UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

RECOMBINANT FELINE INTERFERON OMEGA THERAPY IN CATS NATURALLY INFECTED WITH FELINE IMMUNODEFICIENCY VIRUS: CLINICAL, VIRAL AND IMMUNOLOGICAL RELEVANCE

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TESE DE DOUTORAMENTO EM CIÊNCIAS VETERINÁRIAS

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Porque expressar sentimentos numa outra língua, nunca nos permite transmitir convenientemente o que sentimos, decidi escrever os agradecimentos e a dedicatória em "bom português".

Já lá vão cinco anos desde o verão de 2009…! Estava a acabar o internato em Paris e numa das muitas incursões a Portugal, bati à porta da Professora Rodeia! O futuro era incerto e a minha vontade de ir mais longe também…! Depois de uma conversa em género de desabafo, expressei-lhe o meu interesse pela carreira académica, sobretudo motivada pelo ensino. Sou filho de dois professores e partilhar conhecimento é algo que sempre achei que gostava de fazer na vida. Ali começou esta aventura…

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Resumo

A terapêutica com interferão ómega felino em gatos naturalmente infectados com o vírus da imunodeficiência felina: relevância clinica, virológica e imunitária

Os interferões do tipo I são citoquinas chave do sistema imunitário. Devido às suas propriedades imunomoduladoras, são um recurso terapêutico frequente em diferentes doenças como as infecções retrovirais. O interferão ómega felino (rFeIFN-ω) é o primeiro interferão licenciado para medicina veterinária. Apesar do seu uso no tratamento de infeções retrovirais como o vírus da imunodeficiência felina (FIV) e o vírus da leucemia felina (FeLV), são poucos os estudos que fundamentam o seu benefício clinico. Esta tese visa clarificar as propriedades terapêuticas e imunomoduladoras do protocolo licenciado de rFeIFN-ω (3 ciclos de 5 administrações subcutâneas de 1MU/kg uma vez ao dia a iniciar aos dias 0, 14 e 60) em gatos naturalmente infectados por retrovírus e residentes em gatil. Em detalhe, este trabalho avalia o efeito deste fármaco na melhoria clinica, na excreção de vírus concomitantes, na virémia/provirus e na variação de diferentes marcadores imunitários como proteínas de fase aguda e perfil de citoquinas. Esta tese contempla ainda o desenvolvimento de um protocolo terapêutico alternativo baseado na administração oral de rFeIFN-ω (0.1MU/gato durante 90 dias consecutivos) para uso em gatos FIV-positivos domésticos, os quais apresentam geralmente um quadro clinico subtil e pouco específico.

Os resultados revelaram que o protocolo licenciado induz uma melhoria clinica significativa com redução concomitante das infecções oportunistas e um aumento do perfil de proteínas de fase aguda (APP). O protocolo alternativo revelou-se eficaz na melhoria clinica dos animais tratados, apesar de não induzir alterações significativas do perfil de APPs nem das infecções concomitantes (residuais no grupo de estudo). Ambos os protocolos não induziram alterações na virémia nem no perfil de citoquinas participantes nas respostas T-helper 1 ou T-helper 2 o que sugere que este composto não apresenta propriedades anti-virais nem actua na imunidade adquirida de gatos FIV positivos. Verificou-se contudo um decréscimo dos niveis plasmáticos de Interleucina-6 (citoquina pro-inflamatória) em gatos tratados com o protocolo subcutâneo e uma redução da sua expressão (mRNA) em gatos tratados por vira oral. Tal demonstra que o rFeIFN-ω apresenta propriedades anti-inflamatórias, as quais são mais evidentes aquando do tratamento com o protocolo licenciado. Mais que uma contribuição para um melhor conhecimento do rFeIFN-ω, esta tese explora as suas propriedades imunomoduladoras e valida um novo protocolo oral, o qual poderá ser incluído em futuras guidelines para o tratamento de gatos FIV-positivos.

Palavras chave: felino, interferão ómega felino, imuno-modulação, vírus da imunodeficiência felina, retrovírus

Abstract

Thesis Title: Recombinant feline interferon omega therapy in cats naturally infected with Feline Immunodeficiency Virus: clinical, viral and immunological relevance

Type-I Interferons are well-known cytokines which among their main functions are key components of the host immune response against viral infections. Due to its immune modulation properties, they are commonly used in the therapeutic approach of various diseases such as retroviral infections. Recombinant feline interferon omega (rFeIFN-ω) is the first interferon licensed for use in veterinary medicine. Although it is commonly administered in retroviral infections, namely in Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (FeLV) infected cats, few studies reported its clinical benefits and mechanisms of action. This thesis aims to clarify the main properties of the licensed rFeIFN-ω protocol (3 cycles of 5 daily subcutaneous administrations of 1MU/kg beginning on days 0, 14 and 60) in naturally retroviral infected cats living in an animal shelter, evaluating its effect not only on clinical improvement but also on concurrent viral excretion, viremia/proviral load and various immune biomarkers such as acute phase proteins and cytokine profile. Recognizing the non specific and subtle clinical presentation of the majority of FIV-infected cats, this work also presents and evaluates an alternative oral rFeIFN-ω protocol (0.1MU/cat during 90 days) to be used in client-owned FIV-infected cats.

Results showed that the licensed rFeIFN-ω protocol induces a significant clinical improvement, with a concurrent reduction of opportunistic viral infections and an increase on acute phase proteins (APP) profile. The alternative protocol also revealed an important clinical improvement but without significant changes on opportunistic viral infections (which were of low level in the tested group) or on APP profile. In both protocols, no changes were remarked on viremia neither on T-helper 1/T-helper 2 cytokine profiles meaning that this compound may lack an anti-viral activity for retroviruses in vivo and do not act on the acquired immune response of FIV-positive cats. However, there was a significant reduction of the interleukin-6 plasma levels (pro-inflammatory cytokine) in cats treated with the licensed protocol and a decrease on its mRNA expression in cats treated orally. This shows that rFeIFN-ω can have anti-inflammatory properties, which are more evident in the higher doses of the licensed protocol.

More than contributing for a better knowledge of rFeIFN-ω, this thesis explores its immune modulation properties and validates a new oral protocol which can be included on future FIV-guidelines.

Keywords: feline, interferon therapy, recombinant-feline interferon omega, immune modulation, feline immunodeficiency virus

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List of Abreviations

2-5-OAS - 2´-5´ Oligoadenylate synthase

ABCD – (European) Advisory Board on Cat Diseases

AGP – Alpha-1-glycoprotein

AIDS - Acquired immunodeficiency syndrome

APPs – Acute Phase Proteins

APR – Acute Phase Response

AZT - zidovudine

BID – twice daily

CBC – complete blood count

CNS – central nervous system

CRP – C-reactive protein

CSF – cerebrospinal fluid

CTLs – Cytotoxic T cells

CXCR4 – Chemokine receptor type 4

DM – Diabetes mellitus

DNA- Desoxiribonucleic Acid

ELISA- Enzyme- Linked Immunosorbent Assay

ENV- Envelope

EPO - erythropoietin

FA – Fluorescent Antibodies

FCGS - Feline Chronic Gingivostomatits Syndrome

FCV – Feline Calicivirus

FelV- Feline Leukemia Virus

FeLV-IMHA – FeLV-induced immune-mediated hemolytic anemia

FeSV – Feline Sarcoma Virus

feTHTR1 – feline thiamine transport protein

FISS – Feline Injection Site Sarcoma

FIV - Feline Immunodeficiency Vírus

FIV – Feline Infectious Peritonitis

FPLS – Feline panleukopenia-like syndrome

FPV – Feline parvovirus

Gag – Group specific antigen

G-CSF – Granulocyte Colony-stimulating factor

HIV- Human Immunodeficiency Virus

HuIFN-α- Human Interferon alfa

IFNAR – Interferon Receptor Complex

IFNs – Interferons

IGF-1 – Insulin Growth Factor 1

IL – Interleukin

IN – Integrase

LTR - long terminal repeats

MDS – myelodysplastic syndrome

mRNA- Messenger Ribonucleic Acid

NSAID – Non steroidal anti-inflammatory drug

NK-cells – natural-killer cells

ORF - open reading frames

PAMPs – Pathogen-associated molecular patterns

PBMCs - peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PO group – cats treated with oral rFeIFN-ω

Pol- Polymerase

rFeIFN-ω- Recombinant feline interferon omega

RI – reference interval

RNA- Ribonucleic Acid

RT- Reverse Transcriptase

RT-qPCR – Real-time quantitative PCR

SAA – Serum Amyloid A

SC group – cats treated with the subcutaneous rFeIFN-ω licensed protocol

SE – standard error

SID – once a day

SPE – Serum Protein Electrophoresis

SPF - specific pathogen free

SU- Surface Protein

Th1 – T-helper 1 cells

Th2 – T-helper 2 cells

TLR – toll like receptors

TNF – Tumor necrosis factor

Tregs – T-regulatory cells

VIF- Viral Infectivity Factor

VNAs – Virus Neutralizing Antibodies

Introduction

Theme presentation, justification, objectives

Interferons (IFNs) are key components of the host immune system, being particularly relevant in viral infections (Sadler & Williams, 2008). The large family of IFNs can be divided into different types such as type I-IFNs, commonly used for therapeutic purposes. Among their major functions, type I-IFNs increase and sensitize the immunitary system towards the microbial recognition (Siren, Pirhonen, Julkunen, & Matikainen, 2005), establishing an important link between innate and acquired immunity (Colonna, Trinchieri, & Liu, 2004). Furthermore, they are believed to have some anti-viral properties, blocking viral replication and inducing apoptosis of infected cells (Goodbourn, Didcock, & Randall, 2000; Bracklein, Theise, Metzler, Spiess, & Richter, 2006).

Not only in humans but also in feline medicine, the use of type I-IFNs as immune modulation therapy is common, notably in retroviral infections (Tompkins, 1999; de Mari, Maynard, Sanquer, Lebreux, & Eun, 2004; Domenech, et al., 2011).

Still used in several countries, Human Interferon Alpha (HuIFN-α) was the first interferon used in cats, despite the fact that it is only licensed for humans. In spite of its short term effects, particularly on clinical improvement and increase of the survival time, the development of neutralizing antibodies several weeks after therapy makes HuIFN-α ineffective for long-term immune modulation therapy in cats (Tompkins, 1999; Pedretti, et al., 2006; Hartmann, 2012a). This problem was bypassed by the more recent release of recombinant feline interferon omega (rFeIFN-ω).

RFeIFN-ω is the first interferon compound licensed for use in veterinary medicine. According to the manufacturer's instructions and license, it should be used in three cycles of five daily subcutaneous injections of 1MU/kg, beginning respectively on days 0, 14 and 60. Despite the fact that it was licensed a few years ago, there are not so many studies that support its clinical benefits, particularly in retroviral infections. The first paper described its clinical application dates from 2004 and reported that treated Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV)/FeLV co-infected cats showed a significant improvement and an increased survival time (de Mari, et al., 2004). More recently, another research group showed that rFeIFN-ω did not induce significant changes on parameters such as hypergammaglobulinemia, proviral load and viremia, suggesting an overall effect mainly on the innate immune reaction rather than on the acquired immunity (Domenech, et al., 2011). Further studies are therefore required in order to clarify the mechanisms of action of rFeIFN-ω.

In this sense, the main objective of this work is to explore the main properties of rFeIFN-ω in naturally retroviral infected cats, with special relevance to FIV-infected animals. More than the extension of the current knowledge about the licensed rFeIFN-ω protocol, this work also aims to develop and present a new oral therapeutic protocol, which if successful, can be considered as an alternative immune modulation therapy for FIV-infected cats.

Excluding the literature review (part I), this thesis structurally comprises two parts (part II and III). Part II is based on clinical trials and reports the experimental work which was developed using two specific rFeIFN-ω protocols. The main objectives of the referred experimental work are:

a) To investigate the effect of the licensed rFeIFN-ω protocol on clinical improvement, hematology, biochemistry profile and concurrent viral excretion in naturally retroviral-infected cats living in an animal shelter.

b) To monitor the effect of the licensed rFeIFN-ω protocol on acute phase protein (APP) profile, assessing the role of APPs as potential biomarkers of the innate immune activation in treated animals.

c) To develop and validate a new oral rFeIFN-ω protocol to be used in FIV-infected cats. Recognizing that many FIV-infected cats have a nonspecific clinical presentation and usually does not require a strong immune modulation therapy, this protocol is based on a 10 fold lower dose than the current licensed protocol, to be administered for 3 months (90 continuous days) in these animals. The development of this protocol involves the monitoring of its action on the clinical improvement, hematology, biochemistry profile, concurrent viral excretion and APP profile in treated cats.

d) To assess the effect of the experimental oral rFeIFN-ω protocol on other innate immune parameters such as Mx-protein, a specific biomarker of type-I IFN action.

e) To evaluate proviral load, viremia and cytokine profile [messenger ribonucleic acid (mRNA) expression and concurrent plasma variations] in FIV-infected cats treated with rFeIFN-ω protocols, comparing the main similarities and differences between them. This comparison will allow determining the main mechanisms of action of each rFeIFN-ω protocol, contributing for a better use in clinical practice.

The studies that support this experimental work were converted in five chapters presented on part II. Four of them were submitted/published in international refereed and indexed journals, namely:

Gil, S., Leal, R.O., Duarte, A., McGahie, D., Sepúlveda, N., Siborro, I., Cravo, J., Cartaxeiro, C., Tavares, L., 2013. Relevance of Feline Interferon Omega for Clinical Improvement and

Reduction of Concurrent VIral Excretion in Retrovirus Infected Cats from a Rescue Shelter. Research in Veterinary Science. 2013 Jun;94(3):753-63. doi: 10.1016/j.rvsc.2012.09.025. Epub 2012 Oct 31

*These authors contributed equally to the work

Leal RO[*], Gil S[*], Sepúlveda N, McGahie D, Duarte A, Niza MMRE, Tavares L. 2014 "Monitoring acute phase proteins in retrovirus infected cats undergoing feline interferon omega therapy"*Journal of Small Animal Practice* 2014 Jan;55(1):39-45. doi: 10.1111/jsap.12160. Epub 2013 Nov 27.

[*]These authors contributed equally to the work.

Gil. S*, Leal RO*, McGahie D, Sepúlveda N., Duarte A, Niza MMRE, Tavares L 2014 "Oral Recombinant Feline Interferon-Omega as an alternative immune modulation therapy in FIV positive cats: Clinical and laboratory evaluation" Research in Veterinary Science 2014 Feb;96(1):79-85. doi: 10.1016/j.rvsc.2013.11.007. Epub 2013 Nov 25

*These authors contributed equally to the work.

Leal RO, Gil S, Duarte A, McGahie D, Sepulveda N, Niza MMRE, Tavares L 2014 "Evaluation of Viremia, proviral load and Cytokine profile in naturally FIV-infected cats treated with two different protocols of recombinant feline interferon Omega" (Submitted)

Although it is out of the scope of this thesis, part III extrapolates the use of oral protocol as an alternative to steroid therapy in type II feline diabetes mellitus. It is a report of two clinical cases which illustrates the therapeutic potential of this compound in diseases other than retroviral infections. Despite the fact it is only based on two clinical cases, this study was also published as a case report in an international peer-reviewed journal:

Leal RO, Gil S, Brito MTV, McGahie D, Niza MMRE, Tavares L 2013 "The use of oral Recombinant Feline Interferon Omega in two cats with type II diabetes mellitus and concurrent Feline Chronic Gingivostomatitis Complex". *Irish Veterinary Journal* 2013 Oct 23;66(1):19. Epub 2013 Oct 23

Part I

Literature Review

Part I - Chapter I:
Molecular background of retroviral infections

1.1 Retroviruses and taxonomy

Retroviruses are well described in various species, being part of the family *retroviridae*. The prefix "*Retro"* refers to reverse and is due to the reverse transcriptase, a particular enzyme which characterizes the virions of this family (MacLachland & Dubovi, 2011). The most widely known virus of this family is the human immunodeficiency virus (HIV), which nowadays has a strong healthy impact, being one of the main subjects of scientific research (Murphy, Gibbs, Horzineck, & Studdert, 1999b). In veterinary medicine, animal retroviruses have also been studied mainly because they are excellent comparative models for human acquired immunodeficiency syndrome (AIDS) research, contributing for the advance of the medical science (Murphy, et al., 1999b; Elder, Lin, Fink, & Grant, 2010; Yamamoto, Sanou, Abbott, & Coleman, 2010).

According to the International Committee of Taxonomy of Viruses, retroviruses are classified into two subfamilies: Orthoretrovirinae and Spumaretrovirinae. This last one includes only one genus, the spumavirus which refers to foamy viruses. In the subfamily Orthoretrovirinae, 6 genera are distinguished: *Alpharetrovirinae*, *Betaretrovirinae* and *Gammaretrovirinae*, which have simple structure and are commonly considered simple retroviruses, in opposition to *Deltaretrovirinae*, *Epsilonretrovirinae* and *Lentivirinae* which are complex retroviruses (MacLachland & Dubovi, 2011).

Despite the phylogenetic classification, Lentivirus and Gammaretrovirus are the most important retroviruses in Veterinary Medicine. Lentiviruses include not only the human immunodeficiency viruses (HIV-1 and HIV-2) but also other ones such as FIV. Then, lentiviruses have a strong impact on the immune system of humans and cats, being widely studied in the last decades. Gammaretrovirus, with more simple structure, includes FeLV, which similarly to FIV, have an important clinical impact in Companion Animal Practice namely in feline medicine (Jarrett, 1999; Dunham & Graham, 2008).

Particularly in cats, retroviral infections seem to be ancestral since the feline genome has always had different genetic elements derived from elderly retroviral infections, also called "endogenous retroviruses" which are vertically transmitted by germ line (Roy-Burman, 1995; Dunham & Graham, 2008).

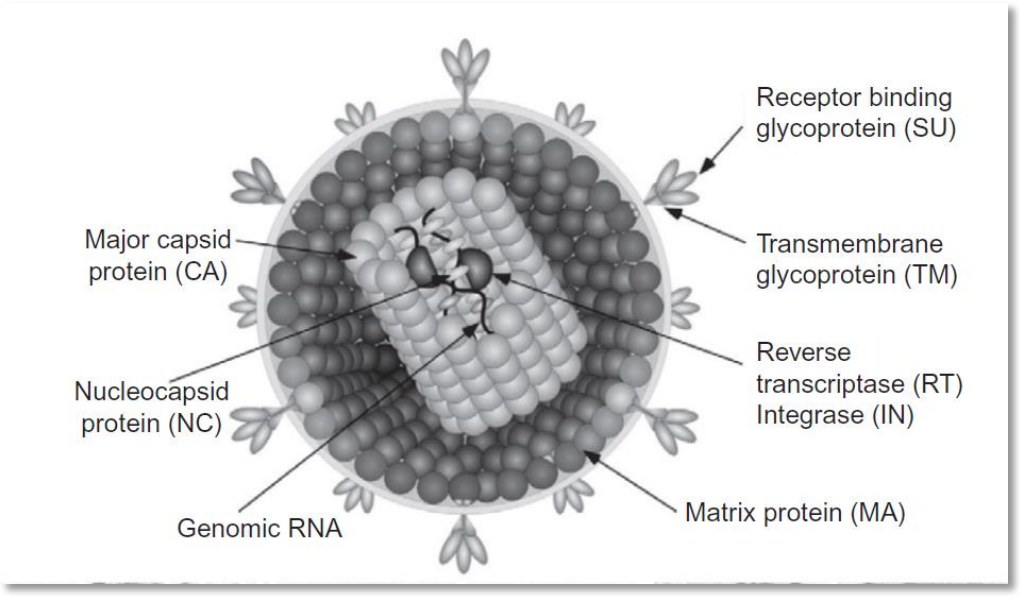
To a better understanding of this work, it is essential to identify the main genetic and molecular basis of retroviruses, giving special relevance to FIV and FeLV, the most important retroviruses in feline practice.

1.2. Genome and molecular basis of retroviruses

1.2.1. The retrovirus virions and their genetic properties

Virions of the family retroviridae are enveloped, having a three-layered structure of 80-100nm of diameter. The inner-layer is the genome of the virion, which is diploid [consisting of a homodimer of two single-stranded ribonucleic acid (RNA)] and includes 30 molecules of reverse transcriptase (RT) in a helical symmetry. It is surrounded by an icosahedral capsid (60nm in diameter) which is involved by an envelope that derived from a host cell membrane (Murphy, et al., 1999b; Goff, 2007; MacLachland & Dubovi, 2011). Schematic diagram of retrovirus virion basic structure is presented on figure 1.

Figure 1: Schematic diagram of a retrovirus virion and its important structures and proteins (MacLachland & Dubovi, 2011).



Retroviruses have different particular findings namely being the only diploid genome (two molecules of single-stranded RNA) and the only viral RNA that requires the host cell enzymes to be synthesized and processed (Murphy, et al., 1999b; Goff, 2007).

The genome of retroviruses contains 3 major genes which encode several proteins:

Gag (group specific antigen): which encodes the virion core (capsid) proteins (Murphy, et al., 1999b).

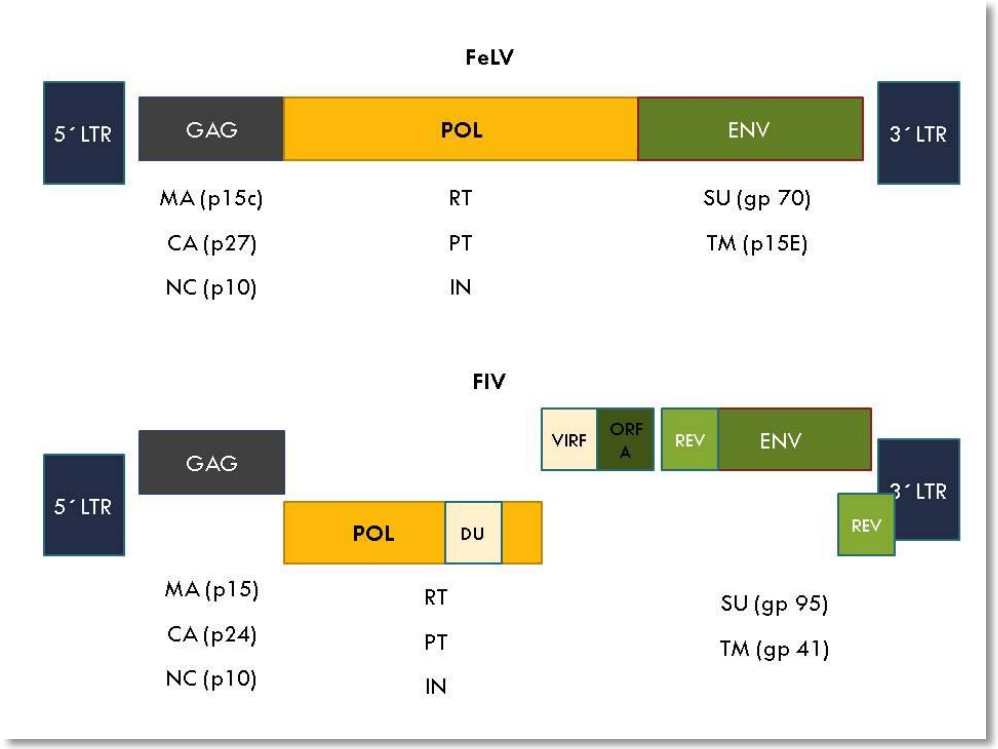
Pol (polymerase): which encodes the protease, the RT enzyme and integrase (IN). The most important of these enzymes is RT, which contains different domains namely a DNA

polymerase (that can have a RNA or DNA template) and a RNase, both particularly relevant for the virus life cycle (Goff, 2007).

Env (*envelope*): that encodes surface/peripheral proteins, determining cell tropism and contributing for pathogenicity (Pancino, Castelot, & Sonigo, 1995; Roy-Burman, 1995; Verschoor, et al., 1995; Johnston, Silva, & Power, 2002).

Despite the described common basal structure, FIV and FeLV are genomically different (figure 2). FeLV virion is a simple genome (with only these three basal genes) while FIV is a complex one as its genome also encodes some accessory genes which strictly regulate the viral cycle and contribute to a productive infection of different cell types (Murphy, et al., 1999b; Dunham & Graham, 2008; Duarte, Gil, Leal, & Tavares, 2012).

Figure 2: Schematic diagram of genomic structure and major proteins of provirus FeLV and FIV. Proviruses are flanked by long terminal repeat regions (LTR) which regulate gene expression. GAG gene encodes for Matrix proteins (MA), capside (CA) and nucleocapsid (NC); POL encodes for Reverse Transcriptase (RT), Protease (PT) and Integrase (IN), ENV encodes for specific Surface protein (SU) and Transmembrane Protein (TM). Being a complex retrovirus, FIV still have accessory genes namely REV, VIF, ORF-A (open reading frame A) and DUTPase (DP). Adapted from (Dunham & Graham, 2008).



In FeLV, the gag gene codes for different proteins namely p10 and p27. This later one is routinely used for rapid diagnostic kits [enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests] due to the fact that it exists in high amounts in the blood stream being also excreted in tears and saliva. Pol codifies the viral RT and env codes for protein gp70 which define the virus subgroup and is crucial to induce immune response. Antibodies against gp70 can neutralize virus, being a relevant protein as a target for vaccine production. P15e interferes with host cell-immune responses and make viral persistence easier (Murphy, et al., 1999b; Dunham & Graham, 2008; Hartmann, 2012b).

In FIV, Env gene codes for two important envelope proteins namely gpSU (gp95) and gpTM (gp41), which are both mediators of virus interaction to the host (Elder, et al., 2010). In detail, gpSU binds to CD134 and gpTM binds to CxCR4 (further detailed) (Shimojima, et al., 2004). Similarly to other lentiviruses such as HIV, different accessory genes are described. Specifically in FIV, the further ones are known (Troyer, Thompson, Elder, & VandeWoude, 2013):

R*ev (Regulator of Expression of Virion Proteins):* that encodes a protein which is associated to the splicing of viral RNA transcripts and their export to the cytoplasm, increasing the efficiency of mRNA translation; its cytoplasmic concentration determines the production of virions (Goff, 2007; MacLachland & Dubovi, 2011).

VIF (Viral infectivity factor): that encodes proteins which determines infectivity and is required on the earlier phases pos-infection (Goff, 2007; MacLachland & Dubovi, 2011).

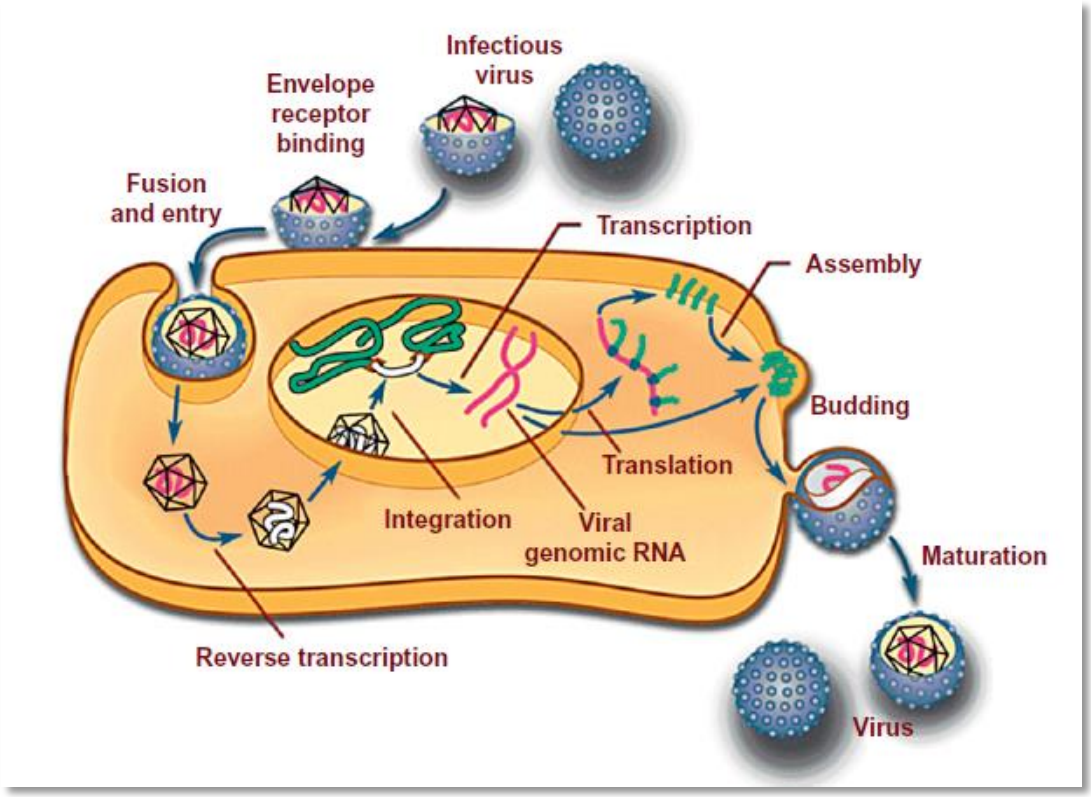
dUTP (*dUTPase protein gene*): present in nonprimate lentiviruses, this enzyme is encoded in the pol gene. dUTP major function is to reduce levels of dUTP that are incorporated into viral DNA and consequently reduce eventual substitution mutations (Goff, 2007).

