UNIVERSITY OF VETERINARY MEDICINE BUDAPEST

Department of Pathology

Retroviruses in koalas – a literature review

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List of abbreviations

ALV - Avian leukosis virus

ARV – Antiretroviral

C. pecorum – Chlamydia Pecorum

Env – Envelope

ESCRT - Endosomal sorting complexes required for transport

FeLV – Feline leukemia virus

FFRV - Flying fox retrovirus

Gag – Group-specific antigen gene

GALV – Gibbon-ape leukemia virus

Glyco-Gag – Glycosylated-gag

GRO – Growth-related oncogene

HE – Haematoxylin and eosin

HIV – Human immunodeficiency virus

HPG - Hervey pteropid gammaretrovirus

HTLV – Human T-cell leukemia virus

IHC – Immunohistochemistry

JSRV - Jaagsiekte sheep retrovirus

KoRV – Koala retrovirus

LTR - Long terminal repeats

MbRV - Melomys burtoni retrovirus

MIRV - Megaderma lyra retrovirus

MMLV – Moloney murine leukemia virus

MMTV - Mouse mammary tumor virus

MuLV – Murine leukemia virus

NHP – Non-human primates

PBMCs – Peripheral blood mononuclear cells

PERV – Porcine endogenous retrovirus

PiT-1 – Sodium-dependent phosphate transporter 1

Pro-pol/Pol - Protease-polymerase

RBD – Receptor binding domain

SU - Surface

TEM – Transmission electron microscopy

THTR1 - Thiamine transport protein 1

TM - Transmembrane

VRA - Variable region A

1. Abstract

Koala retrovirus (KoRV) is a *Gammaretrovirus* that has been identified in both wild koalas in Australia and captive koalas worldwide. The virus is probably a result of trans-species transmission from rodents or bats and are closely related to gibbon-ape leukemia virus (GALV), feline leukemia virus (FeLV) and porcine endogenous retrovirus (PERV). KoRV is one of the major pathogens of koalas, causing immunosuppression, neoplasia, and leukemia. An interesting factor of KoRV is that it exists in both endogenous and exogenous forms and can be divided in up to 10 subtypes (A–J), where co-infection is possible. As well as this, does the virus present variability in genetics and disease prevalence in different koala populations, with Southern and Northern Australian koalas showing great differences. The virus etiology, epidemiology, pathogenesis and its diseases has been greatly researched and studied, especially the last 10 years. This has led to a big improvement in virus detection, treatment, and prevention, where vaccine development has showed great success.

2. Introduction

Koala retrovirus (KoRV) is a virus belonging to the genus *Gammaretrovirus*, that has been identified in both wild and captive koalas (Kinney & Pye, 2016). Koalas (*Phascolarctos cinereus*) are Australian marsupials, only found wild in Australia, but also found captive in different zoos and wildlife parks all around the world (Quigley & Timms, 2020). The koalas in Australia are found throughout the eastern and southern coast, and can be divided into two different groups, the northern koalas in Queensland and New South Wales and the southern koalas in South Australia and Victoria. Although the koalas are all the same species, they show differences in genetics, conservation status and disease prevalence between the different regions of Australia (Fabijan et al., 2020).

The origin of the KoRV is still unclear. KoRV shows strong similarities to the gibbon-ape leukemia virus (GALV), feline leukemia virus (FeLV), porcine endogenous retrovirus (PERV) and human T-cell leukemia virus (HTLV) (Denner & Young, 2013). Regarding the similarities between GALV and KoRV, research has proven that an Australian grassland melomys (*Melomys burtoni*) could be a possible common source between the two of them, since it is unlikely that a direct transmission between the two viruses could be the cause of the sequence similarities (Kinney & Pye, 2016).

Both endogenous and exogenous forms of KoRV have been identified. There have also been several envelope subtypes of KoRV identified, ranging between A to J, where co-infection in koalas is possible. They vary in the receptor types used for host entry and transmission form. All of these characteristics makes KoRV a genetically diverse virus and a significant threat to koala health (Kinney & Pye, 2016; Zheng et al., 2020).

The koalas have experienced substantial population decreases, due to habitat reduction and fragmentation, climate change, wildfires, vehicle collisions, attacks from domestic pets and last, but not least, disease outbreaks. This has resulted in the koalas being officially declared vulnerable by the Australian government and listed as a threatened species in 2012. (Quigley & Timms, 2020). Due to the oncogenic and immunosuppressive capabilities of retroviral infections, KoRV might be regarded as the most significant pathogen to koalas (Fabijan et al., 2020). The diseases associated with KoRV is leukemia, lymphoma and other neoplastic disorders, immunosuppression and hematopoietic disease (Kinney & Pye, 2016). The long-term stability of both wild and captive koala populations are likely affected by KoRV induced lymphoma, leukemia and immunomodulation which stimulate other, severe opportunistic diseases (Denner & Young, 2013). Fatal lymphoid

neoplasia and leukemia are diseases especially common in KoRV-B infected koalas, and has been proposed to have a specific link to KoRV-B (Denner & Young, 2013). It has also been suggested that there is an association between KoRV and *Chlamydia* infection in koalas, and that KoRV may result in an opportunistic *Chlamydia* infection (Kinney & Pye, 2016).

The aim of this literature review is to review what we already know about KoRV, it´s etiology, epidemiology, pathogenesis, and the diseases caused by KoRV, as well as treatment, management and prevention options and future research directions. Both other reviews and case studies has been used to gain the information, with articles specifically regarding KoRV in koalas but also regarding other pathogens that associate with KoRV or koalas.

3. Description of the Koala Retrovirus

3.1. Etiology

Koala retrovirus (KoRV) belongs to the family *Retroviridae*, in the subfamily *Orthoretrovirinae*, genus *Gammaretrovirus.* Retroviruses are positive-stranded RNA viruses which are able to reverse transcribe the genomic RNA into double-stranded DNA, using the viral enzyme reverse transcriptase. To replicate and survive, this DNA copy integrates into the genome of the host, where the integrated DNA copy are then called provirus (Denner & Young, 2013; Kinney & Pye, 2016; Quigley & Timms, 2020).

Like mentioned earlier, the origin of KoRV is still unclear. In 1988, viral particles morphologically consistent with a retrovirus were identified in a female, leukemic koala. The cancerous cells of these koalas were detected with gammaretrovirus-like particles, type C retrovirus to be exact (Canfield et al., 1988). But it wasn't until the year of 2000 that Hanger managed to isolate, recognize and fully sequence the virus, eventually naming it the "koala retrovirus, KoRV" (Hanger et al., 2000). During this time, KoRV showed strong similarities to GALV and was therefore first recognized as a close relative to GALV, as well as having the same disease outcomes in koalas as GALV has in gibbon apes, murine leukemia virus (MuLV) has in mice, FeLV has in cats and HTLV has in humans (Denner & Young, 2013; Hanger et al., 2000). GALV, MuLV and FeLV are all gammaretroviruses, while HTLV is a deltaretrovirus. GALV is an exogenous, oncogenic gammaretrovirus which causes leukemia in gibbons, using the sodium-dependent phosphate transporter (Pit-1) receptor, an orthogolous receptor which KoRV-A also uses (Denner & Young, 2013; Kinney & Pye, 2016). KoRV and GALV show strong sequence similarities, but a direct retroviral transmission is proven to be unlikely. This is because there is a geographic separation between free ranging gibbons and koalas, where overlap does not occur. However, grassland melomys (*Melomys burtoni*), an Australian rodent, could be a possible common source between the two viruses, as these rodents naturally overlap with the koala. Although they do not naturally overlap with the gibbons, the provirus they carry, also a retrovirus, the melomys burtoni retrovirus (MbRV), is very similar to GALV. MbRV is in fact so similar to GALV that it could be considered another strain of GALV, and the provirus contains open reading frames (Kinney & Pye, 2016). Through a screen in Australia of 42 vertebrate species, MbRV was identified with 83% identity to KoRV and 93% identity to GALV (Quigley & Timms,

2020). Phylogenetic analysis published in 2019 also showed that sequences from both GALV and MbRV had a close relation to KoRV (Sarker et al., 2019).

