpesvirus 2* (PhaHV-2) (Vaz *et al.* 2011; Vaz *et al.* 2012). Currently their clinical relevance is currently unclear, although herpesviruses in macropods have been found in animals with conjunctivitis, cloacal ulcerations and respiratory disease (Stalder *et al.* 2015). The presence or interaction with koala retrovirus has not been investigated, nor their associations with lymphoid neoplastic or other neoplastic conditions in the koala.

1.8.6. Transcriptomics

To investigate which viral and cellular mechanisms of oncogenesis are involved in an animal-based cancer, a transcriptomic approach can be used to compare neoplastic tissue with normal tissue. Transcriptome analysis examines mRNA in the cell at one given time point, giving a snapshot of the gene activity within the cell, showing a gene expression profile. RNA-seq is a method that uses Next Generation Sequencing (NGS) to analyse the RNA in given samples, producing reads. Analysis of these reads against the reference genome creates counts of reads against genes. These counts can be compared across groups of tissues and through multiple bioinformatic analyses, produce differential gene expression data.

In koalas, transcriptomes have been analysed from two koalas; liver, heart, lung, brain, kidney, adrenal gland, spleen, uterus and pancreas were sampled from the female koala and bone marrow, kidney, liver, lymph node, salivary gland, spleen and testes were sampled from the male koala (Hobbs *et al.* 2014). From RNAseq data aligned to sequences on the koala genome, Hobbs et al. (2014) produced approximately 15,000 aligned genes from 117,563 protein-coding sequences (Hobbs *et al.* 2014). Using *de novo* assembly and referencing a combination of well-annotated genomes from the gray short-tailed opossum (*Monodelphis domestica*), Tasmanian devil (*Sarcophilus harrisii*) and tammar wallaby (*Macropus eugenii*), they also generated a catalogue of koala gene transcripts (Hobbs *et al.* 2014). This resource is available for browsing online through the koala genome website (koalagenome.org). Stable genes, or reference genes, are genes which can be used to normalise gene expression across samples in qPCR analysis. Stable genes from lymph nodes have been examined in koalas from diverse geographical locations, of which Tmem97 and Hmg20a were shown to be the most stable (Sarker *et al.* 2018). Tmem97 is a protein coding gene for an endoplasmic

reticulum-resistant transmembrane receptor; σ^2 receptor, although it has been shown to be overexpressed in rat and human cancer cell lines (Alon *et al.* 2017). Hmg20a is a protein coding gene for the high mobility group 20a protein and shown to have ubiquitous expression in human tissues (Sumoy *et al.* 2000). These genes have not been investigated in lymphomic koala lymph nodes.

1.9 Conclusion

Koalas from the MLR, SA as one of the southern koala populations, do not appear to have endogenised koala retrovirus (KoRV) within their genome and therefore, are an ideal population for the pathological study of KoRV. Recently investigations of KoRV n the South Australian population has found complexity in the molecular analyses and questions have arisen as to the definitive molecular diagnosis for disease studies in this population. Comparative disease studies between KoRV positive and KoRV negative koalas have not been carried out in this or other populations. Also, KoRV is associated with lymphoma and lymphoid leukaemia and only recently was the first case of lymphoma reported in South Australia. The prevalence and classification of lymphoid neoplasia in this population of koalas is currently undetermined. Overt chlamydiosis has been associated with exogenous KoRV and associations between *Chlamydia pecorum* and both subtypes of Phascolarctid gammaherpesvirus have been found, therefore investigation into pathological consequences of coinfection between these infectious agents warrants investigation. In other species, oncogenesis has also been shown to occur as a result of retroviral infection, but the mechanisms are unknown in koalas. In order to determine if altered gene expression exists, a comparative study of normal and neoplastic cells must be undertaken. The findings of coinfection and cancer development will help guide possible management options for these

koalas and highlight the importance of preventative measures to reduce the impact of KoRV and help to guide clinical and population management decisions. Therefore, the aims of this study are:

- Determine the best molecular techniques to differentiate KoRV positive from KoRV negative koalas within South Australia

- Determine the prevalence of lymphoid neoplasia within South Australian koalas and classify lymphoid neoplasia within South Australian koalas[a],

- Investigate disease, infection and coinfection associations of koala retrovirus, *Chlamydia pecorum* and Phascolarctid gammaherpesviruses within South Australian koalas

- Investigate differential gene expression between KoRV positive lymphoma tissue, KoRV positive lymph node tissue and KoRV negative lymph node tissue for genes of interest in oncogenesis

- Describe any novel presentations of disease in South Australian koalas

[a]As per sample availability and sample preservation.

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Chapter 2: Diagnosis of KoRV in South Australian koalas

This chapter has been submitted to a peer-reviewed journal.

This chapter outlines the complex nature of retroviral infection in South Australian koalas. The initial understanding of koala retrovirus (KoRV) in South Australia was taken from previous studies on northern koalas. Northern koalas have endogenised KoRV within their genome, inherited from one generation to the next. This means that within the northern population an issue of deciphering infected from non-infected has not arisen. Southern koalas are different, they do not have endogenised KoRV within their genome and the infection in these koalas is spread exogenously or horizontally. KoRV has been hypothesised to be spreading throughout koala populations in Australia in a southernly direction, with endogenous and exogenous KoRV and 100% prevalence in Queensland (QLD) & New South Wales (NSW) mainland koalas. However, southern koalas in Victoria (VIC) and South Australia (SA) have been assumed to have only exogenous forms of KoRV, due to their lower prevalence and lower viral and proviral loads.

This chapter focuses on interpretation of molecular studies of KoRV infection in the MLR population. Recent studies have shown variability in proviral PCR analyses and therefore, in acknowledgement of this work, the differentiation of KoRV positive from KoRV negative koalas within the cohort studied in this thesis was necessary. This differentiation is essential to allow pathological associations, therefore with clear demarcation, conclusions can be drawn throughout this thesis. Work in this chapter is based on proviral KoRV (DNA insertions), expression from RNA-seq data, aligned to the koala reference genome, and viral expression (RNA) analysed through two-step RT-qPCR techniques.

Statement of Authorship

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Name of Principal Author (Candidate)	Dr Tamsyn Stephenson
Contribution to the Paper	Conceptualization, methodology, formal analysis, investigation, writing; original draft preparation and writing; review and editing.
Overall percentage (%)	85%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third-party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11 / 03 / 2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i.   the candidate's stated contribution to the publication is accurate (as detailed above);

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Molecular diagnosis of koala retrovirus (KoRV) in South Australian koalas (*Phascolarctos cinereus*)

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2.1 Abstract

Koala retrovirus, a recent discovery in Australian koalas, is endogenised in 100% of northern koalas but has decreased prevalence in southern populations, with lower proviral and viral loads, and an undetermined level of endogenisation. KoRV has been associated with lymphoid neoplasia, predominantly lymphoma. Recent studies have revealed high complexity in southern koala retroviral infections, with a need to clarify what constitutes positive and negative cases. This study aimed to define KoRV infection status in Mount Lofty Ranges koalas in South Australia using RNA-seq and proviral analysis (n = 216). The basis for positivity of KoRV was deemed the presence of central regions of the KoRV genome (*gag* 2, *pol, env* 1 and *env* 2) and based on this, 41% (89/216) koalas were positive, 57% (124/216) negative, and 2% inconclusive. These genes showed higher expression in lymph node tissue from KoRV positive koalas with lymphoma compared with other KoRV positive koalas, which showed lower, fragmented expression. Terminal regions (LTRs, partial *gag* and partial *env*) were present in SA koalas regardless of KoRV status, with almost all (99.5%, 215/216) koalas positive for *gag* 1 by proviral PCR. Further investigation is needed to understand the differences in KoRV infection in southern koala populations.

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2.2 Introduction

The genome of koala retrovirus (KoRV) was sequenced in 2000 (Hanger *et al.* 2000) and was shown to be a type-C gammaretrovirus. It is simple in structure, approximately 8.4 kb long and contains three genes: group-specific antigen gene (*gag*), protease-polymerase gene (*pro-pol* or *pol*) and the envelope gene (*env*), flanked by long terminal repeat (LTR) regions (Hanger *et al.* 2000). Generically, the *gag* gene encodes matrix, capsid and nucleocapsid proteins and is necessary for viral structure (Vogt 1997) and its ability to create budding virions (Shojima *et al.* 2013a); the *pol* gene codes for reverse transcriptase, integrase and protease enzymes; and the *env* gene encodes surface and transmembrane proteins from which cell tropism and receptor affinity arise (Vogt 1997). KoRV variants, denoted A - I, have been classified into three major clades, based on the hypervariable region of the *env* gene; KoRV-A, KoRV-B and KoRV C - I (Stoye 2006; Simmons *et al.* 2011; Simmons *et al.* 2012; Shojima *et al.* 2013b; Xu *et al.* 2013; Shimode *et al.* 2014; Xu *et al.* 2015; Chappell *et al.* 2017). KoRV-A is known to enter the cell via the Pit-1 (SLC20A1) receptor (Oliveira *et al.* 2006), KoRV-B via the THTR1 receptor (Shojima *et al.* 2013a; Xu *et al.* 2013)and the other KoRV subtypes receptors through an unknown mechanism. KoRV has two fundamental methods of transmission: endogenously through the germ line from parent to offspring (Stoye 2006); and exogenously, via horizontal (close contact) or vertical (mother to joey in the

pouch) transmission (Simmons *et al.* 2011; Xu *et al.* 2013). Northern mainland koala populations (from Queensland and New South Wales) have all been found to be positive for endogenous KoRV-A provirus, with exogenous variants KoRV-B through to KoRV-I also present in some koalas (Chappell *et al.* 2017). In contrast, southern koala populations (from Victoria and South Australia) have a lower prevalence of KoRV, primarily KoRV-A, and have lower proviral and viral loads. This has led to the theory that these populations are currently undergoing the process of KoRV endogenisation, with predominantly KoRV-A spreading exogenously through this population (Fiebig *et al.* 2006; Tarlinton *et al.* 2008; Chappell *et al.* 2017; Legione *et al.* 2017; Fabijan *et al.* 2019; Sarker *et al.* 2019; Sarker *et al.* 2020), but this is yet to be confirmed.

Studies of KoRV in South Australian (SA) koalas have been based on detection of KoRV provirus and virus using a PCR amplification of part of the *pol* gene, a highly conserved region of the KoRV genome. Based on this, large numbers of KoRV-free koalas have been found in the two main populations of SA koalas in Kangaroo Island (KI) and the Mount Lofty Ranges (MLR). However more recent studies utilising PCR targeting of *gag* and *env* genes, as well as expression studies of the entire KoRV genome, have identified a more complex KoRV profile in MLR koalas (Sarker *et al.* 2019; Sarker *et al.* 2020), with 38 - 99% positive for nine different proviral targets, and 3% - 51% positive for viral RNA targets (Sarker *et al.* 2020). It has also been shown that MLR KoRV *pol*-negative koalas produced transcripts that aligned to the KoRV genome at positions 1 - 1389 (5' LTR and partial *gag* gene) and 7124 - 8431 (partial *env* gene and 3'LTR), with no transcription of other KoRV genes (Tarlinton *et al.* 2017). Additionally, KoRV *pol*-positive koalas had low transcription of genes between genome positions 1389 and 7124, representing the 5' end of the *gag* gene, the *pol* gene and the 3'end of the *env* gene, unlike Queensland koalas (Tarlinton *et al.* 2017).

It is currently postulated that truncated and potentially defective virus inhibits viral replication in these koalas (Tarlinton *et al.* 2017; Sarker *et al.* 2019). Another recent study has found that terminal sequences from KoRV have, in some cases, recombined with earlier or ancient retroviral elements, such as Phascolarctid endogenous retroelement (PhER) (Lober *et al.* 2018; Yu *et al.* 2019). PhER has been shown to still be active and expressing at least parts of genes (Lober *et al.* 2018; Yu *et al.* 2019), although the clinical significance of this is unknown. This recombination of KoRV with PhER, has been designated as recKoRV and has one prominent subpopulation designated recKoRV-1. RecKoRV-1 has been shown to be present in the MLR population, but not the KI population and notably, within the MLR population recKoRV-1 was shown to be present in KoRV *pol* negative koalas (Lober *et al.* 2018).

In northern koalas, disease states that have been associated with KoRV infection include lymphoid neoplasia (lymphoma and leukaemia) and chlamydiosis (Tarlinton *et al.* 2005; Fabijan *et al.* 2017; Legione *et al.* 2017; Waugh *et al.* 2017). Lymphoid neoplasia and increased severity of chlamydiosis have been found to be associated with high proviral and viral loads in all states (Tarlinton *et al.* 2005; Legione *et al.* 2017; Sarker *et al.* 2020) and with exogenous KoRV-B infection in northern koalas (Shojima *et al.* 2013b; Xu *et al.* 2013; Waugh *et al.* 2017). In the MLR population lymphoma has been found in koalas with KoRV viral and proviral loads comparable to those of northern koalas with neoplastic disease (Sarker *et al.* 2020). No significant association has been identified between KoRV-A status and *Chlamydia pecorum* status in MLR koalas (Fabijan *et al.* 2017; Fabijan *et al.* 2019), despite the exogenous transmission in this population and the suggested increased pathogenicity of exogenous KoRV infections. A study in KoRV positive koalas did show a

positive correlation between KoRV viral load and severity of chlamydiosis (Fabijan *et al.* 2020), indicative of potentially increased pathogenicity with increased viral loads.

KoRV infection in SA koalas is clearly complex, and hence it is important to clarify what KoRV genes should be used to identify an individual as KoRV positive or negative, in order to understand infection prevalence and interpret disease in SA koalas. The aims of this study were to describe the integration and expression of KoRV genes in MLR koalas, so as to recommend guidelines for classification of individuals as positive or negative for infectious, full length KoRV in SA.

2.3 Materials and Methods

Samples

Koalas for this study (n = 216) were sourced from the Mount Lofty Ranges population in South Australia and were deemed unfit for release either due to disease or trauma, and euthanised by veterinarians on welfare grounds. All koalas received a full necropsy at the Veterinary Diagnostic Laboratory, Roseworthy campus, University of Adelaide. Blood samples taken prior to euthanasia were stored in EDTA tubes (Vacuette® Tube, Greiner Bio-One GmBH, Kremsmünster, Austria), or spleen samples were taken postmortem, and stored at -20°C prior to DNA analysis. In koalas that were available for immediate postmortem examination (<1 hour), small $0.5cm^2$ pieces of lymph nodes were placed into RNA Later® (Sigma Aldrich), refrigerated for 24 hours and stored at -80°C prior to RNA analysis. All lymph nodes were examined histologically to determine the presence or absence of lymphoma.

Animal Ethics Approval

The sampling of koalas for this study was approved by the University of Adelaide Animal Ethics Committee, approval number: S-2016-169 and conducted in accordance with the guideline set out in the 'Australian Code for the care and use of animals for scientific purposes 8th edition (2013) (National Health and Medical Research Council: Canberra, 2013).

DNA extraction

DNA from whole blood or spleen was extracted using the QIAMP DNeasy Minikit (Qiagen, Hilgen, Germany) as per manufacturer's instructions. All extracted DNA was quantified using a NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc, United States) and stored at -20°C.

DNA proviral PCR and qPCR

KoRV proviral gene targets *gag* 1, *gag* 2, *env* 1 and *env* 2 (Figure 1) were assessed by conventional PCR using DNA extracted from either whole blood or spleen. KoRV *gag* 1 and 2 were optimised as a multiplex reaction and *env* 1 and 2 reactions were run individually. The KoRV *gag* (1 and 2) 25 µL PCR reactions consisted of 5 µL AllTaq Mastermix (Qiagen, Hilden, Germany), 0.25 mM of each *gag* 1 primer, 0.75 mM of each *gag* 2 primer (Table 1), 3 µL of 1/10 dilution DNA template and 9 mL of PCR-grade water. The individual KoRV *env* 20 µL PCR reactions consisted of 4 µL AllTaq Mastermix (Qiagen, Hilden, Germany), 0.5 mM of each primer (Table 1), 3 µL of 1/10 dilution DNA template and 10 µL of PCR-grade water. The temperature and timing of the PCR reactions for both assays were; initial activation and denaturation of 95°C for 3 minutes; 40 cycles of denaturation at 95°C for 5 seconds; annealing at 60 °C (KoRV *env*) or 62°C (KoRV *gag*) for 15 seconds; and extension

at 72°C for 10 seconds. This was followed by a final extension step of 72°C for 10 seconds. Gel electrophoresis using 1.0% agarose gel was carried out and PCR products visualised under UV light. Samples were loaded in combination with gel red, producing bands at the appropriate product size (Table 1).

KoRV proviral load was measured in DNA extracted from either whole blood or spleen by KoRV *pol* gene qPCR (Table 1, Figure 1). Koala β-actin was used as the reference gene. qPCR reactions of 5µL were set up in triplicate using an Eppendorf epMotion 5075 LH model (Eppendorf, Hamburg, Germany). Each reaction consisted of 2.5µL of Power SYBR green Master Mix (Applied Biosystems, Foster City, CA, USA), 300nM of each primer and 1.5µL of 1/10 or 1/100 dilution of DNA template. qPCRs were run on a 7900HT Sequence Detection System (Applied Biosystems, Singapore). Reaction conditions were; initial denaturation 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds; 60°C for 60 seconds. Melt curve analysis was carried out at 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. Copy number was calculated from a standard curve derived from purified PCR products for both KoRV *pol* and b-actin from koala lymphoma tissue. KoRV proviral load reported as copies/10^3 β-actin copies.
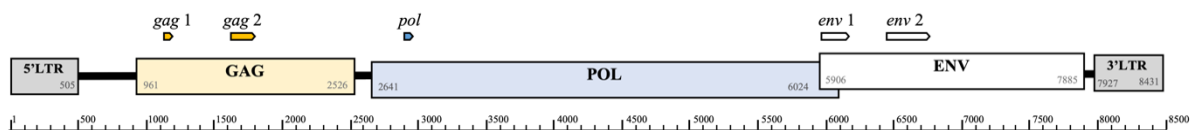
Figure 1: Representation of the KoRV genome (AF151794); showing genes, coordinates and primer locations with block arrows indicating amplicon length

Table 1: PCR targets and primers

Target	Primers	Product size	Reference
Koala β-actin	Fwd 5'- GAGACCTTCAACACCCCAGC -3' Rev 5'- GTGGGTCACACCATCACCAG -3'	111 bp	(Shojima *et al.* 2013b)
KoRV *gag* 1	Fwd 5'- CGGACCAGGTTCCCTACATC -3' Rev 5'- TCGCCCGTTATCTTGACCAG -3'	110 bp	This study
KoRV *gag* 2	Fwd 5'- TTGGCCTTTCTCCTCAGCAG -3' Rev 5'- CCGTGTTGTGATCCCACTGA -3'	290 bp	This study
KoRV *pol*	Fwd 5'-TTGGAGGAGGAATACCGATTACAC-3' Rev 5'-GCCAGTCCCATACCTGCCTT-3'	111 bp	(Tarlinton *et al.* 2005)
KoRV-A *env* 1	Fwd 5'-TCCTGGGAACTGGAAAAGAC-3' Rev 5'-GGGTTCCCCAAGTGATCTG-3'	321 bp	(Waugh *et al.* 2017)
KoRV *env* 2	Fwd 5'-GCCCTCGGCCCTCCTTATTA-3' Rev 5'-GGCAATCTGGAGGCTAGTCAA -3'	522 bp	(Sarker *et al.* 2020)

RNA extraction and sequencing

RNA was extracted from eight SA koalas, two KoRV proviral positive with lymphoma, three KoRV proviral positive and three KoRV proviral negative (except *gag* 1). Tissues were thawed on ice, disrupted with zirconium oxide beads (0.5mm, RNA-free, BioTools, Loganholme, Australia) in a Bullet Blender and Tissue Homogeniser (Next Advance, Troy, NY 12180, USA)) and then cooled on ice. The *mir*Vana™ kit (ThermoFisher Scientific) was used to extract total mRNA as per the manufacturer's protocol. RNA integrity (RIN) was checked using a 2100 BioAnalyser (Agilent Technologies). cDNA libraries were made and sequenced using an Illumina NovaSeq sequencing platform that generated 2 x 150 bp reads (AGRF, Melbourne, Australia).

KoRV gene expression from RNA-seq analysis

The RNA-seq reads were cleaned using Trim_Galore (v0.4.2) (Krueger 2015) and AdapterRemoval (v2.2.1) (Schubert *et al.* 2016) to remove adapters and bases with a Phred score less than 10. Fastqc (v0.11.4) (Andrews 2010) was used to check the quality of the cleaned reads. The reads were aligned to the koala reference genome (GCA_002099425.1 _phaCin_unsw_v4.1) using HISAT2 (v2.1.0) (Kim *et al.* 2015).

BLAST analysis

To determine the presence of KoRV termini regions in the koala reference genome, NCBI standard nucleotide Basic Local Alignment Search Tool (*BLASTn*) (Altschul *et al.* 1990) was used to align the KoRV-A (AF151794.2) reference genome against the koala reference genome (phaCin_unsw_v4.1 reference Annotation Release 100; GCF_002099425.1) with a focus on alignments to the koala genome that only contained the terminal regions of KoRV homology. Also, the central "gap" sequences were recovered from between these terminal alignments from the reference genome and *BLASTn* was then used to align these gap sequences to the nucleotide NCBI database (Coordinators 2018). *BLASTn* was also used to align the Phascolarctos endogenous retroelement (PhER), using coordinates reported in Lober *et al.* (2018), against the koala reference genome. PhER and KoRV alignment results were then compared to find any crossover regions.

Viral two step RT-qPCR analysis

To quantify the difference in KoRV viral gene expression between the three groups, total mRNA was extracted from 15 koalas, five KoRV proviral positive with lymphoma, five KoRV proviral positive and five KoRV proviral negative (except *gag* 1). qPCR set up data is in Appendix S2 - S4. cDNA was reverse transcribed from 250 ng-2500 ng of total RNA using SuperScript IV First Strand Synthesis system (Invitrogen, Thermofisher Scientific) and oligoDT primers as per manufacturer's instructions. cDNA was diluted to a working solution based on starting RNA (equivalent to 1.25 ng/mL RNA). qPCR reactions were run, targeting four KoRV gene regions; *gag* 1, *gag* 2, *pol* and *env* 1, with primers described in Table 1 and targets shown on the KoRV genome in Figure 1. Koala γ-actin was used as the reference gene; forward primer 5'-TGCGCAGCTTCAGATTAAACAA-3', reverse primer 5'-GGCCT CATCACCAACATAACTG-3', due to increased stability in comparison to koala ß-actin

across RNA-seq data for the cohorts. Coefficients of variance (CoV) (Stanton *et al.* 2017) were calculated from gene expression normalised counts (cpm) across all samples with γ-actin CoV calculated as 0.04 (position 289/14435) in comparison to ß-actin CoV 0.06 (position 906/14435). qPCR was optimised for γ-actin and conditions for the qPCR are as follows; 5μL reactions were set up in triplicate using an Eppendorf epMotion 5075 LH model (Eppendorf, Hamburg, Germany). Each reaction consisted of 2.5μL Power SYBR green Master Mix (Applied Biosystems, Foster City, CA, USA). 300nM of each primer (Table 1) and 1.5μL of cDNA template. qPCRs were run on a 7900HT Sequence Detection System (Applied Biosystems, Singapore). Reactions conditions were; initial denaturation 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Melt curve analysis was carried out with conditions of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds.

Relative qPCR statistical analysis

Comparative CT ($2^{(-\Delta\Delta Ct)}$) was calculated for each of the four gene targets studied. The Mann-Whitney U Test was used to determine significant differences between groups. Both calculations were carried out in Microsoft Excel (Version 16.42, 2020).

$$\Delta CT \text{ and } \Delta\Delta CT \text{ equations}$$

$$\Delta CT = \left(Average\ CT_{Gene} - Average\ CT_{Ref}\right)$$

$$\Delta\Delta CT = \Delta CT - \left(Average\ CT_{Gene} - Average\ CT_{Ref}\right)_{KoRV\ negative}$$

$$Fold\ Change = 2^{-(\Delta\Delta CT)}$$

$$Range\ is\ 2^{-(\Delta\Delta CT - sd)}\ to\ 2^{-(\Delta\Delta CT + sd)}$$

2.4 Results

Proviral DNA analyses

The presence/absence of three KoRV genes was determined by PCR using five targets, two in the *gag* gene, one in the *pol* gene and two in the *env* gene, the targets called *gag* 1, *gag* 2, *pol*, *env* 1 and *env* 2 respectively. The majority of koalas (212/216) could be placed into two categories: positive for all gene targets and negative for all gene targets except *gag* 1 (Table 2). The remaining koalas (4/216) showed variation in their gene target results with one koala negative for all targets (Table 2). Individual analyses results are in Appendix S1.

Table 2: Proviral PCR results

KoRV gene target					Count (n=216)	%	Median (range) of proviral load KoRV copies/10^3 ß-actin copies[*]
gag 1	*gag* 2	*pol*	*env* 1	*env* 2			
+	+	+	+	+	89	41.2	41 (0.2-270)
+	-	-	-	-	123	56.9	0
+	-	-	-	+	2	0.9	0
+	-	-	+	-	1	0.5	0
-	-	-	-	-	1	0.5	0

[*]calculated from *pol* gene qPCR

KoRV gene expression from RNA-seq analysis

Figure 2 shows the KoRV expression profiles for the lymph nodes of eight koalas as determined by mapping the RNA-seq reads to a KoRV insertion found in the reference koala genome (scaffold 27:2807277-2815707 of GCA_002099425.1_phaCin_unsw_v4.1 as per Table 2). Every RNAseq sample, regardless of lymphoma and KoRV PCR status, showed substantial coverage across the LTRs and nearby gene regions (called the terminal regions). Two positive koalas with lymphoma (Lymphoma 1 and 2) also showed substantial, comparable coverage across the region between them (herein called the central region). Three koalas negative for lymphoma but PCR positive for all five proviral gene targets (Positive 1,

2 and 3) only showed only low, fragmented coverage across the central region. Of three koalas negative for all PCR targets except *gag* 1, the koalas Negative 1 and 2 showed no coverage across the central region, but Negative 3 showed low, fragmented coverage.
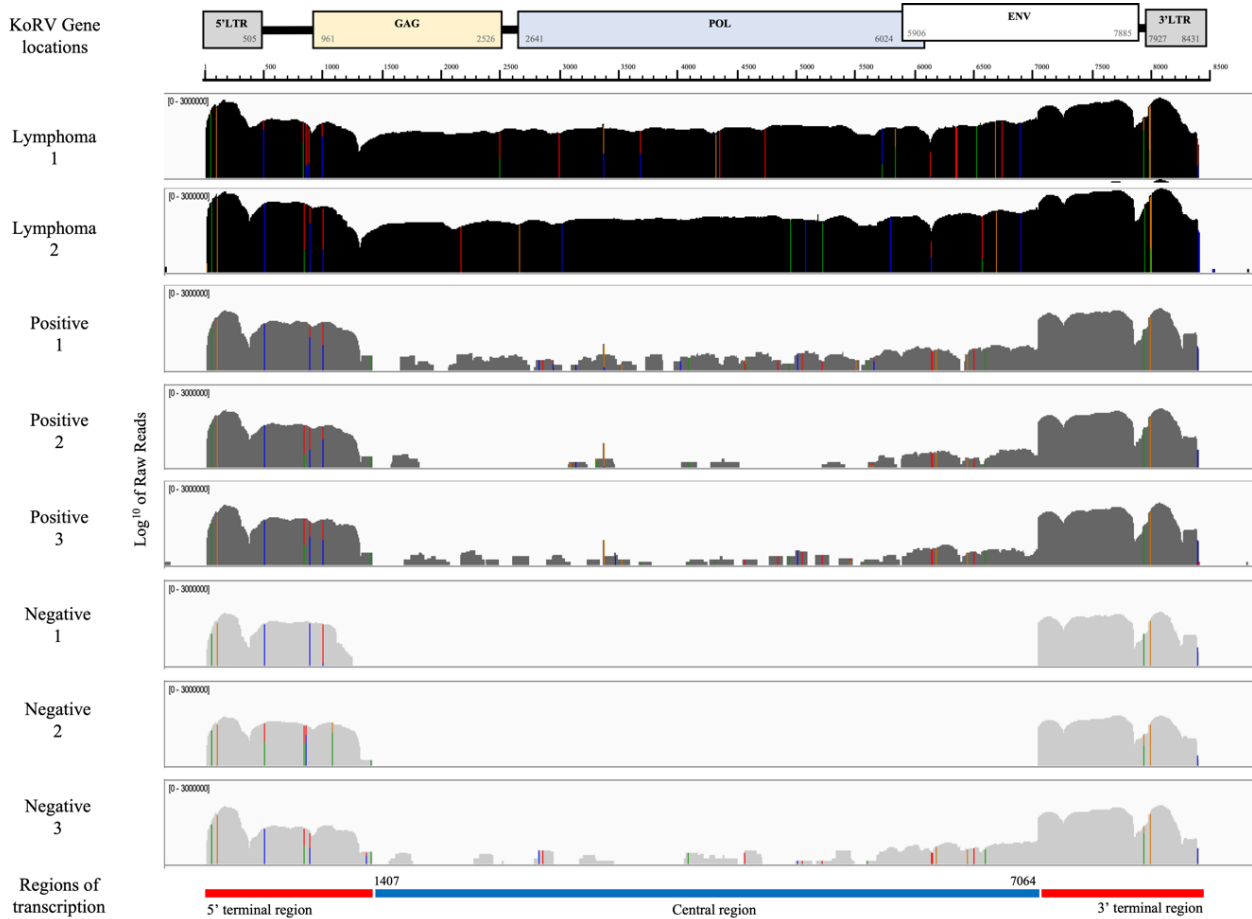
Figure 2: Transcription expression profile (mapped cleaned reads) for two koalas with lymphoma, three KoRV proviral positive and three KoRV proviral negative (except *gag* 1) koalas, demonstrating alignment to the KoRV genome, location of primers for proviral PCRs and transcription regions. All expression is a $\log_{10}$ scale of read counts, black represents KoRV proviral positive koalas diagnosed with lymphoma, dark grey represents KoRV proviral positive koalas and light grey represents KoRV proviral negative (except *gag* 1) koalas. All mapped to scaffold 27; 2807277-2815707 (GCA_002099425.1_phaCin_unsw _v4.1), KoRV-A accession AF151794, as per table 2.

BLAST analysis

Alignment of KoRV-A (AF151794.2) against the reference koala genome (phaCin_unsw _v4.1 reference Annotation Release 100; GCF_002099425.1) using *BLASTn* showed two

types of alignments: nine alignments were to the whole or most of KoRV, but eight only

aligned to the terminal regions of KoRV. The terminal alignments were found in scaffolds,

137, 005, 288, 113, 073, 357, 253 and 101. The terminal region alignment lengths varied

from 1155-2663 bp in the 5' region and 812-2919 bp in the 3' region and are quite similar to

the consistently expressed terminal regions in Figure 2. The central regions within these

scaffolds, from the reference genome, were then aligned using *BLASTn* against the nucleotide

database. Alignments were found to uncharacterised loci in the koala, wombat (*Vombatus*

ursinus) and gray short-tailed opossum (*Monodelphis domestica*) genomes. However,

alignment of PhER (phaCin_unsw_v4.1.fa.scaf00062: 10912078-10920108, as per Lober *et*

al. (2018) using *BLASTn* against the koala reference genome revealed alignment to the same

scaffolds and positions of the central gap region of KoRV. Overall, the *BLASTn* data shows

that the terminal regions are present in several copies in the koala reference genome. It shows

that in the reference genome from a NSW koala, this gap region, in the identified scaffolds,

aligns to PhER (Figure 3), reiterating previous findings (Lober *et al.* 2018).
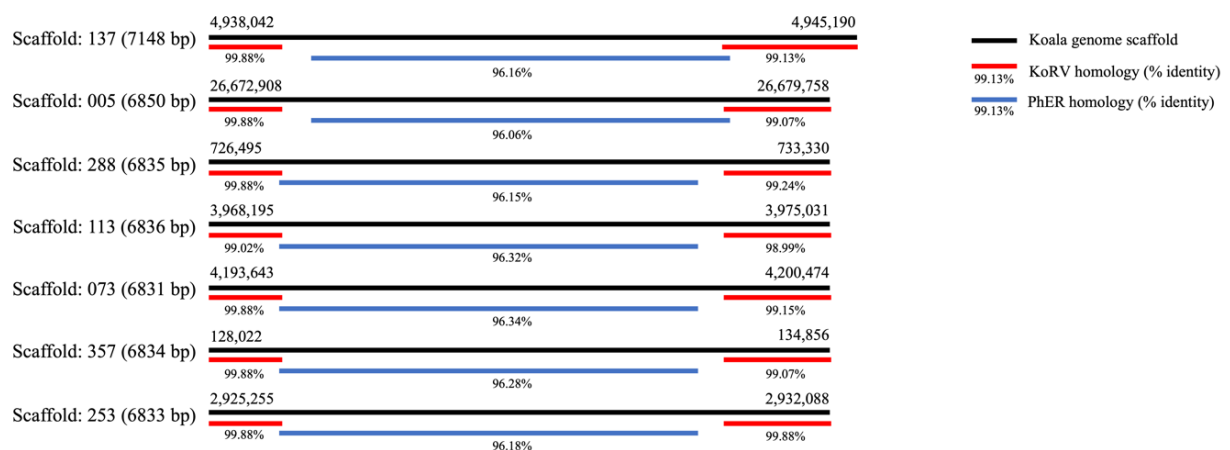
Figure 3: *BLASTn* analysis results showing crossover of the central gap regions from alignment of KoRV termini regions (red) and PhER (blue) to the koala reference genome (black). KoRV is represented by the reference sequence for KoRV-A: AF151794, PhER: phaCin_unsw_v4.1. fa.scaf00062:10912078-10920108, as per Lober et al. 2018 (Lober *et al.* 2018) and the reference koala genome: phaCin_unsw_v4.1 reference Annotation Release 100; GCF_002099425.1

Viral two step RT-qPCR analysis

qPCR for KoRV gene targets *gag* 1, *gag* 2, *pol* and *env* 1 were carried out on RNA from fifteen koala lymph nodes, five KoRV positive with lymphoma (three of which were from a previous study cohort (Fabijan *et al.* 2020)), five KoRV positive and five KoRV negative koalas. Seven of the eight koalas that were previously examined by RNA-seq transcriptome analysis were examined by viral RT-qPCR (all except Negative 3, as not enough material remained). Because *gag* 1 was positive in almost every test, regardless of the sample lymphoma and KoRV status, it was excluded from further analysis. Ten KoRV proviral gene (DNA) positive koalas, including five with lymphoma, were also positive for the three KoRV viral gene (RNA) targets. Five koalas that were KoRV proviral gene (DNA) negative were also negative for the three KoRV viral gene (RNA) targets. Therefore, *gag* 2, *pol* and *env* 1 gene targets had substantially increased expression in koalas that were KoRV proviral positive versus those that were KoRV proviral negative for all three targets, due to the complete lack of expression in KoRV proviral negative koalas (Appendix S5 & S6). Table 3 shows the comparative CT mean fold change ($2^{-\Delta\Delta CT}$) and range ($2^{-(\Delta\Delta CT \pm sd)}$) between KoRV positive and lymphomic koalas. Gene targets *pol* and *env* 1 had significantly increased expression for KoRV proviral and viral positive koalas with lymphoma versus those that were KoRV proviral and viral positive.

Table 3: Comparative CT method results (fold change $2^{-\Delta\Delta CT}$) of KoRV central genes for lymph node RNA from KoRV positive koalas with lymphoma relative to KoRV positive koalas

| KoRV gene | $2^{-\Delta\Delta CT}$ lymphomic koalas relative to positive koalas | | |
	Mean FC[*]	FC Range	*p* value
gag 2	299	47.7-1880.5	0.060
pol	**602**	185.8-1950.2	**0.012**
env1	**242**	42.5-1375.6	**0.012**

FC = Fold change calculated by $2^{-\Delta\Delta CT}$. [*]Bold values are significant (Mann-Whitney U test, p<0.05), more detail of calculations and samples in Appendix S5 & S6

2.5 Discussion

The findings of this study demonstrate the complexity of KoRV status in SA koalas, but clearly show that the central region of KoRV *(gag* 2, *pol*, *env* 1 and *env* 2) is diagnostic for classifying individuals as infectious KoRV positive or negative. The highest expression of all KoRV genes was by koalas with lymphoma, whereas non-lymphomic KoRV positive koalas had variable expression profiles in the central region of the KoRV viral genome. The koalas tested on both proviral (DNA) qPCR and viral (RNA) qPCR analyses demonstrated consistency in results. Gene target *gag* 1 was positive in almost all MLR koalas (215/216) regardless of KoRV status and the terminal sequences inclusive of this are present numerous times in the koala reference genome.

Based on the central region of KoRV, proviral PCR revealed two distinct groupings: 41.2% (89/216) positive and 57.4% (124/216) negative for all targets. These results differ to those of a recent study in which MLR koalas were shown to have varying prevalence of each of the KoRV genes within their proviral DNA, with 79% (77/97) PCR positive for at least one target on each proviral genes, 99% (96/97) positive for *pol* gene target and only 41% (40/97) positive for one of the *env* gene targets (Sarker *et al.* 2020). Viral expression (qPCR) supported our proviral analyses since all KoRV negative koalas showed no expression of *gag* 2, *pol*, and *env* 1 targets. However, in this study there were a low number (1.4 %, 3/216) of koalas that showed mixed results in the proviral PCRs and this could be due to the sensitivity of these assays, as KoRV proviral load can be very low in SA koalas, or potentially represent acute infections.

Gene expression in KoRV positive koalas showed variable depth of coverage across the central region, except for lymphomic koalas in which it was consistently high. High

expression of KoRV was shown directly from lymphomic tissue and supports the ongoing assumption that retroviral oncogenesis occurs in koalas (Hanger 1999; Tarlinton *et al.* 2005; Kinney *et al.* 2016; Fabijan *et al.* 2017). Suppression, latency or subclinical infections of KoRV may explain the fragmentation of alignment to the central region of KoRV in KoRV positive, non-lymphomic koalas. In order for the host to survive, several anti-viral strategies have evolved in response to retroviral infections. Small piwi interacting RNA (piRNA) molecules or small interacting RNA (siRNA) have been found to silence transposons in germinal cells (Tarazona *et al.* 2011; Fu *et al.* 2014; Ahlenstiel *et al.* 2015), DNA methylation can also inhibit transcription and several host genes have been shown to have antiretroviral properties (Goff 2018). piRNAs have been found in koalas, highly expressed within testicular tissue, but also expressed in other tissues including brain, liver and lymph nodes (Yu *et al.* 2019). The inhibition shown from piRNAs through ping-pong modelling in koalas, corresponds to central regions of the KoRV genome (Yu *et al.* 2019). Therefore, piRNAs could be responsible for some of the inhibition and fragmentation of the transcription of central genes in SA koalas as seen in KoRV positive koalas. The low gene expression found in SA koalas differs to that found in previous studies of endogenised Queensland koalas, in which KoRV expression and viral loads are high (Tarlinton *et al.* 2005; Olagoke *et al.* 2018; Fabijan *et al.* 2020; Sarker *et al.* 2020). Cellular genes that encode restriction factors, such as those belonging to the cytidine deaminases, have shown significant antiretroviral properties, including inhibition of viral transcription. Examples of antiretroviral cellular genes are APOBEC3, SAMHD1 and MX2 (Goff 2018). A recent study found the addition of a mouse homolog of APOBEC3 significantly reduces the infectivity of KoRV *in vitro* (Nitta *et al.* 2015). The koala homolog to human or mouse APOBEC3 gene is not present, but APOBEC1, APOBEC2 APOBEC4 and AICDA genes (other cytidine

deaminases) are annotated on the koala genome (NCBI database), with their effect yet to be demonstrated on KoRV infectivity.

From the RNAseq data, in two of the three KoRV proviral (DNA) negative koalas there was no detectable expression from the central region, but the other one koala showed a similar pattern of expression to the KoRV proviral (DNA) positive koalas. Unfortunately, this particular koala did not undergo viral (RNA) gene expression analysis through qPCR. The proviral PCRs were carried on DNA extracted from blood or spleen and RNA-seq with lymph node tissue, this difference could support lymph nodes as the primary target for infection and replication, finding low level transcription in this tissue first before detectable provirus in circulating lymphocytes. Therefore, our tentative conclusion is that this koala was recently infected with the virus only at measurable levels in the greater pool of target cells in the lymph nodes and had not yet spread to or be measurable in other tissues including the blood and spleen. For those KoRV negative koalas without proviral DNA detection or proviral gene expression in the central region of the KoRV genome it would be highly unlikely that these koalas would be able to produce infectious KoRV virions. *Env* genes are responsible for entry into the cells, coding for the receptor binding domain and the transmembrane unit, while *pol* genes are responsible for enzymes including viral polymerase, *gag* genes code for structural proteins and allow for formation of a virion (Balasuriya *et al.* 2017; Greenwood *et al.* 2018). Therefore, without these elements, formation of KoRV virions would be impossible, but it would be necessary to carry out *in vitro* infection studies in cell culture to confirm this.

There were multiple sequences in the koala reference genome that showed strong homology to the KoRV terminal regions but not the central region. Some of these sequences have the

KoRV central region replaced by sequences from the central region of PhER. Possibly these insertions in the reference genome have been generated by recombination between the termini of PhER and KoRV, attributed to recKoRV in previous studies (Hobbs *et al.* 2017; Lober *et al.* 2018). We note that complete KoRV and PhER retroviral sequences only show 72% identity over 102 nucleotides around the 7500bp region on the KoRV genome, which may be enough for recombination to have occurred. Additionally, the lack of KoRV in significant numbers of probable recKoRV positive koalas elicits evolutionary questions for this virus, unable to be answered without greater investigations. A possible alternative explanation for these terminal regions could be due to the historical foundation of this population, reintroduced to SA from very small numbers of VIC koalas. Due to the genetic bottleneck, partial gene loss may have occurred in this population, with endogenisation of a defective version of KoRV in SA koalas. ERVs that have lost the *env* gene tend to have increased proliferation within the host genome, with less defined loci of integration (Magiorkinis *et al.* 2012), therefore correspond to the increased terminal reads seen in this study. Further long read genome sequencing of KoRV positive and negative koalas could help answer some of the questions surrounding these terminal regions.

There is the potential that the terminal retroviral expression in KoRV negative koalas could be inhibiting or reducing subsequent spread of KoRV in SA populations. These elements could also be reducing loads in KoRV positive koalas. Protective antiretroviral properties have been encountered in the study of endogenous retroviruses (ERVs). Some endogenous retroviral elements can inhibit other retroviral entry into cells and therefore increase the resistance to future retroviral infection, such as in feline leukemia virus (FeLV) and murine leukemia virus (MLV) (Mcdougall *et al.* 1994; Nethe *et al.* 2005; Wu *et al.* 2005; Greenwood *et al.* 2018). *Env* regions have been shown to be recruited in response to exogenous retrovirus (XRV) infection to block viral receptors (Stoye 2012) and has been experimentally

demonstrated in mice (Wu *et al.* 2005), cats (Mcdougall *et al.* 1994) and chickens (Robinson *et al.* 1976). The transcribed KoRV fragments from the 5' LTR and 5' *gag* region and from the 3' *env* and 3' LTR could be part of the resistance to future KoRV infection, as previously suggested (Tarlinton *et al.* 2017). This would assume that these elements are endogenised throughout the population, which is likely since the RNA-seq alignment mapping showed presence of these regions in all koalas and proviral PCR analysis showed an overwhelming majority (99.5%, 215/216) of koalas were positive for the 3' *gag* gene target (*gag* 1). *In vitro* studies could explore the ability for KoRV negative lymphocytes to withstand KoRV infection and the effect of transcripts from 5' and 3' regions of KoRV on infection rates. These studies would help distinguish clinical relevance of these KoRV transcripts and expand our understanding of KoRV transmission and cellular response.

2.6 Conclusion

The differentiation of KoRV infection, infectious status and other retroviral integrations is complex in South Australian koala populations. The clinical relevance of KoRV in SA requires the definition between KoRV positive and KoRV negative koalas and this study demonstrated two clear groupings, allowing for future delineation. Future definition of KoRV positive in SA koalas should be a combination of PCR gene targets in the central region of the KoRV genome. Although the commonly used *pol* qPCR should detect positive cases, confirmation with *gag* 2 and *env* 1 gene targets would increase confidence. This study also highlighted the homology to the terminal regions of KoRV in all koalas. Sequencing the termini and gap regions, or potentially long read genome sequencing, of KoRV negative SA koalas could increase knowledge of what these regions are and how they are situated in the genome. *In vitro* cell culture studies using KoRV negative koalas could show whether they

have a level of resistance to KoRV infection. KoRV negative koalas in SA are important for

future and more in-depth understanding of not only infection and transmission, but also

evolution of KoRV.

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Investigations into the high level of lymphoid neoplasia in koalas led to the discovery of viral particles in the bone marrow of a leukaemia animal. This was later sequenced and attributed to a retrovirus, now known as koala retrovirus or KoRV. Further investigations have shown increased viral and proviral loads in koalas with lymphoid neoplastic conditions. South Australian koalas have only recently, since 2014, been reported to have cases of lymphoma within the population. This chapter describes the prevalence of lymphoid neoplasia in this study cohort and also collates past cohorts for classification of lymphoma found in the MLR population.

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By signing the Statement of Authorship, each author certifies that:

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Lymphoma cases in South Australian koalas (*Phascolarctos cinereus*) and their associations with koala retrovirus (KoRV)

Stephenson, T., Speight, K.N., Kovac, J.H., Hemmatzadeh, F., Woolford, L.

3.1 Abstract

Lymphoma, or lymphosarcoma, in koalas has been reported in conjunction with koala retrovirus (KoRV) infection and has been found to occur in approximately 4% of free-ranging and 55% of captive northern koalas (from Queensland or New South Wales). Previous studies in northern populations, where KoRV infections are endogenised, have shown associations with high proviral and viral loads of KoRV, and more recently an association with KoRV-B subtype. In contrast, lymphoma has only been found in a small number of southern koalas, positive for KoRV-A subtype, in a population with lower KoRV infection prevalence and lower proviral loads. In this 2016 - 2019 necropsy study of South Australian koalas from the Mount Lofty Ranges region, the prevalence of lymphoma was found to be very low at 1.2% (3/240). All koalas with lymphoma were infected with KoRV-A, with a mean proviral load of 188 copies/10^3 ß-actin copies (range: 98-241), significantly higher than other koalas in this cohort. All lymphoma koalas were PCR negative for KoRV-B. A review of classification of previous lymphoma cases reported by Fabijan *et al, 2019* (n = 4) in South Australian koalas and current cases (n = 3) demonstrated that abdominal anatomical classification was most common. Also, cytological classification found they were all intermediate to large cell, non-T-cell lymphoma, with a low mitotic index. This study corroborates findings from northern koalas that KoRV plays a significant role in the

pathogenesis of lymphoma. It is likely that the lower prevalence and proviral loads within the southern populations have reduced the presentation of lymphoma.

3.2 Introduction

Lymphoma, or lymphosarcoma, and lymphoid leukaemia are neoplastic diseases of lymphoid origin, disrupting the normal proliferation of immune cell lines (Vail *et al.* 2019). Lymphoma is a solid tumour made up of malignant lymphocytes and originates in lymphoid tissues, particularly lymph nodes. Lymphoid leukaemia originates from the bone marrow or spleen, manifesting with circulating neoplastic lymphocytes; however, circulating malignant lymphocytes can also be found in end-stage lymphoma when the bone marrow or spleen are involved (Valli *et al.* 2020). Lymphomas can derive from B-cell, T-cell or, more rarely, natural killer (NK) cells (Vail *et al.* 2019).

The first published pathological studies reporting lymphoid neoplasia in the koala were in the early 1960s, describing two cases of lymphoid leukaemia in koalas from the Taronga Zoo, NSW collections (Backhouse *et al.* 1960; Heuschele *et al.* 1961). One of these cases, an acute lymphoid leukaemia, was characterised by marked immature lymphocytosis with neoplastic infiltration of sternal bone marrow, peripheral lymph nodes, stomach, kidneys, bladder and caecum (Heuschele *et al.* 1961). As awareness of the disease increased, larger studies were undertaken, a relatively high prevalence of lymphoid neoplasia was found in northern koalas. Of 344 koalas examined between 1981 and 1985 from Queensland and New South Wales, 3.8% had lymphoid neoplasms (Canfield *et al.* 1987). There was no sex predilection to lymphoid neoplasia noted and koalas over two years of age had a greater likelihood of disease (Canfield *et al.* 1987). An investigation of spontaneous proliferations in a wide range

of marsupials found 26/154 koalas were found with neoplastic conditions, of which lymphoma was found in eight koalas, and suspected in an additional three koalas with advanced tissue autolysis (Canfield *et al.* 1990). Between 1982-1994, 5.3% of presentations to the University of Sydney of both free-living and captive koalas were described with lymphoid neoplasia (Spencer *et al.* 1996). The majority of these were multicentric lymphomas, affecting peripheral lymph nodes, liver and spleen (Spencer *et al.* 1996). Both multicentric and alimentary forms are common anatomical classifications in koalas from these studies; (Canfield *et al.* 1987; Spencer *et al.* 1996) affected sites including gastrointestinal tract, thymus, kidney, bladder, brain and bone marrow (Spencer *et al.* 1996). Hanger (1999) reported lymphoid neoplasia in up to 55% of necropsies performed on captive koalas in Queensland (Hanger 1999). Immunotyping of 51 lymphoma cases showed that T-cell lymphomas predominated (26/51, 51%) (Connolly *et al.* 1998). The antibodies that were utilised for this study were anti-human CD3 and CD5 to recognise T-cells, anti-human CD79b to recognise B-cells and anti-koala IgG to recognise plasma cells (Connolly *et al.* 1998). Viral aetiology of these cancers was suspected due to clustering of disease and similarity to spontaneous leukaemias seen in laboratory animals (Heuschele *et al.* 1961; Arundel *et al.* 1977; Canfield *et al.* 1987).

Confirmation of viral association and likely causation occurred with the use of electron microscopy to visualise viral particles in tissues of lymphoid neoplasia (Canfield *et al.* 1988), followed by viral isolation and classification in 2000 (Hanger *et al.* 2000). This virus was defined as koala retrovirus (KoRV), a member of the *Gammaretrovirus* genus (Hanger *et al.* 2000). Since the discovery of KoRV, increased plasma viral loads have been shown to have an association with lymphoid neoplasia (Tarlinton *et al.* 2005). Queensland koalas with lymphoid neoplasia had a significantly higher plasma viral load of 1.6×10^9 copies/mL in

comparison to healthy koalas (7.7×10^7 copies/mL) (Tarlinton *et al.* 2005). Disease studies of koalas have continued to show associations of KoRV with lymphoid neoplasias (Mulot 2014; Fabijan *et al.* 2017; Fabijan *et al.* 2019; Fabijan *et al.* 2020), but the mechanisms of oncogenesis are yet to be determined.

In South Australia (SA) there are several distinct populations of koalas, the largest population is found in the Mount Lofty Ranges (MLR), and other smaller populations on Kangaroo Island (KI), the Eyre Peninsula, the Riverland and in Southeast SA. KoRV prevalence has been investigated in wild-caught koalas from MLR and KI, at 65.3% (n = 75) and 42.4% (n = 170) respectively (Fabijan *et al.* 2019). Within the MLR population the first report of a KoRV positive koalas with lymphoma was described in 2014, and in the 2014 - 2016 study of MLR koalas, the prevalence of lymphoma was 4.3% (4/92) (Fabijan *et al.* 2017; Fabijan *et al.* 2019; Fabijan *et al.* 2020). A recent study of proviral and viral loads in neoplastic koalas showed high proviral loads in both QLD and SA koalas: QLD: 5.25×10^4 copies/10^3 ß-actin copies (3.38×10^4 - 4.78×10^5) comparable with SA koalas which had 2.14×10^5 copies/10^3 ß-actin copies (6.71×10^3 - 4.32×10^5) (Sarker *et al.* 2020).

This necropsy study investigated the prevalence of lymphoma, its relation to KoRV and trends of anatomical, cytological and phenotypical classification in a large cohort of koalas from the MLR population in SA.

3.3 Methods

Koala samples

Between 2016 and 2019, 240 koalas from Adelaide and the surrounding Mount Lofty Ranges were euthanased on welfare grounds and subject to necropsy either soon after death or following a period of frozen storage. Blood samples in EDTA tubes were taken prior to euthanasia for KoRV analyses, or if no blood sample was available a sample of spleen was collected at necropsy. Sampling was run in accordance with animal ethics guidelines and with approval from Adelaide University Animal Ethics Committee (S-2016-169). At necropsy, each koala was assessed for approximate age by tooth wear class (TWC I - VII) (Martin 1981) and body condition score (BCS 1 - 5) (Blanshard *et al.* 2008). Based on necropsy findings, koalas were classed into suspected lymphoma, other non-lymphoma diseases (including diseases such as oxalate nephrosis, pneumonia and mange) and disease free. Histopathological examinations included, but were not restricted to; axillary, inguinal, submandibular and mesenteric lymph nodes, spleen, liver and kidney from all animals where autolysis was not inhibitive of diagnosis. All lymph nodes were assessed for presence of lymphoma. Formalin fixed paraffin embedded tissue sections (4 μM) were routinely processed and stained with haematoxylin and eosin and examined using an Olympus BX43 Microscope with DP25 camera (Olympus corporation, Tokyo, Japan). Lymphoid neoplasms were subject to immunohistochemistry for CD3 (T-cell marker), Pax5 (B-cell marker) at Gribbles Veterinary Pathology, Adelaide and CD79b (B-cell marker) at Veterinary Pathology Diagnostic Services (VPDS), University of Sydney.

KoRV analysis

DNA was extracted from antemortem whole blood (when available) or postmortem spleen samples using QIAMP DNeasy Mini kit (Qiagen, Hilden, Germany) as per manufacturer's instructions. All extracted DNA was measured on NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc, United States) and then stored at -20°C until testing. PCR testing for KoRV provirus was carried out for five proviral targets, two on the *gag* gene, one on the *pol* gene and two *env* location as previously described in Chapter 2, Tarlinton *et al.* (2005) and Waugh *et al.* (2017). Koalas were considered KoRV positive if they were positive for all KoRV proviral gene targets (Chapter 2). KoRV proviral loads were calculated from the *pol* gene and reported as KoRV copies per 10^3 ß-actin copies. Positive control standard curves utilised were purified PCR product from a lymphomic koalas with known copy number. KoRV-B PCR reaction mixes were 4 µL AllTaq Master Mix (Qiagen, Hilden, Germany), 1µM of each of the primers; Fwd 5'-TCCTGGGAACTGGAAA AGAC-3' and Rev 5'- GGCGCAGACTGTTGAGATTC -3' (Waugh *et al.* 2017) and 3µL of 1/10 dilution of DNA template and made up to 20 µL with PCR grade water. Conditions were; initial activation and denaturation of 95°C for 2 mins, followed by 40 cycles of: denaturation at 95°C for 5 secs, annealing at 61°C for 15 secs and extension at 72°C for 10 secs. This was followed by a final extension step of 72°C for 10 secs. Products were visualized under UV light on 1.0% agarose gels with samples loaded in combination with gel red, producing bands 271bp. Positive control was from a known KoRV-B positive NSW koala (*courtesy of D. Higgins*).

Statistical analysis

Association of lymphoma with KoRV infection was examined using Fisher's exact test of independence for current cases (n < 5) and Chi squared analysis was carried out for all reviewed cases (current and previous, n > 5). Odds ratio and relative risk calculations were carried out after chi squared analysis.

Classification of lymphoma cases

A review of all past and present SA lymphoma cases was carried out, where all reports of lymphoma were in koalas from the MLR population. Initial classification of present cases and reclassification of past cases was done using Table 1 for consistency. Table 1 combines the classifications by Spencer *et al.* (1996) (n = 31) and Connolly *et al.* (1998) (n = 51) and pertinent information from the current WHO classification of lymphoma in the dog (Valli *et al.* 2011).

Table 1: Classification and staging of lymphoma in the koala based on anatomical and cytological features (adapted from koala lymphoma; Spencer et al. 1996 and Connolly et al. 1998, with mitotic rates and nuclear size parameters included from WHO classification of canine lymphoma, Valli et al. 2011, updated anatomical terminology from Vail et al. 2019)

Area	Class		Description
Anatomical	Multinodal		Disseminated, bilateral involvement of peripheral lymph nodes with or without other organ involvement
	Abdominal		Lymphoma within the abdominal cavity with the following subgroups; • Alimentary: Gastrointestinal tract, GALT and regional lymph node involvement and associated organs (spleen, liver, pancreas) • Miscellaneous: involvement of other abdominal organs (kidney, adrenal gland, bladder, uterus, ovary) • Combination: Combination of alimentary and miscellaneous subgroups
	Cervicomediastinal		Involvement of lymph nodes of the lower neck, lymph nodes of the chest with or without thymic infiltration
	Atypical		Other lymphomas that do not fit into another class, involvement of non-lymphoid organs such as CNS, conjunctiva, mammary gland
	Mixed		Combination of two or more anatomical classifications
Cytological	Cell population	Monoclonal	>75% same cell size – then divided into small-medium & large, dependent on nuclear size
		Mixed	<75% same cell size
		Immunoblastic	Large cells, eccentric nucleus, prominent nucleoli and abundant cytoplasm
	Cell type (IHC)	B-cell, T-cell or Null type	B-cell markers: CD79b T-cell markers: CD3
	Nuclear size	Koala(Connolly *et al.* 1998)	Small-medium: diameter up to width of 2 erythrocytes Large: diameter greater or equal to width of 3 erythrocytes
		Canine/WHO(Valli *et al.* 2011)	Small: diameter up to width of 1.5 erythrocytes Intermediate: diameter between the width of 1.5 - 2 erythrocytes Large: diameter greater or equal to width of 2 erythrocytes
	Nuclear shape	Non-cleaved	Round nucleus
		Cleaved	Indented or folded nucleus
	Other	Nucleoli	present/absent
		Nuclear chromatin	pattern
		Cytoplasm	Amount and staining pattern
		Mitotic rate Koala(Connolly *et al.* 1998) (mitoses per HPF over 5 HPF)	Low: 0 to 2 Medium: 3 to 5 High: 6+
		Mitotic rate Canine/WHO(Valli *et al.* 2011) (mitoses per HPF over 10 HPF)	Low: 0 to 5 Medium: 6 to 10 High: 11+

3.4 Results

Lymphoma and KoRV Analysis

Based on necropsy findings koalas were classed into suspected lymphoma, other non-lymphoma diseases (including diseases such as oxalate nephrosis, pneumonia and mange – described further in Chapter 3) and disease free. Within this necropsy cohort, 1.2% (3/240) of koalas were found to have lymphoma, 76.3% (183/240) koalas had non-lymphoma diseases and 9.6% (23/240) koalas were disease free (full results shown in Chapter 5). All three koalas with lymphoma were positive for KoRV-A across the five proviral targets; *gag* 1, *gag* 2, *pol*, *env* 1 and *env* 2. In the non-lymphoma disease category 41.5% (76/183) were KoRV positive and in the disease free category 30.4% (7/23) were KoRV positive. The lymphoma cases were all negative for KoRV-B on PCR analysis. All three koalas were male, one young koala, with a TWC of I and two adult koalas, one with a TWC of II and one with a TWC of IV. Body condition score was average to good in these koalas, with two koalas ranking 3/5 and one 4/5. There was no statistical association between KoRV infection and presence of lymphoma in this cohort, *Fisher exact test p value* = 0.068.

A comparison of proviral loads in KoRV positive koalas with lymphoma, non-lymphoma disease and no disease, showed a mean (SD) of 188 (78.4), 54.6 (43.0) and 44 (19.5) copies/10^3 ß-actin copies, respectively (Figure 1). Lymphomic koalas has significantly higher loads in comparison to other diseased koalas and disease free koalas (Student T-Test; two sample assuming unequal variance; lymphoma to other diseases P(T<=t) one-tail = 0.049, lymphoma to disease free P(T<=t) one-tail = 0.044, other diseases to disease free P(T<=t) one-tail = 0.128).
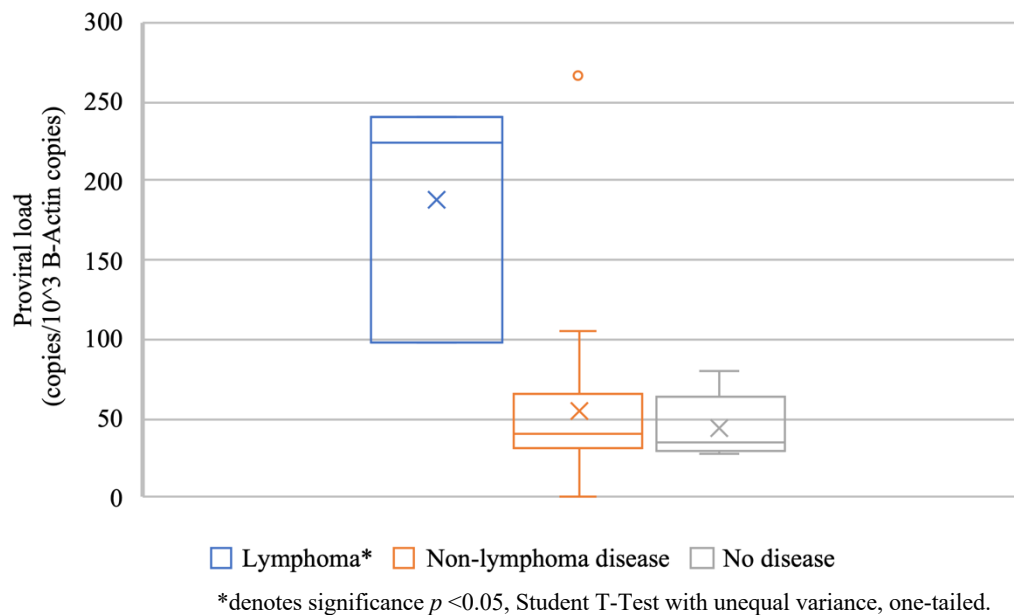
Figure 1: Koala retrovirus proviral load in positive koalas with lymphoma, non-lymphoma disease or disease free cohorts.

Fat Replacement

Peripheral lymph nodes were deemed to be enlarged if they were greater that 20mm in length and mesenteric lymph nodes if they were greater than 12mm in length (Hanger *et al.* 1994a; Hanger *et al.* 1994b). Enlargement of at least one lymph node was noted on necropsy in 52.5% (126/240) of koalas. This was found to be due to the following causes: lymphoma, reactivity, oedema, congestion and or fat replacement. 143 koalas were investigated further regarding fat replacement of lymph node parenchyma, of which 44% (64/143) had at least one enlarged node. Axillary nodes were found to be more prone to fat replacement with 37.1% (13/35) of enlarged axillary nodes having greater than 10% fat replacement. Of these 38.5% (5/13) had marked fat replacement with greater than approximately 66% of the node replaced by fat, 7.7% (1/13) had moderate fat replacement (33% - 66% of the node replaced by fat) and 53.8% (7/13) had mild fat replacement (10% - 33% of the node replaced by fat). Other nodes were less affected by fat replacement, with no mesenteric lymph nodes affected

(See supplementary Table 1). Overall enlargement of lymph nodes due to fat was noted in 18% (20/113) lymph nodes. No incidence of concurrent lymphoid depletion was noted in lymph nodes with fat replacement present.