OrfA (*Open reading frame A*): that modulates viral transcription and encodes accessory proteins with similar functions to HIV vpu (that maturates the viral glycoprotein and is associated with virions release, only present in HIV-1), vpr (a transcriptional enhancer), Tat (increase the efficiency of transcription around 1000-fold and preventing premature end of transcription), and nef [crucial for viral replication in macrophages, encoding a protein which down-regulates the expression of CD4 lymphocytes and Interleukin-2 (IL-2)] (Goff, 2007; MacLachland & Dubovi, 2011; Troyer, et al., 2013).

1.2.2. Life cycle and basic principles

The life cycle of retroviruses is quite simple and is shown on figure 3.

Figure 3: Schematic presentation of replication cycle in retroviruses (MacLachland & Dubovi, 2011).



After binding of envelope surface proteins to specific cellular receptors, the virion enters the cell (by receptor mediated endocytosis), releasing its RNA genome. In the cytoplasm, still inside the capside and due to the action of the viral enzyme “reverse transcriptase”, RNA is copied into cDNA, which is duplicated to produce a double-stranded DNA (Murphy et al., 1999b). During this phase, 300 to 1300 bps are added in each end of the RNA molecule, constituting long terminal repeats (LTRs) which due to their formed secondary structure are important in the replication of retroviruses (Goff, 2007).

Thereafter, DNA enters the nucleus and by non covalent binding of LTRs and it is embedded in the host genome. The integrated DNA is called provirus (Murphy et al., 1999b). In FIV, this process is potentiated by the integrase, an enzyme which determine the site of binding and integration of FIV provirus into the host DNA, influencing the host function (Shibagaki & Chow, 1997; Shibagaki, Holmes, Appa, & Chow, 1997). In FeLV, the integration occurs randomly, also with the help of the integrase (Hartmann, 2012b).

The DNA remains spliced into the host cell for life as provirus (Dunham & Graham, 2008). This nucleic acid is then used for transcription (Murphy, et al., 1999b). Transcription of the

viral genome is done by cellular RNA polymerase which, initiating in the 5´-LTR and finishing in the 3´-LTR produces a new virion RNA. LTRs are important in the initiation of transcription, having promoter regions (such as U3, which encode for positive regulatory elements that enhances viral transcription) (Goff, 2007). Particularly in FeLV, some of these regions are directly involved in viral oncogenesis (Y. Matsumoto, et al., 1992; Nishigaki, et al., 1997). For instance, in FeLV cats U3-LTR upregulates cellular genes (envolved on NFKB pathways) and encharged of the integration of virus, making a specific RNA transcript (Hartmann, 2012b).

All retroviral genomes contain open reading frames (ORF) which are expressed to form precursor proteins that, after translation and viral assembly, form infectious virions (Goff, 2007). The transcription of the retroviral genome is directed into different pathways: a portion of the transcript (corresponding to the truly viral genome) is exported directly to the cytoplasm where it can be packed into new virions; another portion (with identical structure) is exported and submitted to translation, forming Gag and pol; a third part is spliced to form a subgenomic mRNA encoding for *env* proteins and in some complex retroviruses such as FIV, other multiple auxiliary proteins. The gag-pol and env genes are, subsequently, translated separately and, thereafter, their large precursor proteins are cleaved post-translation.

Env protein, is then translated from a distinct mRNA. After transcription, it is processed firstly in the rough endoplasmic reticulum and after it moves to Golgi complex where it suffers glycosylation. Afterwards, it reaches the plasma membrane by unknown mechanisms (Dunham & Graham, 2008; Goff, 2007; MacLachland & Dubovi, 2011; Murphy, et al., 1999b).

Gag-pol poliprotein is transported to the golgi complex where it is processed into several fragments. Once the pol gene encodes for some proteins that are needed at lower levels for viral replication (such as RT and IN), it is not translated in separate but it is expressed as a part of a Gag-pol precursor poliprotein which are thereafter cleaved. Gag poliproteins starts to assembly nucleocapsids on the inner part of the cellular membrane while, by the action of viral proteases, it is cleaved and processed. This process is followed by a binding of nucleocapsids to env proteins which are already fixed in the cellular membrane. Some domains of *gag* protein also interacts with RNA genome, being responsible for packaging viral RNA (Goff, 2007; MacLachland & Dubovi, 2011).

Finally, budding is complete and virion is released from the host cell. These processes are not strict in time meaning that virion continues its maturation during and after the release from the host's cell (Goff, 2007).

1.2.3. The genetic variation in retroviruses

Due to several mutations and recombination processes, retroviruses have an important genetic variation (Dunham & Graham, 2008). Mutations are mainly due to the lack of a 3´-5´exonuclease proof reading activity by RT enzyme. Gag and Pol genes are usually conserved while certain regions of env, particularly those regions which encode proteins that are antibody targets, are highly variable (MacLachland & Dubovi, 2011). This is more evident in FIV rather than FeLV (Dunham & Graham, 2008). Recombination is also frequent (ranging from 1-20% of genome per replication cycle), mainly if host is infected with more than one virus. These mechanisms tend to occur during reverse transcription when RT jump templates and produce duplications, deletions and inversions (MacLachland & Dubovi, 2011). Consequently, mutations, recombination processes and co-infections lead to the emergency of new subtypes, interfering with the phenotype of the virus and its virulence (Carpenter, Brown, MacDonald, & O'Brien S, 1998; Kann, Seddon, Kyaw-Tanner, & Meers, 2007; Shalev, et al., 2009).

FIV

Based on the hypervariable region of the env sequence, they are at least five FIV subtypes (Pancino, et al., 1993): A, B, C, D and E (Duarte & Tavares, 2006; Sellon & Hartmann, 2012a). Due to the constant new arising of different sequences even within the same subtype, this division is not clear and similarly to HIV, sequences are estimated to diverge up to 30% between subtypes and 2.5 to 15% within the same subtype (Sodora, et al., 1994). Furthermore, co-infection with different subtypes and intersubtype recombination is also possible, contributing for FIV variability (Kann, et al., 2007). Therefore, new sequences have been documented worldwide particularly in Texas, Argentina, Portugal and New Zeland (Pecoraro, et al., 1996; Nishimura, et al., 1998; Weaver, Collisson, Slater, & Zhu, 2004; Duarte & Tavares, 2006; Hayward, Taylor, & Rodrigo, 2007).

In general, the most relevant subtypes are FIV-A and FIV-B which were found to be significantly distant between them (Sodora, et al., 1994; Sellon & Hartmann, 2012a). FIV-B is believed to have a low pathogenicity than FIV-A, also revealing a more advanced state of adaptation to the host (Sodora, et al., 1994).

The prevalence of each subtype is different, according to the country and region of the world. In USA and Canada, FIV-A and FIV-B are predominant, despite the fact that others such as FIV-C and FIV-F, a new suggested subtype, are also present (Bachmann, et al., 1997; Reggeti & Bienzle, 2004; Weaver, 2010). In Africa, FIV-A leads the ranking (Kann, et al., 2006) while in South America, namely in Brasil, subtype B is dominant (Caxito, Coelho, Oliveira, & Resende, 2006; Martins, et al., 2008). In Australia the predominant subtype is FIV-A, although FIV-B is also present (Kann, et al., 2006). In the New Zeland FIV-A is more

frequent with documented FIV-C and intersubtype recombination (Hayward, et al., 2007; Hayward & Rodrigo, 2010). In Asia, to be precise in Japan, four FIV subtypes have been also studied (FIV-A, FIV-B, FIV-C and FIV-D) (Nakamura, et al., 2010).

In Europe, subtypes prevalence is also different. In north Europe (namely in Germany), FIV-A is more predominant while in south Europe (Italy, Spain and Portugal), FIV-B leads the subtypes' prevalence (Pistello, et al., 1997; Duarte, Marques, Tavares, & Fevereiro, 2002; Steinrigl & Klein, 2003). Furthermore, some subtypes can even be divided in subgroups which reflects the genetic variation of FIV (Steinrigl & Klein, 2003). This fact occurs particularly in Portugal where previous epidemiological studies revealed an increased viral diversity among FIV infected cats (Duarte, et al., 2002; Duarte & Tavares, 2006). It was described that FIV-B was predominant in the Portuguese feline population (Duarte, et al., 2002). According to the authors, isolated samples appeared to be a subcluster within B subtype, reinforcing the FIV genetic complexity even within subtypes (Duarte, et al., 2002; Duarte & Tavares, 2006).

FeLV

Also based on the env sequence, FeLV can be divided in 3 subtypes (A, B and C) (Dunham & Graham, 2008; Hartmann, 2012b). FeLV-A is the most common in clinical practice and it is transmitted exogenously (horizontally) among the cat population (Dunham & Graham, 2008; Hartmann, 2012b). FeLV-B occurs in about 50% of infected cats and is believed to be due to a recombination between FeLV-A and an endogenous FeLV-related sequence from the feline genome (Shalev, et al., 2009). It is associated with malignancies namely thymic lymphoma (Dunham & Graham, 2008). FeLV C is characterized by point mutations in env, being associated to fatal non regenerative anemia (Dunham & Graham, 2008; Hartmann, 2011). Although not frequently transmissible among cats, it is belived that FeLV-B and FeLV-C may have arisen as a chance in FeLV-A infected cats (Dunham & Graham, 2008).

More recently, a new variant, FeLV-T, has been associated with severe immunodeficiency (Anderson, Lauring, Burns, & Overbaugh, 2000). It is believed to come from multiple mutations in FeLV-A and its nomenclature came from the marked tropism and cytotoxicity for T lymphocytes, causing a severe Immunosupression (Lauring, Anderson, & Overbaugh, 2001; Lauring, Cheng, Eiden, & Overbaugh, 2002; Barnett, Wensel, Li, Fass, & Cunningham, 2003).

1.3. Retroviruses and evolution – a problem in the future?

The particular life cycle and the subsequent genetic variation potentiate the hypothesis that new subtypes may arise in the future. This is particularly true for FIV and may create real problems on diagnosis, therapeutic and prophylactic approaches (Dunham & Graham, 2008).

Therefore, the molecular background in retroviruses is continuously under research, in order to maintain effective prophylactic strategies, avoiding the arising of new subtypes with increased pathogenicity. Only by understanding and following molecular biology of retroviruses, is the scientific committee ready to deal with their clinical properties.

Part I - Chapter II:
Retroviruses – from the immunitary to the clinical perspective

2.1. Epidemiology of Retroviruses

Retroviruses are among the most common infectious diseases in feline practice (Hartmann, 2011). Despite their different physiopathology, FIV and FeLV cause a wide range of clinical signs which can easily overlap making an accurate diagnosis difficult. Although there are different ways to prevent retroviral infections, their identification, isolation and treatment of infected cats are the most effective ones (Levy, et al., 2008). In order to recognize the truly epidemiology of retroviruses it is therefore important to improve all of these strategies.

FIV

In spite of the retrospective studies which suggest its presence in feline population in 1966, FIV was firstly identified twenty years later, when it was isolated in a cattery from California (Pedersen, Ho, Brown, & Yamamoto, 1987; Shelton, et al., 1990). Regarding FIV hosts, domestic cats are the most prone to be persistent infected although cross-infection and concurrent reactive immune responses to lentiviruses from other species such as lions or pumas have been described (VandeWoude, Hageman, O'Brien, & Hoover, 2002; VandeWoude, Hageman, & Hoover, 2003). Transmission from domestic cats to exotic ones was also documented (Nishimura, et al., 1999). Considering that FIV infection of species other than feline is out of the scope of this work, the further detailed clinical and immunitary features relies only on FIV infection in domestic cats.

Since 1986, FIV has been described worldwide with an estimated prevalence up to 29% in some countries such as Japan, relying on an important disease in clinical practice (Ishida, et al., 1989). In North America, its prevalence is around 2.5%, ranging up to 24%, in healthy cats (Levy, Scott, Lachtara, & Crawford, 2006) while in Canada it is described as 4-5%, ranging up to 23% depending on regional location (S. E. Little, 2005; S. Little, Sears, Lachtara, & Bienzle, 2009; Ravi, Wobeser, Taylor, & Jackson, 2010). In Europe, FIV prevalence is variable, even within each country. In general, FIV is more common and well detailed in southern countries, where free-roaming cats are more frequent (Bandecchi, et al., 1992; Peri, et al., 1994; Arjona, et al., 2000; Dorny, et al., 2002; Muirden, 2002). It is more prevalent in sick cats than in healthy cats (Bandecchi, et al., 1992; Sellon & Hartmann, 2012a).

Risk factors such as age, sex, health status and cat life style have been identified being described that intact young-adult male cats with outdoor access are more prone to FIV infection (Ishida, et al., 1989; Levy, et al., 2006; Gleich, Krieger & Hartmann, 2009).

FeLV

Firstly described in 1964 (Jarrett, Crawford, Martin, & Davie, 1964), FeLV is still a feline problematic disease nowadays (Dunham & Graham, 2008). Being one of the most-disease-related deaths reported in cats, his name came from the “contagious tumor” that was firstly associated to it (Addie, et al., 2000; Hartmann, 2011).

FeLV prevalence ranges from 1 to 16% among healthy cats around the world (Arjona, et al., 2000; Bandecchi, Dell'Omodarme, Magi, Palamidessi, & Prati, 2006; Levy, et al., 2006; Solano-Gallego, Hegarty, Espada, Llull, & Breitschwerdt, 2006; Gleich & Hartmann, 2009; Gleich, et al., 2009; Little, et al., 2009). In sick cats, as expected, its prevalence is higher being described as 38% in one study including cats with haemobartonellosis (Harrus, et al., 2002). Concerning its straight relation to neoplasia namely lymphoma, its prevalence in cats with lymphomas is up to 75% (Hartmann, 2012b). With the increase of vaccination and prevention (particulary by removal policy), FeLV prevalence has been decreasing (Lubkin, Romatowski, Zhu, Kulesa, & White, 1996; Hartmann, 2012b). It should not be forgotten that FeLV prevalence is usually based on FeLV p27 antigen detection in blood either by ELISA or immunochromatography techniques. In fact, one study described that 10% of cats were positive for provirus and negative for p27 viremia (Hofmann-Lehmann, et al., 2001). Considering that free antigen can only be detected when animal has productive viremia, prevalence values can be underestimated (Rojko, Hoover, Quackenbush, & Olsen, 1982).

In multi-cat environment, the death rate is around 50% in the first two years after infection and 80% in three years (Hartmann, 2009). In single-cat houses, this rate is lower, although the overall median survival time is estimated on 2.4– 3 years (de Mari, et al., 2004; Levy, et al., 2006; Gleich, et al., 2009; Hartmann, 2011, 2012b).

Concerning risk factors, they are similar to FIV, being documented that free-roaming cats with outside lifestyle have an increased risk (Levy, et al., 2006; Gleich, et al., 2009; Hartmann, 2012b). Despite the fact that FeLV is easier spreaded through social contacts and is a “social friendly disease”, aggressive behavior and a common “male attitude” have an important role as risk factors (Gleich, et al., 2009). Therefore, that previous idea of “social disease” should be reconsidered due to the fact that aggressive cats have showed higher risk of FeLV infection (Gleich, et al., 2009; Goldkamp, Levy, Edinboro, & Lachtara, 2008; Hartmann, 2012b). Gender prevalence is controversial; while some authors defend it tend to be the same in male and females (Lee, Levy, Gorman, Crawford, & Slater, 2002), others refer that male cats are more prone to the disease (Gleich, et al., 2009). Pure breeds have a lesser risk for FeLV infection but it is mainly due to the fact that these animals are usually indoor cats (Hartmann, 2012b). Furthermore, breeders are usually sensitive to retroviral infections and tend to regularly test animals. Regarding age of infection, some authors refer

that adult animals are more prone to FeLV (Levy, et al., 2006), others defend that younger animals are more likely to be infected (Hosie, Robertson, & Jarrett, 1989), and, more recently, it is believed that FeLV infection is age-independent (Gleich, et al., 2009).

2.2 Transmission routes of feline retroviruses

FIV

It is believed that in a natural field, transmission may occur via blood by parenteral inoculation, namely by bite and direct fight wounds, reason why it is more prevalent in adult male cats, commonly involved in "street fights" (Gleich, et al., 2009; Sellon & Hartmann, 2012a). Experimentally, it is described to be transmitted by bite wounds from infected to healthy cats by intravenous, subcutaneous, intraperitoneal and intramuscular routes (Sellon & Hartmann, 2012a).

Its transmission via saliva is discussable. In fact, FIV can be isolated not only in blood lymphocytes, plasma and serum but also in the saliva and salivary epithelium (Matteucci, et al., 1993; Park, Kyaw-Tanner, Thomas, & Robinson, 1995). However, despite the experimental evidence of transmucosal FIV transmission (Moench, et al., 1993), due to the low amount of infectious virus present in naturally FIV-infected cat's saliva, this route is doubtful and considered irrelevant (Matteucci, et al., 1993).

In multi-house cat environments and catteries, horizontal transmission divides scientific community once some authors defend that it depends on the behavioral changes and whether a hierarchy among cats is previously established or not (Dandekar, et al., 1992; Addie, et al., 2000; Hosie, et al., 2009; Sellon & Hartmann, 2012a).

About vertical FIV transmission, it was described not only after experimental inoculation but also in natural infection, being a reliable model of fetal/neonatal HIV infection (O'Neil, Burkhard, & Hoover, 1996; Kolenda-Roberts, et al., 2007; Medeiros, Martins, Dias, Tanuri, & Brindeiro, 2012). Although not fully understood, transmission can occur in the prepartum, intra-partum and post-partum via uterus or placenta route and/or by milk ingestion (Wasmoen, et al., 1992; Sellon, Jordan, Kennedy-Stoskopf, Tompkins, & Tompkins, 1994; O'Neil, Burkhard, Diehl, & Hoover, 1995; O'Neil, et al., 1996; Rogers & Hoover, 1998). Although rare in the natural field, transmission via utero can occur inconsistently meaning that some kittens can become infected while others not (Rogers & Hoover, 1998). Detailing FIV transmission by milk, one study reinforced that virus is concentrated mainly in milk once animals showed higher viral loads in milk than in milk secreting cells or blood cells (Allison & Hoover, 2003). Venereal transmission by seminal route is documented experimentally and

FIV was also isolated from the semen of naturally FIV-infected male cats (Jordan, et al., 1996; Jordan, et al., 1998). Even though, its transmission in the natural field seems to be reduced (Dunham & Graham, 2008).

More than the described transmission routes, other (more uncommon) ones have been documented such as suture materials or cloned FIV provirus. (Rigby, et al., 1997; Sparger, Louie, Ziomeck, & Luciw, 1997; R. Sellon & Hartmann, 2012a).

FeLV

FeLV horizontal transmission is the most common and occurs not only by bite wounds but also by oronasal route and/or direct contact with infected cat's saliva or nasal secretions (Jarrett, et al., 1964; Hardy, et al., 1975; Dunham & Graham, 2008; Cattori, et al., 2009; Hartmann, 2012b). In opposition to FIV, this route is particularly relevant in multi-cat environment where mutual grooming, using common litter areas, and the share of water and food dishes are frequent. Actually, several studies have shown that FeLV RNA can be detected in the saliva, being directly correlated to the viremia and clinical signs (Gomes-Keller, Gonczi, et al., 2006; Gomes-Keller, Tandon, et al., 2006). Furthermore, in early stages of progressive infection, saliva shedding may occur earlier than FeLV antigen p27 detection in blood. However, in established infections, animals with low proviral load may not shed FeLV RNA in the saliva meaning that blood screening is still preferable for the diagnosis and outcome prediction of FeLV-infected cats (Gomes-Keller, Tandon, et al., 2006; Cattori, et al., 2009). Although FeLV can infect various tissues, transmission via urine or feces is discussable. In fact, virus can be isolated from urine and feces of cats with progressive infection (Cattori, et al., 2009). It was even documented that cats which contacted with infected feces developed anti-FeLV antibodies despite having remained negative for provirus and viremia (Gomes-Keller, et al., 2009). Then although less relevant, litter sharing in FeLV-infected cats should be avoided (Gomes-Keller, et al., 2009).

Similarly to FIV, vertical transmission can also occur in FeLV. Kittens can be infected via placenta or when queens licks and nurses them. In fact, it can even occur in queens that are regressively infected (false-negative results on routine tests) due to latent infection which can be reactivated during pregnancy (Hartmann, 2012b).

More than the referred infection routes, others have been described namely iatrogenic transmission by blood transfusions, instruments and contaminated needles (Lutz, et al., 2009). Curiously, one study even reported that cat fleas (*Ctenocephalides felis*) can also be potential vectors of infection (Vobis, D'Haese, Mehlhorn, & Mencke, 2003).

2.3. The host-cells and the retrovirus – the molecular beginning of a long term interaction

The retroviral cycle has direct consequences on the host-cells. In this section, the molecular approach of each virus to the host-cells will be discussed.

FIV

The interaction with the host-cells begins when env proteins contact with them (Hosie, et al., 1998; Willett & Hosie, 1999; Mizukoshi, et al., 2009). As previously stated, these proteins are particularly relevant due to the fact that they are targets of the immune response and their variations are responsible for different disease progression and outcome (Kraase, et al., 2010). To enter the host-cell, retroviruses require specific primary and secondary receptors. The *cell tropism* (defined by the expression of FIV receptors in different cells) is therefore particular (Murphy, et al., 1999b; MacLachland & Dubovi, 2011).

In opposition to HIV where CD4 molecule is the primary receptor, for FIV, the primary receptor is the CD134 (Hosie, Willett, Dunsford, Jarrett, & Neil, 1993) which is expressed in all leukocytes (mainly in CD4 T-cells), macrophages and dendritic cells (Hosie, et al., 1993; de Parseval, Chatterji, Sun, & Elder, 2004; Shimojima, et al., 2004; Willett, et al., 2007; Reggeti, Ackerley, & Bienzle, 2008).

Similarly to HIV, an important co-factor (also described as a secondary receptor) for FIV infection is the chemokine receptor type 4 (CXCR4) (Hosie, et al., 1998; Willett & Hosie, 1999). Although it is only detected in monocyte-derived cells and B-lymphocytes, its gene expression is documented in all the cells particularly in T-lymphocytes, which is consistent with the FIV cell tropism (Willett, Cannon, & Hosie, 2003; Reggeti, et al., 2008; Troth, Dean, & Hoover, 2008).

After FIV enters the host, it replicates rapidly within dendritic cells (that can thereby transmit the virus to lymphocytes), macrophages and CD4+ T-lymphocytes leading to the release of new virions and a viremia peak, 8-12 weeks after infection (Toyosaki, et al., 1993; Beebe, et al., 1994; Sprague, Robbiani, Avery, O'Halloran, & Hoover, 2008). Thereafter, FIV spreads to mononuclear cells in different organs such as bone marrow, lung and intestinal tract (Beebe, et al., 1994; Rogers, Mathiason, & Hoover, 2002; Sandy, Robinson, Bredhauer, Kyaw-Tanner, & Howlett, 2002).

FeLV

FeLV interaction with the host cell is not completely understood and depends on the virus subtype envolved in the infection (Willett, Hosie, Neil, Turner, & Hoxie, 1997; Anderson, et al., 2000; Anderson, Lauring, Robertson, Dirks, & Overbaugh, 2001; Mendoza, Anderson, & Overbaugh, 2006; Rey, Prasad, & Tailor, 2008). FeLV-B, for instance, requires specific cellular sodium-dependent inorganic phosphate transporters called Pit1 and Pit2 (Anderson, et al., 2001; Shojima, Nakata, & Miyazawa, 2006). For FeLV-A, it is believed that the main cell-receptor used is a feline thiamine transport protein (feTHTR1) (Mendoza, et al., 2006). In FeLV-C infections, the cellular receptor required is the FLVCR1, a specific receptor usually present in hematopoietic cells (Tailor, Willett, & Kabat, 1999; Quigley, et al., 2000). The FeLV-T uses a classic membrane receptor (Pit1) and a co-receptor called FeLIX to enter the T-lymphocytes (Anderson, et al., 2000; Shojima, et al., 2006).

Viral replication begins usually in tonsilar lymphocytes and macrophages from the oropharynx tissues spreading, thereafter, to other lymph nodes and blood. Consequently, viremia develops and FeLV spreads to other tissues namely lymphoid, epithelial and myeloid lines where cells tend to divide quickly (Dunham & Graham, 2008; Hartmann, 2011, 2012b).

2.4. Physiopathology of immune suppression in retroviral infections: the immunitary perspective

In this section, physiopathology of FIV and FeLV will be presented and discussed, in order to a better understanding of immunitary pathways that are behind the clinical presentation of retroviral infected cats.

To a better understanding of the immune-suppression induced by retroviruses, it is crucial to deep-in into the different subsets of the immune system. Therefore, a brief description of the main components of this system is further explained.

Although its complexity, the immune system can be divided into two general parts: the nonspecific (innate) response and the specific (acquired) immunity which interact in order to maintain a competent immune system (Kennedy, 2010).

The nonspecific response refers to the innate mechanisms and barriers against pathogenic infections (Kennedy, 2010). Basically, it is managed by the physiologic barriers (such as epithelial tissues, pH changes and digestive enzymes) and cells namely phagocytic cells such as macrophages, dendritic cells or neutrophils which are enrolled in the non specific pro-inflammatory pathways and which response depends on antigen recognition patterns

(pathogen-associated molecular patterns- PAMPs). Dendritic cells are critical in the innate immune response once they serve as presenting-antigen cells to the acquired immunity namely cytotoxic T cells and T-helpers (Pedersen, Dean, Bernales, Sukura, & Higgins, 1998; Roitt & Delves, 2001; Tizard, 2009a, 2009b; Day, 2012; Kennedy, 2010). Despite the fact that they are lymphocytes, a special type of cells that are also involved on the nonspecific response is the natural-killer cells (NK-cells) which do not have antigen-specific receptors (Kennedy, 2010). Although their truly function is still under research, it is believed that they act as an evolutionary bridge between the innate and the acquired immune system (Sun & Lanier, 2009).

The acquired immunity refers to the specific response that recognizes and eliminates specific pathogens (Kennedy, 2010; Day, 2012). It is mainly chiefed by lymphocytes which can be divided into different types taking into account their functions. Among the groups of lymphocytes, B-cells and T-Cells are well known. B-cells develop in the bone marrow and can differentiate into plasma cells (which are enrolled on antibody production) and memory cells. These have specific actions, with special relevance to the cellular and humoral response. On the other hand, T-cells mature in the thymus being divided into two lineages: the cytotoxic T-cells (which express the CD8+ cell marker and are enrolled in the induction of apoptosis in cells displaying nonself antigens) and the T-helper cells (which express the CD4+ cell marker and are the chief cells that modulate the immune-response). In detail, T-helper cells can be divided into various subsets in which T-helper 1 (Th1), T-helper 2 (Th2) and T-regulatory cells (Treg) have important functions. Th1 subset promotes a cell-mediated immunity while Th2 induces the humoral response. Tregs are mainly involved in immune-tolerance mechanisms, preventing immune-mediated lesions (Pedersen, et al., 1998; Roitt & Delves, 2001; Tizard, 2009a, 2009b; Kennedy, 2010; Day, 2012).

FIV

The immune suppression induced by FIV can be explained by different physiopathology changes not only in the acquired immune system but also in the innate response.

Regarding the innate immune changes, lentiviruses reduce neutrophil's function namely chemotaxis, adhesion and migration (Hanlon, et al., 1993; Kubes, et al., 2003; Heit, et al., 2006). Its action in NK and in lymphokine-activated killer cells is discussable once authors defend that FIV reduces its activity in acute infection and others state that it can be increased in asymptomatic animals (Zaccaro, et al., 1995; Zhao, et al., 1995).