It is not just rodents that has been discovered to have a close link to the origin of KoRV. There is a high probability that KoRV is a result of trans-species transmission from bats (Denner & Young, 2013). Previous studies proved that microbats in China, *Megaderma lyra,* was detected with the retrovirus megaderma lyra retrovirus (MIRV), and they showed some similarities to KoRV. More recent studies can identify gammaretroviruses in black-flying foxes, *Pteropus alecto*, found in Queensland. These gammaretroviruses, flying fox retrovirus (FFRV) and hervey pteropid gammaretrovirus (HPG), was phylogenetically grouped closely to both KoRV and GALV (Quigley & Timms, 2020).

Today, we know that the KoRV is a genetically diverse virus, with up to 10 subtypes (A-J) (Figure 1) (Quigley & Timms, 2020; Zheng et al., 2020). The KoRV subtypes show important differences in the cellular receptors used for host cell entry and infection, and like other gammaretroviruses, it is the different receptors that most of the categorizing is based on (Quigley & Timms, 2020; Sarker et al., 2019). KoRV-A was the first variant to be isolated, and has been identified in both wild and captive koalas in Australia, and is currently the most researched subtype (Chappell et al., 2017). KoRV-A was found to utilize the same receptor for cell entry as GALV and FeLV-B, the PiT-1 receptor (Shimode et al., 2014). KoRV-A has later been found in captive koalas in German and Japanese zoos as well (Denner & Young, 2013). More than a decade after the identification of KoRV-A and it´s PiT-1 receptor, two reports came out in 2013, that identified a new subtype of KoRV. Both subtypes were found to utilize another receptor than PiT-1, called the thiamine transport protein 1 (THTR1) (Quigley & Timms, 2020; Xu et al., 2015). The first report was from captive koalas with lymphomas, in Los Angeles Zoo in the USA, and this subtype was designated KoRV-B. The second subtype, found from captive koalas in Kobe Municipal Oji Zoo, in Japan, was also designated KoRV-B. However, it was originally designated KoRV-J, until phylogenetic analysis determined that they should be grouped together under the same name, KoRV-B (Shimode et al., 2014). Due to the heterogeneity in KoRV subtypes occurring in their receptor recognition and receptor binding domains (RBD), within the variable region (VRA) of their envelope proteins, KoRV has shown greater genetic diversity than previously thought (Zheng et al., 2020). The same Kobe study that grouped KoRV-J and -B together, also designated two new, additional subtypes, KoRV-C and KoRV-D. These subtypes were all different to each other in the VRA regions of the RBD (Shimode et al., 2014). Later, through several studies from captive koalas in US zoos, came an interesting

finding of two new KoRV variants, designated KoRV-E and KoRV-F. They also differed from both KoRV-A and KoRV-B in the VRA regions of their envelope RBD, and were found to be using different receptors from both PiT1 and THTR1 (Xu et al., 2015). At last, the subtypes KoRV-G, KoRV-H and KoRV-I were established trough a phylogenetic study of 18 wild southeast koalas, due to the discovery of even more diversity in the amino acid signatures of the VRA regions (Chappell et al., 2017).

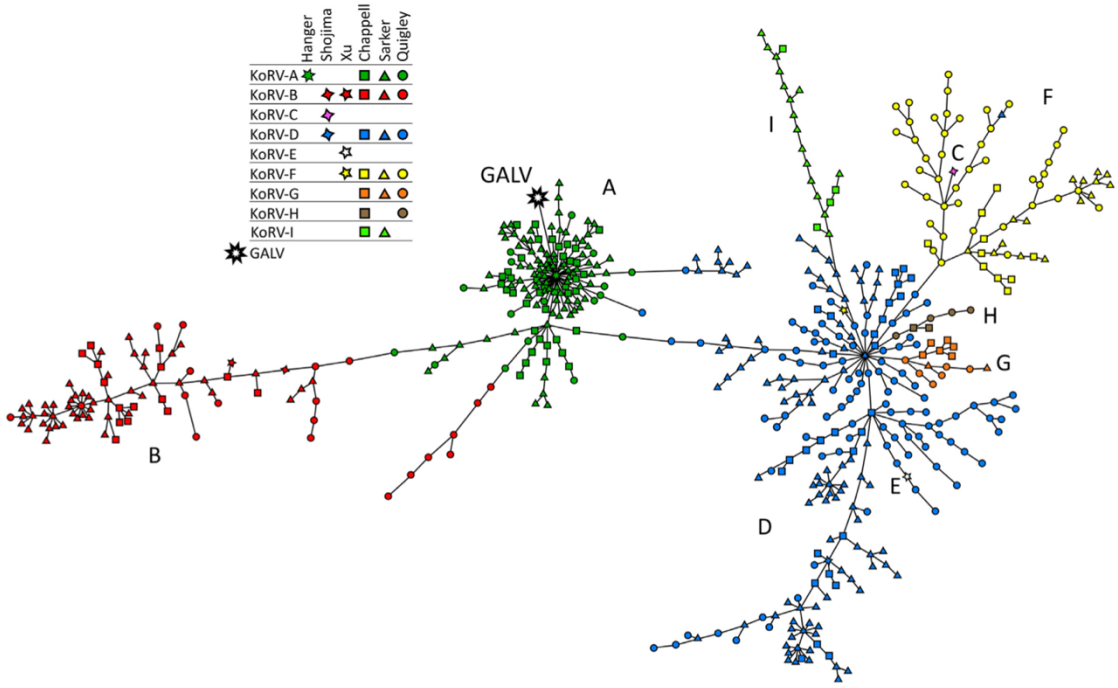

Figure 1. KoRV diversity and the subtypes A-I, visualized by minimum spanning tree. GALV is showed for comparison. (Quigley & Timms, 2020).

In general, the KoRV subtypes can be classified into 3 major groups, based on their differences in receptor binding. The groups are; KoRV-A, which uses the PiT1 receptor, KoRV-B, which uses the THTR1 receptor and KoRV-C-I, which uses unknown receptors (Sarker et al., 2019). Among these, KoRV-A and KoRV-B are considered as the major subtypes (Hashem et al., 2020).

The biology of KoRV is like of a gammaretrovirus, it includes a typical morphology, size and a simple genome organization (Denner & Young, 2013). It is about 8,5 kb long and at each end of a positive-sense, single-stranded RNA genome, it is composed of long terminal repeats (LTRs) at the 5´and 3´ends (Hashem et al., 2021). The LTRs contains three coding genes; the group-specific antigen gene (*gag*), encoding core and structural proteins,

the protease-polymerase (*pro-pol or pol*) gene, encoding reverse transcriptase, protease and integrase, and the envelope (*env*) gene, encoding coat proteins (Hobbs et al., 2014; Quigley & Timms, 2020). The *gag* protein, like other gammaretroviruses, plays a very important part in KoRV budding. This is because *gag* recruits the endosomal sorting complexes required for transport (ESCRT) machinery by interacting with a specific L-domain area in KoRV *gag*, which finally allows virions to be released from infected cells (Quigley & Timms, 2020). KoRV, like other gammaretroviruses, produces two RNA transcripts from its genome: a near-full-length unspliced genome transcript and an *env* mRNA with a single intron (Hobbs et al., 2014; Quigley & Timms, 2020). Studies have proved that the *env* mRNA is 5-fold more abundant than the unspliced transcript (Quigley & Timms, 2020). It is also confirmed that the *env* splice sites are very similar to those of the Moloney Murine Leukemia Virus (MMLV) (Hobbs et al., 2014). Two regions are found in the *env* protein, the surface protein (SU or gp70) and the transmembrane protein (TM or p15E). The TM protein is found to be very important for the KoRV biology because it contains epitopes which are known to be important for neutralizing antibody responses and a major immunosuppressive domain (Quigley & Timms, 2020).