Combination of current and past lymphoma cases

In the 2014 - 2016 postmortem study of MLR koalas, the prevalence of lymphoma was 4.3% (4/92) (Fabijan *et al.* 2020) and in the current cohort was 1.2% (3/240), giving an overall prevalence of lymphoma in MLR koala examined at postmortem to be 2.1% (7/332) between 2014 and 2019. By pooling the two cohorts together a significant association between lymphoma and KoRV infection was found (*Chi squared* = 4.95, *p value* = 0.026, *Odds ratio* = 10.8 and *Relative Risk* = 10.4). This increased number of cases allowed for a review of KoRV association and lymphoma classification in SA koalas.

Classification of Lymphoma in South Australian koalas

Anatomical and cytological classification of the three lymphoma cases from the current 2016 - 2019 cohort and reclassification of four cases of lymphoma from the previous 2014 - 2016 study was undertaken according to the criteria outlines in Table 1. These four cases and one case from the current cohort have been reported (Fabijan *et al.* 2017; Fabijan *et al.* 2019; Fabijan *et al.* 2020), in varying levels of detail. The necropsy reports and lymph node tissue sections were re-evaluated for the cases from 2014 - 2016 and combined with the CD79b and CD3 IHC findings.

Anatomical classification showed six koalas had a mixed presentation, and one was abdominal – combination. Abdominal lymphoma was the most common presentation, with all lymphoma cases involving this region; three lymphoma cases were alimentary and only involved the gastrointestinal tract, associated organs (liver and pancreas) and lymphoid tissue (spleen, lymph nodes), and the other lymphoma cases were combination, also involving other organs of the abdominal cavity, such as the uterus, kidney and adrenal glands. Cervicomediastinal presentation was the next most common with four koalas in this classification, three of these involved infiltration of the thymus. There were three multinodal and two atypical organ involvement cases (Table 2). Atypical presentations included infiltration of the heart and lymphomic serosal nodules on the surface of the diaphragm and parietal pleura. Leukaemia was unable to be evaluated for 4/7 cases due to lack of antemortem blood sample collection and advanced autolysis of bone marrow tissue collected for histology. Leukaemia was not present in the three koalas for which blood samples were obtained. Of these three koalas, two koalas had bone marrow infiltration of lymphoma (one case) and suspected lymphoma (one case, autolysis inhibitive of definitive diagnosis).

Cytological classification was more challenging due to the degree of autolysis in some cases. All cases were monoclonal with four classified as intermediate cell, one as small to intermediate cell, one as intermediate to large cell and one case as large cell, using the canine WHO lymphoma guidelines (Valli *et al.* 2011). The mitotic rate could be assessed in five koalas and was low-medium as per previous koala-based grading (Connolly *et al.* 1998) with the highest mitotic index being 3/HPF. These were all classified as low mitotic index according to the WHO canine grading of lymphoma. From IHC analysis there were two confirmed B-cell lymphomas (CD79b positive) and one null type (Table 3). Four lymphomas were non T-cell origin, with staining of small numbers of non-neoplastic T-cells with CD3, but autolysis and sensitivity of CD79b staining was inconclusive, therefore differentiation

between B-cell and Null cell lymphoma was not possible. Pax5 antibody IHC was attempted on B-cell CD79b positive lymphoma and there was no cross reactivity to the koala lymphocytes.
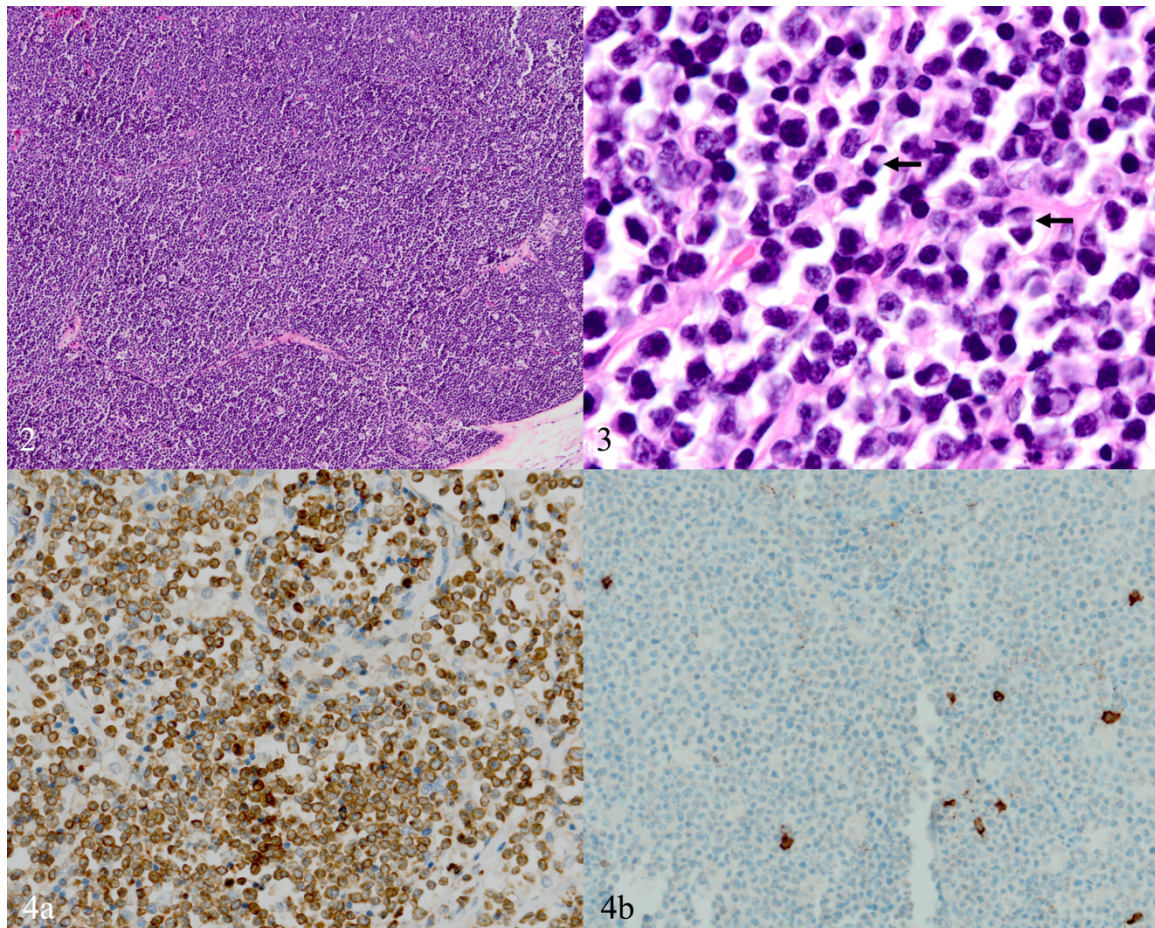
Figure 2-4: Lymphoma histology. 2: Lymphoma effacing lymph node, H&E, 10x. 3: Intermediate cell monoclonal (>75%), low grade (2.2 mitoses/HPF) lymphoma. Medium irregularly round nuclei with variably distinct central nucleoli, vesicular chromatin and scant cytoplasm, mitotic figures (black arrows), H&E, 100x. 4a: Intermediate B-cell lymphoma, diffuse strong CD79b immunostaining, 40x. 4b: Intermediate B-cell lymphoma, diffuse negative staining of neoplastic lymphocytes, strong positive CD3 immunostaining in infiltrating non-neoplastic lymphocytes only, 40x.

Table 2: Anatomical classification and distribution of lymphomic lesions in lymphoma cases in South Australian koalas

Cohort	Case	Classification	Categories	Lymph node involvement	Organ involvement
2014 to 2016	1	Mixed	Multinodal, abdominal (combination) cervicomediastinal	Inguinal, mesenteric (not axillary) (submandibular not examined)	Spleen, liver, bladder, bone marrow, thymus[a]
	2	Mixed	Abdominal (combination), cervicomediastinal, atypical	Mesenteric (not inguinal) (Axillary, submandibular not examined)	Spleen, pancreas, liver, kidney, bladder, heart, ovary, uterus, bone marrow, intestine, adrenal glands[a]
	3	Mixed	Abdominal (combination), cervicomediastinal, atypical.	Not examined	Spleen, liver, kidney, lung (BALT), small intestine[a]
	4	Mixed	Multinodal, abdominal (alimentary), atypical	Axillary, inguinal, submandibular, mesenteric	Testis (Spleen, liver, kidney and thymus also examined)
	5	Mixed	Multinodal, Abdominal (alimentary), cervicomediastinal	Axillary, inguinal, submandibular, caudal aortic (mesenteric not examined)	Spleen, thymus (testes also examined)
2016 to 2019	6	Mixed	Abdominal (alimentary), atypical	Mesenteric (not axillary, submandibular, inguinal)	Spleen, pancreas, nodules on surface of liver, diaphragm and internal thoracic wall (kidney and testes also examined)
	7	Abdominal	Abdominal (combination)	Mesenteric (not axillary, inguinal) (submandibular not examined)	Adrenal glands (kidney, epididymis and penis also examined, marked autolysis)

[a] All the tissues that were examined histologically. Previously reported cases: 1-4 (Fabijan *et al.* 2020), 2 (Fabijan *et al.* 2017), 6 (Fabijan *et al.* 2019)

Table 3: Cytological classification and immunohistochemistry interpretation of lymphoma cases in South Australian koalas

Cohort	Case	Classification	CD3	CD79b	Type
2014 to 2016	1	Intermediate to large cell monoclonal population. Medium to large round nuclei with multiple variably distinct nucleoli, marginalised to vesicular chromatin and scant cytoplasm. Low grade (mitotic index 1.2 mitoses/HPF).	Negative	Negative	Null
	2	Intermediate cell monoclonal population. Medium irregularly indented nuclei with multiple distinct nucleoli, vesicular chromatin and scant cytoplasm. Advanced autolysis inhibited grading.	Negative	inconclusive	Non T-cell*
	3	Large cell monoclonal population. Large irregularly indented nuclei with multiple distinct nucleoli, vesicular chromatin and scant cytoplasm. Connolly: Medium grade, WHO canine: low grade (mitotic index 2.8 mitoses/HPF).	Negative	Weak positive	B-cell
	4	Intermediate cell monoclonal population. Medium round nuclei with multiple variably distinct nucleoli, vesicular chromatin and scant cytoplasm. Low grade (mitotic index <2 mitoses/HPF).	Negative	Positive	B-cell
	5	Intermediate cell monoclonal population. Medium cerebriform nuclei with indistinct nucleoli, vesicular chromatin and scant cytoplasm. Low grade (mitotic index 2.2 mitoses/HPF).	Negative	inconclusive	Non T-cell*
2016 to 2019	6	Small-Intermediate cell monoclonal population. Small round nuclei with indistinct nucleoli, indistinct chromatin and scant cytoplasm. Low grade (mitotic index 0.8 mitoses/HPF), moderate autolysis.	Negative	inconclusive	Non T-cell*
	7	Likely intermediate cell monoclonal population. Advance autolysis inhibiting histopathological evaluation and grading.	Negative	inconclusive	Non T-cell*

*Unable to differentiate between B-Cell and Null cell lymphoma due to CD79b IHC staining inconclusive, due to moderate to advanced autolysis of tissue sections.
Previously reported cases: 1-4 (Fabijan et al. 2020), 2 (Fabijan et al. 2017), 6 (Fabijan et al. 2019)

3.5 Discussion

Lymphoma has only recently been reported in SA koalas and is a rare diagnosis in rescued free-ranging koalas from the MLR, found in only 1.2% (3/240) of koalas examined the current study. Over the past five years only seven cases of lymphoma have been detected and investigated in 332 (2.1%) koala necropsies conducted by our research group. This prevalence is lower than that found in free-ranging koalas examined from NSW, where lymphoid neoplasia was described in 3.8% (13/344) (Canfield *et al.* 1987) in the 1980s and in QLD where lymphoma was observed at 5.1-7.1% (8-11/154) in the 1990s (Canfield *et al.* 1990) and at 7.5% in 2020 (Fabijan *et al.* 2020). Lymphoma in captive QLD koalas has been reported as high as 55.5% (20/36) (Hanger 1999).

The review of all known lymphoma cases from SA koalas showed that anatomical classifications were mixed presentations with abdominal being the most common. Mesenteric lymph nodes were infiltrated with neoplastic cells in 83.3% (5/6) of koalas. Cervicomediastinal involvement was the next most commonly affected site, occurring in 57.1% (4/7) of koalas. There were 50% (3/6) with multinodal presentation. In previous studies from QLD and NSW, multinodal and abdominal were the most common presentations (Canfield *et al.* 1987; Spencer *et al.* 1996). Due to the more cryptic nature of lymphoma in SA koalas the use of abdominal ultrasound or exploratory laparotomy may be necessary to facilitate a definitive pre-euthanasia diagnosis of lymphoma. In addition, future studies investigating lymphoma in SA koalas should examine all lymph nodes and lymphoid tissues.

In regard to cytological and phenotypic classifications, there was a high percentage of intermediate sized non-T-cell (B-cell or null cell), low grade lymphoma. Unfortunately, due to advanced autolysis in some cases, the CD79b marker for B-cell was ineffective. CD79b

can be more sensitive to autolysis in comparison to CD3 due to low dilution of antibody necessary for conclusive staining (*pers comm* S. Lindsay). As part of the IHC for this study, Pax5, a potentially more robust antibody used for identification of B-cell immunophenotype in domestic animals (Valli *et al.* 2020), was used on one previously CD79b positive lymphoma case with lymphomic and normal lymph node present, and there was no cross reactivity, therefore it is not a recommended marker for B-cells in koalas. Interestingly, there was no T-cell lymphoma in the MLR cohort. Immunotyping in NSW and QLD koalas showed just over 50% of koalas had T-cell lymphoma and only 24% were B-cell lymphoma (Connolly *et al.* 1998). Mitotic index in all koalas that could be assessed was low or medium grade, comparable to NSW lymphoma classifications where 76% (25/33) cases were either low or medium grade (Connolly *et al.* 1998). All cases had a low mitotic index according to the WHO classifications for canine lymphoma (Valli *et al.* 2011). Previous grading of mitotic indices was based on the 1986 WHO canine lymphoma scales, therefore if classified to current standards, all koalas in this cohort would have a low-grade mitotic index. A considerable number of these cases were not diagnosed prior to euthanasia. A confounding finding for the sensitivity of lymphoma diagnosis at necropsy was that there were a 37% of enlarged axillary lymph nodes due to mild to marked levels of fat replacement. This increased the size of the lymph nodes but also has the potential to decrease the sensitivity and specificity of diagnosis of lymphoma at necropsy, highlighting the necessity for histopathological confirmation.

In the current study, all lymphoma cases were positive for KoRV, but there were not enough cases to statistically associate lymphoma with KoRV infection. Inclusion of four cases from previously published SA studies (Fabijan *et al.* 2017; Fabijan *et al.* 2019; Fabijan *et al.* 2020) increased the cohort sample size and showed a positive association between lymphoma and KoRV infection, for which koalas with lymphoma were 10.8x more likely to have KoRV.

KoRV proviral load was, on average, significantly higher in those koalas with lymphoma in comparison to other non-lymphoma diseased koalas and disease free koalas, consistent with multiple other studies (Xu *et al.* 2013; Fabijan *et al.* 2017; Fabijan *et al.* 2020; Sarker *et al.* 2020). Unfortunately, the lack of plasma samples for these koalas inhibited investigations into viral load.

KoRV-A, but not KoRV-B was detected in these koalas, which interestingly may coincide with the overrepresentation of B-cell tumours in this population. KoRV-A has been shown to use a sodium-dependent phosphate transporter PiT1 for viral entry into the cell (Oliveira *et al.* 2006; Denner *et al.* 2013). KoRV-B has an altered receptor binding domain and has been shown to utilise a thiamine transporter protein (ThTR1) to gain cellular entry (Shojima *et al.* 2013; Xu *et al.* 2013). *In-vitro* and *in-vivo* studies from mice showed a direct role of PiT1 in cell proliferation and knockout studies show a severe B-cell lymphopaenia, highlighting a fundamental role of PiT1 in B-cell development (Liu *et al.* 2013). Gene transduction studies have shown that increased upregulation of PiT-1 transporter increased viral gene transduction with Gibbon Ape leukaemia virus (GALV) (Sabatino *et al.* 1997), a closely related gammaretrovirus to KoRV (Gallo *et al.* 1978; Hanger *et al.* 2000). Therefore, if there is increased expression of PiT-1 in B-cell development and cell proliferation and KoRV-A targets this receptor, there is potential for greater infiltration of B-cell lymphocytes by KoRV in SA koalas, leading to predominantly B-cell origin lymphomas. Further investigation into possible correlations between PiT-1 expression and lymphoma in KoRV positive SA koalas, along with KoRV related cell culture studies could help differentiate causation.

3.6 Conclusion

Lymphoma is present at a low prevalence in koalas from the Mount Lofty Ranges population in South Australia. Anatomical predilection for abdominal lymphoid organs (spleen or mesenteric lymph nodes) was observed in all MLR lymphoma cases, indicating these organs to be an essential part in the investigation of suspected lymphoma in this population of koalas. Cytologically, neoplasms were monoclonal, medium to large, likely B-cell lymphoma, with a low mitotic index. Despite the lower prevalence of both lymphoma and KoRV in South Australian koalas compared with northern populations, the association of lymphoma with KoRV status and significantly increased proviral load, is now apparent in koalas across Australia.

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The following chapter considers infection status and pathological findings in this 2016 - 2019 necropsy cohort. This large cohort from the Mount Lofty Ranges has allowed for investigation of disease interactions; between pathological findings, KoRV, *Chlamydia pecorum* and Phascolarctid gammaherpesviruses infections. Cooperation of viral or bacterial pathogen can lead to increased prevalence of disease, and investigation of coinfection within this population of koalas can explore clinical relevance of infection and help build a more complete picture of the health status of this koala population. KoRV-associated immunosuppression is likely to play a role in susceptibility to opportunistic infections, but its contribution in the South Australian population still needs greater investigation. *Chlamydia pecorum* is known to be increasing in prevalence and disease severity since it was first reported in the MLR population in 2010, and studies throughout Australia have shown increased severity with higher loads of KoRV. Also, in other species including birds and humans, retroviral oncogenesis is augmented by the presence of herpesviruses, especially gammaherpesviruses. Recently two gammaherpesviruses have been discovered in koalas; phascolarctid gammaherpesvirus 1 (PhaHV-1) and phascolarctid gammaherpesvirus 2 (PhaHV-2). The involvement of PhaHV in oncogenesis and its role in disease of koalas still needs investigation.

Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Dr Tamsyn Stephenson
Contribution to the Paper	Conceptualization, methodology, formal analysis, investigation, writing; original draft preparation and writing; review and editing.
Overall percentage (%)	75%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 11 / 03 / 2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

   i.   the candidate's stated contribution to the publication is accurate (as detailed above);

   ii.   permission is granted for the candidate in include the publication in the thesis; and

   iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Pathological findings associated with koala retrovirus, *Chlamydia pecorum* and Phascolarctid gammaherpesviruses infections in South Australian koalas (*Phascolarctos cinereus*)

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4.1 Abstract

A major threat to koala populations across Australia is disease. Key infectious pathogens include koala retrovirus (KoRV), *Chlamydia pecorum* and more recently described, Phascolarctid gammaherpesvirus (PhaHV). This study examines a large postmortem cohort of South Australian koalas in relation to their disease presentations, infection and coinfection status. Key findings included that reproductive disease was associated with both *C. pecorum* and PhaHV infections, with no koalas with paraovarian cysts having *C. pecorum* infection alone, and 81% (17/21) having *C. pecorum* / PhaHV- 1 and/or 2 coinfections. Furthermore, ocular disease showed no association to a particular pathogen. This study has shown a possible role of Phascolarctid herpesvirus in augmentation of reproductive disease and more work is needed to fully understand the complexity of disease progression in koalas with coinfections. It has also highlighted that other pathogens may need to be considered for koalas presenting with *Chlamydia*-like diseases in South Australia. Koalas with traumatic injuries, especially from road traffic accidents, had negative associations with KoRV, *C. pecorum* and PhaHV infections. These negative associations hold a key message for conservation; every year, significant numbers of koalas are euthanased as a result of traumatic injuries - strategies to reduce road traffic accidents, dog attacks and other trauma events are likely to increase the number of healthy koalas in this population.

4.2 Introduction

Koalas (*Phascolarctos cinereus*) were listed as a vulnerable species in 2016 (Woinarski *et al.*
2016), however the 2019/2020 Australian mega-fires have had further population impacts
with an estimated 11% of habitat burnt (Ward *et al.* 2020). Not only has climate change and
urban development in koala habitat impacted negatively on the koala, but disease has also
contributed to population declines. Two well-researched and key pathogens in koalas are
koala retrovirus (KoRV) and *Chlamydia pecorum*. Another recently described infectious
agent of koalas with poorly understood clinical relevance is Phascolarctid gammaherpesvirus
(PhaHV).

KoRV is a gammaretrovirus that was first discovered in a koala with lymphoid neoplasia
(Hanger *et al.* 2000), and since then has been strongly associated with this neoplastic
condition (Tarlinton *et al.* 2005; Xu *et al.* 2013; Fabijan *et al.* 2017; Fabijan *et al.* 2020). The
genome of KoRV is made up of a *gag* gene, *pro-pol* gene and an *env* gene, flanked by long
terminal repeat regions (LTRs) (Hanger *et al.* 2000). There have been nine variants described
(A-I), based on the hypervariable region within the *env* gene (Shojima *et al.* 2013; Xu *et al.*
2013; Shimode *et al.* 2014; Xu *et al.* 2015). All northern koalas, those from New South
Wales and Queensland, have been shown to have endogenised KoRV-A in their genome. As
a result of all cells containing the KoRV-A provirus, there are significantly higher proviral
loads in northern koalas. Due to the recent endogenisation, KoRV-A is still active and
producing virions in these koalas (Tarlinton *et al.* 2006; Simmons *et al.* 2011; Chappell *et al.*
2017; Sarker *et al.* 2019). Two variants of KoRV (A & B) have been associated with
lymphoid neoplasia (Hanger *et al.* 2000; Tarlinton *et al.* 2005) and increased severity of
chlamydiosis (Xu *et al.* 2013; Legione *et al.* 2017; Waugh *et al.* 2017; Fabijan *et al.* 2020).

Chlamydia pecorum is an intracellular bacterium known to cause urogenital tract disease leading to cystitis and infertility, ocular disease leading to blindness, and pneumonia (Hemsley *et al.* 1997; Blanshard *et al.* 2008; Mackie *et al.* 2016). Cystitis in the koala, can lead to the clinical sign of wet bottom, characterised by mild peri-cloacal urine staining through to more severe urine scalding and ulcerative dermatitis. Reproductive tract disease in females presents with inflammatory disease such as metritis and vaginitis and chronic fibrosis of the oviducts can cause paraovarian cysts in some koalas (Brown *et al.* 1987). Ocular disease presents as a mild to severe conjunctivitis, with or without ocular discharge and can lead to keratitis and blindness (Hemsley *et al.* 1997). Clinical assessment of chlamydiosis can be staged using the criteria developed by Wan *et al.* (2011). In northern koalas, chlamydiosis is common, disease often severe, (Nyari *et al.* 2017; Palmieri *et al.* 2018; Quigley *et al.* 2020) and has been associated with KoRV-B infection in some populations (Waugh *et al.* 2017).

Phascolarctid gammaherpesvirus was first described in Victorian koalas, PhaHV-1 in 2011 (Vaz *et al.* 2011) and PhaHV-2 in 2012 (Vaz *et al.* 2012). In several Victorian populations the prevalence of PhaHV-1 and PhaHV-2 ranged from 7.4 - 45.5% and 0.9 - 54.6%, respectively (Vaz *et al.* 2019). The clinical relevance of PhaHV is yet to be established, although strong associations have been found between infection with PhaHV-2 and coinfection with PhaHV-1 (Vaz *et al.* 2019). Reproductive disease has been found to be associated with both PhaHV-1 and PhaHV-2 infection (Vaz *et al.* 2019). This study also found strong positive association of *C. pecorum* with both PhaHV subtypes, although numbers of overtly affected koalas were too few for conclusions to be drawn as to the clinical impact (Vaz *et al.* 2019). The presence of PhaHV infections in northern koalas has not been reported.

In the southern koalas found in South Australia, KoRV-A is the predominant variant of KoRV (Sarker *et al.* 2019; Sarker *et al.* 2020) and is thought to be circulating exogenously based on low proviral loads (Simmons *et al.* 2012). However recent work has shown small numbers of koalas to have proviral loads similar to that of koalas with endogenised KoRV in Queensland (Simmons *et al.* 2012; Fabijan *et al.* 2019b). The prevalence of KoRV in the Mount Lofty Ranges (MLR), SA was found to be 65.3% in wild-caught koalas (n = 75) (Fabijan *et al.* 2019b), based on PCR targeting the *pol* gene, and in a study of necropsied koalas, 99% (n = 97) were deemed positive, based on at least one positive reaction in multiple PCRs for each of the *gag*, *pol* and *env* genes (Sarker *et al.* 2020). In southern koalas from Victoria, increased urogenital tract disease has been associated with increased KoRV proviral loads in female koalas in Victoria (Legione *et al.* 2017). Association of KoRV with any disease in SA is less clear, with only higher viral loads being associated with severe chlamydiosis (Fabijan *et al.* 2020). Lymphosarcoma has been observed in SA koalas, all of which were positive for KoRV-A (Fabijan *et al.* 2017; Fabijan *et al.* 2019b; Fabijan *et al.* 2020). *C. pecorum* infection was first reported in the MLR population in 2000 (Houlden *et al.* 2000), although overt chlamydiosis was not reported until 2013 (Funnell *et al.* 2013), possibly due to change in circulating strains and pathogenicity (Quigley *et al.* 2018). A 2016 study of necropsied koalas found 88% infection prevalence but low severity of disease in infected koalas (Speight *et al.* 2016). The prevalence in the MLR population was recently found to be 47%, with only 4% showing overt disease (Fabijan *et al.* 2019a). In a study of necropsied MLR koalas, urogenital disease was found to be more common than ocular disease, and no chlamydial pneumonia was reported (Fabijan *et al.* 2019a; Fabijan *et al.* 2019b). Both PhaHV-1 and PhaHV-2 have been found to be circulating within the SA population. The prevalence of PhaHV-1 and PhaHV-2 was found to be 57.5% (46/80) and 40.0% (32/80) respectively in wild caught koalas, and 62.1% (54/87) and 41.4% (36/87),

respectively in necropsied koalas (Kasimov *et al.* 2020). As observed in Victorian koalas, those infected with PhaHV-2 were also likely to be infected with PhaHV-1 (Kasimov *et al.* 2020).

Coinfections between viral and bacterial pathogens can interfere with or augment disease outcomes (Kawaguchi *et al.* 1995; Kumar *et al.* 2018). Both KoRV and PhaHV are viruses that predominantly target immune cells (Hanger *et al.* 2000; Vaz *et al.* 2011) and *C. pecorum* is a bacterial intracellular pathogen of epithelial cells and monocytes (Hemsley *et al.* 1997). In order for each of these infectious pathogens to replicate and survive they have developed mechanisms to increase cellular survival of host cells, therefore inadvertent co-operation between pathogens could be possible allowing each to succeed in their immune cell infection (Eisenreich *et al.* 2019). This co-operation could augment disease processes within the host. Both *C. pecorum* and herpesviruses are opportunistic pathogens which can lay dormant in subclinical or latent states. Their recrudescence and diseased states are possibly secondary to some form of immunocompromise or stressors. KoRV has been associated with causing immunocompromise and been shown to increase risk of chlamydiosis (Legione *et al.* 2017; Waugh *et al.* 2017; Fabijan *et al.* 2019b). Retroviruses have also been known to undergo latency, and KoRV in SA koalas has been shown in some cases to have proviral DNA present without viraemia (Sarker *et al.* 2020). Latent or subclinical disease states can make interpretation of disease interactions more complex.

This paper aims to identify whether associations occur between KoRV, *C. pecorum,* PhaHV-1 and PhaHV-2 in relation to pathological findings in a cohort of necropsied SA koalas.

4.3 Methods

The 240 koalas for this study were sourced from rescued koalas from the Mount Lofty

Ranges in South Australia. They were deemed unfit for release, either due to disease or

trauma, and euthanased by veterinarians on welfare grounds. Postmortem examinations were

carried out on all koalas and gross changes were noted. Demographic data was collected,

including date of euthanasia, tooth wear class I-VII (Martin 1981), body condition score 1-5

(Blanshard *et al.* 2008) and sex of the koala. Scoring of *Chlamydia*-like disease observed

from postmortem and histopathological examinations, was carried out for ocular lesions (0-

3), urogenital lesions (0-3) and adapted from the clinical examination scoring method of Wan

et al. (2011) (Tables 1a and 1b). The ocular and urogenital scores were combined to give an

overall *Chlamydia*-like postmortem disease score from 0 to 6. Grossly abnormal tissues were

sampled and processed for histopathological examination by routine methods.

Table 1: *Chlamydia*-like postmortem disease scores for ocular and urogenital disease, adapted from clinical assessment scores of Wan *et al.* 2011.

Anatomical location	Score	Description
Ocular	0	No detectable disease
	1	Acute or subacute keratoconjunctivitis, inflammation of conjunctiva and/or cornea, without evident hyperplasia
	2	Chronic keratoconjunctivitis, non-active: no discharge, evidence of conjunctival hyperplasia and/or corneal scarring
	3	Chronic keratoconjunctivitis, active: suppurative discharge, conjunctival hyperplasia and redness.
Urogenital	0	No detectable disease
	1	Acute/subacute cystitis (inflammation of mucosa, no thickening of bladder wall), no significant reproductive tissue changes
	2	Chronic cystitis – non-active, thickening of bladder wall. Females: paraovarian cysts without inflammation/thickening of uterine wall
	3	Chronic cystitis – active, thickening of bladder wall, pyuria/suppurative urine, with or without reproductive changes. Females: inflammation or thickening of uterine wall, presence of pus in uterine lumen, with or without paraovarian cysts. Males: Prostatic inflammation present.

For detection of KoRV, *C. pecorum* and PhaHV, several samples were utilised, these included antemortem blood, postmortem splenic tissue and pooled ocular and urogenital swabs (Adelaide University Ethics Committee approval: S-2016-169). Table 2 shows the numbers of samples available for molecular testing. Antemortem blood or postmortem spleen samples were used for KoRV analysis, ocular and urogenital swabs were used for *C. pecorum* analysis, and spleen samples were used for PhaHV analyses. Samples were stored at -20°C prior to DNA extraction and molecular analyses.

Table 2: Sample availability for each pathogen and combination of pathogens

	KoRV	*C. pecorum*	PhaHV	All
KoRV	212			
C. pecorum	167	188		
PhaHV	166	146	177	
All				137

DNA extraction

DNA was extracted using QIAMP DNAEasy Minikit (Qiagen, Hilgen, Germany) as per manufacturer's instructions. All extracted DNA was measured by NanoDrop One spectrophotometer (Thermo Fisher Scientific Inc, United States) and then stored at -20°C until testing.

Molecular analyses

PCR testing for KoRV provirus was carried out for five proviral targets, two on the *gag* gene (PCR), one on the *pol* gene (qPCR) and two *env* locations (PCR) as previously described (Chapter 2). Koalas which were positive in all KoRV proviral gene targets were deemed KoRV positive. *C. pecorum* qPCR was carried out with previously described primers (Hulse *et al.* 2018) and the following conditions; Triplicate repeats of 5µL reactions were run and

consisted of 2.5µL Power SYBR green Master Mix (Qiagen, Hilgen, Germany), 300nM of each primer (Table 3) and 1.5µL of DNA template (1/5 - 1/50 dilution) qPCR conditions were as follows; initial denaturation 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 60 seconds. Melt curve analysis was carried out with conditions of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. Positive samples were deemed by both the CT value and a melt curve which was consistent with a known *C. pecorum* positive control sample. PhaHV PCR used primers targeting the DPOL gene for each variant as previously described (Kasimov *et al.* 2020). Koalas that had appropriately sized bands on gel examination were deemed positive for the individual PhaHV variant.

Statistics

Descriptive statistics, univariable and multivariable statistical analyses were conducted in R (Team 2019) (Version 3.6.0) using summaryTools (Comtois 2020) (version 0.9.7) and tidyverse (Wickham *et al.* 2019) (version 1.3.0) to examine association between infection status, demographic data, pathological conditions occurring in >5 koalas, *Chlamydia*-like postmortem disease scores (Table 1) and comorbidity scores (full list of variables Appendix S7). KoRV and *C. pecorum* associations were analysed based on infection status, and also using copy number levels. PhaHV infection was analysed by each subtype, and as a group when coinfected with KoRV and *C. pecorum*. Timing of euthanasia was analysed based on the month, season and also by breeding season (September to February). Body condition score (BCS) was analysed by score (1-5) and also grouped into good (BCS 4 and 5), compromised (BCS < 3) and severely compromised (BCS < 2. Tooth wear class was analysed by class (I - VII) and also grouped into young (TWC I - II), adult (TWC III - IV), aged (TWC ≥ IV) and senior (TWC ≥ V). Disease data was analysed by syndrome where there were > 5 cases, but also grouped where disease affected one system, for example,

pneumonia and pyothorax were grouped into respiratory disease, and urethritis and cystitis were grouped into lower urinary tract inflammatory disease (LUTD). Ocular and urogenital *Chlamydia*-like postmortem disease scores were treated individually and combined as and overall score. They were also converted to binary variables (positive and negative koalas) with positive koalas having a score of 1 or more. To achieve a morbidity/comorbidity score, analysis was carried out based on the number of different disease processes occurring in each koala (excluding infection status). Koalas with only one morbidity identified had a score of 1, those with two comorbidities identified had a score of two, and so on. Those with morbidity scores of 0 were deemed disease free.

Stepwise backward logistical regression modelling was performed for each infectious agent individually. Variables with a *F-Test P* value of < 0.25 from univariable association assessments were added to the multivariable model. Confounding effects from other variables were assessed and those that influenced the estimate of variables in the multivariable model by ≥ 20% were retained in the final model. Associations between coinfections and female reproductive disease was also assessed using similar stepwise backward logistical regression models, due to the need to investigate these relationships further.

4.4 Results

Pathology findings

Of 240 koalas, 54.6% (131) were males and 45.4% (109) were females. Of the males; 14.5% (19/131) were young (TWC I or II), 64.9% (85/131) were adult (TWC III or IV), and 20.6% (27/131) were senior (TWC V+); 29.8% (39/131) had a compromised body condition score (BCS ≤ 2) and 27.5% (36/131) had a good body condition score (BCS ≥ 4). Of the females;

26.6% (29/109) were young (TWC I or II), 36.7% (40/109) were adult (TWC III or IV), and 35.8% (39/109) were senior (TWC V+); 33.9% (37/109) had a compromised body condition score (BCS < 3) and 35.8% (39/109) had a good body condition score (BCS 4 or 5). For those koalas that had a date of death or euthanasia recorded, 65.5% (146/223) died or were euthanased within the breeding season (September - February).

Within this cohort of necropsied koalas, 90.0% (216/240) had a disease identified by gross and/or histopathological examination (Appendix S1). The most common pathological finding was lower urogenital tract disease (LUTD – cystitis, vaginitis and urethritis) and was found in 51.7% (124/240) koalas. Cystitis was the most common abnormality, with 105 koalas showing inflammation of the bladder mucosa, thickening of the bladder wall, and inflammation of the ureteral opening. Vaginitis was found in 8.3% (9/109) of female koalas, and 21.4% (28/131) of male koalas had penile or prostatic urethritis. Reproductive disease was present in 25.8% (62/240) koalas and was more common in female koalas (endometritis, pyometra, and/or paraovarian cysts) than male koalas (prostatitis and/or epididymitis), with 47.7% (52/109) females and 7.6% (10/131) males presenting with reproductive pathology. Paraovarian cysts were found in 32.1% (35/109), and pyometra and/or endometritis found in 24.8% (27/109) of females. Of the ten males that were found to have reproductive disease, eight had prostatitis and two had epididymitis. There were no cases of orchitis found in this cohort.

Traumatic injury, 35.0% (84/240), was found in a large number of koalas, with 47.6% (40/84) due to road traffic accidents, and 29.8% (25/84) being dog attack victims, other cases of trauma were either aetiology unknown 11.9% (10/84) or previous trauma that was not the reason for euthanasia 10.7% (9/84). Testicular asymmetry in the absence of other disease was observed in 24.4% (32/131) of male koalas. Renal disease was also common in this cohort

with 32.1% (77/240) of koalas showing renal abnormalities. Of these, 77.9% (60/77) were diagnosed with oxalate nephrosis, either based on yellow crystals seen in the kidneys at postmortem or birefringent crystals or crystal ghosts seen on histopathological examination. Parasites were found in 13.3% (32/240) koalas, of which ten koalas were diagnosed with mange caused by *Sarcoptes scabiei,* and 25 koalas with the tapeworm *Bertiella obesa,* which was present in their small intestine at only low levels (1 - 5 worms). Respiratory disease was diagnosed in 9.2% (22/240) of koalas, with 63.6% (14/22) of these having pulmonary actinomycosis (Stephenson *et al.* 2021). Spinal curvature was found in 7.5% (18/240) koalas, showing scoliosis, kyphosis and/or lordosis. Neoplastic disease was found in 3.8% (9/240) of koalas in this cohort, six had lymphoma, including three previously reported (Fabijan *et al.* 2020). Other neoplastic conditions were individual cases of periocular myxoma or myxosarcoma, pulmonary adenocarcinoma and craniofacial tumour (latter not examined by histopathology). There were 14 cases of joint crepitus (presumed arthritis) found through palpation of carpal, elbow, shoulder, hip, stifle and/or tarsal joints.

Disease scoring consisted of *Chlamydia*-like disease scores and morbidity and comorbidity scores. Within this cohort there was a predominance (64.6%, 155/240) of koalas with a positive urogenital *Chlamydia*-like disease score. Only 19.2% (46/240) of koalas scored 1 or more on ocular *Chlamydia*-like disease score and combined (UGT and ocular) *Chlamydia*-like disease scores of 1 or more were observed in 66.3% (159/240) of koalas in this study. Only 10% (24/240) koalas presented with no morbidity present, or a disease free state, 51.7% (124/240) had one morbidity or two comorbidities present and 38.3% (92/240) had three or more comorbidities identified.

Molecular findings

Molecular testing results are shown in Table 3. Male koalas made up the majority of koalas infected with each pathogen, representing 62.9% of KoRV infected koalas, 51.6% of *C. pecorum* infected koalas, 61.9% of PhaHV-1 infected koalas and 64.1% of PhaHV-2 infected koalas. Figure 1 showing a breakdown of infection status in the 137 koalas that were tested for all infectious agents.

Table 3: Molecular testing results for infection and coinfection of KoRV, *C. pecorum*, PhaHV-1 and PhaHV-2

Molecular analysis	Prevalence	Number tested
KoRV	42.0%	212
C. pecorum	50.5%	188
PhaHV-1	54.8%	177
PhaHV-2	36.2%	177
KoRV & *C. pecorum*	20.4%	167
KoRV & PhaHV-1	26.5%	166
KoRV & PhaHV-2	18.1%	166
C. pecorum & PhaHV-1	32.9%	146
C. pecorum & PhaHV-2	18.5%	146
PhaHV-1 & PhaHV-2	24.9%	177
KoRV, *C. pecorum,* PhaHV-1 & PhaHV-2	16.1%	137
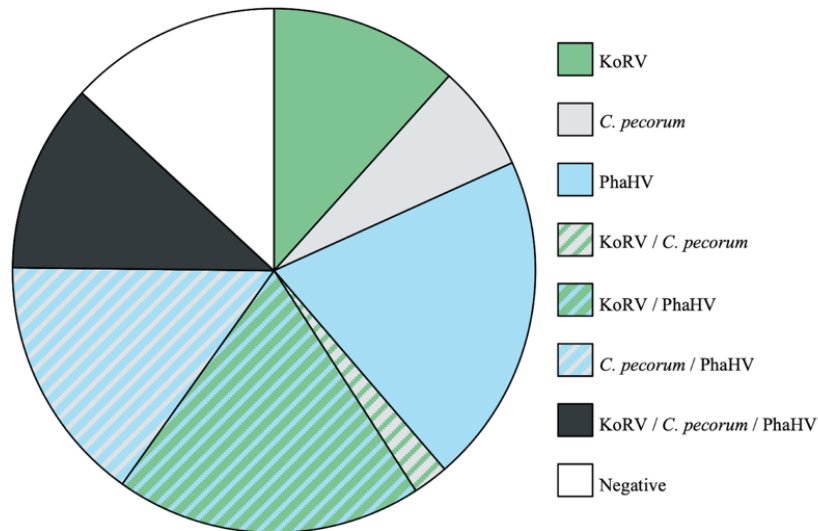
Figure1; Pie chart showing infection proportions of each infectious agent as a single infection or coinfections in koalas tested for all pathogens (n=137).

There were 11.7% (16/137) of koalas that were negative for all three pathogens. Of these 16 koalas, 43.8% (7/16) were males, 56.3% (9/16) were females, 62.5% (10/16) were aged (TWC ≥ IV), 75/0% (12/16) had adequate to excellent body condition (BCS ≥ 3). Over half of these cases (56.3%, 9/16) were euthanased due to trauma.

Pathologies associated with infection status

The percentage of positive cases for each pathological process examined for in this study for KoRV, *C. pecorum*, PhaHV-1 and PhaHV-2 are shown in Figure 2. Notable results include; all (100%) lymphoma cases were infected with KoRV (Fig. 2, A); *C. pecorum* and PhaHV-1 were both highly prevalent in reproductive disease cases (Fig. 2, B and C); and 91% (10/11) cases of joint crepitus (presumed arthritis) were positive for PhaHV-2 (Fig. 2, D). Trauma cases were below the mean prevalence for all infectious agents.
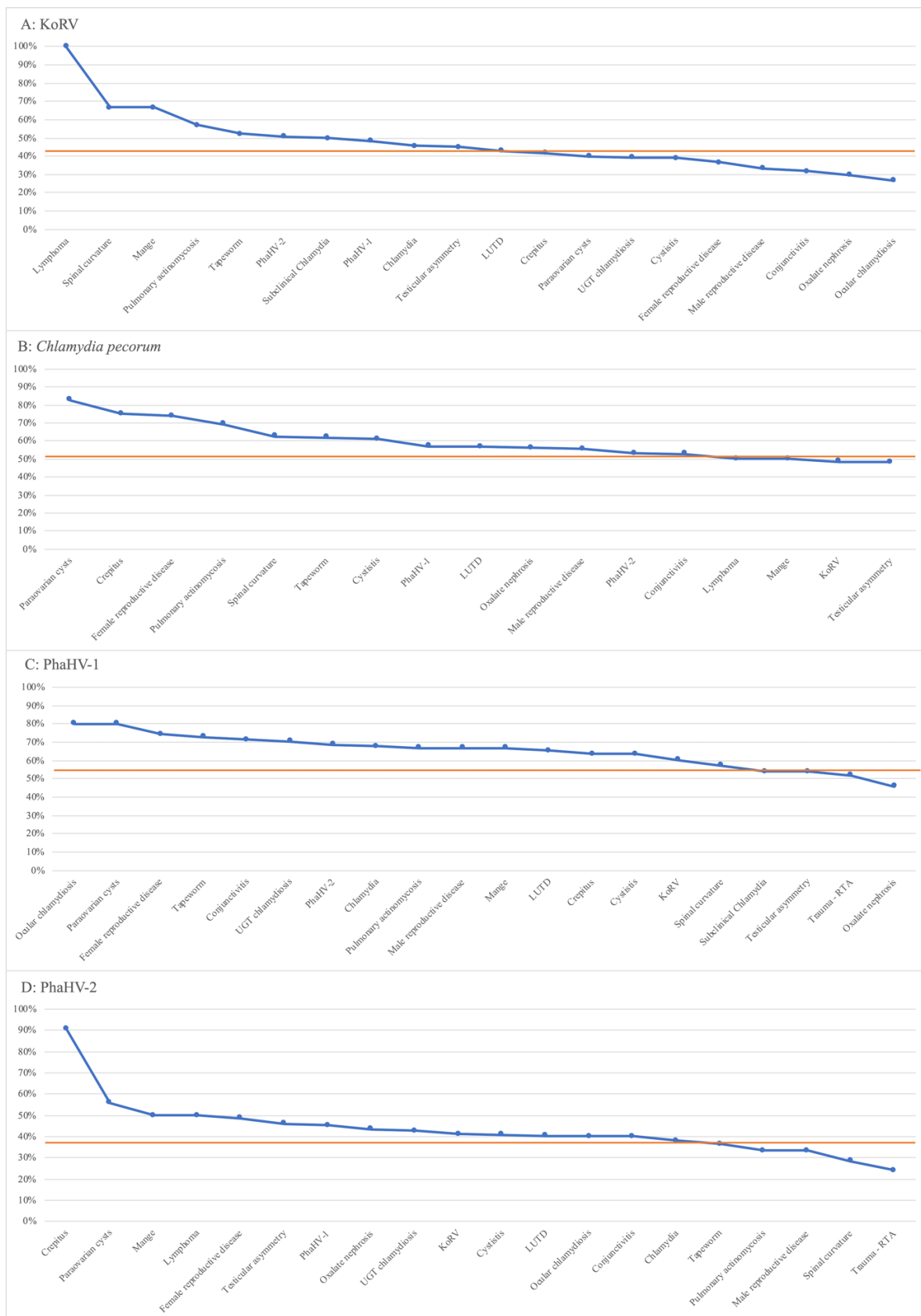
Figure 2; The percentage of positive cases for each pathological finding, or grouped pathologies for A: KoRV, B: *C. pecorum*, C: PhaHV-1 and D: PhaHV-2. Orange lines depict the mean prevalence in the cohort.

Univariate analysis was carried out between infections and coinfections of of KoRV, *C. pecorum* and PhaHV-1 and PhaHV-2. Significant results (*P* value <0.05) are shown for demographics (Figure 3, Appendix S8), infections and pathological findings (Figure 4, Appendix S9) and clinical evaluations (Figure 5, Appendix S10). All infections and coinfections were analysed, red lines show positive associations, blue lines show negative associations, no lines and absent variables mean no significant association was found. From Figure 3 presenting demographic data, there is a cluster of positive associations between infectious agents and aged (TWC ≥ IV), senior (TWC ≥ V) and compromised body condition score (BCS < 3), and negative associations around young (TWC I or II).
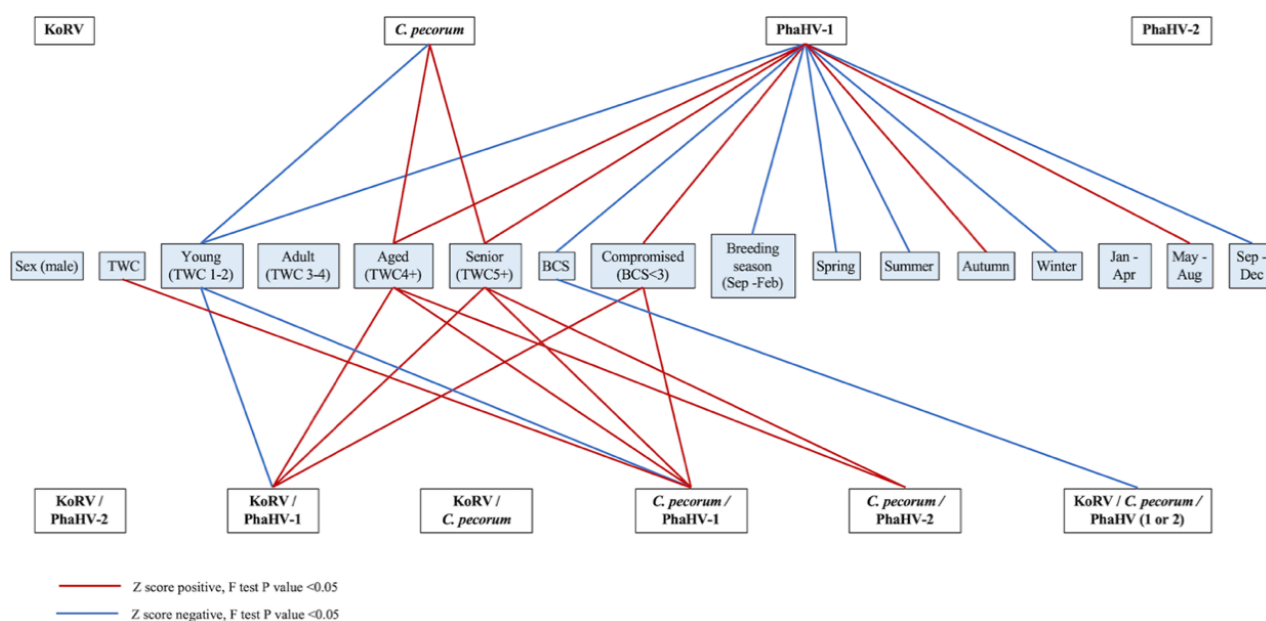
Figure 3: Univariate analyses: significant associations with demographic data (p value < 0.05) and infections and co-infections of KoRV, *C. pecorum* and PhaHV-1 and -2

Pathological findings associated with infection notably show multiple positive associations with reproductive disease, especially female reproductive disease and grouping of negative associations with trauma (Figure 4). KoRV is positively associated with lymphoma in this cohort, with all koalas being KoRV positive. Conjunctivitis was only associated with PhaHV-1 infection in this cohort. Pathological findings associated with coinfection include neoplasia, trauma, reproductive disease, LUTD and arthritis (crepitus). KoRV / PhaHV-2 coinfection was associated with neoplastic conditions, for which 60% (6/10) of cases were lymphoma, all positive for KoRV. Trauma was negatively associated with multiple infections and coinfections
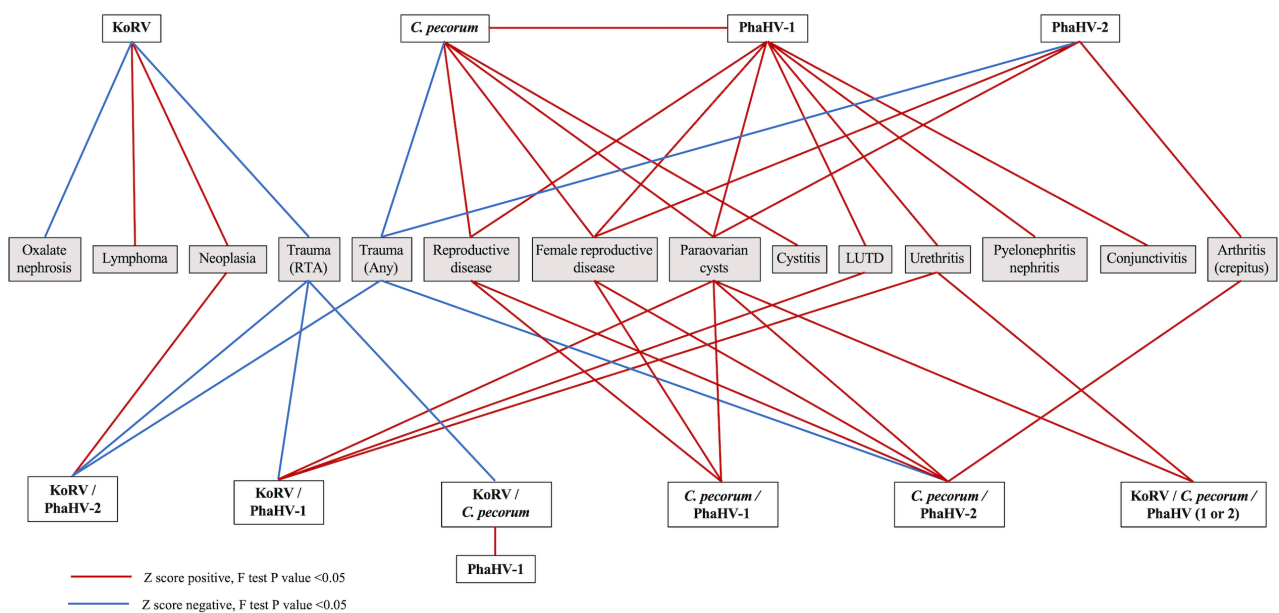
Figure 4:Univariate analyses: significant associations with pathologies (p value < 0.05) and infections and co-infections of KoRV, *C. pecorum* and PhaHV-1 and -2

Disease scores displayed multiple positive associations, particularly with *Chlamydia*-like disease scores (outlined in Table 2). Notably though, no association was found between infection with *C. pecorum* nor *C. pecorum* coinfections and ocular *Chlamydia*-like disease

score. Only *C. pecorum* infection and KoRV / PhaHV-1 co-infection was negatively associated with a disease-free state (comorbidity number = 0). Koalas with three or more comorbidities showed associations with *C. pecorum* infection, PhaHV-1 infection, PhaHV-2 infection, *C. pecorum* and PhaHV-1 co-infection and *C. pecorum* and PhaHV-2 co-infection. KoRV alone was not either positively associated with increasing comorbidity number or negatively associated with koalas that were disease free (Figure 5).
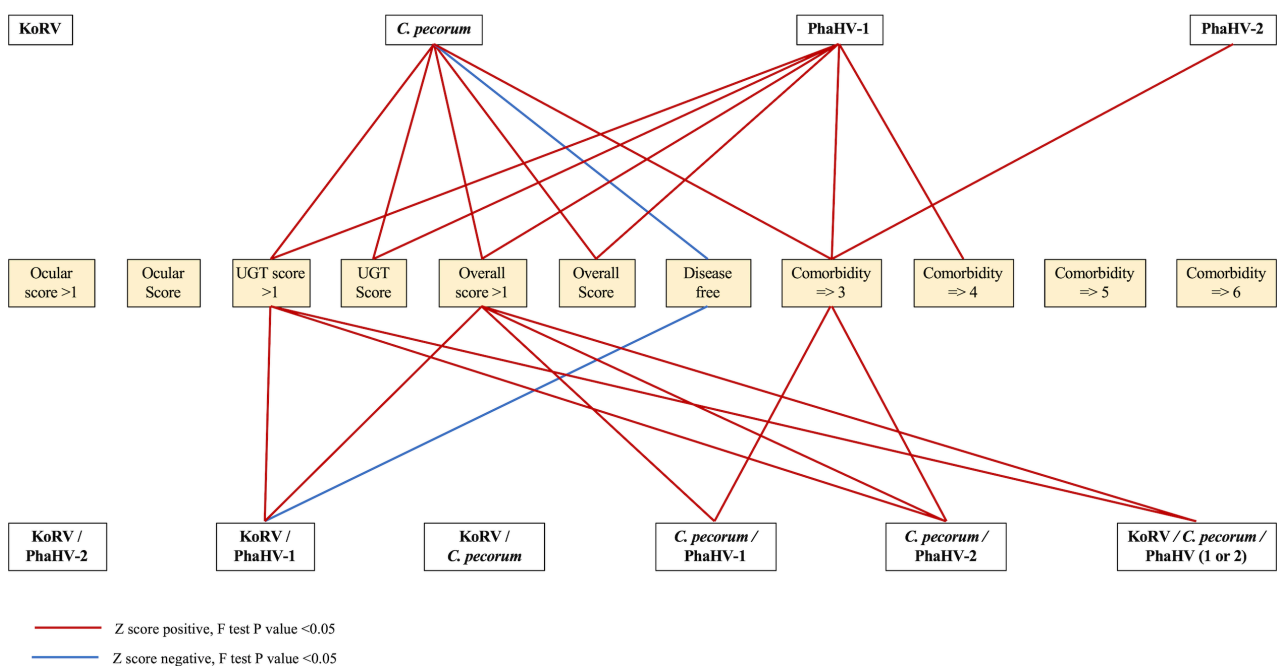
Figure 5: Univariate analyses: Significant associations with clinical evaluation data (p value < 0.05) and infections and co-infections of KoRV, *C. pecorum* and PhaHV-1 and -2

Multivariable modelling of pathological findings, demographical data and clinical evaluation data investigated associations with allowance for confounding influences within the dataset. Covariables were included from the univariate associations with F Test *P* values of < 0.25, and then removed in a backward, stepwise fashion leaving those significantly associated or that significantly influenced the odds of another covariable. Predictors of KoRV infection consisted of one positive predictor; spinal curvature (*Odds Ratio* = 3.54, *CI*: 1.07-11.76, *P* =

0.039), and three negative predictors; oxalate nephrosis (*Odds Ratio* = 0.34, *CI* = 0.17-0.69, *P* = 0.003), koalas that were euthanased due to road traffic accidents (RTA) (*Odds Ratio* = 0.31, *CI* = 0.13-0.72, *P* = 0.006), as well as aged (TWC ≥ IV) koalas (*Odds Ratio* = 0.54, *CI* = 0.30-0.99, *P* = 0.045). Predictors of *C. pecorum* infection consisted of three positive predictors; reproductive disease (male or female) (*Odds Ratio* = 2.39, *CI* = 1.11-5.14, *P* = 0.026), cystitis (*Odds Ratio* = 1.98, *CI* = 1.04-3.75, *P* = 0.037) and senior koalas (TWC ≥ V) (*Odds Ratio* = 2.21, *CI* = 1.06-4.57, *P* = 0.033), and two negative predictors; koalas that were euthanased due to road traffic accidents (*Odds Ratio* = 0.50, *CI* = 0.26-0.97, *P* = 0.041) and young koalas (TWC I or II) (*Odds Ratio* = 0.35, *CI* = 0.12-0.98, *P* = 0.046). Predictors of PhaHV-1 infection consisted of four positive predictors: sex (male) (*Odds Ratio* = 2.48, *CI* = 1.19-5.16, *P* = 0.015), aged (TWC ³ IV) (*Odds Ratio* = 4.19 *CI* = 2.07-8.45, *P* = <0.001), a compromised (BCS < 3) (*Odds Ratio* = 2.26, *CI* = 1.07-4.79, *P* = 0.032) and a positive urogenital *Chlamydia*-like disease score (*Odds Ratio* = 3.18 *CI* = 1.54-6.56, *P* = <0.002). There was one positive predictor of PhaHV-2 infection which was koalas that displayed gross arthritic crepitus at postmortem (*Odds Ratio* = 14.75, *CI* = 1.78-122.15, *P* = 0.013). Trauma and comorbidity score in the PhaHV-2 model confounded the effects from arthritis and were retained in the final model.

Female reproductive disease was further investigated due to the inferences from the univariable analyses, especially possible interactions between *C. pecorum* and PhaHV infections. *C. pecorum* and coinfections of *C. pecorum* / PhaHV-1 and *C. pecorum* / PhaHV-2 were further investigated through multivariate regression. The outcomes are shown in Figure 6, displayed as pink lines, full results Appendix S11. The multivariate analyses calculated predictors of being infected with *C. pecorum* in female koalas were paraovarian cysts, (*Odds Ratio* = 5.71, *CI* = 1.79-18.22, *P* = 0.003) and being senior (*Odds Ratio* = 5.08, *CI* = 1.75-14.72, *P* = 0.003). Predictors of *C. pecorum* / PhaHV-1 coinfection in female

koalas were paraovarian cysts, (*Odds Ratio* = 3.98, *CI* = 1.42-17.31, *P* = 0.044), being senior (*Odds Ratio* = 4.37, *CI* = 1.20 – 15.98, *P* = 0.026), with a confounding factor of a positive overall *chlamydial*-like disease score (*Odds Ratio* = 5.45, *CI* = 0.58-51.37, *P* = 0.138). Predictor of *C. pecorum* / PhaHV-2 coinfection in female koalas was just paraovarian cysts, (*Odds Ratio* = 17.73, *CI* = 3.37-93.14, *P* = 0.001).
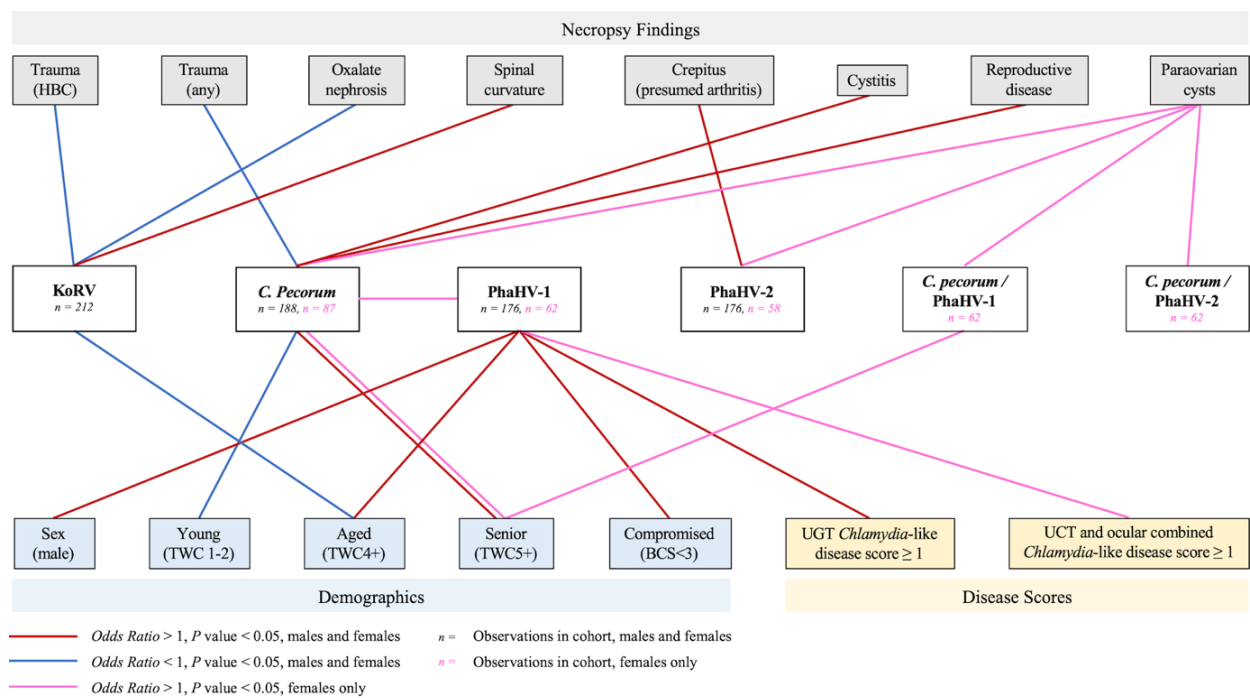
Figure 6: Multivariate backwards stepwise regression model outcomes between infections of KoRV, C. pecorum, PhaHV-1, PhaHV-2 and coinfections of C. pecorum / PhaHV-1 and C. pecorum / PhaHV-2 and necropsy findings, demographic data and disease scoring data. Numbers show total in multivariate analysis for all koalas tested (black) or female koalas tested (pink). Values reported in supplementary Table S5.