Concerning the acquired immune response, various changes have been reported. In FIV infections, lymphocytes tend to progressively lose the ability to respond to antigens mainly due to a several changes on cell surface molecules (such as CD3, CD4 or Major

Histocompatibility Complex II) and cytokine receptors (Ohno, Watari, Goitsuka, Tsujimoto, & Hasegawa, 1992; Rideout, Moore, & Pedersen, 1992; Choi, Yoo, & Collisson, 2000; Nishimura, et al., 2004).

Regarding the CD4+ T cells, as previously stated, after the host's infection, there is an overall reduction in the relative proportion of this lineage in peripheral blood and lymphoid tissues in FIV (Bull, et al., 2003; Dunham & Graham, 2008; Hartmann, 2011). This happens mainly due to a reduction in the production of these cells by bone marrow and thymus due to infection. Secondly, a lysis of the infected cells can be induced by virus *per se*. Thirdly there is a destruction of infected cells due to immune system activity or apoptosis (Bishop, Gruffydd-Jones, Harbour, & Stokes, 1993; Ohno, et al., 1993; Ohno, et al., 1994; Guiot, Rigal, & Chappuis, 1997; Mizuno, et al., 2001; Tompkins, et al., 2002; Alimonti, Ball, & Fowke, 2003; Sellon & Hartmann, 2012a). This loss of CD4+ T cells has a direct implication once these cells promote cell-mediated and humoral response.

About the Treg, under physiological conditions, this subset of cells suppresses antigen-specific and non-specific immune response. During FIV infection, Treg subset is relevant in different phases once it has an increase activity on the inhibition of IFN-γ production by CD8 cells (Mexas, Fogle, Tompkins, & Tompkins, 2008; Petty, Tompkins, & Tompkins, 2008; Fogle, Mexas, Tompkins, & Tompkins, 2010). Therefore, Treg cells impair the immune system to an effective response to infections. Furthermore, Treg cells can even be infected by FIV, acting as a reservoirs of virus mainly during the latent phase of infection (further discussed) (Joshi, Vahlenkamp, Garg, Tompkins, & Tompkins, 2004).

Concerning CD8 cells, they are directly involved on antiviral immunity under physiological condition. Particularly in FIV, the cellular response enrolled by CD8 cells has been documented as more relevant than the humoral response against the viral infection (Bucci, et al., 1998). Within 1-2 weeks after FIV-infection, CD8 specific cytotoxic T cells (CTLs) can be detected in blood, being crucial on the host's antiviral response (Beatty, Willett, Gault, & Jarrett, 1996; Bucci, et al., 1998; Crawford, et al., 2001; Hohdatsu, et al., 2003; Hohdatsu, Nakanishi, Saito, & Koyama, 2005). Apoptosis of not only CD4+ but also CD8+ and B cells tend to occur in lymph nodes, spleen and thymus, being inversely correlated to CD4/CD8 ratio and CD4+ cell count (Holznagel, et al., 1998; Sarli, et al., 1998). Following these changes, an inversion of CD4/CD8 ratio tends to occur usually weeks to months after infection (Ackley, Yamamoto, Levy, Pedersen, & Cooper, 1990; Tompkins, Nelson, English, & Novotney, 1991). It can be justified not only by a decrease on CD4+ subset but also by an increase of CD8, particularly a sub-population called CD8 alpha-bi beta low cells, which are commonly involved in the suppression of viremia (Ackley, et al., 1990; Hoffmann-Fezer, et al., 1992; Willett, Hosie, Callanan, Neil, & Jarrett, 1993). Although discussable, some authors

defend its use as a prognostic tool in infected animals (Walker, Canfield, & Love, 1994). While CD4/CD8 ratio can be supportive indicators of disease progression, there is no direct correlation between this parameter and clinical signs or viremia (Hoffmann-Fezer, et al., 1992; Goto, et al., 2000; Sellon & Hartmann, 2012a).External factors such as the virus sub-type, exposure to other pathogens and cats' age in the moment of infection can change the duration of this phase (Pedersen, Leutenegger, Woo, & Higgins, 2001; Hartmann, 2011).

Although the cytokine profile is not fully understood in FIV-infected cats, several studies have been conducted. In comparison to healthy cats, some authors have showed that FIV-infected animals have an increase of IFN-γ, TNF-α, IL-4, IL-6, IL-10 and IL-2 (Dean & Pedersen, 1998; Lerner, Grant, de Parseval, & Elder, 1998; Liang, et al., 2000; Orandle, et al., 2000; Lehman, et al., 2009). Specifically regarding Th1/Th2 response, it was reported an heterogeneous cytokine profile in lymphoid tissues during the early phase of FIV infection (Dean & Pedersen, 1998). In fact, this heterogeneity of cytokine profile has been reported in multiple studies. Some authors defend that, although there is a punctual increase of IFN-γ, there is an overall reduction on other several Th1 cytokines (IL-2 and IL-12), with concurrent increase in IL-10, a Th2 mediator (Tompkins & Tompkins, 2008). In agreement with these results, other authors documented that in co-infection with *Toxoplasma gondii* or *Listeria monocytogenes*, FIV-infected cats revealed a reduced cell-mediated immunity (Th1) (Levy, et al., 1998). Contradictory, a more recent study have shown that plasma IL-12/23 was elevated in FIV-experimentally infected cats confirming that a Th1 response is present in the early phase of infection (Wood, Troyer, Terwee, & Vandewoude, 2012). Therefore, although there is no clear consensus about a shift from Th1 to Th2 response in FIV infection, this retrovirus induces a cytokine dysregulation with concurrent reduction on transcription levels of several cytokines, leading to an inadequate innate and cell-mediated immune response (Levy, et al., 1998; Kipar, et al., 2004; Tompkins & Tompkins, 2008).

As regards antibody production/humoral response, after the viremia peak, there is a development of anti-FIV antibodies, including virus neutralizing antibodies (VNAs) which can be detected in plasma within 2-4 weeks after infection, although in some animals it can be delayed (Fevereiro, Roneker, Laufs, Tavares, & de Noronha, 1991; Bendinelli, et al., 1995; Sellon & Hartmann, 2012a). These antibodies are produced against different epitopes namely envelope, nucleocapsid and transmembrane proteins (Massi, et al., 1997). However, taking into account that these neutralizing antibodies remain extracellular, they are not effective on viral elimination but only on its neutralization (Del Mauro, et al., 1998; Inoshima, et al., 1998; Mazzetti, et al., 1999). This potentiated humoral response and polyclonal B-cell activity leads to a clinical observed hypergammaglobulinemia (namely an increase of IgG) (Ackley, et al., 1990; Flynn, Cannon, Lawrence, & Jarrett, 1994; Gleich & Hartmann, 2009).

Although IgG is not FIV-specific, its increase is a direct consequence of infection (Flynn, et al., 1994).

FeLV

The overall mechanisms of replication of FeLV are similar to FIV. In FeLV, particularly in cases of persistent viremia, it is well known that both humoral and cellular immune response significantly decreases (Flynn, Dunham, Watson, & Jarrett, 2002; Hartmann, 2012b). However, the physiopathology beneath FeLV-infection is not fully understood.

In a molecular approach and similarly to FIV, various changes can occur in the innate and acquired immune response.

Concerning the innate-immunity, it is described that neutrophils of FeLV-infected cats have decreased functions of chemotaxy and phagocytosis (Hartmann, 2012b). This can be induced by a direct immunesuppresive effect of several viral proteins such as p15E which reduces neutrophils activity and the antigenic response (Copelan, et al., 1983; Lafrado, Lewis, Mathes, & Olsen, 1987).

As regards acquired immunity, various studies have reported a functional depression of both humoral (B-cells) and cellular (T-cells) response in FeLV-infected cats. About cellular response, similarly to FIV, there is a preferential loss of CD4+ cells and an inversion of CD4/CD8 ratio (Quackenbush, et al., 1990; Hoffmann-Fezer, et al., 1996; Dunham & Graham, 2008; Hartmann, 2009, 2011). It is justified by a direct transfer of specific CD4+ lymphocytes towards an effective CD8+ response. In an early phase and even before the development of VNAs, cats develop CTLs which are able to reduce proviral load. They are believed to be enrolled on the recovery of infection and vaccinal protection, being one of the outcome predictors of FeLV (Flynn, Hanlon, & Jarrett, 2000; Flynn, Dunham, Mueller, Cannon, & Jarrett, 2002; Flynn, Dunham, Watson & Jarret, 2002).

Regarding the humoral response, it is described that, although the overall decrease of antibody production, FeLV-infected cats develop virus neutralizing antibodies (VNA) which can give some grade of protection (Russell & Jarrett, 1978; Dunham & Graham, 2008; Hartmann, 2011, 2012b). Usually, the VNA is developed towards envelop and transmembranar proteins such as p15E and gp70 (Elder, et al., 1987; Nick, et al., 1990; Russell & Jarrett, 1978). These VNAs can be transmitted vertically to kittens where they can be highly present (Jarrett, Russell, & Stewart, 1977). Although they do not necessarily interfere on the clinical recovery, VNA is a good indicator of a protective immunity in naturally exposed cats (Dunham & Graham, 2008).

Similarly to what happen in FIV, there is a nonspecific increase of certain immunoglobulins (IgG and IgM) which can lead to the development of antigen-antibody complexes (Hartmann, 2012b). Antigens that lead to immune-complexes are gp70, p27 and p15E proteins (Day, O'Reilly-Felice, Hardy, Good, & Witkin, 1980; Tuomari, Olsen, Singh, & Kraut, 1984). In opposition to FIV, in FeLV-infection there is no marked hypergammaglobulinemia in Serum Protein Electrophoresis (SPE) (Gleich & Hartmann, 2009; Miro, et al., 2007).

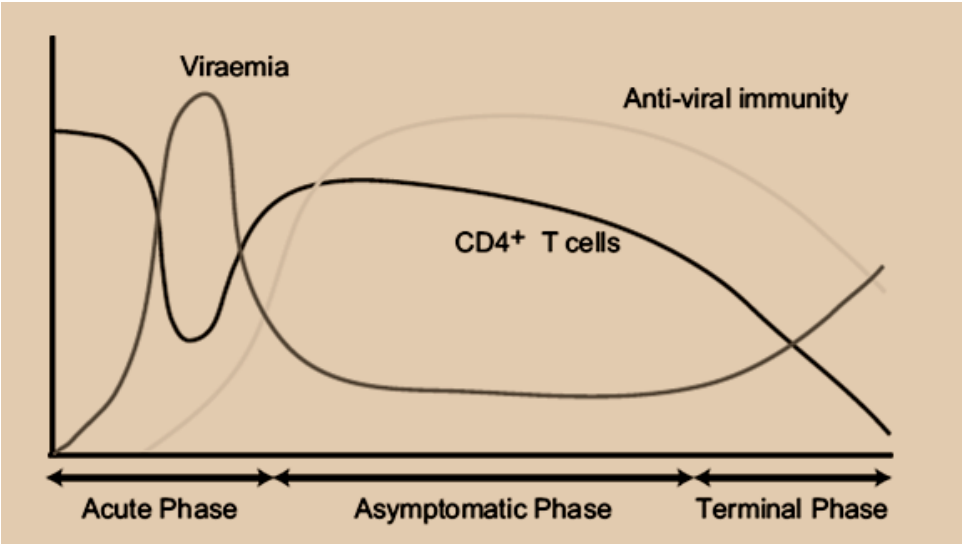
Few studies have been performed on cytokine profile in FeLV-infected cats. It is described that IL-2 and IL-4 are depressed without changes on IL-1 (Linenberger & Deng, 1999). IFN-γ tend to fluctuate and TNF-α is increased in infected cats, causing a pro-inflammatory state and consequently disease (Hartmann, 2012b). The B-cell stimulatory factors (produced by T-cells) are also decreased (Diehl & Hoover, 1992).

2.5. Phases of infection

FIV

In a clinical perspective, viral infection can be divided into different phases (Dunham & Graham, 2008) and are illustrated in figure 4

Figure 4: Time course presentation of the three phases of FIV infection (Dunham & Graham, 2008)



The **acute phase** can occur during several days to weeks after infection and is characterized by an initial viremia peak with concurrent decrease of CD4+ T-lymphocytes. Clinically, at this time of infection the cat can show mild to moderate signs such as anorexia, depression,

fever, enteritis, gingivitis, dermatitis, conjuntivitis and lymphadenopathy. These changes are transient and often subclinical tending to disappear. Even though, the generalized lymphadenopathy can persist due to the increased number and dimension of follicular activity of germinal centers which can last up to weeks or months (Dunham & Graham, 2008; Hartmann, 2011, 2012c; R. Sellon & Hartmann, 2012a).

The **asymptomatic phase** refers to a recovery phase and can occur during many years, in which the cat develops the specific anti-viral immunity and does not present clinical signs. Its duration and clinical presentation depends on other factors such as viral subtype, exposure and age of the host (Pedersen, et al., 2001; Hartmann, 2009, 2011, 2012b, 2012c). During this period, viremia decreases and CD4+ tends initially to increase. Progressively, they slowly decrease leading to an inversion of CD4/CD8 ratio (previously described). Also during this phase, FIV can become latent, particularly in CD4 T cells (McDonnel, Sparger, Luciw, & Murphy, 2012; Murphy, Hillman, Mok, & Vapniarsky, 2012; Murphy, Vapniarsky, et al., 2012; McDonnel, Sparger, & Murphy, 2013). In fact, latency of FIV has been recently studied and is considered an important step of the asymptomatic phase. During this latent period, several conformational and structural changes lead to an inactivation of proviral transcription (McDonnel, et al., 2012; McDonnel, et al., 2013). Consequently, cats can have undetectable viremia despite a positive proviral load in resting and activated CD4+ T-cells (Tomonaga, Inoshima, Ikeda, & Mikami, 1995; Murphy, Vapniarsky, et al., 2012).

After the asymptomatic phase, FIV-infection can progress to a **terminal phase** in which viremia increases due to a progressive failure on antiviral immunity (Dunham & Graham, 2008; Hartmann, 2012c). Consequently, during the terminal phase, there is a significant immunodeficiency which evolutes to severe clinical conditions such as neoplasia, myelosupression or neurologic disease. Due to the similarities to the HIV portrait, this phase is also called “Feline AIDS Stage” (English, et al., 1994; Goto, et al., 2000).

Despite this division, the evolution of the disease and concurrent prediction when an animal can develop towards the terminal stage is not possible. Although animals with viremia levels progress more quickly to the AIDS stage (Diehl, Mathiason-Dubard, O'Neil, & Hoover, 1996), contradictory to what is observed in HIV, cats can recover and re-become asymptomatic (Hartmann, 2012b). Then, the staging process is difficult in naturally FIV-infected cats reason why this classification is purely academic.

FeLV

As stated, FeLV infects various tissues from salivary gland to bone marrow. The progression of disease and concurrent outcome are affected by different factors such as age, viral dose, exposition route or even concurrent diseases (Dunham & Graham, 2008).

Age is a crucial documented factor that affects the grade of immunesuppresion induced by FeLV infection. Neonatal kittens, when infected, can develop the “fading kitten syndrome” (further discussed) that is characterized by an atrophy of the thymus a severe immunesuppression and death. When the infection occurs in the adult cat, there is a progressive resistance and cats can develop regressive infections with mild clinical signs and an “apparent” heathy status. Even though, the majority of cats develop a persistent infection and present the further discussed FeLV-related disease (such as neoplasias like lymphoma or leukemia, severe anemia, enteritis and secondary infections) dying within 3 years after infection (Dunham & Graham, 2008; Hartmann, 2011, 2012b, 2012c).

A few years ago, the division of infection into different phases was supported by viremia results. It was believed that while one third of cats used to become persistently viremic, the other two thirds were able to clear the infection. With the incoming of new diagnostic tools, nowadays, this classification has been restructured. Due to new proviral and viremia findings, FeLV infection can be currently classified in: abortive, regressive, progressive and focal/atypical (Torres, Mathiason, & Hoover, 2005; Dunham & Graham, 2008). The summary of changes of each type of infection is presented on table 1 and further discussed.

Table 1: Main clinical and laboratory changes observed in each stage of FeLV-infection (adapted from Hartmann, 2012b).

Outcome of FeLV infection	P27 FeLV antigen in Blood	Viral RNA in blood (Viremia)	Viral DNA in blood (Provirus)	Viral Shedding	FeLV-Associated Disease
Abortive	Negative	Negative	Negative	Negative	Unlikely
Regressive	Negative	Negative	Positive	Negative	Unlikely
Progressive	Positive	Positive	Positive	Positive	Likely
Atypical/Focal	Negative	Not tested	Not tested	Variable	Unlikely

The **abortive infection** is rare and is characterized by an effective humoral and cell-mediated immune response meaning that the immune system of FeLV-infected cats blocks the infection and cats do not develop viremia. Cats are called “regressor cats”, having high levels of VNAs without detecting FeLV antigen (viral RNA or provirus) at any age (Torres, et al., 2005). This kind of infection is believed to be induced by a low-exposure to FeLV (Major, et al., 2010) although it has been difficult to prove *in vivo*. Recent studies have proven that virus can be detected later in these “regressor cats”, reason why they can have persistent VNAs which reinforce that only a small percentage of these cats can clear FeLV infection

from all the cells. Even though, “regressor cats” are estimated to live the same time as healthy ones (Lutz, et al., 2009).

The **regressive infection** is defined by an effective immune response which blocks FeLV replication before or shortly after bone marrow infection. It tends to happen in older cats coming from animal shelters that clinically exhibit anemia, panleukopenia or purulent inflammatory processes (Suntz, Failing, Hecht, Schwartz, & Reinacher, 2010).

In regressive infection, there is a transient viremia phase which usually lasts for 3-6 weeks, with a maximum of 16 weeks. During this time, animals shed the virus and FeLV p27 can be detected in plasma (Hartmann, 2012b). After this transient viremia, there is no viral replication and shedding, although cats become provirus positive. Due to the induced effective response, cats become protected against new virus and have a low risk of developing FeLV-related diseases (Flynn, et al., 2000; Flynn, Dunham, Watson, et al., 2002; Hartmann, 2012b)

Even in cases where the bone marrow is infected, a small percentage of cats can clear viremia. However, if the viremia persists for longer time, this regression of infection is less probable. In these cases of bone-marrow involvement, cats can develop a “latent infection” where proviral load persists in the bone marrow, there is no viral replication or shedding and viremia is undetectable (Hartmann, 2012b). Even though, infection can be reactivated spontaneously or due to various immunesupressive factors such as stress or steroid therapy (Rojko, et al., 1982). This can justify eventual relapsing viremias and persistent high-titer antibodies during this “latent” period (Hartmann, 2012c). Regressive infection can only be diagnosed with provirus detection by Polymerase Chain Reaction (PCR) or even bone marrow culture.

Previously described as persistent viremia, the **progressive infection** is characterized by an active FeLV-infection in which cats do not have an effective immune response. Consequently, virus replicates firstly in lymphoid tissues and after in bone marrow. Viremia persists for more than 16 weeks and cats are persistently viremic. Moreover, these animals have low levels of VNAs, dying within 3 years after infection. This kind of infection tends to be more frequent in immunosupressed and young cats (Hartmann, 2012b). Particularly in these animals, the FeLV-related diseases are common and a risk factor for mortality (Dunham & Graham, 2008).

In the early phase of infection, progressive or regressive infections are undistinguishable once both are characterized by a similar viremia and proviral load. Afterwards, although both of them are positive to provirus, while in the progressive infection there is a secondary viremia, in regressive infection, viremia is undetected (Cattori & Hofmann-Lehmann, 2008;

Hartmann, 2012c). Therefore, both infections can be distinguished by repeatedly viremia detection (Torres, et al., 2005). If cats test negative 2-3 weeks after infection, it is possible to be a regressive infection (Hartmann, 2012b).

The **atypical infection** accounts for up to 10% of experimental FeLV-infections. This encompasses FeLV-infection of rare locations such as mammary glands, bladder and eyes. In this kind of infection, there is an intermittent low grade production of p27 antigen (Pacitti, Jarrett, & Hay, 1986; Hayes, et al., 1989).

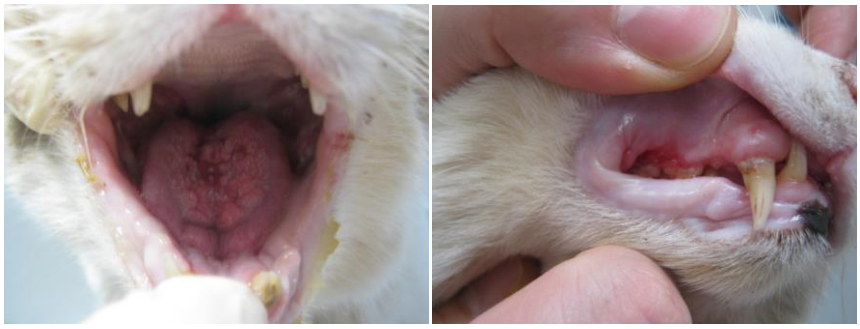
2.6. FIV and FeLV: Clinical and laboratory findings

The clinical presentation of FIV and FeLV can include a wide range of clinical signs and the majority of them are non specific findings observed during routine clinical examination of cats. In both infections, the clinical presentation depends on different factors such as cat's age, time of infection, virus subtypes, route of infection among others. On the further paragraphs, it is discussed in detail, some clinical and laboratory relevant clinical findings in FIV and FeLV infected cats.

FIV

Presented in up to 50% of FIV-infected cats, oral lesions typically include chronic stomatitis, palatitis, gingivitis (figure 5), ulcers or even odontoclastic reabsortion. Being more common in naturally infected than in experimental cats, these lesions lead to oral pain and discomfort particularly during mastication or when the mouth is opened. In severe cases, it can even progress to anorexia. The histopathology analysis usually reveals plasma cells and reactive lymphocyte infiltration with several neutrophils and eosinophils (Hartmann, 2012b). This is believed to be due a chronic antigenic stimulation mainly due to concurrent pathogens, although the truly etiology is unknown (DeBowes, 2009; Hartmann, 2009). In fact, feline calicivirus (FCV) co-infection is common in these animals, worsening the referred oral lesions (Tenorio, Franti, Madewell, & Pedersen, 1991; Reubel, George, Higgins, & Pedersen, 1994).

Figure 5: Severe gingivitis and oral ulcer (tongue) in a FIV-infected cat (original).



Despite being rare, neurologic disease (central or peripheric) can occur in up to 5% of FIV-infected cats and several studies have been performed in order to understand the truly mechanisms of central nervous system (CNS) infection (Dow, Poss, & Hoover, 1990; Dow, Dreitz, & Hoover, 1992; Abramo, Bo, Canese, & Poli, 1995; Bragg, Boles, & Meeker, 2002; Meeker, 2007). Clinical signs are variable and include sleep disturbances, dementia, twitching of face and tong, subclinical forebrain abnormal activity and reduced auditory-evoked potentials (Prospero-Garcia, et al., 1994; Gunn-Moore, Pearson, Harbour, & Whiting, 1996; Steigerwald, Sarter, March, & Podell, 1999; Fletcher, Meeker, Hudson, & Callanan, 2011; Hartmann, 2011). In spite of the fact that the truly physiopathology is unknown, FIV enters the CNS by the blood-cerebrospinal fluid and blood-brain barriers leading to an increase of TNF-α which induces a lymphocyte migration (Fletcher, et al., 2011). Although rare, the presence of concurrent infections such as feline infectious peritonitis, cryptococcosis or toxoplasmosis can worse the neurologic disease remarked in FIV-infected cats (Sellon & Hartmann, 2012a).The analysis of cerebrospinal fluid (CSF) of FIV-infected cats can reveal a high protein content, cellular pleocytosis and an increase of IgG (Dow, et al., 1990).

Ocular disease is also common in FIV-infected cats which can present anterior uveitis, glaucoma or even other rare changes like pars planitis (infiltration of leukocytes into the vitreous), retinal degeneration (focal) and hemorrhages (English, Davidson, Nasisse, Jamieson, & Lappin, 1990; Lappin, et al., 1992; Willis, 2000).

Considering that FIV is a retrovirus and integrates the host-genome, infected cats have up to five times more chances to develop neoplasia than healthy cats (Sellon & Hartmann, 2012a). Among the most frequent tumors in FIV-infected cats, lymphomas (mainly B-cell type) and leukemias lead the ranking (Poli, et al., 1994; Terry, Callanan, Fulton, Jarrett, & Neil, 1995; Callanan, et al., 1996; Gabor, Love, Malik, & Canfield, 2001). Other tumors such as fibrosarcoma, squamous cell carcinoma and mast cell tumors can also occur in FIV-infected cats (Hutson, Rideout, & Pedersen, 1991; Sellon & Hartmann, 2012a). Although a cause-effect theory seems probable, FIV provirus does not correlate to neoplasia occurrence meaning that it probably happens due to a decrease on cell-mediated immunity and a concurrent chronic B-cell stimulation and hyperplasia (Beatty, Callanan, Terry, Jarrett, & Neil, 1998; Beatty, Lawrence, et al., 1998; Diehl & Hoover, 1992). Furthermore, there are some changes on the immunosurveillance mechanisms and an increase of the proliferation of transformed lymphoid cells, reason why lymphoid tumors are commonly associated to FIV-infection (Endo, et al., 1997; Beatty, Lawrence, et al., 1998).

The potentiated humoral response and polyclonal B-cell activation previously described can lead to hypergammaglobulinemia. Consequently, there is also an increase of immune

complexes leading to clinical complications such as uveitis and glomerulonephritis (Poli, et al.,1995; Matsumoto, et al., 1997). More than glomerulonephritis, FIV can also induce glomerulosclerosis and tubulointerstitial infiltrates, decreasing the renal function (Poli, et al., 1993).

Due to the well-recognized immunesuppression, different concurrent opportunistic infections (by viruses, bacterias, protozoas or fungus) have been reported in FIV-infected cats. In fact, FIV-cats can have higher titers of fungal agents in the skin, oropharynx and rectum (Mancianti, Giannelli, Bendinelli, & Poli, 1992), although no correlation was achieved between *Cryptococus* infection, dermatophytes and FIV status (Walker, Malik, & Canfield, 1995; Sierra, Guillot, Jacob, Bussieras, & Chermette, 2000). For toxoplasma infection, studies have reported that although FIV-infected cats have increased antibody titers (Dorny, et al., 2002; Lopes, Cardoso, & Rodrigues, 2008), the FIV-infection induces a worsening of respiratory toxoplasmosis (Davidson, Rottman, English, Lappin, & Tompkins, 1993). Regarding Mycoplasma infections, a positive correlation with FIV infection was achieved, although it is unclear whether there is a causal relationship between both pathogens or if they share the same risk factors (Sykes, Drazenovich, Ball, & Leutenegger, 2007; Bauer, Balzer, Thure, & Moritz, 2008; Macieira, et al., 2008).

More than the described clinical signs, FIV-infected cats can have other clinical presentations which tend to be non specific and correlated to opportunistic infections. Although controversial, diarrhea can occur in FIV cats and seems to be due to a bacterial overgrowth and secondary inflammatory lesions (Papasouliotis, et al., 1998). Also respiratory disease may occur due to secondary bacterial, fungal, protozoal or parasitic infection (Barrs, Martin, Nicoll, Beatty, & Malik, 2000). Therefore, an appropriate screening of concurrent diseases is warrant and imperative in FIV-infected cats.

Concerning the laboratory findings, they are not specific neither pathognomonic of FIV-infection. However, several changes can be observed not only on the hematology profile but also on biochemistry analysis. Regarding the complete blood count (CBC), in the acute phase, animals can show a transitory leucopenia (neutropenia and lymphopenia). In the asymptomatic phase, parameters are usually within the reference ranges (Shelton, Linenberger, Persik, & Abkowitz, 1995; Sellon & Hartmann, 2012a). In the terminal phase, FIV-infected cats can have severe pancytopenias with severe CD4+ cell loss and an inversion on CD4/CD8 ratio (Linenberger, Shelton, Persik, & Abkowitz, 1991; Linenberger & Abkowitz, 1995; Shelton, et al., 1995). Although uncommon (except in the terminal phase), anemia, usually non-regenerative, and thrombocytopenia can also be present in FIV-infected cats (Pedersen, et al., 2001; Fujino, Horiuchi, et al., 2009; Gleich & Hartmann, 2009).