Currently, there is little known information about the stability of infectious KoRV in the environment. However, we can compare it to other similar gammaretroviruses, such as FeLV, which can survive for two days in culture medium but only a couple hours if dried (Zheng et al., 2020).

3.2. Epidemiology

Both endogenous and exogenous forms of transmission are possible with KoRV. Other examples of retroviruses with both transmission forms are the jaagsiekte sheep retrovirus (JSRV), mouse mammary tumor virus (MMTV), FeLV and avian leukosis virus (ALV) (Chappell et al., 2017). KoRV was first recognized as an endogenous retrovirus, with its proviral integration patterns. When a virus is endogenously transmitted, it means that the virus has incorporated into germline cells and is transmitted from parent to offspring in the chromosomal DNA (Quigley & Timms, 2020). KoRV is therefore fixed into the genome of every koala cell and transmitted vertically, in Mendelian fashion (Zheng et al., 2020). Opposite to this type of transmission is exogenous, where the virus is transmitted horizontally between koalas, trough infection of their specific somatic target cells (Denner & Young, 2013; Quigley & Timms, 2020). Exogenous retroviruses can be compared to HIV-

1, which is transmitted through infection of $CD4^+$ T-lymphocytes, and are therefore also horizontally transmitted (Denner & Young, 2013). The transmission capabilities variates within the different KoRV subtypes, and we can therefore organize the KoRV subtypes into either exogenous or endogenous forms (Denner & Young, 2013; Kinney & Pye, 2016). KoRV-A is the only endogenous form of KoRV, from which other subtypes have arisen, and is found in every KoRV-positive koala (Sarker et al., 2019). It is considered endogenous due to their presence in a variety of cells, including sperm cells with multiple provirus copies (Kinney & Pye, 2016). The other KoRV subtypes are usually found in tissues from infected koalas with less than 1 copy per cell, suggesting lack of endogenization into the host genome and therefore considering them "putative somatic insertions" (Kinney & Pye, 2016; Sarker et al., 2019). It has also been reported that KoRV-D and KoRV-E are missing multiple genes that are needed for viral processing. This suggests that they can only be transmitted as defective viruses and only with a replication competent "helper" virus present, as has been reported for other retroviruses (Hobbs et al., 2017; Sarker et al., 2019). Nevertheless, we can categorize the KoRV subtypes into one endogenous subtype, which is KoRV-A, and another nine exogenous subtypes, which includes KoRV-B-J (Hashem et al., 2021).

The process of KoRV endogenization into koalas is estimated to have started about 49,000 years ago, which in evolutionary terms, is fairly recent, especially compared to other retroviruses which have been endogenized into the mammalian genomes for millions of years (Hashem et al., 2020, 2021). Previous investigation and research has proven that the northern koalas population had KoRV completely endogenously incorporated into its genome, but southern koalas, however, seemed to still be under the process of endogenization (Hashem et al., 2021). Considering that some regional wild koala populations in Australia still do not report 100% prevalence, it can suggest that KoRV may currently be in the process of endogenizing the genome of southern koalas. This provides an interesting and exciting opportunity for scientists to examine the active endogenization of a retrovirus into a host genome in real time (Denner & Young, 2013; Hashem et al., 2020; Kinney & Pye, 2016).

The mother koala is called dam, the father koala is called sire and their offspring is called joey (Quigley & Timms, 2020). Previous studies have mostly reported that KoRV-B transmits infection from dam-to-offspring via *de novo* (horizontal) infection, in contrast to KoRV-A which transmits infection via genetic inheritance (vertical infection) (Hashem et al., 2020; Xu et al., 2013). *De novo* infection from dam-to-offspring could be through uterine fluids, milk or pap (which is thought to originate from the dam´s caecum) (Hashem et al.,

2020). However, a study reported a KoRV-B-positive joey from a KoRV-B-positive dam and a KoRV-B-negative sire. The joey was only 6 weeks old when it died in the mother´s pouch. KoRV-B could have been transmitted from the dam through the milk ingested in the pouch, however, it also suggests that *in utero* infection is a possibility (Xu et al., 2013). KoRV-B was reported to have a 3% annual transmission rate for adult-to-adult koala contact per year, and a dams-to-joey annual transmission rate of 100%. However, a recent study reported for the first time a KoRV-B-negative joey from parents which were both KoRV-B-positive, which questions the 100% transmission rate for dam-to-joey (Hashem et al., 2020).

3.3. Pathogenesis

There are substantial differences between the pathogenicity and disease prevalence between the northern and southern koalas, and different KoRV subtypes also contribute to variation in prevalence between the different koala populations (Sarker et al., 2019). Northern koalas are vulnerable to extinction, and their population numbers are dropping quickly. They have a high disease prevalence, and the two major pathogens to koalas, *Chlamydia pecorum* (*C. pecorum*) and KoRV, are both highly prevalent in this population. KoRV-A is 100% prevalent in northern koalas and is an active endogenous infection. In the same population, *C. pecorum* infection is reported to be up to 90% prevalent and they also have a high prevalence of severe, overt chlamydial disease. With contrast to the northern koalas, the southern koala population are considered overabundant and have a much lower infection and disease prevalence than the northern koalas (Fabijan et al., 2020). Recent PCR studies of KoRV has suggested that southern koalas have a low prevalence of KoRV (15–20%) (Sarker et al., 2019), and infection there is thought to spread mainly exogenously (Fabijan et al., 2020). This can explain the lower frequency of chlamydial disease in the southern koalas compared to the northern koala population (Fabijan et al., 2020; Waugh et al., 2017). KoRV-B, which is presumed to only transmit infections exogenously, is commonly reported as the cause of lymphomas, leukemias and, finally, lymphoid neoplasia in koalas, which in the northern koalas, is the most commonly reported neoplasia (Fabijan et al., 2020; Quigley & Timms, 2020). In a PCR study of both captive and wild koalas, there was a much higher incidence of leukemia, lymphoma and other neoplasia in koalas infected with non-A-subtypes of KoRV (Zheng et al., 2020). Worst of all health outcomes is seen in koalas infected with several KoRV subtypes. A study of six captive koalas in a Japanese zoo showed that the koalas infected with several subtypes had poorer health outcomes than those infected with only one single subtype. This was seen on the increased WBC counts, which

indicated a leukemic condition at the time of blood sampling, in contrast to the clinically healthy-looking koalas infected with the single subtype, KoRV-A (Hashem et al., 2021). It has also been reported that captive koalas only developed leukemia/lymphoma when they were co-infected with both KoRV-A and KoRV-B, and do not with only KoRV-A infection (Waugh et al., 2017).