Further breakdown investigating the infection status of female koalas with paraovarian cysts can be seen in Figure 7. It demonstrates the majority of cases occur with coinfection by *C. pecorum* / PhaHV. There were no cases of paraovarian cysts with only a *C. pecorum* infection.

Figure 7: Infection status of female koalas based on presence or absence of paraovarian cysts
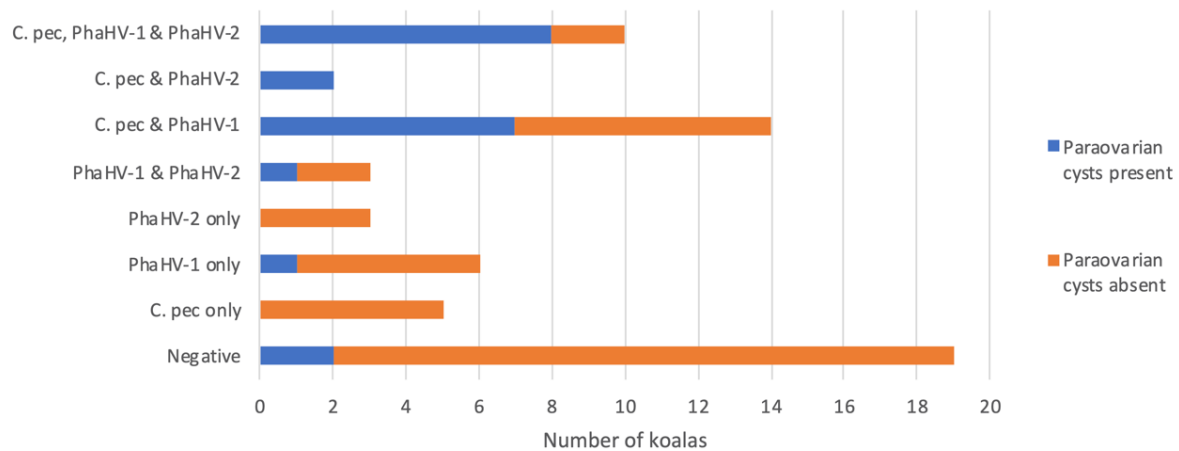
4.5 Discussion

The prevalence of koala retrovirus, *Chlamydia pecorum* and Phascolarctid gammaherpesviruses have previously been studied within the South Australian koala population, however pathological findings associated with coinfections involving all three infectious agents have not previously been reported. KoRV prevalence in this study was lower than previous reports in wild-caught and euthanased cohorts (Fabijan *et al.* 2019b; Fabijan *et al.* 2020; Sarker *et al.* 2020) and there were limited disease associations. *C. pecorum* prevalence was similar to a previous study of euthanased SA koalas (Fabijan *et al.* 2019a) and associations with urogenital tract disease were identified. PhaHV prevalence expanded on recent findings (Kasimov *et al.* 2020) and associations with reproductive disease in females were found for PhaHV-2. Possible influence or augmentation of *C. pecorum* / PhaHV coinfection on the presence of reproductive disease and LUTD was found. Coinfection was found in just under half of the koalas tested for all pathogens (48.2%, 66/137), and coinfection with all three pathogens was found in 16.1% (22/137).

A significant association was found for coinfection with *C. pecorum* and PhaHV-1, for which there was a significant association for female koalas from the multivariate analyses. For the 62 female koalas tested for both *C. pecorum* and PhaHV-1, 50.0% (31/62) were positive for *C. pecorum*, 53.2% (33/62) positive for PhaHV-1 and coinfection was found in 60.0% (24/40) of infected koalas. of genes associated with oxalate nephrosis or spinal curvatures, through increased transcription or disruption of genetic sequence, and could be a factor in their associations with KoRV infection. However, South Australian koalas have gone through a genetic bottleneck, therefore this confounding factor could also be influencing these findings. Trauma was not only negatively associated with KoRV but was also found to be negatively associated with all pathogens except PhaHV-1. This highlights not only that healthier koalas are more likely to suffer traumatic injuries due to increased movement and reduced likelihood of compromise, but also that trauma cases can serve as a control group in postmortem disease studies of koalas.

KoRV was not associated with any other conditions, including lymphoma or neoplastic conditions in this cohort using multivariate modelling, likely due to low prevalence of these conditions in SA koalas, but it should be noted that all koalas that had lymphoma in this cohort were infected with KoRV, supportive of the key role of KoRV in lymphoma development show in other studies (Tarlinton *et al.* 2005; Xu *et al.* 2013; Fabijan *et al.* 2017; Fabijan *et al.* 2020). However, univariate analysis did show association between lymphoma and KoRV infection and also neoplastic conditions and KoRV / PhaHV-2 coinfection. Herpesviruses have been shown to augment retroviral oncogenesis in chickens (Mays *et al.* 2019) and humans (da Silva *et al.* 2011), but this needs further analysis in koalas to draw any conclusions on viral cooperation in oncogenesis. Retroviruses are also known to cause immunosuppression (Levy 2011) and therefore would likely be associated with greater

comorbidity. KoRV has previously been shown to be associated with dysregulation of cytokine expression, therefore possible reduction in immune response and susceptibility to Chlamydial infection in NSW koalas (Maher *et al.* 2016). In South Australian koalas, KoRV was not associated with increased comorbidity or overt chlamydiosis, although *C. pecorum*, PhaHV-1 and PhaHV-2 were more likely to be present in koalas with three or more comorbidities.

Reproductive disease was highlighted within the univariate analyses by strong associations observed with *C. pecorum,* PhaHV-1, PhaHV-2 and their coinfections. There were no observed associations with KoRV, as has been found in northern koalas (Waugh *et al.* 2017; Quigley *et al.* 2018). Further multivariate modelling showed that the main pathogen associated within this cohort and reproductive disease was, unsurprisingly, *C. pecorum*, which has long been known to cause urogenital tract inflammatory diseases (Brown *et al.* 1987; Hemsley *et al.* 1997). However, analyses with just the female koalas revealed a possible role for PhaHV-2 or coinfection of *C. pecorum* / PhaHV. This emphasises the need to investigate potential roles of other pathogens in *Chlamydia*-associated diseases and points out the fact that these clinical signs are potentially not pathognomonic for *C. pecorum,* as supported by original descriptions of koalas with reproductive disease (Brown *et al.* 1987). There has been previous acknowledgement of disease presentation the is associated with *Chlamydia* without *Chlamydia* positive PCR results (Wan *et al.* 2011; Nyari *et al.* 2017; Legione *et al.* 2018; Quigley *et al.* 2018), although whether this is due to involvement of other pathogens or whether the chlamydial infection is cleared prior to testing remains unclear. In this study 21/62 koalas had paraovarian cysts and were tested for both *C. pecorum* and PhaHV. None of these koalas were infected with only *C.* pecorum and 17/21 were infected with *C. pecorum* and one or both of PhaHV1 or 2. In other species, herpesviruses is

known to cause reproductive lesions, such as bovine herpesvirus 1 (BoHV-1) (Graham 2013), bovine herpesvirus 4 (BoHV-4) (Donofrio *et al.* 2008), equine herpesvirus 1 (EHV-1) (Holz *et al.* 2019) and equine herpesvirus 3 or equine coital exanthema (EHV-3). BoHV-1 has shown to cause infectious pustular vulvo-vaginitis, necrotising endometritis and cystic corpora lutea (Graham 2013). BoHV-4 uterine disease has shown to be augmented by bacterial infection, which induces upregulation of genes necessary for BoHV-4 replication (Donofrio *et al.* 2008), therefore maybe *C. pecorum* and PhaHV are interacting in order to cause disease in koalas. Additionally, herpesvirus recrudescence upon stress or immunocompromise might relate to increased ability of detection with reproductive disease presence. Also, the recently discovered expression of NTDPase by PhaHV (Vaz *et al.* 2018), may reduce the inflammatory response (Robson *et al.* 2006; Vaz *et al.* 2018) and allow for immune evasion of PhaHV. PhaHV samples analysed were from spleen and not directly from the tissues affected, therefore, analysis of reproductive tissues for herpesvirus DNA could add support to the role of PhaHV in reproductive disease.

Conjunctivitis is also currently presumed to be caused by *C. pecorum* in koalas, however only 50% (18/36) of cases were *C. pecorum* positive in this cohort. Within the univariate analysis PhaHV-1 was associated with overt ocular disease, for which 71% (25/35) tested positive, but *C. pecorum* was not associated. This may be due to the low number of conjunctivitis cases within this cohort, but it does warrant further investigation of lesions in order to clarify the role of PhaHV in ocular disease. Possible confounding limitations were that PhaHV infection status was determined by spleen tissue in this cohort, not from direct sampling of ocular lesions, and *C. pecorum* was pooled ocular and urogenital swabs testing. Other species have herpesvirus-related ocular disease, such as cats (Maes 2012), dogs (Ledbetter 2013) and crocodiles (Shilton *et al.* 2016), therefore there is potential for PhaHV

to cause ocular lesions. Interestingly, hatchling crocodiles with conjunctival or pharyngeal disease had high odds of detecting both herpesvirus and *Chlamydiaceae* from their lesions, showing possible augmentation of disease (Shilton *et al.* 2016).

Crepitus, which was assumed to be due to arthritic changes within the joint, was diagnosed by joint manipulation on postmortem examination and was associated with PhaHV-2 infection. This model is influenced by the comorbidity scores and negatively influenced by koalas euthanased due to trauma. The koalas in the arthritic group they are an older subset of koalas, with a median TWC of IV (range III - VI), therefore age is likely to be a potential factor in the pathogenesis of arthritis. However, herpesviruses have been implicated in viral arthritis, both experimentally (Webb *et al.* 1973) and found in synovial fluid of arthritic joints in humans (Burgos *et al.* 2015).

Influence of sex, on likelihood of infection or coinfection showed that male koalas were more likely to be positive for PhaHV-1. This association has been shown previously in VIC koalas (Stalder *et al.* 2015) and may be due to the greater movement of male koalas through the population (Menkhorst *et al.* 2019). Age (TWC) was associated with both chlamydial and PhaHV-1 infections and *C. pecorum* / PhaHV-1 coinfection (females), with infection more likely to occur in older koalas. *C. pecorum* was also less likely to occur in young koalas. These findings are consistent with the lifelong nature of infection with *Chlamydia* or PhaHV-1 and increased transmission during sexually mature years. Previous studies have also found this association with age for PhaHV-1 (Stalder *et al.* 2015; Vaz *et al.* 2019; Kasimov *et al.* 2020), however epidemiological studies of *C. pecorum* infection in QLD koalas, showed high transmission to young animals and no association with age (Nyari *et al.* 2017). This may be due to the severity of disease in QLD koalas and the increased potential of transmission to

joeys. KoRV was less likely to occur in aged koalas (TWC ≥ IV) and PhaHV-2 did not have any association with age, suggesting either the possibility of infection at a young age (mother to joey), the pathogen causing mortality, or the ability to overcome infection with time. Koalas with a compromised condition (BCS < 3) were associated with PhaHV-1 infection, but no other combination of pathogens. PhaHV infection has been reported to be more likely in emaciated koalas (Vaz *et al.* 2019). The association with BCS is understandable due to herpesviruses replicating more avidly when immunocompromise or stress occurs (Ackermann 2006), therefore increasing the likelihood of detecting infection/shedding. Notably, PhaHV-2 was not associated with a compromised BCS and may represent different recrudescence triggers.

For those koalas tested for all pathogens, only 11.7% (16/137) were negative for all. Whilst this is only a small number of koalas, when extrapolated to a population of 100,000 or more koalas, this SA population could have significant numbers of koalas without KoRV, *C. pecorum* and PhaHV infections. There is potential for this study to underestimate this number, due to the fact that this study cohort is a euthanased cohort. These koalas were mostly female, older koalas which had adequate to excellent body condition, with their main cause for presentation being euthanased due to trauma. This, again, highlights the need for ongoing efforts to protect this cohort of koalas.

4.6 Conclusion

In conclusion, this study found that in SA koalas, the threat of disease predominantly came from *C. pecorum* infection and reproductive disease, with disease augmentation possibly

occurring with PhaHV. This study highlights the need for further investigation into disease associations and possibly causation of PhaHV, especially for its role in reproductive disease pathogenesis. The use of the *Chlamydia* vaccine (Nyari *et al.* 2019) and potentially the development of a PhaHV vaccine or therapeutic (Vaz *et al.* 2018) in this population would also reduce the impact of major disease threats. The negative association of key pathogens with trauma cases holds a key message for conservation, in that significant numbers of koalas are euthanased as a result of direct human interaction and are amongst the healthiest, therefore strategies to reduce motor vehicle accidents, dog attacks and other trauma events are likely to increase the number of healthy koalas in the population.

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Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations

The conception of this paper was derived from the necessity for understanding whether Phascolarctid herpesviruses were present and at what level in the Mount Lofty Ranges koala population. This work was part of an Honours project conducted by Vasilli Kasimov and was set up to support this PhD and with myself as co-supervisor.

The samples collected were from the postmortem examinations I carried out and from wild caught koalas for which I was paramount in the organisation, anaesthesia and sample collection from. Although I am not the primary author on this publication, my role from its conception to final acceptance was considerable. Vasilli carried out the molecular work and analysis under the guidance of several colleagues, including myself.

This initial work allowed for expansion of my coinfection study in Chapter 4.

Statement of Authorship

Title of Paper	Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations
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Principal Author

Name of Principal Author	Vasilli Kasimov
Contribution to the Paper	Methodology, formal analysis, investigation, writing; original draft preparation and writing; review and editing.
Overall percentage (%)	50%
Signature	Date 5/3/2021

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author (Candidate)	Dr Tamsyn Stephenson
Contribution to the Paper	Conceptualization, methodology, investigation, writing; review and editing and funding acquisition.
Overall percentage (%)	40%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Signature	Date 14·4·21

Please cut and paste additional co-author panels here as required.

viruses

MDPI

Identification and Prevalence of Phascolarctid Gammaherpesvirus Types 1 and 2 in South Australian Koala Populations

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Abstract: To determine Phascolarctid gammaherpesviruses (PhaHV) infection in South Australian koala populations, 80 oropharyngeal swabs from wild-caught and 87 oropharyngeal spleen samples and swabs from euthanased koalas were tested using two specific PCR assays developed to detect PhaHV-1 and PhaHV-2. In wild-caught koalas, active shedding of PhaHV was determined by positive oropharyngeal samples in 72.5% (58/80) of animals, of which 44.8% (26/58) had PhaHV-1, 20.7% (12/58) PhaHV-2 and 34.5% (20/58) both viral subtypes. In the euthanased koalas, systemic infection was determined by positive PCR in spleen samples and found in 72.4% (63/87) of koalas. Active shedding was determined by positive oropharyngeal results and found in 54.0% (47/87) of koalas. Koalas infected and actively shedding PhaHV-1 alone, PhaHV-2 alone or shedding both viral subtypes were 48.9% (23/47), 14.9% (7/47) and 36.2% (17/47), respectively. Only 45.9% (40/87) were not actively shedding, of which 40.0% (16/40) of these had systemic infections. Both wild-caught and euthanased koalas actively shedding PhaHV-2 were significantly more likely to be actively shedding both viral subtypes. Active shedding of PhaHV-2 had a significant negative correlation with BCS in the euthanased cohort, and active shedding of PhaHV-1 had a significant positive relationship with age in both wild-caught and euthanased cohorts.

Keywords: Phascolarctid gammaherpesvirus; koala; South Australia; PhaHV-1; PhaHV-2

1. Introduction

The *herpesviridae* family contains widely prevalent double-stranded DNA viruses, classified into three subfamilies: *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae*. They have been found to infect many species across the animal kingdom, including all mammalian and avian species investigated. The persistent and often lifelong infection of herpesviruses has allowed them to co-evolve with their animal hosts, which may have led to an adaptability advantage over other infectious diseases and contributed to the survival strategy of the virus [1].

Several herpesviruses have been characterised in Australian marsupials which include Macropodid herpesvirus-1 (MaHV-1, alphaherpesvirus) detected from oral and genital mucous membrane lesions in Parma wallabies (*Notamacropus parma*) during a mortality event in 1975 [2] Macropodid herpesvirus-2 (MaHV-2, *alphaherpesvirinae*) isolated from quokka kidney cells (*Setonix brachyurus*) [3], Macropodid herpesvirus-3 (MaHV-3, gammaherpesvirus) and Macropodid herpesvirus-4 (MaHV-4, *alphaherpesvirinae*) from a variety of tissues including whole blood, mammary

covered gammaherpesviruses, Phascolarctid gammaherpesviruses-1 [4] (PhaHV-1) and PhaHV-2, detected in the liver, spleen and nasal scrapings from various koalas (*Phascolarctos cinereus*) [5–7]. Recently, Stalder et al. [8] conducted a surveillance study on a range of Australian marsupials (*n* = 278) and detected six additional novel herpesviruses (one alphaherpesvirus and five gammaherpesviruses); three in common wombats (*Vombatus ursinius*) (VoHV1–3), one in swamp wallabies (*Wallabia bicolor*) (MaHV-5), one in Tasmanian devils (*Sarcophilus harrisii*) (DaHV-2) and one in Southern brown bandicoots (*Isoodon obesulus*) (PeHV-1).

Gammaherpesviruses such as Epstein Barr virus (EBV) and Kaposi sarcoma-associated herpesvirus (KHV) in humans, ovine herpesvirus-2 (OVH-2) infections in sheep and cattle, and the recently discovered novel gammaherpesviruses found in koalas (PhaHV-1 and PhaHV-2) are lymphotropic by nature, initially infecting epithelial cells and then establishing a latent infection within B-cells and T-cells which are densely populated within the host spleen and lymph nodes [9]. These viruses typically lay dormant within host lymphocytes and prevent the cell from dying via the translation of effector proteins which interfere with natural cell pathways, enabling evasion of the host's immune system. Eventually, due to external or environmental stressors, compromised immunity or other factors, a recrudescence of infection may occur causing the virus to suddenly replicate rapidly. This causes the cell to lyse, permitting new virions to be actively shed and be transmissible through respiratory and sexual transmission pathways [10–12].

Currently, PhaHV infection and prevalence has only been described in Victorian koala populations. PhaHV-1 and 2 were detected in 10.1% (10/99) and 23.2% (23/99) of surveyed koalas, respectively, with only one koala (1/99) being co-infected with both viral subtypes. Vaz et al. (2019) [7] also conducted a survey on 810 koalas from various populations across Victoria, with PhaHV prevalence ranging from 1 to 55%. PhaHV DNA has been detected from conjunctival, nasal, oropharyngeal, cloacal and prepuce swabs [8].

The clinical significance of PhaHV is still under scrutiny, with correlations reported between PhaHV infection and "wet bottom" in koalas, a clinical manifestation of *Chlamydia pecorum* infection [7,8]. No other direct correlations between disease and PhaHV infection have been reported in koalas. Vaz et al. [5] describe severe lymphoid depletion in both lymph nodes and spleen of infected koalas. These koalas also had other comorbidities, such as chronic dermatitis caused by *Sarcoptes scabiei*, chronic interstitial nephritis and cystitis, bilateral conjunctivitis, pulmonary congestion, enlarged nodular spleens and airway haemorrhages [5]. Splenic lymphoid area has been reported to be positively associated with koala retrovirus (KoRV) viral loads, and disease-free koalas have been shown to have small numbers or absence of periarteriolar lymphoid sheaths or splenic lymphoid follicles [13]. Cystitis and conjunctivitis are common findings in clinical chlamydiosis [14]. Conditions observed in PhaHV-positive koalas are similar to those observed during the Macropodid herpesvirus 1 (MaHV-1) outbreak in 1975 [2], in which infected wallabies displayed signs of conjunctivitis, pneumonia, splenic and hepatic necrosis [2,3].

Given the relatively high prevalence of PhaHV in Victoria, we hypothesised that both viral subtypes occur and are actively being shed within the South Australian koala population. Moreover, we expected that a significant relationship exists between age and PhaHV infection, due to the lifelong nature of infection and the increased likelihood of infection with time. We also expected there to be a relationship between poor body condition of koalas and shedding of the virus, since stress and being immunocompromised increases the likelihood of recrudescence in other species, resulting in active shedding, including from the oropharynx [15–17]. Most gammaherpesviruses shed virions from epithelial cells, therefore DNA detected from swabs of these tissues is most likely to confirm active shedding [18]. The latent stage of infections occurs within lymphocytes, which are also densely populated within the spleen [19,20], therefore infection status could be determined through DNA extracted from spleen samples.

The primary objectives of this study were to determine if either of the PhaHV viral subtypes were prevalent in South Australian koala populations, the percentage of koalas actively shedding the virus

and whether active shedding of either viral subtype has significant correlations with factors such as age, body condition score or sex.

2. Materials and Methods

2.1. Animal Ethics

This study was approved by the University of Adelaide Animal Ethics Committee and conducted in accordance with the guideline set out in the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition (2013) (National Health and Medical Research Council: Canberra, 2013). Animal ethics approval number for wild-caught koalas: S-2018-022 (granted 6 April 2018) and for the euthanased examination cohort: S-2016-169 (granted 9 January 2017); DEW scientific permit number: Y26054-7 (granted 7 September 2017).

2.2. Sample Collection

Two cohorts of koalas were used for this study: wild-caught and euthanased koalas, both from the Mount Lofty Ranges koala population in South Australia. The wild-caught cohort was considered to represent a random sample to investigate active shedding of virus, and the euthanased cohort, euthanased on welfare grounds, enabled investigations of systemic PhaHV infection status.

2.3. Wild-Caught Koalas

As part of a larger koala health surveillance project, wild-caught koalas were sourced from three national parks or reserves in the Mount Lofty Ranges, in Morialta, Cleland and Belair. They were caught by the flag technique, which utilized a large pole with a flag attached to the far end. The flag end of the pole was used to direct the koala down the tree. Once the koala was close to the ground, it was restrained and taken to a field hospital where it was anaesthetised and samples collected, including oropharyngeal swabs. Oropharyngeal swabs were used for PhaHV testing due to their increased rate of positivity [8] and suitability in the field. Further demographic data were recorded and included tag identification, sex, tooth wear class (TWC I-VII) [21] and body condition score (BCS 1–5) [22]. Global positioning system (GPS) data and tagged trees were recorded so koalas could be released at their point of capture. Oropharyngeal swabs were placed in sealed plastic bags and kept on ice before storage at −80 °C within 12 h of sampling. Samples were kept in the −80 °C freezer for up to 12 months until tested.

2.4. Euthanased Cohort

For koalas that had been euthanased on welfare grounds, sex, TWC [21] and BCS [22] were recorded. Oropharyngeal swabs and spleen samples were collected and stored at −20 °C until tested. Spleen samples were collected to determine systemic infection status due to gammaherpesvirus latency in immunological cells [11,12,18,23,24].

2.5. DNA Extraction

DNA was extracted from both oropharyngeal swabs and spleen tissue samples using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The concentration of the extracted DNA was measured using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific Inc, Waltham, MA, USA). A working solution of 20 ng/µL of DNA from the extracted stock solutions was prepared for PCR tests.

2.6. Quality Control

The koala beta (β)-actin gene was screened via qPCR, as a quality control, from extracted oropharyngeal and spleen DNA samples to confirm adequate DNA was extracted, adopting the same protocol described by Shojima et al. [25]. Any samples negative for β-actin were removed from the study due to a lack of quality for further testing. DNA samples were run in triplicate in a 5 µL reaction.

The DNA copy number was derived from a standard curve from the purified PCR product from a South Australian koala. Negative control contained no DNA template.

2.7. Molecular Diagnostics (Conventional PCR)

Specific primers were designed for PhaHV-1 and PhaHV-2 based on the published DPOL gene (Table 1), due to both viral subtypes only having a 60% nucleotide pairwise identity [6]. Primers were confirmed to be specific via NCBI Primer Blast, Sanger sequencing and by testing the primer sets on each of the PhaHV subtypes (Table 2). PCR reactions were run in 20 µL of volume which included 0.5 µM of forward and reverse primer, 5 µL of 4× AllTaq Master Mix solution (QIAGEN, Hilden, Germany), 5 µL of 20 ng/µL DNA template and 8.5 µL of ultrapure water. PCR conditions were initial activation and denaturation of 95 °C for 2 min, followed by 34 cycles of denaturation at 95 °C for 5 s, annealing at 61 °C (PhaHV-1) or 64 °C (PhaHV-2) for 15 s and extension at 72 °C for 10 s. This was followed by a final extension step of 72 °C for 10 s.

Table 1. PCR primers, products and annealing temperatures.

Target	Primer Name	Primer Sequence	Product Bp	Annealing Temp	Reference
β-actin (QC)	β-actin-Fwd	5′ GAGACCTTCAACACCCCAGC 3′	111	60 °C	Shojima et al. (2013) [25]
	β-actin-Rev	5′ GTGGGTCACACCATCACCAG 3′			
PhaHV-1	VK-PhaHV-1-Fwd	5′ CGGCATCCTCCCCTGTTTAA 3′	220	61 °C	Current study
	VK-PhaHV-1-Rev	5′ GCCCCTACATTCAACGAACA 3′			
PhaHV-2	VK-PhaHV-2 Fwd	5′ CGCACTCTAAGCTGTCCCTT 3′	330	64 °C	Current study
	VK-PhaHV-2 Rev	5′ TTTCGAGCATCATGCGTCCT 3′			

Table 2. Results from AGRF Sanger sequencing, showing primer sets used, sample number, type of sample (oropharyngeal or spleen), query and identity to published GenBank sequences (accession numbers: JN585829.1, JQ996387.1).

Primers	Sample	PhaHV-1 (JN585829.1)	PhaHV-2 (JQ996387.1)	Source	Query Cover	Per Ident
VK-PhaHV-1	K18-051	+		Oro	100%	100%
	K18-051	+		Spleen	100%	100%
	K18-064	+		Oro	100%	100%
	K18-064	+		Spleen	100%	100%
VK-PhaHV-2	K18-043		+	Oro	100%	100%
	K18-043		+	Spleen	100%	100%
	K18-044		+	Oro	100%	100%
	K18-044		+	Spleen	100%	100%

2.8. Statistical Analyses

Binary Logistic Regression analyses (performed using IBM SPSS Statistics 23) were used to determine any significant relationships between infection of either PhaHV subtype, coinfections of PhaHV, BCS, TWC and sex. Compromised BCS was considered as BCS 1 to 3 out of 5 (1–3/5) (emaciated, poor, or fair muscle condition), and good BCS as 4–5/5. Variables with *p*-values of ≤0.05 were considered statistically significant.

A Pearson correlation coefficient (r) analysis was conducted on both wild-caught and euthanased cohorts (*n* = 80 and *n* = 87, respectively) to show correlations between covariates. Covariates analysed within each cohort were as follows; within the euthanased cohort: infected (PhaHV DNA detected in the spleen); Oro_Phas1, Oro_Phas2 and Oro_coinfection (PhaHV-1 DNA only, PhaHV-2 DNA only, or both PhaHV subtypes DNA detected from oropharyngeal swab, respectively); Spleen_Phas1, Spleen_Phas2 and Spleen_coinfection (PhaHV-1 DNA only, PhaHV-2 DNA only, or

both PhaHV subtypes DNA detected from spleen tissue, respectively); TWC (tooth wear class) [21]; BCS (body condition score) [22], BCS_compromised (BCS 1–3/5) and sex. Covariates within the wild-caught koalas: infected (PhaHV-1 or PhaHV-2 DNA detected from oropharyngeal swab), Oro_Phas1, Oro_Phas2 and Oro_coinfection, TWC; BCS; BCS_compromised and sex. The results were visualized on a correlation heatmap using R version 3.0.1 using ggplot2 and ggcorrplot packages.

3. Results

3.1. PhaHV-1 and PhaHV-2 Specific PCR Test

The newly designed primer sets designated, VK-PhaHV-1 and VK-PhaHV-2, were both specific in detecting PhaHV-1 and PhaHV-2, respectively. Samples were confirmed in Sanger Sequencing (Australian Genome Research Facilities), both PhaHV-1 and PhaHV-2 showing 100% identity to GenBank sequence identifications (Table 2) (accession numbers: JN585829.1, JQ996387.1).

3.2. Wild-Caught Cohort of Koalas

Approximately three-quarters, 72.5% (58/80), of the wild-caught koalas from the Mount Lofty Ranges were actively shedding PhaHV. These were shown to be actively shedding just PhaHV-1 (44.8% (26/58)), just PhaHV-2 (20.7% (12/58)) or both viral subtypes (34.5% (20/58)). Only 27.5% (22/80) of the wild sampled koalas were not actively shedding either viral subtype (Table 3).

Table 3. Prevalence of active shedding of Phascolarctid gammaherpesvirus (PhaHV) viral subtypes in the two cohorts, wild-caught and euthanased.

Type of Infection	Wild-Caught		Euthanased	
	n	*%*	*n*	*%*
Active Shedding	58	73%	47	54%
Active Shedding Only PhaHV-1	26	33%	23	26%
Active Shedding Only PhaHV-2	12	15%	7	8%
Coinfected Shedding	20	25%	17	20%
No active shedding	22	28%	40	46%
TOTAL	80		87	

Infection of PhaHV-1 only had a significant positive correlation with TWC (*p*-value = 0.023; r = 0.2; *n* = 80) (Figure 1A). The proportion of koalas in each TWC for each status of infection is shown in Figure 2. There were no significant associations between splenic infection with PhaHV and koalas with a compromised BCS (*p*-value = 0.203; *n* = 80) or with sex (*p*-value = 0.776; *n* = 80).
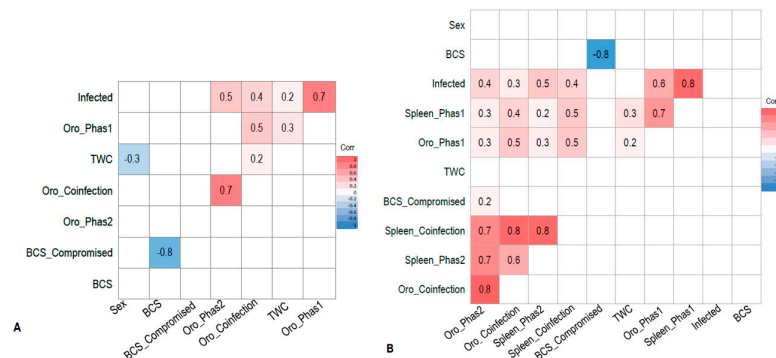
Figure 1. (**A**) Pearson's correlation matrix displaying interactions between covariates in wild-caught koalas from the Mount Lofty Ranges, South Australia (*n* = 80) portrayed on a heat map. Values closer to

1.0 indicate a stronger positive correlation between the two variables. Coloured tiles contain a *p*-value ≤ 0.05. White tiles correspond to an insignificant correlation (*p*-value > 0.05) and are excluded from the model. Variable "BCS_compromised" uses compromised koalas (BCS 1–3/5) as the reference value. (**B**) Pearson's correlation matrix displaying interactions between covariates from euthanased koalas sourced from various wildlife hospitals in Adelaide (*n* = 87), portrayed on a heat map. Values closer to 1.0 indicate a stronger positive correlation between the two variables. Coloured tiles contain a *p*-value ≤0.05. White tiles correspond to an insignificant correlation (*p*-value > 0.05) and are excluded from the model. Variable "BCS_compromised" uses compromised koalas (BCS 1–3/5) as the reference value. Acronym definitions are provided in Section 2.8 (statistical analysis).
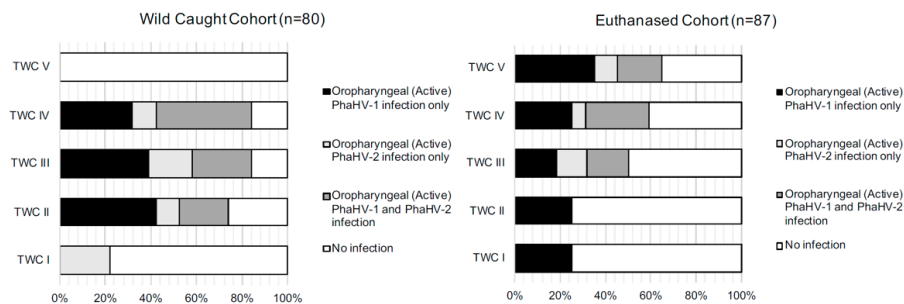
Figure 2. Histograms displaying the percentage of koalas actively shedding PhaHV-1 and PhaHV-2 and noninfected koalas within each tooth wear classification (TWC) group, for wild-caught and euthanased cohorts from South Australia.

3.3. Euthanased Cohort of Koalas

It was found that 72.4% (63/87) of euthanased koalas were infected with PhaHV. Active shedding of the virus occurred in 54.0% (47/87) of koalas in the euthanased cohort, with active shedding of only PhaHV-1 in 48.9% (23/47), only PhaHV-2 in 14.9% (7/47), or both subtypes simultaneously in 36.2% (17/47) of koalas. All koalas had matched positive spleen samples which confirmed infection (Table 3). Furthermore, 18.4% (16/87) of koalas were systemically infected with the virus but not actively shedding virus. The remaining 27.6% (24/87) of koalas were not infected at either the oropharyngeal or splenic site.

There was a strong correlation between splenic coinfection with both PhaHV-1 and PhaHV-2, as being co-infected significantly increased the likelihood of actively shedding (positive oropharyngeal sample) both viral subtypes (*p*-value < 0.01; r = 0.80; *n* = 87) (Figure 1B). A significant positive correlation exists between active PhaHV-1 and PhaHV-2 infection, as koalas were 3.5 times more likely to be actively shedding PhaHV-1 if infected with PhaHV-2 (*p*-value = 0.006; r = 0.3; Exp (B) = 3.538) (Figure 1B). There was a high probability of koalas actively shedding PhaHV-1 (Exp (B) = 28.51) and PhaHV-2 (Exp (B) = 92.49) when co-infected with both viral subtypes in the spleen (Figure 3). Similarly, to the wild-caught koalas, the euthanased cohort had a significant positive correlation between TWC and PhaHV-1 infection (Figure 1A,B) (*p*-value = 0.05; r = 0.2; *n* = 87). However, contrary to wild-caught koalas, a significant positive correlation exists between active shedding of PhaHV-2 and a compromised BCS (*p*-value = 0.04; r = 0.2; *n* = 97) (Figure 1B), with euthanased koalas actively shedding PhaHV-2 being 3.5 times more likely to have a compromised BCS (Exp (B) = 3.514). No significant association between compromised BCS and active shedding of PhaHV-1 or sex was found (*p*-value = 0.2 and 0.16, respectively).
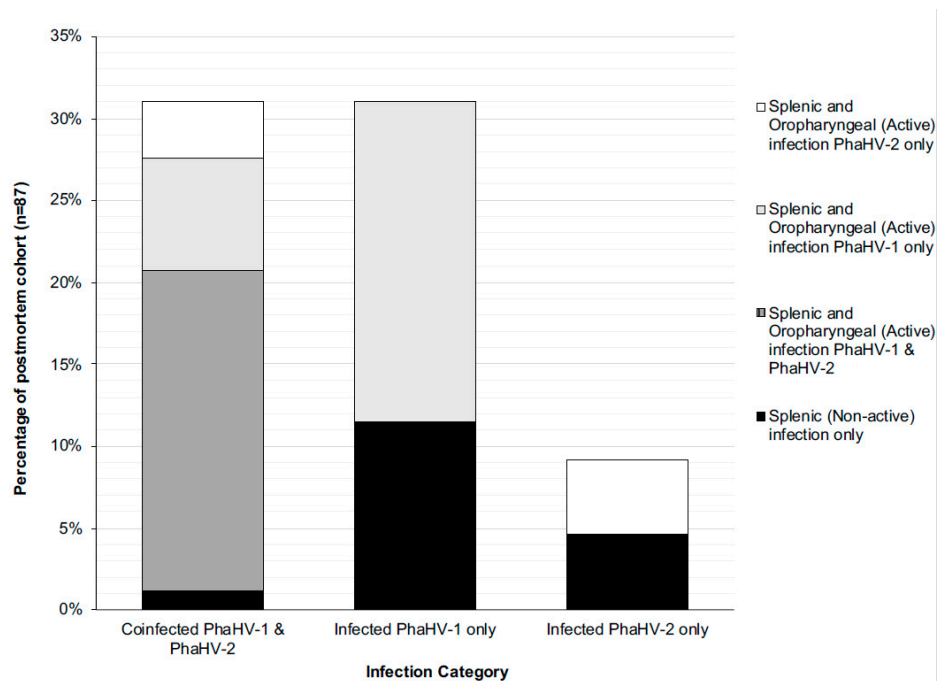
Figure 3. Histogram of infection status within the euthanased cohort (*n* = 87), displaying the following categories: coinfection with both viral subtypes (demonstrated by a dual splenic infection, and actively shedding both or singular viral subtypes, or not shedding); infected with just PhaHV-1 (demonstrated by a PhaHV-1 splenic infection and shedding or not shedding PhaHV-1); infected with just PhaHV-2 (demonstrated by a PhaHV-2 splenic infection and shedding or not shedding PhaHV-2).

4. Discussion

In this study, oropharyngeal and spleen samples were tested, as previous papers [5,6] succeeded in detecting and isolating the same viral subtypes from these sites. In the wild-caught cohort, only oropharyngeal swabs could be collected, whereas in the euthanased cohort, both oropharyngeal and spleen could be collected. As previously mentioned, it has been assumed that the PCR-positive oropharyngeal swabs are most likely to confirm active shedding [18], whilst latent infections occur within lymphocytes which are densely populated within the spleen [19,20]. In the euthanased cohort, koalas that were positive for PhaHV in both spleen tissue and oropharyngeal swab samples were deemed as infected and actively shedding the virus. Koalas only positive in the spleen were classified as being infected.

4.1. Active Shedding

In this study, the prevalence of PhaHV active shedding in wild-caught koalas was 72.5% (58/80) and was higher than that of the euthanased koalas at 54% (47/87). The rate of active shedding was higher than initially expected when compared with other herpesvirus cases found in other koalas in Australia. Stalder et al. [8] surveyed 99 captive and wild koalas in Victoria in 2015 and detected PhaHV-1 and 2 in 10.1% (10/99) and 23.2% (23/99) of koalas, respectively. Another study in Victoria found the prevalence of PhaHV-1 and PhaHV-2 was 17% and 22%, respectively, and an overall prevalence of 33% (*n* = 810), with different populations ranging from 1 to 55% [7]. Mainland populations in Victoria had 22–46% and 31–55% test positive for PhaHV-1 and PhaHV-2 [7], respectively. These numbers are more

consistent with the prevalence in Mount Lofty Ranges koalas, with active shedding of PhaHV-1 and PhaHV-2 at 57.5% (46/80) and 40% (32/80), respectively, in the wild-caught koalas and 46% (40/87) and 27.5% (24/87), respectively, in euthanased koalas. These may represent populations with similar high densities with greater opportunity for infection spread. Differences in prevalence of viral shedding between PhaHV-1 and PhaHV-2 may show that PhaHV-2 is less established within the SA koala population than PhaHV-1, or that PhaHV-2 may have a lower tendency to actively shed than PhaHV-1. This may be due to the genetic differences between the viral subtypes, as PhaHV-2 only shares 60% pairwise identity with PhaHV-1 [6] and may contribute to higher expression of immune suppressor genes in PhaHV-2. Latency studies would need to be carried out for this theory to be concluded.

A significant relationship and positive correlation existed between active shedding of PhaHV-1 and TWC in both the wild-caught cohort (p-value = 0.023, r = 0.3) and the euthanased cohort (p-value = 0.05, r = 0.2), whereby increasing age increased the likelihood of PhaHV-1 infection. This relationship was expected: as age increased, the probability of infection increased due to the likelihood of exposure to the virus and the lifelong nature of the disease. In contrast, there was no correlation between active shedding of PhaHV-2 and TWC in either cohort. The study by Vaz et al. [7] also showed similar results regarding PhaHV-1 infection correlation with age and PhaHV-2 spread more evenly across age groups. This study proposed that PhaHV-1 infection may be more likely due to sexual contact between adult koalas, whilst PhaHV-2 infection may occur from close contact between mother and joey [7]. Vaz et al. showed repeatable detection of both PhaHV-1 and PhaHV-2 in koala cloacal regions [7,8], therefore, viral transmission between mother and joey may not only occur in the pouch, but potentially during parturition and/or from the consumption of a unique maternal faeces known as "pap". This faecal complex contains tannin protein complex-degrading enterobacteria (T-PCDE) essential for digestion of gum leaves [26] and may also be contaminated with other diseases such as *Chlamydia pecorum* and PhaHV-1 and PhaHV-2. Kent et al. [27] conducted a survey on sexually transmitted mustelid gammaherpesvirus 1 (MusHV-1) in European badgers (*Meles meles*) and investigated the prevalence of infection in adults and cubs; the high proportion of infected cubs showed a strong likelihood of vertical transmission.

Body condition score is a commonly used and effective indicator of the health status of an animal [18]. In our study, koalas with a BCS of 1–3/5 (emaciated, poor, or fair muscle condition) were considered compromised, whilst koalas with BCS 4–5/5 were considered in good condition. It was hypothesised that koalas with a low BCS were more likely to be actively shedding if infected, due to being unable to keep the herpesvirus in a latent, suppressed state. Active shedding of PhaHV-2 had a significant correlation with a compromised BCS in the euthanased koala cohort (p-value = 0.04; r = 0.2; n = 87), but not in the wild-caught cohort. This was likely confounded by the spread of koalas in each BCS category with a right-skewed bias towards healthy koalas in the wild-caught cohort and a more normal distribution in the euthanased cohort (Figure 4). Surprisingly, compromised BCS had no significant correlation with infection or active shedding of PhaHV-1 in either cohort. The lack of relationship between compromised BCS and active shedding of PhaHV-1 is likely due to apparently healthy animals being able to actively shed herpesviruses without developing clinical disease [10]. The potential to asymptomatically shed gammaherpesviruses or have subclinical disease has been demonstrated in other reservoir hosts [11]. In a case study of ovine herpesvirus-2 (OvHV-2; gammaherpesvirus), Li et al. [10] took a series of nasal swabs and discovered that healthy lambs between 6 and 9 months of age had an active shedding prevalence of 61% (n = 56). Gammaherpesviruses can be more likely to cause disease in susceptible, nonreservoir hosts, as seen with cattle infected with OvHV-2 causing malignant catarrhal fever (MCF) [28].
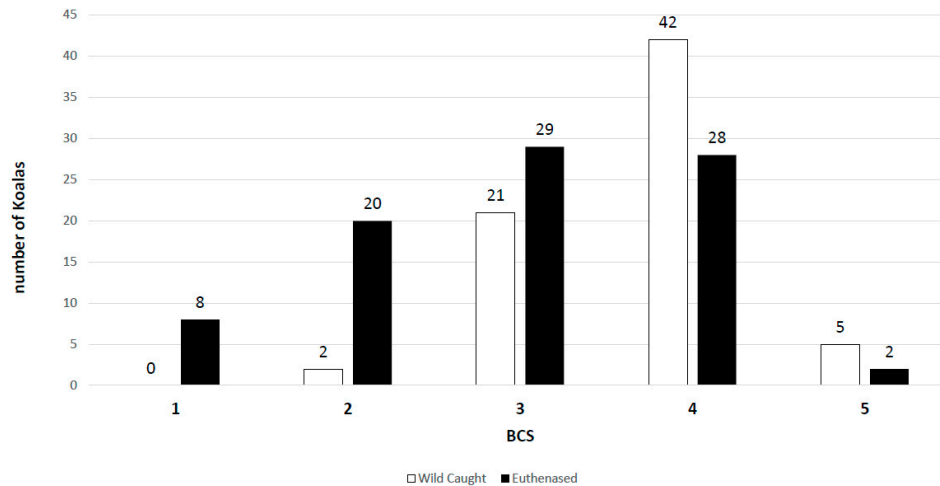
Figure 4. Histogram displaying the distribution of koalas in each cohort within their assigned body condition score (BCS).

4.2. Systemic Infection and Active Shedding

The majority of infected euthanased koalas were actively shedding the virus (74.6%; 47/63), whilst only 25.4% (16/63) of infected koalas were not actively shedding. There was a strong positive correlation between splenic coinfection of both viral subtypes and active shedding of the virus (0.8) (Figure 1B). In the euthanased cohort, koalas infected with PhaHV-2 were 3.5 times more likely to be coinfected with PhaHV-1 (Exp (B) = 3.538; *p*-value = 0.006; *n* = 87) and as a result were 1.5 times more likely to be actively shedding PhaHV-1 with every incremental increase in TWC (Exp (B)) = 1.514; *p*-value = 0.05; *n* = 87). This suggests that viral coinfection highly predisposes koalas to active shedding of the virus. Potentially, increased expression of viral effector proteins from both PhaHV-1 and PhaHV-2 could lead to greater immunosuppression, initiating recrudescence. A study investigating herpesvirus coinfection in humans showed that infection of EBV and HHV-7 were also shown to promote HHV-6 infection and disease [29–32] however, this may just be a result of severe immunosuppression that triggers viral coinfection, as stated by Handous et al. [33]. PhaHV-2 had a significant correlation with a compromised BCS, suggesting that PhaHV-2 may be an indicator of other underlying health conditions such as immunosuppression, stress and coinfection of other diseases including PhaHV-1, *Chlamydia pecorum* or koala retrovirus.

In our study, there was no significant relationship between sex and infection status. Yet Stalder et al. [8] showed a significant relationship between infection and sex, with male koalas more likely to have an infection. Vaz et al. [7] showed that females without young were more likely to be infected than those with young; they also found a correlation between PhaHV and "wet bottom", a clinical sign of overt chlamydiosis. The association of potentially infertile females with PhaHV may be confounded by chlamydial infection, a known cause of reproductive pathology in koalas [34].

4.3. Sites of Active Viral Shedding

In our study, most oropharyngeal swabs from infected (spleen positive) koalas were positive for Phascolarctid gammaherpesvirus, suggesting that significant active shedding is from rostral epithelial tissues (nasal, oropharyngeal). This finding differs to that of Vaz et al. [7], which found that rostral (ocular, nasal, oropharyngeal) swabbing was less likely to pick up an infection in comparison with caudal (urogenital, cloacal) swabbing. Samples from caudal epithelial tissues were not tested in our

study, therefore, shedding from different epithelial sites needs to be investigated further to provide tissue tropism insights. One concern about the interpretation of active shedding is the contamination of the oropharyngeal swabs with cells within which latency has been established, that is, circulating mononucleocytes. Nevertheless, due to the tissue tropism of herpesviruses, it is less likely to detect virus in epithelial swabs when the animals are only infected in latent forms [15,35,36]. It is also highly likely that the detected viruses in epithelial cells are actively shed viruses [37].

4.4. Potential Areas for Further Research

This study focused on the presence and prevalence of Phascolarctid herpesviruses within the mainland South Australian koala population. Further research is needed to understand the association of PhaHV with other infections—primarily koala retrovirus (KoRV) and Chlamydial infection—since PhaHV may be playing a role in the augmentation of clinical disease or be shed secondary to them. Since PhasHV-2 had an association to koalas with a compromised BCS and increased PhasHV-1 shedding, this could be suggestive of an increase in pathogenicity in this subtype. Investigation into disease presentations, haematology and immune function markers with and without PhaHV infection may shed some light on the effect of these viruses. Serological studies could be conducted to investigate the koalas' response to PhaHV infection, since the koalas' immune system has often been perceived as "immunologically lazy" [38,39]. These studies would help to further determine the clinical significance, host response to infection and impact of these newly discovered viruses on koala populations.

5. Conclusions

We showed there was a high prevalence of PhaHV infection in koalas in the Mount Lofty Ranges population, with more than two-thirds of both wild-caught and euthanased cohorts actively shedding the virus. PhaHV-1 had a greater prevalence within the SA koala populations, with more koalas actively shedding PhaHV-1 than PhaHV-2.

Despite being less prevalent, koalas coinfected with PhaHV-2 were more likely to also be infected and actively shedding PhaHV-1. Neither of the viral subtypes were shown to have any significant relationship with BCS in wild-caught koalas; however, PhaHV-2 infection had a significant correlation with BCS in euthanased koalas. PhaHV-1 infection was also shown to be positively correlated with TWC in both cohorts, whilst sex had no significant correlation with either viral subtype in both cohorts.

The clinical significance of these recently discovered Phascolarctid herpesviruses is still unknown, and additional investigation into the pathogenicity, clinical signs of the virus and coinfection with other pathogens is important. Uncovering the significance of PhaHV will help determine the health status and guide the management of koala populations.

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Chapter 5: Key genes and pathways and their potential role in KoRV-associated lymphoma in South Australian Koalas

As has been shown in. both Chapter 3 and Chapter 4, the most important role of KoRV in this SA population is that of inducing lymphoma. Although a relatively small percentage of koalas infected with KoRV go on to develop lymphoma in this cohort, investigation of genetic oncogenesis can help to understand this disease process. KoRV expression, as seen in Chapter 3, was significantly higher in lymphomic koalas, therefore its ability to influence cellular gene transcription is increased. Chapter 5 investigates through the use of RNA-seq data which genes are dysregulated by KoRV in koalas with lymphoma.

Key Genes and Pathways and their potential role in KoRV-associated lymphoma in South Australian Koalas

5.1 Abstract

Retroviral incitement of neoplasia is well known. Several studies have found an association of KoRV with lymphoid neoplasia in koalas, with viral particle visualised in leukaemic bone marrow. KoRV proviral load and gene expression is significantly higher in lymphomic tissue than KoRV positive lymph nodes. Differential gene expression showed significant upregulation of oncogenes, inclusive of MYB, MYCL and FLT3. These genes are key genes in oncogenic pathways in other gamma retroviruses, inclusive of feline leukaemia virus (FeLV) and murine leukaemia virus (MuLV). Pathway analysis showed inhibition of Th1/Th2 signalling and NF-κB signalling pathways in lymphomic koalas, likely suppressing the immune system and increasing likelihood for oncogenesis. This study identifies candidate genes of interest in KoRV-induced oncogenesis and shows immunosuppression in the pathogenesis of lymphoma in koalas.

5.2 Introduction

The study of retroviruses, RNA viruses that rely on integration into the host DNA to replicate, has contributed significantly into the understanding of the development of cancer and discovery of oncogenes in a wide range of species (Levy 2011; Beemon *et al.* 2012). Several mechanisms are utilised by oncogenic retroviruses to elicit malignant disease in their host, including expression of oncogenes, insertional mutagenesis, activation of cellular

microRNAs, immunosuppression and through the effect of expression of accessory genes like *tax* and *HBZ* (Levy 2011; Beemon *et al.* 2012).

Viral oncogenes (v-*onc*) have been identified in the genomes of more complex retroviruses. These retroviruses have incorporated host cellular genes into their genomes through readthrough and oncogene capture, which once replicated are then inserted back into the host genome, increasing the expression quickly. Increased expression of the gene by the host then leads to tumour production by various mechanisms (Levy 2011). Identification of viral oncogenes (v-*onc*) has led to the discovery of the cellular genes (c-*onc*) which has facilitated a greater understanding of cellular pathways in general. Complex retroviruses with their known v-*onc* include the following: simian sarcoma virus (SSV) and v-*sis* in woolly monkeys, feline sarcoma virus (FeSV), v-*fms* and v-*kit* in cats, Rous sarcoma virus (RSV) and v-*src,* avian myeloblastosis virus (AMV) and v-*myb* both in chickens and Abelson murine leukaemia virus (Abl-MuLV) and v-*abl* in mice (Beemon *et al.* 2010).

Insertional mutagenesis occurs when the integration of the provirus into the host DNA is in close vicinity to a cellular gene associated with cell proliferation, growth or cell death, altering its regulation of transcription (Levy 2011). Study of retroviral species that cause cancer due to their insertion sites has led to many discoveries of genes involved in cell growth, differentiation and survival (Levy 2011). The site of integration of the retrovirus will disrupt the DNA structure at that site, resulting for example, in separation of exons within cellular genes and production of proteins with altered function, reduction of mRNA stability through separation of regulatory elements and promoter enhancement from viral LTR regions (Beemon *et al.* 2012). Viral LTR regions increase transcription rates of the viral genome and, depending on integration site, can increase cellular gene transcription and disrupt regulation

of protein production (Beemon *et al.* 2012). LTR regions have been shown, in other species infected with oncogenic retroviruses, to incorporate enhancer regions and thereby increase prevalence of disease (DesGroseillers *et al.* 1984; Athas 1994; Chen *et al.* 1994; Ghosh *et al.* 2000). Avian leukosis virus (ALV) is a specific example of a retrovirus that causes disease through insertional mutagenesis. In ALV induced bursal lymphoma there is an upregulation of MYC gene and overexpression of a transcription factor that has a multitude of functions including driving cell proliferation (Kato *et al.* 1992). Also genes MYB and TERT have been shown to be involved in mutagenesis of ALV (Clurman *et al.* 1989), with TERT also a candidate in lymphoma oncogenesis in bovine leukemia virus (BLV) (Hemmatzadeh *et al.* 2015). MYC and MYB have also been implicated in tumorigenesis in MuLV and feline leukemia virus (FeLV) (Levy 2011). It has been found in some species that retroviral insertion near MYB leading to B-cell lymphomas (Kanter *et al.* 1988; Jiang *et al.* 1997).

MicroRNAs (miRNAs) are associated with post transcriptional regulation of gene expression and RNA silencing and are thought to be critical for cellular processes for growth, differentiation and apoptosis (Landais *et al.* 2007). Retroviruses can integrate into these loci and can cause overexpression of the miRNA. Landais *et al.* (2007) have described murine retroviral integration and upregulation of KIS2, with overexpression of MiR-106-363 cluster miRNAs in transformed tumour cells with possible disruption of tumour suppression pathways (Landais *et al.* 2007). MiRNAs have also been implicated in the progression of chronic lymphocytic leukaemia in humans and expression profiles of these miRNAs can distinguish between normal and malignant B-cells in this disease (Calin *et al.* 2004). In ALV, miR-155 is another upregulated microRNA responsible for repression of tumour suppressor genes (Levy 2011).

Immunosuppression has been associated with retroviruses since the human immunodeficiency virus (HIV) and AIDS epidemic almost 40 years ago and since then immunosuppression has been found with multiple retroviral infections (Denner 1998). A highly conserved candidate domain within the retroviral genome was sought through investigations of multiple genera of retroviruses. This resulted in the identification of the immunosuppressive domain (isu) within the transmembrane protein which was found to be highly conserved across retroviral genomes, such as FeLV, PERV and MuLV (Denner 1998). *In vitro* experiments found that the peptides corresponding to this region inhibited several lymphocytic reactions, decreased lymphocyte proliferation and altered cytokine activity (Denner 1998). Immunosuppression has subsequently been association with increased risk of malignant disease. A common complication of HIV induced AIDS is the formation of cancers and it is suggested that these cancers may arise as a consequence of inadequate immune surveillance, altered immune response or reactivation of an oncogenic virus (Levy 2011).

In koalas, koala retrovirus (KoRV) is a recently described gammaretrovirus for which KoRV-A, the most prevalent variant, has become endogenised in northern populations of koalas. KoRV was first recognised following investigation of lymphoid neoplasia and suspicion of viral aetiology. KoRV has been found in all populations of koalas throughout Australia, with prevalence and transmission differing from northern to southern koalas. Northern koalas harbour endogenous KoRV within their genome with exogenous variants transmitted through the population. Southern koalas only found with exogenous variants, predominantly exogenous KoRV-A (Simmons *et al.* 2012). High proviral and viral loads of KoRV (Tarlinton *et al.* 2005) and exogenous infections of both KoRV-A and KoRV-B have been positively associated with the incidence of lymphoid neoplasia (Xu *et al.* 2013; Fabijan *et al.*

2017; Sarker *et al.* 2020a). Increased transcription potential has been shown in KoRV-F due

to the five repeats in the U3 region, but there has not been an inference to its pathogenicity

(Xu *et al.* 2015). Immunosuppression is also likely to occur with KoRV, with altered

cytokine expression and immune parameters (Maher *et al.* 2016; Maher *et al.* 2019), in

combination with a highly conserved immunosuppressive domain (Fiebig *et al.* 2006), similar

to that of FeLV, MuLV and PERV. The cellular pathways affected by KoRV infection,

particularly in koalas with lymphoma, are yet to be described. The most likely way that

KoRV is inciting neoplastic change in koalas is through insertional mutagenesis, due to the

lack of change in its genome size and longevity of infection prior to malignancy.

The aim of this study was to identify dysregulated genes that may initiate transition to

neoplasia and investigate enriched cellular pathways in KoRV positive koalas diagnosed with

lymphoma. Furthermore, differential gene expression in KoRV negative against KoRV

positive koalas was investigated to evaluate possible impacts of infection.

5.3 Materials and methods

Samples

Koalas for this study (n = 9) were selected on the basis of previous examinations for disease

and KoRV status (as described in chapters 2, 3 and 4). Eight koalas were from the Mount

Lofty Ranges in SA, two were KoRV positive and diagnosed with lymphoma, three were

KoRV positive without lymphoma and three were KoRV negative based on *pol* qPCR

proviral investigations (Chapter 2). Lymph nodes for RNA extraction were collected within 1

hour of euthanasia and placed in RNALater® (Sigma). Lymphoma tissue samples

(lymphomic lymph nodes) from SA koalas were unable to be collected in a timely fashion,

since all cases were frozen prior to necropsy, an issue compounded by the low prevalence of

lymphoma in South Australia (see Chapter 4). In addition, frozen archived tissue from a KoRV positive NSW koala with lymphoma, B00310, also with endogenised KoRV-A, was sourced to increase numbers in the lymphoma cohort. B00310 was housed in the collection at Perth Zoo and the clinical and postmortem examination was carried out by Dr Simone Vitale, Perth Zoo and histopathology examined by Dr David Forshaw, Veterinary Pathologist, Animal Health, Albany. Details of koalas used are presented in Appendix S12.

RNA extraction and sequencing

RNA was extracted from koala lymph nodes from KoRV negative and KoRV positive koalas (allocated as per Chapter 2) and lymphomic lymph nodes from KoRV positive koala. Tissues were thawed on ice, disrupted with zirconium oxide beads (0.5mm, RNA-free, BioTools, Loganholme, Australia) in a Bullet Blender and Tissue Homogeniser (Next Advance, Troy, NY 12180, USA)) and then cooled on ice. Total mRNA was extracted using the *mir*Vana™ kit (ThermoFisher Scientific) as per the manufacturer's protocol. RNA integrity was checked using a 2100 BioAnalyser (Agilent Technologies). Only three replicates from each cohort could be chosen, therefore this was done on quality of RNA which had minimal or no degradation and limited or no genomic DNA contamination. cDNA libraries were made and sequenced using an Illumina sequencing platform, generating 2 x 150 bp reads (AGRF, Melbourne, Australia).

Transcriptome analysis

The RNA-Seq reads were cleaned using Trim_Galore (v0.4.2) (Krueger 2015) and AdapterRemoval (v2.2.1) (Schubert *et al.* 2016) to remove adapters and bases with a Phred score less than 10. Fastqc (v0.11.4) (Andrews 2010) was used to quality check the cleaned reads. The reads were aligned to the koala reference genome (GCA_002099425.1_phaCin_

unsw_v4.1) using HISAT2 (v2.1.0) (Kim *et al.* 2015). Annotation was carried out using

Ensembl release 97 along with manually added annotation for Koala retrovirus A (KoRV A

NCBI Accession AF151794) and PhER (Lober *et al.* 2018) (Table 1). The featureCounts

function of the R package Rsubread (Liao *et al.* 2019) was used to count reads mapped onto

annotated features. Differential gene expression analysis was carried out with limma (Ritchie

et al. 2015) and edgeR (Robinson *et al.* 2010; McCarthy *et al.* 2012), and the function

voomWithQualityWeights was used to deal with outlier samples. Genes were considered to be

differentially expressed if they had a $\log_2$ fold change (logFC) greater than 2 or less that -2 and

adjusted *P* value less than 0.05. Due to the current annotation quality of the koala genome,

some genes did not have corresponding symbols and gene identifications other than that of the

koala Ensemble number. In order to increase the power of onward processing in IPA, koala

Ensemble gene identifiers were mapped to corresponding human gene identifiers increasing

the number of genes that could be identified with symbols.

Table 1: Alignment coordinates for additional annotation of the koala reference genome with
KoRV (AF151794) genes and PhER.

Region	Number	Scaffold	Start	Stop	bp
KoRV 5'LTR	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2807277	2808240	502
KoRV *gag* 1	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2808242	2808777	536
KoRV *gag* 2	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2808778	2809803	1026
KoRV *pol*	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2809921	2813257	3336
KoRV *env* 1	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2813306	2814277	971
KoRV *env* 2	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2814278	2815160	883
KoRV 3'LTR	NW_018343978.1	phaCin_unsw_v4.1.fa.scaf00027	2815204	2815707	504
PhER	Sequence as per Lober *et al.* (2018)	phaCin_unsw_v4.1.fa.scaf00062	10912078	10920108	8030

Analysis in IPA

Integrated Pathway Analysis (IPA) (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis) was used to integrate the differential gene expression into cellular pathways, explore key genes and their direct networks. The gene symbol was used as the identification. The Qiagen Knowledgebase gene expression dataset was used to discover similar gene expression profiles from other diseases and disorders. It was used to create gene of interest connection pathways to describe outcomes from these experiments.

5.4 Results

RNA quality control was carried out to assess the RNA integrity (RIN) and the amount of RNA present (ng/µL). The mean RIN was 7.8 (sd: 2.35; range 2.4-10) and mean concentration was 140 ng/µL (sd: 199.8; range 5-577 ng/µL). The 9 selected samples (Appendix S12) were chosen on their availability, KoRV proviral results, RIN and lack of genomic DNA contamination. Supplementary material (Appendix S13) shows the Bioanalyser results containing RNA quality parameters for these samples. Samples K16-019, K16-069 and B00310 were lymphoma (KoRV positive) samples, K17-073, K17-080 and K18-005 were KoRV positive samples, K17-005, K17-019 and K18-004 were KoRV negative samples chosen for onward transcriptome analyses. Alignment of the transcriptomes reads for these samples are shown in Table 2 and demonstrate an overall adequate to excellent depth and percentage of successful alignments.

Table 2: Sample read depths and successful alignment data. Analysis from Subread, reporting paired end read alignments for each sample, successful alignments of these to the reference genome and percent aligned.