Biochemistry parameters tend to be within the reference range in FIV-infected cats. As referred, cats can show an hyperproteinemia mainly due to hyperglobulinemia (Miro, et al., 2007; Gleich & Hartmann, 2009). Renal function can also be affected and proteinuria can be present with or without mild to moderate azotemia (Poli, et al., 1993; Baxter, Levy, Edinboro, Vaden, & Tompkins, 2012). Among other unexpected findings, FIV-infected cats can have hyperglycemia, hypertriglyceridemia, hypocholesterolemia and a slightly increase on activated partial thromboplastin time without evident coagulopathy (Hart & Nolte, 1994; Hofmann-Lehmann, Holznagel, Ossent, & Lutz, 1997).

FeLV

In spite of the prospected low survival time of FeLV-infected cats, it has been increasing due to an improvement on veterinary care and an early recognition of the infection. Regarding its clinical presentation, authors reported that the most relevant clinical findings in FeLV-cats are concurrent opportunistic co-infections, present in up to 15% of cases, anemia (11%), lymphoma (6%), "-cytopenias" (5%) and myeloproliferative disorders (4%)(Cotter, 1991; Hartmann, 2012b). A brief description of the important clinical and laboratory signs of FeLV-infection is further performed.

Similarly to FIV, there are several physiopathology mechanisms that potentiate the immunesuppression observed in FeLV-infected cats. The overall deregulated immune response namely the loss of T-cell suppressor activity can lead to the formation of antibody complexes and the onset of immune-mediated diseases (Gleich & Hartmann, 2009; Hartmann, 2011, 2012b, 2012c). The more common are: auto-immune hemolytic anaemia (Kohn, Weingart, Eckmann, Ottenjann, & Leibold, 2006), glomerulonephritis (Anderson & Jarrett, 1971), uveitis (Brightman, Ogilvie, & Tompkins, 1991) and polyarthritis (where FeLV accounts for 20% of polyarthritis feline cases) (Hartmann, 2012b).

After the infection of the bone marrow, FeLV induces a well-known myelosuppression that can lead to hematopoietic and myeloproliferative diseases. These include non-regenerative and regenerative anemia, neutropenia, thrombocytopenia, thrombocytophaty and pancytopenia (Hartmann, 2012b). Particularly FeLV-C can cause pure red cell aplasia that is a rare hematological disease (Quackenbush, et al., 1990; Shelton & Linenberger, 1995). Detailing changes observed in hematopoetic cell lines, it is well known that anemia is common in FeLV-infected cats. It is mainly non-regenerative and is caused not only by a direct effect of the virus on hematopoietic and stromal cells but also due to a high concentration of pro-inflammatory cytokines that induce a chronic-disease anemia (Hartmann, 2012b). Cats usually show macrocytosis without reticulocitosis, which is mainly due to a mitotic defect induced by FeLV during erythropoiesis. A regenerative anemia can also occur and is mainly hemolytic, secondary to hemotropic mycoplasma infections

(George, Rideout, Griffey, & Pedersen, 2002; Harrus, et al., 2002). Even though, blood loss due to concurrent thrombocytopenia and FeLV-induced immune-mediated hemolytic anemia (FeLV-IMHA) are also described causes of regenerative anemia in FeLV-infected cats (Tasker, Murray, Knowles, & Day, 2010). Due to a decrease on platelet production, either by leukemia secondary infiltration or due to a direct bone marrow suppression, and a reduction on its life-span, thrombocytopenia can occur in infected cats (Hartmann, 2012b). Also immune-mediated thrombocytopenia is described concurrently with IMHA (Hartmann, 2011). On the other hand, due to a change on size, shape and function induced by direct replication of FeLV in platelets, animals can also develop thrombocytopathy (Hartmann, 2011). Regarding leukocytes, as stated, FeLV induces a moderate to severe lymphopenia, with progressive loss of CD4+ and inversion of CD4/CD8 ratio (Quackenbush, et al., 1990; Hoffmann-Fezer, et al., 1996). Also neutropenia can occur mainly due to a direct myeloid hypoplasia of the granulocyte precursors (Shelton & Linenberger, 1995). In fact, an immune-mediated cause cannot be excluded once in several cases, neutropenia improves after steroid therapy ("glucocorticoid-responsive neutropenia") (Hartmann, 2012b). In cases of severe leucopenia (<3000 cells/µl), an FeLV associated enteritis, also known as feline panleukopenia-like syndrome (FPLS) or myeloblastopenia can occur. It is characterized by a destruction of the intestinal crypts in a similar way to panleukopenia infection, reason why it is called FPLS (Hartmann, 2011, 2012b, 2012c). Although it is unclear whether this syndrome comes from FeLV or a possible concurrent co-infection with feline parvovirus (FPV) (Lutz, et al., 1995) this clinical presentation should always be considered. More than the described hematopoietic changes, FeLV-infected cats can also develop myeloproliferative diseases. They are secondary to bone marrow infiltration and tissue-replacement which causes myelofibrosis and or the myelodysplastic syndrome (MDS). While the first refers to abnormal proliferation of fibroblasts after bone marrow overstimulation, the last one is characterized by a dysplastic bone marrow with concurrent peripheral blood cytopenias (Hisasue, et al., 2009).

In a similar way to FIV, also FeLV-infected cats are more prone to develop neoplasia. The most commons are lymphoma and leukemia (Jarrett, Laird, & Hay, 1973; Hardy, et al., 1980; Hartmann, 2011). Others such as fibrosarcoma (Ellis, et al., 1996) or feline olfactory neuroblastomas have also been associated to FeLV (Schrenzel, Higgins, Hinrichs, Smith, & Torten, 1990). The relationship between FeLV and neoplasia is justified by somatically acquired insertional mutagenesis (Tsatsanis, et al., 1994; Forman, Pal-Ghosh, Spanjaard, Faller, & Ghosh, 2009; Fujino, Liao, et al., 2009).

In a clinical approach, lymphoma is the most common FeLV-related neoplasia. This tumor can be induced in kittens after experimental (Jarrett, et al., 1973) and naturally FIV-infected cats have a higher risk to develop it (Essex, et al., 1975). In the same perspective, around

80% of cats suffering from lymphoma or leukemia were FeLV positive (Cotter, Hardy, & Essex, 1975; Hardy, et al., 1980; Shelton, et al., 1990). More recently however, these percentages have been reducing mainly due to an overall decrease of FeLV prevalence (Louwerens, London, Pedersen, & Lyons, 2005). This can be explained not only by an increased vaccination policy but also by a more strict epidemiological control (Hartmann, 2012b). In opposition to FIV, FeLV associated lymphomas are mainly of T-cell type (Hardy, Zuckerman, MacEwen, Hayes, & Essex, 1977). This association can be explained by the fact that FeLV infects mainly T-cells, immature thymocytes and monocytes (Hartmann, 2011, 2012c). Concerning the different clinical presentation of FeLV-associated lymphoma, it is described that mediastinal lymphoma, common in young cats, is associated to FeLV-infection in 80-90% of cases whilst this retrovirus is detected in 50% of cats with multicentric lymphoma (Hartmann, 2011, 2012c). Intestinal lymphoma, more often in older cats, occurs concurrently with FeLV in a lower prevalence (6 up to 25-30%) (Hartmann, 2011, 2012c). Other cases such as extranodal, miscellaneous or atypical lymphoma account for up to 20% of prevalence in FeLV-infected cats (Taylor, et al., 2009; Hartmann, 2011, 2012c).

As previously referred, leukemia is also an important FeLV-related disease, involving not only the lymphoid tissue but also other hematopoietic cells. It is estimated that more than 50% of cats with feline leukemia are FeLV-positive (Hisasue, et al., 2009). Similarly to FIV, this is mainly due to several changes occurring during virus integration namely changes on LTR such as the presence of tandem direct repeats (Hisasue, et al., 2009).

Also fibrosarcoma has been associated to FeLV-infection particularly to vaccination against this virus. Although the association has been discussable and is apparently not significant (Ellis, et al., 1996), fibrosarcoma is caused by a feline sarcoma virus (FeSV) which is a recombinant virus that aroused from a recombination of FeLV-A with oncogenes. In this sense, FeLV-A is a helper-virus, supplying proteins that are required for FeSV replication.

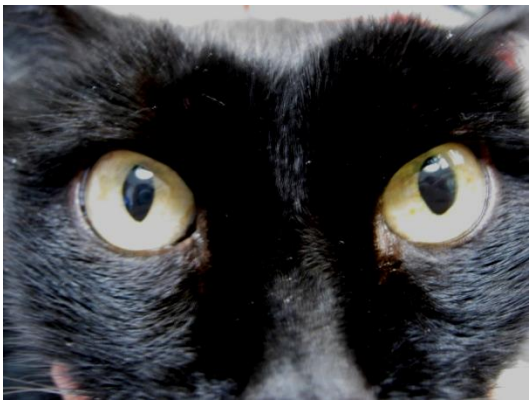
Considering the immune suppression status induced by FeLV, it seems predictable that infected animals are more prone to develop concurrent opportunistic infections. However, studies failed on proving this association. Therefore, not all the concurrent infections can be associated to FeLV and no correlation was established between concurrent co-infections of FeLV-infected cats with Leishmania or Mycoplasma (Macieira, et al., 2008; Hartmann, 2011, 2012c).

FeLV-infection can also induce reproductive disorders. Recognizing that FeLV can be vertically transmitted, queens can abort and if neutropenic, can easily develop bacterial endometritis. Fetal reabsortion can also occur and, if the pregnancy progresses, kittens can have neonatal death. In fact, either by vertical transmission or by direct exposure, when kittens are infected, they die within the first two weeks of live mainly due to the “fading kitten

syndrome”**,** characterized by dehydration, failure to nurse, hypothermia and thymic atrophy (Hartmann, 2011, 2012c).

In line with the previously stated for FIV, FeLV infection can also lead to neurologic disease, which is commonly associated to CNS lymphoma. However, the virus by itself can induce neuropathy and particularly FeLV-C shows a high tropism for the CNS (Fails, Mitchell, Rojko, & Whalen, 1997; Mitchell, et al., 1997). Common clinical signs are hyperesthesia, vocalization, paresia, anisocoria (figure 6), mydriasis, central blindness or even urinary incontinence (Carmichael, Bienzle, & McDonnell, 2002). These signs can be explained by different physiopathology mechanisms namely a neuronal death induced by an increase on intracellular free calcium in response to FeLV-env proteins (Hartmann, 2012b).

Figure 6: Anisocoria in a FeLV-infected cat(original)



2.7. Diagnosis of FIV and FeLV

More than the clinical and laboratory findings, the diagnosis of retroviral infections should be supported by other complementary exams that allow a correct assessment of the disease.

In virology, diagnosis methods usually rely on five techniques: 1) Virus isolation in cell cultures; 2) Electronic microscopy; 3) detection of specific viral antigens by different methods such as ELISA, immunofluorescence or even immunohistochemistry 4) detection of nucleic acid (by PCR or in-situ hybridization) 5) serology (detection of antibodies) (Evermann, Sellon, & Sykes, 2012).

In this section, a brief description of the main diagnostic techniques used for FIV and FeLV diagnosis will be performed.

FIV

In general, the clinical suspicion of FIV-infection can be reinforced by serology and/or virus detection.

Serology (Antibody testing)

As mentioned, FIV-infected cats can develop VNA within 2-4 weeks although some of them can have a slight delay until 60 days. Taking into account that FIV-infection is persistent, VNA also persists for life (Sellon & Hartmann, 2012a).

Antibodies (usually against p24 antigen) can be detected by ELISA or by rapid immunomigration-type assays, currently available as easy-to-use tests in the clinical practice (Hartmann, Werner, Egberink, & Jarrett, 2001; Hartmann, et al., 2007; Hartmann, 2011, 2012c; Sellon & Hartmann, 2012a). Although these tests have a relatively high sensitivity, it has been improved by adding other FIV-protein antigens (Rosati, et al., 2004). Even though, a false positive result can occur. When a positive result is obtained, a confirmatory test should always be performed, mainly when animal lives in a low-risk area. When a negative result is achieved, it is reliable once the test is highly sensitive (Sellon & Hartmann, 2012a).

Virus detection

In contrast to FeLV where antigen detection is reliable, FIV-infected cats have low viral loads during most of their lives reason why antigen-based ELISA are not useful in these cases. The classical virus isolation is possible but expensive, time-consuming and requiring expertise knowledge. Consequently, with the income of improved ELISA, this method became unpractical (Sellon & Hartmann, 2012a).

Virus detection can be performed by proviral load or viremia measurements using PCR technology, or even more recently real-time quantitative PCR (RT-qPCR). The first allows the detection of amplifier products using a gel electrophoresis whilst the second one permits a quantification (absolute or relative) taking into account the fluorescence detection that is proportional to the number of target copies amplified. Among its multiple benefits, this technique allows to distinguish between vaccinated and naturally infected cats once the commercialized vaccine does not induce provirus production (Diehl, Mathiason-DuBard, O'Neil, & Hoover, 1995; Uhl, Heaton-Jones, Pu, & Yamamoto, 2002; R. Sellon & Hartmann, 2012a). Although false-positives can occur, PCR is a sensitive technique that requires specialized knowledge (Bienzle, et al., 2004; Arjona, et al., 2007). One limitation to its use in FIV diagnosis is the marked genetic variability of the virus that can complicate its detection, once designed primers and probes cannot detect all of the subtypes. Other limitation is the use of conventional PCR in cats with low proviral loads. With the onset of RT-qPCR, low

proviral loads can be easier detected since conventional PCR could be negative in these situations (Sellon & Hartmann, 2012a).

When should a cat be tested for FIV?

According to common feline practitioners' guidelines, every cat must be tested for retroviruses and considering that the compliance for testing is lower than expected, the test should never be delayed for other consultations (Goldkamp, et al., 2008). Even exclusively indoor cats (where the FIV-prevalence seems to be very low) must be tested not only because FIV status can influence its quality of live but also due to the possible contact that indoor cats can have with others during their life (namely new incoming cats or escape/evasion scenarios) (Hosie, et al., 2009; R. Sellon & Hartmann, 2012a).

When an FIV-infection is suspected in adult cats, they should be immediately tested. In catteries, the mother status should always be tested once mothers can transmit the virus vertically and, mother VNAs can be transferred to kittens by colostrum, giving false-positives (Levy, et al., 2008).

In kittens, the age cut-off for testing is discussable. All the FIV-positive kittens younger than 16 weeks should be retested after this age. This is mainly due to the fact that antibodies production may take up to 12 weeks after infection. Consequently, even in adults, when there was a possible contact with infected cats, tests should only be performed around 3 months after that date (Dunham & Graham, 2008; Levy, et al., 2008). In kittens, results should be always considered. When the kitten is negative, it is likely to be truly FIV-negative. However, it can have been recently infected without time to develop antibodies being warrant to repeat the test within 60-90 days for confirmation. If a kitten tests positive, it may be truly infected (vertical infection is possible but improbable) or it may have maternal antibodies which were transferred passively (in vaccinated or infected mothers). Therefore, the 6 months old seems to be a reasonable cut-off for FIV test once maternal antibodies do not interfere by this age (Levy, et al., 2008; Sellon & Hartmann, 2012a).

In several countries where FIV-vaccination is commercialized (not in Portugal), the antibodies detection to evaluate whether an animal is infected or not becomes a problem once antibodies from vaccinated cats cannot be distinguished from those produced in response to naturally infection (Crawford & Levy, 2007). Moreover, the vaccinated-induced antibodies can persist for more than 3 years and vaccinated queens can transmit them, persisting in kittens even after the weaning phase (Sellon & Hartmann, 2012a). To bypass this diagnostic challenge, a test that detects antibodies against multiple FIV-antigens were

developed, allowing the distinction between vaccinated and infected animals with high accuracy (97-98%) (Kusuhara, et al., 2007).

When a positive result is firstly achieved, as stated, it must be confirmed by other test or technique. In case of ELISA, a second ELISA antibody (from different manufacturer) can be performed (Hartmann, et al., 2007). However, other techniques such as viral culture, virus isolation, western blot, immunofluorescence or PCR can be used for FIV diagnosis (Dunham & Graham, 2008).

Despite the fact that it is not routinely performed nowadays, virus culture is considered the gold standard technique for FIV identification. Both western-blot and immunofluorescence are based on anti-FIV antibodies detection. Even though they are less specific than the ELISA screening tests, particularly in vaccinated animals (Sellon & Hartmann, 2012a).

FeLV

Various techniques have been developed in order to detect FeLV infection. Nowadays, FeLV diagnosis relies on virus detection (either one specific antigen, viral RNA or some components of proviral DNA) although other methods such as immunofluorescence [Fluorescent antibodies (FA) for FeLV antigen cell-bound detection], or virus isolation can be considered (Dunham & Graham, 2008; Hartmann, 2012b).

Virus detection

In a similar way to FIV, clinicians have easy-to-use tests that, based on rapid immunomigration-type or ELISA assays, allow a rapid diagnosis of FeLV antigen. The current antigen detected is p27 (a capsid protein produced in large amounts by virus infected cells) (Dunham & Graham, 2008; Hartmann, 2012b). The first ELISA was licensed for use in 1979 but, although its sensitivity, this kit had a low specificity, reason why other researchers developed another ELISA directed to three different epitopes of p27 antigen and in which cross reaction is minimal (Dunham & Graham, 2008; Hartmann, 2012b). Thenceforward, various ELISAs and immunochromatographic/rapid immunomigration assays were used. Briefly, these last techniques follow the ELISA principles in which color is obtained as a result of an immunologic reaction (Robinson, et al., 1998). All the widely used kits in-practice detect p27. The colorimetric ELISA is the most widely used due to the fact that it is very sensitive detecting low levels of this free soluble antigen in wholeblood, plasma or serum. Being a sensitive test, it becomes positive in early infection, even before the invasion of the bone marrow (Jarrett, Golder, & Stewart, 1982). Consequently, this test can detect animals with transient or persistent viremia (Barr, 1996). Various comparative studies have been perfomed particularly in Europe in order to assess sensitivity and sensibility of commercial kits (Hartmann, et al., 2001; Hartmann, et al., 2007; Pinches, et al., 2007; Sand, Englert,

Egberink, Lutz, & Hartmann, 2010). In general, positive predictive values are estimated in 80% while negative predictive values are around 100% (Hartmann, et al., 2001; Hartmann, et al., 2007). Several authors have also developed ELISA kits using tears and saliva (Hawkins, 1991; Babyak, Groves, Dimski, & Taboada, 1996). However, blood seems to be the most accurate biological sample for FeLV-test (Hawkins, 1991; Babyak, et al., 1996; Hartmann, 2012b). Despite the method used, similarly to FIV, when a positive result is obtained other confirmatory tests must be performed (Hartmann, et al., 2007). In fact, other alternative techniques such as immunofluorescence, virus isolation or PCR are available for FeLV diagnosis.

Immunofluorescence/Direct FA testing detects cell-associated p27 antigen in infected blood cells being performed directly on blood or bone marrow smears (Hardy & Zuckerman, 1991; Dunham & Graham, 2008). Immunofluorescence becomes positive after bone marrow infection, identifying progressive infections (Hartmann, 2012b). However, recognizing that bone marrow infection occurs lately in the physiopathology of infection, this test should not be performed as a screening test in cats within the first weeks of viremia but only to confirm positive results or predict the outcome (Hartmann, 2011, 2012b, 2012c).

Virus isolation detects the presence of entire virions indicating viremia (O. Jarrett, Laird, Hay, & Crighton, 1968). Although it is relevant to assess FeLV-infection, virus isolation can take up to 10 days and expertise knowledge which preclude this technique.

Being highly sensitive, PCR technology allows the detection (Conventional PCR) and quantification (RT-qPCR) of viral nucleic acid sequences, either viral RNA or proviral DNA in various biological samples such as blood, bone marrow or even saliva (Hartmann, 2012b). Similarly to FIV, minor strain variations can lead to false-negative results meaning that primers and probe's choice must be careful. Various authors defend that PCR should always be performed when regressive infection is suspected, namely in cats with lymphoma and bone-marrow disease (Jackson, Haines, Meric, & Misra, 1993; Jackson, Haines, Taylor, & Misra, 1996). With the development of RT-qPCR, the quantification of proviral load and viremia has been helpful on the assessment of the immune response and the truly amount of virus in animals (Hofmann-Lehmann, et al., 2001; Gomes-Keller, Gonczi, et al., 2006). In fact, RT-qPCR is particularly relevant in FeLV-infected cats with negative antigen tests. However, in clinical practice, the clinical relevance of cats which test negative for viremia but positive for provirus is discussable once, being aviremic, they do not shed virus and are unlikely to develop disease (Hartmann, 2012b). More recently, authors have proved that FeLV RNA and DNA were stable for more than 2 months in the saliva stored at room temperature suggesting that this biological samples can be useful to test FeLV in a noninvasive approach (Gomes-Keller, Gonczi, et al., 2006; Gomes-Keller, Tandon, et al.,

2006). However, some of these animals tested positive in blood and negative in saliva meaning that blood is still more accurate for this purpose.

Antibody Detection

In opposition to FIV, antibodies against FeLV-antigens are not persistent and tend to decrease. Consequently, antibody detection is not useful (Hartmann, 2012b). Moreover, antibodies detection does not distinguish between vaccinated and naturally-infected cats. Once FeLV-vaccination does not prevent infection, many cats may have been vaccinated and be regressively infected at the same time, developing antibodies (Hofmann-Lehmann, et al., 2008; Hartmann, 2012b). Also in abortive phase, FeLV-infected cats can have circulating antibodies without detectable viremia or provirus (Major, et al., 2010). Consequently, antibody detection is useless in the diagnosis of FeLV infection being only useful as a screening test to assess vaccination efficacy (Langhammer, Hubner, Kurth, & Denner, 2006; Hartmann, 2012b).

When should a cat be tested for FeLV?

In a similar way to FIV, the specific guidelines of the American Association of Feline Practitioners reinforce that all the cats (sick or healthy) must be tested in order to avoid the viral spreading (Levy, et al., 2008). In multi-cat environment, when a cat is tested positive, all the cats in the house must be tested (Lutz, et al., 2009).

Concerning the age recommended to test, due to the fact that first screening tests usually detect antigen, there is no interference with maternal antibodies or vaccination status and cats can be tested at any age. Even though, in kittens the maternal transmission may test negative for weeks to months after delivery, reason why some authors recommend testing kittens twice in 12-weeks intervals (Dunham & Graham, 2008; Hartmann, 2012c).

Also in cats with known recent exposure or after adoption, test must be done immediately and when the test is negative, another should be performed after 28 days (Levy, et al., 2008). In particular household mixed catteries (where healthy cats live with FeLV-infected ones), an annual screening is strongly recommended as well as blood-donor cats and animals with access to outdoors, particularly in cat-dense neighborhoods (Levy, et al., 2008).

Vaccination usually does not interfere with the FeLV-tests once these are based on antigen tests. However, test must be performed before vaccination and not after once FeLV-vaccinal antigens can cross-react and give false positive results. Furthermore, it is not known for how long, reason why test must be safely done before (Levy, et al., 2008; Lutz, et al., 2009).

It is important to consider the reliability of the tests used hence misdiagnosis and false-positive results can lead to euthanasia of FeLV-negative cats. Then, as stated, it is always

recommended to retest animals at least a second time either to confirm negativity or to better define the infection (Levy, et al., 2008; Lutz, et al., 2009). In fact, if a cat tested positive, a second result (90 days after) may help differentiating between regressive and progressive infection. If the cat is still positive, a third test 10 weeks later can be useful to confirm a progressive infection. Even though, some authors defend that using FA is a hypothesis. Only 3-9% of positive cats to FA have transient viremia meaning that, if it is positive in FA, cat is very likely under a progressive infection (Jarrett, et al., 1982; Hardy & Zuckerman, 1991; Jarrett, Pacitti, Hosie, & Reid, 1991; Hartmann, 2012b). In personal author's viewpoint, with the RT-qPCR resource, these recommendations can be bypassed by quantifying and monitoring proviral load and viremia not only in blood but also in bone marrow. Although there are no studies about it, it will be strongly helpful in the future to distinguish between regressive and progressive infection. In cases of (re) testing animals previously vaccinated for FeLV, it is important to refer that, as stated, vaccination does not tend to interfere with test results. Even though, rare exceptions should be considered such as testing immediately after vaccination (Levy, et al., 2008). Considering that the test is routinely performed before and not after vaccination, this is not a common clinical problem.

2.8. Therapeutic approach to Retroviral Infections: Anti-virals and immune modulators – general considerations

When a cat is suspected of being infected with retroviruses, the first therapeutic approach is always supportive and symptomatic. It consists on the major therapeutic basis and, until the diagnosis is established, never forgetting other differential diagnosis. It should not be forgotten that each cat is an individual clinical case and supportive therapy must be directed to the main chief complaints. Fluid therapy and antibiotics are usually performed in order to prevent dehydration and opportunistic infections. With the exception of particular hemolytic disorders, glucocorticoids and immune-supressive drugs should always be avoided in retroviral infected cats due to the obvious immune suppression. For the same reason, supportive therapy and nurse care usually take longer periods than in healthy cats (Dunham & Graham, 2008; Hartmann, 2012a, 2012b; Sellon & Hartmann, 2012a).

Cats infected with FIV usually do not show severe clinical signs. Authors reported that FIV-infected cats live lesser than healthy cats with a life span of 4.9 towards 6 years, respectively (Hartmann, 2009; Sellon & Hartmann, 2012a). Nowadays, with the improvement of veterinary care and prophylactic policies, most of them die in old-age and due to a cause unrelated to the infection. Therefore, FIV-infected cats can have an excellent quality of life and must not be submitted to euthanasia only due to its infection (Hosie, et al., 2009).

In opposition to FIV infection, FeLV-infected cats die within the first 5 years after infection, with a median survival time of 2.4 years (Addie, et al., 2000). This low life expectancy is related to the onset of FeLV-related diseases. For instance, hematological disease and neoplasia such as lymphoma or leukemia can increase the morbidity and mortality rate. A correct diagnosis must be performed and the respective therapy and prognosis must be discussed with owners (Hartmann, 2012b).

More than the common drugs used for supportive therapies, antivirals and immune modulators can be administered in retroviral infected cats. Considering that most of the drugs are licensed for human use, there is a lack of well-controlled clinical trials in cats and their efficacy is not entirely clear (Hartmann, 2012a). In the further paragraphs, some of these compounds and their concurrent use will be discussed.

Antivirals

In contrast to antibiotics, antivirals do not induce a complete elimination of infection once they only inhibit some steps of the replication cycle, being ineffective during the latent phase. Moreover, viral replication is more dependent on the cell's host than bacterias (Murphy, Gibbs, Horzineck, & Studdert, 1999a). With the exception of several immune modulators which have antiviral activity, there are not antivirals licensed for use in veterinary medicine meaning that, all the truly antiviral compounds are licensed for humans namely in HIV therapies (Hartmann, 2012a). Interestingly, most of its use specifically in cats comes from previous studies in which FIV-infected cats are used as HIV experimental models. Particularly in this specie, antivirals have an overall low efficacy and induce significant toxic effects that should be considered (Dunham & Graham, 2008; Hartmann, 2012a).

In general, antivirals can be divided into several classes, taking into account the step of viral cycle in which they act. Academically, various classes are considered, such as: a) RT inhibitors b) Non-nucleoside RT inhibitors; c) inhibitors of DNA/RNA synthesis; d) inhibitors of nucleotide synthesis; e) antagonists/homologues of specific receptors; f) neuraminidase inhibitors; g) ion channel blockers; h) peptides. Particularly in retrovirology, only RT inhibitors and antagonist receptors are commonly used. More than these ones, there are different antivirals that have been studied in retroviral infections namely non nucleoside RT inhibitors (Suramin), inhibitors of nucleotide synthesis (Foscarnet, Ribavarine) or ion channel blockers (amantadine) (Dunham & Graham, 2008; Hartmann, 2012a). However, recognizing that their clinical efficacy is unclear, for this discussion, only RT inhibitors and antagonist receptors will be considered.

The **RT inhibitors/nucleoside analogues** are the most common antiviral drugs. The majority are nucleoside analogues which, acting as anti-metabolits, are “false substrates” that bind to RT enzyme and block its activity (Hartmann, 2012a).