The wide variety in prevalence between the northern and southern koalas has led to speculations that KoRV is currently transmitted across Australia´s koala population in a "northern to southern" transmission wave. However, a PCR study showed that both northern and southern koala populations had the full range of KoRV subtypes, in both DNA and RNA forms. Even KoRV-B, which earlier was thought to have a low prevalence in the southern koalas, was found in every koala they investigated. On the other hand, the KoRV-B in the southern koalas were found with a lower absolute copy number, and non-KoRV-A in the northern koalas were found with a higher replication efficiency, which could indicate that either viral or host factors protects the southern koala population from KoRV-replication (Sarker et al., 2019).

KoRV has had concerns about its zoonotic potential to humans, due to its ability to infect a variety of different cells and because both KoRV-B and -E showed the ability to infect human cell lines using the different THTR1 and other receptors (Zheng et al., 2020). Recently, a study was done on human kidney cells (HEK293T cells) to demonstrate cellular response to KoRV infection, and the result was that KoRV infection does indeed induce antiviral and oncogenic responses in human cell lines, and therefore supporting the hypothesis that it is a oncogenic virus (Sarker et al., 2020). Some gammaretroviruses use glycosylated-*gag* (*glyco-gag*), which is the alternative form of the *gag* protein, in order to overcome host limitation factors like APOBEC3. The study found that, even though the koala genome contains genes that appear to be APOBECs, there was no evidence that KoRV expresses *glyco-gag* in human cell cultures and KoRV infectivity was restricted to human APOBEC3G and mouse APOBEC3. Therefore, zoonotic transmission of KoRV was proven to be unlikely (Quigley & Timms, 2020).

4. Infection and disease

Infections caused by retroviruses can result in a variety of outcomes for the host species, but not all retroviruses are pathogenic. Examples of some of the non-pathogenic retroviruses are the foamy viruses (Denner & Young, 2013). Some retroviral infections can even be beneficial, for example syncytin, a retrovirus that has endogenized into the human genome and plays a highly important role for the human placental morphogenesis (Kinney & Pye, 2016). KoRV, on the other hand, is considered to be a very dangerous pathogen for the koalas and can cause severe diseases and poor health outcomes. Especially since KoRV exists in both endo- and exogenous forms, and because koalas can be infected with several subtypes at the same time (Hashem et al., 2021). The virus can induce certain types of cancers, like leukemia and lymphoma, and immunodeficiency, which can predispose the koalas to opportunistic infections, such as chlamydial disease (Fabijan et al., 2020). These health issues have been reported in both wild and captive koalas (Hashem et al., 2021). In general, KoRV-infection in koalas cause diseases which are strikingly similar to those caused by FeLV-infection in cats. Some of the reported diseases in both FeLV-infected cats and KoRV-infected koalas are lymphoma, leukemia, anemia, mesothelioma, craniofacial tumors, chlamydiosis, rhinitis/pneumonia, stomatitis, gingivitis, cryptococcosis and toxoplasmosis (Denner & Young, 2013).

4.1. Neoplasia

When KoRV was found in 1988 in the neoplastic cells in the bone marrow of an adult, female, emaciated and lethargic koala, it was originally identified as a pathogen for leukemia, and since then the association between KoRV and neoplastic diseases has been maintained (Canfield et al., 1988; Kinney & Pye, 2016). Leukemia is also linked to disease caused by many other retroviruses, like MuLV, FeLV, GaLV and HTLV (Denner & Young, 2013). One of the many studies that has proved the link between KoRV infection and neoplasia, and that this can cause severe outcomes for koalas, is a PCR and blood test investigation of both wild and captive koalas (126 in total) infected with KoRV. The result was that 16 koalas (12,7 %) had died from leukemia-lymphoma at the end of the study, 7 (5,6%) had died from other cancers, 37 (29,4%) had died from other causes and the remaining 66 (52,4%) were alive and clinically healthy (Zheng et al., 2020). With a total of 18,3% koalas dying of neoplasia, it is obvious that it is a major threat to the koala population, and that KoRV likely has a say in it. Especially in the northern koala populations, where

more than 60% of mortality of the captive koalas are caused by leukemia and lymphoma (Xu et al., 2013).

As a result of neoplastic bone marrow invasion, koalas can develop lymphoid leukemia or lymphoma. Non-lymphoid tissues, with diffuse infiltration or defined foci, may also be impacted (Fabijan et al., 2017). Lymphoma can either be T-cell or B-cell lymphoma, and is characterised by single or multiple solid tumours, impacting all of the lymphoid tissue or specific lymph nodes (Ito et al., 2019).

Lymphoma was reported to occur in koalas with the highest KoRV proviral and viral loads, in a study published by Fabijan and colleagues in 2020. The study compared southern and northern koala populations, all infected with KoRV. Both populations had koalas with lymphoma not only affecting lymph nodes, thymus and spleen, but also involved the bladder, non-lymphoid bone marrow, gastrointestinal tract, liver, pancreas, heart, lungs, kidney and bladder, adrenal gland and/or brain (Figure 2) (Fabijan et al., 2020). The study also reported more evidence for KoRV-induced oncogenesis and suggested that the main locations for KoRV replication could be in lymphocytes and splenic lymphoid tissue (Fabijan et al., 2020).

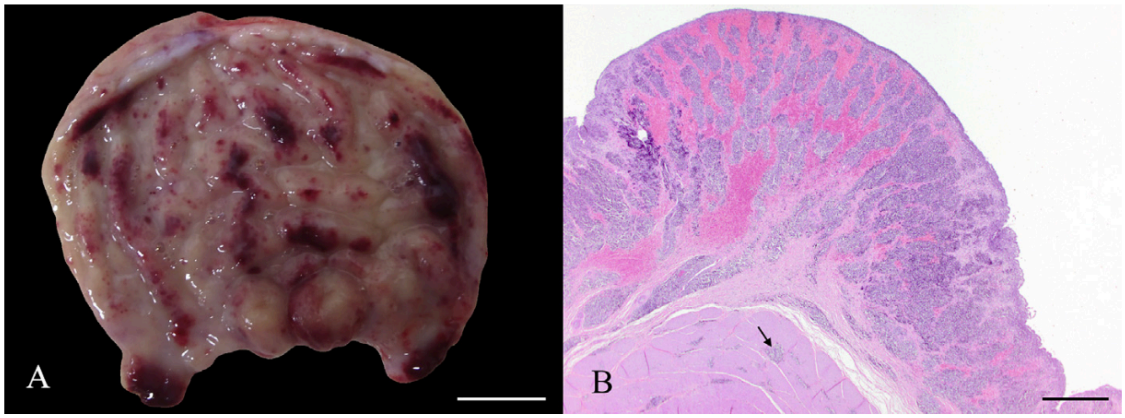

Figure 2. Lymphoma in a 4-year-old male koala from South Australia. A) Picture of the infiltrated bladder wall. Thickened, with pale mucosal surface and irregular, red pattern. Bar: 1 cm. B) Histopathology of the bladder mucosa and submucosa, with haematoxylin and eosin (HE) stain. There is loss of normal architecture and infiltration of neoplastic cells. Arrow: infiltration of neoplastic cells into muscularis. Bar: 1 cm. (Fabijan et al., 2020)

It has also become clear that KoRV-B is the dominating subtype of KoRV which can be linked to leukemia, lymphoma and other neoplasia in koalas. It has been suggested that the reason for this, could be that they have a greater number of the enhancer regions for viral transcription, found in the U3 region of the LTR region. Compared to KoRV-A which has only one of these enhancer regions, KoRV-B has four copies, and KoRV-F has five. Since KoRV replicates by randomly inserting into the host genome, the result of an upregulation of viral transcription could be a higher non-specific host transcription, which finally could increase the risk for malignant outcomes. This would be the outcome of all non-KoRV-A subtypes, due to the higher copy number of the enhancer region. However, like mentioned earlier, KoRV-B also seems to be able to transmit from dam-to-joey, unlike the other non-KoRV-A subtypes which only transmits infection horizontally (Xu et al., 2013, 2015). In the end, all these theories are suggestions, and exactly how KoRV-B causes leukemia and neoplasia is not fully understood.