Sample	Group	Total alignments	Successful alignments	Percent
K16-019	Lymphoma	174,086,869	85,106,984	48.9%
K16-069	Lymphoma	237,299,059	132,113,483	55.7%
B00310	Lymphoma	165,989,148	77,145,170	46.5%
K17-073	KoRV Positive	133,726,184	69,035,908	51.6%
K17-080	KoRV Positive	42,214,027	23,590,823	55.9%
K18-005	KoRV Positive	70,327,655	34,262,618	48.7%
K17-005	KoRV Negative	221,067,114	103,024,814	46.6%
K18-004	KoRV Negative	36,734,022	23,451,217	63.8%
K17-019	KoRV Negative	51,666,580	27,618,985	53.5%

From consultation the desired depth of read is >50,000,000, adequate depth of reads is >25,000,000

KoRV expression

Figure 1 shows the alignment of cleaned reads to the integrated KoRV provirus in the reference genome. This figure is an adaptation of that used in Chapter 2, showing the addition of the Perth Zoo lymphoma case (B00310) in the lymphoma cohort. Chapter 2 discussed the eight South Australian koalas that were used for transcriptome analyses and assigned KoRV status. Chapter 2 also highlights the expression of KoRV within one of the proviral negative koalas (K17-019, Negative 3). Appropriate categorisation of KoRV status is important to the onward interpretation of DGE results, therefore adequate explanation of how each DGE analysis was carried out is paramount. K17-019 was not excluded from further analysis, but adaptations of groupings for DGE were explored
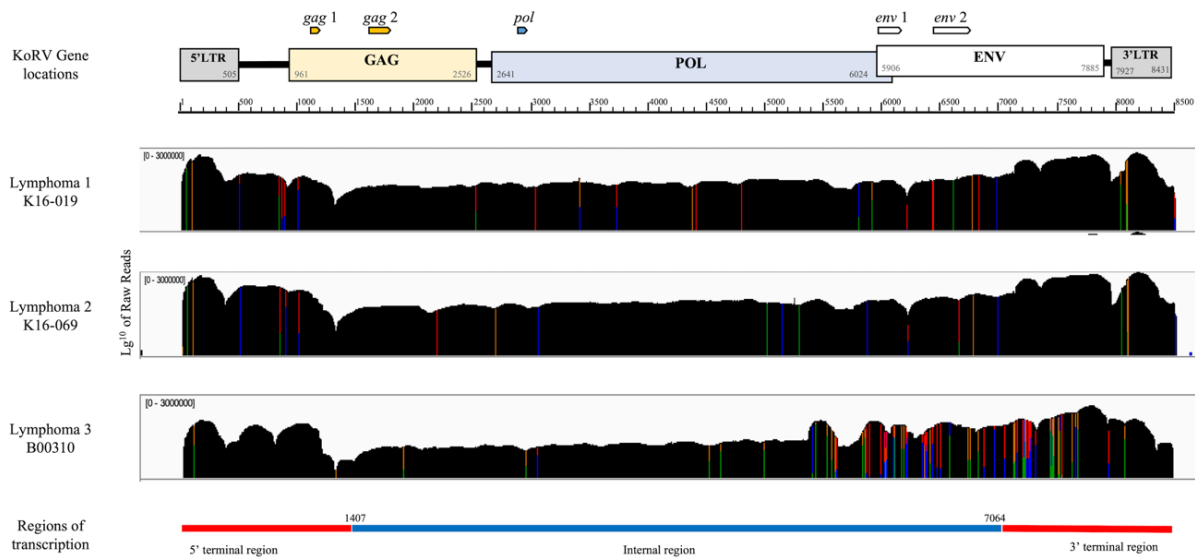
Figure 1: Alignments of cleaned reads against the integrated KoRV genome within the koala reference genome

Differential KoRV gene expression from RNA seq data

Reads from RNA-seq data were normalised, aligned to the gene segments (as per Table 1) and log Counts per Million reads (CPM) were calculated. Differential expression was found in *gag* 1, *gag* 2, *pol*, *env* 1, *env* 2 and 3'LTR expression between KoRV positive koalas diagnosed with lymphoma (n = 3) and KoRV negative koalas (n = 3) (Figure 3). Differential expression was also seen in *gag* 2, *pol* and *env* 1 between KoRV positive koalas diagnosed with lymphoma (n = 3) and KoRV positive koalas (n = 3) ($\log_2$FC $\geq$ 2 or $\leq$ -2, adjusted *P* value <0.05). No differential expression for KoRV genes was observed between KoRV positive (n = 3) and KoRV negative (n = 3) koalas.

This highlights the significant increase in KoRV gene expression from the internal region of the KoRV genome in lymphomic animals and supports the alignment data from Figure 2. The assignment of koalas to groups for this differential expression was based on provirus detection.
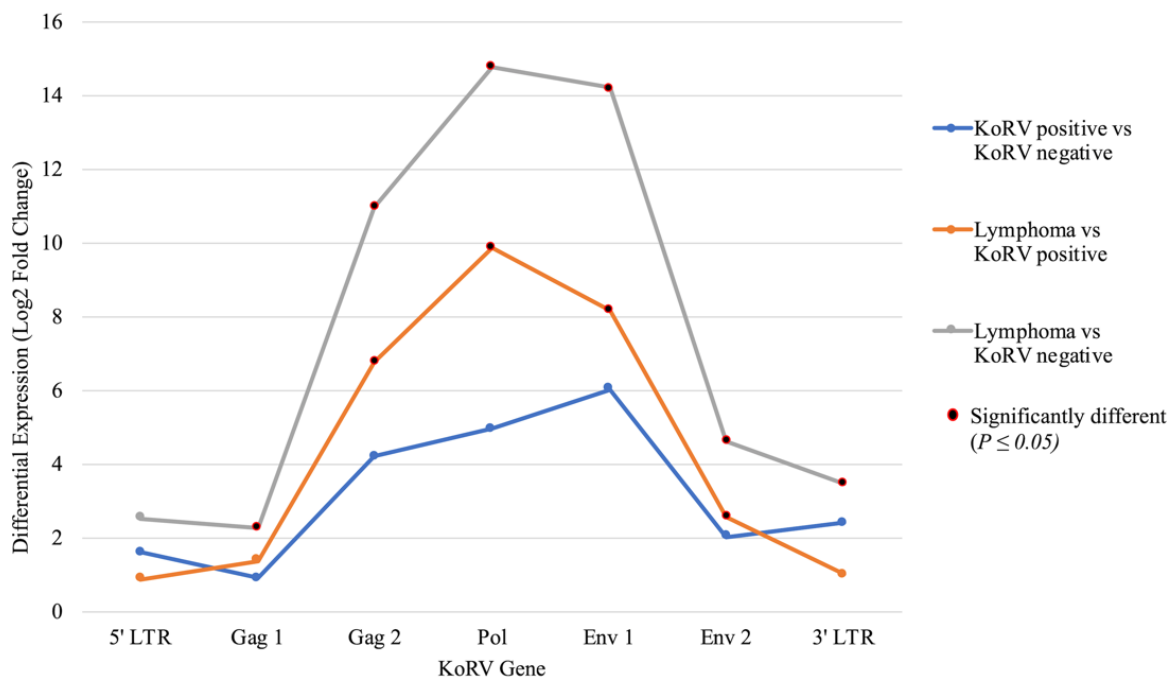
Figure 2: KoRV gene expression from RNA-seq data

Analyses of biological variance

The biological coefficient of variance of the data, grouped as per proviral DNA results, was calculated to be 0.533 (53.3%). This is a relatively high biological variance, likely due to the small number of replications able to be processed and the inability to control which samples were able to be processed. Ideal environmentally and physiologically controlled animal experimentation may give a biological coefficient closer to 10% (McCarthy *et al.* 2012). The mean variance trend, sample specific weights and biological coefficients were plotted for the

samples and whole cohort and are reported in supplementary material (Appendix, S14 and S15). Briefly, KoRV negative and KoRV positive koalas more closely aligned together, but with moderate degree of spread. The lymphoma group are more dispersed, although these are distinct from both the KoRV positive and KoRV negative koalas.

Differential gene expression (DGE)

14,438 genes and protein coding regions were aligned and counted against the koala genome. 12,510 of these genes had corresponding symbol identifiers. The DGE was calculated between cohorts and there were 1207 differentially expressed genes between KoRV positive koalas diagnosed with lymphoma and KoRV negative koalas, 244 upregulated and 963 downregulated. There were 939 differentially expressed genes between KoRV positive koalas diagnosed with lymphoma and KoRV positive koalas, 223 upregulated and 716 downregulated. There was no DGE between KoRV positive and KoRV negative koalas. The 14,438 genes were plotted to show $\log_2$FC against $\log_{10}$(adj P value) to visually assess the upregulation (red) and downregulation (blue) of each gene or protein coding region in the DGE (Figure 3, on the next page). Two of the three most highly upregulated genes were koala retroviral genes or partial genes; *pol* and *env* 1, $\log_2$FC of 9.88 (adj. P value 0.0007) and 8.19 (adj. P value 0.00009), respectively, as can be seen in the top right corner of the volcano plot (Figure 3).

Figure 3: Volcano plot of gene expression with significantly upregulated genes shown in red and significantly downregulated genes shown in blue. The top 50 significant genes are labelled.

Examining the influence of K17-019

Due to the atypical transcription alignment of K17-019 against the KoRV genome in the internal region (as seen in Chapter 2), further analyses were carried out to investigate the importance of this koala's status. DGE was also calculated between KoRV positive and KoRV negative with K17-019 included in the KoRV positive group, KoRV positive and KoRV negative with K17-019 excluded, lymphoma and KoRV negative with K17-019 excluded and lymphoma against KoRV positive with K17-019 included in the KoRV positive group. Table 3 shows the changes in DGE in all these comparisons.

Table 3: Differential gene expression with K17-019 (negative 3) in different groupings.

Groups compared	Up*	Not sig	down*
positive vs negative (K17-019 included in the KoRV negative group)	0	14438	0
positive vs negative (K17-019 excluded)	0	14438	0
positive vs negative (K17-019 included in the KoRV positive group)	0	14437	1
lymphoma vs negative (K17-019 included in the KoRV negative group)	244	13231	963
lymphoma vs negative (K17-019 excluded)	351	13165	801
lymphoma vs positive (K17-019 included in the KoRV negative group)	223	13499	716
lymphoma vs positive (K17-019 included in the KoRV positive group)	214	13567	658

*Genes are considered differentially expressed when the log$_2$FC expression ≥ 2.0 or ≤ -2.0 and the adjusted *P* value < 0.05. Colours the same as the DGE analyses used in Figure 4.

Removal of K17-019 from the negative group did not change the DGE outcomes between KoRV positive and KoRV negative but decreases the negative replicates to only two. Reassigning K17-019 to the KoRV positive cohort only increases the DGE to 1 gene, which was adiponectin (ADIPOQ). Removal of K17-019 from the KoRV negative cohort in the comparison with lymphoma increased the significant genes by 66 and also increased the upregulated genes by 107 genes and decreased the downregulated genes by 162. Reassigning K17-019 to the KoRV positive cohort in the comparison with lymphoma decreased the significant genes by 68 and decreased both the upregulated genes by 9 and the downregulated genes by 58.

Crossover genes

Due to the variance in differential expression, only crossover genes were examined, to eliminate the effect of K17-019 in relation to finding genes paramount to KoRV-induced oncogenesis. The below Venn diagram (Figure 4) used genes able to be processed by the IPA software which have corresponding gene identifications to human genes, revealing 391 common genes differentially expressed in lymphomic animals when compared across cohorts.
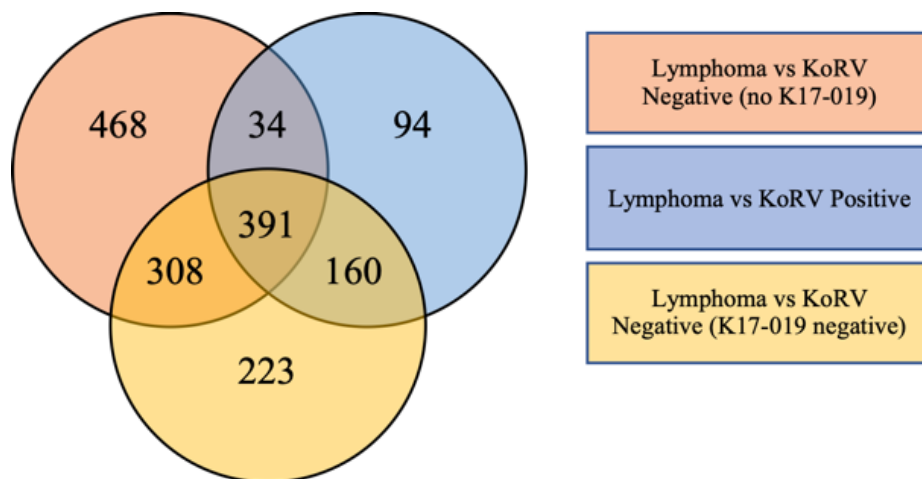
Figure 4: Venn diagram of differential genes between groupings
Shading of diagram is the same as in shading in Table 5.

Gene descriptors from Tocris, NCBI Entrez and UniProt online databases from the 391 common genes were assessed for possible contribution to oncogenesis. Genes of interest are those with gene functions related to oncogenesis, particularly transcription regulation, cell cycle regulation or immunomodulation have been highlighted in Figure 5 by bold text.

Figure 5: Heatmap of expression (CPM) for 391 genes of interest from the comparison of lymphoma vas KoRV negative or KoRV positive koalas

On the basis of gene description and function analysis 49 genes of interest were identified: GPX2, DNTT, MSI1, MMP9, MYB, MUTYH, LOXL2, CDK6, FLT3, CXXC5, GSTP1, PLXNC1, FAM83D, CIB1, FGF8, APEX1, PHLPP2, PRF1, ETV3, STARD8, PLAGL1, VCAM1, IL6R, LYST, UBASH3A, SLCO2A1, ITK, IL7R, NFATC2, IRF8, CD28, SH2D1A, CD247, NCAM1, EOMES, KLRK1, KLRK1, TNFRSF9, TRAV4, NOD2, TRAF3, SH2D3C, NMT2, TNFAIP3, NR3C1, NEDD9, SPHK1, GATA3 and NFATC1. Collated in supplementary material are gene descriptions, $\log_2$FC, and whether gene expression is consistent with increased likelihood of oncogenesis or immunosuppression (Appendix S16). Differentially expressed genes in lymphomic koalas corresponding to increased likelihood of oncogenesis comprised 11 upregulated genes ($\log_2$FC > 2.0): GPX2, DNTT, MSI1, MMP9, MYB, MUTYH, LOXL2, CDK6, FLT3, CXXC5 and GSTP1; and four downregulated genes ($\log_2$FC < -2.0): PRF1, ETV3, STARD8 and PLAGL1. DGE in lymphomic koalas corresponding to increased likelihood of immunosuppression were 19 genes which were all downregulated ($\log_2$FC < -2.0): VCAM1, IL6R, LYST, UBASH3A, SLCO2A1, ITK, IL7R, NFATC2, IRF8, CD28, SH2D1A, CD247, NCAM1, EOMES, KLRK1, KLRK1, TNFRSF9, TRAV4 and PRF1.

These 391 genes were also explored in IPA and the Qiagen Ingenuity Database, examining for associations with specific IPA terms; B cell neoplasm, lymphocytic cancer, lymphoid cancer, T cell non-Hodgkin lymphoma, tumorigenesis of lymphocytes, B cell cancer and B cell lymphoproliferative (Figure 6). Ninety five genes differentially expressed in lymphomic koalas were associated with these conditions, and their combined expression was predicted to increase the likelihood of all these diseases.
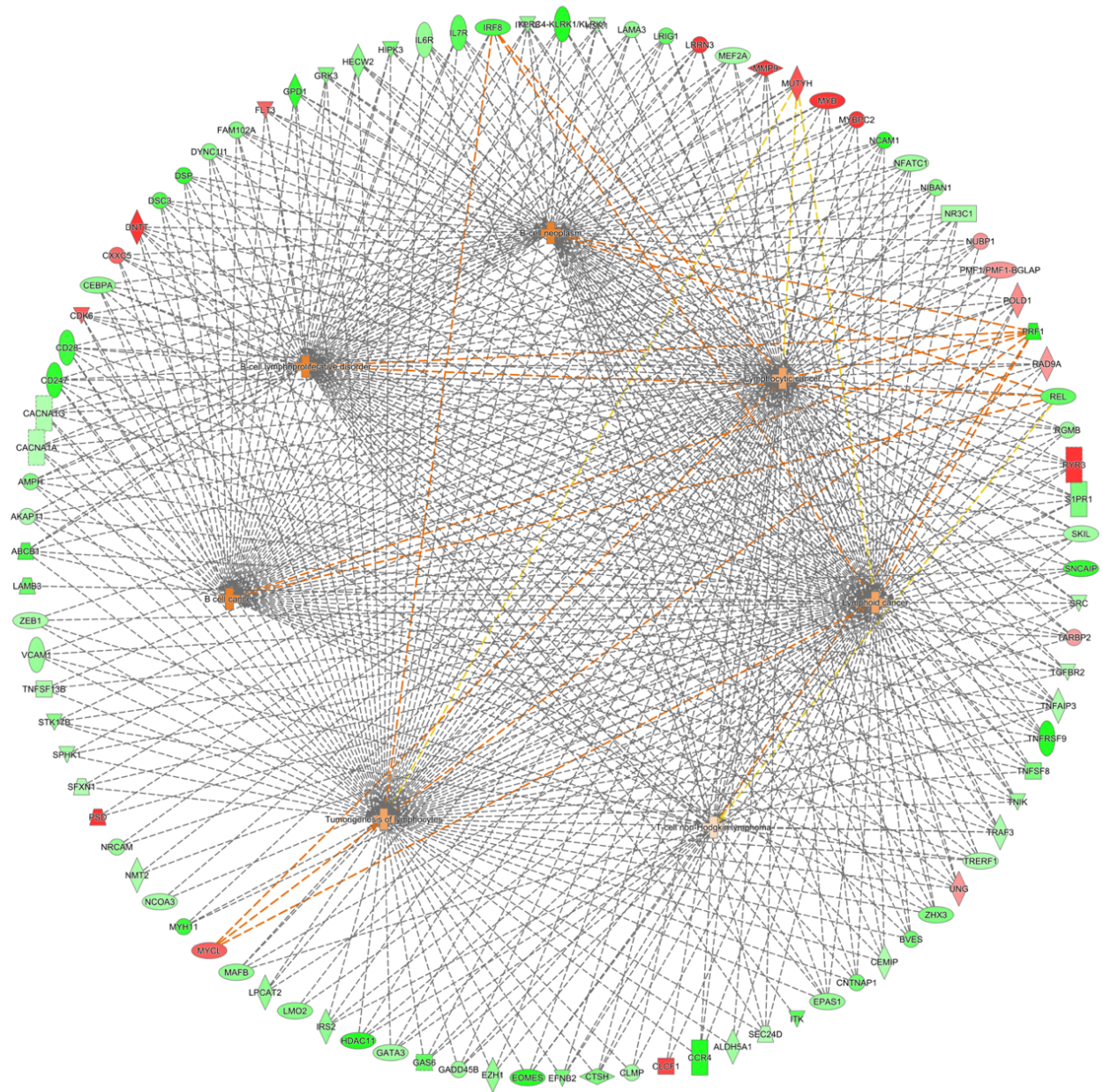
Figure 6: Connection of 95 genes to B cell neoplasm, lymphocytic cancer, lymphoid cancer, T cell non-Hodgkin lymphoma, tumorigenesis of lymphocytes, B cell cancer and B cell lymphoproliferative disorder using IPA disease function database. Prediction of increased likelihood of disease for all disorders (orange). Genes coloured red were upregulated and those coloured green were downregulated, intensity of colour related to increased upregulation or downregulation respective of colour

Oncogenes and tumour suppressor genes

The DGE was also explored in relation to known oncogenes and tumour suppressor genes,

Figure 7 shows those oncogenes for which expression was increased and the tumour

suppressor genes for which expression was decreased in lymphomic koalas. The highlighted,

dark yellow or dark blue, were those genes significantly upregulated or downregulated, respectively (adjusted *P* value < 0.05), Log$_2$FC is on the x axis.
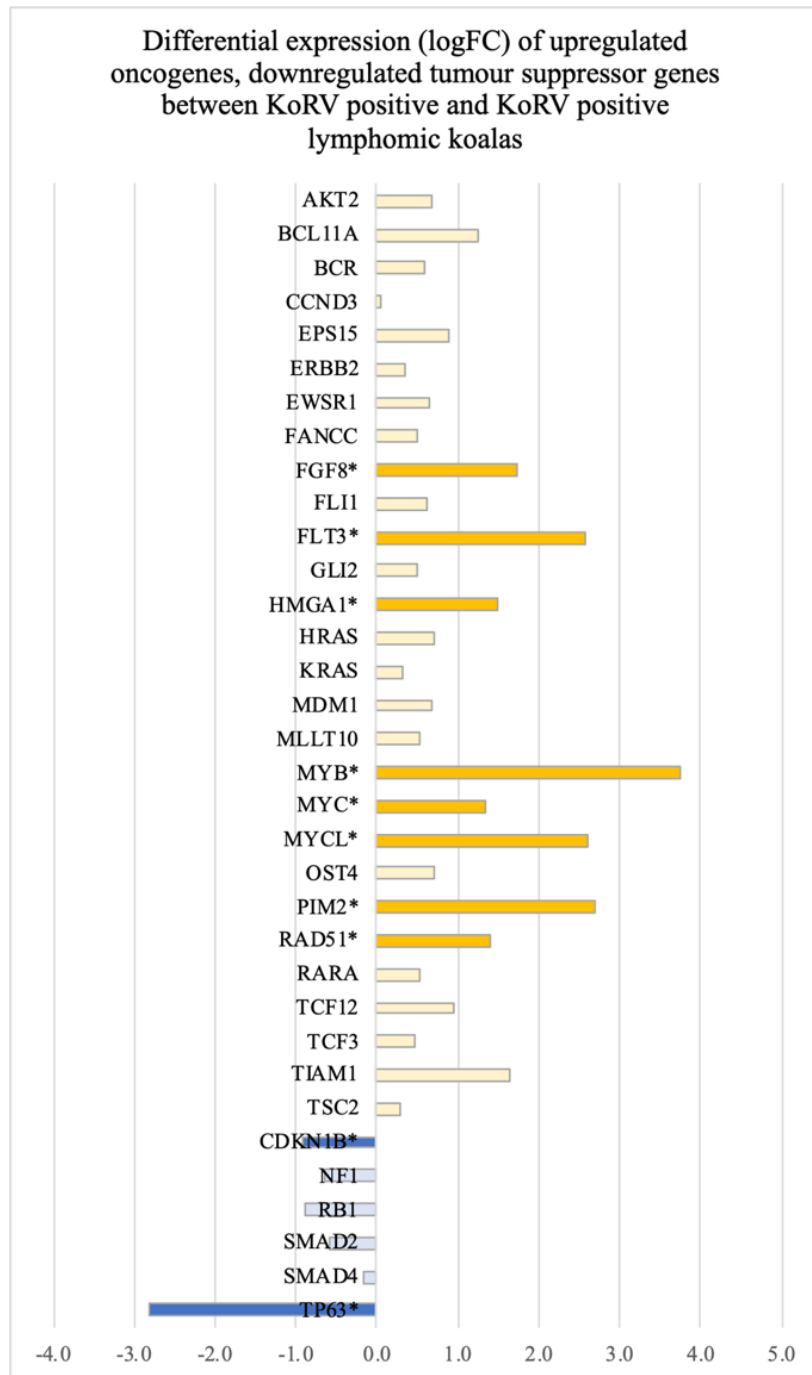
Figure 7: Differential expression (log2FC) of upregulated oncogenes and downregulated tumour suppressor genes from lymphoma against KoRV positive koalas. *indicates significantly dysregulated (adj. *P* value < 0.05)

Correlating these genes with those within the 391 genes of interest highlight key genes which may be responsible for induction and transformation to malignancy. These are MYB, MYCL and FLT3. Between KoRV positive koalas diagnosed with lymphoma and KoRV positive koalas, MYB was significantly upregulated with a Log_2FC of 3.8, MYCL was significantly upregulated with a Log_2FC of 2.6 and FLT3 was significantly upregulated with a Log_2FC of 2.6.

Gene descriptions for these three key genes, collated from Tocris, NCBI Entrez gene and UniProt databases showed key oncogenic factors for each gene. MYB is a known oncogene with abnormal expression, mutation or translocation in leukaemias and lymphomas of humans, cats, mice and others (Tsatsanis *et al.* 1994; Fujino *et al.* 2008; Beemon *et al.* 2012; Baron *et al.* 2014; Johnson *et al.* 2014). It is a transcription activator with a recognition sequence of 5'-YAAC[GT]G-3' and plays a significant role in the control of proliferation and differentiation of haemopoietic cells (Lieu *et al.* 2009; Sarvaiya *et al.* 2012; Nakano *et al.* 2016). FLT3 is a receptor tyrosine kinase that regulates haematopoiesis, mutations and dysregulation of which have been associated with acute myeloid and lymphoblastic leukaemia (Carow *et al.* 1996; Reilly 2003). MYCL is a recognised proto-oncogene previously named basic helix-loop-helix (BHLH) transcription factor. It is involved with DNA-binding transcription and protein dimerization (Klapproth *et al.* 2010). It can be seen from Figure 8 that each of these genes have considerable direct, experimentally observed gene interactions in relation to cancer, from the collation of data on the IPA Knowledge Base (Qiagen ®).

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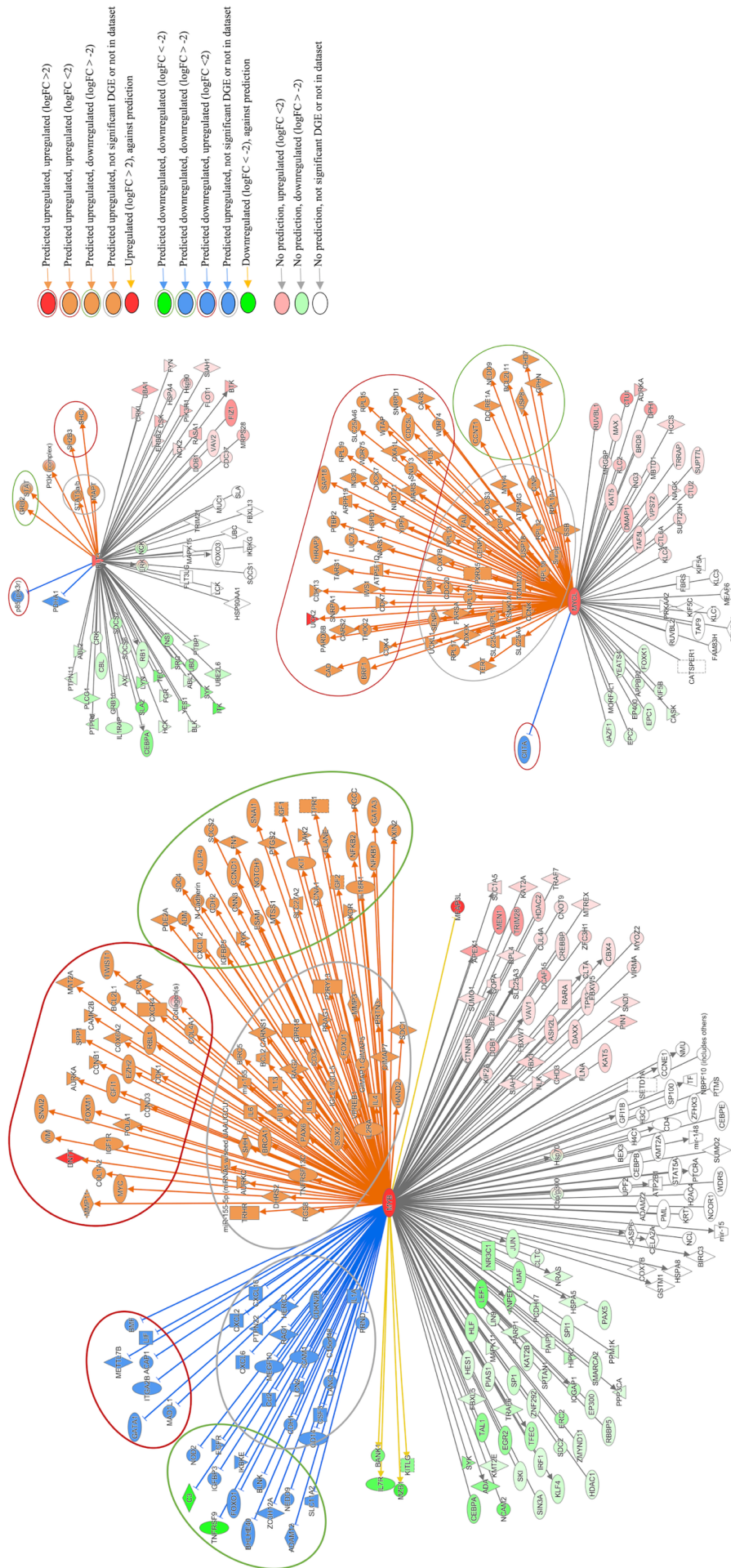
Figure 8: Experimentally observed direct downstream interactions to the Ingenuity Knowledge Base (Qiagen®) in cancer for (a) MYB, (b) FLT3 and (c) MYCL. IPA pathways grow function with overlay of log2FC values from Lymphoma *vs* KoRV positive koalas (original cohort).

Genes of interest within Figure 8 are predicted and observed upregulated and downregulated genes. Those with a $\log_2$FC of > 2 and < -2 between lymphoma and KoRV positive koala samples were: C3, DNTT, TNFRSF9 and UCK2. These genes are also within the top 50 upregulated and top 50 downregulated genes of the lymphoma against KoRV positive cohort. Those genes in Figure 8 predicted to be upregulated with an observed $\log_2$FC of < 2, > 0.5 were ARPP19, ATP5F1D, AURKA, BCL2L1, BRF1, CAD, CAMK2B, CARS1, CARS2, CCNB1, CCND3, CDC5L, CDK1, CDK13, CDK4, CDK7, Col1A2, COL4A1, Collagens, COX6A2, CXCR4, DOCK7, EZH2, FOXM1, GFI1, HSPD1, IGF1R, INO80, IWS1, LUC7L3, MAT2A, MMP11, MYC, NUDT21, OXA1L, PARD6B, PCNA, POLA1, PTBP2, PUS1, RBL1, RPL15, RPL19, SAP18, SH2B3, SHC1, SLC25A46, SNAI2, SNRPA1, SNRPD1, SNU13, SPP1, TARS1, THOC2, THRAP3, TWIST1, VIM, WDR74, WDR75, WTAP, YIPF1. Those genes in Figure 8 predicted to be downregulated with an observed $\log_2$FC of <0.5, > -2 were ADAM12, BHLHE40, BLNK, EGFR, FOX01, IGFBP3, IKBE, NEDD9, NOD2, SLC11A2 and ZC3H12A. When comparing these genes with the 391 commonly differentially expressed genes DNTT, NEDD9, NOD2, TNFRSF9 and UCK2 are common to both lists.

Enriched pathways

Enriched pathways within this comparative analysis of lymphoma and KoRV positive non-lymphomic koalas support environment immunosuppression in lymphomic koalas. Several pathways in both the innate and adaptive immune response have been shown to be downregulated (predicted inhibition shown in blue), both in the calculated summary (Figure 9) and enriched canonical pathways (Figure 10).
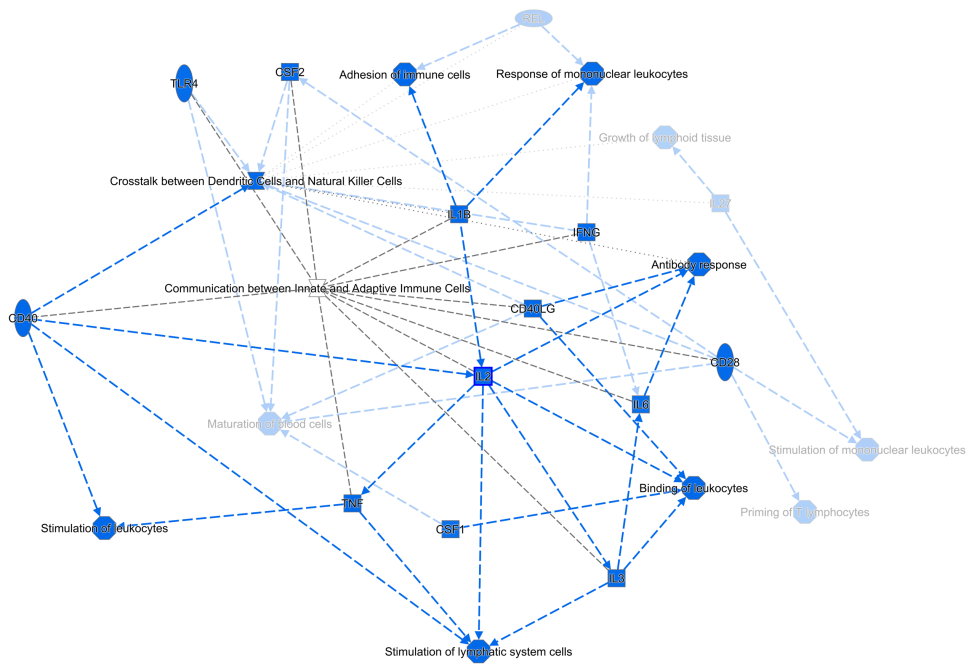
Figure 9: IPA enriched pathway summary graphic highlighting the inhibition of immune pathways and downregulated immune genes.
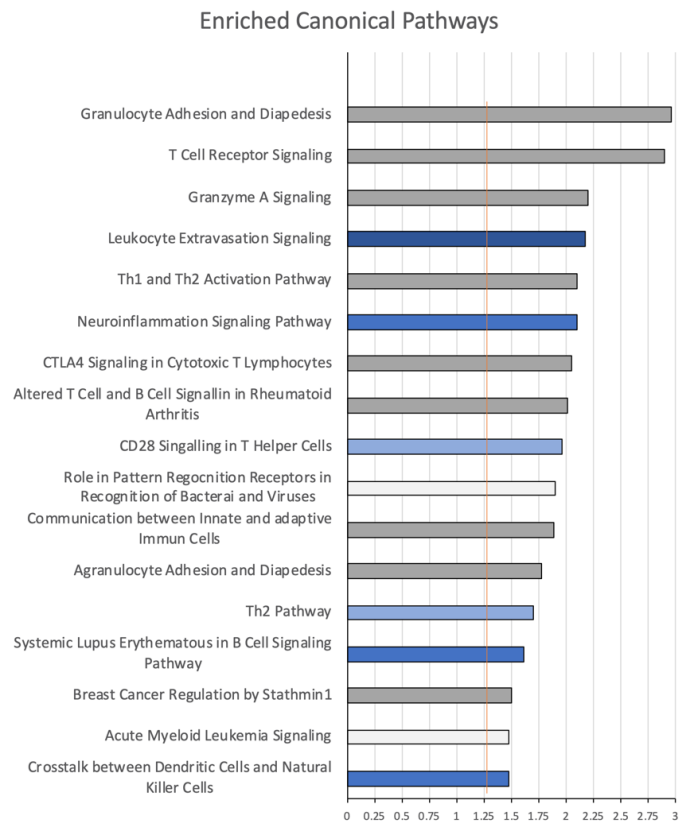
Figure 10: Enriched canonical pathways in the analysis of lymphoma vs KoRV positive koalas. The shown enriched canonical pathways are involved with immune function, cell cycle, growth and differentiation and cancer, blue demonstrates predicted downregulation and grey indicates IPA analysis unable to currently predict outcome, log(p value) on x-axis, threshold of significance is the orange line.

Figure 11 (on next page) shows some of the canonical pathways overlayed with expression ($\log_2$FC) from the lymphoma and KoRV positive analysis, with further predicted activation or inhibition through IPA MAP analysis. MAP analysis draws upon the Qiagen Ingenuity Database (Qiagen ®) and will predict inhibition or activation of the genes in your dataset based on large input of published knowledge, datasets and datamining. There are several genes and gene clusters repetitive within these pathways, including NF-κB, IKK, IκB, interleukins (especially IL2, IL4 and IL10), cytokines, and complement (especially C3 and CD28). As seen in Figure 11, the downstream effect of the inhibition and downregulation of these genes may inhibit immune responses and contribute to immunosuppression.

Figure 11: Genes and predicted gene interactions within enriched pathways from the DGE analysis of KoRV positive koalas diagnosed with lymphoma (n = 3) against KoRV positive koalas (n =3). Pathways produced in IPA canonical pathway analysis

5.5 Discussion

Key findings from the analysis of RNAseq data from KoRV positive koalas with lymphoma, KoRV positive koalas and KoRV negative koalas are: the high differential expression of KoRV genes in lymphomic tissue; the differential expression of key oncogenes in lymphomic koalas; the enrichment and inhibition of multiple immune pathways in KoRV positive koalas with lymphoma; and the lack of differential gene expression (DGE) between KoRV negative and non-lymphomic KoRV positive koalas in this cohort.

Since the discovery of KoRV, its association with lymphoid neoplasia has been established with multiple studies showing the presence of virions in neoplastic bone marrow (Canfield *et al.* 1988) high proviral and viral loads in koalas diagnosed with lymphoid neoplasia (Tarlinton *et al.* 2005; Xu *et al.* 2013; Fabijan *et al.* 2017; Sarker *et al.* 2020a). In this study we observed KoRV *env* 1 and *pol* genes to be significantly upregulated in lymphomic koalas, suggesting that upregulated KoRV transcription may be important in the pathogenesis of lymphoma in koalas. Retroviral transcription relies on host polymerases to replicate the provirus (Rabson *et al.* 1997), therefore the high expression of *pol* transcripts in lymphoma tissue would mean high virion production. In bovine leukaemia virus (BLV), *env* gene expression was found to not always be present in lymphoma tissue (Hemmatzadeh *et al.* 2008) with potential silencing of viral expression (Merimi *et al.* 2007), therefore suggestive of initial viral induction of transformation to malignancy, rather than continued overexpression of virus as found in this KoRV study. This may in part be due to the simple structure of KoRV in comparison to the oncogene-carrying BLV. High viraemia in KoRV may increase the rate of mutagenesis and recombination, with possible induction of

oncogenesis through evasion of the immune system or immunosuppression (Denner *et al.* 2013).

In the analysis of KoRV positive with lymphoma and KoRV positive koalas there were 939 genes that were significantly differentially expressed with log_2FC of > 2 or < -2. Based on the other cohort analyses against the other cohorts tested, previous retroviral associated genes and Ingenuity Database® (prior knowledge), it is likely that there are three key genes that play a role in the pathogenesis of KoRV-induced lymphoma, these are MYB, MYCL and FLT3. MYB is necessary for the normal function and development of red and white blood cells (Sumner *et al.* 2000; Thomas *et al.* 2005; Vegiopoulos *et al.* 2006; Lieu *et al.* 2009) and the oncogenic function of MYB was first reported chickens infected with avian myeloblastosis virus (AMV). AMV is an alpharetrovirus known to cause acute myeloblastic leukaemia (AML) (Perbal 2008). Since the investigation into MYB in relation to induction of AML by AMV, other species and other neoplastic conditions have been associated with the dysregulation of MYB. Acute transforming oncogenic retroviruses AMV and the avian E26 leukaemia virus contain v-*myb* within their genome (Shen-Ong 1990; Engelke *et al.* 1994). Others, such as murine leukaemia virus (MuLV), feline leukaemia virus (FeLV) and avian leukosis virus (ALV) also have dysregulation through insertion. ALV induces B-cell lymphomas through integration in the c-*myb* locus (Clurman *et al.* 1989). MuLV insertions and proviral hybridization with the MYB gene in BALB/c mice increased activation of MYB possibly creating a pre-leukaemic state, prior to other proto-oncogene dysregulation (Belli *et al.* 1995). MYB is noted as a weak transformer of cells and has been shown to cooperate with other signalling molecules, such as KIT to drive leukaemogenesis (Ferrao *et al.* 1997). Gene targets of MYB have also shown to include other oncogenes such as MYC (Schmidt *et al.*

2000) and BCL2 (Frampton *et al.* 1996), and MYB gene activation is key in multiple leukaemic and lymphomic transformations (Pattabiraman *et al.* 2013).

MYCL is a member of the MYC family of genes, with c-MYC being a well-studied oncogene of the family of transcription regulators, especially in retroviral induced tumorigenesis (Uren *et al.* 2005). MYCN is another member of this family, with research showing its activation in MLV-induced leukaemias. MYC and MYCN have been shown to have strong collaborative mutagenesis properties with expression of *Pim* serine/threonine kinases (PIM1, PIM2, PIM3) (Mikkers *et al.* 2002; Uren *et al.* 2005)and in FeLV, c-MYC and PIM1 are two of six clonal insertion sites (CIS) demonstrating a significant role in FeLV lymphanogenesis (Fujino *et al.* 2008). MYC and MYB have also been shown to have synergistic properties, augmenting retroviral-induced lymphomas (Hanlon *et al.* 2003). Both these findings have been seen in this study, with increased expression of PIM2, MYC, MYCL and MYB, therefore these interactions may be playing key roles in tumorigenesis in KoRV-induced lymphomas.

Fms-like tyrosine kinase 3 (FLT3) is a tyrosine kinase receptor in the same family as receptors such as KIT and FMS (Ullrich *et al.* 1990). KIT and FMS are viral oncogenes in feline sarcoma virus (FeSV) (Levy 2011), and all of these genes have been shown to be upregulated through proviral insertion in the gene loci. MuLV-like endogenous retroviral fusion with FLT3 has also been found to promote B-cell acute lymphoblastic leukaemia in mice (Johnson *et al.* 2014) and has been reported upregulated in the majority of humans with acute myeloid leukaemia and acute B-cell leukaemia (Carow *et al.* 1996; Reilly 2003).

Other genes identified within this DGE analysis may hold interest for future consideration. In this study there was high upregulation of MMP9, a zinc-dependent matrix metalloproteinase, known to enhance cancer metastasis and lymphangiogenesis in mice and cell culture (Chang *et al.* 2001; Yoo *et al.* 2011). MMP9 is also upregulated in HEK293T cells infected with KoRV (Sarker *et al.* 2020b). MMP9 expression in non-small cell lung cancer has been shown to be GABA receptor (GABR$_{A3}$) expression dependent (Liu *et al.* 2016). This is also consistent with findings in this study, for which expression of GABRR2, a GABA receptor of the GABA-A family, is significantly upregulated. PIM2, a serine/threonine kinase, was also significantly upregulated, and i been shown to have similar attributes to that of its family member and oncogene PIM1. PIM1 is dysregulated in Moloney MuLV-induced T-cell lymphomas (Uren *et al.* 2005; Maeda *et al.* 2008). PIM2 has also been shown to have anti-apoptotic properties, promoting cell survival in multiple myeloma cell lines (Asano *et al.* 2011) and an independent promoter of leukocyte growth and survival (Hammerman *et al.* 2005).

Multiple immune pathways were enriched within this dataset, with a significant proportion predicted to be inhibited, leading to the downregulation of immune response, antiviral response and inflammation. Development of neoplasia as a result of retroviral immunosuppression has been demonstrated in humans with AIDS, and in rhesus macaques (*Macaca mullata*) with simian immunodeficiency virus (SIV) (Levy 2011). The lymphomas from these infections are B-cell origin, multicentric, widespread and associated with Epstein-Barr Virus (EBV) or simian homologue of EBV (Levy 2011). AIDS non-Hodgkin lymphoma (AIDS-NHL) has multiple subtypes, but all have cytokine dysregulation, mutations in oncogenes and tumour suppressor genes, coinfection with EBV or Kaposi's Sarcoma herpesvirus (KSHV) and low CD4+ cell counts (Bernstein *et al.* 2006; Levy 2008). CD4+

cells differentiate into different T-helper cells, dependent of the antigen stimulation, therefore reduction of these cells, as seen in AIDS, SIV-induced immunodeficiency, MuLV induced murine immunodeficiency (MAIDS) would lead to inhibition of Th1 and Th2 pathways (Mosier *et al.* 1985; Morse *et al.* 1989; Bernstein *et al.* 2006; Klapproth *et al.* 2009; Levy 2011). Cytokine dysregulation in lymphomic koalas is suggested in this study, with CCR4, CCR7, CCR8, CD28, CD247, CXCR5, GATA3, IL10, ILR6, ILR7, IRF6, IRF8, TNFSF8 (CD30LG) all being significantly downregulated ($\log_2$FC < -2, adj. *P* value < 0.05). Combining these effects predicted inhibition of Th1/Th2 signalling and NF-κB signalling pathways. There is an AIDS-like syndrome seen in koalas, in the form of hypocellularity of bone marrow, lymphoid depletion of the spleen, lymphopaenia and neutropaenia, along with diseases suggestive of immunocompromise are reported in association with KoRV (Hanger 1999; Hanger *et al.* 2003). NF-κB pathways have shown contradictory responses to various retroviral infections and cellular transformation: for example, in v-ABL transformed cells, both inhibition and activation of NF-κB pathways have been demonstrated (Wilson *et al.* 2010). Activation of NF-κB have also been shown to increase the rate of apoptosis in MYC-transduced murine and human lymphomas (Klapproth *et al.* 2009). Inhibition of the NF-κB immune pathways have shown to be critical in the malignant progression of MYC-induced tumours (Klapproth *et al.* 2009). It is a possibility that the inhibition of NF-κB pathways are also critical in oncogenesis in koalas, due to upregulation of MYC, MYB and MYCL to avoid NF-κB-induced apoptosis of lymphoma cells.

This study found no differential expression between KoRV negative and KoRV positive koalas. This is likely to be a due to the biological variance of the koalas in both groups or could represent limited differences in the effect of KoRV in low proviral load koalas compared with KoRV negative koalas. Another factor influencing this would be the

classification of the negative 3 sample (K17-019). These factors could strongly affect the DGE classifications in this analysis. This sample was negative in all its proviral testing (as reported in Chapter 2), but from the RNAseq alignment to the KoRV genome, it showed homology and similarity to the KoRV positive koalas. It is most likely that this was an acute infection, and therefore not detected in proviral DNA with the multiple PCRs carried out. Unfortunately, removing this from the negative group reduced the replication in that group down to two, which is less that ideal to draw conclusions from. However, placing negative 3 (K17-019) into the KoRV positive group, showed only adiponectin (ADIPOQ) with differential expression. Adiponectin is mainly expressed in adipose tissue, but has been demonstrated to have some anti-inflammatory effects, inhibiting NF-κB and affecting IL10 in macrophages (Ouchi *et al.* 2007). Proviral expression is low in SA koalas overall, and therefore may influence the impact of KoRV at the individual level (Tarlinton *et al.* 2017). Investigation of koalas that had higher proviral loads, for which the cases are more limited in SA, may tease out some possible immune related cellular pathways that KoRV impacts upon, prior to development of lymphoid neoplasia.

One way to counteract and reduce biological variance, which is a considerable hurdle in disease studies with wild species, can be through increased replication of samples. Unfortunately, the opportunity to extract RNA from lymphoma tissue in koalas, particularly in free-ranging South Australian koalas, was minimal. This limited the ability find clear genes affected by KoRV in the incitement of lymphoma, and to find differential expression in KoRV positive and KoRV negative koalas. However, this is the first study to directly compare lymph node tissues in lymphomic, KoRV positive and KoRV negative koalas and DGE analysis revealed possible insights into the pathogenesis of lymphoma in koalas and

review of these selected genes supports their potential role. Further analyses of qPCR gene expression studies in multiple animals may also shed more light on their involvement.

5.6 Conclusion

Three genes, MYB, MYCL and FLT3 are significant candidates in the pathogenesis of KoRV-associated lymphoma in SA koalas. Exploring these genes in a greater number of samples through qPCR gene expression studies and examining for common sites of viral insertion (CIS) from these, and greater numbers of, koalas would increase certainty to their contribution if gene expression was consistently increased and KoRV proviral promotion of transcription was found. Inhibition of immune pathways in lymphomic tissue was also a key finding of this study and would enhance the transition to malignancy through the evasion of the immune system. Although there is an inability to decipher the progression of oncogenesis from this cross-sectional analysis, previous studies have suggested inhibition of NF-κB is needed prior to tumorigenesis. Overall, the interplay of MYB, MYCL and FLT3 oncogene expression and immunosuppression are likely to be paramount in the formation of KoRV-induced lymphoma in koalas.

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This chapter contains two related papers, the first is the main paper and the second is a support paper.

1. *Pulmonary actinomycosis in South Australian koalas, Published in Veterinary Pathology in January 2021 (First authorship)*

2. *Hypertrophic osteopathy secondary to pulmonary actinomycosis in South Australian koalas, Published in the Australian Veterinary Journal in January 2021 (Second authorship)*

This chapter highlights novel pathologies found in this cohort of necropsies, demonstrating the continued need for broad studies into koala disease. Without large cohorts like these the emergence of new or unrecognised diseases, along with new pathogens, may not be found. This chapter reports two new disease presentations found in koalas, the first - pulmonary actinomycosis, the second - hypertrophic osteopathy.

There were 17 cases of pulmonary actinomycosis report in this paper, 15 from this study cohort and two from external veterinarians. Culture discovered a novel species for which the closest known bacteria is *Actinomyces timonensis*. Further biochemical, morphological and genomic data is needed to fully describe this new species of *Actinomyces*. Pulmonary actinomycosis was not associated with KoRV infection.

The second part to this chapter is a support paper, first author was Dr Joanna Griffith, which collates a subset of these 17 cases and shows four of them to have hypertrophic osteopathy secondary to pulmonary actinomycosis. Since the writing and publication of this paper, there have been at least a further three koalas in the MLR population with this disease presentation.

Statement of Authorship

Title of Paper	Pulmonary Actinomycosis in South Australian Koalas (Phascolarctos cinereus)
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Principal Author

Name of Principal Author (Candidate)	Dr Tamsyn Stephenson
Contribution to the Paper	Conceptualization, investigation, methodology, formal analysis, writing; original draft preparation, writing; review and editing and funding acquisition.
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

   i.   the candidate's stated contribution to the publication is accurate (as detailed above);

   ii.   permission is granted for the candidate in include the publication in the thesis; and

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Original Article

Pulmonary Actinomycosis in South Australian Koalas (*Phascolarctos cinereus*)

Tamsyn Stephenson[1] , **Ken Lee[1], Joanna E. Griffith[1],
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and Lucy Woolford[1]**

Abstract

Pneumonia has been reported in both free-ranging and captive koalas and a number of causative agents have been described. Between 2016 and 2019, 16 free-ranging and 1 captive koala (*Phascolarctos cinereus*) from the Mount Lofty Ranges of South Australia were identified with pyogranulomatous lobar pneumonia, which involved the left caudal lobe in 14/17 (82%) cases. Within lesions, numerous gram-positive or gram-variable, non-acid-fast filamentous bacteria were observed in association with Splendore-Hoeppli phenomenon. Culture yielded growth of anaerobic bacteria, which were unidentifiable by MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry) analysis in 5/5 cases. Sequencing of the bacterial 16S rRNA gene identified a novel *Actinomyces* species in 4 samples, confirming a diagnosis of pulmonary actinomycosis. Concurrent examination of resin lung casts from healthy koalas suggested greater laminar flow of air to the left caudal lung lobe in koalas. *Actinomyces* spp. have been reported as commensals of the oral microbiome in other species, and an association with similar pulmonary lesions in other species. Considering the predilection for involvement of the left caudal lung lobe, aspiration is suggested as the likely cause in some cases of pulmonary actinomycosis in koalas. Pulmonary actinomycosis has not been previously described in koalas and further work needs to be undertaken in order to classify this organism within the *Actinomyces* genus.

Keywords

actinomycosis, *Actinomyces*, etiology, pneumonia, *Phascolarctos cinereus*, Marsupialia

Pneumonia has been reported in both free-ranging and captive koalas with varying prevalence between the states of Australia, ranging from 0% ($n = 44$) in free-ranging koalas of Victoria,[43] 4% (3/85) in free-ranging koalas in South Australia,[54] to 21% ($n = 28$) in captive koalas in New South Wales.[3]

Pathogens reported to cause pneumonia in koalas include *Bordetella bronchiseptica*,[14,39] *Chlamydia* spp.,[9,17,35] and *Cryptococcus gattii* (previously *Cryptococcus neoformans var. gattii*).[14,27] *B. bronchiseptica* has been identified in outbreaks of pneumonia within captive koala colonies in Queensland[39] and New South Wales,[13,14] either as a primary pathogen or secondary to other diseases such as cryptococcosis or chlamydiosis.[14] Association of *Chlamydia* spp. (*C. pecorum*, *C. pneumoniae*) with a rhinitis/pneumonia complex of koalas has been well documented.[11,46] In 1984, when *C. pecorum* was categorized as *C. psittaci*, 1 in 12 koalas diagnosed with rhinitis-pneumonia complex had *C. psittaci* cultured from nasal samples.[8] However, chlamydial pneumonia had not been definitively reported as a primary agent until recently when Mackie et al identified *C. pecorum* as the cause of pneumonia in a juvenile koala.[35] *Cryptococcus gattii* can cause an extensive fungal pneumonia, which can disseminate to other tissues,

including the central nervous system.[27] Cryptococcal infections have been reported in koalas in Queensland[39] and New South Wales,[13,27] but not in Victoria or South Australia. Other documented infections associated with pneumonia in koalas include *Pseudomonas aeruginosa*,[13,44] *Nocardia asteroides*,[57] *Staphylococcus epidermidis*,[57] *Staphylococcus aureus*,[8] *Mycobacterium ulcerans*,[41] α-hemolytic *Streptococcus* spp.,[8] *Corynebacterium* sp. and other unidentified diptheroids,[8] *Pasteurella* spp.,[8] *Acinetobacter lwoffii*,[8] *Aspergillus* spp.,[8] and a parasitic pneumonia associated with *Marsupostrongylus* sp.[38]

This study describes the gross and histopathological features and the microbiological investigations of the first reported

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cases of pulmonary actinomycosis in koalas and proposes its pathogenesis and significance.

Materials and Methods

Routine postmortem examinations were conducted between October 2016 and August 2019 on free-ranging and captive koalas from the Mount Lofty Ranges and surrounding suburbs of Adelaide, South Australia. Koalas were euthanized based on veterinary decision, due to clinical findings indicating a poor prognosis for rehabilitation. Bodies were submitted to the University of Adelaide as part of a broader study into koala pathology (Adelaide University Animal Ethics Committee approval S-2016-169). Sex, tooth wear class (TWC) as an estimate of age,[37] and body condition score (BCS)[9] of koalas were determined. From the broader study, cases with grossly evident lung lesions were identified. Representative tissue samples were placed in 10% buffered formalin for histopathological investigation. When postmortem examinations were performed within 24 hours of death, Amies dry swabs (Copan Italia) were taken from lung lesions for microbiological culture. Blood samples taken during clinical assessment or postmortem spleen samples were collected for koala retrovirus analysis.

Histopathology

Tissues were processed routinely, embedded in paraffin, and sections cut at 4 μm. Slides were stained with hematoxylin and eosin (17/17), Gram (17/17), periodic acid–Schiff (PAS; 17/17), modified Ziehl-Neelsen (15/17), Alcian blue (13/17), and Giemsa (13/17). Formalin-fixed paraffin-embedded (FFPE) tissue from animals that had undergone advanced autolysis ($n = 4$) were not subjected to all special stains.

Microbiological Investigations

Swabs of lung lesions (5/17) were initially plated on sheep blood agar and MacConkey agar plates (Thermo Scientific) and grown both aerobically and anaerobically. No koalas that had microbiological investigations performed had received antimicrobial therapy prior to euthanasia. Unidentified anaerobic colonies were subcultured for purity and identification on anaerobic agar (Thermo Scientific) in aerobic and anaerobic conditions, as well as on actinomyces agar (Thermo Scientific) in anaerobic conditions. Anaerobic conditions were achieved by placing AnaeroGen 2.5 L pack (Thermo Scientific) and CampyGen 2.5 L pack (Thermo Scientific) in a sealed culture jar with an anaerobic indicator and incubated at 37.0 °C for 48 to 72 hours. The isolates were analyzed by MALDI-TOF-MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry). Isolates that were not identifiable by MALDI-TOF-MS analysis or not speciated to a previously identified commensal of the marsupial oral microbiome or previously known respiratory pathogen were further investigated by PCR (polymerase chain reaction) for 16S rRNA. DNA was extracted from pure cultures using QIAMP DNAEasy Minikit (Qiagen) as per manufacturer's instructions. Conventional PCR was run on 8 samples with primers to the 16S rRNA sequence.[58] Purified PCR products were sent for Sanger Sequencing at Australian Genome Research Facility, and BLAST (NCBI) was used to align the sequence to any known bacteria.

Resin Study

In order to assist the understanding of lesion distribution, resin studies of bronchial anatomy were undertaken in 4 koala cadavers, acquired following euthanasia due to non-respiratory morbidities. Tracheal and bronchial washes were carried out with heparinized saline (3×) and then water until the wash ran clear. Epoxy resin (Barnes) was syringed into the airways in situ via a transverse incision through the cervical trachea and allowed to cure overnight with the koala in an upright position. The lungs were removed from the body and the tissue digested in a 5% w/v sodium hydroxide bath. A rotary tool (Dremel) was used to isolate the bifurcation of the trachea and the branching of the lobar bronchi from the left and right primary bronchi in the resin cast.

PCR Analyses

DNA was extracted from either whole blood collected antemortem (when available) or spleen using QIAMP DNAEasy Minikit (Qiagen) as per manufacturer's instructions for koala retrovirus PCR analysis. DNA was extracted from affected FFPE lung tissue using QIAMP DNA FFPE Tissue Kit (Qiagen) as per manufacturer's instructions for *Chlamydia pecorum* PCR analysis. All extracted DNA was measured on NanoDrop One spectrophotometer (Thermo Fisher Scientific) and then stored at −20 °C until testing. The 20 μL PCR reaction consisted of 4 μL AllTaq Mastermix (Qiagen), 3 μL of 1/10 dilution DNA template, and 10 μL of PCR-grade water. For detection of koala retrovirus proviral DNA 0.5 μM of forward primer 5′-TCCTGGGAACTGGAAAAGAC-3′ and 0.5 μM of reverse primer 5′-GGGTTCCCCAAGTGATCTG-3′[56] was added (321 bp amplicon). For detection of *C. pecorum* OMP gene, 1 μM of forward primer 5′-CCAAGCATAATCGTAACAA-3′ and 1 μM of reverse primer 5′-CGAAGCAAGATTCTTGTC-3′[24] was added (140 bp amplicon). PCR conditions were the following: initial activation and denaturation of 95 °C for 2 minutes, followed by 40 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C for 15 seconds, and extension at 72 °C for 10 seconds. This was followed by a final extension step of 72 °C for 10 seconds. Electrophoresis was used to separate the PCR product on 1.0% agarose gels with samples loaded in combination with gel red, visualized with UV light.

Table 1. Case Details of Mount Lofty Ranges Koalas With Pyogranulomatous Lobar Pneumonia.

Koala[a]	TWC[b]	BCS[c]	Sex	KoRV[d]	*C. pecorum*[e]	Lobe distribution[f]	Gram stain[g]
1	5	3	Female	Positive	Negative	LCa, RM	Positive
2	6	3	Female	Negative	Negative	LCa, RM	Positive/variable
3	5	3	Female	Negative	Negative	RCr, RM	Variable/negative
4	4	3	Male	Negative	Negative	LCa, RM	Positive/variable
5	2	3	Female	Negative	Positive	LCa, RM	Positive/variable
6	5	3	Female	Positive	Positive	LCa, LCr	Variable
7	4	3	Male	Positive	Negative	LCa, RCa	Positive
8	6	3	Male	Negative	Negative	LCa	Positive
9	4	5	Female	Positive	Positive	LCa, RM	Positive
10	4	1	Male	Positive	Positive	LCa	Positive/variable
11	3	3	Male	Positive	Negative	LCa	Variable/negative
12	5	3	Male	Positive	Negative	LCa, RM	Positive
13	5	4	Male	Negative	Negative	LCa, RM	Positive
14	6	2	Female	Positive	Positive	LCa, LCr, RCa, RM, RCr	Positive
15	3	3	Female	n/d	Negative	RCa, RM, RCr	Positive
16	3	3	Male	n/d	Negative	RCa, RM, RCr	Positive
17	2	4	Male	n/d	Negative	LCa, LCr	Positive

Abbreviations: TWC, tooth wear class; BCS, body condition score; KoRV, koala retrovirus; PCR, polymerase chain reaction; FFPE, formalin-fixed paraffin-embedded.
[a]Bold type denotes cases subjected to culture.
[b]Tooth wear class.[36]
[c]Body Condition Score.[9]
[d]KoRV proviral DNA PCR on whole blood or spleen tissue.
[e]*Chlamydia pecorum* PCR on FFPE lung tissue.
[f]LCa = left caudal, LCr = left cranial, RCa = right caudal, RM = right middle, RCr = right cranial.
[g]Gram stain attributes of filamentous bacteria associated with Splendore-Hoeppli phenomena.

Statistical Analysis

Chi-squared analyses were carried out in Microsoft Excel v. 16.31 to find any interactions of sex, TWC, BCS, koala retrovirus or *C. pecorum* status with pulmonary actinomycosis.

Results

Gross Pathology Findings

Nine male and 8 female koalas were diagnosed with lobar pneumonia (Table 1); there was no statistical association with sex ($P = .81$). There were 5 young adult koalas (TWC II/III), 9 adult koalas (TWC IV/V), and 3 aged koalas (TWC VI/VII). No juvenile koalas were represented. Older koalas represented 12/17 (71%) of affected koalas (TWC $\geq$IV), although this was not statistically significant ($P = .09$). A low BCS ($\leq$3) was found in 14/17 (82%) affected koalas ($P = .01$). Comorbidities were present in all koalas: 3 presented with additional lesions of the upper respiratory system including ulcerative stomatitis ($n = 1$) and fungal (candidiasis) glossitis ($n = 2$). Lesions consistent with urogenital chlamydiosis were evident in the majority of koalas ($n = 14$) and included mild-moderate ulcerative cystitis ($n = 10$), paraovarian cysts ($n = 4$), urethritis ($n = 1$), prostatitis ($n = 1$), conjunctivitis ($n = 5$), and mild "wet bottom" (pericloacal urine staining) ($n = 3$).

The lobar pneumonia involved the left caudal lung lobe in 14/17 (82%) of the affected koalas. Of these 14 cases, 3/14 had only the left caudal lung lobe affected, 6 had both the left caudal lung lobe and the right middle lung lobe predominantly involved (Fig. 1), and 5 had involvement of 2 or more lung lobes. Only 3/17 (18%) cases had no involvement of the left caudal lung lobe (Table 1).

Resin Study

Resin casts demonstrated that the left primary bronchus followed a caudal path and did not branch until deep into the left lung. The right primary bronchus branched much earlier in a more lateral direction from the trachea, into lobar bronchi (Fig. 2 and Fig. 3).