In FIV, the more common RT inhibitor used is zidovudine (AZT) that has been proven to inhibit viral replication in vitro and in vivo (Hartmann, 1995a). This compound decreases viremia, improves CD4/CD8 ratio, reduces the clinical signs (namely neurologic disease and stomatitis) and potentiates quality of life in treated animals (Hartmann, et al., 1992; Hartmann, 1995a, 1995b). Even though, AZT only delays viremia peak and immunitary changes without preventing viral replication or infection (Hartmann, et al., 1992; Meers, et al., 1993; Hayes, Wilkinson, Frick, Francke, & Mathes, 1995; Hartmann, Ferk, North, & Pedersen, 1997; Hayes, Phipps, Francke, & Mathes, 2000). Moreover, virus can become resistant to AZT therapy and mutations can occur in 6 months after therapy (Smith, Remington, Preston, Schinazi, & North, 1998; McCrackin Stevenson & McBroom, 2001; Hartmann, 2012a). To avoid this, an experimental trial associated AZT with another RT inhibitor (lamivudine) showing severe adverse effects (Arai, Earl, & Yamamoto, 2002).

As in all therapies, AZT has secondary effects such as shiny hair coat, vomit and anorexia which should be considered. Also CBC should be closely monitored in cats under AZT therapy once it can induce a non regenerative anemia, particularly when given in higher doses (Smyth, et al., 1994). Then, a weekly CBC is recommended and if the values are stable after the first month, a monthly control is warrant. In fact, hematocrit tends to decrease in the first 3 weeks of therapy but in the majority of cases it increases without withdrawing the therapy. Only if CBC decreases up to 20%, AZT must be discontinued until results back to normal. Obviously, cats with pancytopenia must not be treated and cats with chronic kidney failure should have the dose adjusted (Hartmann, 2012a; Sellon & Hartmann, 2012a).

In FeLV, several studies have described the use of RT inhibitors such as didanosine and zalcitabine (Hoover, et al., 1989; Tavares, Roneker, Postie, & de Noronha, 1989; Polas, et al., 1990). Regarding AZT, more than the *in-vitro* studies, it was shown to be useful in FeLV-infected cats particularly when is started in less than 1 week after challenge, protecting from bone marrow infection and persistent viremia (Hartmann, et al., 1992; Hartmann, 2012a). More studies are warrant in order to fully understand the potential use of RT inhibitors in naturally retroviral infected cats.

Other antivirals that can be used in retroviral infections are **antagonists/homologous of receptors** namely byciclams such as plerixafor/AMD 3100 which bind either to viruses (homologous) or to cell-receptors (antagonists), inhibiting the virus-cell interaction. With the exception of several drugs, these compounds are strongly selective for HIV meaning that they are not used in veterinary medicine (Hartmann, 2012a).

As stated, CXCR4 is a secondary receptor of FIV infection, which blockage has been studied. Byciclams selectively block lentivirus ligation to CXCR4, preventing the entrance into the cell (Schols, et al., 1997; Donzella, et al., 1998; Egberink, et al., 1999). In between these compounds, plerixaflor (AMD3100) has been shown to effectively reduce proviral load without significant clinical improvement in naturally infected cats (Hartmann, Stengel, Klein, Egberink, & Balzarini, 2012). Although without clinical significance, AMD3100 can decrease magnesium levels meaning that magnesium and calcium must be closely monitored in cats under therapy (Hartmann, et al., 2012).

The blockage of CD134 has also been researched using specific antibodies anti-CD134. Authors concluded that higher concentration of antibodies anti-CD134 reduces viral load and improved clinical signs of FIV-infected cats (Grant, Fink, Sundstrom, Torbett, & Elder, 2009).

Immune modulators

Taking part of the whole group of the "immune therapy", by definition, immune modulators are compounds which interfere with the immune system. They are commonly used in different clinical situations particularly in canine and feline viral infections. It is believed that immune modulators restore several functions of the immune system allowing a better management of opportunistic infections and a better clinical recovery. Some of these compounds can even have a direct antiviral effect (Hartmann, 2012a). In between the well-known immune modulators, **interferons** and **growth factors/hormones** will be further discussed, mainly due to its current use in retroviral infections.

IFNs are widely used in retroviral infections and, being the main subject of this work, their truly action will be discussed on Part I- chapter III.

Despite the fact that **Growth factors/Hormones** do not have an antiviral effect, these compounds act as immune modulators once they can alter bone marrow function and, consequently, impair a disruption of hematopoiesis by viral infections. Although they were cloned, hematopoietic growth factors are not licensed for use in veterinary medicine. As a consequence, human compounds are often used, limiting its long term applicability due to the development of neutralizing antibodies (Hartmann, 2012a). The most commonly cytokines and growth factors used in veterinary medicine are filgastrim, sargramostim, erythropoietin and insulin-like growth factor-1.

Filgrastim is a granulocyte colony-stimulating factor (G-CSF) that has been administered in FIV and FeLV-infected cases, although its efficacy is doubtful mainly when viruses are replicating in actively dividing cells (Kraft & Kuffer, 1995). In FIV, studies revealed that this compound increased viral load while in FeLV, its effects are doubtful and divides author's opinion (Arai, Darman, Lewis, & Yamamoto, 2000; Hartmann, 2012b). When used, it can

induce a short-term increase in neutropenia which, after 10 days to 7 weeks, can reappear due to the development of neutralizing antibodies. Therefore, its usage is less frequent nowadays (Hartmann, 2012b).

Sargramostim is another growth factor which induces the proliferation and differentiation of myeloid and erythroid progenitors namely granulocyte and monocyte lines. Its use is described in FIV cats that developed neutrophilia with therapy (Arai, et al., 2000). Similarly to filgrastim, viral load increased in these animals meaning that, in both cases, there is a potentiated expression of the virus by infected lymphocytes. Consequently, it is contra-indicated in FIV infected cats (Hartmann, 2012a).

Although it cannot be classified as cytokine, erythropoietin (EPO) is a well-known hormone directly involved on erythropoiesis. It is currently used in different diseases namely non regenerative anemias caused by endogenous EPO deficiency. Authors reported that its use in FIV-infected cats induced an increase in different cell-lines, without increasing viral load. Therefore, it is considered to be used in FIV-infected cats showing cytopenias (Arai, et al., 2000). Also in FeLV, despite the fact that endogenous EPO is usually increased in cats with anemia, its exogenous administration is recommended once it increases all the cell lines (Ogilvie, 1995; Arai, et al., 2000). Even though, several FeLV-infected cats can be resistant to EPO therapy hence bone marrow stromal cells are infected either by FeLV or other concurrent pathogens. In these cases, blood transfusion should be considered (Hartmann, 2012b).

In both infections, treatment must be done until the HT increases up to 30% and a concurrent iron supplementation can be beneficial. Considering that the available exogenous EPO is a recombinant –human product, cats can develop anti-EPO antibodies in 25-30% of cases around 6-12 months after the onset of therapy (Hartmann, 2012b).

Insulin-growth factor-1 (IGF-1) is also a hormone with distinct functions namely the thymus stimulation and T-lymphocyte proliferation. Authors have reported its use in FIV-infected cats describing an increased thymus with potentiated regeneration of the cortical and reduced B-cells (Woo, Dean, Lavoy, Clark, & Moore, 1999). Once viral load did not increased, IGF-1 use in FIV-cats must be studied once it can be an alternative therapy in young cats.

More than the referred compounds, there are other immune modulators that have been tested in retrovirology.

Although not yet used in feline medicine, there are several cytokines such as IL-2 that activates T-helper cells and neutrophils. It is currently used in HIV patients meaning that IL-2 can be considered a possible immune modulator for FIV cats in the near future (Meuer, Dumann, Meyer zum Buschenfelde, & Kohler, 1989; Hartmann, 2012a).

There are also various natural compounds that have been used as immune modulators in retroviral infections. Examples are *Acemannan, Staphylococus Protein A and propionibacterium acnes* (McCaw, et al., 2001; Hartmann, 2012a). Even though, neither are studies that support its use nor there is a conclusive evidence of their benefits. Particularly in FIV, also the use of antioxidative compounds has been shown to improve CD4/CD8 ratio without changing viral load. This means that, hence FIV-infected cats have a basal oxidative stress with concurrent decrease of glutathione peroxidase activity, the use of antixodatives should be further studied (Webb, Lehman, & McCord, 2008).

In sum, although not always based on a well-documented science, there are different antivirals and immune modulators that are currently used in FIV and FeLV-infections in order to impair viral replication and maximizing the main functions of the depressed immune system.

2.9. Considerations about management and prognosis of retroviral infections

As previously referred, FIV and FeLV management is mainly based on supportive therapy, control of concurrent infections, prevention of new diseases (by vaccination) and a direct therapeutic approach when required and taking always into consideration the individual clinical signs of the cat. Even though, general considerations must be taken about management and prognosis of retroviral infections.

FIV

As general recommendations for FIV cats, it is always reinforced that intact cats must be neutered and animals should be kept indoor not only to reduce exposure to different antigens but also to avoid the spreading of infection. Infected cats should be fed with good diets and owners must be instructed to be alert to the main clinical signs of the disease progression. In cats with severe oral disease, dental cleaning and antibiotherapy are imperative. According to the evolution of the disease, other therapies must be considered namely dental extraction or antivirals. Steroid therapy should always be avoided, being the last therapeutic resource. Usually each therapy takes longer than for healthy cats and during hospitalization, FIV-infected cats should be placed in individual cages. FIV-infected cats must be submitted to a veterinary check-up, at least two-three times per year, including CBC, chemistry panel and urine analysis (Levy, et al., 2008; Hosie, et al., 2009).

As stated, the horizontal transmission is discussable in multi-house environments. However, it is recommended to neuter cats and also to avoid the introduction of new animals because

it may lead to fights and potentiate transmission. In a similar fashion, in animal shelters the same policy should be applied. Cats should be housed individually (unless from the same household) and if animals must be segregated, it should be done with the smallest number of animals possible. According to the recent European Advisory Board on Cat Diseases (ABCD) guidelines for FIV, euthanasia should be considered in particular cases of very symptomatic cats which are suspected of having an advanced stage of disease. In breeding catteries, FIV is usually not a problem hence cats are kept indoors and frequently tested so, this is not a common disease associated to this environment (Levy, et al., 2008; Hosie, et al., 2009).

Regarding the current vaccination of FIV-infected cats with core vaccines, it is described that, although the immune suppression, these cats can develop an immune response to various antigens (Dawson, et al., 1991; Lawrence, Callanan, Willett, & Jarrett, 1995). Nowadays, it is recommended that FIV-infected cats must be vaccinated regularly (every 6 months instead of the common annual boosters). Inactivated vaccines should be preferred once modified live virus vaccines can induce disease (Richards, et al., 2006). Whilst some authors defend that vaccination helps stabilizing CD4 cell counts (Reubel, Dean, George, Barlough, & Pedersen, 1994), others defend that vaccination can decrease CD4/CD8 ratio and can potentiate the expression of FIV-receptors and the viral production (Lehmann, et al., 1992). However, taking into account that these animals are more prone to opportunistic infections, core vaccination must always be considered and seems to be more beneficial than harmful (Sellon & Hartmann, 2012a).

The prognosis of FIV-infected cats is favorable, particularly when owners are aware of the disease and the basic care. In fact, FIV can progress from the asymptomatic through the terminal phase and, once CD4+-Tcells decrease in number and in its efficacy to respond to antigens, opportunistic infections and severe immunodeficiency worse the clinical portrait (Burkhard & Dean, 2003; Dunham & Graham, 2008).

FeLV

It is believed that, although the low life expectancy of FeLV-infected cats, a good management may increase it. Consequently, the decision of euthanasia or therapy should always be carefully taken (Hartmann, 2012b).

In multi-cat environment, similarly to FIV, when a cat tests positive all the cats which contact with this one must be tested. If some of them test negative, owners must be aware of the risk of cohabitation of these animals. In fact, the risk of transmission seems to be lower than expected because, if negative cats have been previously exposed to FeLV-positive shedding virus, they are likely to be immune to disease. Even though, viral shedding can occur.

Despite the fact that infected cats should be separated from others, if owners refuse it, uninfected cats should be vaccinated (although it does not provide a good protection under these circumstances) (Levy, et al., 2008; Hosie, et al., 2009).

As for FIV, in single-cat environment, the cat should live indoor and must be fed with appropriate good nutrition. Intact animals must be neutered and veterinary check-ups are recommended at least twice a year (Levy, et al., 2008). In cases of hospitalization, similarly to FIV, the direct contact among FeLV-infected cats and other cats must be avoided. Therefore, FeLV-infected cats must be isolated in individual cages, although they can cohabit (with the adequate measures) at the same ward of other hospitalized cats. Some authors defend that FeLV-infected cats should not be placed in a "contagious unit" once these animals are immune suppressed and can easily be infected by other pathogens (Sellon & Hartmann, 2012a).

Regarding vaccination of FeLV-infected cats, for the same reasons described for FIV, vaccination with core vaccines is recommended, in a more frequent plan (boosters every 6 months) and avoiding modified live vaccines (Levy, et al., 2008; Hartmann, 2012b) .

Regarding prognosis, it is usually affected by different parameters such as the virus, the host's genetics and the presence of concurrent infections or FeLV-related disease (Hartmann, 2012b). More than 50% of infected cats remain asymptomatic for 2 years; around 20% of infected cats die within the first 2 years after the onset of the follow-up and other 20% develop severe illness after infection (Hartmann, 2009).

2.10. Preventing retroviral infections: the relevance and problematic of vaccination

The most important preventive methods of retroviral infections rely on efficient testing and removal strategies (Hartmann, 2009). More recently, vaccination is also an important preventive tool. From years to years, different research groups have been working and developed vaccines for both retroviruses (Dunham & Graham, 2008). In Portugal, only FeLV vaccination is currently commercialized. However, in the further paragraphs, not only FeLV but also FIV vaccines will be discussed.

FIV Vaccines

In a similar fashion to HIV, the development of FIV vaccine has been very difficult mainly because the infection and evasion mechanisms developed by the virus to the immune system (Dunham, 2006). Different types of vaccines have been tested namely inactivated

vaccines, mutated virus vaccines, cell-infected vaccines or more recently, DNA vaccines, sub-unit vaccines and bacterial vectors (Kohmoto, et al., 1998; Lockridge, et al., 2000; Broche-Pierre, Richardson, Moraillon, & Sonigo, 2005; Dunham, 2006; Dunham & Graham, 2008; Freer, et al., 2008; Pistello, et al., 2005).

Later than for FeLV, the first FIV licensed vaccine is based on cell-infected and inactivated and was released in 2002 in the USA (Uhl, et al., 2002). Nowadays it is commercialized in Canada, Australia and New Zeland. However, it is not available in other countries such as Portugal. The licensed inactivated vaccine (Fel-O-Vax) was produced using a feline cell-line infected with two FIV subtypes (specifically subtype A and D) and adding an adjuvant. This vaccine has been effective in various therapeutical trials and the protection is achieved for around 48 weeks(Pu, et al., 2001; Kusuhara, et al., 2005; Pu, et al., 2005; Huang, Conlee, Gill, & Chu, 2010). Although it does not induce a protective immunity to all the subtypes, some studies have reported some protection against the subtype B (Huang, et al., 2004; Kusuhara, et al., 2005; Pu, et al., 2005; Huang, et al., 2010). In spite of the clinical benefits of vaccination, several studies have proven that it can induce an upregulation of several receptors such as CD134 on lymphocytes (Dunham, et al., 2006; Huisman, Martina, Rimmelzwaan, Gruters, & Osterhaus, 2009). Furthermore, in cases of vaccination of previously infected cats, it can lead to an increase on viral load and accelerate the disease progression. To avoid these situations, all the cats must be tested before vaccination (Sellon & Hartmann, 2012a).

FeLV vaccines

The first FeLV-vaccines dated from the 70's years and were based on live-tumoral cells which, despite being effective, caused neoplasia in vaccinated animals (Hartmann, 2012b). Also based on the same cells, inactivated vaccines were produced and were ineffective once they induced a severe immune suppression. With the development and improvement of inactivated vaccine's industry, the first FeLV vaccine was licensed and released in 1985 (Dunham & Graham, 2008).

Nowadays, 6 FeLV vaccine types are licensed for use. They are based on: entire inactivated virions, inactivated gp70, feline oncornavirus cell membrane antigens, extracts of infected cell-cultures, recombinant proteins (namely p45), DNA vaccines or live canarypox recombinant vaccine (which expresses Gag, Env and proteases) (Osterhaus, et al., 1985; Dunham & Graham, 2008; Hartmann, 2012b). With the exception of this last type, all of the mentioned vaccines have an adjuvant such as cytokines, kemokines and co-stimulators (Dunham & Graham, 2008). These adjuvants improve the immune response to vaccines. In detail, one study reported that a DNA vaccine of FeLV (containing gag, pol and env genes)

with adjuvants such as IL-12 and IL-18 were effective on prevention of persistent and transient viremia (L. Hanlon, et al., 2001).

Routine vaccination guidelines recommend two SC doses for initial protection and an annual or tri-annual booster (Richards, et al., 2006; Jirjis, et al., 2010; Scherk, et al., 2013). In experimental infected cats, all the vaccines tested did not show to prevent transitory viremia, viral replication or even the integration of provirus (Hofmann-Lehmann, et al., 2006). However, although further studies are required to fully understand its protective role, it is believed that vaccination can limit viral replication, reducing viremia and proviral load (Hofmann-Lehmann, et al., 2006; Hofmann-Lehmann, et al., 2007). Considering that there is no increase on VNAs before viral challenge in vaccinated animals, it seems reasonable to state that VNAs do not seem to be the main basis of this protective immunity (Hawks, et al., 1991; Pedersen, 1993). Then, this can be possibly due to the effect of CTLs and cell-mediated immunity which is stimulated by vaccination (Flynn, et al., 2000; L. Hanlon, et al., 2001).

As many other vaccines, FeLV vaccine efficacy and safety is not completely understood. Indeed, various studies have been relating FeLV vaccination and the development of feline injection site sarcomas (FISS) between 4 months to 2 years after vaccination (with a median around 1 year) (Kirpensteijn, 2006; Dunham & Graham, 2008). Although it cannot be confirmed, it is possible that the granulomatous inflammation induced at the injection site may justify this relationship (Carroll, Dubielzig, & Schultz, 2002). To make eventual therapy excision and amputation easier, particularly in USA, several guidelines recommend to administer FeLV vaccine in the left rear leg and rabies on the right ("right for rabies, left for leukemia") (Richards, et al., 2006; Shaw, et al., 2009). However, several findings confirmed that, despite this policy, FISS still occurred in the interscapular region and this association has been discredited (Shaw, et al., 2009). Even though, considering a possible correlation with the adjuvant used, vaccines without adjuvant is always preferred (Hartmann, 2012b).

Concerning the practical decisions about when and whether a cat should be vaccinated or not, it is reasonable to defend that only cats at risk of infection should be vaccinated. In general, according to the recent guidelines, vaccination should be done in two initial booster separated from 4 weeks, followed by a 2-3 year booster plan. Household cats living exclusively indoor do not require this vaccine (Hartmann, 2012b; Scherk, et al., 2013).

2.11. Retroviral infections and the public health perspective

Although neither FIV nor FeLV are zoonosis, they have been discussed about their potential effect in public health.

Regarding FIV, only one study reported that after performing an autologous transfusion with infected in-vitro peripheral blood mononuclear cells (PBMCs) with an FIV-isolate, monkeys developed disease (Johnston, Olson, Rud, & Power, 2001). Even though, no relation was established between FIV and HIV infections. Humans who were bitten by FIV-infected cats or professionals who were iatrogenically exposed to the virus did not develop antibodies against it (Butera, et al., 2000; Sellon & Hartmann, 2012a). Therefore, FIV is not a truly concern in the public health perspective.

Also for FeLV, its transmission to humans is discussable. In fact, virus does not grow in human bone marrow cell lines (Morgan, Dornsife, Anderson, & Hoover, 1993). However, one study have documented a possible causal relationship between FeLV infected cats and childhood leukemia (Bross & Gibson, 1970). Surprisingly, veterinarians were shown to be more prone to die from leukemia rather than a control population from other professions (Blair & Hayes, 1980; Hartmann, 2012b). However, no viremia or bone marrow infection with FeLV was detected in humans with leukemia (Nowotny, et al., 1995). This relation can be explained by the natural immune suppression of humans with leukemia, which can be potentiated by the direct contact with cats particularly when animals have zoonotic infections. In spite of these particular findings, FeLV is not a zoonotic disease being irrelevant for the public health policies (Hartmann, 2012b).

Part I - Chapter III:
The role of Interferon in Veterinary Medicine

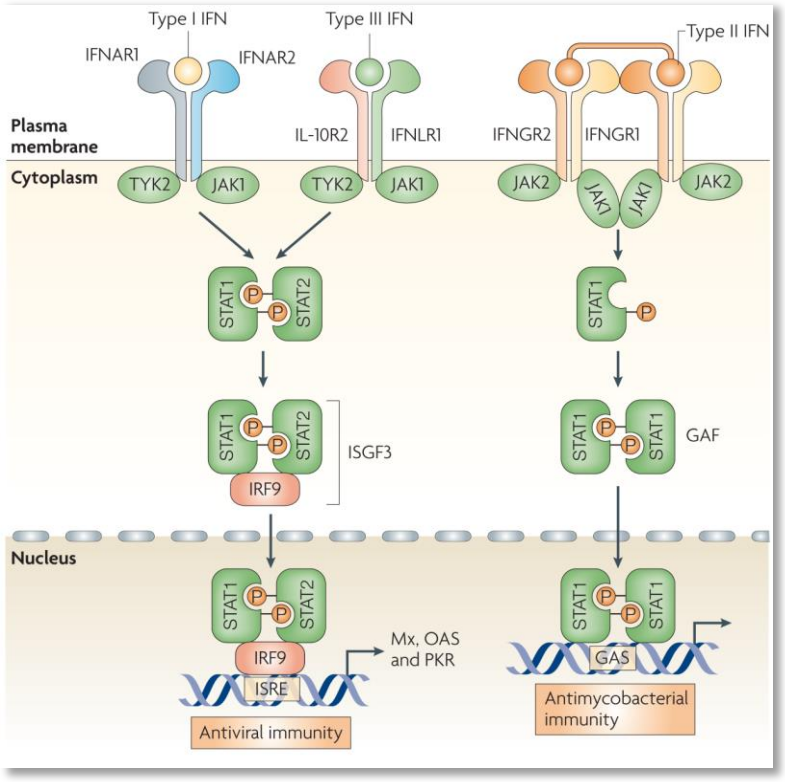
3.1. Interferon: molecular features and actions

Interferons (IFNs) are polypeptides produced by cells of vertebrates after the direct contact with various pathogens such as viruses, bacterias or even molecules such as double chain RNA or bacteria (Hartmann, 2012a). Being a family of cytokines, they are key components on the activation of the innate immune system being particularly relevant in viral infections (Sadler & Williams, 2008).

In a molecular point, IFNs can be divided into three classes (I, II and III), taking into account the receptor complex where they bind (Sadler & Williams, 2008). Type I IFNs are well-studied. They bind to a receptor complex (IFNAR), composed of two sub-units namely IFNAR1 and IFNAR2, ubiquitously expressed. When type I IFN binds to this receptor complex, it activates a signal transduction pathway that induces more than 300 IFN-stimulated genes with different antiviral properties (Der, Zhou, Williams, & Silverman, 1998). For this reason, this class of IFNs seems to be particularly relevant in viral infection (Dupuis, et al., 2003; U. Muller, et al., 1994; Sadler & Williams, 2008). Among their major functions, type I-IFNs act as positive feedback inducers, increasing the expression of Toll-like receptors (TLRs) and sensitizing cells to microbial recognition (Siren, et al., 2005). They establish a truly relation between innate and adaptive immunity, inducing the differentiation of important cells (Colonna, et al., 2004). They are believed not only to block viral replication but also to slow the growth of infected cells making them more susceptible to apoptosis (Goodbourn, et al., 2000; Bracklein, et al., 2006).

Type II IFNs bind to another receptor named IFNGR. The single known type II IFN is IFN-γ, which is produced by natural killers and T-cells after activation and is involved on the immune response but mainly to other pathogens than viruses (Goodbourn, et al., 2000; Sadler & Williams, 2008). Type III IFN refers to another class in which IFN-λ is included that binds to IL10-receptor 2 (IL-10R2) and IFNLR1 and seem to be involved mainly on the regulation of the antiviral response (Haller, Kochs, & Weber, 2007; Sadler & Williams, 2008). Their schematic representation and the respective mechanisms of action are illustrated on figure 7.

Figure 7: Different Interferon types and main cellular mechanisms of action (in Sadler 2008).



3.2. The therapeutic role of interferon in retroviral infections: from immune modulation to antiviral therapy

Due to its referred actions, type I-IFNs are well-studied and are frequently used as part of the therapeutic approach in various diseases not only in humans but, more recently, in veterinary medicine.

In humans, around 13 subtypes of type I-IFNs are known (Sadler & Williams, 2008). Among them, HuIFN-α also called leukocyte interferon is particularly relevant once it can be produced by recombinant DNA technology and is commercially available as an immune modulator drug (Hartmann, 2012a).

In veterinary medicine, the most relevant type I IFN is rFeIFN-ω which was recently developed and is currently licensed for use as an immune modulator not only in cats but also in dogs. Although it is commonly used, in countries where it is still unavailable, HIFN-α is an immune modulator resource in small animal medicine.

In the further paragraphs, both compounds and the respective therapeutic actions will be discussed.

The use of Human Interferon-alpha (HuIFN-α) in retroviral infections

Among their major functions, it was showed that HuIFN-α inhibits oncogenic mutations induced by retroviruses, it restrains viral nucleic acid synthesis and suppress protein production. Due to these anti-viral and immune modulation properties, HuIFN-α is used in various human diseases such as myeloid leukemia, papilomatosis and HIV.

For the same reasons, HuIFN-α has been also used in feline medicine namely in FIV, FeLV, Feline Herpesvirus (FHV-1), FCV and Feline Coronavirus (FCoV) viral infections (Fulton & Burge, 1985; Weiss & Oostrom-Ram, 1989; Zeidner, et al., 1990; Collado, Doménech, Gómez-Lucía, Tejerizo, Miró, 2006; Pedretti, et al., 2006).

Two distinct protocols can be distinguished in feline medicine: the parenteral high dose [(10^4-10^6 IU/kg once a day (SID)] and the oral low dose (1-50IU/kg SID). Considering its species-specific potential, when the high dose protocol is administered there is a development of neutralizing antibodies, which occurs within 3-7 weeks after the onset of therapy. Due to this reason, the immune modulator action of HuIFN-α is limitative and long-.term ineffective. In contrast, when HuIFN-α is given orally, it can be administered for a longer period without the arising of neutralizing antibodies. This can be explained mainly by the lower dose and the route of administration (Zeidner, et al., 1990).

Concerning retroviral infections, until the release of rFeIFN-ω, HuIFN-α were used frequently in FIV and FeLV-infected cats. Nowadays, it is still an immune modulator resource in countries in which rFeIFN-ω is not available.

In FIV-infected cats, there are not many control-studies about the truly effect of HuIFN-α. In detail, the recommended high dose protocol was efficient for up to 6-7 weeks, the time at which antibodies developed (Hartmann, 2012a). Its parenteral use seemed to have a more efficient antiviral effect rather that the oral protocol (Schellekens, Geelen, Meritet, Maury, & Tovey, 2001). Although more commonly applied in clinical practice, the oral protocol (recommended for FIV on the dosage of 50IU/cat SID in cycles of 7 days alternating with 7 days off for 6 months followed by a withdrawal of therapy during 2 months and a repeated cycle of 6 months), is destroyed in the GI tract by gastric acid, trypsine and other proteolytic enzymes (Hartmann, 2012a). Consequently, it is not absorbed and cannot be detected on blood samples (Cantell & Pyhala, 1973). However, it improves the clinical status of animals and increases the survival time, which seems to be related to a direct local stimulation of the oral lymphoid tissue (Tompkins, 1999; Pedretti, et al., 2006). In fact, it seems to directly stimulate the lymphoid local tissue and pharyngeal area, leading to a cascade of immune

modulation with up-regulation of IFN-α and a downregulation of IL-4, which can spread for a systemic effect (Tompkins, 1999). Even though, viral load did not change. This means that the observed clinical improvement was mainly due to a reduction on opportunistic infections (Pedretti, et al., 2006).

In FeLV, it was described that HuIFN-α has an in vitro antiviral activity. It was shown that this compound inhibits viral replication in a FeLV-infected cell line (FL74 cell-line) by decreasing RT activity, increasing apoptosis in infected cells but without affecting viral protein expression (Collado, et al., 2007). In clinical practice, similarly to FIV therapy, two protocols can be performed: the high-dosage SC injection (10^4-10^6 IU/kg SID) or the low-dosage oral protocol (1-50IU/kg SID) (Hartmann, 2012b). In a similar fashion to FIV, high parenteral doses lead to the development of neutralizing antibodies. Similarly to what was previously referred for FIV, when given orally, its mechanisms of action is mainly in the local tissue, being also destroyed by the GI tract and undetected in blood (Tompkins, 1999). The success of low-dose protocol is mainly based on mimic natural defense processes (Cummins, Tompkins, Olsen, Tompkins & Lewis, 1988).