A case study was done on a wild koala from the southern Australia, infected with KoRV-A and severe reproductive chlamydiosis and diagnosed with lymphoma. The koala originally came to the hospital for right hindlimb lameness and was later identified with right stifle crepitus (Figure 3) and enlarged superficial lymph nodes containing a high number of atypical lymphocytes, indicating lymphoma. Lymphoma was later confirmed with histopathology from the bone marrow, mesenteric lymph nodes and ovary with further infiltration of other tissues (Fabijan et al., 2017).

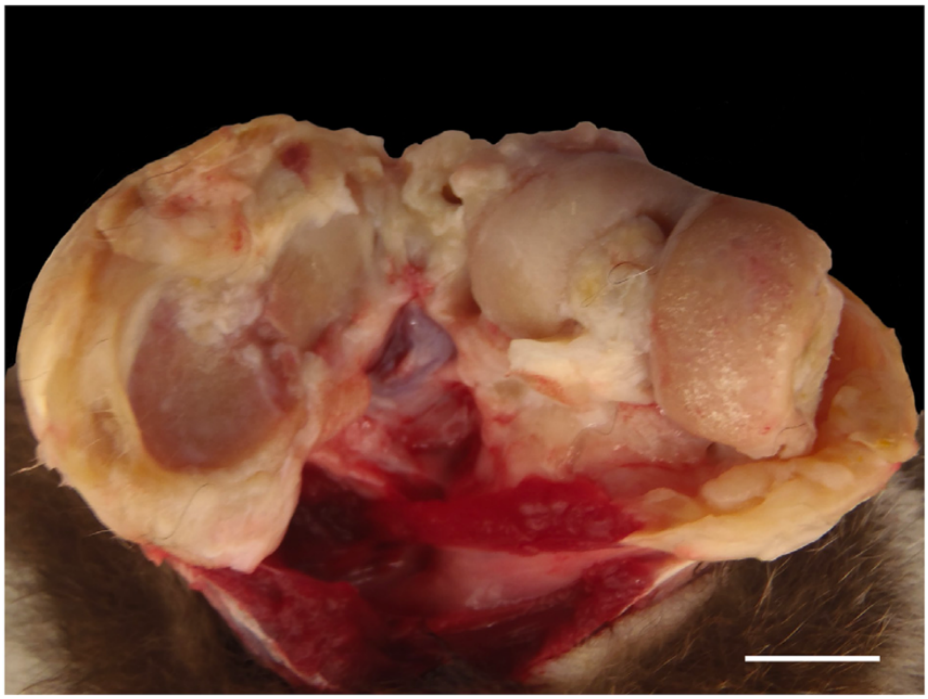

Figure 3. Right stifle of KoRV-infected koala with lymphoma. Over the medial femoral condyle, extensive cartilage degeneration has occured and exposure of subchondral bone. Bar: 1 cm. (Fabijan et al., 2017)

Another case study investigated myelogenous leukemia in a KoRV-infected, female koala with diabetes mellitus, which came in with epistaxis, anemia, leykocytosis and tachypnea. Scattered, atypical, large myeloid cells, as well as other leukocytes and erythroblasts was seen on a blood smear (Figure 4). Hematology showed an increased white blood cell count (up to $295\times10^2/\mu L$), severe regenerative anemia, thrombocytopenia, hypokalemia and raised aspartate transaminase. Like seen in the other case study, this koala too had enlarged systemic lymph nodes, and pathology also revealed a fragile liver with several masses and hemorrhages in its substance and ascites. Both cervical and axillary lymph nodes had acinous masses of variable sizes. According to histopathology, both vasculature and surrounding tissues in the organs were seen with atypical myeloid cells, along with myelocytic and metamyelocytic cells. The koalas eventually got hypothermia, convulsions and died. The author suggested that the myelogenous leukemia was caused by KoRV (Ito et al., 2019).

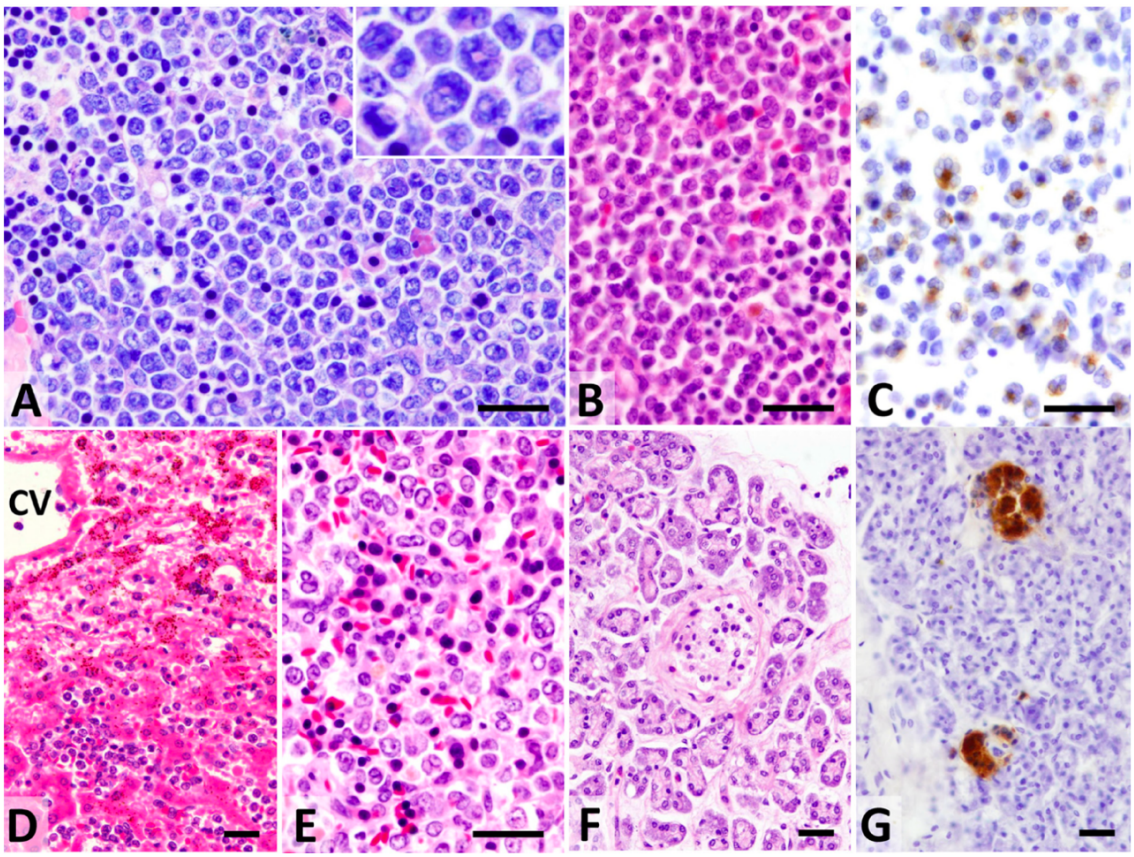

Figure 4. Histopathological findings of leukemic koala with diabetes mellitus. A) Axillary lymph node, Giemsa stain: atypical myeloid cells. B) Axillary lymph node, HE stain: atypical myeloid cells. C) Immunohistochemistry (IHC): myeloperoxidase in the cytoplasm, expressed by atypical myeloid cells. D) Liver, HE stain: lipofuscin in the cytoplasm. E) Extramedullary hematopoiesis. F) Pancreas, HE stain: atrophic Langerhans islands and interstitial fibrosis. G) IHC: insulin in the cytoplasm, expressed by the atrophic Langerhans cells. (Ito et al., 2019)

4.2. Immunosuppression

The main function of the immune system is to protect the body from pathogens. Like all marsupials, the koalas have a short gestation period, and the new-born joeys are born at an early embryonic stage. They are therefore born without a fully developed immune system, as the complete development and maturation of immune tissues occurs while they are in in the mother's pouch. At birth they lack the ability to form an adaptive immune response, and are therefore dependent on the innate immune system and their mother's milk which provide the passive immunity (Hobbs et al., 2014; Kinney & Pye, 2016; Madden et al., 2018).