Histopathology

All pneumonias were characterized by a marked extensive chronic-active neutrophilic, histiocytic, and lymphocytic pneumonia (pyogranulomatous pneumonia), with variable multinucleate giant cell formation, necrosis, and fibrosis. There was concurrent intralesional Splendore-Hoeppli material with associated filamentous bacteria (Table 1 and Fig. 4 and Fig. 5). Most (16/17) cases had concurrent mild pleuritis or pleural fibrosis of the affected lobe. Filamentous bacteria were non-acid-fast) and gram-positive or gram-variable (Fig 5). Either gram-positive cocci or gram-negative rods were also observed in 3/17 (18%) cases. In 2 cases there was plant material present (PAS positive) within inflamed and consolidated airways, confirming concurrent aspiration pneumonia. No other slides had PAS-positive elements. Alcian blue stain for detection of
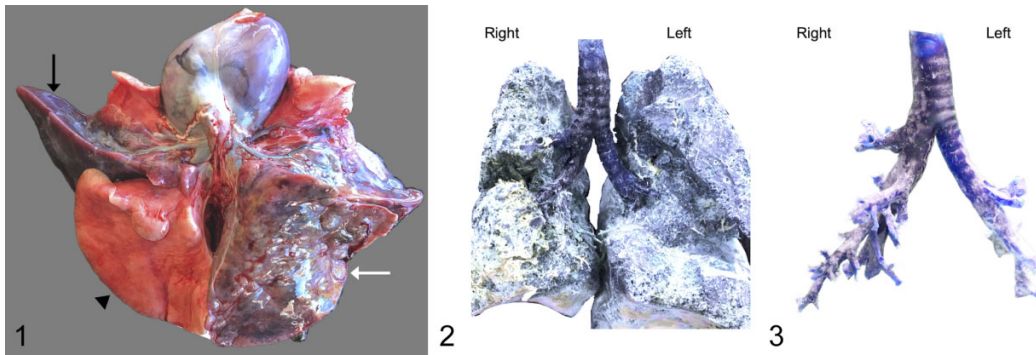
Figure 1. Pulmonary actinomycosis, lung, koala, case 13. Ventral view, heart reflected cranially. There is lobar pneumonia predominantly affecting the left caudal lobe (white arrow) and right middle lobe (black arrow). The right caudal lobe (arrow head) is normal. **Figures 2–3**. Normal, tracheal and bronchial resin cast, koala. **Figure 2**. Entry of bronchi into the lungs. **Figure 3**. The primary left bronchus had a more direct path to the left caudal lobe, in contrast to the branching of the primary right bronchus.
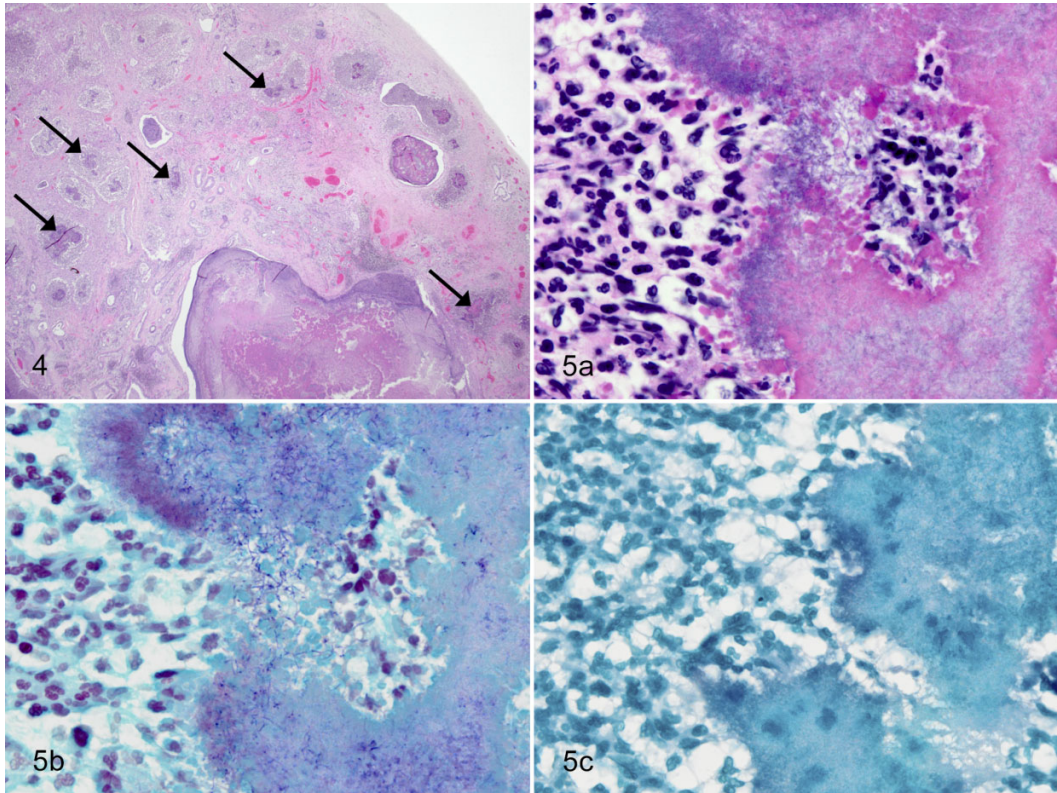
Figures 4–5. Pulmonary actinomycosis, lung, koala. **Figure 4**. Case 6. There is extensive marked chronic-active bronchopneumonia with Splendore-Hoeppli reactions (arrows). **Figure 5**. Case 13. Splendore-Hoeppli phenomenon with associated filamentous bacteria (a, HE) that are gram-positive (b, Gram stain), and non-acid-fast (c, modified Zhiel-Neelsen stain).

Table 2. 16S rDNA PCR Sequencing Results of the Anaerobic Isolates.

Case	Isolate	Bacterial species	Query cover	% Ident
4	1	***Actinomyces timonensis***	99	**94.29**
10	1	*Pseudomonas strutzeri*[a]	99	89.95
	2	***Actinomyces timonensis***	99	**94.92**
	3	*Fusobacterium* clone	99	95.25
15	1	***Actinomyces timonensis***	99	**86.79**
16	1	*Porphyromonus gingivalis* identified on MALDI-TOF-MS		
	2	***Actinomyces timonensis***	99	**95.07**
17	1	*Fusobacterium nucleatum*	99	98.9
	2	*Solobacterium moorei*	98	96.63

Abbreviations: PCR, polymerase chain reaction; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry.
Note: The boldface text highlights isolates that have homology to *Actinomyces*.
[a]From culture investigations, this bacterium grew anaerobically and formed black pigmented colonies. These characteristics are consistent with *Prevotella heparinolytica* as identified on MALDI-TOF-MS analysis (Table S2). This culture did not grow under aerobic conditions and as *P. strutzeri* is a facultative aerobe, this also supports the identification as *Prevotella* spp.

Cryptococcus spp. was negative. Giemsa stain did not identify *Chlamydia* spp. within tissue section. No viral inclusions were observed (Supplemental Table S1).

Microbiological Investigations

Microbiological culture was performed on swabs from 5 koalas. Culture showed no growth aerobically, and either pure or mixed growth in anaerobic cultures. Two of the 9 anaerobic subcultures subjected to MALDI-TOF-MS analysis were identified: Case 10—*Prevotella heparinolytica*; and Case 16—*Porphyromonas gingivalis*. The remaining 7 were not identified with MALDI-TOF MS (Supplemental Table S2) and were identified by PCR amplification and sequencing of the 16S rRNA gene (Table 2).

BLAST analysis (NCBI) from 16S rRNA gene sequencing of pure subcultures ($n = 8$) from 5 cases of pyogranulomatous lobar pneumonia identified 4 cases in which the isolate shared approximately 95% genetic sequence with *Actinomyces timonensis* (Table 2). The closest identified species of other culture isolates were *Fusobacterium nucleatum*, *Fusobacterium* spp., and *Solobacterium moorei*.

Of 14 koalas tested for koala retrovirus A, 8 (57%) were positive (Table 1). Koala retrovirus status was not significantly associated with pulmonary actinomycosis ($P = .59$, $n = 14$).

Of cases tested by PCR for *C. pecorum*, 5 (29%) were positive (Table 1). Detection of *C. pecorum* was not significantly associated with pulmonary actinomycosis ($P = .09$, $n = 17$).

Discussion

The results of this study showed pulmonary actinomycosis in this cohort of koalas was characterized by marked pyogranulomatous lobar pneumonia, most commonly affecting the left caudal lobe. Splendore-Hoeppli phenomenon and intralesional gram-variable non-acid-fast filamentous bacteria, consistent with actinomycosis, were present in all cases. Microbiological culture and 16S rRNA analysis demonstrated a gram-variable, filamentous bacterium with approximately 95% sequence homology to *Actinomyces timonensis* in 4 out of 5 cases subjected to testing. Although the histological lesions in case 17 were consistent with this pathogen, the negative culture may reflect the difficulty in isolating *Actinomyces* spp.[34] These findings support that a significant pathogen in these pneumonia cases is a novel *Actinomyces* species, due to less than 97% similarity to *Actinomyces timonensis*. Pulmonary actinomycosis has not been described in the koala previously, but the observed lesions were consistent with actinomycotic lesions observed in other species.[49,53]

Actinomyces is an anaerobic or facultative aerobic, gram-positive, filamentous bacteria, which is non-spore forming and non-motile.[47] *Actinomyces* species can be found in normal healthy microbiota of the human oropharynx and gastrointestinal tract[55,60] and on nasal, oral, or oropharyngeal mucosal surfaces of other animals[15,47] and are most often associated with opportunistic infections.[47] Pulmonary actinomycosis is rare in animals but has been described in a small number of free-ranging species including 2 chamois (*Rupicapra rupicapra*)[49] and a black-tufted marmoset (*Callithrix penicillata*)[53] and in a captive red kangaroo (*Osphranter rufus*).[28]

The pathogenesis of pulmonary actinomycosis in the koala is unknown and assumed to be non-contagious. In humans, aspiration has been reported as a leading initiator of pulmonary actinomycosis[55,60] with parenchymal or lobar pneumonia often affecting the lower lung lobes.[19,60] Pulmonary actinomycosis in koalas showed a similar lesion distribution, with caudal lung lobes, especially the left caudal lung lobe, affected in the majority of cases, consistent with the bipedal-like position of the koala when it climbs and rests. The lung resin study showed that the left primary bronchus followed a caudal path and did not branch until deep into the left lung, whereas the right primary bronchus branched in a more lateral direction from the trachea prior to entering the lung parenchyma, then into lobar bronchi. A less acute angle of bifurcation creates greater laminar flow.[30] Therefore, if aspiration were to occur, plant material or bacteria from the oropharynx could seed within the left caudal lung lobe more readily. Aspiration pneumonia has been described in koalas,[22] with potentially greater risk of occurrence in captive syringe-fed koalas or hand-raised joeys.[9] In the current study, there were no juvenile koalas and only one captive animal. Aspiration pneumonia, as evidenced by pulmonary inflammation around leaf fragments, was found in 2 of the 17 cases presented here, one of which had a less extensive or low-grade pulmonary actinomycosis and the other had more extensive pulmonary actinomycosis with all lung lobes affected.

Anaerobes are frequently isolated in cases of aspiration pneumonia in other species, including in humans.[4,20,21,48] The non-*Actinomyces* isolates identified in the current study have been described as opportunistic infections from the oral microbiome: *Porphyromonas* spp.,[4] *Prevotella* spp.,[48]

Fusobacterium spp.,[20] and *Solobacterium* spp.[21] In humans, concomitant species are often cultured in addition to the *Actinomyces* sp., and could potentiate the pathogenicity of the *Actinomyces* infection.[34] Investigation into these co-cultured bacteria may provide further insight into the pathogenesis of pulmonary actinomycosis in koalas. *Prevotella* and *Porphyromonas* are common isolates from the oral microbiome of many animals,[5,6,18,51] and *Prevotella intermedia*, *Prevotella nigrescens*, *Porphyromonas gingivalis*-like, *Porphyromonas salivosa*, *Porphyromonas endontalis*, and *Porphyromonas loveana* sp. nov. have been isolated from the oral microbiome of koalas.[1,6,7,42] *Porphyromonas* spp. have also been isolated more frequently in ageing koalas with periodontal disease.[6] We speculate that the isolate from case 16 might be *Porphyromonas gingivalis*-like species[42] or *Porphyromonas loveana.*[7] *Fusobacterium necrophorum* is commonly isolated from macropod progressive periodontal disease,[5,6,40] but has also been isolated from healthy red-necked wallabies (*Notamacropus rufogriseus*), eastern grey kangaroos (*Macropus giganteus*), and red kangaroos,[2,40] demonstrating that *Fusobacterium* spp. are within the macropod oral microbiome. *Fusobacterium nucleatum* is a commensal bacterium of the oral cavity and an opportunistic pathogen in humans.[20] *Solobacterium moorei* has been identified in humans to be associated with halitosis[21] and root canal infections.[50] A similar isolate has been identified in canine and feline periodontal disease,[25] and as sequencing showed less than 97% homology, it is likely that this is also a novel marsupial species. Poor dental hygiene has been increasingly reported in koalas[12,45] and has been recognized as a predisposing factor for pulmonary actinomycosis in humans.[55,60] Dental health scoring was not carried out with this cohort and would be a useful addition to the further investigation of koala pulmonary actinomycosis. A recent study identified increasing periodontal disease with age of koalas,[12] and given that this study showed a trend of pulmonary actinomycosis in older koalas, an association with dental disease is possible.

A reduced body condition score (emaciated, poor or fair) was significantly associated with the presence of pulmonary actinomycosis. It is likely that pulmonary actinomycosis would be a debilitating disease leading to reduced food intake in affected koalas and subsequent loss of condition, due to the fine metabolic balance koalas have between intake and output.[26,29,31–33] Also, koala retrovirus has been associated with the presence of disease[16,52,56,59] and changes in immune function.[23,36] However, analysis of this cohort did not show any association between koala retrovirus and pulmonary actinomycosis, similar to the findings for koala retrovirus and periodontal disease.[12] Other pathogens, such as *C. pecorum*, *Bordetella bronchiseptica*, or *Cryptococcus* spp., cannot be ruled out as initiating pathogens, inciting micro-anaerobic conditions needed for *Actinomyces* to proliferate, although investigations using PCR, culture, and special stains did not find such associations. *C. pecorum* was found in 5 FFPE lung tissue sections and may contribute to the pathogenesis in these koalas or be due to pathogen presence in circulating mononucleocytes.[10] The majority of koalas presented with comorbidities

associated with overt chlamidiosis, which may reflect the reduced fitness or compromised state of these koalas.

This case series presents the first reports of pulmonary actinomycosis in koalas. The pathogenesis likely involves aspiration. Currently this has only been recognized in South Australian koalas, which might indicate an increased susceptibility in this subpopulation. Anatomical or dental microbial origins, among others, warrant exploration as potential risk factors.

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i.   the candidate's stated contribution to the publication is accurate (as detailed above);

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iii.   the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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EDUCATION, ETHICS & WELFARE

EDUCATION, ETHICS & WELFARE

CASE REPORT

Hypertrophic osteopathy in South Australian koalas (*Phascolarctos cinereus*) with concurrent pulmonary actinomycosis

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Pulmonary actinomycosis is described in 17 South Australian koalas necropsied between 2016 and 2019. From these cases, four koalas had secondary hypertrophic osteopathy. Plain radiographical and computed tomography images demonstrated periosteal reaction on multiple appendicular skeletal bones in all cases, including scapula, humerus, ulna, radius, ilium, femur, tibia, fibula, metacarpus, metatarsus and phalanx. Grossly, periosteal surfaces of the metaphyses and diaphyses of long bones were thickened and roughened; microscopically, this was characterised by bi-layered proliferation of well-differentiated trabecular bony spicules oriented perpendicular to the cortex (pseudocortices) and separated by vascular connective tissue, typical for hypertrophic osteopathy. Well characterised in domestic species and rarely reported in marsupials, this is the first radiographical and pathological characterisation of hypertrophic osteopathy in koalas, associated with pulmonary actinomycosis in all cases.

Hypertrophic osteopathy (syn. hypertrophic osteodystrophy, hypertrophic osteoarthropathy, Marie's disease) (HO) is a condition of periosteal new bone formation on the diaphyses and metaphyses of the appendicular skeleton, usually bones of the limbs, and – less frequently – the axial skeleton. It is most commonly associated with a chronic inflammatory or neoplastic process, frequently in the chest cavity.[1] The condition has been described in humans,[2] domestic species (examples include dogs,[3] cats,[4,5] horses[6] and cattle[7]) and a variety of wild animals (reviewed by Thorsson in 2015[8]). In marsupials, HO has been described in one captive red kangaroo (*Osphranter rufus*) in association with pulmonary actinomycosis;[9] a captive wallaroo (*Osphranter robustus*) with severe necrotising pleuro-pneumonia associated with a heavy mixed growth of *Morganella morganii*, β-haemolytic *Streptococcus* and *Bacteroides* spp.[10]; and a wild brush-tailed possum (*Trichosurus vulpecula*) with a primary osteosarcoma involving the oesophageal wall.[11] Pulmonary actinomycosis is rare in free-ranging animals but has been described in non-marsupial species, including chamois (*Rupicapra rupicapra*)[12] and a black-tufted marmoset (*Callithrix penicillata*),[13]

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without the sequelae of HO. In South Australia, pulmonary actinomycosis has recently been described in a case series of 17 koalas (Stephenson et al, accepted 2020[14]). Lesions of pulmonary actinomycosis are characterised by pyogranulomatous inflammation and striking intralesional Splendore-Hoeppli material with associated Gram-positive non-acid fast filamentous bacteria.[9,15]

Here, we describe a case series of four wild South Australian koalas (*Phascolarctos cinereus victor*) presenting with HO associated with pulmonary actinomycosis. HO is previously undescribed in koalas.

Methods

As part of a larger study of koala disease (University of Adelaide Animal Ethics Committee approval number S-2016-169), koalas euthanased for reasons of disease or trauma were opportunistically collected for necropsy from wildlife groups, veterinarians and wildlife parks. In 2018–2019, one long-term captive from a local wildlife park and three wild koalas presenting via these methods were diagnosed with HO using combinations of clinical examination, diagnostic imaging (plain radiography, computed tomography [CT]) and necropsy. Animals were aged, and their condition was scored as previously described (Table 1).[16,17]

Radiography

Plain radiographs were taken of cases 1, 2 and 4. Cases 1 and 2 were sent for further evaluation by CT. CT was performed on entire animals using a Philips™ Ingenuity 128 scanner (Koninklijke Philips N. V). CT images were acquired using exposure variables of 120 kV and 98–113 mA and a rotation time of 0.5 s. Images were acquired in axial 0.67-mm slice thickness with a 0.34-mm reconstruction interval using a sharp YD algorithm and were reconstructed into 3.0-mm axial, coronal and sagittal planes and viewed in the bone window. Three-dimensional (3D) images were created in Osirix Lite v 11.0.2 (©Pixmeo Sarl, DICOM Viewer) with 3D surface-rendering bone settings and refined in MeshLab.[18] Another wild adult male koala, euthanased due to sarcoptic mange and polydipsia, also underwent CT, described as a control animal.

Necropsy and histopathology

After euthanasia with pentobarbitone sodium, case 1 was stored at −20°C for 40 days and then defrosted for 48 h at room temperature, and case 2 was stored at 4°C for 24 h. Case 3 was examined within 1 h of euthanasia, and case 4 was stored at 4°C and examined within

Table 1. Demographic data and HO distribution. Both cases 1 and 2 had a full computed tomography investigation. Case 3 did not have radiographs; therefore, the skeletal extent of the HO was not examined fully. Case 4 did not have complete coverage by plain radiography of the skeletal system

Koala	Status	Gender	TWC[a]	BCS[b]	HO observed (radiographically or grossly)									
					S	H	R	U	MC	I	F	T	Fi	MT
Case 1	Free ranging	M	2	4[c]	•	•	•	•		•	•	•	•	
Case 2	Free ranging	M	3	3	•	•	•	•	•	•	•	•	•	•
Case 3	Captive	M	5	4	ND	ND	ND	ND	ND	ND	•	ND	ND	ND
Case 4	Free ranging	F	3	3		•	•	•	•		•			

[a] Tooth wear class.[16]
[b] Body condition score as per Blanshard.[17]
[c] BCS described as 'adequate'. Key to HO observed, either radiographically or grossly at necropsy in the bones: F, femur; Fi, fibula; H, humerus; I, pelvis (ileum); MC, metacarpi; MT, metatarsi; ND, not determined; R, radius; S, scapula; T, tibia; U, ulna.

24 h. All animals underwent a standard midline necropsy.[16] Representative soft-tissue and bone samples were placed in 10% buffered formalin. After fixation, bone samples were decalcified with 10% formic acid for up to 2 weeks before processing. Tissues were stained routinely with haematoxylin and eosin and examined with light microscopy. Sections of lung were also prepared with Gram and modified Zeihl-Neelsen stains. Routine microbial aerobic and anaerobic cultures of lung lesions and 16S rRNA gene sequencing of anaerobic colonies were carried out in three cases, as described previously in Stephenson et al (accepted 2020).[14]

Results

Clinical examination (summarised in Table 1)

Case 1 was a male koala assessed to be a young adult with a moderate body condition score and suspected to have been hit by a motor vehicle. This koala was presented with an inability to climb with a clenched forepaw and inappetence, but no signs of respiratory disease were evident. Case 2 was an adult male koala with a mildly compromised body condition. This koala was presented with an inability to walk or climb; all paws were swollen (Figure 1) and hot to the touch; and there was bilateral corneal ulceration, but no signs of respiratory disease were evident. Case 3 was a castrated captive male koala, approximately 8 years of age based on husbandry records, with adequate body condition. He was no longer on display due to scoliosis and kyphosis and displayed a reluctance to climb. He had a history of renal disease and bouts of respiratory disease in 2011, 2017 and 2018, and a 'cough' was noted 1 month before euthanasia. Case 4 was an adult female koala with mildly compromised body condition. This koala was presented with an inability to climb, sitting at the base of a tree for 2 days, and abdominal breathing. Cases 1 and 2 were referred and had radiographic investigations (plain and CT). Figure 2 shows plain radiographic findings, and Figure 3 shows CT findings. All cases are summarised in Table 1.

Gross postmortem

All koalas had lobar consolidation of the pulmonary tissue. Cases 1 and 3 had greater consolidation in the left lung lobe fields, with

Figure 1. Swollen forepaw of koala, case 2 (photo credit: Carolyn Vince).

case 3 having prominent lobar demarcation, with the left caudal and right middle affected. Cases 2 and 4 had greater consolidation in the right lung lobes. There were multifocal firm grey-yellow small nodules throughout the affected lung tissue, as described in Stephenson et al (accepted 2020).[14] Gross bone examination showed irregular periosteal thickening of the long bones, as well as prominent periosteal vascularisation, most apparent in the diaphysis (Figure 4). Comorbidities included bilateral colloidal goitre and fungal glossitis consistent with oral candidiasis in case 1; fungal glossitis consistent with oral candidiasis in case 2; severe scoliosis of the thoracic spine and kyphosis of the thoracolumbar spine in case 3; and in case 4, no other abnormalities were recorded at postmortem.

Microbiology

Microbiological examination and identification are fully described in Stephenson et al (accepted 2020).[14] Two of the three lung swabs demonstrated isolates belonging to the *Actinomyces* family.

Histopathology

Pulmonary. All cases had neutrophilic, histiocytic and lymphocytic bronchopneumonia with striking Splendore-Hoeppli phenomenon and associated Gram-positive filamentous bacteria that were modified Zeihl-Neelsen-negative. Cases 1, 2 and 4 had concurrent pleuritis, and case 3 had occasional multinucleate giant cells, haemosiderin-laden macrophages, areas of fibrosis and type II pneumocyte hyperplasia and concurrent pleural fibrosis. All cases were consistent with pulmonary actinomycosis as reported in Stephenson et al (accepted 2020).[14]

Long bone. All cases were diagnosed with HO, demonstrated by a marked increase in thickness of cortical bone caused by a bi-layered proliferation of new bone (pseudocortices), characterised by spicules of well-differentiated trabecular bone oriented perpendicular to the original cortex, which varied mildly in length. These were covered by a thin layer of fibrovascular connective tissue and, often, by a second, less-organised layer of irregularly proliferating spicules of osteoid and cartilage at various phases of ossification, interspersed with loose fibrovascular tissue (Figure 4).

Figure 2. (A) Radiographs from unaffected control koala, hindlimb. (B) Hypertrophic osteopathy, hindlimb, case 1: plain radiograph showing periosteal reaction on femur, tibia, fibula and metatarsal bones (white arrows) and thickening of cortical bone (black arrowheads).

Discussion

HO is an unusual phenomenon whereby periosteal proliferation and periostitis occurs, usually along the long bones of the limbs, resulting in spiculated exostoses. Clinically affected animals have hot swollen limbs, with pain and reduction in movement. HO is divided into primary (idiopathic) HO, now recognised in people to occur as a result of mutations in 15-hydroxyprostaglandin dehydrogenase,[19] and secondary HO, which is most commonly associated with intrathoracic disease.[1] This series includes the first report of secondary HO in koalas. All described cases had intrathoracic disease, specifically pulmonary actinomycosis.

The proposed pathogenesis of HO involves abnormal vascularisation, hypoxia and chronic inflammation, leading to the release of vascular endothelial growth factor (VEGF). Dickinson et al[20] hypothesised that abnormal pulmonary vasculature allows megakaryocytes to enter systemic circulation. Together with platelets, megakaryocytes impact capillaries, interacting with vascular endothelial cells and causing
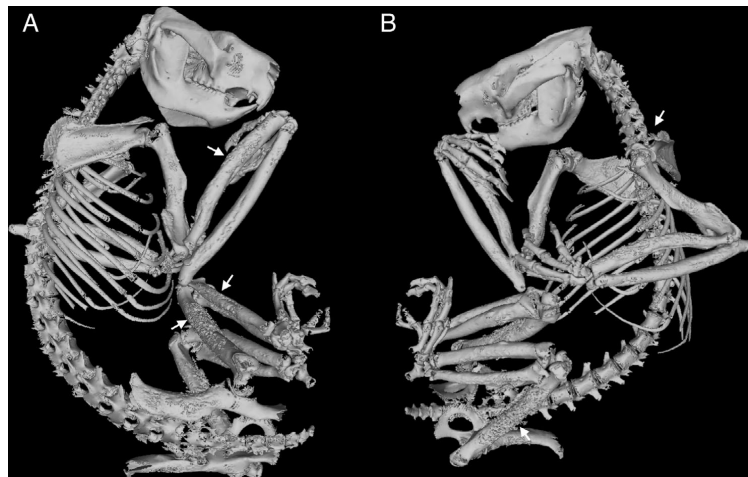
Figure 3. CT images of case 2 showing the extensive irregular periosteal reaction and proliferation throughout the axial and appendicular skeletal system (some areas depicted by white arrows). (A) Left lateral side down, (B) right lateral side down.
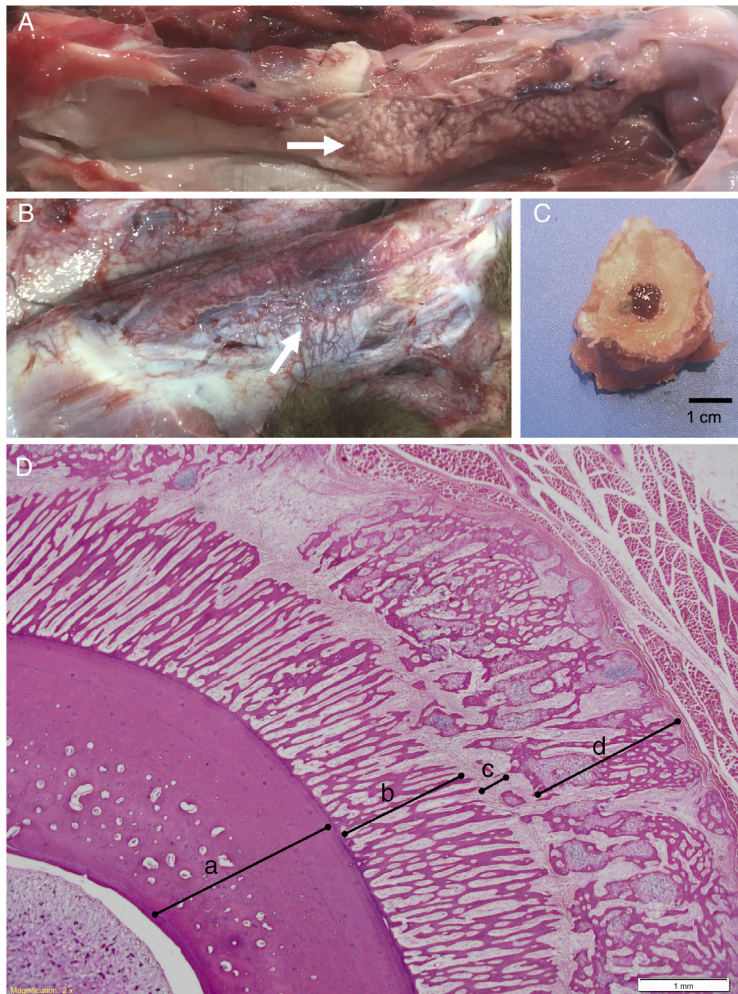
Figure 4. (A–C) Gross appearance of hypertrophic osteopathy. (A) Multifocal to coalescing irregular bony periosteal proliferations along the length of the diaphysis, humerus (white arrow, case 2). (B) Prominent highly vascular and congested connective tissue covering periosteal surfaces (white arrow, case 1). (C) Transverse cut through diaphysis showing marked thickening of humerus (case 2). (D) Histological appearance of hypertrophic osteopathy. There is a marked increase in thickness caused by a bilayered proliferation of cortical bone. The first layer, closest to original cortical bone (a), comprises spicules of well-differentiated trabecular bone oriented perpendicular to the original cortex, which vary mildly in length (b). These are covered by a thin layer fibrovascular connective tissue (c) and a second, less-organised layer of irregularly proliferating spicules of osteoid and islands of cartilage at various phases of ossification, interspersed with loose fibrovascular tissue (d). Femur diaphysis, koala, case 2, haematoxylin and eosin stain.

local hypoxia and the release and activation of inflammatory and growth-promoting factors such as platelet-derived growth factor and VEGF. VEGF stimulates local angiogenesis; capillary permeability and oedema; and proliferation of connective tissue, smooth muscle and bone.[21] Inflammatory factors also potentially play a role in the development of HO. Prostaglandin E2 (PGE2) is thought to act directly by increasing the transcription of VEGF in osteoblasts and indirectly by activating endothelial cells, which themselves increase transcription of VEGF. Furthermore, circulating prostaglandins are thought to undergo first-pass catabolism in the lungs. Aberrant pulmonary vasculature, as occurs in pulmonary disease, is hypothesised to result in higher circulating levels of PGE2, contributing to the development of HO.[22]

Inflammatory lung disease (pneumonia or tuberculosis) was the most common cause of HO in a review of the disease in wild animals, accounting for 15 of 31 cases.[8] All cases in the present study had concurrent pulmonary actinomycosis (Stephenson et al, accepted 2020).[14] The pathogenesis of pulmonary actinomycosis in koalas is in need of further investigation, although aspiration is suspected. The pathogenesis is unknown for pulmonary actinomycosis in other wildlife species, including red kangaroo,[9] black-tufted marmoset[13] and chamois.[12] In humans, aetiology is suspected to be aspiration, with oral hygiene, alcoholism, smoking and underlying respiratory disorders as risk factors.[23]

Morbidity and mortality in koalas across their natural range is relatively well investigated compared with many wildlife species. A

survey of pathology records by koala research groups in Queensland, n = 519[24] and n = 75;[25] NSW, n = 127[26] and n = 28;[27] SA, n = 85;[28] and Victoria, n = 44[29] has been published, with no cases of HO reported. Similarly, no cases of HO were identified in a database of 1391 submissions to the Veterinary Pathology Diagnostic Service (University of Sydney) of mostly (87%) NSW-origin koalas (unpublished, D Higgins). The absence of previous reports suggests that HO is rare in koalas and other marsupials, both captive and free ranging, with the literature only demonstrating three reported cases.[9-11] A captive red kangaroo (*O. rufus*) was reported to have HO secondary to pulmonary actinomycosis,[9] as reported in this case series. These four cases of HO in South Australian koalas presented over a 15-month period (27 April 2018–28 July 2019). Although advanced imaging has increased the ability for this disease to be characterised, plain radiography was sufficient to diagnose the disease when performed and interpreted by experienced koala veterinarians or those who had HO on their list of differentials. This apparent sudden increase in the incidence of cases may suggest an emerging disease syndrome or South Australian veterinarians' improving confidence and willingness to examine and treat koalas.

Most koalas presented on the ground or had trouble climbing, with minimal compromise to body condition. Initial examination of all cases failed to diagnose HO, but presenting signs such as swellings on the limbs or an inability to climb could be presumptively attributed to trauma or weakness associated with other diseases and caused a syndrome such as HO to not be considered. Two of these four cases lacked overt respiratory signs, which has been reported in other species,[6,9,30] Possible hindrances to the clinical detection of HO may include inexperience of veterinarians examining koalas and/or a lack of time and resources to perform diagnostic tests indicated for wild animal cases in a private practice setting. Diagnostic work-up and treatment of wildlife by private veterinary practices are often largely absorbed by the practice, and paying clients may take priority over wildlife cases. One survey of Australian veterinarians identified a lack of time and/or lack of knowledge and skills as the biggest barriers to appropriate treatment of wildlife.[31] Koalas are frequently victims of motor vehicle strike or dog attack[28,32] and, in the Adelaide region, renal failure due to oxalate nephrosis;[28,33] therefore, primary focus can be concentrated on these common morbidities. During necropsy, long bones might not be routinely examined, so it is possible that historic cases of HO in koalas have gone undiagnosed. However, the florid clinical, radiographical and pathological signs of HO in these four cases were readily identified with comprehensive clinical examination and necropsy.

Improving awareness of this uncommon condition will enhance diagnostic accuracy and HO detection. Lameness, demonstrated by koalas as difficulty ambulating and inability to climb, was a unifying clinical sign, and clinical assessment of koalas unable to climb should include radiographic investigations. Detection of elevated serum alkaline phosphatase (ALP) activity may increase clinical suspicion, as has been reported demonstrated in other species with HO.[3,8] A limitation of this study is that ALP analysis was not performed in these koalas; biochemistry analysis is frequently confined to the assessment of azotaemia due to high prevalence of oxalate nephrosis in South Australian koalas and resource constraints (S Craig personal communication). Finally, detection of thoracic lesions at

necropsy should prompt examination of long bones, either through postmortem radiography or transverse sectioning and histopathology.

Conclusion

HO has previously been unreported in koalas and should be considered a differential for lameness, difficult ambulation and/or inability to climb. In all cases, HO was associated with pulmonary actinomycosis. Considering no previously reported cases in the extensive necropsy series of koalas in other states of Australia, and of 85 necropsied koalas in SA, HO secondary to pulmonary actinomycosis may be an emerging disease in South Australian koalas. Routine orthogonal radiography covering the skeletal system or, if possible, CT imaging facilities are paramount for diagnosis, allowing visualisation of HO in koalas and intrathoracic lesions.

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Pathogenesis of infectious agents in South Australian koalas is a relatively new, but important field of investigation to understand the disease threats to this iconic species across Australia. The prevalence of KoRV and *Chlamydia pecorum* in South Australian koala populations provided opportunity for evaluation of the pathogenic effects of infection. Pathological and infectious disease studies across a large cohort of MLR (SA) koalas presenting for necropsy between 2016 - 2019, has allowed more confident differentiation between KoRV positive and KoRV negative koalas (Chapter 2). This clear demarcation of infection status allowed for pathological associations with KoRV to be identified throughout this thesis. High transcription levels across the full KoRV genome were found in isolated RNA from lymphomic lymph node tissue and low transcription levels across the internal region of the KoRV genome in non-lymphomic KoRV positive koalas. This study also highlighted the presence of terminal regions of homology to KoRV in KoRV negative koalas, a previously observed phenomenon (Tarlinton *et al.* 2017) that will require further analysis. Chapter 3 described the association of KoRV with lymphoma and further classified lymphoma in SA koalas, based on anatomical and histological features, and estimated a prevalence of lymphoma in the free-ranging MLR koala population. Chapter 4 explored the association of KoRV with *C. pecorum* and Phascolarctid gammaherpesviruses (PhaHV-1 and PhaHV-2) and described pathological findings. This study found limited association of KoRV infection with various disease presentations in MLR koalas, however a potential augmentation of female urogenital chlamydiosis by PhaHV was identified. Another key finding was the lack of disease in trauma (road traffic accident) koalas, which highlighted a need for the reduction of detrimental anthropogenic effects in conservation. Chapter 5 used RNAseq data to identify promising key genes and pathways in oncogenesis in KoRV positive

koala with lymphoma. Three key oncogenes, MYB, MYCL and FLT3, were found to be differentially expressed in lymphomic koalas. There was also enrichment and downregulation of several immune pathways, which suggested an induction of immunosuppression. As a result of the extensive nature of the necropsy study, several conditions were identified in SA koalas for the first time. Two such conditions are pulmonary actinomycosis and hypertrophic osteopathy, described in Chapter 6.

7.1 Internal region of KoRV genome indicative of infection in SA koalas

In northern koalas, KoRV infection status has been reliably diagnosed on the presence of either a proviral *pol* gene or *env* gene target (Tarlinton *et al.* 2005; Simmons *et al.* 2011; Shimode *et al.* 2014; Chappell *et al.* 2017; Fabijan *et al.* 2017; Legione *et al.* 2017; Waugh *et al.* 2017; Fabijan *et al.* 2019b). However, recent studies on KoRV infections in southern populations have found variability in proviral target results causing confusion in infection status (Sarker *et al.* 2020a). To clarify this issue, proviral, viral and RNAseq analyses of KoRV infections were directly compared in a cohort of South Australian koalas (Chapter 2) and showed that koalas in the Mount Lofty Ranges (MLR), and likely those from other southern populations, can be reliably diagnosed based on gene targets in the internal region of the KoRV genome. Positive koalas (89/216) had all of the internal gene targets (including *pol*) and in negative koalas (124/216) all were absent. Only a small number of cases showed ambiguous results and combining all targets from this internal region (shown with gene targets *gag* 2, *pol*, *env* 1 and *env* 2) increased the confidence in a positive or negative result. The proviral analyses correlated well with viral gene expression in 10 positive and 5 negative koalas by RT-qPCR analysis and transcription alignment also showed internal regions of transcription in six positive koalas and no transcription in two negative koalas. For the one proviral KoRV negative case that showed KoRV gene alignment in RNAseq, an acute

infection may be responsible for some difficulties experienced in the collation of viral

expression and proviral analyses but would likely only account for a small number of false

negatives. False positives may occur only if the analyses is based on part or all of the termini

regions of the KoRV genome, as MLR koalas have been shown to have regions of homology

to KoRV at both termini, regardless of infection status. Proviral PCR analyses showed that

99.5% (215/216) of koalas tested were positive for the *gag* 1 gene target which was within

the terminal region of homology at the 5' end. The origin of this homologous region is

unknown and discussed further later in this chapter but should not contribute to the

classification of KoRV infection status in SA koalas. It is therefore recommended that

internal region gene targets only should be used for infection status in SA koalas. Another

means to increase the sensitivity and confidence would be to adapt the current conventional

PCRs to qPCRs, to give proviral loads for multiple targets. This would also allow for melt

curve analysis to confirm PCR products are consistent with known positive amplicons,

although would be unlikely to distinguish homologous regions from true infections.

One reason that SA koalas have more variability in their KoRV infection, and potentially

more difficult to diagnose, is that, as seen from the RNAseq alignment against the KoRV

genome in Chapter 2, the viral expression across the central region is fragmented and low in

KoRV positive animals without lymphoma. Supporting this, KoRV proviral and viral loads in

the southern populations have been found to be lower than those in northern koalas, which

have high viral expression. This has previously been attributed to the exogenous nature of the

infection, with a reduced number of host cells replicating the provirus (Simmons *et al.* 2012;

Wedrowicz *et al.* 2016). Inhibition of transcription caused by suppressive cellular genes,

latency of retroviruses and/or Piwi-interacting RNAs (piRNAs) may also responsible for

these fragmented central alignments of viral transcripts to the KoRV genome, as discussed in

Chapter 2. These mechanisms of suppression may have a greater effect in SA koalas due to exogenous infections having lower proviral loads and being more likely to elicit host immune responses. Alternatively, RNA-seq processing algorithms could play a role in the fragmentation of reads with some alignments lower than the threshold with low read numbers being below the counts per million needed to register. This theory is potentially supported by the qPCR positivity of the KoRV positive koalas tested in RNAseq and in qPCR, being positive in qPCR and not showing corresponding transcript alignment in the RNAseq data, shown in Figure 1.
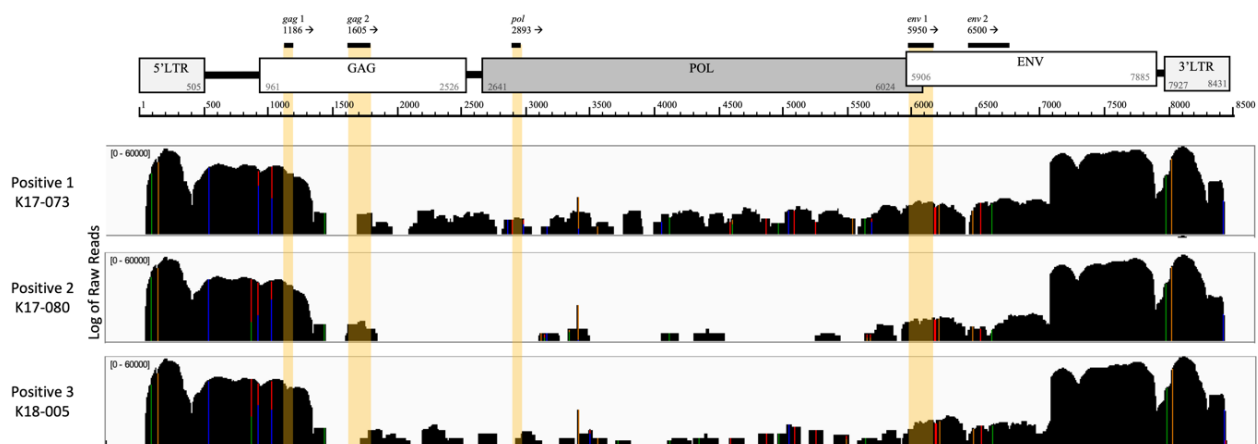
Figure 1: KoRV positive koala RNAseq alignment against the KoRV genome, with highlighted KoRV qPCR analysis amplicons (yellow)

As can be seen, *gag* 2 and *pol* PCR amplicons did not correspond to visualised transcript alignment, yet these koalas were qPCR positive from the same samples. This could also be a factor of read depth in the RNA samples, where the read depth in KoRV positive samples was, 100M (positive 1, K17-073), 32M (positive 2, K17-080) and 51M (positive 3, K18-005) reads. It can be seen from these that the reads aligned to the positive 1 with a depth of 100M reads, has less fragmentation of alignment in comparison to positive 2 with a depth of 32M reads. Although this trend is there, the sample size is inadequate to draw conclusive evidence

from. It has been shown that the non-discovery rate (NDR) for genes was negatively associated with the read depth (Tarazona *et al.* 2011). This, in turn, is suggestive of low read transcripts potentially being lost to the noise of RNAseq (Tarazona *et al.* 2011). This demonstrates that targeted viral RNA analysis for KoRV in southern koalas with low level infections is more sensitive than that of RNAseq alignment when analysing KoRV within the whole transcriptome data. Further bioinformatics analysis, harvesting only sequences that align to the KoRV genome may help reduce the potential loss of low-level reads or RNA extractions with greater depth (> 100M reads, likely > 150M reads) from KoRV positive koalas, but both these investigations were beyond the timeframe of this thesis.

7.2 Transcripts align to the termini of the KoRV genome in all SA koalas

All koalas in this study, irrespective of their KoRV status, expressed transcripts that aligned to terminal regions of the KoRV genome. These regions were inclusive of the long terminal repeats (LTRs), partial *gag* gene and partial *env* gene, and was found through transcriptome analysis and high read counts to these regions were identified in all nine koalas using transcriptome analysis. Examination of the reference genome showed multiple insertions of these terminal regions across the genome, with approximately 4000 - 5000 nucleotide distance between them. This 4000 - 5000 nt gap region, when extracted from the reference genome aligned (*BLASTn*) to an endogenous retroelement called PhER, Phascolarctid Endogenous Retroelement (Lober *et al.* 2018). The combination of KoRV and PhER in the reference genome and other koalas has been allocated the term recKoRV (Lober *et al.* 2018). Unfortunately, the internal gap regions from the South Australian koalas were unable to be analysed within the timeframe of this thesis, therefore confirmation of the presence of PhER or recKoRV is yet to be confirmed. Truncated retroviral sequences, some of which align to

the terminal regions, have also been seen in both northern (Hanger 1999; Lober *et al.* 2018; Yu *et al.* 2019) and southern (Tarlinton *et al.* 2017; Lober *et al.* 2018) koalas. The mechanisms behind this finding in KoRV is not fully understood but could be potentially due to the insertion of fragments of KoRV during replication, suppression of internal regions or degradation of endogenous retroviruses (Tarlinton *et al.* 2017; Lober *et al.* 2018; Nikolai *et al.* 2018; Yu *et al.* 2019).

Multimapping, increased numbers and homology could increase the alignment of reads to the LTR region. Multimapping of reads may well exaggerate the LTR region expression, where the nucleotide sequences are similar between koala retroviruses (Ishida *et al.* 2015; Lober *et al.* 2018) and identical between parts of the KoRV 5'LTR and the 3'LTR (Ishida *et al.* 2015). Ishida *et. al.* studied ten endogenous KoRV proviruses from six koalas, three northern and three southern koalas and found seven LTR haplotypes which were identical between 5' and 3' LTRs (Ishida *et al.* 2015). From studies in other species, it is well reported that replication strategies for retroviruses result in multiple random, semi-targeted or targeted locations within the genome rather than a single integration of the retrovirus in one particular location (Boeke *et al.* 1997). The LTR region is particularly favoured for multiple insertions and homologous insertion of two LTRs without the proviral genes can occur on insertion into the host DNA (Benachenhou *et al.* 2009; Stoye 2012). In humans, the number of single LTRs are approximately tenfold higher than that of complete provirus and all species analysed have a similar deposition of LTRs within their genomes (Benachenhou *et al.* 2009). Retroviruses, such as feline leukaemia virus (FeLV) have been shown to have duplications or triplications of LTRs, significantly increasing transcription and pathogenicity (Chandhasin *et al.* 2005). These LTR factors have the potential to amplify the read counts and mapping to each LTR region and could explain the findings of our analysis.

Endogenous retroviral recombination with KoRV can increase transcription of crossover regions of KoRV. ERVs other than KoRV have been demonstrated within the koala genome, four of which show active expression: PhER (Lober *et al.* 2018), ERVL.1, ERV.1 and ERVK.14 (Yu *et al.* 2019) Transcriptome mapping in SA KoRV negative koalas have very similar regions of expression to that of recKoRV-1. Lober *et. al.* (2018) demonstrated multiple possible recombination events with ERV PhER, which was reiterated in our *BLAST* analysis. RecKoRV-1 is thought to be a more recent combination of KoRV 5' LTR and *gag* sequences to position 1,177, PhER central region and then KoRV *env* region from 7,617 and inclusive of the 3'LTR (Lober *et al.* 2018). Within the analysis of the ERVs PhER and recKoRV there were considerable number of breakpoints where recombination with KoRV had occurred, highlighting the potential recent occurrence of these recombinations (Lober *et al.* 2018). From our transcriptome mapping it would seem likely that there was a consistent significant decrease of reads after position 1230 or 1420 and a significant increase of reads after position 7064 within the SA population. If recombination between KoRV and PhER has occurred in SA koalas then the peaks of aligned transcription at either end of the KoRV genome would not be found in KoRV negative koalas; therefore, either these recombinations are potentially evolutionarily older than KoRV, or there is homology to something else in the koala genome, which is currently actively transcribing. A greater number of samples from KoRV negative koalas would improve confidence. KoRV could be a recent recombination product between these regions and a close retroviral species, potentially from a transspecies infection. The closest retroviral relative is that of an Australian native mouse (*Melomys burtoni*). Melomys burtoni retrovirus (MbRV) has 74 - 84% homology of 4 amplicons tested to KoRV and 90 - 94% nucleotide identity to Gibbon ape leukemia virus (GALV), (Simmons *et al.* 2014) the previously known closest retrovirus to KoRV (Hanger *et al.* 2000). Also,

flying foxes have recently been investigated for retroviral sequences and have demonstrated a clade of viruses closely related to KoRV, possibly closer than MbRV and GALV, with Hervey pteroid gammaretrovirus (HPG) from the Australian black flying fox (*Pteropus alecto*) showing similar cell tropism as KoRV and GALV (Hayward *et al.* 2020). Whole genome sequencing with long reads of SA KoRV negative koalas would be necessary to further elucidate KoRV evolution. Integration sites for the KoRV retroelements may reveal similarities or differences between koalas, allowing estimation of how long these retroelements have been present in the SA koala population. LTR analysis of KoRV positive northern koalas has shown ten identical 5' and 3' LTRs in endogenised KoRV, which demonstrate the earliest occurrence of integration into the germline would have been an estimated 22,200 - 49,900 years ago (Ishida *et al.* 2015). Furthermore, comparison to NSW and QLD koalas, along with ancient koala DNA may also reveal whether there are common integrations sites across populations.

ERVs have four main fates over time: disruption, genetic drift, partial gene loss and recombination (Boeke *et al.* 1997). Due to the haploid nature of endogenised inheritance of KoRV, PhER and recKoRV, with koalas rarely having sequences on both chromosomes, some SA koalas may have gene loss, or just have a similar endogenous ERV to recKoRV (Lober *et al.* 2018). Retrotransposon DNA has also been shown to account for 44% of the koala genome (Yu *et al.* 2019). Complete disruption to non-pathogenic viral DNA tends to occur over considerable lengths of time and is unlikely to be occurring for KoRV, due to its recent endogenisation (Boeke *et al.* 1997; Avila-Arcos *et al.* 2013). The analysis of genetic drift and integration would need more thorough investigation through SA koala genome analysis. Partial gene loss may have occurred in this SA population, with endogenisation and loss of central KoRV genes within a founder koala, thus this bottlenecked population could have inherited this defective version of KoRV. ERVs that have lost the env gene tend to have

increased proliferation within the host genome, with less defined loci of integration (Magiorkinis *et al.* 2012). The terminal regions in KoRV negative koalas could have increased proliferation and transcription potential, resulting in the increased read counts seen on transcriptome analysis. With 99.5% (215/216) koalas tested in this study containing genomic regions homologous to the KoRV 5' *gag* (*gag* -1) PCR amplifiable sequence, it is reasonable to consider that these elements are endogenous within this population. RNAseq and qPCR analysis also showed that there was transcription of the 5' *gag* region in all SA koalas, and there was only a 5x (range 0.8-38) fold change ($2^{-\Delta\Delta CT}$) between KoRV positive and KoRV negative koalas, indicating similar levels of expression in both groups.

7.3 High level KoRV gene transcription is present in lymphomic koalas, but low prevalence of lymphoma in SA koalas

KoRV has previously been associated with the development of lymphomic and leukaemic disease, with prior studies showing that lymphomic koalas have high plasma viral loads and high proviral loads from spleen or whole blood (Tarlinton *et al.* 2005; Fabijan *et al.* 2017; Sarker *et al.* 2020a). To complement these findings, this study found all koalas with lymphoma had KoRV, higher proviral loads and they showed high level transcription across the entire viral genome in lymphomic tissue. KoRV had a positive association to lymphoma ($\chi^2 p$ value: 0.005) but was not found to have a significant association in the multivariate analysis, likely due to the low prevalence of lymphoma in this cohort (1.2%, 3/240). KoRV infection was found in 41.2% (89/216) of SA koalas, with a mean proviral load of 59.2 copies/10^3 ß-actin copies (range: 0.2 – 270 copies/10^3 ß-actin copies). Lymphoma koalas were all KoRV positive and had an average of 188 copies/10^3 ß-actin copies (range: 98 – 241 copies/10^3ß-actin copies). Lymphomic koalas within this study showed high transcriptions across the whole KoRV genome, and significantly higher viral loads in targeted RT-qPCR for

gag 2, *pol* and *env* 1 genes in comparison to KoRV positive koalas. This is the first report of high viral load directly from lymphomic tissue and supports retroviral PCR positivity found in leukaemic lymphocytes and tissue culture (Hanger 1999). This also is reflective of the clonal nature of these cancerous cells, with active transcription in every cell, therefore increased alignment of reads across the whole genome, equating to increased viral loads. This is strong evidence for KoRV being the initiator of mutagenesis and the cause of lymphoma in koalas.

Classification of collected koala lymphoma cases from MLR population (Chapter 3) showed a dominance of abdominally located intermediate to large cell non-T cell lymphomas. Only two cases were identified as B-cell origin. This lack of definitive immunotyping in other cases was likely due to the advance autolytic stage of the tissue at fixation and the decreased sensitivity of the CD79b antibody (*pers. comm*. S Lindsay). Immunotyping from NSW and QLD lymphoma cases demonstrated just over 50% were T-cell origin and 24% were B-cell origin (Connolly *et al.* 1998). In humans, the incidence of HIV-AIDS related neoplasia is increased in comparison the general population and almost all AIDS-related lymphomas are B-cell lymphoma (Cesarman 2011). Interestingly, in SA koalas, KoRV-A is the detectable KoRV variant, which uses a sodium-dependent phosphate transporter, PiT1 for viral entry (Oliveira *et al.* 2006; Denner *et al.* 2013). *In vitro* and *in vivo* studies have shown a fundamental role of PiT1 in B-cell development (Liu *et al.* 2013). Therefore, there is potential for greater infection of B-cell lymphocytes by KoRV in SA koalas, leading to predominantly B-cell origin lymphomas.

In FeLV there are several pathways from infection, and cats that succumb to FeLV diseases are those with persistently high antigenaemic, and therefore viraemic states (Beatty 2014) (Figure 2). FeLV-A variant is the most common exogenously transmitted variant of FeLV,

although occasionally FeLV-A and FeLV-B can transmit together. The persistently high replicative state of persistently antigenaemic cats can lead to greater mutation and development of increased FeLV variants, each of which has greater association with disease than FeLV-A alone. FeLV-B is associated with lymphoid neoplastic conditions, which can occur in 10 - 20% of persistently infected cats. Using a simple model, like in Figure 2, this would equate to 3 - 6% of cats infected with FeLV-B developing lymphoid neoplasia.
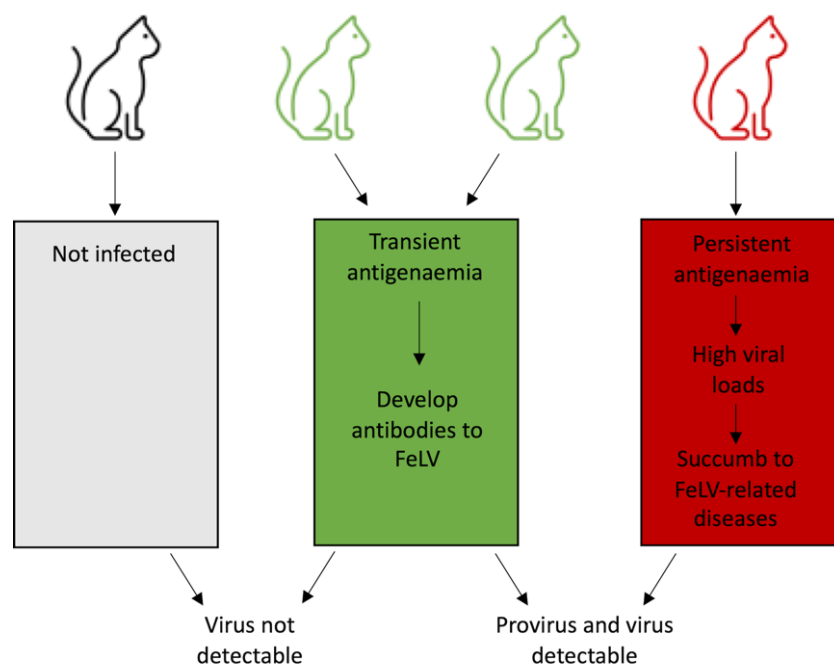
Figure 2: Outcome pathways for cats in FeLV infection, with approximately one third of those infected having persistent high viral load infections and succumbing to FeLV-related disease, adapted with author's permission from Beatty, 2014.

In the 2016 - 2019 cohort 86 koalas were KoRV positive, of which three developed lymphoma. Using the simplified FeLV model above, if one third of koalas were persistently viraemic, that would be approximately 28 koalas, and 10 - 20% of these would be 3 - 6 koalas (Figure 3). Therefore, it would be likely to see 3 - 6 koalas developing lymphoid neoplasia, for which this study found 3 koalas with lymphoma. This consistent finding with

FeLV, demonstrates a possible link in the pathogenesis of both these diseases. Other studies have shown a lack of viraemia in KoRV exogenously infected koalas (Sarker *et al.* 2019), increased chlamydiosis severity with high viraemic states (Sarker *et al.* 2020a) and KoRV-A and KoRV-B's association to lymphoid neoplastic conditions (Shojima *et al.* 2013; Fabijan *et al.* 2017).
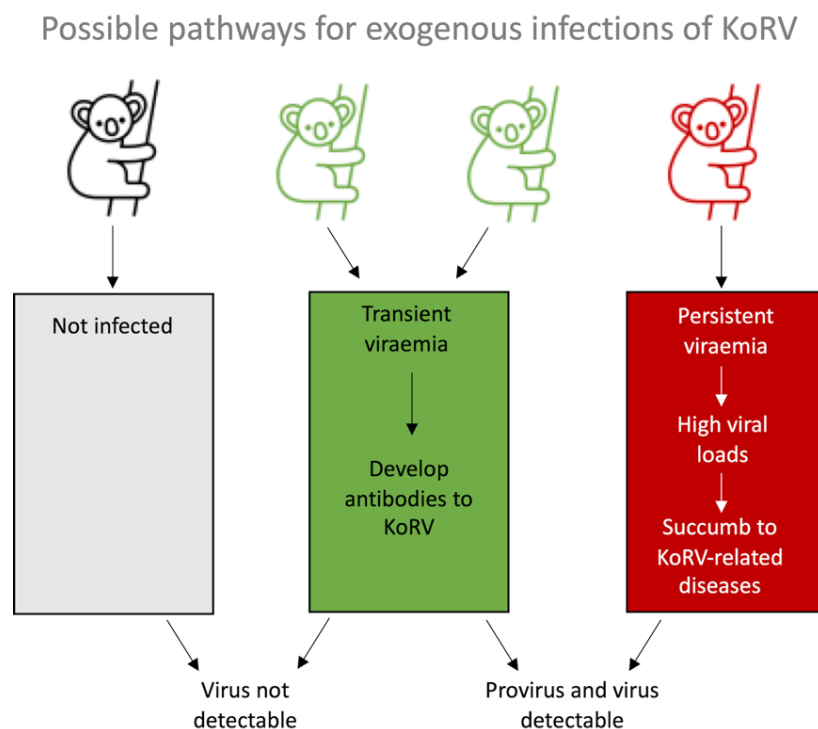
Figure 3: Potential outcome pathways for koalas exogenously infected with KoRV, adapted from that of FeLV (Figure 2).

A counterargument to the consistency of the FeLV-KoRV similarity is that the 2016-2019 cohort studied in this thesis is a disease-biased study. A disease-biased study should show higher numbers than those in the general population and hence overestimate the impact of KoRV. If this is the case, then the implications are that KoRV is currently having minimal impact on the South Australian population. Maintaining the low impact of KoRV and not following in the footsteps of northern koalas would mean taking steps now to mitigate the

role of KoRV in this population. If the persistency of viraemia was paramount to succumbing to KoRV-related diseases, then the advancement of the KoRV vaccine and its ability to reduce viral load in infected koalas (Olagoke *et al.* 2018; Olagoke *et al.* 2020) could be a way of mitigating these impacts. The use of the vaccine could be deemed essential for captive populations where mortality rate is largely attributed to lymphoid neoplasia (Hanger 1999). Also, longitudinal studies of KoRV virus load, targeting captive southern koalas, or monitoring exogenous KoRV infections of northern koalas, would be beneficial in order to explore this persistent viraemic state. Captive koalas would not only be beneficial due to their availability and increased lifespan, but also the high rate of lymphoid neoplasia seen in this cohort (Hanger 1999).

7.4 Oncogenes in KoRV-associated lymphoma

Three key genes of interest in the oncogenesis of lymphoma in koalas were discovered, based on their significant upregulation in lymphomic tissue. These were MYB, MYCL (a member of the MYC family) and FLT3. These genes have been shown to be dysregulated and part of oncogenesis in FeLV, MuLV, ALV, ATLV and others (Kanter *et al.* 1988; Tsatsanis *et al.* 1994; Carow *et al.* 1996; Frampton *et al.* 1996; Hanlon *et al.* 2003; Johnson *et al.* 2014; Nakano *et al.* 2016). In a study of the immunogenetics of the koala and the inhibition of KoRV expression from piRNAs, it was found that these inhibitory clusters were enriched for the binding motif for A-MYB (Yu *et al.* 2019), therefore if expression of MYB was to increase, it could be assumed that these inhibitory molecules would be supressed. Key interactions of MYB and MYCL with proto-oncogene MYC have also been discovered (Schmidt *et al.* 2000), which could potentiate the oncogenic effects of KoRV. A recent study which examined the integration of KoRV into the koala genome shows multiple insertions specific to tumour tissue, for which MYB and MYC showed KoRV insertions within

transcription enhancement range (McEwen *et al.* 2021). This recent discovery shows more evidence that these particular genes could be significant in KoRV-associated lymphoid neoplasia. FLT3 has shown to be a negative regulator of IL-10 (Astier *et al.* 2010), a significantly downregulated interleukin in this study. IL-10 deficient mice develop colitis (Kuhn *et al.* 1993), which is noteworthy due to a previous association of typhlocolitis with immunodeficient diseases of koalas being identified (Hanger 1999). IL-10 was also the only significantly different cytokine in KoRV-B infection baseline cell culture studies, for which it was significantly higher in the KoRV-B negative group (Maher *et al.* 2016). It was also significantly upregulated on mitogen stimulation in KoRV-B koalas (Maher *et al.* 2016). Increased expression of IL-10 has also shown immunosuppressive actions, contradictory to this and other studies, with viral upregulation of IL-10 being favourable for persistent infection (Rojas *et al.* 2017; Vahl *et al.* 2017). Therefore, IL-10 would be an interesting cytokine to investigate further. Another important gene for future investigation is MMP9, a matrix metalloproteinase whose expression has shown to augment angiogenesis and growth of tumours (Chang *et al.* 2001). This gene was upregulated in both the HEK293T cell infection study (Sarker *et al.* 2020b), and in this lymphoma study. MMP9 expression in non-small cell lung cancer has been shown to be GABA receptor (GABR$_{A3}$) expression dependent (Liu *et al.* 2016), and a similar mechanism in koalas may be supported by the finding of increased GABA receptor expression (GABRR2) in lymphomic tissue.