In a practical approach, one study compared the effect of high dose HuIFN-α protocol, an antiviral (AZT) and a combination of both drugs in FeLV-positive cats with high levels of virus but pre-symptomatics (Zeidner, et al., 1990). This study showed that HuIFN-α alone or in combination with AZT was associated to a significant decrease on seric p27 for 2 weeks after the beginning of therapy. Then, the development of anti-HuIFN-α-antibodies 3-7 weeks later made the therapy unsuccessful. Regarding clinical improvement, however, HuIFN-α high dose therapy did not induce a significant improvement of clinical signs (Hartmann, 2012a).

Another placebo therapeutic trial evaluated the effect of two oral doses of HuIFN-α in FeLV-cats (0.5IU/cat in 8 cats and 5IU/cats in 5 cats PO SID in cycles of 7 days alternating with 7 days without therapy during 1 month) (Cummins, et al., 1988). No changes were observed on the viremia levels between groups. However, treated animals revealed a clinical improvement and an increase on survival time. Surprisingly, cats treated with 0.5IU/cat showed a better overall response. More recently, a larger study showed that 69 FeLV-infected cats treated with 30IU/kg SID in alternated weeks had a longer survival time than the historic control groups (Hartmann, 2012a; Weiss, Cummins, & Richards, 1991). Conversely, other study on ill-client owned FeLV-infected cats failed to prove that cats treated either with low dose HuIFN-α protocol (30 IU/cat 7 days in alternated weeks) alone or in combination with other immune modulator (Staph A), revealed a significant clinical improvement or changes on survival time (McCaw, et al., 2001).

In sum, despite the fact that higher protocols may have a short-term benefits, the use of oral low dose HuIFN-α protocol is more often performed in feline medicine due to the fact that it

can mimic the immune response towards the non-self interferon, having an evident immune-modulator effect (Schellekens, et al., 2001).

The use of Recombinant-Feline Interferon Omega in Retroviral infections

As it is called, rFeIFN-ω is a recombinant product produced by a baculovirus which contains the feline IFN sequence. This baculovirus replicates in silkworms after their infection, leading to the production of rFeIFN-ω which is therafter purified. It is currently the only interferon produced for veterinary medicine. Although it is still not available worldwide, it is currently licensed in Europe, Australia and in some Asiatic countries such as Japan (Hartmann, 2012a).

In spite of its recent development, rFeIFN-ω has multiple functions. *In vitro*, it was shown to have important antiviral, anti-tumoral, antiproliferative and anti-colony effects. In clinical practice, it has been used in different situations namely in viral infections (including FIV and FeLV), feline vaccinal sarcomas and other neoplasias (Priosoeryanto, Tateyama, Yamaguchi, & Uchida, 1995; Tateyama, et al., 1995).

Although the similar pharmacokinetic properties between HuIFN-α and rFeIFN-ω, this last one does not induce the production of neutralizing antibodies in cats, once it is a feline product. Consequently, it can be used for longer periods in cats (Hartmann, 2012a).

Since its release, various protocols have been developed, without the report of any adverse effects. In general, for acute canine and feline infections, the recommended starter protocol is $2.5x10^6$ IU/kg IV or SC, SID during 3 consecutive days. In chronic infections, the 10^6 IU/kg SID in cycles of 5 consecutive days is the described protocol (Hartmann, 2012a). Although these general recommendations, in feline medicine, various protocols have been applied in different viral diseases.

Research results have shown that rFeIFN-ω has an anti-viral effect for FHV-1, FCV, FCoV and FPV *in vitro* (Mochizuki, Nakatani, & Yoshida, 1994; Siebeck, et al., 2006).

Concerning its routine use in feline medicine, *in vivo*, it was shown to be effective in FHV-1 (Haid, et al., 2007). In FCV infections, one study described that its oral administration in a low dose (0.1MU/cat SID 90 days) was as effective as steroid therapy in cats with refractory feline gengivostomatitis complex (Hennet, Camy, McGahie, & Albouy, 2011). In FCoV, rFeIFN-ω did not change the mean survival time of treated cats in comparison to a placebo control (Ritz, Egberink, & Hartmann, 2007). Although studies about its action in panleukopenia cases are scarce, it was documented that rFeIFN-ω induces a subtle improvement of clinical signs in dogs infected with parvovirus (Ishiwata, Minagawa, & Kajimoto, 1998).

Specifically regarding retroviral infections, the recommended protocol consists of 3 cycles of 5 daily injections of 1 MU/kg beginning on days 0, 14 and 60, respectively (de Mari, et al., 2004; Hartmann, 2012a). It was shown that rFeIFN-ω inhibits FeLV viral replication *in vitro*, decreasing viability and increasing apoptosis of infected cells (Rogers, Merigan, Hardy, Old, & Kassel, 1972). Although studies *in vivo* are scarce, it was proven that the referred subcutaneous rFeIFN-ω protocol induces an increase on survival time in FeLV and FIV/FeLV co-infected animals (de Mari, et al., 2004).

More recently, a spanish group evaluated the effect of the licensed protocol in FIV and FeLV infected cats having showed that it does not significantly change viremia, proviral load, RT activity or CD4/CD8 ratio (Domenech, et al., 2011). Even though, a significant clinical improvement was documented. This means that rFeIFN-ω does not seem to have an anti-viral effect *in-vivo* and the observed clinical improvement must be related to a potential immune modulation of the innate immune system (Domenech, et al., 2011). Further studies are then required to fully understand the truly action of rFeIFN-ω in retroviral infected cats.

PART II

EXPERIMENTAL WORK

PART II - CHAPTER I:

Relevance of feline interferon omega for clinical improvement and reduction of concurrent viral excretion in retrovirus infected cats from a rescue shelter

Chapter I: Relevance of Feline Interferon omega for clinical improvement and reduction of concurrent viral excretion in retrovirus infected cats from a rescue shelter

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Abstract

Feline Immnunodeficiency (FIV) and Feline Leukemia (FeLV) viruses are common infectious agents in stray cats and shelter environments. Recombinant feline interferon-ω (rFeIFNω) has shown an antiviral action not only against FIV and FeLV but also against herpesvirus (FHV-1) and calicivirus (FCV).

16 naturally infected FIV/FeLV cats were followed during rFeIFNω therapy in order to monitor clinical signs and to correlate with excretion of concomitant viruses (FCV, FHV-1, feline coronavirus (FCoV) and parvovirus (FPV)). Cats were submitted to clinical evaluations and concomitant virus excretion assessement.

Comparing D0 to D65, 10/16 cats improved clinical scores. Of the 10 cats positive for FHV-1 on D0, 4 were negative and 6 reduced viral loads. Of the 11 FCoV positive cats, 9 reduced viral loads. The 13 FCV positive cats and the FPV positive cat were negative on D65.

In conclusion, rFeIFNω improves clinical signs and reduces concurrent viral excretion in naturally infected retroviral cats.

Keywords: Feline Immunodeficiency Virus, Feline Leukemia Virus, Interferon, Therapy, shelter, Feline Herpesvirus, Feline Coronavirus, Feline Calicivirus

Introduction

FIV and FeLV are two important retroviruses that infect domestic cats (Hosie, et al., 2009; Lutz, et al., 2009). Their prevalence differs according to geographic regions and indoor/outdoor status (Norris, et al., 2007; Gleich, et al., 2009). Common risk factors are geriatric cats, male gender, mixed breeding, aggressive behavior, co-infection with other retroviruses and outdoor contact with non-hierarchical cat communities (Murray, Roberts, Skillings, Morrow, & Gruffydd-Jones, 2009). Even with more sensitive diagnostic tests and a good therapeutic approach, retroviruses remain a problem among animal rescue shelters (Hosie, et al., 2009; Lutz, et al., 2009).

The clinical signs observed in cats infected with these retroviruses are nonspecific and mainly due to immune suppression (Hartmann, 2011). In FeLV infected cats, clinical signs usually develop in viraemic animals some months to years after the infection (Lutz, et al., 2009). In FIV infected cats, most of the clinical signs are not directly caused by the viraemia, but they result from secondary infections (Gleich & Hartmann, 2009). Furthermore, some FIV-infected cats may even show clinical signs that result from imbalanced stimulation of parts of the immune response, such as immune-mediated glomerulonephritis (Hosie, et al., 2009). Although many retroviral infected cats go on to develop clinical signs, others may remain in good health for several years (Hosie, et al., 2009).

The immune suppression induced by retroviruses may predispose cats to clinical infection with multiple opportunistic agents to which they would normally be resistant. Moreover, it is also possible to trigger an exacerbated response to some common bacterial, fungal and protozoal pathogens, occasionally (Dunham & Graham, 2008; Reche, et al., 2010). In rescue cat shelters, viruses such as FHV-1, FCV, FCoV and FPV are also important infectious agents to consider. They are particularly exacerbated when incoming animals are introduced to the shelters. However, even in stable resident shelters, intermittent excretion of these viruses may contribute to continuous spreading to the environment. These concurrent viral infections are potentiated in retroviral infected shelters and easily contribute to a general worsening of the clinical condition of infected cats (Addie, et al., 2009; Radford, et al., 2009; Thiry, et al., 2009; Truyen, et al., 2009). General management of retroviral infected cats should include an isolation policy, neutering and regular health check-ups, particularly in rescue shelters. For symptomatic cats, supportive general treatment should always be considered (Hosie, et al., 2009; Lutz, et al., 2009). Antiviral and immune modulation therapies are important options that should also be considered (Collado, Doménech, Gómez-Lucía, Tejerizo, Miró, 2006).

Recognizing the similarity between FIV and HIV, there are multiple drugs such as zidovudine, fozivudine or human Interferon-α, commonly used in humans, that can be

applied in retroviral infected cats. However some of these drugs can have significant side effects (Domenech, et al., 2011; Fogle, Tompkins, Campbell, Sumner, & Tompkins, 2011). Interferons are a family of species-specific compounds that act not only as anti-viral drugs but also as immune modulators and anti-tumor agents (Gerlach, et al., 2009; Tompkins, 1999). They can be classified in type I or type II IFNs according to their biological properties (Pestka, Krause, & Walter, 2004; Collado, Doménech, Gómez-Lucía, Tejerizo, Miró, 2006).

Type I IFNs are produced by virally infected cells and have immunomodulating effects (Domenech, et al., 2011). This is due to interaction with specific cell-receptors and concurrent induction of the expression of specific genes that encode cytokines involved in innate immunity. Moreover, type I IFNs also have anti-viral effects, anti-proliferative and anti-inflammatory actions (Domenech, et al., 2011; Gerlach, et al., 2009; Gerlach, Schimmer, Weiss, Kalinke, & Dittmer, 2006). Type II IFNs are mainly immunomodulatory with only a low level of anti-viral effects, meaning that they are less useful in clinical practice. Currently there are two important IFNs used in veterinary medicine: Human Interferon-α and Recombinant Feline Interferon-ω (rFeIFNω), both of them type I IFNs (Collado, Doménech, Gómez-Lucía, Tejerizo, Miró, 2006).

Although it was proven that Human Interferon-α increases survival time in FIV and FeLV-cats (Weiss, et al., 1991; Pedretti, et al., 2006), the development of specific neutralizing antibodies may decrease its efficiency (Zeidner, et al., 1990; Muller, 2002). More recently, rFeIFNω was licensed for use in veterinary medicine, namely for treatment of canine parvovirus and feline retroviral (FIV and FeLV) infections. As an homologous feline molecule, it has a good safety index and does not induce production of neutralizing antibodies (de Mari, et al., 2004). However, despite its license, there is limited published information about the use of rFeIFNω in retroviral infections. Initially, its use in asymptomatic FIV cats was described (Caney S., 2003) but the first conclusive results were provided by a study that revealed a clinical improvement and an increased survival time in FeLV and co-infected symptomatic cats (de Mari, et al., 2004). A more recent study demonstrated that rFeIFNω improves the clinical condition and haematologic parameters not only in FeLV but also in FIV infected cats (Domenech, et al., 2011). While there are few in-vivo studies to support its expected benefits, the use of rFeIFNω has been extended to other viral infections namely FHV-1, FCoV, FCV and FPV (Ishida, Shibanai, Tanaka, Uchida, & Mochizuki, 2004; Paltrinieri, Crippa, Comerio, Angioletti, & Roccabianca, 2007; Ritz, et al., 2007; Thiry, et al., 2009; Hennet, et al., 2011).

This study aims to evaluate the role of rFeIFNω on clinical improvement of naturally infected FIV and FeLV cats living in a rescue shelter and to clarify whether this therapy also reduces concurrent viral excretion of FCV, FHV-1, FCoV and FPV.

Materials and Methods

Animals

Sixteen neutered domestic short-hair cats (11 males and 5 females), living in a Lisbon animal shelter and previously determined as FIV and/or FeLV positive status were selected for the study. Nevertheless, at inclusion, all the cats were retested to confirm their FIV/FeLV infections by ELISA (ViraCHEK/FIV and ViraCHEK/FeLV, Synbiotics).

The cats were living in good conditions, in agreement with current ethical and welfare standards. All the procedures involving the manipulation of these animals were consented and approved not only by the Committee for Ethics and Animal Welfare of the Faculty of Veterinary Medicine - Technical University of Lisbon (CEBEA) but also by the clinical direction of the referred animal shelter (União Zoófila de Lisboa).

Taking into account that animals' origins were unknown, age and past information were considered irrelevant for this study. Nevertheless, all the animals were adults with ages estimated in a range of 3 to 8 year old. Animals were housed in two different catteries, correlated with their FIV or FeLV status. Due to previous shelter facilities, Co-infected animals were housed in the FeLV cattery. Subsequently, cats were divided in three different groups according to their retroviral status: FIV positive cats (n=7; 5 males and 2 females), FeLV positive cats (n=6; 4 males and 2 females) and Co-infected animals (n=3; 2 males and 1 female). Based on previous studies (de Mari, et al., 2004), the inclusion criteria were the following: 1) cats of any age, breed or sex (heterogeneous population), 2) cats that showed at least one clinical sign potentially related to retroviral infections, 3) cats that had previously had a positive rapid immune-migration FIV/FeLV test result. Exclusion criteria were: 1) cats that showed clinical signs of malignancy (such as Lymphoma or Lymphoid leukemia), 2) cats having received immunomodulating drugs (such corticotherapy) during the 4 weeks before the study 3) cats having received antibiotics or non-steroidal anti-inflammatory drugs during the 2 weeks before the study and 4) cats that did not complete the therapeutic protocol.

Products

Vials of rFeIFNω (Virbagen Omega; Virbac) were reconstituted with the accompanying saline diluent according to the manufacturer's recommendations immediately before each treatment.

Treatment Protocol

Based on assumptions derived from two previously published double arm trials with rFeIFNω (de Mari, et al., 2004; Domenech, et al., 2011), a single arm study was performed.

All the animals were treated with rFeIFNω, according to the licensed protocol (3 cycles of injections at Day (D) 0, D14 and D60. Each treatment cycle consists of 5 subcutaneous injections: 1MU/kg once per day for 5 days.

Treatment was administered by two veterinary clinicians from the research team.

Assessments before therapy were designated in our report as D0 and considered representative of the stage of each animal before treatment.

Supportive Treatment

Despite the exclusion criteria applied, some animals needed supportive treatment during therapy. Consequently, potentiated amoxicillin, hepatic protectants (ursodeoxycholic acid, Sylimarin or S-Adenilmethionine) and/or fluid therapy were allowed. Antibiotics (other than potentiated amoxicillin), corticosteroids and non-steroidal anti-inflammatory drugs were not permitted to avoid any possible immunomodulation effects.

Clinical evaluation and Scoring

At D0 (before therapy), D10, D30 and D65 after the beginning of the protocol, all the cats were submitted to regular clinical evaluations.

In order to reduce subjectivity, the findings of the clinical evaluations were scored according to a clinical-score scale (table 2). This scale included the most important clinical parameters typically presented in retroviral infections (figure 8) such as oral ulcers/gingivitis (score 0-2), caudal stomatitis/palatitis (score 0-2), ophthalmic abnormalities (score 0-2), lymphadenopathy (score 0-2), ocular and nasal discharge (score 0-2), mucous membrane color (score 0-2), coat appearance (score 0-1), body score (score 0-2), faecal appearance (score 0-1) and concurrent diseases/co-morbidities (score 0-2). After each parameter was assessed, a sum score of 11 criteria was obtained for each animal to reflect the overall clinical condition of the animal. These scores were then compared at each time point.

Table 2: Clinical Score - scale used for cats' clinical evaluation

Clinical Parameter	Classification
Oral Ulcers/ Gingivitis	0 – No evidence of oral lesions +1 – mild to moderate oral lesions +2 – severe oral gingivitis
Caudal Stomatitis/ Palatitis	0 – No evidence of caudal stomatitis +1 – mild to moderate hyperemia and caudal stomatitis +2 – severe hyperemia and caudal stomatitis
Ophthalmology abnormalities	0 – no evidence of ophthalmology changes +1 – mild conjunctival hyperemia (mainly unilateral), mild keratitis +2 – severe conjunctival hyperemia (mainly bilateral), active keratitis
Lymphadenopathy	0 – no evidence of lymphadenopathy + 1 – mild localized lymphadenopathy +2 – generalized lymphadenopathy
Ocular discharge	0 – no evidence of ocular discharge +1 – serous ocular discharge +2 – muco-purulent ocular discharge
Nasal discharge	0 – no evidence of nasal discharge +1 – serous nasal discharge +2 – muco-purulent nasal discharge
Pale Mucous membranes	0 – no evidence of pale mucous membranes +1 - mild pale mucous membranes +2 – severe pale mucous membranes
Dry Coat/Seborrhea	0 – normal coat condition +1 – dry coat and/or seborrhea
Body Condition Score	0 – normal or fat: body condition score 4/6 to 6/6 +1 – mildly reduced body condition score 3/6 +2 – underweight animal with a body score of 1/6-2/6
Faecal appearence	0 – no evidence of diarrhea +1 – clinical evidence of diarrhea
Concurrent diseases or Co-morbidities	0 – no evidence of concurrent diseases +1 – clinical evidence of concurrent disease +2 – severe prostration/ global weakness

Figure 8: Clinical parameters observed in some cats evaluated in the study namely subcutaneous abscess (A – Cat 1), oral ulcers/gingivitis (B – Cat 3), caudal stomatitis (C – Cat 10) and ocular and nasal discharge (D – Cat 13).

Blood Sample Collection and Treatment

Blood samples were collected by venipuncture of the jugular vein at the same time as the clinical evaluations (D0, 10, 30, 65). Samples were analysed for complete blood-cell count (CBC), hepatic enzymes (alanine-transaminase, aspartate-transaminase) and renal function (creatinine, urea). CBC and biochemistry was performed on Cell-Dyn 3700 (Abbott diagnostics division) and Kone Optima 4.2 (Kemia Cientifica) respectively.

To allow a better evaluation and simpler blood sample collections, cats were submitted to mild tranquilization with 0.2-0.5 mg/kg of butorphanol solution (Dolorex, Intervet Portugal), sub-cutaneously.

Survey of concomitant pathogens.

Cats from all groups were checked for FIV antibodies and FeLV antigen by ELISA using serum or plasma at D0 (ViraCHEK/FIV and ViraCHEK/FeLV, Synbiotics).

At each time point (D0, 10, 30, 65) oral swabs for the feline respiratory tract viruses (FHV-1; FCV) and rectal swabs for the digestive tract viruses (FPV, FCoV), were also obtained. Oral and rectal swabs were processed for viral DNA/RNA extraction (QIAamp MinElute Virus Spin Kit, Qiagen, Portugal).

The determination and quantification of concurrent viral excretion were performed using the diagnostic procedures available in the Virology Laboratory of the Faculty of Veterinary Medicine – Technical University of Lisbon.

Screening and quantification of FHV-1 was assessed by RT-qPCR amplification (Applied 7300 instrument, Applied Biosystems), using a specific gene expression kit (TaqMan gene expression Kit - Applied Biosystems). Primers and Cycling conditions are described in tables 3 and 4. Serial tenfold dilutions of the recombinant plasmid DNA were used to estimate the FHV-1 target copy number, using a specific software (7300 System SDS software) and generating a standard curve obtained with 10^1- 10^6 DNA dilution of recombinant plasmids, with a correlation efficiency of r^2=0.997.

FCV presence was assessed using conventional reverse transcriptase PCR (Desario, et al., 2005; Wilhelm & Truyen, 2006) using 20ng of viral RNA. Primers and Cycling conditions are described in tables 3 and 4. A live vaccine (Fevaxyn Pentofel, Pfizer) was used as a positive control.

Table 3: Primer nucleotide sequences used for the amplification of FCV, FHV-1, FPV and FCoV.

Virus	Sequence
FCV [a]	Primer forward: GNA AAG CWC AAC AAA TTG AATT Primer reverse: CHTGTACCCTYTGCTCAAG
FHV-1 [b]	Primer forward: ACGTGGTGAATTATCAGCTGAAG Primer reverse: AAGGTATGGTGCGGCAAATC Probe*: TGCTGCCTATATCACCGCCCACTATCAA*
FPV [b]	Primer forward: CAGGAAGATATCCAGAAGGA Primer reverse: GGTGCTAGTTGATATGTAATAAACA
FCoV [b]	Primer forward: TGGTCATCGCGCTGTCTACT Primer reverse: AGGGTTGCTTGTACCTCCTATTACA Probe*:TTGTACAGAATGGTAAGCAC*

[a] Wilhelm et al., 2006; [b] In house designed

Table 4: PCR and Real Time PCR amplification conditions

Virus	Reverse transcription	Initial denaturation	Denaturation	Annealing	Extension	Last Extension
FCV	42ºC/30mn	95ºC/5mn	95ºC/30sec 35 Cycles	52ºC/30sec 35 Cycles	68ºC/30sec 35 Cycles	68ºC/10mn
FHV-1		95ºC/10mn	95ºC/15sec	60ºC/1mn 45 Cycles		65ºC-95ºC (0.1ºC/s ramp rate)
FPV		95ºC/10mn	94ºC/30 sec 40 Cycles	50ºC/1 mn 40 Cycles	68ºC/1mn 40 Cycles	68ºC/10mn
FCoV	48ºC/15mn	95ºC/10 mn	95ºC/15sec	60ºC/1mn 45 Cycles		65ºC-95ºC (0.1ºC/s ramp rate)

FPV presence was assessed using conventional PCR (Desario, et al., 2005; Wilhelm & Truyen, 2006). 10 ng of viral DNA was used to amplify sequences within the VP2 gene. Primers and cycling conditions are described in tables 3 and 4.

For screening and quantification of FCoV, primers and probe (TaqMan, Applied Biosystems) were chosen using a specific software (Primer Express, Applied Biosystems), within the 177 bp fragment included in the 3' UTR region of FCoV (Herrewegh, et al., 1995), previously cloned in the pGEM plasmid (Duarte, Veiga, & Tavares, 2009). FCoV quantification was assessed by one step RT-qPCR (TaqMan RNA-to-CT™ 1-Step Kit, Applied Biosystems), using cycling conditions described in table 4. Serial tenfold dilutions of the recombinant plasmid DNA were used to estimate the FCoV target copy number, using the referred specific software and generating a standard curve with a correlation efficiency of r^2=0.997.

Statistical analysis

To compare clinical scores and viral loads between different treatment days, the non parametric Friedman Test was applied. The significance level was set at 5%. For the remaining analyses, the mean values and the respective standard errors were reported.

Results

Clinical evaluation and Scoring

Cats were submitted to clinical evaluation at D0, 10, 30 and 65 after starting therapy with rFeIFNω using a score scale (table 2).

Oral ulcers and gingivitis were the most frequent clinical sign at D0 in all the groups, Caudal stomatitis was also a prevalent finding at the beginning of the study, particularly with FIV positive cats. These were also the clinical signs that improved most consistently with therapy.

Concerning the other parameters, they were variable during therapy and nonspecific fluctuations were observed. The detailed individual clinical scores are recorded in tables 5-7.

Considering the total values of clinical scores over the course of the study, FIV and Co-infected cats improved homogeneously during therapy. Conversely, FeLV cats showed important fluctuations during therapy meaning that, at D10 and 30, the cats' mean scores worsened. Despite these results, comparing total scores of D0 and D65, a global improvement was observed.

Comparing D0 and D65, the beginning and end of treatment respectively, the overall improvement in clinical scores (indicating a better clinical condition) for the 16 naturally retroviral infected cats is statistically significant ($p=0.00066$, Friedman Test). In particular, 10 cats improved their clinical conditions while 6 cats maintained the same clinical status. No cats experienced worsening of their scores.

Regarding the FIV group, the clinical improvement was also statistically significant ($p = 0.025$, Friedman Test). In particular, 4 out of 7 cats showed a marked improvement (final score > 50% better than initial), 1/7 revealed a mild to moderate improvement (final score up to 50% better than initial) and 2/7 remained stable. Concerning FeLV infected cats, the clinical improvement was not statistically relevant ($p= 0.32$, Friedman Test). Nevertheless, it is observed that 3/6 showed a mild to moderate improvement and 3/6 remained with the same initial score. Two out of three co-infected cats showed a marked improvement and one out of three remained stable. However, due to the low number of animals in this group, these results were not statistically significant ($p=0.16$, Friedman Test).

Table 5: Total group and detailed individual clinical score values for each parameter evaluated in FIV cats during rFeIFNω therapy.

DETAILED INDIVIDUAL CLINICAL SCORES																												
	Cat 1				Cat 2				Cat 3				Cat 4				Cat 5				Cat 6				Cat 7			
Clinical Parameter/Day	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65
Oral Ulcers	2	2	2	1	2	2	1	0	2	2	2	2	2	2	1	1	1	1	1	1	2	2	1	0	1	1	1	1
Caudal Stomatitis	2	1	1	1	0	0	0	0	0	0	0	0	1	1	1	1	2	2	2	0	1	1	1	0	0	0	0	0
Ophthalmological abnormalities	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lymphadenopathy	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ocular discharge	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nasal discharge	2	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mucous membranes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dry coat/seborrhea	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Body Condition	2	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0	2	2	1	0	1	1	1	1	0	0	0	0
Faecal Appearance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0
Concurrent Diseases	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	0	0	0	0
TOTAL (individual)	**13**	**10**	**8**	**6**	**2**	**2**	**1**	**0**	**2**	**2**	**2**	**2**	**3**	**3**	**2**	**2**	**7**	**7**	**5**	**2**	**5**	**5**	**4**	**2**	**1**	**1**	**1**	**1**

TOTAL GROUP CLINICAL SCORES				
Day	**0**	**10**	**30**	**65**
Total (sum)	**33**	**30**	**23**	**15**

Table 6: Total group and detailed individual clinical score values for each parameter evaluated in FeLV cats during rFeIFNω therapy

DETAILED INDIVIDUAL CLINICAL SCORES																								
	Cat 8				Cat 9				Cat 10				Cat 11				Cat 12				Cat 13			
Clinical Parameter/ Day	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65	0	10	30	65
Oral Ulcers	2	2	2	1	0	0	0	0	2	2	1	1	1	1	1	1	2	2	2	0	2	2	2	2
Caudal Stomatitis	0	0	0	0	0	0	0	0	2	2	2	1	0	0	0	0	0	0	0	0	0	0	0	0
Ophthalmological abnormalities	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	2	0	1	1	1	1	1
Lymphadenopathy	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Ocular discharge	0	0	1	0	0	0	1	1	1	0	1	0	0	0	0	0	0	2	1	0	1	0	0	0
Nasal discharge	0	0	1	0	1	1	1	1	1	1	1	0	0	0	0	0	0	2	1	0	2	2	2	0
Mucous membranes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0
Dry coat/seborrhea	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	1	0	1	1	1	1
Body Condition	0	0	1	1	1	0	0	0	0	0	2	1	0	0	0	0	0	2	2	1	1	0	0	0
Faecal Appearance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	1	0	0	0	0
Concurrent Diseases	0	0	0	0	1	1	1	1	0	0	1	1	1	1	1	0	0	0	0	0	2	2	2	2
TOTAL (Individual)	**2**	**2**	**5**	**2**	**3**	**2**	**3**	**3**	**6**	**5**	**11**	**4**	**2**	**2**	**2**	**1**	**3**	**14**	**9**	**3**	**11**	**8**	**6**	**6**

TOTAL GROUP CLINICAL SCORES				
Day	**0**	**10**	**30**	**65**
Total (sum)	**27**	**33**	**36**	**19**

Table 7: Total group and detailed individual clinical score values for each parameter evaluated in Co-Infected cats during rFeIFNω therapy.