KoRV, like many other retroviruses such as HIV, FeLV and MuLV, can cause immunosuppression in their host (Fiebig et al., 2006). The immunosuppression caused by retroviruses makes the host more vulnerable to opportunistic diseases like fungal infections, commonly cryptococcosis, and bacterial infections, commonly tuberculosis. KoRV is known to cause an opportunistic chlamydial infection and disease, which throughout the years have been thoroughly researched (Waugh et al., 2017). *Chlamydia* is an obligate intracellular bacterium, and koalas are infected with either *Chlamydia pecorum* or *Chlamydia pneumoniae*, which cause keratoconjunctivitis, urinary tract disease, reproductive disease, rhinitis and pneumonia (Kinney & Pye, 2016). *C. pecorum* is the primary pathogenic species in koalas, and infection can even lead to death (Waugh et al., 2017). *Chlamydia* is immunity dependent on both cell-mediated and humoral immunity (Kinney & Pye, 2016). Some *Chlamydia*-infected koalas can be asymptomatic for a long time, while others develop clinical disease. However, host or environmental factors are likely involved in the variation in clinical outcomes in koalas (Waugh et al., 2017).

When studying KoRV's effect on the immune system, cytokines are of big importance, because they are immune regulators generated by both the innate and adaptive immune system. This means that the cytokines are excellent markers for assessing the local and systemic immune responses produced by KoRV, or by other intracellular pathogens. For the Th-1 immune response the cytokines IFNγ and IFNα acts as markers, for Th-2 immune response IL-10, IL-4 and IL-6 and finally for the Th17 immune response IL-17A. In the koala there has been identified four cell markers: CD4, CD8β, CLEC1B and CLEC4E. CD4 and CD8β are of the biggest importance as they make it possible to differentiate between classic T-helper cells and cytotoxic T-cells (Madden et al., 2018).

It is unclear exactly how KoRV affects the immune system, and what we know is highly based on the fact that northern koalas have the same diseases as those seen in FeLV infected cats (Maher et al., 2019). Both FeLV-infected cats and HIV-1-infected humans are known to have a decreased number of $CD4^+$ cells, however it is not known if this happens in KoRV-infected koalas. Like mentioned earlier, we known that for retroviruses, the TM proteins, with its immunosuppressive domain, is involved in the mechanism for immunopathogenesis. The sequence for this immunosuppressive domain is identical in the three retroviruses, KoRV, FeLV and MuLV. By incubating human peripheral blood mononuclear cells (PBMCs) with sucrose-gradient purified KoRV, an increase in IL-10, IL-6, growth-related oncogene (GRO) and MCP-1 was observed. These markers are recognized

for lymphocyte proliferation inhibition, and similar alterations have been seen in both HIV and FeLV, which can cause immunodeficiencies *in vivo* (Fiebig et al., 2006). Studies have also revealed an upregulation of key cytokines generated by mitogen-stimulated lymphocytes in koalas infected with KoRV-B. One of the cytokines was IL-17A, which earlier was thought to be a marker for chlamydial disease in koalas (Madden et al., 2018). IL-17A was in a cytokine study of northern koalas found to be highly upregulated in KoRV-B infected koalas, which corresponds with IL-17A as a marker for the severity of chlamydial disease and the pathogenesis in northern koalas. However, in the southern koalas, there was a downregulation of IL-17A and IFNγ in KoRV-positive koalas, which possibly could be explained by the ongoing KoRV endogenization. KoRV-positive southern koalas has also been reported to have an increased amount of periodontitis, which is inflammation of the gums caused by bacteria in the dental plaque (Quigley & Timms, 2020).

4.3. Infection and disease in joeys

Unfortunately, there is little research done on KoRV infections and disease status in joeys, as most of the previous studies has focused on adult koalas (Hashem et al., 2020). Like mentioned earlier, new-born joeys are born at an early embryonic stage. Right after birth, they climb up from the dam´s cloaca to her pouch, where it attaches to one of her teats. Here it stays for a period of six to eight months, where most of the development happens. This makes possible treatment and vaccination procedures harder to complete, as well as any research and study on their health or the diseases they could be suffering from (Hobbs et al., 2014; Kinney & Pye, 2016).

Recently, a study was done on a deceased 6-month-old male joey, that had been ejected from its dam´s pouch. Both sire and dam were KoRV-A and KoRV-B positive, and the joey was found with KoRV-A, but negative for KoRV-B. Both parents seemed healthy on haematological examinations. In the post mortem examination of the joey, cause of death was unclear. Histopathology of tissues could also not be obtained. However, fluid was found in the thoracic and peritoneal cavity (Figure 5), which could be consistent with neoplasia, such as lymphoma and leukemia, as the cause of death (Hashem et al., 2020).