7.5 Immunosuppression likely a prerequisite to lymphoid neoplasia in KoRV-associated pathogenesis

Immune pathways, inclusive of leukocyte extravasation signalling, Th2 pathway, CD28 signalling in T helper cells and crosstalk between dendritic cells and natural killer cells were all shown to be enriched with overall inferred downregulation of these pathways (IPA

analysis). Key immune genes such as IL10, C3 and CD28 were significantly downregulated in lymphomic koalas and are likely to be key genes in KoRV-associated immunosuppression. After acute infections and initial immune response, progression to HIV-related disease shows signs of immune senescence and reduced function (Brenchley *et al.* 2003; Betts *et al.* 2006). Extrapolation of pathogenesis in HIV-related disease may suggest that KoRV-associated lymphoma may be precipitated by reduced immune function. Immunosuppression has also been shown to cause recrudescence in viral shedding in regressive FeLV infected cats (Helfer-Hungerbuehler *et al.* 2015; Hofmann-Lehmann *et al.* 2020). Acute infection with KoRV has been shown to significantly upregulate various cytokines, interleukins and interleukin receptors in HEK293T cells (Sarker *et al.* 2020b), of which CCL5 is significantly down regulated in this lymphoma study. CCL5 is a potent inhibitor of HIV produced by CD8$^+$ cells (McBrien *et al.* 2018), and its protein complex, RANTES, exhibits dose-dependent inhibition of HIV-1, HIV-2 and SIV (Cocchi *et al.* 1995). This finding supports a role for this gene in the control of KoRV infection in individual animals, and maybe with the reduction of CCL5 expression, proviral and viral loads could increase, further research is indicated.

CD4:CD8 ratios are markers of T helper cells to cytotoxic T-cells, with CD4 molecules present on T helper and CD8 molecules on cytotoxic T-cell. CD4$^+$ T helper cells recognise major histocompatibility complex (MHC) II class proteins and with the presence of correct stimuli can increase activation of B-cells. CD8$^+$ Cytotoxic T-cells recognise MHC I class proteins and with the presence of correct stimuli increases the cytotoxic effects of these cells (Alberts *et al.* 2002). There was downregulation of Th1 and Th2 cytokines in lymphomic tissues from the koalas in this study, therefore supporting suppression of both pathways. KoRV positive koalas have been shown to have a lower CD4:CD8 ratio than KoRV negative (Maher *et al.* 2019). Acute infections of HIV and SIV produce a peak viraemia followed by a

decline in the level of viraemia. This viraemia can be influenced by the CD4:CD8 ratios, with possible delineation between the development of latency or chronic viraemia; which is more susceptible to onward progression to disease (Mellors *et al.* 1996; McBrien *et al.* 2018; Zanoni *et al.* 2020). CD8$^+$ T-cells have also been established to exert inhibitive control on HIV and SIV infected cells (McBrien *et al.* 2018). The shift from T helper 1 (Th1) to T helper 2 (Th2) cells has previously been implicated in retroviral immunosuppression (Clerici *et al.* 1993), although more recent work has shown dysregulation of both Th1 and Th2 cytokines in lentiviral infections (Tompkins *et al.* 2008) and in KoRV-B infection (Maher *et al.* 2016). Further investigation of these ratios in lymphomic koalas could help differentiate the T cell roles in KoRV-associated neoplasia.

Immunosuppression is associated in many species with opportunistic disease and has been found to play a role in retroviral associated disease, with extrapolation to KoRV and overt chlamydiosis (Connolly 2000; Cesarman 2011; Levy 2011; Blinov *et al.* 2013; Higgins *et al.* 2014; Madden *et al.* 2018; Robbins *et al.* 2020). Differences have been seen in progression of chlamydiosis between MHC allele types in koalas, with recognition of presumed protective alleles *Dab* 10 and *UC* 01:01, and susceptibility allele *DBb* 04 (Robbins *et al.* 2020). Since the MLR population has experienced significant bottlenecks, one may assume the MHC allele diversity is low, however, it would be useful to investigate MHC allelic diversity and type in relation to progression of disease since disease severity is low in this population. A lack of genetic diversity in the MLR population (Houlden *et al.* 1999; Masters *et al.* 2004), should reduce the fitness of this population, yet the disease severity is much less than other populations. However, non-host factors could also play a role, with different *C. pecorum* strains associated with disease progression (Quigley *et al.* 2018; Robbins *et al.* 2020), as have different variants of KoRV (Waugh *et al.* 2017; Quigley *et al.* 2019; Sarker *et al.* 2020a),

with more work is continuing in this area. Therefore, comparative immunogenetics, genetic diversity and disease variant analyses are needed to fully understand chlamydiosis manifestations in different populations.

7.6 Role of PhaHV in KoRV oncogenesis

There were only six lymphoma cases from South Australian koalas analysed in Chapter 4 for coinfection with PhaHV. Two of them were positive for PhaHV-1 and three of them were positive for PhaHV-2. PhaHV-1 was much more prevalent in the population than PhaHV-2 and Chi squared analysis did not show any association with lymphoma when looked at individually, but coinfection of KoRV and PhaHV-2 showed a significantly positive relationship (*Odds ratio*: 5.93 (1.14 - 30.90), *p* value: 0.002). A possible link between lymphoma in KoRV positive koalas and PhaHV could be similar to that found in human studies that have shown augmentation of retroviral oncogenesis by gammaherpesviruses, namely Epstein-Barr Virus (EBV, human herpesvirus 4, HHV4) or Kaposi sarcoma herpesvirus (KSV, human herpesvirus 8, HHV8) in humans with HIV-AIDS, as well as homologs to these in macaques (Bruce *et al.* 2012). EBV in AIDS patients have shown increased risk of developing multiple forms of lymphoma; non-Hodgkin lymphoma (NHL), Hodgkin lymphoma (HL), Burkitt lymphoma (BL), diffuse large B-cell lymphoma (DLBCL), plasmablastic lymphoma (PBL) and primary effusion lymphoma (PEL). PEL is also characterised by the presence of KSV, with greater than 80% of cases concurrently infected with EBV (Nador *et al.* 1996; Cesarman 2011). EBV has been shown to express BHRF1 a viral homolog to BCL2 (Kelly *et al.* 2009), an antiapoptotic gene associated with follicular lymphoma. Molecular pathogenesis of mutagenesis in KSV has shown three viral gene products contribute to transformation, LANA, vCYC and vFLIP (Cesarman 2011). LANA has been shown to inactivate p53 and stabilise c-*myc* (Friborg *et al.* 1999; Bubman *et al.*

2007). vCYC is a cyclin protein that can modulate the cell cycle and increase phosphorylation of tumour suppressor gene Rb, when complexed with CDK6 (Godden-Kent *et al.* 1997; Mann *et al.* 1999). vFLIP activates NF-κB and inhibits apoptosis (Guasparri *et al.* 2004). Studies in FIV and FeLV have shown possible gammaherpesvirus involvement in FIV associated lymphomas (Aghazadeh *et al.* 2018). *Felis catus* gammaherpesvirus 1 (FcaGHV1) has been recently discovered in cats and although not found to be associated with lymphoma and was shown to decrease life expectancy in cats with lymphoma (McLuckie *et al.* 2018). FcaGHV1 was also found to have significantly higher loads in cats with feline immunodeficiency virus (FIV) (Beatty *et al.* 2014). In FIV-associated lymphoma FcaGHV1 has been shown to express viral genes with homology to those in KSV; LANA and vFLIP (Aghazadeh *et al.* 2018), highlighting a potential role in mutagenesis. It may be possible to do further analyses of the transcriptome from koala K16-069, which was PhaHV-2 positive. The transcriptome could be searched for homologs to LANA, vCYC and vFLIP and see if there was gene expression present and compared to the koala that was PhaHV-1 positive (K17-073). It could be possible, with known PhaHV LANA, vCYC and vFLIP sequences from the transcriptome data (if found), to investigate targeted qPCR on known PhaHV-2 positive and negative, lymphomic and non-lymphomic koalas. Another interesting host gene to look at is the c-MYC gene, since it has been shown to play a role in herpesvirus oncogenesis. LANA expression has been associated with a herpesvirus homolog to mircroRNA-155, a key activation miRNA of c-*myc* (Bubman *et al.* 2007). This would be interesting since MYCL, a member of the MYC family of genes, may play a key role in KoRV-associated oncogenesis from the findings of this study.

7.7 Coinfection of *C. pecorum* and PhaHV is associated with the presence of paraovarian cysts

There was a correlation between *C. pecorum* and PhaHV coinfection and the presence of paraovarian cysts. There was a stronger association of PhaHV-2 than PhaHV-1 with paraovarian cysts, and a greater association of PhaHV-1 than PhaHV-2 with *C. pecorum* infection. It was also found that of those koalas with paraovarian cysts that were tested for both *C. pecorum* and PhaHV that these koalas were either coinfected with *C. pecorum* and PhaHV or were only PhaHV positive, no koalas were only *C. pecorum* positive (Chapter 4). PhaHV has also shown strong correlations to coinfection with *C. pecorum* in Victorian koalas, with increased likelihood of reproductive disease in female koalas (Vaz *et al.* 2019). *C. pecorum* has had extensive research into the pathogenesis of urogenital disease in female koalas and infection has been correlated with reproductive disease with resultant inflammation and fibrosis responsible for the progression to paraovarian cysts (Hemsley *et al.* 1997; Higgins *et al.* 2005). The severity of chlamydiosis has been increasing in the MLR population of koalas, as found in the 2014 - 2016 study (Fabijan *et al.* 2019a; Fabijan *et al.* 2020) in comparison to 2011 - 2014 study (Speight *et al.* 2016). Severity of chlamydiosis in this study has shown a similar overall picture to the 2014 - 2016 study, with only 14.7% (14/95) *C. pecorum* positive koalas showing no clinical signs of disease and 12% (11/92) in 2014 - 2016. This study has highlighted a possible augmentation by PhaHV and needs further investigation into the presence of PhaHV in affected tissues to corroborate these associations. This could be achieved through molecular testing of paraovarian cystic fluid and further histopathological examination of multiple sections of reproductive tract to examine for inclusion bodies. It should be noted that, inclusion bodies, although pathognomonic for disease, these inclusion bodies can be transient (Heuschele *et al.* 2001; King 2001; Montali

2001). It must also be considered that paraovarian cysts are a chronic lesion and likely to be causing some compromise of the koala, in turn leading to expression or shedding of herpesvirus. In this study koalas with paraovarian cysts had a mean BCS of 2.54 (SD: 1.06), and a median BCS of 3, with no koalas having a BCS of 5. Therefore, further investigation into the role of PhaHV in reproductive pathology is needed to differentiate between recrudescence due to immunocompromise and stress, or direct involvement in the pathogenesis of paraovarian cyst formation.

7.8 Other disease associations with PhaHV-1 and PhaHV-2

Conjunctivitis and arthritis are two other pathologies in koalas that have possible PhaHV involvement. PhaHV-1 had a high prevalence in conjunctivitis cases, although this was not statistically significant due to low numbers of conjunctivitis cases. Herpesviruses have been associated with both reproductive disease and ocular disease pathogenesis in other species, such as cows (Donofrio *et al.* 2008; Graham 2013), horses (Holz *et al.* 2019) and cats (Maes 2012). In marsupials, associations have been found with respiratory disease (Wilcox *et al.* 2011) and a lack of pouch young or non-lactation (Stalder *et al.* 2015). Also, there was a significant association of PhaHV-2 with the presence of crepitus and presumed arthritis, (p = 0.013). Herpesvirus replication has been found in equine synovial epithelium with focal necrosis (Blunden *et al.* 1995). Examination for inclusion bodies in synoviocytes PhaHV-2 positive, arthritis positive koalas and molecular analysis of synovial fluid in arthritic koalas would help assess the contribution of PhaHV-2 to arthritic pathogenesis. Investigations into whether herpesvirus associations are due to direct involvement in pathogenesis or to recrudescence from stress and immunosuppression is necessary.

Anecdotally carers and those treating koalas in South Australia have said that "it's always the healthy ones that get hit by cars". In this study, 40 koalas were victims of RTAs, a prevalence of 16.7% and 25 (10.4%) were victims of a dog attack. The associations seen in Chapter 4 show that there are negative associations with trauma and infection with KoRV and *C. pecorum* through multivariate analysis. Using chi squared analysis to analyse trauma as the dependent variable there are a greater number of negatively associated variable, see table 1. Positive associations of note, koalas with a good body condition score (BCS 4 or 5) were 4.4 times (*CI*:2.1 - 8.9) more likely and those koalas found to be free from disease at postmortem examination were 10.2 times (*CI*:4.1 - 25.4) more likely to be victims of an RTA (Table 1).

Table 1: Significantly associated demographic and disease variables with koalas that were victims of road traffic accidents (RTA), n = 40

Variable	N	Pearson's Chi squared value	*P* value (1 sided)	Odds ratio	Confidence interval
Compromised BCS (<3)	2	15.74	0.000	0.090	0.021-0.382
Good BCS (4+)	24	18.47	0.000	4.382	2.159-8.896
KoRV positive	9/37	5.737	0.012	0.382	0170-0.856
C. pecorum positive	11/31	3.363	0.050	0.478	0.215-1.064
Conjunctivitis	3	4.688	0.019	0.279	0.082-0.948
Oxalate Nephrosis	2	10.240	0.000	0.129	0.030-0.552
Respiratory Disease	0	4.844	0.015	0.890	0.848-0.934
Spinal curvature	0	3.892	0.033	0.910	0.871-0.951
Disease free	14	33.333	0.000	10.231	4.122-25.394

The negative associations with disease and positive associations with health highlight the significance of this subpopulation. Koalas in an overabundant population like the South Australian population, need freedom of movement to find food and mates. Mitigation strategies to reduce the anthropogenic impact are crucial in protecting the healthy subpopulation in the Mount Lofty Ranges. It is also crucial that legislation and policy reflect this anthropogenic contribution to population decline and highlight the impacts on the

healthier subpopulations not just in SA, but also around Australia (Taylor-Brown *et al.* 2019).

In the Mount Lofty Ranges population critical data could be collated on blackspots for RTAs, initiating protective measures, such as alert systems responsive to animal presence, education of drivers and increased social awareness would all help reduce mortalities from RTAs. Dynamic road signs to tell people to go slower in koala zones have showed a decrease in speed, especially for those signs displaying a koala (Blacker *et al.* 2019). Also projects such as "Leave it", overseen by Griffith University, have seen a decrease in dog attacks due to the training and awareness campaigns (David *et al.* 2019). These kinds of public engagement and increased awareness could help the healthy koalas survive when living in close proximity to humans.

7.10 Two newly described conditions; Pulmonary actinomycosis and hypertrophic osteopathy

A novel bacterial species was discovered associated with a previously undescribed respiratory disease presentation. Pulmonary actinomycosis was identified in 15 koalas in the necropsy cohort, with a prevalence of 6.3%, (Chapter 6) and also includes two other cases collated from Adelaide veterinarians. This was a relatively high prevalence for a new pathology and 16S RNA gene molecular analysis and sequencing shown on average a 95% homology to *Actinomyces timonensis*, but further full genome sequencing and biochemical characterisation of the isolates are needed for complete speciation and naming of the novel species of *Actinomyces.* This is currently ongoing through our research group and collaborators but is beyond the timeframe and scope for this thesis. Pulmonary actinomycosis is a non-infectious disease with a likely aetiology of aspiration. It presented as a lobar bronchopneumonia, predominantly found with left caudal lung lobe involvement. Histopathological examination showed the presence of Splendore Hoeppli phenomenon with

association of Gram positive or Gram variable, modified Zeil Neelson negative, filamentous bacteria. Two koalas from this study and two koalas from collaborators were found to have secondary hypertrophic osteopathy (Chapter 6). Two koalas underwent CT examination whish revealed extensive periosteal reactions on both the axial and appendicular skeleton, and histopathology confirmed the extent of bone pathology. Exploration of these novel presentations with other key pathogens, KoRV, *C. pecorum* and PhaHV did not reveal any statistical associations.

7.11 Conclusion

This study has advanced the understanding of the health and disease status of koalas in the Mount Lofty Ranges population in South Australia. It has described molecular proviral analyses for definitive diagnosis of KoRV and shown highly expressed host-genomic regions of homology to KoRV in almost all SA koalas. This requires further investigations as to its origin but could either be a defunct endogenised KoRV from the founding koalas, or part of the KoRV evolution, arising prior to KoRV itself. These termini could also reduce infection rates in SA koalas, potentially inhibiting or increasing resistance to viral entry. Investigation into the presence of PhaHV in SA koalas showed and association with *C. pecorum* infection and disease presentation. Further work investigating the pathogenesis of reproductive disease in SA koalas is needed, since, unlike the northern koala populations, *C. pecorum* is potentially not the sole infective agent in *Chlamydia*-like disease. The lymphoma prevalence is very low in this population but shows similarities in pathogenesis pathways to that of FeLV, with key genes such as MYB and MYC being implicated in the initiation of oncogenesis. Immunosuppression is also a key finding in oncogenesis of KoRV, and it is likely that this is necessary prior to tumorigenesis, due to the apoptotic nature of NF-κB in the presence of MYB and MYC. This pathway to lymphoma is very similar to those seen in

HIV, FIV and SIV, of which herpesvirus augmentation has been seen in some tumours. Although only 50% of lymphoma cases were positive for PhaHV-2, this interaction warrants further investigation. Additionally, koalas within this necropsy cohort were found to have previously undescribed conditions; pulmonary actinomycosis and hypertrophic osteopathy, which advances the knowledge of disease presentations in koalas across Australia. Finally, the reduced likelihood of koalas euthanased due to road traffic accidents presenting with concurrent disease or infection demonstrates that these koalas are the healthiest in the MLR population. Therefore, greater steps to protect this subpopulation from tragic anthropogenic fatalities needs to be taken, not only from local signage, education and training perspectives, but state and federal legislative changes to protect wildlife from the detrimental effects of humans. Overall, the value of the MLR population for harbouring koalas with lower prevalence of disease from infectious pathogens should be considered on a national level.

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Appendix

S1: Koala sex, TWC, BCS, molecular analysis and pathological findings

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K16-069	m	1	4	1		0	1	Lymphoma, testicular asymmetry
K16-092	f	3	5	0	1	1	1	Urogenital Chlamydiosis (cystitis & endometritis), trauma (RTA)
K16-093	f	5	4	0	1	0	0	Urogenital Chlamydiosis (cystitis), trauma (RTA)
K16-095	m	3	4	0	0	0	0	Cystitis, pyelonephritis, trauma (RTA)
K16-096	m	3	3	0	1			Urogenital Chlamydiosis (cystitis, prostatitis), arthritis, dental disease
K16-097	f	5	3	1	1			Subclinical chlamydia, pulmonary actinomycosis
K16-098	m	4	4	1				Trauma (RTA)
K16-099	f	4	1	0	0	0	0	Cystitis, trauma (DA)
K16-100	f	6	2	0	1	0	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), arthritis, colloidal goitre, thromboembolic disease
K16-101	f	2	0	0		0	1	Oxalate nephrosis, trauma (RTA)
K16-102	f	4	4	0	0			Cystitis, conjunctivitis
K16-103	f	6	2	0	1	1	0	Ocular Chlamydiosis, pulmonary actinomycosis
K16-104	m	4	3	1	0			Trauma (DA), spinal curvature, testicular asymmetry
K16-105	f	4	4		1			Urogenital Chlamydiosis (cystitis, vaginitis, paraovarian cysts), trauma (RTA)
K16-106	f	2	3	0	0			Cystitis, vaginitis, trauma (RTA)
K16-108	f	2	5	0	0			Cystitis, trauma (other)
K16-109	f	6	2	0	1			Urogenital Chlamydiosis (cystitis, pyometra), dental disease
K16-110	m	4	3	0	0			Trauma (RTA)
K16-111	m	6	3	0	1			Urogenital Chlamydiosis (cystitis), arthritis, dental disease
K17-001	f	6	3	0	0			Paraovarian cysts, conjunctivitis, dermatitis
K17-002	f	2	4	0		0	1	Oxalate nephrosis, trauma (DA)
K17-003	m	4	3	1		0	1	Oxalate nephrosis
K17-005	f	3	3	0				Cystitis, endometritis, conjunctivitis, oxalate nephrosis, craniofacial tumour
K17-006	f	3	2	1	0	0	0	Conjunctivitis, oxalate nephrosis
K17-007	m	2	0	1				Oxalate nephrosis
K17-008	f	2	2	0	1			Urogenital Chlamydiosis (cystitis, pyometra), oxalate nephrosis
K17-009	m	3	1	1	1			Urogenital Chlamydiosis (cystitis)
K17-010	f	5	2	0	1			Urogenital Chlamydiosis (cystitis), pulmonary actinomycosis
K17-011	m	1	2		0			Pneumonia
K17-015	f	5	1		1			Urogenital Chlamydiosis (cystitis, pyometra, paraovarian cysts), dental disease
K17-016	m	4	3	0		1	0	Cystitis, conjunctivitis, tapeworm, pulmonary actinomycosis
K17-018	m	3	5	1				Trauma (RTA)
K17-021	f	4	4					Cystitis, oxalate nephrosis
K17-022	f	2	2	0	0			Cystitis, pulmonary actinomycosis
K17-023	f	1	4	0				Trauma (RTA)

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K17-024	f	4	4	0	0	1	0	Cystitis, conjunctivitis, oxalate nephrosis, pyelonephritis
K17-025	m	3	3		1	0	0	Urogenital Chlamydiosis (cystitis, prostatitis), trauma (other)
K17-026	m	2	3	1	0			Spinal curvature, tapeworm, mange, dermatitis
K17-027	f	4	3	0	1			Urogenital Chlamydiosis (cystitis, endometritis), oxalate nephrosis
K17-028	f	3	4	1	1			Urogenital Chlamydiosis (cystitis, vaginitis), trauma (other), dental disease
K17-029	m	2	1					Trauma (DA)
K17-030	m	2	5	0	1	0	1	Ocular Chlamydiosis, prostatitis
K17-031	f	5	4	0	0	0	0	Trauma (RTA)
K17-032	m	2	4	0		0	0	Trauma (RTA)
K17-033	f	1	3	0		0	0	Oxalate nephrosis
K17-034	f	5	3	1	0	1	1	Cystitis, vaginitis
K17-035	f	2	4	0		0	0	Oxalate nephrosis, trauma (DA)
K17-036	f	5	1	1	1	0	0	Urogenital Chlamydiosis (cystitis), oxalate nephrosis, trauma (DA)
K17-037	m	3	4	0		1	0	Trauma (DA)
K17-038	m	3	3	0		0	0	Trauma (DA)
K17-039	f	2	4	0	0	0	0	Trauma (RTA)
K17-040	m	3	4	0		1	0	Trauma (RTA)
K17-041	m	4	3	0	0	1	1	Cystitis, epididymitis, dental disease
K17-042	f	5	3	1	1			Urogenital Chlamydiosis (cystitis), pulmonary actinomycosis
K17-043	f	1	3	0	1	0	0	Subclinical chlamydia, oxalate nephrosis
K17-044	m	4	4	0		1	0	Oxalate nephrosis
K17-045	m	3	3	1		0	0	Trauma (RTA)
K17-046	m	2	2	0	0			Oxalate nephrosis, testicular asymmetry
K17-047	m	4	3	0	0	1	0	Cystitis, epididymitis, trauma (RTA)
K17-048	m	3	5	0	0	0	1	Cystitis, thromboembolic disease
K17-049	m	5	4	0	0	0	0	Trauma (DA), testicular asymmetry
K17-050	m	5	3	1	1	1	1	Subclinical chlamydia, trauma (DA), dental disease, testicular asymmetry
K17-051	f	2	3	1	0	0	0	Vaginitis, trauma (other), tapeworm
K17-052	m	4	3	1	1	0	0	Subclinical chlamydia, oxalate nephrosis, spinal curvature, lymphoma
K17-053	f	2	4	1	0	0	0	Pyometra, trauma (RTA)
K17-054	f	2	4	1	0	0	0	Vaginitis, oxalate nephrosis, pneumonia
K17-055	m	5	3	0	0	0	0	Dental disease, dermatitis, testicular asymmetry
K17-056	m	4	4	1	1	1	1	Urogenital Chlamydiosis (cystitis), spinal curvature, testicular asymmetry
K17-057	m	4	2	0	1	1	0	Subclinical chlamydia, pyelonephritis, dental disease
K17-058	m	6	1	0	1			Urogenital Chlamydiosis (cystitis)
K17-059	f	3	4	1	0	0	0	Cystitis, spinal curvature
K17-060	f	1	4	1		0	0	Trauma (DA)

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K17-061	m	4	2	1	0	1	1	Cystitis, conjunctivitis, pulmonary actinomycosis
K17-062	m	3	3	0	1			Urogenital Chlamydiosis (cystitis), oxalate nephrosis
K17-063	f	4	4	0	0			Oxalate nephrosis
K17-064	m	1	4	0		0	1	Oxalate nephrosis
K17-065	m	3	3	1		0	1	Oxalate nephrosis
K17-066	m	4	3	0	1			Ocular Chlamydiosis, testicular asymmetry
K17-067	m	6	3	0	1	0	1	Urogenital Chlamydiosis (cystitis), arthritis, pulmonary actinomycosis
K17-068	m	4	5	0	1			Ocular Chlamydiosis, tapeworm
K17-069	f	4	4	1	0	1	0	Cystitis, pyometra, paraovarian cysts, pulmonary actinomycosis, colloidal goitre
K17-070	f	5	2	0	0	0	0	Cystitis, paraovarian cysts, conjunctivitis
K17-071	f	1	4	1	0	0	0	Cystitis, trauma (other)
K17-072	f	4	4	0	0	1	0	Urogenital Chlamydiosis (cystitis, pyometra)
K17-073	m	4	0	1	1	1	0	Urogenital Chlamydiosis (cystitis), pulmonary actinomycosis
K17-074	m	5	4	0	0			Cystitis, prostatitis, conjunctivitis, trauma (RTA)
K17-075	f	6	3	x	1			Urogenital Chlamydiosis (cystitis, endometritis), oxalate nephrosis
K17-076	m	2	4	0	0	0	0	Cystitis, trauma (RTA)
K17-077	m	4	3	1	1			Urogenital Chlamydiosis (cystitis), trauma (other), testicular asymmetry
K17-078	m	4	3	0				Cystitis, conjunctivitis, trauma (other)
K17-079	m	3	2	1	0	0	1	Cystitis, urethritis, pyelonephritis, pulmonary actinomycosis
K17-080	f	4	2	1	1			Urogenital Chlamydiosis (cystitis, endometritis, paraovarian cysts)
K18-001	m	2	3	0		0	0	Oxalate nephrosis
K18-002	f	5	3					Pyometra, paraovarian cysts, arthritis, dental disease, colloidal goitre, thromboembolic disease
K18-003	m	2	4	0		1	1	Oxalate nephrosis
K18-004	f	5	3	0		0	0	Endometritis, conjunctivitis, trauma (other)
K18-005	m	5	3	1	1			Urogenital Chlamydiosis (cystitis, prostatitis), trauma (other), mange, dermatitis, pulmonary actinomycosis
K18-006	m	1	2	0				Conjunctivitis, mange, dermatitis
K18-007	f	2	2	1		1	0	Cystitis, endometritis
K18-008	m	4	4	1				Prostatitis, trauma (RTA)
K18-009	f	5	3	1				Cystitis, endometritis, pyometra, conjunctivitis, thromboembolic disease
K18-010	f	2	3	0				Endometritis, conjunctivitis, oxalate nephrosis, trauma (other)
K18-011	m	4	3	1		0	0	Pyothorax, testicular asymmetry
K18-012	m	3	3	0		1	1	Conjunctivitis, oxalate nephrosis, dental disease
K18-013	f	6	3	1		1	1	Cystitis, paraovarian cysts, conjunctivitis, dental disease
K18-014	f	2	4	0		0	0	Oxalate nephrosis
K18-015	m	5	3	1		1	1	Cystitis, urethritis, oxalate nephrosis, arthritis

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K18-016	f	2	3	1		1	1	Pyometra, paraovarian cysts, mange
K18-017	f	3	4					Cystitis, trauma (RTA)
K18-018	m	5	1	1				Cystitis, spinal curvature, cryptorchidism
K18-019	m	3	1	1				Conjunctivitis, renal fibrosis, dental disease, tapeworm, testicular asymmetry
K18-020	m	3	1					Cystitis, spinal curvature
K18-021	f	4	3			0	0	Cystitis, paraovarian cysts
K18-023	f	5	2	0		1	1	Endometritis, paraovarian cysts, pyelonephritis, trauma (other), tapeworm
K18-024	m	4	4	1		1	0	Cystitis, urethritis, tapeworm
K18-025	m	4	3			0	0	Conjunctivitis, dental disease
K18-026	f	1	2	1	0	0	0	Oxalate nephrosis, pyelonephritis
K18-027	m	3	2	1	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis), spinal curvature, dental disease
K18-028	f	2	4		0	0	1	Cystitis, vaginitis, endometritis, conjunctivitis
K18-029	m	3	1		0	1	0	Cystitis, conjunctivitis, pyelonephritis, spinal curvature
K18-030	m	5	3	0	1	0	0	Urogenital Chlamydiosis (cystitis), oxalate nephrosis, spinal curvature, pulmonary actinomycosis
K18-031	f	4	2	0	0	1	0	Oxalate nephrosis
K18-032	m	1	1	0	0	1	0	Testicular asymmetry
K18-033	f	5	4		1	1	1	Urogenital Chlamydiosis (cystitis, vaginitis, paraovarian cysts), tapeworm, colloidal goitre
K18-034	f	4	3	1	0	0	0	Trauma (RTA), colloidal goitre
K18-035	f	4	2	0	0	0	0	Colloidal goitre
K18-036	m	3	3	0				Oxalate nephrosis, trauma (other)
K18-037	f	1	4	0				Conjunctivitis, oxalate nephrosis, pyelonephritis
K18-038	f	3	3	0				Paraovarian cysts
K18-039	f	7	1	x	0	0	0	Cystitis, conjunctivitis, colloidal goitre
K18-040	f	4	2	0	0	1	1	Cystitis, vaginitis, endometritis, paraovarian cysts, conjunctivitis, arthritis
K18-041	m	3	3		1	1	0	Urogenital Chlamydiosis (cystitis, urethritis), oxalate nephrosis, spinal curvature, dermatitis, testicular asymmetry
K18-042	m	4	3	1	0	0	1	Pneumonia
K18-043	f	6	3	0	1	1	1	Urogenital Chlamydiosis (cystitis)
K18-044	m	4	2	0	0	1	1	Urethritis, dental disease, dermatitis
K18-045	f	4	2	1	0	1	1	Cystitis, conjunctivitis, oxalate nephrosis
K18-046	f	5	2	0	1	1	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis, pyelonephritis
K18-047	m	3	2	1	0	1	0	Urethritis, prostatitis, conjunctivitis
K18-048	m	3	4	0	0	1	0	Cystitis, trauma (RTA)
K18-049	m	4	4		0	1	1	Cystitis, arthritis
K18-050	f	3	2	0	1	1	0	Urogenital Chlamydiosis (cystitis, endometritis, pyometra), oxalate nephrosis
K18-051	f	4	3	0	0	1	0	Cystitis, pyelonephritis, trauma (other), dental disease

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K18-052	m	4	5	1	0	1	0	Trauma (RTA)
K18-053	f	6	3	0	1	1	1	Ocular and Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis
K18-054	f	4	2	0	1	1	0	Urogenital Chlamydiosis (cystitis, paraovarian cysts), spinal curvature
K18-055	f	4	4	x	1	1	1	Ocular and Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis
K18-056	m	4	2	1	1	1	1	Urogenital Chlamydiosis (cystitis, urethritis), arthritis, tapeworm, periorbital myxoma/myxosarcoma, testicular asymmetry
K18-057	m	5	1	1	1	1	1	Urogenital Chlamydiosis (cystitis, urethritis), spinal curvature, testicular asymmetry
K18-058	f	4	2	0	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis), oxalate nephrosis, pyelonephritis, dental disease, thromboembolic disease
K18-059	m	4	1	0	0	1	1	Urethritis, oxalate nephrosis, pyelonephritis, trauma (other), testicular asymmetry
K18-061	m	3	1	1	0	1	0	Urethritis, testicular asymmetry
K18-062	m	4	2	0	1	1	0	Urogenital Chlamydiosis (cystitis, urethritis)
K18-063	f	5	4	0	1	1	0	Urogenital Chlamydiosis (cystitis, endometritis)
K18-064	m	5	1	1	0	1	1	Trauma (other), arthritis, dental disease
K18-065	m	5	3	1	0	1	1	Spinal curvature
K18-066	m	3	3	0	0	0	0	Oxalate nephrosis
K18-067	m	5	3	1	0	1	1	Urethritis, conjunctivitis, trauma (DA), testicular asymmetry
K18-068	m	4	3	0	0	1	0	Trauma (RTA)
K18-069	m	4	4	0	1	0	0	Urogenital Chlamydiosis (cystitis, urethritis), trauma (RTA), testicular asymmetry
K18-070	f	5	1	1	1	1	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), dental disease
K18-071	f	5	1	0	1	1	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), thromboembolic disease
K18-072	f	4	2	1	1	1	0	Subclinical chlamydia, oxalate nephrosis
K18-073	m	4	3	0	0	1	1	Urethritis, conjunctivitis, trauma (RTA), testicular asymmetry
K18-074	m	3	4	0	1	0	0	Urogenital Chlamydiosis (cystitis), trauma (RTA)
K18-075	f	5	2	1	1	1	0	Urogenital Chlamydiosis (cystitis, paraovarian cysts), pyelonephritis, trauma (RTA)
K18-076	m	4	4	0	1	1	0	Urogenital Chlamydiosis (cystitis), pyelonephritis, trauma (RTA), tapeworm
K18-077	m	3	3	1	0	0	0	Mange
K18-078	m	4	4	1	1	1	1	Urogenital Chlamydiosis (cystitis, urethritis), trauma (other)
K18-079	m	3	3	0	0	1	1	Oxalate nephrosis, tapeworm
K18-080	m	3	4	0	0	0	1	Trauma (RTA), testicular asymmetry
K18-081	f	1	4	0	0	0	1	Oxalate nephrosis, bronchoalveolar adenocarcinoma
K18-082	m	5	4	1	1	1	0	Urogenital Chlamydiosis (cystitis), trauma (DA), colloidal goitre
K18-083	f	2	3	0	0	0	0	Oxalate nephrosis, trauma (other)
K18-084	m	3	3	1	0	1	1	Mange, dermatitis
K18-085	m	5	3	0	0	1	0	Trauma (DA)
K18-087	m	5	2	0	1	1	0	Subclinical chlamydia, dental disease
K18-088	f	4	3	0	0	0	0	Cystitis, oxalate nephrosis, spinal curvature

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K18-089	f	4	4	0	0	0	0	Oxalate nephrosis, tapeworm
K18-090	f	3	3	0	1	0	0	Ocular and Urogenital Chlamydiosis (cystitis), mange, dermatitis
K18-091	m	4	1	0	1	0	0	Urogenital Chlamydiosis (cystitis, urethritis), oxalate nephrosis, spinal curvature, testicular asymmetry
K18-092	m	3	4	1	1	0	0	Urogenital Chlamydiosis (cystitis), conjunctivitis, dental disease
K18-093	f	6	1	1	0	0	0	Endometritis, dental disease
K18-094	f	3	3	1	1	0	0	Subclinical chlamydia, tapeworm
K18-095	f	5	1	0	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis, paraovarian cysts)
K18-096	f	6	1	1	1	1	0	Urogenital Chlamydiosis (cystitis, paraovarian cysts)
K18-097	m	3	3	0	1	0	1	Urogenital Chlamydiosis (cystitis, urethritis), arthritis, testicular asymmetry
K18-098	m	5	2	1	0	1	0	Cystitis, urethritis, conjunctivitis, trauma (other)
K18-099	m	3	3	1	0	0	0	Urethritis, testicular asymmetry
K18-100	m	4	4	1	0	1	0	Urethritis, trauma (DA), testicular asymmetry
K18-101	f	4	1	0	1	0	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis, arthritis
K18-102	m	5	3	0	1	1	1	Subclinical chlamydia, trauma (RTA), testicular asymmetry
K18-103	m	5	2	0	0	0	0	Conjunctivitis, dental disease, tapeworm
K18-104	f	6	2	1	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis, paraovarian cysts), pulmonary actinomycosis
K18-105	f	4	3	0	0	0	0	Paraovarian cysts, trauma (DA)
K18-106	m	3	3	0	1	0	0	Subclinical chlamydia, oxalate nephrosis, testicular asymmetry
K18-107	m	3	2	1	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis, urethritis), arthritis, tapeworm
K18-108	m	3	2	1	1	1	0	Subclinical chlamydia, renal fibrosis, trauma (other), testicular asymmetry
K18-109	m	2	3	0	0	0	0	Trauma (RTA), testicular asymmetry
K18-110	m	3	3	0	1	0	0	Urogenital Chlamydiosis (cystitis), trauma (DA)
K18-111	f	2	4	1	0	0	1	Trauma (DA)
K18-112	f	5	3	0	0			Endometritis, trauma (RTA)
K18-113	m	3	2	0	1	0	1	Urogenital Chlamydiosis (cystitis, urethritis), renal fibrosis, testicular asymmetry
K18-114	m	1	1	1	1	0	0	Subclinical chlamydia
K18-115	f	5	1		0			Cystitis, dental disease, mange
K18-116	f	5	4		1			Urogenital Chlamydiosis (cystitis, paraovarian cysts)
K18-117	f	2	4		1			Urogenital Chlamydiosis (cystitis, endometritis, paraovarian cysts), trauma (DA)
K18-118	f	5	4		1			Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis
K18-119	f	6	3		1			Ocular and Urogenital Chlamydiosis (cystitis, paraovarian cysts), dental disease, dermatitis, pneumonia, thromboembolic disease
K18-120	f	3	3		0			Oxalate nephrosis
K18-121	f	4	3		0			Trauma (RTA)
K18-122	m	3	1	1	1	1	0	Urogenital Chlamydiosis (cystitis, urethritis), tapeworm, mange, dermatitis

Koala	Sex	TWC	BCS	KoRV positive	*Chlamydia* positive	PhaHV1	PhaHV2	Pathological findings
K18-123	m	2	3	1	0	1	0	Renal fibrosis, trauma (DA)
K18-124	f	3	4	0	0	1	0	Trauma (DA)
K18-125	f	3	4	1	1	1	1	Urogenital Chlamydiosis (cystitis, paraovarian cysts), oxalate nephrosis, pyelonephritis
K18-126	m	4	4	0	0	1	0	Urethritis, renal fibrosis, trauma (RTA)
K18-127	m	5	3	x	1	1	1	Ocular Chlamydiosis, renal fibrosis, tapeworm
K18-128	m	4	2	1	1	1	1	Urogenital Chlamydiosis (cystitis, urethritis), oxalate nephrosis, pyelonephritis, spinal curvature, tapeworm
K18-129	f	5	4	1	1	1	0	Urogenital Chlamydiosis (cystitis), renal fibrosis, trauma (DA), tapeworm
K18-130	m	3	4	1	0	0	0	Cystitis, trauma (DA), tapeworm
K18-131	m	3	3	0	1	1	1	Ocular and Urogenital Chlamydiosis (cystitis), trauma (RTA)
K18-132	m	3	4	1	0	1	1	Cystitis, trauma (RTA), tapeworm
K18-133	f	4	1	0	1	1	0	Urogenital Chlamydiosis (cystitis, endometritis, paraovarian cysts) , conjunctivitis
K18-134	m	3	2	0	1	0	0	Subclinical chlamydia, trauma (other), tapeworm
K18-135	f	2	4	1	1	1	0	Urogenital Chlamydiosis (cystitis), oxalate nephrosis
K18-136	m	7	2	0	0			Urethritis, testicular asymmetry
K18-137	m	4	3	0	1	1	1	Ocular Chlamydiosis, tapeworm, mange, pneumonia
K18-138	m	4	4	1	1	1	1	Urogenital Chlamydiosis (cystitis, urethritis), pneumonia
K18-139	m	3	4		0			Cystitis, urethritis, pneumonia
K18-140	m	1	4	1	0	0	1	Oxalate nephrosis
K18-141	f	5	2	0	1	1	0	Ocular and Urogenital Chlamydiosis (cystitis, endometritis, paraovarian cysts)
K18-142	m	5	2	1	0	1	1	Cystitis, oxalate nephrosis
K18-143	m	5	3	0	1	0	0	Urogenital Chlamydiosis (cystitis), trauma (DA), spinal curvature
K18-144	m	3	2	0	0	1	0	Cystitis, trauma (DA), tapeworm, testicular asymmetry
K18-145	m	5	3	0	1	1	0	Urogenital Chlamydiosis (cystitis, prostatitis), trauma (RTA), tapeworm
K18-146	m	5	3	0	1	1	0	Subclinical chlamydia, oxalate nephrosis, pyelonephritis, trauma (RTA)
K19-049	m	3	3	1		1	1	Lymphoma

Tooth wear class (TWC) based on Martin (1981) and body condition score (BCS) based on Blanshard *et al.* (2008), positive for molecular analysis = 1, negative = 0.

S2: qPCR set up for Gamma actin and KoRV genes; amplification curves
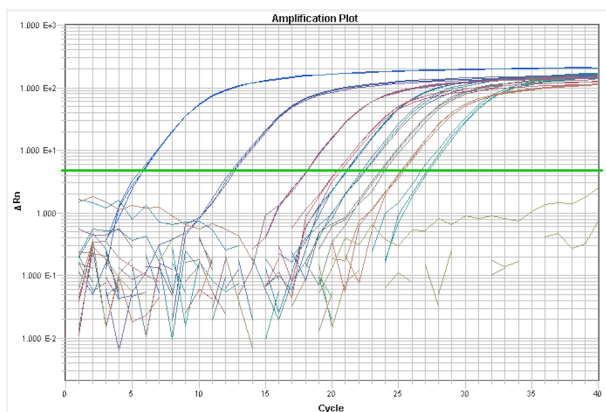
A: Gamma-Actin qPCR Amplification curves for std curve and sensitivity
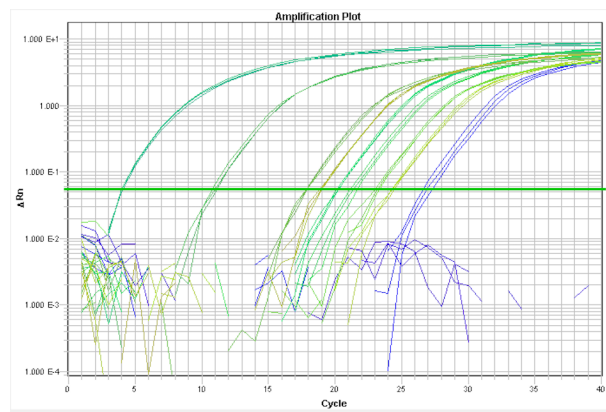
B: KoRV *env-A* qPCR Amplification curves for std curve and sensitivity
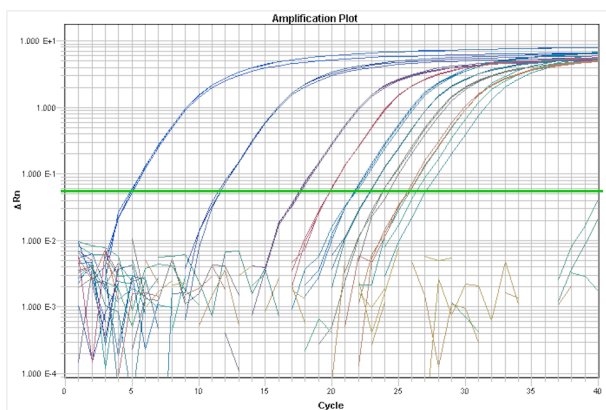
C: KoRV *gag-1* qPCR Amplification curves for std curve and sensitivity
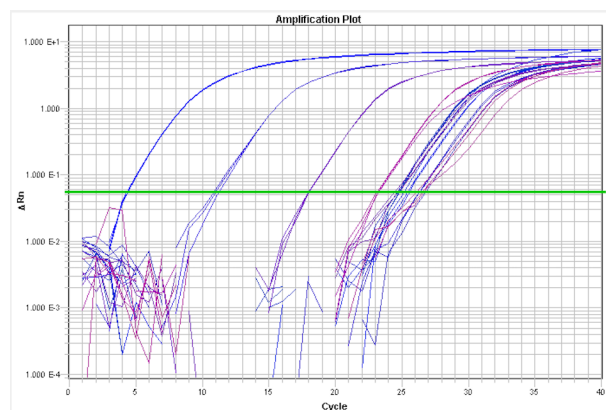
D: KoRV *gag-2* qPCR Amplification curves for std curve and sensitivity

S3: qPCR set up for Gamma actin and KoRV genes; melt curve analysis
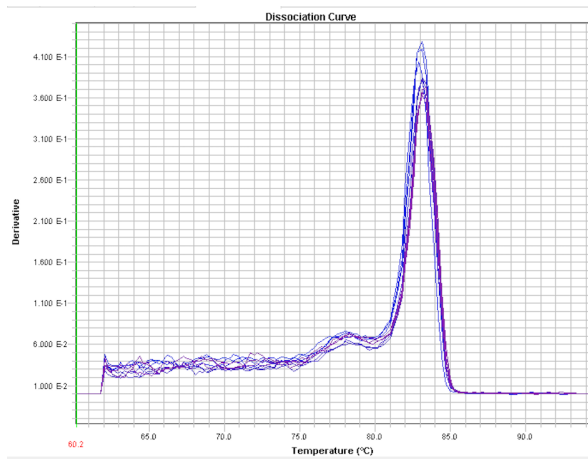
A: Gamma-Actin qPCR melt curve analysis
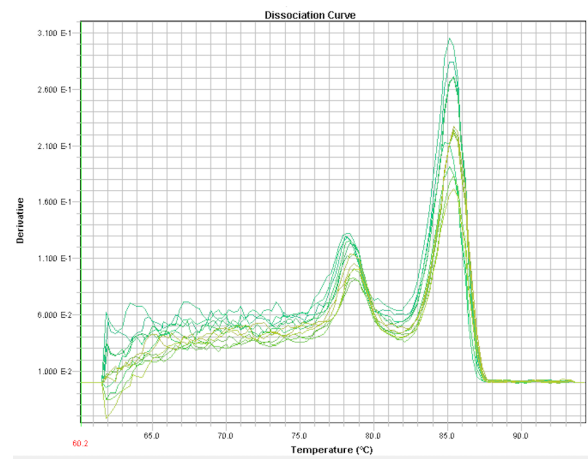
B: KoRV *env-A* qPCR melt curve analysis
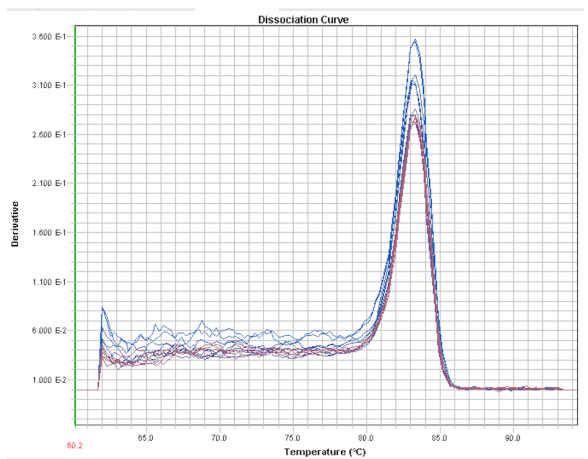
C: KoRV *gag-1* qPCR melt curve analysis
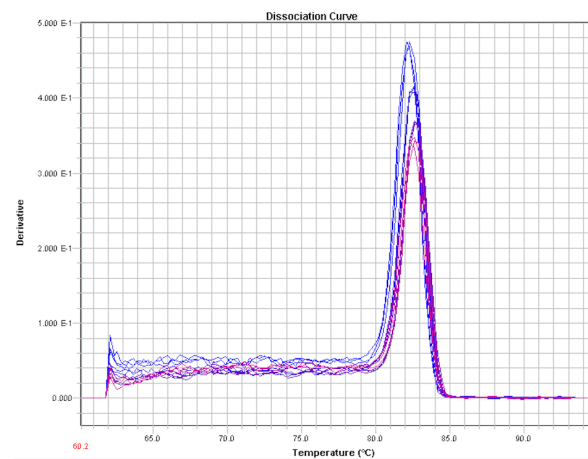
D: KoRV *gag-2* qPCR melt curve analysis

S4: qPCR set up for Gamma actin and KoRV genes; standard curves

$y = -1.8023x + 21.885$
$R^2 = 0.9849$

$y = -2.5778x + 26.646$
$R^2 = 0.9583$

$y = -3.9157x + 36.222$
$R^2 = 0.9979$

$y = -2.5994x + 25.978$
$R^2 = 0.9334$

$y = -3.1866x + 30.347$
$R^2 = 0.997$

CT value

Amplicon copy number 10^X

- ACTG1
- KoRV_GAG1
- Pol
- KoRV_ENV
- KoRV_GAG2
- Linear (ACTG1)
- Linear (KoRV_GAG1)
- Linear (Pol)
- Linear (KoRV_ENV)
- Linear (KoRV_GAG2)
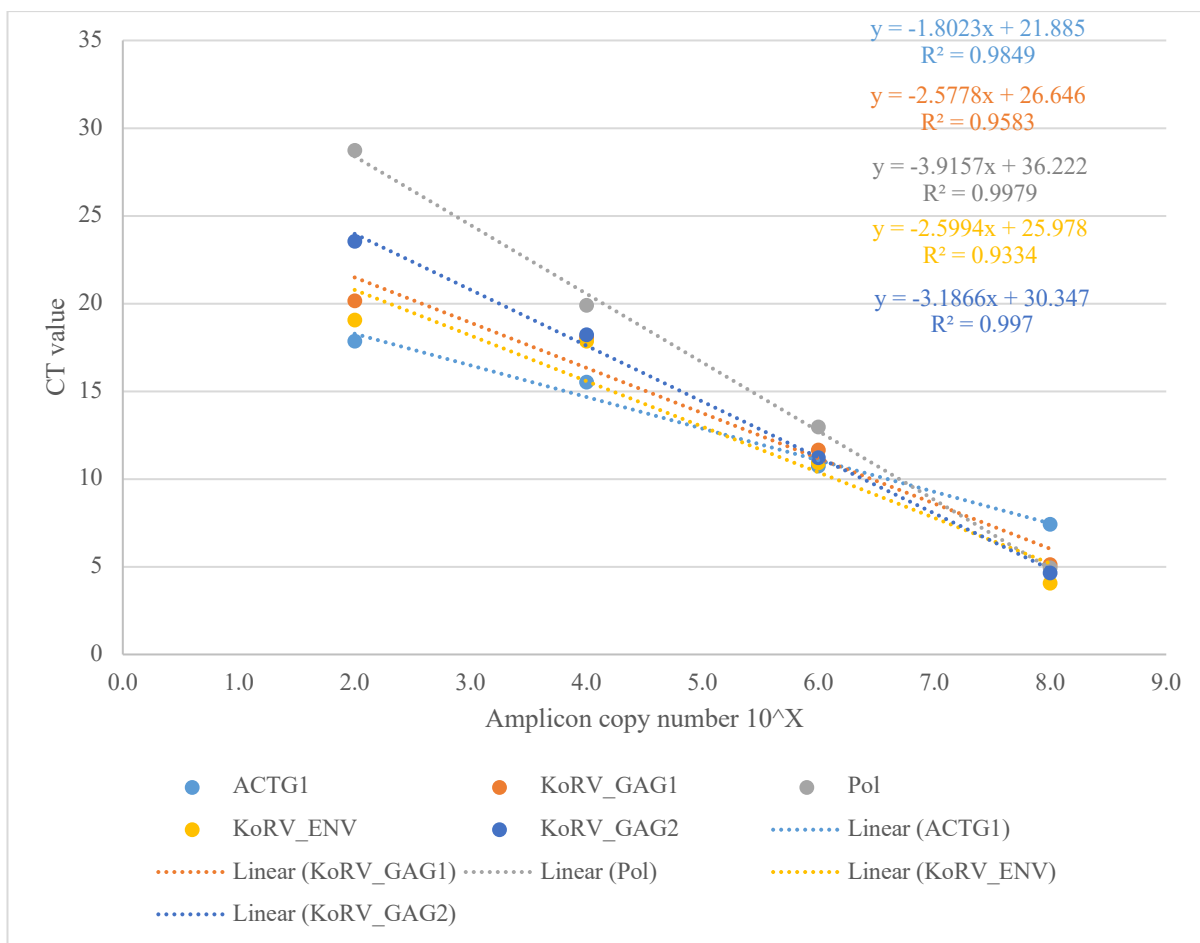
S5: Qualitative PCR results for cDNA from KoRV negative, KoRV positive koalas and koalas diagnosed with lymphoma, using KoRV negative as the control group

Sample and gene	KoRV Gene Average C_T	ACT-G Average C_T	ΔC_T KoRV Gene - ACT-G	$\Delta\Delta C_T$ ΔC_T KoRV group -ΔC_T KoRV Negative (control)	$2^{-\Delta\Delta CT}$ Fold difference in KoRV gene relative to KoRV negative* mean (range)	Z score	P val
Negative *gag* 1	22.21	18.19	4.02	0.00	1 (0.2-5.2)		
Positive *gag* 1	22.14	20.57	1.57	-2.45	5 (0.8-38)	-1.25	0.211
Lymphoma *gag* 1	20.01	20.88	-0.87	-4.89	**77** (3.8 – 220.1)	**-2.09**	**0.036**
Negative *gag* 2	40.00	18.19	21.81	0.00	1 (0.1-8.1)		
Positive *gag* 2	27.27	20.57	6.70	-15.11	**35303** (1279.2-974294.7)	**-2.51**	**0.012**
Lymphoma *gag 2*	19.36	20.88	-1.52	-23.33	**35266696** (2318694.0-119326373)	**-2.51**	**0.012**
Negative *pol*	40.00	18.19	21.81	0.00	1 (0.1-8.1)		
Positive *pol*	30.41	20.57	9.84	-11.97	**3999** (263.9-60595.2)	**-2.51**	**0.012**
Lymphoma *pol*	21.49	20.88	0.61	-21.20	**2407462** (743156.5-7798999.3)	**-2.51**	**0.012**
Negative *env* 1	40.00	18.19	21.81	0.00	1 (0.1-8.1)		
Positive *env* 1	26.04	20.57	5.47	-16.34	**82705** (6287.7-1087841.2)	**-2.51**	**0.012**
Lymphoma *env* 1	18.44	20.88	-2.45	-24.25	**80134588** (4278039.5-350930484)	**-2.51**	**0.012**

*bold values are significant (Mann-Whitney U test, p<0.05)

S6: Qualitative PCR results for cDNA from KoRV positive koalas and koalas diagnosed with lymphoma, using KoRV positive as the control group

Sample and gene	KoRV Gene Average C_T	ACT-G Average C_T	ΔC_T KoRV Gene - ACT-G	$\Delta\Delta C_T$ ΔC_T KoRV group -ΔC_T KoRV positive (control)	$2^{-\Delta\Delta CT}$ Fold difference in KoRV gene relative to KoRV negative* mean (range)	Z score	P val
Positive *gag* 1	22.14	20.57	1.57	0.00	1 (0.1-7.0)		
Lymphoma *gag* 1	20.01	20.88	-0.87	-2.44	5.4 (0.9-32.2)	-1.46	0.144
Positive *gag* 2	27.27	20.57	6.70	0.00	1 (0.0-27.6)		
Lymphoma *gag 2*	19.36	20.88	-1.52	-8.23	299 (47.7-1880.5)	-1.88	0.060
Positive *pol*	30.41	20.57	9.84	0.00	1 (0.1-15.2)		
Lymphoma *pol*	21.49	20.88	0.61	-9.23	**602** (185.8-1950.2)	**-2.51**	**0.012**
Positive *env* 1	26.04	20.57	5.47	0.00	1 (0.1-13.2)		
Lymphoma *env* 1	18.44	20.88	-2.45	-7.92	**242** (42.5-1375.6)	**-2.51**	**0.012**

*bold values are significant (Mann-Whitney U test, p<0.05)

S7: Variable descriptions for disease, demographical and score groupings

Variable	Explanation
Month	Month of the year euthanasia/death occurred
Breeding season	Euthanasia/death occurred in the breeding season (Sept-Feb)
Spring	Euthanasia/death occurred in the spring (Sept-Nov)
Summer	Euthanasia/death occurred in the summer (Dec-Feb)
Autumn	Euthanasia/death occurred in the Autumn (Mar-May)
Winter	Euthanasia/death occurred in the winter (June-Aug)
Jan-April	Euthanasia/death occurred in Jan-Apr
May-August	Euthanasia/death occurred in May-Aug
Sept-Dec	Euthanasia/death occurred in Sep-Dec
Monthly rainfall	Monthly average rainfall at Mount Lofty
Monthly mean min	Monthly mean maximum temp (^{o}C) at Mount Lofty
Monthly mean max	Monthly mean minimum temp (^{o}C) at Mount Lofty
Sex	Male or female
TWC	Tooth wear class (I-VII)
Aged	Tooth wear class >III
TWC I-II	Young: Tooth wear class I-II
TWC III-IV	Adult: Tooth wear class III-IV
TWC- V+	Senior: Tooth wear class V-VII
BCS	Body Condition Score 1-5
comp <3	Compromised: Body Condition Score <3
BCS<2	Severely compromised: Body Condition Score <2
BCS 2 or 3	Body Condition Score 2 or 3
BCS 4 or 5	Good Body Condition Score 4 or 5
KoRV positive	KoRV proviral PCR positive
KoRV Proviral Load	KoRV proviral qPCR load (copies/10^3 B-actin copies)
KoRV 0-10	KoRV proviral qPCR load 0-10
KoRV 11-50	KoRV proviral qPCR load 11-50
KoRV 51-100	KoRV proviral qPCR load 51-100
KoRV >100	KoRV proviral qPCR load >100
Chlamydia positive	*C. pecorum* qPCR positive from ocular and/or urogenital
Chlam load	*C. pecorum* qPCR load (copies/uL)
Chlam+ and load <10	*C. pecorum* qPCR load <10 copies/uL
Chlam + and load >10	*C. pecorum* qPCR load >10 copies/uL
PhaHV-1	Positive =1, negative = 0, for Phascolarctid gammaherpesvirus 1
PhaHV2	Positive =1, negative = 0, for Phascolarctid gammaherpesvirus 2
PhaHV either	Positive =1, negative = 0, for Phascolarctid gammaherpesvirus either 1 or 2
PhaHV both	Positive =1, negative = 0, for Phascolarctid gammaherpesvirus both 1 and 2
Overt Chlamydiosis	*C. pecorum* positive qPCR and associated clinical signs
Subclinical Chlamydia	*C. pecorum* positive qPCR and no associated clinical signs
Ocular Chlamydiosis	*C. pecorum* positive qPCR and associated ocular clinical signs
UGT Chlamydiosis	*C. pecorum* positive qPCR and associated urogenital clinical signs
Ocular and UGT Chlamydiosis	*C. pecorum* positive qPCR and associated clinical signs
UGT score	Urogenital Chlamydia-like disease rank (0-3)
Ocular Score	Ocular Chlamydia-like disease rank (0-3)
Overall score	Combined urogenital and ocular chlamydia-like disease rank (0-6)
positive UGT score	Positive urogenital chlamydia-like disease rank
positive Ocular score	Positive ocular chlamydia-like disease rank
positive Overall score	Positive combine urogenital and ocular chlamydia-like disease rank
Severe UGT score (3)	Urogenital chlamydia-like disease rank of 3
Severe Ocular score (3)	Ocular chlamydia-like disease rank of 3
Severe Overall score (3+)	Combined chlamydia-like disease rank of 3 or more
LUTD	Lower urogenital tract disease; urethritis, cystitis and/or vaginitis

Variable	Explanation
urethritis	Positive for urethritis
Cystitis	Positive for cystitis
vaginitis	Positive for vaginitis
endometritis	Positive for endometritis
pyometra	Positive for pyometra
Female repro disease	Female reproductive disease; positive for endometritis, pyometra and/or paraovarian cysts
Paraovarian cysts	Positive for paraovarian cysts
Male repro	Male inflammatory reproductive disease; positive for prostatitis and/or epididymitis
Epididymitis	Positive for epididymitis
Prostatitis	Positive for prostatitis
Repro disease male or female	Male or female reproductive disease present
Conjunctivitis	Positive for conjunctivitis
Oxalate nephrosis	Positive for Oxalate nephrosis
Pyelonephritis, nephritis	Positive for pyelonephritis and/or nephritis
Renal fibrosis	Positive for renal fibrosis
Renal	Renal disease present; positive for oxalate nephrosis, renal fibrosis, pyelonephritis and/or nephritis
Trauma - RTA	Positive for trauma – road traffic accident
Trauma - Dog attack	Positive for trauma - dog attack
Trauma - other	Positive for trauma - other
Trauma	Positive for any kind of trauma
Spinal curvature	Positive for scoliosis, lordosis and/or kyphosis
Arthritis (crepitus)	Positive for arthritis (by detection of crepitus)
Dental disease	Positive for severe dental disease (inclusive of; tooth root abscess, sever gum recession and gingivitis, tooth fracture and associated inflammation)
Variable	Explanation
Tapeworm	Positive for tapeworm
Mange	Positive for mange
Parasitic	Parasitic disease present; positive for mange and/or tapeworm
Dermatitis	Positive for dermatitis
Pneumonia	Positive for pneumonia
Pulmonary actinomycosis	Positive for pulmonary actinomycosis
Respiratory	Respiratory disease present; positive for pneumonia, pyothorax and/or pulmonary actinomycosis
Colloidal Goitre	Positive for Colloidal Goitre
Thromboembolic disease	Positive for thromboembolic disease
Lymphoma	Positive for lymphoma, lymphosarcoma
Neoplasia	Neoplastic disease present
Testicular asymmetry	Testicular asymmetry present with no associated disease
Testicular Genetic Disease	Testicular genetic disease; testicular asymmetry, cryptorchidism, testicular hypoplasia/aplasia
Comorbidity number	Comorbidity number - level of disease present
Comorbidity = 0 (disease free)	Comorbidity = 0 (disease free)
Comorbidity >=3	Comorbidity - level of disease present >=3
Comorbidity >=4	Comorbidity - level of disease present >=4
Comorbidity >=5	Comorbidity - level of disease present >=5
Comorbidity >=6	Comorbidity - level of disease present >=6