DETAILED INDIVIDUAL CLINICAL SCORES												
	Cat 14				Cat 15				Cat 16			
Clinical Parameter/Day	0	10	30	65	0	10	30	65	0	10	30	65
Oral Ulcers	2	2	2	0	0	0	0	0	2	2	1	1
Caudal Stomatitis	0	0	0	0	0	0	0	0	2	2	1	1
Ophthalmological abnormalities	0	0	0	0	0	0	0	0	0	0	0	0
Lymphadenopathy	0	0	0	0	0	0	0	0	0	0	0	0
Ocular discharge	0	0	0	0	0	0	0	0	0	0	0	0
Nasal discharge	0	0	0	0	0	0	0	0	0	0	0	0
Mucous membranes	0	0	0	0	0	0	0	0	0	0	0	0
Dry coat/seborrhea	0	0	0	0	0	0	0	0	1	1	1	0
Body Condition	0	0	0	0	1	1	1	1	0	0	0	0
Faecal Appearance	0	0	0	0	0	0	0	0	1	1	1	0
Concurrent Diseases	0	0	0	0	0	0	0	0	1	1	1	1
TOTAL (Individual)	**2**	**2**	**2**	**0**	**1**	**1**	**1**	**1**	**7**	**7**	**5**	**3**

TOTAL GROUP CLINICAL SCORES				
Day	**0**	**10**	**30**	**65**
Total (Sum)	**10**	**10**	**8**	**4**

Haematology

Although some mild fluctuations were observed, hematologic parameters remained within reference ranges during therapy.

One FIV positive cat developed a very mild anemia at D65, which was clinically irrelevant. One FeLV positive cat revealed a moderate anemia also at D65. Mean values for red-blood cells concentration (with standard error (± SE)) are shown in figure 9.

Despite some irrelevant occasional variations, all the animals had normal leucocyte levels during therapy. Mean values (± SE) are displayed in Fig. 10.

Figure 9: Average ± Standard Error of red blood cell count variation in FIV, FeLV and Co-infected cats under treatment with rFeIFNω (reference range is in between 5,0-10,0x10^6/µl, which is represented by continuous black lines). p = 0.32, Friedman Test for comparison of D0 and D65.

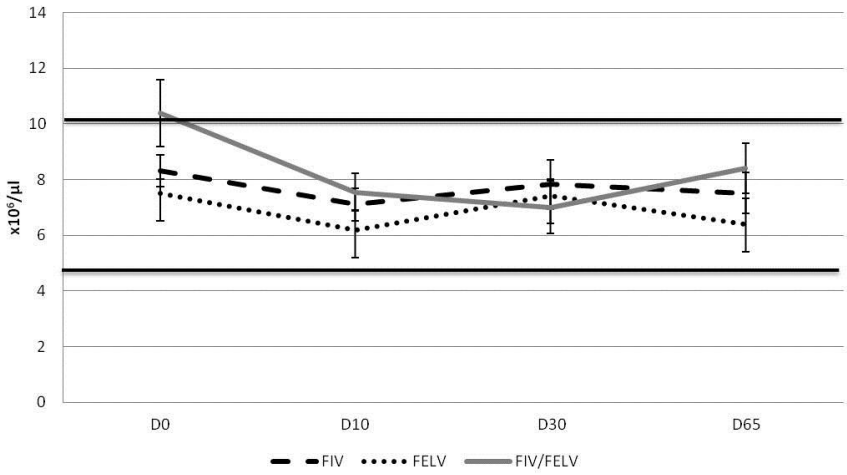
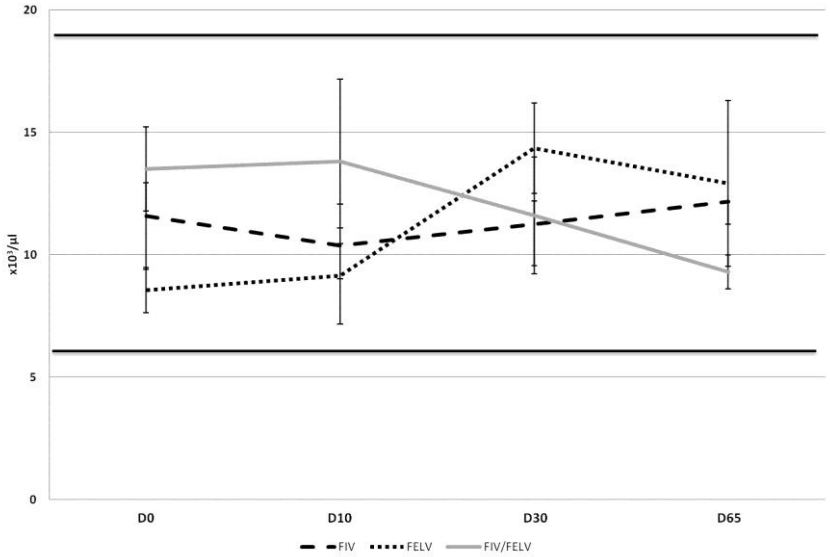


Figure 10: Average ± Standard Error of white blood cell count variation in FIV, FeLV and Co-infected cats under treatment with rFeIFNω (reference range is in between 5,5-19,5x10^3/µl, which is represented by continuous black lines). p = 0.62, Friedman Test for comparison of D0 and D65.



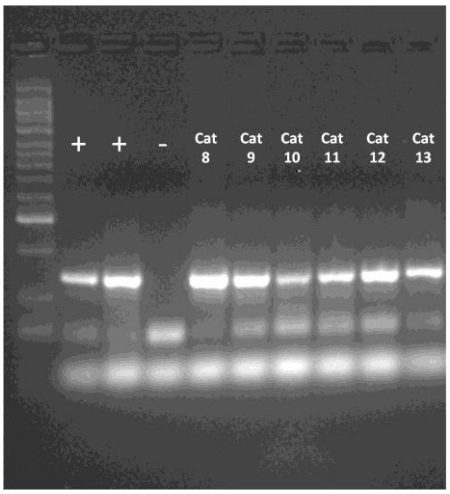
Biochemistry analysis

In all cats, renal parameters and liver enzymes remained stable and within reference range during therapy.

Survey of concomitant pathogens

Oral swabs for FCV detection were processed for viral RNA as described above. Amplification showed that prevalence of FCV shedding was 13/16 (4/7 FIV, 6/6 FeLV (Figure 11) and 3/3 Co-infected) at D0 and 5/16 (1/7 FIV, 3/6 FeLV and 1/3 Co-infected) at D10. At D30 and D65 all the cats tested negative. All the FIV cats that tested positive for FCV (4/7) had gingivostomatitis in contrast with FeLV and Co-infected groups in which not all the FCV positive animals showed this clinical sign.

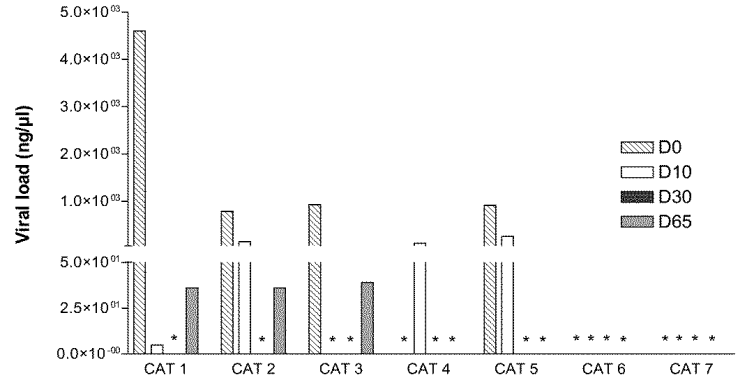
Figure 11: FCV PCR amplification for FeLV group on D0. (+) vaccine positive control. (-) negative control. Cats are listed as presented in table 6.



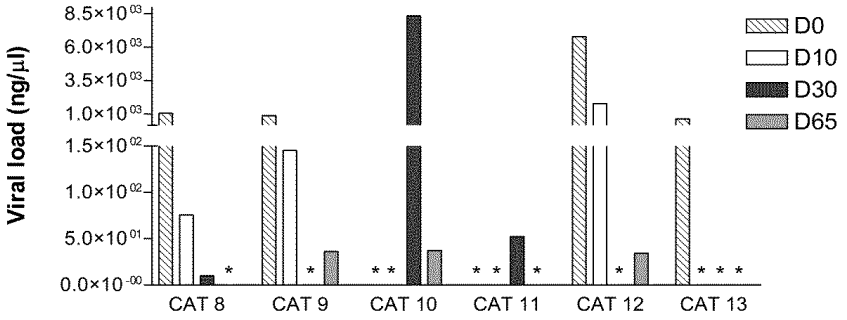
As previously described, oral swabs for FHV-1 were processed for viral DNA extraction. Quantification of FHV-1 revealed 10/16 positive cats (4/7 FIV, 4/6 FeLV and 2/3 Co-infected) at D0. Detailed results are summarized in figure 12. Comparing D0 with D65, 3 of the 4 FIV infected cats reduced their FHV-1 excretion and 1 cat tested negative (figure 12). Also in this group, one cat showed a punctual excretion on D10 but tested negative on D65. In FeLV group, on D65 2 of the 4 FHV-1 positive cats tested negative. The other two were still excreting, although at a lower level than D0 (figure 12). Also in this group, 2 cats revealed a punctual FHV-1 excretion on D30 but on D65 one reduced and the other tested negative. In the Co-infected cats, 2/3 cats were FHV-1 positive. On D65, 1 cat was still excreting FHV-1, at lower levels than D0, and the other cat tested negative (figure 12).

Figure 12: Real-Time PCR viral load quantification (ng/µl) of FHV-1 excretion in FIV, FeLV and co-infected cats under rFeIFNω therapy (D0, D10, D30, D65). p-values are respectively: p=0.046; p=0.18; p=0.16 (Friedman Test). Overall p = 0.0066 (Friedman Test). (*) refers to zero values.

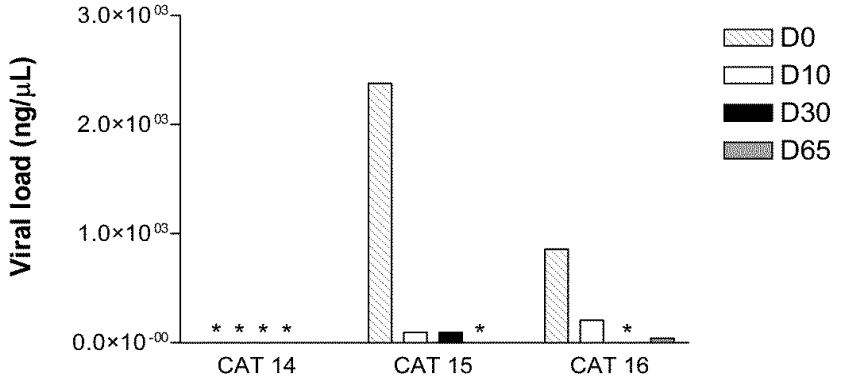
FIV Group/ FHV-1 Excretion



FeLV Group/ FHV-1 Excretion



Co-Infected Group/ FHV-1 Excretion

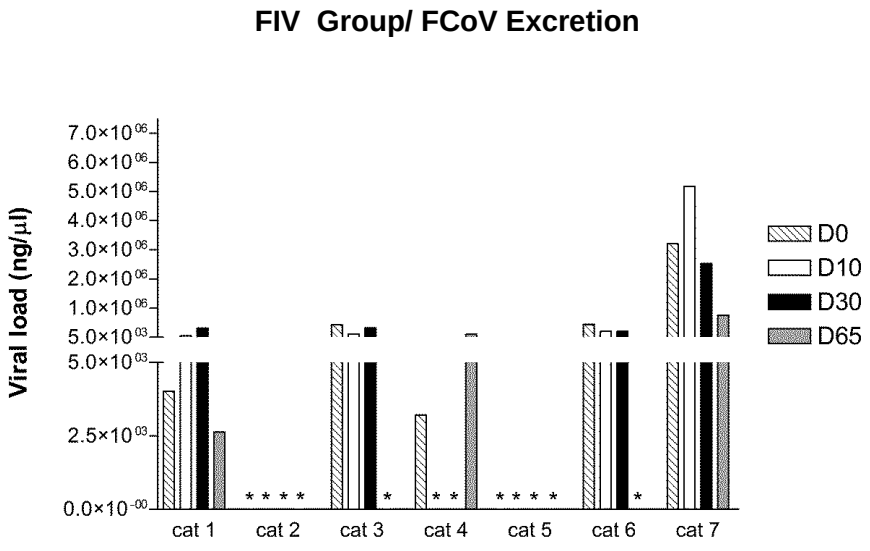


Comparing D0 with D65, the overall reduction of FHV-1 excretion is statistically significant for the 16 naturally retroviral infected cats (p= 0.0066, Friedman Test). When correlating FHV-1 viral status with individual clinical scores, it is observed that 5/16 cats (31%; Confidence Interval 95% =11%; 59%) showed a concurrent clinical improvement and a reduction in FHV-1 excretion.

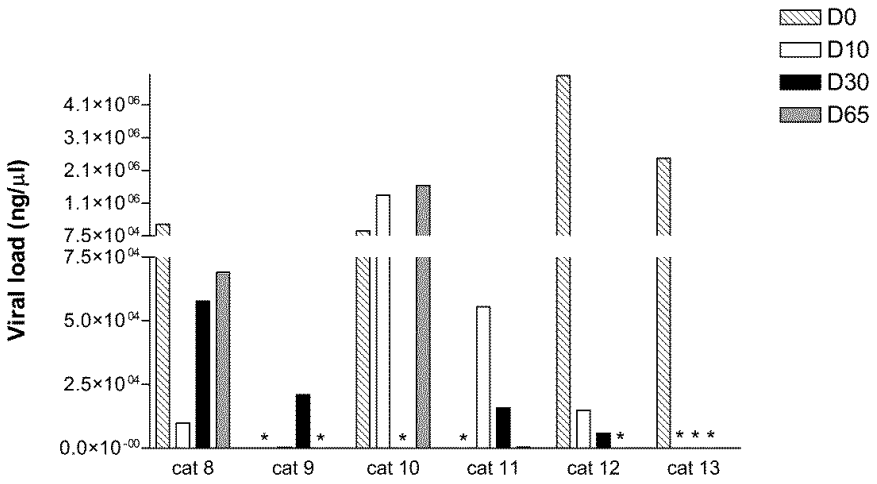
Rectal swabs used for screening and quantification of FCoV showed 11/16 positive (5/7 FIV, 4/6 FeLV and 2/3 Co-infected) on D0. On D65, nine of them decreased their viral excretion. In detail, 4 FIV infected cats reduced their FCoV viral excretion while 1 cat increased it (figure 13). In the FeLV group, comparing D0 with D65, 3 of the 4 FCoV infected cats reduced their viral load, while 1 enhanced it. Two cats which were negative on D0, revealed punctual excretions on D10 and D30 but decreased to zero or values near zero at D65. Both Co-infected cats which were FCoV positive on D0 reduced viral excretion at D65. Interestingly the one Co-Infected cat which was negative for FCoV excretion at D0 revealed an increased excretion at D30, which reduced at D65 (Figure 13). Comparing D0 with D65, overall group results are not statistically significant (p = 0.17, Friedman Test).

Correlating FCoV excretion with individual clinical scores, it is observed that 5/16 cats (31%; Confidence Interval 95% =11%; 59%) showed a concurrent clinical improvement and a reduction in FCoV excretion.

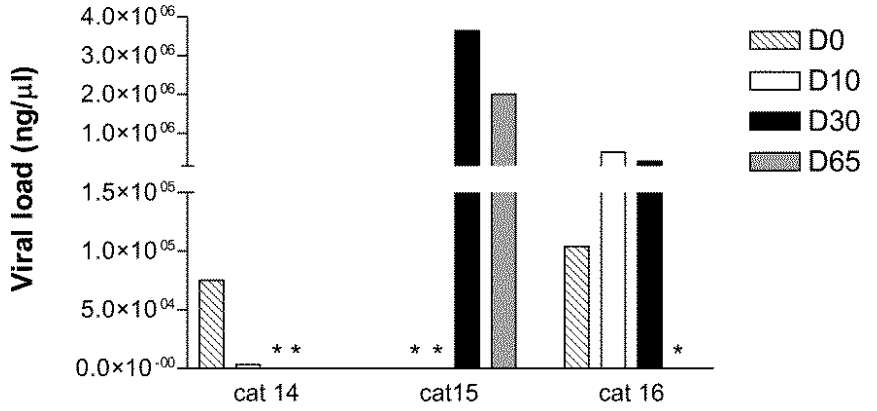
Figure 13: Real-Time PCR viral load quantification (ng/µl) of FCoV excretion in FIV, FeLV and co-infected cats under rFeIFNω therapy (D0, D10, D30, D65). p-values are respectively: p=0.18; p=0.65; p=0.56 (Friedman Test). Overall p = 0.17 (Friedman Test). (*) refers to zero values.



FeLV Group/ FCoV Excretion



Co-Infected Group/ FCoV Excretion



Rectal swabs were also used for FPV screening. At D0 only one FeLV infected cat tested positive by conventional PCR for parvovirus excretion. From D10 till the end of therapy all cats remained negative.

Discussion and Conclusions

FIV/FeLV naturally infected cats were followed before, during and after rFeIFNω treatment. Parameters such as clinical evaluation, haematological analysis, and viral loads were assessed in each individual cat before therapy (at D0). These assessments were designated as D0 and were representative of the stage of each animal before therapy.

As rFeIFNω is a licensed product and its efficacy had been described in multiple double arm studies (de Mari, et al., 2004; Domenech, et al., 2011), a single arm trial was performed in

order to extend our understanding of the improvement of retroviral infected cats under this therapy. A control other than the D0 results, such as a group without treatment or placebo was considered of limited interest and ethically controversial in the context. The control group would be eventually important if we were studying experimental infected animals, where all the cats should be at the same stage of infection. In this case, we have a heterogeneous group of cats and there was difficult to know the point and stage of infection. Even more, these cats were located in a shelter environment where the inclusion of a placebo group was even more controversial ethically. Indeed, the whole point is the therapy in the shelter environment. It is believed that this study reflects the crude reality of cat shelters. In this sense, the decision of establishing a control group before therapy was deliberated and assumed. In our viewpoint, measurements on D0 in the shelter environment are potentially more representative than a placebo with different stages of infection group. Therefore, each cat at day 0 was considered its best untreated control.

Regarding the length of the study, it would be also interesting to evaluate viral excretion a few weeks after the end of the therapy (D65). However, the inclusion of new animals in the catteries could not be excluded after this time-point, being impossible to consider a reliable follow-up of the group viral excretion, when re-infections could occur. Therefore, although the animals were followed after this time-point, sample collections for viral excretion were concluded at this time point.

In accordance with previous studies (de Mari, et al., 2004; Domenech, et al., 2011), the clinical condition of the majority of the cats improved with rFeIFNω treatment. This improvement was more pronounced in cats with higher initial clinical scores. 6/16 cats were mildly symptomatic at the start of the study and remained stable. No cat got worse at the end of the study than at the start. When compared with FeLV cats, FIV cats presented a more evident general improvement. The fluctuations observed with the FeLV cats during the course of the study contributed to the worsening of clinical scores at interim time points for certain cats. Recognizing that rFeIFNω is an immune-modulator, a possible explanation refers to the fact that FeLV cats have a more compromised immune response in comparison to FIV cats (Pardi, Hoover, Quackenbush, Mullins, & Callahan, 1991; Gleich & Hartmann, 2009). This is mainly due to the fact that FeLV infected cats seem to have a more pronounced defect of helper T-cells. Consequently, these animals have a reduced humoral immune response (Gleich & Hartmann, 2009).

Despite the low number of animals, the co-infected group also showed a good improvement when compared with FeLV cats. In those animals, rFeIFNω also seems to have an important imunomodulatory effect, resulting in significant improvement.

The most evident clinical sign at inclusion in all groups was the oral ulcers and gingivitis/gingivostomatitis, both of which improved during rFeIFNω therapy. Although the condition has a multifactorial etiology, rFeIFNω is well known as a prescribed therapy for Feline Chronic Gingivostomatits Syndrome (FCGS) (Dowers, et al., 2010) and these results corroborate the relevance of its use. However it is interesting to note that all previously published studies on the use of rFeIFNω in the management of FCGS have focused on retrovirus negative cats (Hennet, et al., 2011), and this is the first time, to our knowledge, that evidence has been presented for efficacy in FeLV or FIV positive cats.

Previous studies suggested that rFeIFNω is effective on retrovirus-induced anemia in cats (de Mari, et al., 2004) and improves haematological profiles (Domenech, et al., 2011). In this study, all the cats had normal haematologic values on D0 and consequently no significant changes were observed during therapy. Even the FeLV cats, which are often lymphopenic (Gleich & Hartmann, 2009), were normal at baseline and showed no relevant changes during treatment. Two cats (1/7 FIV and 1/6 FeLV) developed anaemia at D65. Nevertheless, both cats were closely monitored after the end of the study and two weeks later CBC were repeated and showed normal values suggesting a temporary irrelevant fluctuation of the values.

Renal function and transaminases were also assessed. All the cats had a normal biochemistry profile and this did not significantly change during therapy. According to the European Medicines Agency's published scientific discussion, rFeIFNω may lead to a temporary leucopenia, thrombocytopenia, anaemia and an increase in alanine aminotransferase. Other than the mild short-term anaemia and sporadic clinical irrelevant trombocytopenias, none of these other side-effects were observed.

One of the main goals of this study was the evaluation of concurrent viral excretion in cats under rFeIFNω therapy. Some authors have suggested that FIV-positive cats that are co-infected with other viruses such as FCV and/or FeLV seem to have a higher prevalence of oral infections and severe oral lesions (Tenorio, et al., 1991). This agrees with the presented results of this study. In fact, all the FIV cats that tested positive for FCV (4/7) had gingivostomatitis. In the FeLV group, 4 of the 6 FCV positive cats had severe gingivostomatitis, 1/6 had mild gingivitis while 1/6 had no observed oral lesions. For Co-infected cats, all 3 animals tested FCV positive and 2 of them had oral signs.
FCV status can also be associated with the extension of gingivitis to the palate and the mucosa lateral to the palatoglossal arches (caudal stomatitis/palatitis). Three out of four FIV/FCV positive cats presented this sign. Even with a low sample size, these findings agree with a previous study which reported that a greater proportion of cats with caudal stomatitis

are FCV positive (Hennet, Boucraut-Baralon C., 2005). In contrast, in FeLV and Co-infected animals, only one cat in each group presented with caudal stomatitis.

Recently, a study described the use of rFeIFNω by oral administration in refractory cases of caudal stomatitis (Hennet, et al., 2011). To the authors' knowledge, there are no studies that describe the use of rFeIFNω, in its licensed protocol, in concurrent retroviral and FCV-infected cats. 13/16 animals were positive for FCV at D0 (4/7 FIV, 6/6 FeLV and 3/3 Co-infected). At D30 and D65, all cats tested negative for FCV, meaning that rFeIFNω, administered according to this protocol, was associated with a remission of FCV excretion in these cats. Furthermore, only 3 of those cats revealed persistent oral lesions at the end of therapy. The remaining FCV positive animals improved their gingivostomatitis and palatitis. Although FCGS may have a multifactorial etiology, the improvement of oral clinical signs was evident and agreed with the observed reduction in FCV excretion. It is likely that the observed improvement of both the clinical signs and the shedding of FCV was due to a combination of the immunomodulating and the antiviral effects of the treatment. This is especially important in animal shelters where gingivostomatitis is frequently associated with active FCV infection, and animals may also be more likely to experience some stress-induced reduction in immune competence.

Recognizing that FHV-1 is ubiquitous in catteries (Thiry, et al., 2009), its prevalence among retroviral infected cats was expected to be elevated. At D0, 10/16 animals tested positive. During rFeIFNω therapy, a reduction of FHV-1 excretion was observed ($p=0.0066$, Friedman Test). Curiously, shedding of FHV-1 was even completely suppressed in 4 animals during therapy. Typical respiratory and ocular signs were not very remarkable in these shelter cats. However, the concurrent clinical improvement and reduction in FHV-1 excretion observed in some cats (31%) lead to conclude that, in particular cases, the reduction of FHV-1 shedding may contribute for a clinical improvement. Despite these good clinical findings for animal shelters, FHV-1 may remain latent and the suppression of viral excretion may not lead to complete cure (Dowers, et al., 2010). Also in these animals, this effect may be temporary. Therefore, not only a clinical follow-up but also a reduction in input/output of animals within catteries should be considered.

It has been reported that 70% of the feline population in catteries are FCoV positive (Addie, et al., 2009). As expected, in our study 11/16 cats tested positive at D0. Infected cats may shed high viral loads of FCoV without showing relevant symptoms (Foley, Poland, Carlson, & Pedersen, 1997; Meli, et al., 2004). Despite promising results in an initial study using rFeIFNω therapy in cats with clinical feline infectious peritonitis (Ishida, et al., 2004) a further one was not able to reveal a significant clinical improvement (Ritz, et al., 2007). However, viral excretion was not assessed. To our knowledge, this is the first study that describes the effect of rFeIFNω on FCoV excretion levels in naturally FIV and FeLV infected cats. Despite

some fluctuations which can explain non statistical significant results (p >0.05, Friedman test), viral excretion decreased in most animals (9/11 positive FCoV cats) showing that rFeIFNω therapy seems to be helpful in this situation.

As remarked for FHV-1, several cats showed a concurrent clinical improvement and a reduction in FCoV excretion (31%). Therefore, FCoV status may also contribute for the better clinical condition observed in these cases.

Not only in FCoV but also in FHV-1, viral excretion is not a continuous process and some fluctuations were observed. In fact, some cats showed punctual excretions of both viruses namely on D30. A possible explanation may reside in the licensed rFeIFNω protocol. It is worth noting that D30 is the only follow-up time point during this study at which the animal had not experienced rFeIFNω administration on the previous days.

In the FPV screening, only one cat tested positive on conventional PCR and it was negative 10 days after treatment. In fact, Parvovirus PCR detection is not species-specific. As recently reported (Clegg, et al., 2011), healthy cats may shed canine parvovirus, being an important reservoirs of this virus namely in animal shelters. Due to lack of clinical signs, in agreement with this study, this cat was assumed to be a subclinical carrier.

This study corresponds to the common reality of animal shelters where the overflow of cats and continuous resident rotation (stray animals that are introduced and others that are adopted) may contribute to potentiate different ubiquitous viral infections. Concerning these results, rFeIFNω seems to be able to contribute to the management of this reality by improving clinical signs and decreasing concurrent viral excretion. In summary, rFeIFNω therapy may be beneficial in naturally retroviral infected cats, particularly in the shelter/rescue context, where prevalence of concomitant infections is higher.

Part II - Chapter II

Monitoring acute phase proteins in retrovirus infected cats undergoing feline interferon omega therapy

Chapter II: Monitoring acute phase proteins in retrovirus infected cats undergoing feline interferon omega therapy

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Parts of this work were presented in abstract on the 21st European College of Veterinary Internal Medicine (ECVIM) Congress, September 2011 (Annexe IV), on the 11th International Feline Retrovirus Research Symposium, August 2012 (Annexe V) and on the 22nd ECVIM Congress, September 2012 (Annexe VI).

Abstract

Objectives: Recombinant feline interferon-ω (rFeIFN-ω) is an immunomodulator currently used in the treatment of different retroviral diseases including feline immune deficiency virus (FIV) and feline leukemia virus (FeLV). Although its mechanism of action remains still unclear, this drug appears to potentiate the innate response. Acute phase proteins (APPs) are one of the key components of innate immunity and studies describing their use as a monitoring tool for the immune system in animals undergoing rFeIFN-ω are lacking. This study aimed to determine whether rFeIFN-ω therapy influences APP concentrations namely serum amyloid A (SAA), alpha-1-glycoprotein (AGP) and C-reactive protein (CRP).

Methods: A single arm study was performed using sixteen cats, living in an Animal Shelter, naturally infected with retroviruses and subjected to the rFeIFN-ω licensed protocol. Samples were collected before (D0), during (D10, D30) and after therapy (D65). SAA and CRP were measured by specific ELISA kits and AGP by single radial immunodiffusion.