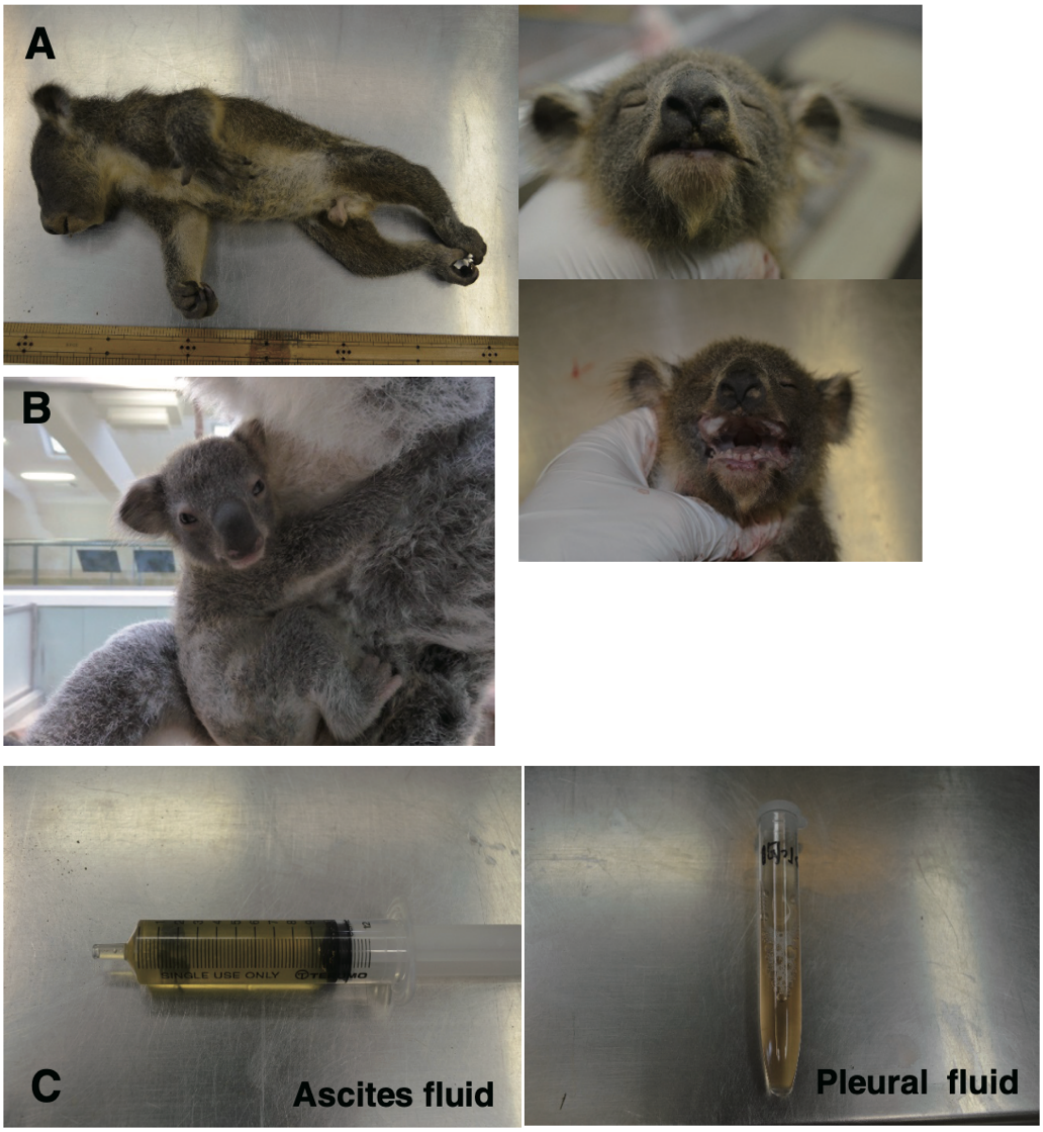

Figure 5. The 6 months old deceased joey. B) A healthy 7-month-old joey and his mother, to compare. C) Ascites fluid collected from the deceased joey´s peritoneal cavity, and pleural fluid collected from its thoracic cavity. (Hashem et al., 2020)

Another study shows a 1-month-old joey, ejected from its dam´s pouch. Both the dam and joey were positive for KoRV-B. Unfortunately, the cause of death could not be found with necropsy, due to the age of the joey (Xu et al., 2013).

5. Diagnosis

Endogenous retroviruses can insert into the germ line cells of the host, and all vertebrate species that has been previously studied, contain endogenous retroviruses. Most of these species contain the retrovirus in an inactive form, often trough mutations and deletions. Koalas, however, contain KoRV in their genome as full-length replication competent genome, and are actively transcribed (Tarlinton et al., 2005).

Hanger detected KoRV, as type C retrovirus-like particles, with transmission electron microscopy (TEM) in lymphoma tissues from a leukemic koala, from blood from captive koalas and in mitogen stimulated PBMCs. PCR amplified provirus from blood and tissues of the captive koalas, reverse transcriptase-PCR and Southern blot analysis also demonstrated the retrovirus (Hanger et al., 2000). A couple years later, quantitative real-time reverse transcriptase PCR was used to examine the association between KoRV and leukaemia and lymphoma. Viraemia was demonstrated using real-time PCR to detect KoRV genomic RNA in koala plasma and showed a significant increase in koalas suffering from leukemia and/or lymphoma. Detection of KoRV happened by using the KoRV *pol* gene, where real-time PCR primers and probes were designed for this specific region. Diagnosis of leukemia and lymphoma in the koalas was based on pathology and increased white blood cell count (Tarlinton et al., 2005). The KoRV *pol* gene was the only region used for KoRV detection, until the differentiation of KoRV subtypes was possible. Then, PCR primers was designed to amplify part of the VRA and TM domain of the KoRV *env* gene. This made it possible to differentiate between KoRV-A and KoRV-B (Waugh et al., 2017). The *env* gene of retroviruses are usually the most variable area of the virus because it encodes the protein that is the most exposed to the host´s immune response, as it is located externally to the virus membrane (Sarker et al., 2019). Using the KoRV *env* gene, with PCR and next generation sequence strategy, at least 9 KoRV subtypes have been identified, ranging between subtypes A to I (Sarker et al., 2019; Zheng et al., 2020). PCR targeting the *gag* gene has also been utilized for KoRV detection in recent studies (Stephenson et al., 2021). Primer sets has also been made for the entire KoRV genome, making it possible to use PCR for complete KoRV genomes but also to detect defective KoRV variants (Xu et al., 2015).

In the southern koala population, where KoRV is spread horizontally through close contact of koalas, some koalas have never been infected with KoRV. This makes it even more important to have an accurate diagnosis method. In a study of southern koalas in the Mount Lofty Ranges, KoRV positive and negative cases could be easily identified using

RNA sequencing and proviral analysis. The presence of central regions of KoRV genome (*gag* 2, *pol*, *env* 1 and *env* 2) was the basis for diagnosing KoRV positive koalas. By using all three gene targets together, the confidence of detecting KoRV is highly increased. When comparing KoRV positive koalas suffering from lymphoma with other KoRV positive koalas, the genes also showed an increased expression in lymph node tissue in the lymphoma-suffering koalas, and a decreased expression in the other KoRV positive koalas (Stephenson et al., 2021).

The antibody response is usually a very useful diagnostic technique for detecting retrovirus infections, as antibody responses often have been observed in individuals infected with exogenous retrovirus. Unfortunately, studies have suggested that koalas could be tolerant, as none of the koalas tested positive for specific antibodies against KoRV-A, when Western blot analyses were done (Fiebig et al., 2015).

6. Treatment and disease control

6.1. Antiretroviral medications

When it comes to the use of medications for treating KoRV infection, researchers and studies often compare KoRV to another retrovirus, the lentivirus HIV. Both viruses cause a lot of the same disease outcomes, like immunosuppression, and like some of the many KoRV subtypes, HIV is also an exogenous retrovirus that transmits disease horizontally (Denner & Young, 2013). One of the biggest accomplishments in modern medicine has been the development of antiretroviral (ARV) medications for the treatment of HIV infection. Many of these medicines have quite wide antiretroviral activity and could therefore be active against KoRV infection as well (Lifson, 2014). The replication cycle of retroviruses includes several steps that gives potential opportunities for treatment options. For anti-HIV medications, the target of the replication cycle steps include: binding of cell free virions to receptors and co-receptors on the surface of the target cell, inhibition of fusion of the membranes of the virion and the host cell, prevention of virion contents to enter the cytoplasm and reverse transcription of the viral RNA genome into DNA, inhibition of integration of reverse transcribed viral DNA into host cell chromosomes and, finally, blocking of the transcription, translation, virion assembly, -budding and -maturation (Lifson, 2014). Growing experience with the use of anti-HIV drugs in non-human primates (NHP) models have revealed key considerations and potential limitations that can aid the efforts to utilize the medications for koalas suffering from KoRV infection. Important considerations are potency against the target virus, drug delivery, pharmacokinetics, toxicity and treatment sustainability. The link between the drug's mechanism of action and targets regarding the disease process's pathophysiology might be the most essential consideration factor. (Lifson, 2014). Many of the challenges with treating koalas are based on their arboreal lifestyle and their unique diet, which almost exclusively consists of eucalyptus foliage (Hobbs et al., 2014; Kinney & Pye, 2016). When treating animals other than humans, the most common administration routes for successful drug delivery is either orally or subcutaneously. With the koalas' unique diet and absorption from a gastrointestinal tract that differs from primates, the choices for oral administration are very limited. There are difficulties with palatability, acceptance and compatibility of the drug and the food items, not to mentions that most ARVs needs to be given more than once per day, which is time consuming for the staff. Subcutaneous injections are therefore the preferred method of administrations, as it requires