S8: Univariate significant demographic data

Infection	Demographic association	Demo + Infection+	Demo + Infection -	Demo - Infection +	Demo - Infection -	Total	Estimate	Std Error	Z value	Pr...z..	F test p value
C. pecorum	Senior (TWC³ V)	41	18	54	75	188	1.15	0.33	3.44	0.00	0.00
C. pecorum	Young (TWC I or II)	6	22	89	71	188	-1.53	0.49	-3.13	0.00	0.00
C. pecorum	Aged (TWC³ IV)	62	44	33	49	188	0.74	0.30	2.47	0.01	0.02
PhaHV 1	Aged (TWC³ IV)	65	24	32	56	177	1.56	0.33	4.78	0.00	0.00
PhaHV 1	Young (TWC I or II)	6	27	91	53	177	-2.04	0.48	-4.23	0.00	0.00
PhaHV 1	Compromised (BCS <3)	41	17	56	63	177	1.00	0.34	2.92	0.00	0.00
PhaHV 1	Senior (TWC³ V)	33	13	64	67	177	0.98	0.37	2.63	0.01	0.01
PhaHV 1	Months September - December	35	45	62	35	177	-0.82	0.31	-2.66	0.01	0.01
PhaHV 1	Breeding Season (September - February)	41	52	44	23	160	-0.89	0.33	-2.67	0.01	0.01
PhaHV 1	Season Autumn	33	15	52	60	160	0.93	0.36	2.56	0.01	0.01
PhaHV 1	Season Spring	33	43	52	32	160	-0.75	0.32	-2.33	0.02	0.03
PhaHV 1	Months May - August	30	13	67	67	177	0.84	0.37	2.23	0.03	0.03
KoRV & C. pecorum	Severely compromised (BCS <2)	8	12	26	121	167	1.13	0.51	2.24	0.02	0.03
KoRV & PhaHV 1	Aged (TWC³ IV)	29	55	15	67	166	0.86	0.37	2.34	0.02	0.02
KoRV & PhaHV 1	Compromised (BCS <3)	21	35	23	87	166	0.82	0.36	2.26	0.02	0.03
KoRV & PhaHV 1	Senior (TWC³ V)	17	27	27	95	166	0.80	0.38	2.10	0.04	0.05
KoRV & PhaHV 1	Young (TWC I or II)	4	28	40	94	166	-1.09	0.57	-1.93	0.05	0.05
C. pecorum & PhaHV 1	Aged (TWC³ IV)	37	43	11	55	146	1.46	0.40	3.66	0.00	0.00
C. pecorum & PhaHV 1	Senior (TWC³ V)	23	19	25	79	146	1.34	0.39	3.48	0.00	0.00
C. pecorum & PhaHV 1	Young (TWC I or II)	1	19	47	79	146	-2.43	1.04	-2.33	0.02	0.00
C. pecorum & PhaHV 1	Compromised (BCS <3)	25	30	23	68	146	0.90	0.36	2.49	0.01	0.02
C. pecorum & PhaHV 2	Senior (TWC³ V)	13	29	14	90	146	1.06	0.44	2.40	0.02	0.02
C. pecorum & PhaHV 2	Aged (TWC³ IV)	20	60	7	59	146	1.03	0.48	2.17	0.03	0.03

S9: Univariate significant disease data

Infection	Disease association	Disease + Infection+	Disease + Infection -	Disease - Infection +	Disease - Infection -	Total	Estimate	Std..Error	z.value	Pr...z.	F_test_pvalue
KoRV	Lymphoma	6	0	83	123	212	16.96	979.61	0.02	0.99	0.00
KoRV	Trauma (RTA)	9	28	80	95	212	-0.96	0.41	-2.34	0.02	0.02
KoRV	Neoplasia	7	2	82	121	212	1.64	0.81	2.02	0.04	0.04
KoRV	Oxalate Nephrosis	16	38	73	85	212	-0.71	0.34	-2.11	0.03	0.04
C. pecorum	Paraovarian cysts	24	5	22	36	87	2.06	0.56	3.67	0.00	0.00
C. pecorum	Female reproductive disease	31	10	15	31	87	1.86	0.48	3.86	0.00	0.00
C. pecorum	Reproductive disease (male or female)	36	14	59	79	188	1.24	0.36	3.44	0.00	0.00
C. pecorum	Cystitis	56	36	39	57	188	0.82	0.30	2.76	0.01	0.01
C. pecorum	PhaHV 1 infection	48	36	23	39	146	0.82	0.34	2.38	0.02	0.02
C. pecorum	Trauma	25	40	70	53	188	-0.75	0.31	-2.39	0.02	0.02
PhaHV 1	Female reproductive disease	25	9	12	28	74	1.87	0.52	3.60	0.00	0.00
PhaHV 1	Paraovarian cysts	20	5	17	32	74	2.02	0.58	3.46	0.00	0.00
PhaHV 1	Lower urogenital tract infection	58	31	39	49	177	0.85	0.31	2.77	0.01	0.01
PhaHV 1	Overt Chlamydiosis	42	17	42	45	146	0.97	0.36	2.71	0.01	0.01
PhaHV 1	Reproductive disease (male or female)	29	11	68	69	177	0.98	0.39	2.50	0.01	0.01
PhaHV 1	Urogenital Chlamydiosis	39	16	45	46	146	0.91	0.36	2.51	0.01	0.02
PhaHV 1	Chlamydia infection	48	23	36	39	146	0.82	0.34	2.38	0.02	0.02
PhaHV 1	Conjunctivitis	25	10	72	70	177	0.89	0.41	2.17	0.03	0.04
PhaHV 1	Urethritis	20	6	40	37	103	1.13	0.52	2.17	0.03	0.04
PhaHV 1	Pyelonephritis or nephritis	14	4	83	76	177	1.16	0.59	1.98	0.05	0.05
PhaHV 2	Arthritis (crepitus)	10	1	54	112	177	3.03	1.06	2.86	0.00	0.00
PhaHV 2	Paraovarian cysts	14	11	9	40	74	1.73	0.55	3.17	0.00	0.00
PhaHV 2	Trauma	15	50	49	63	177	-0.95	0.35	-2.72	0.01	0.01
PhaHV 2	Female reproductive disease	16	18	7	33	74	1.43	0.54	2.66	0.01	0.01
KoRV & C. pecorum	PhaHV 1 infection	22	56	5	54	137	1.45	0.53	2.72	0.01	0.00
KoRV & C. pecorum	Trauma (RTA)	33	105	7	23	168	-2.17	1.04	-2.10	0.04	0.01

S9: Univariate significant disease data; table continued

Infection	Disease association	Disease + Infection+	Disease + Infection -	Disease - Infection +	Disease - Infection -	Total	Estimate	Std..Error	z.value	Pr...z..	F_test_pvalue
KoRV & PhaHV 1	Urethritis	14	11	15	57	97	1.58	0.50	3.17	0.00	0.00
KoRV & PhaHV 1	Lower urinary tract infection	29	52	15	70	166	0.96	0.37	2.61	0.01	0.01
KoRV & PhaHV 1	Paraovarian cysts	9	13	6	41	69	1.55	0.62	2.52	0.01	0.01
KoRV & PhaHV 1	Trauma (RTA)	3	26	41	96	166	-1.31	0.64	-2.05	0.04	0.04
KoRV & PhaHV 2	Trauma (RTA)	1	28	29	108	166	-2.02	1.04	-1.94	0.05	0.03
KoRV & PhaHV 2	Neoplasia	4	4	26	132	166	1.62	0.74	2.20	0.03	0.04
KoRV & PhaHV 2	Trauma	6	57	24	79	166	-1.06	0.49	-2.17	0.03	0.04
C. pecorum & PhaHV 1	Female reproductive disease	18	10	6	28	62	2.13	0.60	3.56	0.00	0.00
C. pecorum & PhaHV 1	Paraovarian cysts	15	6	9	32	62	2.18	0.61	3.56	0.00	0.00
C. pecorum & PhaHV 1	Reproductive disease (male or female)	19	15	29	83	146	1.29	0.41	3.16	0.00	0.00
C. pecorum & PhaHV 2	Paraovarian cysts	10	11	2	39	62	2.88	0.85	3.40	0.00	0.00
C. pecorum & PhaHV 2	Female reproductive disease	11	17	1	33	62	3.06	1.09	2.82	0.00	0.00
C. pecorum & PhaHV 2	Arthritis (crepitus)	6	4	21	115	146	2.11	0.69	3.06	0.00	0.00
C. pecorum & PhaHV 2	Reproductive disease (male or female)	12	22	15	97	146	1.26	0.45	2.78	0.01	0.01
C. pecorum & PhaHV 2	Trauma	5	48	22	71	146	-1.09	0.53	-2.06	0.04	0.05
KoRV, C. pecorum & PhaHV	Urethritis	7	16	6	50	79	1.29	0.63	2.07	0.04	0.05
KoRV, C. pecorum & PhaHV	Paraovarian cysts	6	13	3	36	58	1.71	0.78	2.20	0.03	0.05

S10: Univariate significant scoring data

Infection	Score association	Score + Infection+	Score + Infection -	Score - Infection +	Score - Infection -	Total	Estimate	Std Error	Z value	Pr...z...	F_test pvalue
C. pecorum	Overall chlamydia score ≥ 1	80	55	15	38	188	1.30	0.35	3.71	0.00	0.00
C. pecorum	Comorbidity score ≥ 3	51	25	44	68	188	1.15	0.31	3.69	0.00	0.00
C. pecorum	Urogenital chlamydia score ≥ 1	77	56	18	37	188	1.04	0.34	3.09	0.00	0.00
C. pecorum	Disease free (Comorbidity score 0)	3	10	92	83	188	-1.31	0.68	-1.93	0.05	0.05
PhaHV 1	Overall chlamydia score ≥ 1	76	37	20	43	176	1.49	0.34	4.41	0.00	0.00
PhaHV 1	Urogenital chlamydia score ≥ 1	73	36	23	44	176	1.36	0.33	4.13	0.00	0.00
PhaHV 1	Comorbidity score ≥ 3	48	18	49	62	177	1.22	0.34	3.62	0.00	0.00
PhaHV 1	Comorbidity score ≥ 4	24	8	73	72	177	1.08	0.44	2.46	0.01	0.02
PhaHV 2	Comorbidity score ≥ 3	32	34	32	79	177	0.84	0.32	2.61	0.01	0.01
KoRV & PhaHV 1	Urogenital chlamydia score ≥ 1	36	64	7	58	165	1.54	0.45	3.41	0.00	0.00
KoRV & PhaHV 1	Overall chlamydia score ≥ 1	36	66	7	56	165	1.47	0.45	3.26	0.00	0.00
KoRV & PhaHV 1	Disease free (Comorbidity score 0)	1	17	43	105	166	-1.94	1.04	-1.86	0.06	0.04
C. pecorum & PhaHV 1	Comorbidity score ≥ 3	29	30	19	68	146	1.24	0.37	3.38	0.00	0.00
C. pecorum & PhaHV 1	Overall chlamydia score ≥ 1	41	61	7	37	146	1.27	0.46	2.76	0.01	0.00
C. pecorum & PhaHV 2	Comorbidity score ≥ 3	18	41	9	78	146	1.34	0.45	2.96	0.00	0.00
C. pecorum & PhaHV 2	Overall chlamydia score ≥ 1	25	77	2	42	146	1.92	0.76	2.53	0.01	0.00
C. pecorum & PhaHV 2	Urogenital chlamydia score ≥ 1	23	77	4	42	146	1.14	0.57	1.99	0.05	0.04
KoRV, *C. pecorum* & PhaHV	Urogenital chlamydia score ≥ 1	19	73	3	42	137	1.29	0.65	1.99	0.05	0.05
KoRV, *C. pecorum* & PhaHV	Overall chlamydia score ≥ 1	19	74	3	41	137	1.26	0.65	1.93	0.05	0.05

S11: Multivariate analysis of pathological findings with infection and coinfection status.

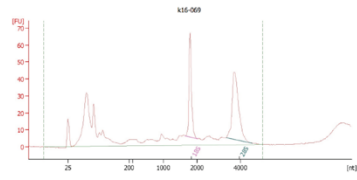| Infection	Observations	Predictor		Odds Ratio	CI	p	Cox & Snell's R^2 / Nagelkerke's R^2
KoRV	212 (all)	Aged (TWC ≥ IV)		0.54	0.30 – 0.99	**0.045**	0.094 / 0.126
		Trauma (RTA)		0.31	0.13-0.72	**0.006**	
		Oxalate nephrosis		0.34	0.17-0.69	**0.003**	
		Spinal curvature		3.54	1.07-11.76	**0.039**	
C. pecorum	188 (all)	Reproductive disease		2.39	1.11 – 5.14	**0.026**	0.172 / 0.229
		Senior (TWC ≥ V)		2.21	1.06 – 4.57	**0.033**	
		Young (TWC ≤ II)		0.35	0.12 – 0.98	**0.046**	
		Cystitis		1.98	1.04 – 3.75	**0.037**	
		Trauma		0.50	0.26 – 0.97	**0.041**	
C. pecorum	87 (female)	Paraovarian cyst		5.71	1.79 – 18.22	**0.003**	0.261 / 0.349
		Senior (TWC ≥ V)		5.08	1.75 – 14.72	**0.003**	
PhaHV-1	176 (all)	Aged (TWC ≥ IV)		4.19	2.07 – 8.45	**<0.001**	0.227 / 0.303
		Sex male		2.48	1.19 – 5.16	**0.015**	
		Positive urogenital chlamydia-like disease rank		3.18	1.54 – 6.56	**0.002**	
		Compromised (BCS ≤ 2)		2.26	1.07 – 4.79	**0.032**	
PhaHV-1	62 (female)	Positive overall (OC + UGT) chlamydia-like disease rank		8.50	1.55 – 46.71	**0.014**	0.308 / 0.411
		C. pecorum positive		6.19	1.82 – 21.03	**0.003**	
PhaHV-2	176 (all)	Arthritis (crepitus)		14.75	1.78 – 122.15	**0.013**	0.123 / 0.169
		Trauma		0.56	0.25 – 1.25	0.157	
		Comorbidity score (tabled against score of 1)	Score of 2	1.42	0.57 – 3.54	0.458	
			Score of 3	1.88	0.70 – 5.00	0.209	
			Score of ≥ 4	1.70	0.61 – 4.76	0.313	
			Score of 0	0.79	0.18 – 3.48	0.754	
PhaHV-2	58 (female)	Paraovarian cyst		4.95	1.42 – 17.31	**0.012**	0.107 / 0.156
C. pecorum & PhaHV-1	62 (female)	Paraovarian cyst		3.98	1.04 – 15.26	**0.044**	0.313 / 0.425
		Positive overall (OC + UGT) chlamydia-like disease rank		5.45	0.58 – 51.37	0.138	
		Senior (TWC ≥ V)		4.37	1.20 – 15.98	**0.026**	
C. pecorum & PhaHV-2	62 (female)	Paraovarian cyst		17.73	3.37 – 93.14	**0.001**	0.226 / 0.361

S12: Table of Koala groupings based on proviral KoRV analyses and lymphoma status, demographical data and postmortem findings

Sample	Group	Sex	TWC	BCS	Postmortem findings
K16-019	Lymphoma	m	1	3	Multicentric lymphoma, B-cell origin
K16-069	Lymphoma	m	1	4	Multicentric lymphoma, metastases pancreas, non T-cell origin. Emaciation, asymmetrical testicles
B00310	Lymphoma	f	4	1	Multicentric, metastatic lymphoma and lymphoid leukaemia, T-cell origin. Peripheral extensive emphysema.
K17-073	KoRV Positive	m	4	0	Multifocal to coalescing bronchial interstitial pyogranulomatous pneumonia, Splendore hoeppli present, mild focal pleuritis – Pulmonary actinomycosis. Mild mixed colitis with focal neutrophilic ulceration. reactive lymph nodes, diffuse hepatic atrophy with foci of fibrosis, consistent with emaciated state. Moderate ulcerative cystitis with moderate rump staining.
K17-080	KoRV Positive	f	3	2	mild to moderate rump staining, mild ulcerative cystitis, paraovarian cysts, mild lymphoplasmacytic endometritis
K18-005	KoRV Positive	m	5	3	Marked acanthosis and orthokeratotic and segmentally parakeratotic hyperkeratosis with numerous intracorneal mites. Intracorneal pustules and mild to moderate mixed perivascular and interstitial dermatitis - indicative of secondary bacterial infection (Mange). Multifocal to coalescing bronchial interstitial pyogranulomatous pneumonia, Splendore hoeppli present – Pulmonary actinomycosis. Splenic, hepatic congestion. Mild chronic-active prostatitis. lymphoplasmacytic superficial, cystitis, inflammation. Mild reactivity of axillary and inguinal lymph nodes. dislocated R hip/femoral head ligament ruptured
K17-005	KoRV Negative	f	3	3	Purulent conjunctivitis and uveitis of R eye, craniofacial tumour, mild enlargement all LNN, possible ON with pitting of cortical surface, moderate ulcerative cystitis, ulcerative endometritis, lactating and pouch young present
K18-004	KoRV Negative	f	5	3	mild rump staining, previous trauma to rib cage (callus formation), mild-moderate enlargement of inguinal and axillary lymph nodes – due to fat infiltration. LNN, poor gut fill, mild ulcerative cystitis, moderate-severe lymphoplasmacytic histiocytic, neutrophilic endometritis, Associated unilateral pyometra (gross finding). mild lymphoplasmacytic conjunctivitis with superficial subdermal fibrosis.
K17-019	KoRV Negative	f	4	4	focal mild perioral keratosis and dermatitis, mild wet bottom, renal, pulmonary and splenic congestion, pancreatic haematoma, moderate ulcerative cystitis, active ovaries

TWC = tooth wear class (Martin, 1981) a marker of aging in southern koalas, BCS = body condition score, based on muscle mass of supraspinatus and infraspinatus muscles (Blanshard *et al.* 2008).

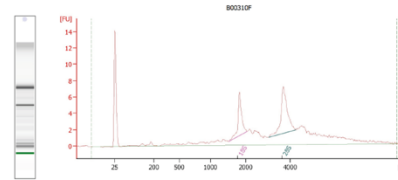S13: RNA quality analysis for the chosen lymphoma, KoRV positive and KoRV negative koala samples

Lymphoma:

KoRV Positive:

KoRV Negative:

S14: Variance analyses showing the mean-variance trend, sample specific weightings and biological variance for each gene in the cohort
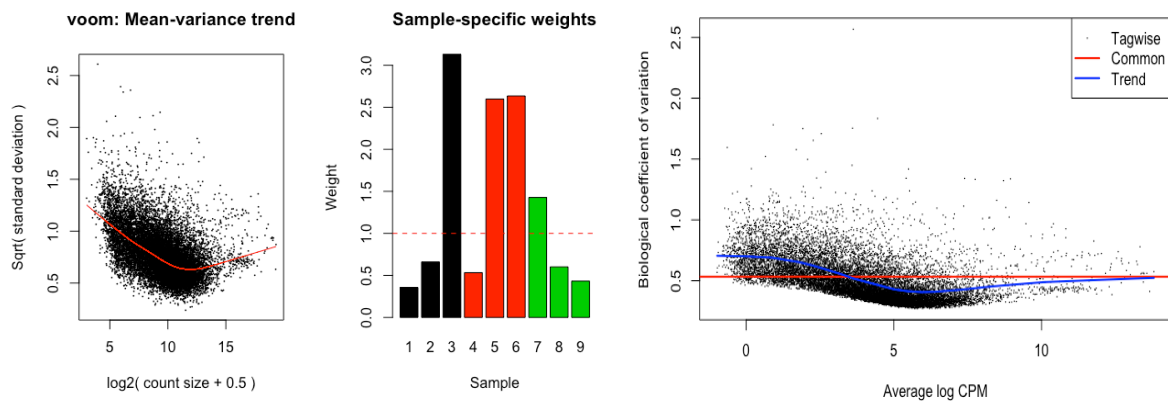
Sample-specific weights: 1 = K17-005, 2 = K17-019, 3 = K18-004, 4 = K17-073, 5 = K17-080, 6 = K18-005, 7 = B00310, 8 = K16-019, 9 = K16-069

S15: Variance of RNA-seq samples: Spread of data measured for each koala with numbers representing groupings, in A: the original cohort assignment (KoRV negative (K17-005, K18-004 and K17-019), KoRV positive (K17-073, K17-080 and K18-005) and lymphoma (K16-019, K16-069 and B00310) and B: the K17-019 reassigned cohort (KoRV negative (K17-005, and K18-004), KoRV positive (K17-019, K17-073, K17-080 and K18-005) and lymphoma (K16-019, K16-069 and B00310)

A: Original Cohort Data   B: K17-019 assigned to the KoRV positive cohort
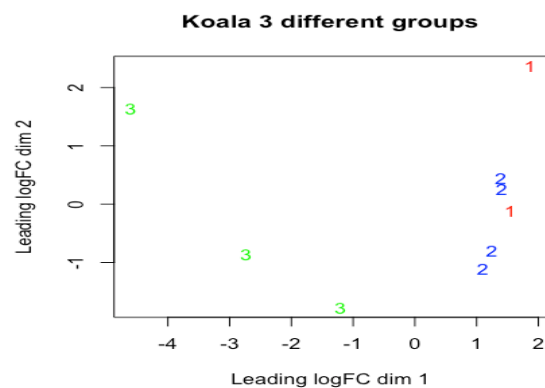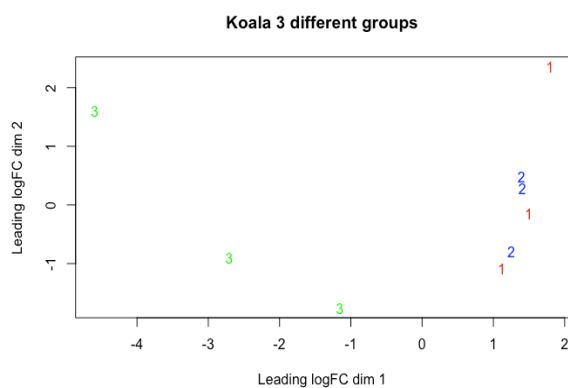
1 = negative, 2 = positive, 3 = lymphoma, second chart – reassignment of K17-019 to the KoRV positive group.

S16: Gene descriptions, LogFC, oncogenesis and immunosuppression correlation and groupings for 91 genes of interest

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
GPX2	9.800	The protein encoded by this gene belongs to the glutathione peroxidase family, members of which **catalyze the reduction of organic hydroperoxides and hydrogen peroxide (H2O2) by glutathione, and thereby protect cells against oxidative damage**. Several isozymes of this gene family exist in vertebrates, which vary in cellular location and substrate specificity. This isozyme is predominantly expressed in the gastrointestinal tract (also in liver in human), is localized in the cytoplasm, and whose preferred substrate is hydrogen peroxide. **Overexpression of this gene is associated with increased differentiation and proliferation in colorectal cancer**. This isozyme is also a selenoprotein, containing the rare amino acid selenocysteine (Sec) at its active site. Sec is encoded by the UGA codon, which normally signals translation termination. The 3' UTRs of selenoprotein mRNAs contain a conserved stem-loop structure, designated the Sec insertion sequence (SECIS) element, that is necessary for the recognition of UGA as a Sec codon, rather than as a stop signal. Alternatively spliced transcript variants have been found for this gene. [provided by RefSeq, Jul 2016]	yes	oncogenesis	up	> 2FC
DNTT	7.952	This gene is a member of the DNA polymerase type-X family and encodes a template-independent DNA polymerase that catalyzes the addition of deoxynucleotides to the 3'-hydroxyl terminus of oligonucleotide primers. In vivo, the encoded protein is expressed in a restricted population of normal and **malignant pre-B and pre-T lymphocytes** during early differentiation, where it generates antigen receptor diversity by synthesizing non-germ line elements (N-regions) at the junctions of rearranged Ig heavy chain and T cell receptor gene segments. Alternatively spliced transcript variants encoding different isoforms of this gene have been described. [provided by RefSeq, Jul 2008]	yes	oncogenesis	up	> 2FC
MSI1	6.482	This gene encodes a protein containing two conserved tandem RNA recognition motifs. Similar proteins in other species function as RNA-binding proteins and play central roles in **posttranscriptional gene regulation**. Expression of this gene has been correlated with the grade of the **malignancy and proliferative activity in gliomas and melanomas**. A pseudogene for this gene is located on chromosome 11q13. [provided by RefSeq, Jul 2008]	yes	oncogenesis	up	> 2FC
MMP9	4.303	Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and **metastasis**. Most MMP's are secreted as inactive proproteins which are activated when cleaved by extracellular proteinases. The enzyme encoded by this gene degrades type IV and V collagens. Studies in rhesus monkeys suggest that the enzyme is **involved in IL-8-induced mobilization of hematopoietic progenitor cells from bone marrow, and murine studies suggest a role in tumor-associated tissue remodeling**. [provided by RefSeq, Jul 2008] Matrix metalloproteases (matrix metalloproteinase, MMPs), also called matrixins, are zinc-dependent endopeptidases and the major proteases in ECM degradation. MMPs are capable of degrading several extracellular molecules and a number of bioactive molecules. May play an essential role in local proteolysis of the extracellular matrix and in leukocyte migration. Could play a role in bone osteoclastic resorption. Cleaves KiSS1 at a Gly-.Leu bond. Cleaves type IV and type V collagen into large C-terminal three quarter fragments and shorter N-terminal one quarter fragments. Degrades fibronectin but not laminin or Pz-peptide.	yes	oncogenesis	up	> 2FC
MYB	3.755	This gene encodes a protein with three HTH DNA-binding domains that functions as a transcription regulator. This protein plays an essential role in the regulation of hematopoiesis. **This gene may be aberrantly expressed or rearranged or undergo translocation in leukemias and lymphomas, and is considered to be an oncogene**. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Jan 2016] Transcriptional activator; DNA-binding protein that specifically recognize the sequence 5'-YAAC[GT]G-3'. Plays an important role in the control of proliferation and differentiation of hematopoietic progenitor cells.	yes	oncogenesis	up	> 2FC
CLCF1	3.041	**Cytokine with B-cell stimulating capability. Binds to and activates the ILST/gp130 receptor.**	no	immunosuppression	up	> 2FC
ELMO3	2.959	The protein encoded by this gene is similar to a C. elegans protein that functions in phagocytosis of apoptotic cells and in cell migration. Other members of this small family of engulfment and cell motility (ELMO) proteins have been shown to interact with the dedicator of **cyto-kinesis 1 protein to promote phagocytosis** and effect cell shape changes. [provided by RefSeq, Jul 2008] Involved in cytoskeletal rearrangements required for phagocytosis of apoptotic cells and cell motility. Acts in association with DOCK1 and CRK. Was initially proposed to be required in complex with DOCK1 to activate Rac Rho small GTPases. May enhance the guanine nucleotide exchange factor (GEF) activity of DOCK1 (By similarity).	no	immunosuppression	up	> 2FC
WIF1	2.829	The protein encoded by this gene functions to **inhibit WNT proteins**, which are extracellular signaling molecules that play a role in embryonic development. This protein contains a WNT inhibitory factor (WIF) domain and five epidermal growth factor (EGF)-like domains and is thought to be involved in mesoderm segmentation. **This gene functions as a tumor suppressor gene and has been found to be epigenetically silenced in various cancers.** [provided by RefSeq, Jun 2010]	no	oncogenesis	up	> 2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
MUTYH	2.804	This gene encodes a DNA glycosylase involved in **oxidative DNA damage repair**. The enzyme excises adenine bases from the DNA backbone at sites where adenine is inappropriately paired with guanine, cytosine, or 8-oxo-7,8-dihydroguanine, a major oxidatively damaged DNA lesion. The protein is localized to the nucleus and mitochondria. This gene product is thought to play a role in signaling apoptosis by the introduction of single-strand breaks following oxidative damage. **Mutations in this gene result in heritable predisposition to colorectal cancer,** termed MUTYH-associated polyposis (MAP). Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Apr 2017]	yes	oncogenesis	up	> 2FC
LOXL2	2.749	Mediates the post-translational oxidative deamination of lysine residues on target proteins leading to the formation of deaminated lysine (allysine) (PubMed:27735137). Acts as a transcription corepressor and specifically mediates deamination of trimethylated 'Lys-4' of histone H3 (H3K4me3), a specific tag for epigenetic transcriptional activation (PubMed:27735137). Shows no activity against histone H3 when it is trimethylated on 'Lys-9' (H3K9me3) or 'Lys-27' (H3K27me3) or when 'Lys-4' is monomethylated (H3K4me1) or dimethylated (H3K4me2) (PubMed:27735137). Also mediates deamination of methylated TAF10, a member of the transcription factor IID (TFIID) complex, which induces release of TAF10 from promoters, leading to inhibition of TFIID-dependent transcription (PubMed:25959397). LOXL2-mediated deamination of TAF10 results in transcriptional repression of genes required for embryonic stem cell pluripotency including POU5F1/OCT4, NANOG, KLF4 and SOX2 (By similarity). Involved in epithelial to mesenchymal transition (EMT) via interaction with SNAI1 and participates in repression of E-cadherin CDH1, probably by mediating deamination of histone H3 (PubMed:16096638, PubMed:27735137, PubMed:24414204). During EMT, involved with SNAI1 in negatively regulating pericentromeric heterochromatin transcription (PubMed:24239292). SNAI1 recruits LOXL2 to pericentromeric regions to oxidize histone H3 and repress transcription which leads to release of heterochromatin component CBX5/HP1A, enabling chromatin reorganization and acquisition of mesenchymal traits (PubMed:24239292). Interacts with the endoplasmic reticulum protein HSPA5 which activates the IRE1-XBP1 pathway of the unfolded protein response, leading to expression of several transcription factors involved in EMT and subsequent EMT induction (PubMed:28332555). **Involved in E-cadherin repression following hypoxia, a hallmark of EMT believed to amplify tumor aggressiveness, suggesting that it may play a role in tumor progression** (PubMed:20026874). When secreted into the extracellular matrix, promotes cross-linking of extracellular matrix proteins by mediating oxidative deamination of peptidyl lysine residues in precursors to fibrous collagen and elastin (PubMed:20306300). Acts as a regulator of sprouting angiogenesis, probably via collagen IV scaffolding (PubMed:21835952). Acts as a regulator of chondrocyte differentiation, probably by regulating expression of factors that control chondrocyte differentiation (By similarity).	yes	oncogenesis	up	> 2FC
CDK6	2.661	**CDK6, along with its partner CDK4, are key players in cell cycle progression.** The complex has been implicated in a number of **cancer types,** and is the focus of therapeutic research and development. One targeted therapy for CDK inhibition is palbociclib, which may slow the growth of advanced stage breast cancers. It has also been shown, in mouse, that CDK inhibition may sensitize mutant PIK3CA tumors to PI3K inhibitors. The protein encoded by this gene is a member of the CMGC family of serine/threonine protein kinases. This kinase is a catalytic subunit of the protein kinase complex that is important for **cell cycle G1 phase progression and G1/S transition.** The activity of this kinase first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. This kinase, as well as CDK4, has been shown to phosphorylate, and thus regulate the activity of, tumor suppressor protein Rb. **Altered expression of this gene has been observed in multiple human cancers.** A mutation in this gene resulting in reduced cell proliferation, and impaired cell motility and polarity, and has been identified in patients with primary microcephaly. [provided by RefSeq, Aug 2017] Cdks (cyclin-dependent kinases) are heteromeric serine/threonine kinases that control progression through the cell cycle in concert with their regulatory subunits, the cyclins. Although there are 12 different cdk genes, only 5 have been shown to directly drive the cell cycle. Serine/threonine-protein kinase involved in the control of the cell cycle and differentiation; promotes G1/S transition. Phosphorylates pRB/RB1 and NPM1. Interacts with D-type G1 cyclins during interphase at G1 to form a pRB/RB1 kinase and controls the entrance into the cell cycle. Involved in initiation and maintenance of cell cycle exit during cell differentiation; prevents cell proliferation and regulates negatively cell differentiation, but is required for the proliferation of specific cell types (e.g. erythroid and hematopoietic cells). Essential for cell proliferation within the dentate gyrus of the hippocampus and the subventricular zone of the lateral ventricles. Required during thymocyte development. Promotes the production of newborn neurons, probably by modulating G1 length. Promotes, at least in astrocytes, changes in patterns of gene expression, changes in the actin cytoskeleton including loss of stress fibers, and enhanced motility during cell differentiation. Prevents myeloid differentiation by interfering with RUNX1 and reducing its transcription transactivation activity, but promotes proliferation of normal myeloid progenitors. Delays senescence. Promotes the proliferation of beta-cells in pancreatic islets of Langerhans. May play a role in the centrosome organization during the cell cycle phases (PubMed:23918663).	yes	oncogenesis	up	> 2FC
MYCL	2.617	MYCL (MYCL Proto-Oncogene, BHLH Transcription Factor) is a Protein Coding gene. Diseases associated with MYCL include Apocrine Adenosis Of Breast and Lower Lip **Cancer.** Gene Ontology (GO) annotations related to this gene include DNA-binding transcription factor activity and protein dimerization activity. An important paralog of this gene is MYCN.	yes	oncogenesis	up	> 2FC

Appendix 24

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
FLT3	2.564	**FLT3 is an important cytokine receptor** involved in normal hematopoiesis. Mutations in this gene are common in **acute myeloid leukemia (AML)** and screening for mutations in this gene has been recommended by the World Health Organization in patients with AML, particularly in cases of cytogenetically normal AML (CN-AML). FLT3 mutations commonly co-occur with mutations such as NPM1 that are associated with CN-AML and likely modulate prognostic impact. While FLT3-ITD mutations have been associated with poorer prognosis in AML, the prognostic impact of FLT3-TKD mutations are still up for debate. This gene encodes a class III receptor tyrosine kinase that **regulates hematopoiesis**. This receptor is activated by binding of the fms-related tyrosine kinase 3 ligand to the extracellular domain, which induces homodimer formation in the plasma membrane leading to autophosphorylation of the receptor. The activated receptor kinase subsequently phosphorylates and activates multiple cytoplasmic effector molecules in pathways involved in apoptosis, proliferation, and differentiation of hematopoietic cells in bone marrow. Mutations that result in the constitutive activation of this receptor **result in acute myeloid leukemia and acute lymphoblastic leukemia**. [provided by RefSeq, Jan 2015] FMS-like receptor tyrosine kinase-3 (FLT3) is a member of the class III RTK (receptor tyrosine kinase) family and is expressed primarily in hematopoietic progenitor cells. Its expression in these cells means that FLT3 has an important role in the pathogenesis of AML. Tyrosine-protein kinase that acts as cell-surface receptor for the cytokine FLT3LG and regulates differentiation, proliferation and survival of hematopoietic progenitor cells and of dendritic cells. Promotes phosphorylation of SHC1 and AKT1, and activation of the downstream effector MTOR. Promotes activation of RAS signaling and phosphorylation of downstream kinases, including MAPK1/ERK2 and/or MAPK3/ERK1. Promotes phosphorylation of FES, FER, PTPN6/SHP, PTPN11/SHP-2, PLCG1, and STAT5A and/or STAT5B. Activation of wild-type FLT3 causes only marginal activation of STAT5A or STAT5B. Mutations that cause constitutive kinase activity promote cell proliferation and resistance to apoptosis via the activation of multiple signaling pathways.	yes	oncogenesis	up	> 2FC
CXXC5	2.538	May indirectly participate in activation of the NF-kappa-B and MAPK pathways. Acts as a mediator of BMP4-mediated modulation of canonical Wnt signaling activity in neural stem cells (By similarity). **Required for DNA damage-induced ATM phosphorylation, p53 activation and cell cycle arrest. Involved in myelopoiesis. Transcription factor.** Binds to the oxygen responsive element of COX4I2 and represses its transcription under hypoxia conditions (4% oxygen), as well as normoxia conditions (20% oxygen) (PubMed:23303788). May repress COX4I2 transactivation induced by CHCHD2 and RBPJ (PubMed:23303788). Binds preferentially to DNA containing cytidine-phosphate-guanosine (CpG) dinucleotides over CpH (H=A, T, and C), hemimethylated-CpG and hemimethylated-hydroxymethyl-CpG (PubMed:29276034). The protein encoded by this gene is a retinoid-inducible nuclear protein containing a CXXC-type zinc finger motif. The encoded protein is involved in **myelopoiesis**, is required for DNA damage-induced p53 activation, regulates the differentiation of C2C12 myoblasts into myocytes, and negatively regulates cutaneous wound healing. Several transcript variants encoding the same protein have been found for this gene. [provided by RefSeq, Nov 2015]	yes	oncogenesis	up	> 2FC
GSTP1	2.479	Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Based on their biochemical, immunologic, and structural properties, the soluble GSTs are categorized into 4 main classes: alpha, mu, pi, and theta. This GST family member is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to **function in xenobiotic metabolism and play a role in susceptibility to cancer**, and other diseases. [provided by RefSeq, Jul 2008]	yes	oncogenesis	up	> 2FC
EMP3	2.354	Probably involved in cell proliferation and cell-cell interactions. The protein encoded by this gene belongs to the PMP-22/EMP/MP20 family of proteins. The protein contains four transmembrane domains and two N-linked glycosylation sites. It is thought to be **involved in cell proliferation, cell-cell interactions and function as a tumor suppressor**. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Sep 2015]	no	oncogenesis	up	> 2FC
FAM83D	1.867	**Probable proto-oncogene** that regulates cell proliferation, growth, migration and epithelial to mesenchymal transition. Through the degradation of FBXW7, may act indirectly on the expression and downstream signaling of **MTOR, JUN and MYC** (PubMed:24344117). May play also a role in cell proliferation through activation of the ERK1/ERK2 signaling cascade (PubMed:25646692). May also be important for proper chromosome congression and alignment during mitosis through its interaction with KIF22.	yes	oncogenesis	up	not > 2FC

Appendix 25

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
SUV39H1	1.803	This gene encodes an evolutionarily-conserved protein containing an N-terminal chromodomain and a C-terminal SET domain. The encoded protein is a histone methyltransferase that trimethylates lysine 9 of histone H3, which results in transcriptional gene silencing. Loss of function of this gene disrupts heterochromatin formation and may cause chromosome instability. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Aug 2013] Histone methyltransferase that specifically trimethylates 'Lys-9' of histone H3 using monomethylated H3 'Lys-9' as substrate. Also weakly methylates histone H1 (in vitro). H3 'Lys-9' trimethylation represents a specific tag for epigenetic transcriptional repression by recruiting HP1 (CBX1, CBX3 and/or CBX5) proteins to methylated histones. Mainly functions in heterochromatin regions, thereby playing a central role in the establishment of constitutive heterochromatin at pericentric and telomere regions. H3 'Lys-9' trimethylation is also required to direct DNA methylation at pericentric repeats. SUV39H1 is targeted to histone H3 via its interaction with RB1 and is **involved in many processes, such as repression of MYOD1-stimulated differentiation, regulation of the control switch for exiting the cell cycle and entering differentiation, repression by the PML-RARA fusion protein, BMP-induced repression, repression of switch recombination to IgA and regulation of telomere length.** Component of the eNoSC (energy-dependent nucleolar silencing) complex, a complex that mediates silencing of rDNA in response to intracellular energy status and acts by recruiting histone-modifying enzymes. The eNoSC complex is able to sense the energy status of cell: upon glucose starvation, elevation of NAD(+)/NADP(+) ratio activates SIRT1, leading to histone H3 deacetylation followed by dimethylation of H3 at 'Lys-9' (H3K9me2) by SUV39H1 and the formation of silent chromatin in the rDNA locus. Recruited by the large PER complex to the E-box elements of the circadian target genes such as PER2 itself or PER1, contributes to the conversion of local chromatin to a heterochromatin-like repressive state through H3 'Lys-9' trimethylation. Lysine methyltransferases are enzymes that catalyze the transfer of methyl groups from S-adenosylmethionine (SAM) to the lysine residues on histones, particularly histones H3 and H4. **The dysregulation of this methylation is critical in the development of cancer.**	variable	oncogenesis	up	not > 2FC
CIB1	1.746	This gene encodes a member of the EF-hand domain-containing calcium-binding superfamily. The encoded protein interacts with many other proteins, including the platelet integrin alpha-IIb-beta-3, DNA-dependent protein kinase, presenilin-2, focal adhesion kinase, p21 activated kinase, and protein kinase D. The encoded protein may be involved in **cell survival and proliferation, including cancer** and Alzheimer's disease. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Apr 2013] [Microbial infection] Involved in keratinocyte-intrinsic immunity to human beta-papillomaviruses (HPVs).|||Isoform 2]: **Plays a regulatory role in angiogenesis and tumor growth** by mediating PKD/PRKD2-induced vascular endothelial growth factor A (VEGFA) secretion.||Calcium-binding protein that plays a role in the regulation of numerous cellular processes, such as cell differentiation, cell division, cell proliferation, cell migration, thrombosis, angiogenesis, cardiac hypertrophy and apoptosis. Involved in bone marrow megakaryocyte differentiation by negatively regulating thrombopoietin-mediated signaling pathway. Participates in the endomitotic cell cycle of megakaryocyte, a form of mitosis in which both karyokinesis and cytokinesis are interrupted. Plays a role in integrin signaling by negatively regulating alpha-IIb/beta3 activation in thrombin-stimulated megakaryocytes preventing platelet aggregation. Up-regulates PTK2/FAK1 activity, and is also needed for the recruitment of PTK2/FAK1 to focal adhesions; it thus appears to play an important role in focal adhesion formation. Positively regulates cell migration on fibronectin in a CDC42-dependent manner, the effect being negatively regulated by PAK1. Functions as a negative regulator of stress activated MAP kinase (MAPK) signaling pathways. Down-regulates inositol 1,4,5-trisphosphate receptor-dependent calcium signaling. Involved in sphingosine kinase SPHK1 translocation to the plasma membrane in a N-myristoylation-dependent manner preventing TNF-alpha-induced apoptosis. Regulates serine/threonine-protein kinase PLK3 activity for proper completion of cell division progression. Plays a role in microtubule (MT) dynamics during neuronal development; disrupts the MT depolymerization activity of STMN2 attenuating NGF-induced neurite outgrowth and the MT reorganization at the edge of lamellipodia. Promotes cardiomyocyte hypertrophy via activation of the calcineurin/NFAT signaling pathway. Stimulates calcineurin PPP3R1 activity by mediating its anchoring to the sarcolemma. In ischemia-induced (pathological or adaptive) angiogenesis, stimulates endothelial cell proliferation, migration and microvessel formation by activating the PAK1 and ERK1/ERK2 signaling pathway. **Promotes also cancer cell survival and proliferation.** May regulate cell cycle and differentiation of spermatogenic germ cells, and/or differentiation of supporting Sertoli cells.	yes	oncogenesis	up	not > 2FC
FGF8	1.724	The protein encoded by this gene is a member of the fibroblast growth factor (FGF) family. **FGF family members possess broad mitogenic and cell survival activities,** and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, **tumor growth and invasion.** This protein is known to be a factor that supports androgen and anchorage independent **growth of mammary tumor cells. Overexpression of this gene has been shown to increase tumor growth** and angiogenesis. The adult expression of this gene is restricted to testes and ovaries. Temporal and spatial pattern of this gene expression suggests its function as an embryonic epithelial factor. Studies of the mouse and chick homologs revealed roles in midbrain and limb development, organogenesis, embryo gastrulation and left-right axis determination. The alternative splicing of this gene results in four transcript variants. [provided by RefSeq, Jul 2008]	yes	oncogenesis	up	not > 2FC

Appendix 27

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
SIRT7	1.704	NAD-dependent protein-lysine deacylase that can act both as a deacetylase or deacylase (desuccinylase, depropionylase and deglutarylase), depending on the context (PubMed:22722849, PubMed:26907567, PubMed:30653310, PubMed:31542297). Specifically mediates deacetylation of histone H3 at 'Lys-18' (H3K18Ac) (PubMed:22722849, PubMed:30420520). In contrast to other histone deacetylases, displays strong preference for a specific histone mark, H3K18Ac, directly linked to control of gene expression (PubMed:22722849, PubMed:30653310). H3K18Ac is mainly present around the transcription start site of genes and has been linked to activation of nuclear hormone receptors; SIRT7 thereby acts as a **transcription repressor** (PubMed:22722849). Moreover, H3K18 hypoacetylation has been reported as a marker of **malignancy** in various cancers and seems to maintain the transformed phenotype of cancer cells (PubMed:22722849). Also able to mediate deacetylation of histone H3 at 'Lys-36' (H3K36Ac) in the context of nucleosomes (PubMed:30653310). Also mediates deacetylation of non-histone proteins, such as ATM, CDK9, DDX21, DDB1, FBL, FKBP5/FKBP51, RAN, RRP9/U3-55K and POLR1E/PAF53 (PubMed:24207024, PubMed:26867678, PubMed:28147277, PubMed:28886238, PubMed:28426094, PubMed:30540930, PubMed:31075303, PubMed:30944854, PubMed:28790157). Enriched in nucleolus where it stimulates transcription activity of the RNA polymerase I complex (PubMed:16618798, PubMed:19174463, PubMed:24207024). Acts by mediating the deacetylation of the RNA polymerase I subunit POLR1E/PAF53, thereby promoting the association of RNA polymerase I with the rDNA promoter region and coding region (PubMed:16618798, PubMed:19174463, PubMed:24207024). In response to metabolic stress, SIRT7 is released from nucleoli leading to hyperacetylation of POLR1E/PAF53 and decreased RNA polymerase I transcription (PubMed:24207024). Required to restore the transcription of ribosomal RNA (rRNA) at the exit from mitosis (PubMed:19174463). Promotes pre-ribosomal RNA (pre-rRNA) cleavage at the 5'-terminal processing site by mediating deacetylation of RRP9/U3-55K, a core subunit of the U3 snoRNP complex (PubMed:26867678). Mediates 'Lys-37' deacetylation of Ran, thereby regulating the nuclear export of NF-kappa-B subunit RELA/p65 (PubMed:31075303). Acts as a regulator of DNA damage repair by mediating deacetylation of ATM during the late stages of DNA damage response, promoting ATM dephosphorylation and deactivation (PubMed:30944854). Suppresses the activity of the DCX (DDB1-CUL4-X-box) E3 ubiquitin-protein ligase complexes by mediating deacetylation of DDB1, which prevents the interaction between DDB1 and CUL4 (CUL4A or CUL4B) (PubMed:28886238). Activates RNA polymerase II transcription by mediating deacetylation of CDK9, thereby promoting 'Ser-2' phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (PubMed:28426094). Deacetylates FBL, promoting histone-glutamine methyltransferase activity of FBL (PubMed:30540930). Acts as a regulator of mitochondrial function by catalyzing deacetylation of GABPB1 (By similarity). Regulates Akt/AKT1 activity by mediating deacetylation of FKBP5/FKBP51 (PubMed:28147277). Required to prevent R-loop-associated DNA damage and transcription-associated genomic instability by mediating deacetylation and subsequent activation of DDX21, thereby overcoming R-loop-mediated stalling of RNA polymerases (PubMed:28790157). In addition to protein deacetylase activity, also acts as protein-lysine deacylase (PubMed:27436229, PubMed:27997115, PubMed:31542297). Acts as a protein depropionylase by mediating depropionylation of Osterix (SP7), thereby regulating bone formation by osteoblasts (By similarity). Acts as a histone deglutarylase by mediating deglutarylation of histone H4 on 'Lys-91' (H4K91glu); a mark that destabilizes nucleosomes by promoting dissociation of the H2A-H2B dimers from nucleosomes (PubMed:31542297). Acts as a histone desuccinylase: in response to DNA damage, recruited to DNA double-strand breaks (DSBs) and catalyzes desuccinylation of histone H3 on 'Lys-122' (H3K122succ), thereby promoting chromatin condensation and DSB repair (PubMed:27436229). Also promotes DSB repair by promoting H3K18Ac deacetylation, regulating non-homologous end joining (NHEJ) (By similarity). Along with its role in DNA repair, required for chromosome synapsis during prophase I of female meiosis by catalyzing H3K18Ac deacetylation (By similarity). Involved in transcriptional repression of LINE-1 retrotransposon via H3K18Ac deacetylation, and promotes their association with the nuclear lamina (PubMed:31226208). Required to stabilize ribosomal DNA (rDNA) heterochromatin and prevent cellular senescence induced by rDNA instability (PubMed:29728458). Acts as a negative regulator of SIRT1 by preventing autodeacetylation of SIRT1, restricting SIRT1 deacetylase activity (By similarity). This gene encodes a member of the sirtuin family of proteins, homologs to the yeast Sir2 protein. Members of the sirtuin family are characterized by a sirtuin core domain and grouped into four classes. The functions of human sirtuins have not yet been determined; however, yeast sirtuin proteins are known to regulate epigenetic gene silencing and suppress recombination of rDNA. Studies suggest that the human sirtuins may function as intracellular regulatory proteins with mono-ADP-ribosyltransferase activity. The protein encoded by this gene is included in class IV of the sirtuin family. [provided by RefSeq, Jul 2008] Silent information regulator (Sir2)-like family deacetylases (also known as sirtuins) are a group of enzymes closely related to histone deacetylases. These enzymes can be found in the cytoplasm, mitochondria or nucleus of the cell and are ubiquitously expressed.	variable	oncogenesis	up	not > 2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
TARBP2	1.609	(Microbial infection) **Binds to the HIV-1 TAR RNA which is located in the long terminal repeat (LTR) of HIV-1**, and stimulates translation of TAR-containing RNAs (PubMed:2011739, PubMed:11438532, PubMed:12475984). This is achieved in part at least by binding to and inhibiting EIF2AK2/PKR, thereby reducing phosphorylation and inhibition of EIF2S1/eIF-2-alpha (PubMed:11438532). May also promote translation of TAR-containing RNAs independently of EIF2AK2/PKR (PubMed:12475984). Mediates recruitment of FTSJ3 methyltransferase to HIV-1 RNA, **leading to 2'-O-methylation of the viral genome, allowing HIV-1 to escape the innate immune system** (PubMed:30626973).‖Required for formation of the RNA induced silencing complex (RISC). Component of the RISC loading complex (RLC), also known as the micro-RNA (miRNA) loading complex (miRLC), which is composed of DICER1, AGO2 and TARBP2. Within the RLC/miRLC, DICER1 and TARBP2 are required to process precursor miRNAs (pre-miRNAs) to mature miRNAs and may subsequently dissociate from DICER1 and TARBP2. May also play a role in the production of short interfering RNAs (siRNAs) from double-stranded RNA (dsRNA) by DICER1. HIV-1, the causative agent of acquired immunodeficiency syndrome (AIDS), contains an RNA genome that produces a chromosomally integrated DNA during the replicative cycle. Activation of HIV-1 gene expression by the transactivator Tat is dependent on an RNA regulatory element (TAR) located downstream of the transcription initiation site. The protein encoded by this gene binds between the bulge and the loop of the HIV-1 TAR RNA regulatory element and activates HIV-1 gene expression in synergy with the viral Tat protein. Alternative splicing results in multiple transcript variants encoding different isoforms. This gene also has a pseudogene. [provided by RefSeq, Jul 2008]	yes	vrial infection	up	not > 2FC
APEX1	1.534	The APEX gene encodes the major AP endonuclease in human cells. It encodes the APEX endonuclease, a DNA repair enzyme with apurinic/apyrimidinic (AP) activity. Such AP activity sites occur frequently in DNA molecules by spontaneous hydrolysis, by DNA damaging agents or by DNA glycosylases that remove specific abnormal bases. The AP sites are the most frequent pre-mutagenic lesions that can prevent normal DNA replication. Splice variants have been found for this gene; all encode the same protein. Disruptions in the biological functions related to APEX are associated with many various **malignancies** and neurodegenerative diseases.[provided by RefSeq, Dec 2019] Multifunctional protein that plays a central role in the cellular response to oxidative stress. The two major activities of APEX1 are DNA repair and redox regulation of transcriptional factors. Functions as a apurinic/apyrimidinic (AP) endodeoxyribonuclease in the DNA base excision repair (BER) pathway of DNA lesions induced by oxidative and alkylating agents. Initiates repair of AP sites in DNA by catalyzing hydrolytic incision of the phosphodiester backbone immediately adjacent to the damage, generating a single-strand break with 5'-deoxyribose phosphate and 3'-hydroxyl ends. Does also incise at AP sites in the DNA strand of DNA/RNA hybrids, single-stranded DNA regions of R-loop structures, and single-stranded RNA molecules. Has a 3'-5' exoribonuclease activity on mismatched deoxyribonucleotides at the 3' termini of nicked or gapped DNA molecules during short-patch BER. Possesses a DNA 3' phosphodiesterase activity capable of removing lesions (such as phosphoglycolate) blocking the 3' side of DNA strand breaks. May also play a role in the epigenetic regulation of gene expression by participating in DNA demethylation. Acts as a loading factor for POLB onto non-incised AP sites in DNA and stimulates the 5'-terminal deoxyribose 5'-phosphate (dRp) excision activity of POLB. Plays a role in the protection from granzymes-mediated cellular repair leading to cell death. Also involved in the DNA cleavage step of class switch recombination (CSR). On the other hand, APEX1 also exerts reversible nuclear redox activity to regulate DNA binding affinity and transcriptional activity of transcriptional factors by controlling the redox status of their DNA-binding domain, such as the FOS/JUN AP-1 complex after exposure to IR. Involved in calcium-dependent down-regulation of parathyroid hormone (PTH) expression by binding to negative calcium response elements (nCaREs). Together with HNRNPL or the dimer XRCC5/XRCC6, associates with nCaRE, acting as an activator of transcriptional repression. Stimulates the YBX1-mediated MDR1 promoter activity, when acetylated at Lys-6 and Lys-7, leading to drug resistance. Acts also as an endoribonuclease involved in the control of single-stranded RNA metabolism. **Plays a role in regulating MYC mRNA turnover** by preferentially cleaving in between UA and CA dinucleotides of the MYC coding region determinant (CRD). In association with NMD1, plays a role in the rRNA quality control process during cell cycle progression. Associates, together with YBX1, on the MDR1 promoter. Together with NPM1, associates with rRNA. Binds DNA and RNA.	yes	oncogenesis	up	not > 2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
NOD2	-1.592	Involved in gastrointestinal immunity. Upon stimulation by muramyl dipeptide (MDP), a fragment of bacterial peptidoglycan, binds the proximal adapter receptor-interacting RIPK2, which recruits ubiquitin ligases as XIAP, BIRC2, BIRC3, INAVA and the LUBAC complex, triggering activation of MAP kinases and activation of NF-kappa-B signaling. **This in turn leads to the transcriptional activation of hundreds of genes involved in immune response.** Required for MDP-induced NLRP1-dependent CASP1 activation and IL1B release in macrophages (PubMed:18511561). Component of an autophagy-mediated antibacterial pathway together with ATG16L1 (PubMed:20637199). Plays also a role in sensing single-stranded RNA (ssRNA) from viruses. **Interacts with mitochondrial antiviral signaling/MAVS,** leading to activation of interferon regulatory factor-3/IRF3 and expression of type I interferon (PubMed:19701189). This gene is a member of the Nod1/Apaf-1 family and encodes a protein with two caspase recruitment (CARD) domains and six leucine-rich repeats (LRRs). The protein is primarily expressed in the peripheral blood leukocytes. It plays a role in the immune response to intracellular bacterial lipopolysaccharides (LPS) by recognizing the muramyl dipeptide (MDP) derived from them and activating the NFKB protein. Mutations in this gene have been associated with Crohn disease and Blau syndrome. Alternatively spliced transcript variants encoding distinct isoforms have been found for this gene. [provided by RefSeq, Jun 2014]	yes	immunosuppression	down	not < -2FC
FES	-1.607	Tyrosine-protein kinase that acts downstream of cell surface receptors and plays a role in the regulation of the actin cytoskeleton, microtubule assembly, cell attachment and cell spreading. Plays a role in FCER1 (high affinity immunoglobulin epsilon receptor)-mediated signaling in mast cells. **Acts down-stream of the activated FCER1 receptor and the mast/stem cell growth factor receptor KIT.** Plays a role in the regulation of mast cell degranulation. Plays a role in the regulation of cell differentiation and promotes neurite outgrowth in response to NGF signaling. Plays a role in cell scattering and cell migration in response to HGF-induced activation of EZR. Phosphorylates BCR and down-regulates BCR kinase activity. Phosphorylates HCLS1/HS1, PECAM1, STAT3 and TRIM28. **This gene encodes the human cellular counterpart of a feline sarcoma retrovirus protein with transforming capabilities.** The gene product has tyrosine-specific protein kinase activity and that activity is required for maintenance of cellular transformation. Its chromosomal location has linked it to a specific translocation event identified in patients with **acute promyelocytic leukemia** but it is also involved in normal hematopoiesis as well as growth factor and cytokine receptor signaling. Alternative splicing results in multiple variants encoding different isoforms.[provided by RefSeq, Jan 2009]	no	oncogenesis	down	not < -2FC
TGFBR2	-1.620	The protein encoded by this gene is a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with TGF-beta receptor type-1, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and **regulate the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis.** Mutations in this gene have been associated with Marfan Syndrome, Loeys-Deitz Aortic Aneurysm Syndrome, and **the development of various types of tumors.** [provided by RefSeq, Aug 2017] Alternatively spliced transcript variants encoding different isoforms have been characterized.	no	oncogenesis & immunosuppression	down	not < -2FC
CEMIP	-1.627	Mediates depolymerization of hyaluronic acid (HA) via the cell membrane-associated clathrin-coated pit endocytic pathway. Binds to hyaluronic acid. Hydrolyzes high molecular weight hyaluronic acid to produce an intermediate-sized product, a process that may occur through rapid vesicle endocytosis and recycling without intracytoplasmic accumulation or digestion in lysosomes. Involved in hyaluronan catabolism in the dermis of the skin and arthritic synovium. Positively regulates epithelial–mesenchymal transition (EMT), and **hence tumor cell growth, invasion and cancer dissemination.** In collaboration with HSPA5/BIP, **promotes cancer cell migration** in a calcium and PKC-dependent manner. May be involved in hearing.	no	oncogenesis	down	not < -2FC
PHLPP2	-1.641	Protein phosphatase involved in **regulation of Akt and PKC signaling.** Mediates dephosphorylation in the C-terminal domain hydrophobic motif of members of the AGC Ser/Thr protein kinase family; specifically acts on 'Ser-473' of AKT1, 'Ser-660' of PRKCB isoform beta-II and 'Ser-657' of PRKCA. **Akt regulates the balance between cell survival and apoptosis through a cascade that primarily alters the function of transcription factors that regulate pro- and antiapoptotic genes.** Dephosphorylation of 'Ser-473' of Akt triggers apoptosis and decreases cell proliferation. Also controls the phosphorylation of AKT3. Dephosphorylates STK4 on 'Thr-387' leading to STK4 activation and apoptosis (PubMed:20513427). Dephosphorylates RPS6KB1 and is involved in regulation of cap-dependent translation (PubMed:21986499). **Inhibits cancer cell proliferation and may act as a tumor suppressor.** Dephosphorylation of PRKCA and PRKCB leads to their destabilization and degradation. Dephosphorylates RAF1 inhibiting its kinase activity (PubMed:24530606).	yes	oncogenesis	down	not < -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
TNFSF13B	-1.696	The protein encoded by this gene is a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. This cytokine is a ligand for receptors TNFRSF13B/TACI, TNFRSF17/BCMA, and TNFRSF13C/BAFFR. **This cytokine is expressed in B cell lineage cells, and acts as a potent B cell activator.** It has been also shown to play an **important role in the proliferation and differentiation of B cells.** Alternatively spliced transcript variants encoding distinct isoforms have been identified. [provided by RefSeq, Mar 2011] [Isoform 3]: **Acts as a transcription factor** for its own parent gene, in association with NF-kappa-B p50 subunit, at least in autoimmune and **proliferative B-cell diseases.** The presence of Delta4BAFF is essential for soluble BAFF release by IFNG/IFN-gamma-stimulated monocytes and for B-cell survival. It can directly or indirectly regulate the differential expression of a large number of genes **involved in the innate immune response and the regulation of apoptosis.**||Cytokine that binds to TNFRSF13B/TACI and TNFRSF17/BCMA. TNFSF13/APRIL binds to the same 2 receptors. Together, they form a 2 ligands -2 receptors pathway involved in the stimulation of B- and T-cell function and the regulation of humoral immunity. A third B-cell specific BAFF-receptor (BAFFR/BR3) promotes the survival of mature B-cells and the B-cell response.||Isoform 2 seems to inhibit isoform 1 secretion and bioactivity.	no (o), yes (i)	oncogenesis & immunosuppression	down	not <-2FC
SH2D3C	-1.702	This gene encodes an adaptor protein and member of a cytoplasmic protein family involved in cell migration. The encoded protein contains a putative **Src homology** 2 (SH2) domain and guanine nucleotide exchange factor-like domain which allows this signaling protein to form a complex with scaffolding protein Crk-associated substrate. Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Nov 2011] [Isoform 1]: **Important regulator of chemokine-induced, integrin-mediated T lymphocyte adhesion and migration**, acting upstream of RAP1 (By similarity). Required for tissue-specific adhesion of T lymphocytes to peripheral tissues (By similarity). Required for basal and CXCL2 stimulated serine-threonine phosphorylation of NEDD9 (By similarity). May be involved in the regulation of T-cell receptor-mediated IL2 production through the activation of the JNK pathway in T-cells (By similarity).||[Isoform 2]: May be involved in the BCAR1/CAS-mediated JNK activation pathway.||Acts as an adaptor protein that mediates cell signaling pathways involved in cellular functions such as cell adhesion and migration, tissue organization, and the regulation of the immune response (PubMed:12432078, PubMed:20881139). Plays a role in integrin-mediated cell adhesion through BCAR1-CRK-RAPGEF1 signaling and activation of the small GTPase RAP1 (PubMed:20881139). Promotes cell migration and invasion through the extracellular matrix (PubMed:20881139). Required for marginal zone B-cell development and thymus-**independent type 2 immune responses** (By similarity). Mediates migration and adhesion of B cells in the splenic marginal zone via promoting hyperphosphorylation of NEDD9/CASL (By similarity). Plays a role in CXCL13-induced chemotaxis of B-cells (By similarity). Plays a role in the migration of olfactory sensory neurons (OSNs) into the forebrain and the innervation of the olfactory bulb by the OSN axons during development (By similarity). Required for the efficient tyrosine phosphorylation of BCAR1 in OSN axons (By similarity).	yes	immunosuppression	down	not <-2FC
NMT2	-1.711	Adds a myristoyl group to the N-terminal glycine residue of certain cellular and viral proteins. This gene encodes one of two N-myristoyltransferase proteins. N-terminal myristoylation is a lipid modification that is involved in regulating the function and localization of signaling proteins. **The encoded protein catalyzes the addition of a myristoyl group to the N-terminal glycine residue of many signaling proteins, including the human immunodeficiency virus type 1 (HIV-1) proteins, Gag and Nef.** Alternative splicing results in multiple transcript variants. [provided by RefSeq, Apr 2015]	yes	immunosuppression	down	not <-2FC

Appendix 30

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
TRAF3	-1.665	The protein encoded by this gene is a member of the TNF receptor associated factor (TRAF) protein family. TRAF proteins associate with, and mediate the signal transduction from, members of the TNF receptor (TNFR) superfamily. This protein participates in the signal transduction of CD40, a TNFR family member important for the **activation of the immune response**. This protein is found to be a critical component of the **lymphotoxin-beta receptor (LTbetaR) signaling complex**, which induces NF-kappaB activation and cell death initiated by LTbeta ligation. Epstein-Barr virus encoded latent infection membrane protein-1 (LMP1) can interact with this and several other members of the TRAF family, **which may be essential for the oncogenic effects of LMP1**. Several alternatively spliced transcript variants encoding three distinct isoforms have been reported. [provided by RefSeq, Dec 2010]	yes	immunosuppression	down	not < -2FC
SRC	-1.692	This gene is **highly similar to the v-src gene of Rous sarcoma virus**. This **proto-oncogene** may play a role in the regulation of embryonic development and cell growth. The protein encoded by this gene is a tyrosine-protein kinase whose activity can be inhibited by phosphorylation by c-SRC kinase. Mutations in this gene could be involved in the **malignant progression of colon cancer**. Two transcript variants encoding the same protein have been found for this gene. [provided by RefSeq, Jul 2008] Non-receptor protein tyrosine kinase which is activated following engagement of many different classes of cellular receptors including immune response receptors, integrins and other adhesion receptors, receptor protein tyrosine kinases, G protein-coupled receptors as well as cytokine receptors. Participates in signaling pathways that control a diverse spectrum of biological activities including **gene transcription, immune response**, cell adhesion, cell cycle progression, apoptosis, migration, and transformation. Due to functional redundancy between members of the SRC kinase family, identification of the specific role of each SRC kinase is very difficult. SRC appears to be one of the primary kinases activated following engagement of receptors and plays a role in the activation of other protein tyrosine kinase (PTK) families. Receptor clustering or dimerization leads to recruitment of SRC to the receptor complexes where it phosphorylates the tyrosine residues within the receptor cytoplasmic domains. Plays an important role in the regulation of cytoskeletal organization through phosphorylation of specific substrates such as AFAP1. Phosphorylation of AFAP1 allows the SRC SH2 domain to bind AFAP1 and to localize to actin filaments. Cytoskeletal reorganization is also controlled through the phosphorylation of cortactin (CTTN) (Probable). When cells adhere via focal adhesions to the extracellular matrix, signals are transmitted by integrins into the cell resulting in tyrosine phosphorylation of a number of focal adhesion proteins, including PTK2/FAK1 and paxillin (PXN) (PubMed:21411625). In addition to phosphorylating focal adhesion proteins, SRC is also active at the sites of cell-cell contact adherens junctions and phosphorylates substrates such as beta-catenin (CTNNB1), delta-catenin (CTNND1), and plakoglobin (JUP). Another type of cell-cell junction, the gap junction, is also a target for SRC, which phosphorylates connexin-43 (GJA1). SRC is implicated in regulation of pre-mRNA-processing and phosphorylates RNA-binding proteins such as KHDRBS1 (Probable). Also plays a role in PDGF-mediated tyrosine phosphorylation of both STAT1 and STAT3, leading to increased DNA binding activity of these transcription factors (By similarity). Involved in the RAS pathway through phosphorylation of RASA1 and RASGRF1 (PubMed:11389730). Plays a role in EGF-mediated calcium-activated chloride channel activation (PubMed:18586953). Required for epidermal growth factor receptor (EGFR) internalization through phosphorylation of clathrin heavy chain (CLTC and CLTCL1) at 'Tyr-1477'. Involved in beta-arrestin (ARRB1 and ARRB2) desensitization through phosphorylation and activation of GRK2, leading to beta-arrestin phosphorylation and internalization. Has a critical role in the stimulation of the CDK20/MAPK3 mitogen-activated protein kinase cascade by epidermal growth factor (Probable). Might be **involved not only in mediating the transduction of mitogenic signals at the level of the plasma membrane but also in controlling progression through the cell cycle via interaction with regulatory proteins in the nucleus** (PubMed:7853507). Plays an important role in osteoclastic bone resorption in conjunction with PTK2B/PYK2. Both the formation of a SRC-PTK2B/PYK2 complex and SRC kinase activity are necessary for this function. Recruited to activated integrins by PTK2B/PYK2, thereby phosphorylating CBL, which in turn induces the activation and recruitment of phosphatidylinositol 3-kinase to the cell membrane in a signaling pathway that is critical for osteoclast function (PubMed:8755529, PubMed:14585963). Promotes energy production in osteoclasts by activating mitochondrial cytochrome C oxidase (PubMed:12615910). Phosphorylates DDR2 on tyrosine residues, thereby promoting its subsequent autophosphorylation (PubMed:20100835, PubMed:16186108). **Enhances DDX58/RIG-1-elicited antiviral signaling** (PubMed:19419966). Phosphorylates PDPK1 at 'Tyr-9', 'Tyr-373' and 'Tyr-376' (PubMed:14585963). Phosphorylates RUNX3 and COX2 on tyrosine residues, TNK2 on 'Tyr-284' and CBL on 'Tyr-731' (PubMed:20100835, PubMed:21309750). Phosphorylates BCAR1 at 'Tyr-128' (PubMed:22710723). Phosphorylates CBLC at multiple tyrosine residues, phosphorylation at 'Tyr-341' activates CBLC E3 activity (PubMed:20525694). Involved in anchorage-independent cell growth (PubMed:19307596). Required for podosome formation (By similarity). Src kinases consist of eight non-receptor tyrosine kinases (Src, Fyn, Yes, Lck, Lyn, Hck, Fgr and Blk) that interact with the intracellular domains of growth factor/cytokine receptors, GPCRs and integrins. Members of the Src kinase family have a very similar domain structure.	no	oncogenesis	down	not < -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
TNFAIP3	-1.721	This gene was identified as a gene whose expression is rapidly induced by the tumor necrosis factor (TNF). The protein encoded by this gene is a zinc finger protein and ubiqitin-editing enzyme, and has been shown to inhibit NF-kappa B activation as well as TNF-mediated apoptosis. The encoded protein, which has both ubiquitin ligase and deubiquitinase activities, is **involved in the cytokine-mediated immune and inflammatory responses.** Several transcript variants encoding the same protein have been found for this gene. [provided by RefSeq, Jul 2012] Ubiquitin-editing enzyme that contains both ubiquitin ligase and deubiquitinase activities. **Involved in immune and inflammatory responses signaled by cytokines, such as TNF-alpha and IL-1 beta, or pathogens via Toll-like receptors (TLRs)** through terminating NF-kappa-B activity. Essential component of a ubiquitin-editing protein complex, comprising also RNF11, ITCH and TAX1BP1, that ensures the transient nature of inflammatory signaling pathways. In cooperation with TAX1BP1 promotes disassembly of E2-E3 ubiquitin protein ligase complexes in IL-1R and TNFR-1 pathways; affected are at least E3 ligases TRAF6, TRAF2 and BIRC2, and E2 ubiquitin-conjugating enzymes UBE2N and UBE2D3. In cooperation with TAX1BP1 promotes ubiquitination of UBE2N and proteasomal degradation of UBE2N and UBE2D3. Upon TNF stimulation, deubiquitinates 'Lys-63'-polyubiquitin chains on RIPK1 and catalyzes the formation of 'Lys-48'-polyubiquitin chains. This leads to RIPK1 proteasomal degradation and consequently termination of the TNF- or LPS-mediated activation of NF-kappa-B. Deubiquitinates TRAF6 probably acting on 'Lys-63'-linked polyubiquitin. Upon T-cell receptor (TCR)-mediated T-cell activation, deubiquitinates 'Lys-63'-polyubiquitin chains on MALT1 thereby mediating disassociation of the CBM (CARD11:BCL10:MALT1) and IKK complexes and preventing sustained IKK activation. Deubiquitinates NEMO/IKBKG; the function is facilitated by TNIP1 and leads to inhibition of NF-kappa-B activation. Upon stimulation by bacterial peptidoglycans, probably deubiquitinates RIPK2. Can also inhibit I-kappa-B-kinase (IKK) through a non-catalytic mechanism which involves polyubiquitin; polyubiquitin promotes association with IKBKG and prevents IKK MAP3K7-mediated phosphorylation. Targets TRAF2 for lysosomal degradation. In vitro able to deubiquitinate 'Lys-11'-, 'Lys-48'- and 'Lys-63' polyubiquitin chains. Inhibitor of programmed cell death. Has a role in the function of the lymphoid system. Required for LPS-induced production of proinflammatory cytokines and IFN beta in LPS-tolerized macrophages.	yes	immunosuppression	down	not < -2FC
PLXNC1	-1.742	This gene encodes a member of the plexin family. Plexins are transmembrane receptors for semaphorins, a large family of proteins that regulate axon guidance, cell motility and migration, and the **immune response.** The encoded protein and its ligand regulate melanocyte adhesion, and viral semaphorins may modulate the immune response by binding to this receptor. The encoded protein may be a **tumor suppressor protein for melanoma.** Alternatively spliced transcript variants have been observed for this gene. [provided by RefSeq, Jan 2011] Receptor for SEMA7A, for smallpox semaphorin A39R, vaccinia virus semaphorin A39R and for herpesvirus Sema protein. Binding of semaphorins triggers cellular responses leading to the rearrangement of the cytoskeleton and to secretion of IL6 and IL8 (By similarity).	yes	oncogenesis & immunosuppression	down	not < -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
NR3C1	-1.761	This gene encodes glucocorticoid receptor, which can function both as a transcription factor that binds to glucocorticoid response elements in the promoters of glucocorticoid responsive genes to activate their transcription, and as a regulator of other transcription factors. This receptor is typically found in the cytoplasm, but upon ligand binding, is transported into the nucleus. It is involved in inflammatory responses, cellular proliferation, and differentiation in target tissues. Mutations in this gene are associated with generalized glucocorticoid resistance. Alternative splicing of this gene results in transcript variants encoding either the same or different isoforms. Additional isoforms resulting from the use of alternate in-frame translation initiation sites have also been described, and shown to be functional, displaying diverse cytoplasm-to-nucleus trafficking patterns and distinct transcriptional activities (PMID:15866175). [provided by RefSeq, Feb 2011] [Isoform 10]: Has transcriptional activation activity.|||[Isoform Alpha]: Has transcriptional activation and repression activity (PubMed:15866175, PubMed:19248771, PubMed:20484466, PubMed:23820903, PubMed:11435610, PubMed:15769988, PubMed:17635946, PubMed:19141540, PubMed:21664385). Mediates glucocorticoid-induced apoptosis (PubMed:23303127). Promotes accurate chromosome segregation during mitosis (PubMed:25847991). May act as a tumor suppressor (PubMed:25847991). May play a negative role in adipogenesis through the regulation of lipolytic and antilipogenic gene expression (By similarity).|||[Isoform Alpha-2]: Has lower transcriptional activation activity than isoform Alpha. Exerts a dominant negative effect on isoform Alpha trans-repression mechanism (PubMed:20484466).|||[Isoform Alpha-B]: More effective than isoform Alpha in transcriptional activation, but not repression activity.|||[Isoform Alpha-C1]: Has transcriptional activation activity (PubMed:15866175, PubMed:23820903). Has transcriptional repression activity (PubMed:23303127). Mediates glucocorticoid-induced apoptosis (PubMed:23303127, PubMed:23820903).|||[Isoform Alpha-C2]: Has transcriptional activation activity.|||[Isoform Alpha-D2]: Has transcriptional activation activity.|||[Isoform Alpha-D1]: Has transcriptional activation activity of all isoforms created by alternative initiation (PubMed:15866175, PubMed:23820903).|||[Isoform Alpha-D3]: Has lowest transcriptional activation activity of all isoforms created (PubMed:23303127).|||[Isoform Beta]: Acts as a dominant negative inhibitor of isoform Alpha (PubMed:7769088, PubMed:8621628, PubMed:20484466). Has intrinsic transcriptional activity independent of isoform Alpha when both isoforms are coexpressed (PubMed:19248771, PubMed:26711253). Loses this transcription modulator function on its own (PubMed:20484466). Has no hormone-binding activity (PubMed:8621628). May play a role in controlling glucose metabolism by maintaining insulin sensitivity (By similarity). Reduces hepatic gluconeogenesis through down-regulation of PEPCK in an isoform Alpha-dependent manner (PubMed:26711253).|||[Isoform GR-P]: Increases activity of isoform Alpha.||Receptor for glucocorticoids (GC) (PubMed:27120390). Has a dual mode of action: as a transcription factor that binds to glucocorticoid response elements (GRE), both for nuclear and mitochondrial DNA, and as a modulator of other transcription factors. Affects inflammatory responses, cellular proliferation and differentiation in target tissues. Involved in chromatin remodeling (PubMed:9590696). Plays a role in rapid mRNA degradation by binding to the 5' UTR of target mRNAs and interacting with PNRC2 in a ligand-dependent manner which recruits the RNA helicase UPF1 and the mRNA-decapping enzyme DCP1A, leading to RNA decay (PubMed:25775514). Could act as a coactivator for STAT5-dependent transcription upon growth hormone (GH) stimulation and could reveal an essential role of hepatic GR in the control of body growth (By similarity). Glucocorticoid receptors (GRs) are nuclear hormone receptors of the NR3C class, which also includes mineralocorticoid, progesterone and androgen receptors. They exist as homodimers coupled to Hsp90 or HMGB proteins, which are shed upon activation.	yes	immunosuppression	down	not < -2FC
PDK2	-1.788	This gene encodes a member of the pyruvate dehydrogenase kinase family. The encoded protein phosphorylates pyruvate dehydrogenase, down-regulating the activity of the mitochondrial pyruvate dehydrogenase complex. Overexpression of this gene may play a role in both cancer and diabetes. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Dec 2010] Kinase that plays a key role in the regulation of glucose and fatty acid metabolism and homeostasis via phosphorylation of the pyruvate dehydrogenase subunits PDHA1 and PDHA2. This inhibits pyruvate dehydrogenase activity, and thereby regulates metabolite flux through the tricarboxylic acid cycle, down-regulates aerobic respiration and inhibits the formation of acetyl-coenzyme A from pyruvate. Inhibition of pyruvate dehydrogenase decreases glucose utilization and increases fat metabolism. Mediates cellular responses to insulin. Plays an important role in maintaining normal blood glucose levels and in metabolic adaptation to nutrient availability. Via its regulation of pyruvate dehydrogenase activity, plays an important role in maintaining normal blood pH and in preventing the accumulation of ketone bodies under starvation. Plays a role in the regulation of cell proliferation and in resistance to apoptosis under oxidative stress. Plays a role in p53/TP53-mediated apoptosis.	no	oncogenesis	down	not < -2FC

Appendix 33

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
ETV1	-1.911	This gene encodes a member of the ETS (E twenty-six) family of **transcription factors**. The ETS proteins regulate many target genes that modulate biological processes like cell growth, angiogenesis, migration, proliferation and differentiation. All ETS proteins contain an ETS DNA-binding domain that binds to DNA sequences containing the consensus 5'-CGGA[AT]-3'. The protein encoded by this gene contains a conserved short acidic transactivation domain (TAD) in the N-terminal region, in addition to the ETS DNA-binding domain in the C-terminal region. This gene is involved in chromosomal translocations, which result in multiple fusion proteins including EWS-ETV1 in **Ewing sarcoma** and at least 10 ETV1 partners (see PMID: 19657377, Table 1) in **prostate cancer**. In addition to chromosomal rearrangement, **this gene is overexpressed in prostate cancer, melanoma and gastrointestinal stromal tumor**. Multiple alternatively spliced transcript variants encoding different isoforms have been identified. [provided by RefSeq, Jul 2016] Transcriptional activator that binds to DNA sequences containing the consensus pentanucleotide 5'-CGGA[AT]-3'.	no	oncogenesis	down	not <-2FC
GATA3	-1.990	This gene encodes a protein which belongs to the GATA family of **transcription factors**. The protein contains two GATA-type zinc fingers and is an important **regulator of T-cell development** and plays an important role in endothelial cell biology. Defects in this gene are the cause of hypoparathyroidism with sensorineural deafness and renal dysplasia. [provided by RefSeq, Nov 2009] GATA3 (GATA Binding Protein 3) is a Protein Coding gene. Diseases associated with GATA3 include Hypoparathyroidism, Sensorineural Deafness, And Renal Disease and Hypoparathyroidism. Among **its related pathways are** Preimplantation Embryo and **C-MYB transcription factor network**. Gene Ontology (GO) annotations related to this gene include DNA-binding transcription factor activity and transcription factor binding. An important paralog of this gene is GATA2. Transcriptional activator which binds to the enhancer of the T-cell receptor alpha and delta genes. Binds to the consensus sequence 5'-AGATAG-3'. **Required for the T-helper 2 (Th2) differentiation process following immune and inflammatory responses.**	yes	immunosuppression	down	not <-2FC
NFATC1	-1.991	The product of this gene is a component of the nuclear factor of activated T cells DNA-binding transcription complex. This complex consists of at least two components: a preexisting cytosolic component that translocates to the nucleus upon T cell receptor (TCR) stimulation, and an inducible nuclear component. Proteins belonging to this family of **transcription factors play a central role in inducible gene transcription during immune response**. The product of this gene is an inducible nuclear component. It functions as a major molecular target for the immunosuppressive drugs such as cyclosporin A. Multiple alternatively spliced transcript variants encoding distinct isoforms have been identified for this gene. Different isoforms of this protein may **regulate inducible expression of different cytokine genes**. [provided by RefSeq, Jul 2013] Plays a role in the inducible expression of cytokine genes in T-cells, especially in the **induction of the IL-2 or IL-4 gene transcription**. Also controls gene expression in embryonic cardiac cells. Could regulate not only the activation and proliferation but also the differentiation and programmed death of T-lymphocytes as well as lymphoid and non-lymphoid cells (PubMed:10358178). Required for osteoclastogenesis and regulates many genes important for osteoclast differentiation and function (By similarity). NFAT (Nuclear Factor of Activated T-Cells) proteins are transcription factors that are integral for the development and function of the immune system. Five NFAT isoforms have been identified to date, NFATC1-C4 and NFAT5.	yes	immunosuppression	down	not <-2FC
MRAS	-2.000	Serves as an important signal transducer for a novel upstream stimuli in **controlling cell proliferation**. Activates the MAP kinase pathway. This gene encodes a member of the **Ras** family of small GTPases. These membrane-associated proteins function as signal transducers in multiple processes including cell growth and differentiation, and dysregulation of Ras signaling has been associated with **many types of cancer**. The encoded protein may play a role in the tumor necrosis factor-alpha and MAP kinase signaling pathways. Alternatively spliced transcript variants encoding multiple isoforms have been observed for this gene. [provided by RefSeq, Nov 2011]	no	oncogenesis	down	<-2FC
VCAM1	-2.024	This gene is a member of the Ig superfamily and encodes a cell surface sialoglycoprotein expressed by cytokine-activated endothelium. This type I membrane protein mediates leukocyte-endothelial cell adhesion and signal transduction, and may play a role in the development of artherosclerosis and rheumatoid arthritis. Three alternatively spliced transcripts encoding different isoforms have been described for this gene. [provided by RefSeq, Dec 2010] Important in cell-cell recognition. Appears to function in leukocyte-endothelial cell adhesion. Interacts with integrin alpha-4/beta-1 (ITGA4/ITGB1) on leukocytes, and mediates both adhesion and signal transduction. The VCAM1/ITGA4/ITGB1 interaction may **play a pathophysiologic role both in immune responses and in leukocyte emigration to sites of inflammation.**	yes	immunosuppression	down	<-2FC
IL6R	-2.125	This gene encodes a subunit of the **interleukin 6 (IL6) receptor complex**. Interleukin 6 is a potent pleiotropic cytokine that **regulates cell growth** and differentiation and plays an important role in the **immune response**. The IL6 receptor is a protein complex consisting of this protein and interleukin 6 signal transducer (IL6ST/GP130/IL6-beta), a receptor subunit also shared by many other cytokines. Dysregulated production of IL6 and this receptor are implicated in the pathogenesis of many diseases, **such as multiple myeloma, autoimmune diseases and prostate cancer.** Alternatively spliced transcript variants encoding distinct isoforms have been reported. A pseudogene of this gene is found on chromosome 9. [provided by RefSeq, May 2011]	yes	immunosuppression	down	<-2FC
ETV3|	-2.215	**Transcriptional repressor** that contribute to growth arrest during terminal macrophage differentiation by repressing target genes involved in Ras-dependent proliferation. **Represses MMP1 promoter** activity.	yes	oncogenesis	down	<-2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
NEDD9	-1.802	The protein encoded by this gene is a member of the CRK-associated substrates family. Members of this family are adhesion docking molecules that mediate protein-protein interactions for signal transduction pathways. This protein is a focal adhesion protein that acts as a scaffold to **regulate signaling complexes important in cell attachment, migration and invasion as well as apoptosis and the cell cycle. This protein has also been reported to have a role in cancer metastasis.** Alternative splicing results in multiple transcript variants. [provided by RefSeq, Aug 2012] Docking protein which plays a central coordinating role for tyrosine-kinase-based signaling related to cell adhesion. May function in transmitting growth control signals between focal adhesions at the cell periphery and the mitotic spindle in response to adhesion or growth factor signals initiating cell proliferation. **May play an important role in integrin beta-1 or B cell antigen receptor (BCR) mediated signaling in B- and T-cells.** Integrin beta-1 stimulation leads to recruitment of various proteins including CRK, NCK and SHPTP2 to the tyrosine phosphorylated form. Required for correct adhesion and migration of T-cells (PubMed:17174122).	yes	immunosuppression	down	not < -2FC
TACC1	-1.870	This locus may **represent a breast cancer candidate gene.** It is located close to FGFR1 on a region of chromosome 8 that is amplified in some breast cancers. Several transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Jun 2017] Involved in transcription regulation induced by nuclear receptors, including in T3 thyroid hormone and all-trans retinoic acid pathways (PubMed:20078863). Might promote the nuclear localization of the receptors (PubMed:20078863). Likely involved in the processes that **promote cell division** prior to the formation of differentiated tissues.	no	oncogenesis	down	not < -2FC
SPHK1	-1.893	The protein encoded by this gene catalyzes the phosphorylation of sphingosine to form sphingosine-1-phosphate (S1P), a lipid mediator with both intra- and extracellular functions. Intracellularly, S1P regulates proliferation and survival, and extracellularly, it is a ligand for cell surface G protein-coupled receptors. This protein, and its product S1P, play a key role in TNF-alpha signaling and the NF-kappa-B activation pathway important in **inflammatory, antiapoptotic, and immune processes.** Phosphorylation of this protein alters its catalytic activity and promotes its translocation to the plasma membrane. Alternative splicing results in multiple transcript variants encoding different isoforms. [provided by RefSeq, Sep 2017] Catalyzes the phosphorylation of sphingosine to form sphingosine 1-phosphate (SPP), a lipid mediator with both intra- and extracellular functions. Also acts on D-erythro-sphingosine and to a lesser extent sphinganine, but not other lipids, such as D,L-threo-dihydrosphingosine, N,N-dimethylsphingosine, diacylglycerol, ceramide, or phosphatidylinositol (PubMed:20577214, PubMed:23602659, PubMed:29662056, PubMed:24929359, PubMed:11923095). In contrast to proapoptotic SPHK2, has a negative effect on intracellular ceramide levels, enhances cell growth and inhibits apoptosis (PubMed:16118219). **Involved in the regulation of inflammatory response** and neuroinflammation. Via the product sphingosine 1-phosphate, stimulates TRAF2 E3 ubiquitin ligase activity, and promotes activation of NF-kappa-B in response to TNF signaling leading to **IL17 secretion** (PubMed:20577214). In response to TNF and in parallel to NF-kappa-B activation, negatively regulates RANTES induction through p38 MAPK signaling pathway (PubMed:23935096). Involved in endocytic membrane trafficking induced by sphingosine, recruited to dilate endosomes, also plays a role on later stages of endosomal maturation and membrane fusion independently of its kinase activity (PubMed:28049734, PubMed:24929359). In Purkinje cells, seems to be also involved in the regulation of autophagosome-lysosome fusion upon VEGFA (PubMed:25417698).||Has serine acetyltransferase activity on PTGS2/COX2 in an acetyl-CoA dependent manner. The acetyltransferase activity increases in presence of the kinase substrate, sphingosine. During neuroinflammation, through PTGS2 acetylation, promotes neuronal secretion of specialized preresolving mediators (SPMs), especially 15-R-lipoxin A4, which results in an increase of phagocytic microglia. Sphingosine kinase (SphK) mediates the conversion of sphingosine to sphingosine-1-phosphate, a key sphingolipid signaling molecule involved in cell growth, survival, differentiation and motility. Two mammalian isoforms have been cloned - SPHK1 and SPHK2.	yes	immunosuppression	down	not < -2FC
NFATC3	-1.905	The product of this gene is a member of the nuclear factors of activated T cells DNA-binding transcription complex. This complex consists of at least two components: a preexisting cytosolic component that translocates to the nucleus upon T cell receptor (TCR) stimulation and an inducible nuclear component. Other members of this family participate to form this complex also. The product of this gene plays a role in the **regulation of gene expression in T cells and immature thymocytes.** Several transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq, Nov 2010] **Acts as a regulator of transcriptional activation.** Plays a role in the **inducible expression of cytokine genes in T-cells, especially in the induction of the IL-2** (PubMed:18815128). Along with NFATC4, involved in embryonic heart development (By similarity). NFAT (Nuclear Factor of Activated T-Cells) proteins are transcription factors that are integral for the development and function of the immune system. Five NFAT isoforms have been identified to date, NFATC1-C4 and NFAT5.	yes	immunosuppression	down	not < -2FC

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EZH1	-2.218	EZH1 is a component of a noncanonical Polycomb repressive complex-2 (PRC2) that mediates methylation of histone H3 (see MIM 602812) lys27 (H3K27) and functions in the maintenance of embryonic stem cell pluripotency and plasticity (Shen et al., 2008 [PubMed 19026780]).[supplied by OMIM, Mar 2009] Polycomb group (PcG) protein. Catalytic subunit of the PRC2/EED-EZH1 complex, which methylates 'Lys-27' of histone H3, leading to **transcriptional repression** of the affected target gene. Able to mono-, di- and trimethylate 'Lys-27' of histone H3 to form H3K27me1, H3K27me2 and H3K27me3, respectively. Required for embryonic stem cell derivation and self-renewal, suggesting that it is involved in safeguarding embryonic stem cell identity. Compared to EZH2-containing complexes, it is less abundant in embryonic stem cells, has weak methyltransferase activity and plays a less critical role in forming H3K27me3, which is required for embryonic stem cell identity and proper differentiation. Lysine methyltransferases are enzymes that catalyze the transfer of methyl groups from S-adenosylmethionine (SAM) to the lysine residues on histones, particularly histones H3 and H4. **The dysregulation of this methylation is critical in the development of cancer.**	variable	oncogenesis	down	< -2FC
STARD8	-2.236	This gene encodes a member of a subfamily of Rho GTPase activating proteins that contain a steroidogenic acute regulatory protein related lipid transfer domain. The encoded protein localizes to focal adhesions and may be involved in regulating cell morphology. **This protein may also function as a tumor suppressor.** [provided by RefSeq, Mar 2010] Accelerates GTPase activity of RHOA and CDC42, but not RAC1. Stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLCD1.	yes	oncogenesis	down	< -2FC
MITF	-2.282	**Transcription factor** that regulates the expression of genes with essential roles in cell differentiation, proliferation and survival. Binds to M-boxes (5'-TCATGTG-3') and symmetrical DNA sequences (E-boxes) (5'-CACGTG-3') found in the promoters of target genes, such as BCL2 and tyrosinase (TYR). Plays an important role in melanocyte development by regulating the expression of tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1). Plays a critical role in the differentiation of various cell types, such as neural crest-derived melanocytes, mast cells, osteoclasts and optic cup-derived retinal pigment epithelium. The protein encoded by this gene is a transcription factor that contains both basic helix-loop-helix and leucine zipper structural features. The encoded protein regulates melanocyte development and is responsible for pigment cell-specific transcription of the melanogenesis enzyme genes. Heterozygous mutations in the this gene cause auditory-pigmentary syndromes, such as Waardenburg syndrome type 2 and Tietz syndrome. [provided by RefSeq, Aug 2017]	no	oncogenesis	down	< -2FC
MAFB	-2.299	Acts as a **transcriptional activator or repressor** (PubMed:27181683). Plays a pivotal role in regulating lineage-specific hematopoiesis by repressing ETS1-mediated transcription of erythroid-specific genes in myeloid cells. Required for monocytic, macrophage, osteoclast, podocyte and islet beta cell differentiation. Involved in renal tubule survival and F4/80 maturation. Activates the insulin and glucagon promoters. Together with PAX6, transactivates weakly the glucagon gene promoter through the G1 element. **SUMO modification controls its transcriptional activity and ability to specify macrophage fate.** Binds element G1 on the glucagon promoter (By similarity). **Involved either as an oncogene or as a tumor suppressor, depending on the cell context.** The protein encoded by this gene is a basic leucine zipper (bZIP) transcription factor that plays an important role in the regulation of lineage-specific hematopoiesis. The encoded nuclear protein represses ETS1-mediated transcription of erythroid-specific genes in myeloid cells. This gene contains no introns. [provided by RefSeq, Jul 2008]	variable	oncogenesis	down	< -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
CEBPA	-2.307	AML with mutated CEBPA' is a provisional entity in the WHO classification of **acute myeloid leukemia (AML)** and is recommended to be tested for in patients with AML. CEBPA mutations are particularly associated with cytogenetically normal AML (CN-AML). CEBPA is an intronless gene that is required for granulocyte formation in mice. N-terminal nonsense mutations result in a dominant negative C/EBP-alpha protein while C-terminal mutations reduce the DNA-binding potential of this **transcription factor**. CEBPA mutations are associated with a favorable prognosis, **however, NPM1 and FLT3 mutations should also be assessed in CN-AML patients as concurrent mutations may have prognostic implications.** Isoform 3: Can act as dominant-negative. Binds DNA and have transactivation activity, even if much less efficiently than isoform 2. Does not inhibit cell proliferation (PubMed:14660596).||Isoform 4: Directly and specifically enhances ribosomal DNA transcription interacting with RNA polymerase I-specific cofactors and inducing histone acetylation.||Transcription factor that coordinates proliferation arrest and the differentiation of myeloid progenitors, adipocytes, hepatocytes, and cells of the lung and the placenta. Binds directly to the consensus DNA sequence 5'-T[TG]NNNGNAA[TG]-3' acting as an activator on distinct target genes (PubMed:11242107). During early embryogenesis, plays essential and redundant functions with CEBPB. Essential for the proper development of the liver and the lung (By similarity). Necessary for terminal adipocyte differentiation, is required for postnatal maintenance of systemic energy homeostasis and lipid storage (By similarity). To regulate these different processes at the proper moment and tissue, interplays with other transcription factors and modulators. Downregulates the expression of genes that maintain cells in an undifferentiated and proliferative state through E2F1 repression, which is critical for its ability to induce adipocyte and granulocyte terminal differentiation. Reciprocally E2F1 blocks adipocyte differentiation by binding to specific promoters and repressing CEBPA binding to its target gene promoters. Proliferation arrest also depends on a functional binding to SWI/SNF complex (PubMed:14660596). In liver, regulates gluconeogenesis and lipogenesis through different mechanisms. To regulate gluconeogenesis, functionally cooperates with FOXO1 binding to IRE-controlled promoters and regulating the expression of target genes such as PCK1 or G6PC. To modulate lipogenesis, interacts and transcriptionally synergizes with SREBF1 in promoter activation of specific lipogenic target genes such as ACAS2. **In adipose tissue, seems to act as FOXO1 coactivator accessing to ADIPOQ promoter through FOXO1 binding sites** (By similarity).	no	oncogenesis	down	<-2FC
LMO2	-2.432	LMO2 encodes a cysteine-rich, two LIM-domain protein that is required for yolk sac erythropoiesis. The LMO2 protein has a central and crucial role in hematopoietic development and is highly conserved. The LMO2 transcription start site is located approximately 25 kb downstream from the 11p13 T-cell translocation cluster (11p13 ttc), where a number **T-cell acute lymphoblastic leukemia-specific** translocations occur. Alternative splicing results in multiple transcript variants encoding different isoforms.[provided by RefSeq, Nov 2008] Acts with TAL1/SCL to regulate red blood cell development. **Also acts with LDB1 to maintain erythroid precursors in an immature state.**	no	oncogenesis	down	<-2FC
LYST	-2.478	This gene encodes a protein that regulates intracellular protein trafficking in endosomes, and may be involved in pigmentation. Mutations in this gene are associated with Chediak-Higashi syndrome, a lysosomal storage disorder. Alternative splicing results in multiple transcript variants, though the full-length nature of some of these variants has not been determined. [provided by RefSeq, Apr 2013] Adapter protein that regulates and/or fission of intracellular vesicles such as lysosomes (PubMed:11984006, PubMed:25216107). Might regulate trafficking of effectors involved in exocytosis (PubMed:25425525). In cytotoxic T-cells and natural killer (NK) cells, has role in the regulation of size, number and exocytosis of lytic granules (PubMed:26478006). In macrophages and dendritic cells, regulates phagosome maturation by controlling the conversion of early phagosomal compartments into late phagosomes (By similarity). **In macrophages and dendritic cells, specifically involved in TLR3- and TLR4-induced production of pro-inflammatory cytokines by regulating the endosomal TLR3- TICAM1/TRIF and TLR4- TICAM1/TRIF signaling pathways** (PubMed:27881733).	yes	immunosuppression	down	<-2FC
CX3CR1	-2.483	Receptor for the CX3C chemokine fractalkine (CX3CL1); binds to CX3CL1 and mediates both its adhesive and migratory functions (PubMed:9390561, PubMed:23125415). **Acts as coreceptor with CD4 for HIV-1 virus envelope protein** (in vitro) (PubMed:9726990). Isoform 2 and isoform 3 seem to be more potent HIV-1 coreceptors than isoform 1 (PubMed:14607932). Fractalkine is a transmembrane protein and chemokine involved in the adhesion and migration of leukocytes. The protein encoded by this gene is a receptor for fractalkine. The encoded protein also is a coreceptor for HIV-1, and some **variations in this gene lead to increased susceptibility to HIV-1 infection and rapid progression to AIDS. Four** transcript variants encoding two different isoforms have been found for this gene. [provided by RefSeq,Jan 2010]	variable	vrial infection	down	<-2FC
TNFSF8	-2.584	The protein encoded by this gene is a cytokine that belongs to the **tumor necrosis factor (TNF)** ligand family. This cytokine is a ligand for TNFRSF8/CD30, which is a cell surface antigen and a marker for **Hodgkin lymphoma** and related hematologic malignancies. The engagement of this cytokine expressed on B cell surface plays an inhibitory role in modulating Ig class switch. This cytokine was shown to enhance cell proliferation of some lymphoma cell lines, while to induce cell death and reduce cell proliferation of other lymphoma cell lines. The pleiotropic biologic activities of this cytokine on different CD30+ **lymphoma** cell lines may play a pathophysiologic role in **Hodgkin's and some non-Hodgkin's lymphomas.** Two transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Nov 2011]	variable	oncogenesis	down	<-2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
PLAGL1	-2.725	This gene encodes a C2H2 zinc finger protein that functions as a **suppressor of cell growth. This gene is often deleted or methylated and silenced in cancer cells.** In addition, overexpression of this gene during fetal development is thought to be the causal factor for transient neonatal diabetes mellitus (TNDM). Alternative splicing and the use of alternative promoters results in multiple transcript variants encoding two different protein isoforms. The P1 downstream promoter of this gene is imprinted, with preferential expression from the paternal allele in many tissues. [provided by RefSeq, Nov 2015] Acts as a transcriptional activator (PubMed:9722527). Involved in the transcriptional regulation of type 1 receptor for pituitary adenylate cyclase-activating polypeptide.	yes	oncogenesis	down	<-2FC
CTSH	-2.751	The protein encoded by this gene is a lysosomal cysteine proteinase important in the overall degradation of lysosomal proteins. It is composed of a dimer of disulfide-linked heavy and light chains, both produced from a single protein precursor. The encoded protein, which belongs to the peptidase C1 protein family, can act both as an aminopeptidase and as an endopeptidase. **Increased expression of this gene has been correlated with malignant progression of prostate tumors.** Alternate splicing of this gene results in multiple transcript variants encoding different isoforms. [provided by RefSeq, Jan 2016] Important for the overall degradation of proteins in lysosomes.	no	oncogenesis	down	<-2FC
UBASH3A	-2.754	Interferes with CBL-mediated down-regulation and degradation of receptor-type tyrosine kinases. Promotes **accumulation of activated target receptors, such as T-cell receptors, EGFR and PDGFRB, on the cell surface.** Exhibits negligigle protein tyrosine phosphatase activity at neutral pH. May act as a dominant-negative regulator of UBASH3B-dependent endocytic pathways by functionally sequestering dynamin via its SH3 domain. This gene encodes one of two family members belonging to the T-cell ubiquitin ligand (TULA) family. Both family members **can negatively regulate T-cell signaling.** This family member can facilitate growth factor withdrawal-induced apoptosis in T cells, which may occur via its interaction with AIF, an apoptosis-inducing factor. Alternative splicing of this gene results in multiple transcript variants. [provided by RefSeq, Aug 2011]	yes	immunosuppression	down	<-2FC
SLCO2A1	-2.762	This gene encodes a prostaglandin transporter that is a member of the 12-membrane-spanning superfamily of transporters. The encoded protein may be involved in **mediating the uptake and clearance of prostaglandins in numerous tissues.** [provided by RefSeq, Dec 2011] May mediate the release of newly synthesized prostaglandins from cells, the transepithelial transport of prostaglandins, and the clearance of prostaglandins from the circulation. Transports PGD2, as well as PGE1, PGE2 and PGF2A.	yes	immunosuppression	down	<-2FC
RASGRP3	-2.829	The protein encoded by this gene is a guanine nucleotide exchange factor that **activates the oncogenes HRAS and RAP1A.** Defects in this gene have been associated with systemic lupus erythematosus and **several cancers.** [provided by RefSeq, Mar 2017]	no	oncogenesis	down	<-2FC
SLA2	-2.899	This gene encodes a member of the SLAP family of adapter proteins. The encoded protein may play an important receptor-proximal **role in downregulating T and B cell-mediated responses** and inhibits antigen receptor-induced calcium mobilization. This protein interacts with Cas-Br-M (murine) ecotropic retroviral transforming sequence c. Two transcript variants encoding distinct isoforms have been identified for this gene. [provided by RefSeq, Jul 2008] Adapter protein, which **negatively regulates T-cell receptor (TCR) signaling.** Inhibits T-cell antigen-receptor induced activation of nuclear factor of activated T-cells. May act by linking signaling proteins such as ZAP70 with CBL, leading to a CBL dependent degradation of signaling proteins.	no	immunosuppression	down	<-2FC
KLF5	-2.945	**Transcription factor** that binds to GC box promoter elements. Activates the transcription of these genes. This gene encodes a member of the Kruppel-like factor subfamily of zinc finger proteins. The encoded protein is a transcriptional activator that binds directly to a specific recognition motif in the promoters of target genes. This protein acts downstream of multiple different signaling pathways and is regulated by post-translational modification. **It may participate in both promoting and suppressing cell proliferation. Expression of this gene may be changed in a variety of different cancers** and in cardiovascular disease. Alternative splicing results in multiple transcript variants. [provided by RefSeq, Nov 2013]	variable	oncogenesis	down	<-2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
REL	-3.160	This gene encodes a protein that belongs to the **Rel homology** domain/immunoglobulin-like fold, plexin, transcription factor (RHD/IPT) family. Members of this family regulate genes involved in apoptosis, inflammation, the immune response, and oncogenic processes. **This proto-oncogene plays a role in the survival and proliferation of B lymphocytes.** Mutation or amplification of this gene is associated with **B-cell lymphomas, including Hodgkin's lymphoma.** Alternative splicing results in multiple transcript variants encoding different isoforms. [provided by RefSeq, Apr 2014] **Proto-oncogene** that may play a role in **lymphopoiesis.** NF-kappa-B is a pleiotropic transcription factor which is present in almost all cell types and is involved in many biological processed such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. NF-kappa-B is a homo- or heterodimeric complex formed by the Rel-like domain-containing proteins RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL and NFKB2/p52. The dimers bind at kappa-B sites in the DNA of their target genes and the individual dimers have distinct preferences for different kappa-B sites that they can bind with distinguishable affinity and specificity. Different dimer combinations act as transcriptional activators or repressors, respectively. NF-kappa-B is controlled by various mechanisms of post-translational modification and subcellular compartmentalization as well as by interactions with other cofactors or corepressors. NF-kappa-B complexes are held in the cytoplasm in an inactive state complexed with members of the NF-kappa-B inhibitor (I-kappa-B) family. In a conventional activation pathway, I-kappa-B is phosphorylated by I-kappa-B kinases (IKKs) in response to different activators, subsequently degraded thus liberating the active NF-kappa-B complex which translocates to the nucleus. The NF-kappa-B heterodimer RELA/p65-c-Rel is a transcriptional activator.	no	oncogenesis	down	< -2FC
SFRP1	-3.243	Soluble frizzled-related proteins (sFRPS) function as modulators of Wnt signaling through direct interaction with Wnts. They have a role in **regulating cell growth and differentiation** in specific cell types. SFRP1 decreases intracellular beta-catenin levels (By similarity). Has antiproliferative effects on vascular cells, in vitro and in vivo, and can induce, in vivo, an angiogenic response. In vascular cell cycle, delays the G1 phase and entry into the S phase (By similarity). In kidney development, inhibits tubule formation and bud growth in metanephroi (By similarity). Inhibits WNT1/WNT4-mediated TCF-dependent transcription. This gene encodes a member of the SFRP family that contains a cysteine-rich domain homologous to the putative Wnt-binding site of Frizzled proteins. Members of this family act as soluble modulators of Wnt signaling; epigenetic silencing of SFRP genes leads to deregulated activation of the **Wnt-pathway which is associated with cancer.** This gene may also be involved in determining the polarity of photoreceptor cells in the retina. [provided by RefSeq, Sep 2009]	no	oncogenesis	down	< -2FC
GAS6	-3.251	(Microbial infection) Can bridge virus envelope phosphatidylserine to the TAM receptor tyrosine kinase Axl to mediate viral entry by apoptotic mimicry (PubMed:21501828). Plays a role in Dengue cell entry by apoptotic mimicry (PubMed:23084921). Plays a role in Vaccinia virus cell entry by apoptotic mimicry (PubMed:21501828). Plays a role in ebolavirus and marburgvirus cell entry by apoptotic mimicry (PubMed:17005688).||Ligand for tyrosine-protein kinase receptors AXL, TYRO3 and MER whose signaling is implicated in cell growth and survival, cell adhesion and cell migration. GAS6/AXL signaling plays a role in various processes such as endothelial cell survival during acidification by preventing apoptosis, optimal cytokine signaling during human natural killer cell development, hepatic regeneration, gonadotropin-releasing hormone neuron survival and migration, platelet activation, or regulation of thrombotic responses. This gene encodes a gamma-carboxyglutamic acid (Gla)-containing protein thought to be involved in the stimulation of cell proliferation. **This gene is frequently overexpressed in many cancers** and has been implicated as an adverse prognostic marker. Elevated protein levels are additionally associated with a variety of disease states, including venous thromboembolic disease, systemic lupus erythematosus, chronic renal failure, and preeclampsia. [provided by RefSeq, Aug 2014]	no	oncogenesis	down	< -2FC
ITK	-3.291	This gene encodes an intracellular tyrosine kinase expressed in T-cells. The protein contains both SH2 and SH3 domains which are often found in intracellular kinases. It is thought to play a role in **T-cell proliferation and differentiation.** [provided by RefSeq, Jul 2008] ITK (IL-2-inducible T-cell kinase) is a member of the Tec family of nonreceptor tyrosine kinases that are critically important for the growth, differentiation and activation of myeloid-, mast- and B-cells. **ITK has a critical role in T-cell growth, signaling and function.** Tyrosine kinase that plays an essential role in regulation of the **adaptive immune response.** Regulates the development, function and differentiation of conventional T-cells and nonconventional NKT-cells. When antigen presenting cells (APC) activate T-cell receptor (TCR), a series of phosphorylation lead to the recruitment of ITK to the cell membrane, in the vicinity of the stimulated TCR receptor, where it is phosphorylated by LCK. Phosphorylation leads to ITK autophosphorylation and full activation. Once activated, phosphorylates PLCG1, leading to the activation of this lipase and subsequent cleavage of its substrates. In turn, the endoplasmic reticulum releases calcium in the cytoplasm and the nuclear activator of activated T-cells (NFAT) translocates into the nucleus to perform its transcriptional duty. Phosphorylates 2 essential adapter proteins: the linker for activation of T-cells/LAT protein and LCP2. Then, a large number of signaling molecules such as VAV1 are recruited and ultimately lead to lymphokine production, T-cell proliferation and differentiation (PubMed:12186560, PubMed:12682224, PubMed:21725281). Phosphorylates TBX21 at 'Tyr-530' and mediates its interaction with GATA3 (By similarity).	yes	immunosuppression	down	< -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
IL7R|	-3.313	The protein encoded by this gene is a **receptor for interleukin 7 (IL7)**. The function of this receptor requires the interleukin 2 receptor, gamma chain (IL2RG), which is a common gamma chain shared by the receptors of various cytokines, including interleukins 2, 4, 7, 9, and 15. This protein has been shown to **play a critical role in V(D)J recombination during lymphocyte development. Defects in this gene may be associated with severe combined immunodeficiency (SCID).** Alternatively spliced transcript variants have been found. [provided by RefSeq, Dec 2015] Receptor for interleukin-7. Also acts as a receptor for thymic stromal lymphopoietin (TSLP).	yes	immunosuppression	down	< -2FC
NFATC2	-3.475	This gene is a member of the nuclear factor of activated T cells (NFAT) family. The product of this gene is a DNA-binding protein with a **REL-homology region (RHR)** and an NFAT-homology region (NHR). This protein is present in the cytosol and only translocates to the nucleus upon T cell receptor (TCR) stimulation, where it becomes a member of the nuclear factors of activated T cells transcription complex. This complex plays a central role in **inducing gene transcription during the immune response.** Alternate transcriptional splice variants encoding different isoforms have been characterized. [provided by RefSeq, Apr 2012] Plays a role in the **inducible expression of cytokine genes in T-cells, especially in the induction of the IL-2, IL-3, IL-4, TNF-alpha or GM-CSF.** Promotes invasive migration through the activation of GPC6 expression and WNT5A signaling pathway. NFAT (Nuclear Factor of Activated T-Cells) proteins are transcription factors that are integral for the development and function of the immune system. Five NFAT isoforms have been identified to date, NFATC1-C4 and NFAT5.	yes	immunosuppression	down	< -2FC
IRF8	-3.630	Interferon consensus sequence-binding protein (ICSBP) is a transcription factor of the interferon (IFN) regulatory factor (IRF) family. Proteins of this family are composed of a conserved DNA-binding domain in the N-terminal region and a divergent C-terminal region that serves as the regulatory domain. The IRF family proteins bind to the IFN-stimulated response element (ISRE) and regulate expression of genes stimulated by type I IFNs, namely IFN-alpha and IFN-beta. **IRF family proteins also control expression of IFN-alpha and IFN-beta-regulated genes that are induced by viral infection.** [provided by RefSeq, Jul 2008] **Plays a role as a transcriptional activator or repressor** (PubMed:25122610). Specifically binds to the upstream regulatory region of type I IFN and IFN-inducible MHC class I genes (the interferon consensus sequence (ICS)). **Plays a negative regulatory role in cells of the immune system.** Involved in CD8(+) dendritic cell differentiation by forming a complex with the BATF-JUNB heterodimer in immune cells, leading to recognition of AICE sequence (5'-TGAnTCA/GAAA-3'), an immune-specific regulatory element, followed by cooperative binding of BATF and IRF8 and activation of genes (By similarity). Positively regulates macroautophagy in dendritic cells (PubMed:29434592).	yes	immunosuppression	down	< -2FC
PTPN3	-3.645	May act at junctions between the membrane and the cytoskeleton. Possesses tyrosine phosphatase activity. The protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that **regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation.** This protein contains a C-terminal PTP domain and an N-terminal domain homologous to the band 4.1 superfamily of cytoskeletal-associated proteins. P97, a cell cycle regulator involved in a variety of membrane related functions, has been shown to be a substrate of this PTP. This PTP was also found to interact with, and be regulated by adaptor protein 14-3-3 beta. Several alternatively spliced transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Feb 2009]	no	oncogenesis	down	< -2FC
CD28	-3.830	The protein encoded by this gene is essential for **T-cell proliferation and survival, cytokine production, and T-helper type-2 development.** Several alternatively spliced transcript variants encoding different isoforms have been found for this gene.[provided by RefSeq, Jul 2011] **Involved in T-cell activation, the induction of cell proliferation and cytokine production and promotion of T-cell survival. Enhances the production of IL4 and IL10 in T-cells** in conjunction with TCR/CD3 ligation and CD40L costimulation (PubMed:8617933). Isoform 3 **enhances CD40L-mediated activation** of NF-kappa-B and kinases MAPK8 and PAK2 in T-cells (PubMed:15067037).	yes	immunosuppression	down	< -2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
þH2D1A	-3.869	Cytoplasmic adapter regulating receptors of the signaling lymphocytic activation molecule (SLAM) family such as SLAMF1, CD244, LY9, CD84, SLAMF6 and SLAMF7. In SLAM signaling seems to cooperate with SH2D1B/EAT-2. Initially it has been proposed that association with SLAMF1 prevents SLAMF1 binding to inhibitory effectors including INPP5D/SHIP1 and PTPN11/SHP-2 (PubMed:11806999). However, by simultaneous interactions, recruits FYN which subsequently phosphorylates and activates SLAMF1 (PubMed:12458214). Positively regulates CD244/2B4- and CD84-mediated natural killer (NK) cell functions. Can also promote CD48-, SLAMF6 -, LY9-, and SLAMF7-mediated NK cell activation. In the context of NK cell-mediated cytotoxicity enhances conjugate formation with target cells (By similarity). May also regulate the activity of the neurotrophin receptors NTRK1, NTRK2 and NTRK3. This gene encodes a protein that plays a major role in the bidirectional **stimulation of T and B cells.** This protein contains an SH2 domain and a short tail. It associates with the signaling lymphocyte-activation molecule, thereby acting as an inhibitor of this transmembrane protein by blocking the recruitment of the SH2-domain-containing signal-transduction molecule SHP-2 to its docking site. This protein can also bind to other related surface molecules that are expressed on activated T, B and NK cells, thereby modifying signal transduction pathways in these cells. Mutations in this gene cause lymphoproliferative syndrome X-linked type 1 or Duncan disease, a rare immunodeficiency characterized by extreme susceptibility to infection with Epstein-Barr virus, with symptoms including severe mononucleosis and **malignant lymphoma. Multiple transcript variants encoding different isoforms have been found for this gene. [provided by RefSeq, Jul 2008]**	yes	immunosuppression	down	<-2FC
CD247	-4.120	The protein encoded by this gene is **T-cell receptor zeta,** which together with T-cell receptor alpha/beta and gamma/delta heterodimers, and **with CD3-gamma, -delta and -epsilon, forms the T-cell receptor-CD3 complex.** The zeta chain plays an important role in coupling **antigen recognition to several intracellular signal-transduction pathways. Low expression of the antigen results in impaired immune response.** Two alternatively spliced transcript variants encoding distinct isoforms have been found for this gene. [provided by RefSeq, Jul 2008] Part of the TCR-CD3 complex present on T-lymphocyte cell surface that plays an **essential role in adaptive immune response.** When antigen presenting cells (APCs) activate T-cell receptor (TCR), TCR-mediated signals are transmitted across the cell membrane by the CD3 chains CD3D, CD3E, CD3G and CD3Z. All CD3 chains contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic domain. Upon TCR engagement, these motifs become phosphorylated by Src family protein tyrosine kinases LCK and FYN, resulting in the activation of downstream signaling pathways (PubMed:2470098, PubMed:7509083). CD3Z ITAMs phosphorylation creates multiple docking sites for the protein kinase ZAP70 leading to ZAP70 phosphorylation and its conversion into a catalytically active enzyme (PubMed:7509083). Plays an important role in intrathymic T-cell differentiation. Additionally, participates in the activity-dependent synapse formation of retinal ganglion cells (RGCs) in both the retina and dorsal lateral geniculate nucleus (dLGN) (By similarity).	yes	immunosuppression	down	<-2FC
NCAM1	-4.323	(Microbial infection) Acts as a receptor for rabies virus.||This protein is a cell adhesion molecule involved in neuron-neuron adhesion, neurite fasciculation, outgrowth of neurites, etc. This gene encodes a cell adhesion protein which is a member of the immunoglobulin superfamily. The encoded protein is involved in cell-to-cell interactions as well as cell-matrix interactions during development and differentiation. The encoded protein has been shown to be involved in development of the nervous system, and for cells involved in the expansion of T cells and dendritic cells which play **an important role in immune surveillance.** Alternative splicing results in multiple transcript variants. [provided by RefSeq, Jun 2011] Cell adhesion molecules (CAMs) are a large family of transmembrane proteins that are involved in the binding of a cell to another cell or to the extracellular matrix. They have roles in cell proliferation, differentiation, motility, trafficking, apoptosis and tissue architecture.	yes	immunosuppression	down	<-2FC
TSPAN8	-4.328	The protein encoded by this gene is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. Most of these members are cell-surface proteins that are characterized by the presence of four hydrophobic domains. The proteins **mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility.** This encoded protein is a cell surface glycoprotein that is known to complex with integrins. **This gene is expressed in different carcinomas.** The use of alternate polyadenylation sites has been found for this gene. [provided by RefSeq, Jul 2008]	no	oncogenesis	down	<-2FC
PRF1	-4.407	Plays a **key role in secretory granule-dependent cell death, and in defense against virus-infected or neoplastic cells.** Plays an important role in killing other cells that are recognized as non-self by the immune system, e.g. in transplant rejection or some forms of autoimmune disease. Can insert into the membrane of target cells in its calcium-bound form, oligomerize and form large pores. Promotes cytolysis and apoptosis of target cells by facilitating the uptake of cytotoxic granzymes. This gene encodes a protein with structural similarities to complement component C9 that is important in immunity. This protein forms membrane pores that allow the release of granzymes and subsequent cytolysis of target cells. Whether pore formation occurs in the plasma membrane of target cells or in an endosomal membrane inside target cells is subject to debate. Mutations in this gene are associated with a variety of human disease including diabetes, multiple sclerosis, **lymphomas,** autoimmune lymphoproliferative syndrome (ALPS), aplastic anemia, and familial hemophagocytic lymphohistiocytosis type 2 (FHL2), a rare and lethal autosomal recessive disorder of early childhood. [provided by RefSeq, Aug 2017]	yes	oncogenesis & immunosuppression	down	<-2FC

Symbol	logFC	Description (Tocris, Uniprot, NCBI Entrez Gene)	Expected direction	oncogenesis or immune suppression	up or down	FC group
EOMES	-4.486	This gene belongs to the TBR1 (T-box brain protein 1) sub-family of T-box genes that share the common DNA-binding T-box domain. The encoded protein is a transcription factor which is crucial for embryonic development of mesoderm and the central nervous system in vertebrates. **The protein may also be necessary for the differentiation of effector CD8+ T cells which are involved in defense against viral infections.** A similar gene disrupted in mice is shown to be essential during trophoblast development and gastrulation. Alternative splicing results in multiple transcript variants. [provided by RefSeq, May 2013] Functions as a transcriptional activator playing a crucial role during development. Functions in trophoblast differentiation and later in gastrulation, regulating both mesoderm delamination and endoderm specification. Plays a role in brain development being required for the specification and the proliferation of the intermediate progenitor cells and their progeny in the cerebral cortex. **Also involved in the differentiation of CD8+ T-cells during immune response regulating the expression of lytic effector genes.**	yes	immunosuppression	down	<-2FC
KLRK1	-5.167	**Natural killer (NK) cells are lymphocytes that can mediate lysis of certain tumor cells and virus-infected cells without previous activation.** They can also regulate specific humoral and cell-mediated immunity. NK cells preferentially express several calcium-dependent (C-type) lectins, which have been implicated in the regulation of NK cell function. The NKG2 gene family is located within the NK complex, a region that contains several C-type lectin genes preferentially expressed in NK cells. **This gene encodes a member of the NKG2 family.** The encoded transmembrane protein is characterized by a type II membrane orientation (has an extracellular C terminus) and the presence of a C-type lectin domain. It binds to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins, where ligand-receptor interactions can result in the activation of NK and T cells. **The surface expression of these ligands is important for the recognition of stressed cells by the immune system, and thus this protein and its ligands are therapeutic targets for the treatment of immune diseases and cancers.** Read-through transcription exists between this gene and the upstream KLRC4 (killer cell lectin-like receptor subfamily C, member 4) family member in the same cluster. [provided by RefSeq, Dec 2010]	yes	immunosuppression	down	<-2FC
TNFRSF9	-5.971	The protein encoded by this gene is a member of the **TNF-receptor superfamily.** This receptor **contributes to the clonal expansion, survival, and development of T cells.** It can also induce proliferation in peripheral monocytes, enhance T cell apoptosis induced by TCR/CD3 triggered activation, and regulate CD28 co-stimulation to promote Th1 cell responses. **The expression of this receptor is induced by lymphocyte activation.** TRAF adaptor proteins have been shown to bind to this receptor and transduce the signals leading to activation of NF-kappaB. [provided by RefSeq, Jul 2008]	yes	immunosuppression	down	<-2FC
TRAV4	-6.494	T cell receptors **recognize foreign antigens** which have been processed as small peptides and bound to **major histocompatibility complex (MHC) molecules** at the surface of antigen presenting cells (APC). Each T cell receptor is a dimer consisting of one alpha and one beta chain or one delta and one gamma chain. In a single cell, the T cell receptor loci are rearranged and expressed in the order delta, gamma, beta, and alpha. If both delta and gamma rearrangements produce functional chains, the cell expresses delta and gamma. If not, the cell proceeds to rearrange the beta and alpha loci. This region represents the germline organization of the T cell receptor alpha and delta loci. Both the alpha and delta loci include V (variable), J (joining), and C (constant) segments and the delta locus also includes diversity (D) segments. The delta locus is situated within the alpha locus, between the alpha V and J segments. During T cell development, the delta chain is synthesized by a recombination event at the DNA level joining a D segment with a J segment; a V segment is then joined to the D-J gene. The alpha chain is synthesized by recombination joining a single V segment with a J segment. For both chains, the C segment is later joined by splicing at the RNA level. Recombination of many different V segments with several J segments provides a wide range of antigen recognition. Additional diversity is attained by junctional diversity, resulting from the random addition of nucleotides by terminal deoxynucleotidyltransferase. Five variable segments can be used in either alpha or delta chains and are described by TRAV/DV symbols. Several V and J segments of the alpha locus are known to be incapable of encoding a protein and are considered pseudogenes. [provided by RefSeq, Aug 2016]	yes	immunosuppression	down	<-2FC

S17: IPA Summary for analysis of differential gene expression between KoRV positive lymph node tissue and lymphoma tissue, LogFC greater than two-fold, either direction, adjusted *p* value < 0.05

INGENUITY
PATHWAY ANALYSIS

Analysis Name: Lymphoma v KoRV Positive K17out
Analysis Creation Date: 2020-07-07
Build version: exported
Content version: 52912811 (Release Date: 2020-06-01)

Top Canonical Pathways

Name	p-value	Overlap
Tryptophan Degradation to 2-amino-3-carboxymuconate Semialdehyde	1.56E-04	50.0 % 3/6
Granulocyte Adhesion and Diapedesis	1.12E-03	6.1 % 11/180
T Cell Receptor Signaling	1.31E-03	7.6 % 8/105
NAD biosynthesis II (from tryptophan)	1.57E-03	25.0 % 3/12
Atherosclerosis Signaling	4.30E-03	6.3 % 8/127

Top Upstream Regulators

Upstream Regulators

Name	p-value	Predicted Activation
TNF	3.55E-13	Inhibited
IL2	8.59E-13	Inhibited
beta-estradiol	1.15E-11	Inhibited
lipopolysaccharide	1.77E-11	Inhibited
IL12 (complex)	8.03E-11	Inhibited

Causal Network

Name	p-value	Predicted Activation
etanercept	1.86E-15	Activated
PDLIM5	6.11E-15	Activated
WNT16	2.65E-14	
SGK1	3.27E-14	
etanercept	5.50E-14	Activated

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value range	# Molecules
Cancer	2.77E-05 - 7.07E-26	442
Organismal Injury and Abnormalities	2.77E-05 - 7.07E-26	448
Gastrointestinal Disease	2.77E-05 - 3.05E-23	415
Dermatological Diseases and Conditions	1.66E-05 - 1.27E-19	296
Endocrine System Disorders	1.69E-05 - 2.45E-18	382

Molecular and Cellular Functions

Name	p-value range	# Molecules
Cellular Development	2.65E-05 - 1.12E-17	187
Cellular Function and Maintenance	1.86E-05 - 1.12E-17	187
Cellular Growth and Proliferation	2.43E-05 - 1.12E-17	179
Cellular Movement	2.56E-05 - 5.92E-16	154
Cell Morphology	1.57E-06 - 7.95E-13	104

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PATHWAY ANALYSIS

Physiological System Development and Function

Name	p-value range	# Molecules
Hematological System Development and Function	2.65E-05 - 1.20E-20	153
Tissue Morphology	2.65E-05 - 1.20E-20	140
Lymphoid Tissue Structure and Development	2.65E-05 - 2.03E-18	120
Cell-mediated Immune Response	2.56E-05 - 1.12E-17	73
Embryonic Development	1.18E-05 - 1.12E-17	121

Top Regulator Effect Networks

ID	Regulators	Disease & Functions	Consistency Score
1	CARD9,CD44,CSF2,IFI16,Ige,IL1,IL18,IL1B,RELA,STAT1 (+2 more)	Adhesion of blood cells,Binding of leukocytes (+9 more)	45.867
2	CD40LG,CD44,CSF2,IFI16,Ige,IL1,IL18,IL1B,PSMB11 (+2 more)	Adhesion of blood cells,Binding of leukocytes (+10 more)	43.275
3	AIP,CARD9,CD40LG,CD44,CEBPA,CSF2,CX3CL1,ERG,GATA1 (+13 more)	Adhesion of blood cells,Binding of leukocytes (+18 more)	43.205
4	CARD9,CD3,CD40LG,CD44,CSF2,GATA1,IFI16,Ige,IL1 (+8 more)	Adhesion of blood cells,Binding of leukocytes (+11 more)	42.573
5	CARD9,CD44,CSF2,IFI16,Ige,IL1,IL18,IL1B,RELA,STAT1 (+1 more)	Adhesion of blood cells,Binding of leukocytes (+13 more)	41.293

Top Analysis-Ready Molecules

Expr Log Ratio

Molecules	Expr. Value	Chart
GPX2	↑ 9.800	
DNTT	↑ 7.952	
LRRN3	↑ 7.810	
NMRK2	↑ 7.295	
OBSL1	↑ 7.217	
MSI1	↑ 6.482	
SV2C	↑ 6.471	
TBC1D21	↑ 6.465	
HSPB7	↑ 6.420	
INHA	↑ 6.098	

Expr Log Ratio

Molecules	Expr. Value	Chart
SYT4	↓ -7.778	
IL1F10	↓ -7.429	
HOXB9	↓ -7.115	
CNN1	↓ -6.765	
CTSG	↓ -6.711	
TRAV4	↓ -6.494	
CCR4	↓ -6.177	
TMPRSS3	↓ -6.146	
TNFRSF9	↓ -5.971	
GZMA	↓ -5.604	

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PATHWAY ANALYSIS

Appendix 44

S18: Box link

Excel file with IPA gene expression data for all genes, upregulated and downregulated between lymphoma and KoRV positive koalas, excel file inclusive of IPA enriched canonical pathways and IPA Disease and Function gene analysis.

Link:

https://universityofadelaide.box.com/s/68atugyqdnvg1qti85jpeu4a06mafutv