less staff time, is faster and more convenient and ensures full bioavailability. However, daily injections can be challenging, as well as the volume injected, compatibility with other drugs and reactions at the local injection site (Lifson, 2014). Another important factor to consider regarding ARV drugs, is the goal of the treatment. With HIV treatment, unfortunately, the antiretroviral medications cannot completely eradicate the virus from the body, so the treatment consist of a lifelong process of suppressing the replication of HIV. The goal is to prevent progression of clinical disease and to prevent transmission of HIV, by blocking *de novo* infection of $CD4^+$ T-cells (Becerra et al., 2016; Kinney & Pye, 2016). Since KoRV is endogenously transmitted, the virus would be present at the moment of birth in the offspring, and the treatment with ARV drugs would start immediately to prevent clinical disease progression. However, this would not be a convenient treatment plan, as the new-born joeys spend their very first half year of their life in the mother´s pouch, attached to one of her teats. Due to this situation, the localization of the joey and its small size, both oral and subcutaneous administrations would be nearly impossible (Kinney & Pye, 2016).

6.2. Vaccines

Since antiretroviral treatment is limited and unpractical to use in koalas, vaccination seems to be the best option for prevention and management (Olagoke et al., 2020). There is also an increased need for a vaccine that can work therapeutically to prevent disease progression in already KoRV-infected koalas, as all koalas in the northern population seems to be endogenously infected with KoRV (Olagoke et al., 2020). In contrast to development of ARV medicines, HIV research is no longer useful for the development of a successful KoRV-vaccine in koalas, as all previous efforts into making a HIV-vaccine has failed. However, the vaccine against FeLV, the gammaretrovirus that causes disease in cats, has been of much more use for the development of an effective vaccine against KoRV. From the FeLV-vaccine a template was made, that has been very useful for the KoRV-vaccine development. Retrovirus vaccines development often use the envelope protein p15E and its epitopes, and this has become an important part of the KoRV vaccine development (Denner & Young, 2013; Kinney & Pye, 2016). Vaccination in koalas with recombinant envelope protein-based anti-KoRV vaccine have showed to be successful and with no vaccine-associated side effects. In both KoRV-infected and KoRV-free southern koalas, the vaccine induced a strong humoral immune response and showed a significant increase in anti-KoRV IgG leves and neutralizing antibodies. In exogenously KoRV-infected koalas it had a highly

important therapeutic effect by reducing the viral load (Olagoke et al., 2018). As mentioned earlier, koalas have seemed to be in a state of tolerance when testing for KoRV-A antibodies, indicating that therapeutic immunization of koalas infected with KoRV-A will fail. This has caused concerns for koala vaccination (Fiebig et al., 2015). However, vaccination of endogenously infected koalas with the recombinant KoRV *env* protein combined with a Tri adjuvant, showed a complete clearance of KoRV-A in the plasma. This means that the already KoRV-A infected koalas can benefit from this vaccine as a prophylactic measure, by boosting the natural anti-KoRV antibodies and increasing the amounts of different epitopes to be recognized. At the same time, the vaccine can induce antibodies that are cross-reactive against several subtypes of KoRV (Olagoke et al., 2020).

6.3. Disease control and future prevention

KoRV is in the process of becoming endogenized in the genome of all koalas, and if this happens, the process is irreversible and KoRV will be transmitted to all subsequent generations. To avoid this, the uninfected koalas must be separated from contact with the infected koalas, either by isolation or quarantine. However, this is only possible with the southern koala populations, as the northern koalas already have been fully endogenized by KoRV (Denner & Young, 2013).

Another way to prevent severe disease in koalas, is to control and manage *Chlamydia* infection. This is the most serious pathogen koalas suffer from, and one of the biggest reasons for the decline in koala populations. Together with KoRV, which supress the immune system and exacerbates chlamydial pathogenesis, the two pathogens cause a serious threat to the koalas. The most practical solution to this seems to be the development of a chlamydial vaccine specific to koalas (Madden et al., 2018).

8. Summary

Koalas are listed as a threatened species for several reasons, but it is apparent that KoRV plays a major role in this threat, both for free Australian koalas and captive koalas worldwide. KoRV can be divided into several subtypes that uses different receptors, are transmitted differently, and cause different disease outcomes. KoRV-A, one of the main subtypes of KoRV, uses the PiT1 receptor and is the only endogenous form of KoRV, transmitting infection vertically. The other nine subtypes, KoRV-B-J, uses the THTR1 receptor or other unknown receptors, and are exogenous forms which transmits infection horizontally. KoRV-B, the other major subtype of KoRV, is known for causing lymphoma, leukemia and lymphoid neoplasia in koalas. KoRV also causes immunosuppression in koalas, which predisposes them to other severe diseases such as those caused by *Chlamydia*.

Wild living koalas are only found in Australia, but there are major differences between the northern and southern koala populations in Australia. There are huge, interesting variations in the KoRV subtypes and transmission ways that dominates and the disease prevalence. The northern koala populations are clearly the most exposed, as the koala population is declining in a high rate. They are associated with a high disease prevalence, with both KoRV and *Chlamydia* having a high prevalence. KoRV-A is 100% prevalent and KoRV is here an active endogenous infection, resulting in KoRV being fixed into the genome of every single northern koala. The southern koala populations have a much lower prevalence of KoRV and *Chlamydia*. KoRV is mainly transmitted exogenously here, and they seem to be under the process of becoming endogenized by KoRV, resulting in some southern koalas never being infected by KoRV.

Both KoRV diagnosis, treatment and prevention has improved drastically the last few years. Diagnosis of KoRV is mainly done by detection with the help of PCR, using the *pol*, *gag* and *env* genes. This has also made it possible to detect different subtypes of KoRV and defective KoRV variants. The use of ARV drugs for the treatment of KoRV infection is possible, however they can only be administered orally or subcutaneously to koalas. Due to their arboreal lifestyle and unique diet, as well as being borne underdeveloped and attached inside their mother's pouch for so long, ARV drugs seem very complicated and nearly impossible for koalas. Therefore, vaccination is most likely the best option, not only for prophylactic purpose, but also for therapeutic purpose to prevent disease progression. Every year Australian koalas are taken into care then released back into the wild, and during this period they could be vaccinated. Recombinant envelope protein-based anti-KoRV vaccine

has been a great success in koalas, resulting in an a strong immune response and increased production of anti-KoRV IgG levels and neutralizing antibodies. In already KoRV-A infected koalas, the vaccine showed a complete clearance of KoRV-A in the plasma. The trial also revealed no vaccine-associated side effects in the vaccinated koalas.

KoRV is a complex and genetically diverse virus. The endogenization of KoRV in koalas seems to be unavoidable, and the long-term effects of this is currently unknown. Koalas may after time evolve and adapt to the endogenization. However, the presence of exogenous forms, with co-infection possible, may complicate the severity of the disease outcomes. Much research has been done about KoRV and its diseases, although considering the severity of the diseases and the threat koalas are experiencing as a species, further investigation should be made. There is still a lot to learn about the pathogenesis and the pathology behind the diseases, which in the future can aid the development of detection methods, medicines and vaccines.

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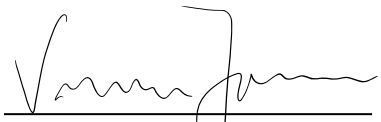

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