Results: All the APPs significantly increased in cats undergoing rFeIFN-ω therapy (D0/D65: $p < 0.05$)

Clinical Significance: APPs appear to be reasonable predictors of innate-immune stimulation and may be useful in the individual monitoring of naturally retroviral infected cats undergoing rFeIFN-ω therapy.

Keywords: Alpha-1-glycoprotein, C-reactive protein, feline interferon omega, retrovirus, serum amyloid A

Introduction

The acute phase response (APR) is one of the main reactions of the innate host defense system (Baumann & Gauldie, 1994; Paltrinieri, 2008). It refers to a nonspecific and complex phenomenon that occurs in the early stages of inflammation, preceding the development of the acquired immune response (Baumann & Gauldie, 1994; Ceron, Eckersall, & Martynez-Subiela, 2005). The APR is a consequence of the production and release of several cytokines, of which interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α are the most relevant (Martínez-Subiela S, 2001; Paltrinieri, 2008). These mediators induce various changes in the body including fever, leucocytosis and a modulation of protein synthesis by hepatocytes (Baumann & Gauldie, 1994; Ceciliani, Giordano, & Spagnolo, 2002).

Positive APPs, which increase during inflammation, are believed to act as immunomodulators, contributing in different ways to reinforce the body's innate defenses during inflammation (Petersen, Nielsen, & Heegaard, 2004; Eckersall & Bell, 2010). The most relevant and well described positive APPs in cats are AGP and SAA (Petersen, et al., 2004; Ceron, et al., 2005; Eckersall & Bell, 2010; Paltrinieri, 2008).

AGP is believed to act as an immunomodulator and anti-inflammatory protein because it down-regulates the neutrophilic response secondary to inflammation, stimulates the production of IL-1R antagonists by macrophages, reduces platelet aggregation and lymphoid proliferation and modulates the production of anti-inflammatory cytokines by the circulating lymphocytes (Hochepied, Berger, Baumann, & Libert, 2003). Its increase in different cat infectious diseases has been described (Duthie, Eckersall, Addie, Lawrence, & Jarrett, 1997; Paltrinieri, Giordano, Ceciliani, & Sironi, 2004; Paltrinieri, Giordano, Tranquillo, & Guazzetti, 2007; Paltrinieri, Metzger, et al., 2007). In retroviral infected cats, while a previous study reported its increase (Duthie, et al., 1997), a more recent one found that FIV positive cats have lower concentrations of AGP than healthy ones (Korman, et al., 2012).

SAA is a small protein that appears to be the precursor of amyloid protein A, a major protein of alpha-amyloid which is potentially involved in a variety of chronic inflammatory diseases (Uhlar & Whitehead, 1999). Among its major functions, SAA acts as a scavenger of oxidized metabolites, protecting tissues from excessive damage induced by inflammation (He, Shepard, Chen, Pan, & Ye, 2006). As with AGP, its measurement has been reported in different feline diseases (Kajikawa, Furuta, Onishi, Tajima, & Sugii, 1999; Sasaki, et al., 2001; Sasaki, et al., 2003; Giordano, Spagnolo, Colombo, & Paltrinieri, 2004; Tamamoto, Ohno, Ohmi, Goto-Koshino, & Tsujimoto, 2008; Tamamoto, Ohno, Ohmi, Seki, & Tsujimoto, 2009).

CRP was the first APP described and it is considered to be a major protein in different species such as humans and dogs (Schultz & Arnold, 1990; Ceron, et al., 2005). Among the major functions in the immune-system, CRP is involved in the activation of the classical complement pathway, the enhancement of phagocytosis or even the modulation of polymorphonuclear cells (Schultz & Arnold, 1990). Because CRP does not appear to be involved in the feline acute phase reaction, it has not been very well studied or documented in the cat (Ceron, et al., 2005). In human medicine, several studies describe its increase in HIV positive patients (Jahoor, et al., 1999; Treitinger, et al., 2001), even after immunomodulation therapy with exogenous IL-2 (Barbai, et al., 2010). Despite the similarity between HIV and FIV (Hosie, et al., 2009; Hartmann, 2011), the CRP behavior in FIV positive cats undergoing immunomodulating therapy remains unknown.

Recombinant feline interferon-omega (rFeIFN-ω; Virbagen, Virbac) is an immunomodulating drug that plays an important role in the therapeutic approach for various feline diseases including cat retrovirus infections (Collado, Doménech, Gómez-Lucía, Tejerizo, Miró, 2006). There are only a few studies describing the clinical improvement of retroviral infected cats with rFeIFN-ω therapy (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013) and little is known about the immunological bases that support these findings. Because of reported clinical improvement, increased survival time and reduction of concurrent viral excretion, rFeIFN-ω appears to be involved in the innate response (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013).

Studies that describe the use of APPs as a clinical monitoring tool for the immune system in animals undergoing IFN-therapy are scarce. Therefore, the main objective of this study was to determine whether rFeIFN-ω therapy influences APPs (namely SAA, AGP and CRP) in naturally retroviral infected cats and whether these parameters may be good predictors of the innate-immune stimulation.

Material and Methods

Animals

Sixteen naturally retroviral infected cats living in an animal shelter (União Zoófila, Lisbon) were selected for the study. All of the cats were accustomed to the shelter environment having lived there for at least 8 weeks before the start of the protocol. In accordance with previous studies (de Mari, et al., 2004; Gil, et al., 2013), the inclusion criteria included the following: 1) cats of any age, breed or sex (heterogeneous population), 2) cats that showed at least one clinical sign potentially related to retroviral infections, 3) cats that had previously shown a positive rapid immune-migration FIV and/or FeLV test result. Exclusion criteria were: 1) cats with any type of malignancy/neoplasia (such as lymphoma or lymphoid

leukaemia), 2) cats having received immunomodulating drugs (such as corticosteroids) during the 4 weeks prior to the study 3) cats having received antibiotics or non-steroidal anti-inflammatory drugs during the 2 weeks prior to the study and 4) cats that did not complete the therapeutic protocol. Initially, all cats were retested to confirm their FIV/FeLV infections by using commercially available ELISA (ViraCHEK/FIV and ViraCHEK/FeLV, Synbiotics).

Animals were housed in two different catteries, according to their FIV or FeLV status. Animals concurrently infected with FIV and FeLV were housed in the FeLV cattery. All of the cats in each cattery were treated and during the study, no incoming animals were allowed. For the purposes of analysis, cats were divided in three different groups according to their retroviral status: FIV positive cats (n=7), FeLV positive cats (n=6) and co-infected animals (n=3).

The cats were living in good conditions, in agreement with current ethical and European welfare standards. All the procedures involving the manipulation of these animals were consented and approved not only by the Local Committee for Ethics and Animal Welfare (CEBEA-Faculty of Veterinary Medicine/Technical University of Lisbon) but also by the clinical director of the animal shelter.

Treatment Protocol

Based on assumptions derived from two previously published double arm trials with rFeIFN-ω (de Mari, et al., 2004; Domenech, et al., 2011), a single arm study was performed. In this study model, a time point prior to therapy is considered to be the animal's own control. In this study, assessments before therapy were designated as D0 and considered representative of the stage of each animal before treatment.

All the animals were treated with rFeIFN-ω, according to the licensed protocol (3 cycles of injections at Day (D) 0, D14 and D60. Each treatment cycle consists of 5 subcutaneous injections: 1MU/kg once per day for 5 days). Vials of rFeIFN-ω (Virbagen Omega; Virbac) were reconstituted with the accompanying saline diluent according to the manufacturer's recommendations immediately before each treatment. Treatment was administered by two veterinary clinicians from the research team of the project where this study is inserted.

Supportive Treatment

Despite the exclusion criteria applied, some animals needed supportive treatment during therapy. Consequently, potentiated amoxicillin, hepatic protectants (ursodeoxycholic acid, sylimarin or S-adenylmethionine) and/or fluid therapy were allowed. Although any antibiotic may have direct immunomodulator effects, potentiated amoxicillin was allowed taking into account its empirical use and frequent administration in retrovirus infected cats with

suspected bacterial infections. Antibiotics (other than potentiated amoxicillin), corticosteroids and non-steroidal anti-inflammatory drugs were not permitted to avoid any possible immunomodulation effects.

Blood collection and analysis

Blood samples were collected by venipuncture of the jugular vein at 4 specific time points namely: before (D0), during (D10, D30) and after therapy (D65).

To allow a better evaluation and simpler blood sample collections, cats were submitted to mild sedation with 0.2-0.5 mg/kg of butorphanol solution (Dolorex, Intervet Portugal), subcutaneously.

Serum samples were collected after clotting of the sample had occurred by centrifugation of the (5000g, 10min), and were subsequently frozen at -20ºC until analysed.

SAA and CRP were measured by specific ELISA kits (Phase SAA Multispecies/ Tridelta and Cat CRP ELISA/Kamiya Biomedical Company, respectively). AGP was determined by single radial immunodiffusion (Feline AGP, SRID, Tridelta). All the measurements were performed according the manufacturer's instructions.

Statistical Analysis

Statistical evaluation was performed using R Statistical Software. Because of the small sample size, non parametric statistical tests were used. Kruskall-Wallis Tests were applied to assess differences among groups at each time point. When differences were observed, a Pairwise comparison was applied. To assess group variations during time, a Friedman Rank Sum Test was used. The non-parametric tests applied took into account not only the magnitude but also the predominant 'sign' (positive or negative) of the effect. Significance was set at $p < 0.05$.

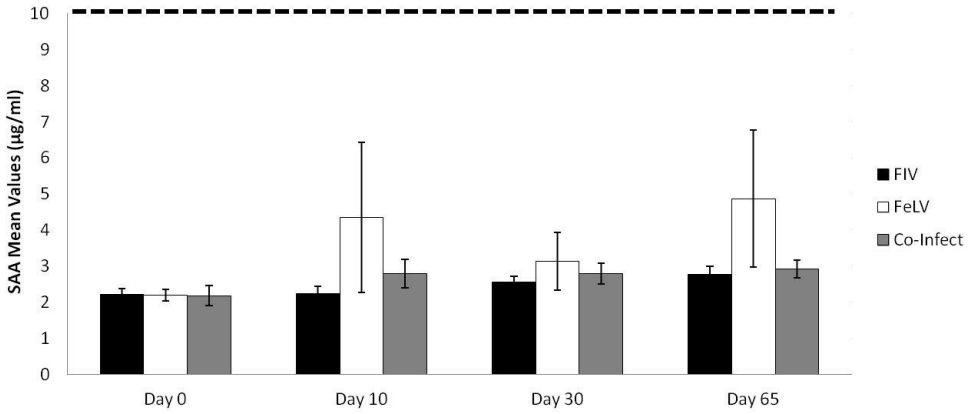
Results

All sixteen cats completed the licensed therapeutic protocol. No statistical differences between groups were observed apart from CRP on D65 and AGP on D30.

For SAA, groups were similar at all time points and all the cats behaved similarly. Therefore, for analysis over time, the groups were all considered together. Global results are presented in figure 14. A significant increase of SAA concentration was observed (P=0.0005; increased SAA in 15 animals and decreased SAA in one). On D65, mean SAA concentration was 1.6

times higher than D0. All values remained below the upper limit of the reference interval (RI) of 10µg/mL.

Figure 14: Mean ± standard error (SE) of serum concentrations of serum amyloid A (SAA) in naturally retroviral infected cats, before (D0) during (D10, D30) and after (D65) rFeIFNω therapy. The reference interval (RI) is < 10 µg/ml. The observed increase was statistically significant (Friedman test D0 versus D65 p= 0.0005).



For AGP, the three groups were also similar at all time points, apart from D30 (p=0.016), where co-infected and FeLV cats showed higher mean values than FIV cats (FIV versus co-Infected: p = 0.029; FIV versus FeLV: p = 0.067; Felv versus co-infected: p = 0.12) (figure 15). There was a significant overall increase (1.7 times) of AGP concentrations (P=0.012; AGP increased in 13 cats and decreased in 3) from D0 to D65. Basal values were within the RI (260-580 µg/ml). During therapy, mean values of FeLV and co-infected cats exceeded the the upper limit of the RI while for FIV, all the results remained within the RI.

For CRP, the three groups were statistically indistinguishable during the study apart from D65 (p=0.019), where there was a statistically significant difference. At this time point, co-infected and FeLV cats had higher CRP concentrations than FIV cats (FIV versus co-Infected: p = 0.009; FIV versus FeLV: p = 0.052; Felv versus co-infected: p = 0.36). Results of CRP values are presented in figure 16. From D0 to D65 there was an increase in CRP concentrations in all the cats (P=0.0001; increased CRP in 16 cats). This increase was approximately 1.8 times the baseline value. Values on D30 and D65 were above the upper limit of the RI (38-186 µg/ml) for FIV, FeLV and co-infected cats.

Figure 15: Mean ± standard error (SE) serum concentrations of alpha-glycoprotein-1 (AGP) in naturally retroviral infected cats, before (D0) during (D10, D30) and after (D65) rFeIFNω therapy. The horizontal line represents the upper limit of the reference interval (260-580 µg/ml). The observed increase was statistically significant (Friedman test D0 versus D65 p= 0.012). Groups are statistically similar except at D30 (*). Kruskall-Wallis p=0.016; Pairwise Comparison: FIV versus Co-Infected: p = 0.029; FIV versus FeLV: p = 0.067; Felv versus Co-infected: p = 0.12.

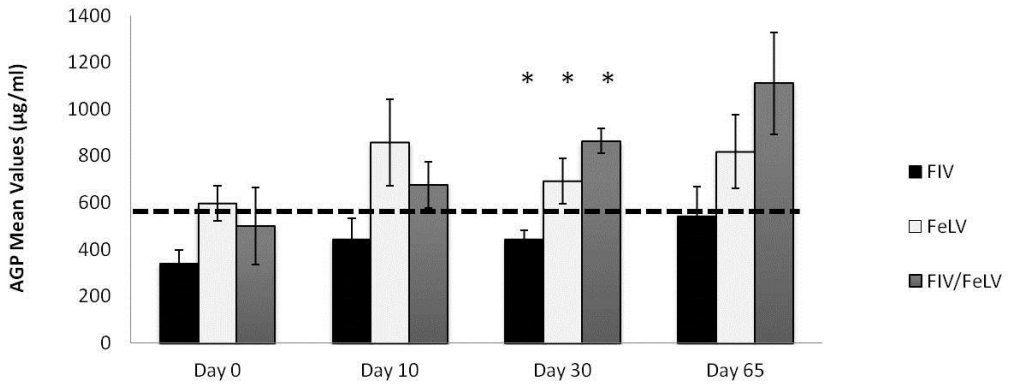
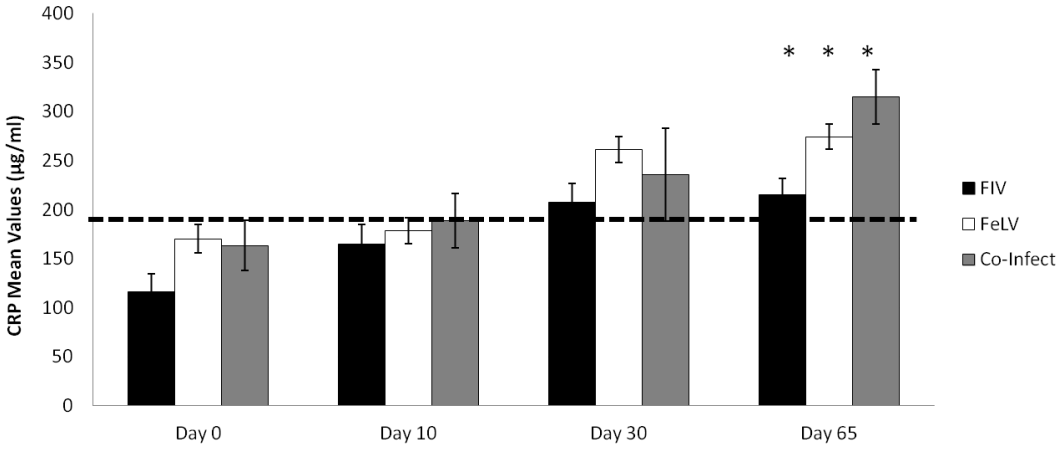


Figure 16: Mean ± Standard Error (SE) of serum concentrations of c-reactive protein (CRP) in naturally retroviral infected cats, before (D0) during (D10, D30) and after (D65) rFeIFNω therapy. The horizontal line represents the upper limit of the reference interval (38-186 µg/ml). The observed increase was statistically significant (Friedman test D0 versus D65 p= 0.0001). Groups are similar except at D65 (*). Kruskall-Wallis p=0.019; Pairwise Comparison: FIV versus Co-Infected: p = 0.009; FIV versus FeLV: p = 0.052; Felv versus Co-infected: p = 0.36.



Only one cat of the FeLV group (cat 9) received supportive therapy at D65, namely intravenous fluids, because of suspected otitis/vestibular syndrome and mild-dehydration. This fact was considered as a co-morbidity factor and was included in the clinical-evaluation and considered in the clinical score. This animal also received potentiated amoxycilin but after the end of the study. The APP profile of this cat was similar to the wider group.

Data related to clinical signs and concurrent viral loads (namely calicivirus, herpesvirus, coronavirus and parvovirus) in these cats over the same time period have been previously reported (Gil, et al., 2013). Herpesvirus and coronavirus viral load were assessed by RT-qPCR, while calicivirus and parvovirus status were determined by conventional-PCR (Gil, et al., 2013). These results are summarized in table 8 which also includes the detailed APP results. Clinical signs improved and concurrent viral excretion decreased in the majority of the cats. Only two cats increased coronovirus excretion but they remained asymptomatic. All the cats that were positive for calicivirus and a single cat that was excreting parvovirus became negative at the end of the study meaning an overall significant decrease of concurrent viral infections. Simultaneously, the majority of cats had increased APP concentrations.

Table 8: Individual variation of clinical scores, concurrent viral excretion and acute phase proteins in FIV, FeLV and FIV/FeLV cats treated with rFeIFN-ω. Comparing D0 (before) to D65 (end of the therapy): (↓) refers to a decrease of the parameter; (↑) refers to an increase of the parameter; (→) refers to a stable parameter; (-) refers to negative samples; (*) refers to intermittent excretion during therapy, despite a negative result at D0 and D65. (BN) refers to animals that were positive at D0 and became negative with therapy. For Clinical Scores, a reduction on the parameter refers to a clinical improvement.

Cat	**FIV/FeLV Status**	**Clinical Scores (D0 vs D65)**	**Concurrent Viral Excretion (individual tendency D0 versus D65)**				**Acute Phase Proteins (individual tendency D0 versus D65)**		
			Calicivirus	Herpesvirus	Coronavirus	Parvovirus	SAA	AGP	CRP
1	FIV	↓	**BN**	↓	↓	-	↑	↑	↑
2	FIV	→	-	↓	-	-	↑	↑	↑
3	FIV	↓	-	↓	↓	-	↑	↑	↑
4	FIV	↓	**BN**	*	↑	-	↑	↑	↑
5	FIV	↓	-	↓	-	-	↑	↑	↑
6	FIV	↓	**BN**	-	↓	-	↓	↑	↑
7	FIV	→	**BN**	-	↓	-	↑	↓	↑
8	FeLV	→	**BN**	↓	↓	-	↑	↑	↑
9	FeLV	→	**BN**	↓	*	-	↑	↑	↑
10	FeLV	↓	**BN**	*	↑	-	↑	↑	↑
11	FeLV	↓	**BN**	*	*	-	↑	↑	↑
12	FeLV	→	**BN**	↓	↓	-	↑	↓	↑
13	FeLV	↓	**BN**	↓	↓	-	↑	↓	↑
14	FIV/FeLV	↓	**BN**	-	↓	-	↑	↑	↑
15	FIV/FeLV	→	**BN**	↓	*****	-	↑	↑	↑
16	FIV/FeLV	↓	**BN**	↓	↓	**BN**	↑	↑	↑

Discussion

This study describes the changes observed in serum concentrations of three different APPs in 16 cats naturally infected with retroviruses and undergoing rFeIFN-ω therapy. A single-arm study was considered and performed as this is likely to be the most reliable approach when studying naturally infected animals, for which time of infection and sub-types of the virus are uncertain. In this study rFeIFN-ω was used according to a licensed protocol, approved for veterinary use in feline retrovirus infections. Although only a few studies have been published, clinical improvement of cats undergoing rFeIFNω therapy has been well documented in double arm trials (de Mari, et al., 2004; Domenech, et al., 2011). This study provides further information regarding parameters complementary to the main clinical signs and concurrent viral excretion data previously published (Gil, et al., 2013), clarifying the physiopathology phenomena behind immune-stimulation.

As several authors have suggested (Ceron, et al., 2005) the concentration of APPs may be within the RI even in animals with disease. As such measurement of these parameters for monitoring inflammation stimulus is not without difficulty. Using each individual animal as its own reference was considered to be a practical and reasonable approach to bypass this problem (Ceron, et al., 2005). Therefore, as in previous human studies that evaluated APPs (Wasunna, et al., 1995; Barbai, et al., 2010), animals acted as their own controls and the time-point before therapy (D0) was considered the baseline value for each cat. Subsequently, it was possible to study the individual trends during therapy.

Although there is no consensus on upper and lower limit values for the measured APP, the suggested ranges for each kit were used. Particularly for AGP and CRP, cats had higher mean values than the upper limit at some time points. However, previous studies describe higher values in healthy cats (Kajikawa, et al., 1999; Selting, et al., 2000), which if adapted would expand the recommended RI. Only the comparison between individual values before (D0), during and after therapy (D65) with particular relevance to differences between D0 and D65 were considered relevant for the study, whether they were above the RI or not.

For concentrations of SAA and AGP, the three groups studied (FIV, FeLV and co-infected animals) behaved similarly, demonstrating increased values during rFeIFN-ω therapy. Groups were not statistically different with the exception of AGP on D30. This particular variation is secondary to the fact that the FIV group demonstrated lower AGP values compared to FeLV and co-infected cats. Despite the low number of animals a possible explanation may be sub-clinical diseases leading to a particular alteration at this time point. However, considering that the statistical difference was only evident at this particular time-point, this finding was not considered biologically significant. According to previous studies which reported that AGP and SAA are good predictors of immunomodulation (Kajikawa, et

al., 1999; Hochepied, et al., 2003)**,** these results support the hypothesis that rFeIFN-ω therapy may modulate pro-inflammatory innate mechanisms. These APPs may be a useful monitoring tool for demonstrating modulation of the innate immune response.

For CRP, with the exception of D65 the three groups of cats were also similar throughout the study. The particular variation at D65 was due to the fact that FeLV and co-infected cats had higher values than FIV cats. As for AGP at D30, this may have been due to various causes such as sub-clinical uncontrolled infections in the shelter or even a natural progression of retroviral disease. From the beginning (D0) until the end of the treatment (D65) a significant increase of CRP concentrations was noted. Although CRP has not been considered as a useful biomarker of inflammation in cats, this study shows that this APP behaves similarly to SAA and AGP. Therefore, in contrast with previous studies, CRP may also have value as a biomarker of feline inflammation, being increased in a similar magnitude to the other APPs measured.

A recent study described the evolution of different APPs, namely SAA and AGP, in FIV and non-FIV cats following *Mycoplasma haemofelis* and *Candidatus Mycoplasma Haemominutum* infections (Korman, et al., 2012). This study revealed that pre-existing FIV infection did not significantly affect the acute phase response to mycoplasma. Despite remaining within the RI, FIV positive cats demonstrated lower concentrations of AGP than non-FIV cats. This contrasted with previous studies suggesting that AGP was increased in FIV cats (Duthie, et al., 1997). Taking all of this into account, it seems reasonable to consider that FIV cats are able to develop an efficient acute phase, which may lead to a rise of APPs. Regarding the more conventional assessments of the immune-system in retroviral infected cats, a previous study concluded that the hyperglobulinaemia commonly observed in FIV cats may be due to hyperactivation of B-cells which is more evident when the disease progresses (Gleich & Hartmann, 2009). In contrast, and due to a progressive defect of helper T-cells, FeLV cats do not usually present with hyperglobulinaemia being more prone to have severe cytopenias (Gleich & Hartmann, 2009). According to this study, the increase of APPs was similar in FIV and FeLV, suggesting that the innate immune stimulation must have the same basis in these two groups. No studies were performed to correlate gamma-globulins with APPs in retroviral infected cats, and whether rFeIFN-ω interferes with serum protein electrophoresis profiles remains unclear.

It has been previously demonstrated that rFeIFNω results in an overall clinical improvement not only of FIV but also FeLV and co-infected cats (Gil, et al., 2013). Clinical signs, evaluated by a score-scale, decreased in the 3 groups (FIV, FeLV and co-infected) meaning an overall improvement of symptomatic cats. In total, 10/16 improved their clinical signs while 6/16 remained stable. Furthermore, a significant decrease in excretion of other viruses was also

observed concomitantly (Gil, et al., 2013). Correlating the APP profile with these previous results, it was observed that APP serum concentrations increased in cats with concurrently improved clinical signs and reduced viral excretion. This finding was consistent even in cats with low clinical scores and in those which scores remained stable with therapy. Recognizing that APPs may be increased in different situations such as chronic infections and severe inflammation (Ceron, et al., 2005; Paltrinieri, 2008), this concurrent clinical improvement and the decrease in the loads of other viruses reinforce and sustain the hypothesis that interferon therapy potentiates the immune response and may involve a beneficial APP increase in treated animals.

In conclusion, all the measured APPs significantly increased, revealing a potential innate immune response in naturally infected cats during rFeIFN-ω therapy. In humans it has been described that the administration of IL-2 in HIV patients induced an increase of CRP, which was positively correlated to an increase of CD4+ cell count (Barbai, et al., 2010). These findings reported a possible involvement of CRP in the IL2-induced immune-stimulation (Barbai, et al., 2010). Conversely, it was previously described that CD4/CD8 ratios do not change in cats under rFeIFN-ω (Domenech, et al., 2011). Therefore, the true mechanism by which rFeIFN-ω induces an increase in APP remains unclear. In order to further characterise the immune response during rFeIFN-ω therapy, further studies are required to correlate these findings with other parameters such as the cytokine profile. For now, the results of this study suggest that APPs may be promising predictors of innate-immune stimulation in naturally retroviral infected cats undergoing rFeIFN-ω therapy. In the future, they could be combined together in an APP-panel that may help with the individual monitoring and assessment of rFeIFN-ω therapy.

Part II - Chapter III

Oral recombinant feline interferon-omega as an alternative immune modulation therapy in FIV positive cats: clinical and laboratory evaluation

Chapter III: Oral recombinant feline interferon-omega as an alternative immune modulation therapy in fiv positive cats: clinical and laboratory evaluation

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Abstract

Recombinant-Feline Interferon-Omega (rFeIFN-ω) is an immune-modulator licensed for use subcutaneously in Feline Immunodeficiency virus (FIV) therapy. Despite oral protocols have been suggested, little is known about such use in FIV-infected cats. This study aimed to evaluate the clinical improvement, laboratory findings, concurrent viral excretion and acute phase proteins (APPs) in naturally FIV-infected cats under oral rFeIFN-ω therapy (0.1MU/cat rFeIFN-ω PO, SID, 90 days). 11 FIV-positive cats were treated with oral rFeIFN-ω (PO Group). Results were compared to previous data from 7 FIV-positive cats treated with the subcutaneous licensed protocol (SC Group). Initial clinical scores were similar in both groups. Independently of the protocol, rFeIFN-ω induced a significant clinical improvement of treated cats. Concurrent viral excretion and APP`s variation were not significant in the PO group. Oral rFeIFN-ω can be an effective alternative therapy for FIV-infected cats, being also an option for treatment follow-up in cats submitted to the licensed protocol.

Keywords

Feline Immunodeficiency Virus, Recombinant-Feline Interferon Omega, immune-modulation, oral therapy,

Introduction

Recombinant Feline Interferon-Omega (rFeIFN-ω, Virbagen Omega®, Virbac) is an immune-modulator drug licensed for use in Europe, Australia and some Asian countries. Among its main therapeutic indications, it is frequently used in the management of Feline Immunodeficiency Virus (FIV) and Feline Leukemia Virus (Hosie, et al., 2009; Lutz, et al., 2009). rFeIFN-ω therapy induces clinical improvement of retroviral infected cats (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013), and increases their survival time (de Mari, et al., 2004). Slightly changes in some clinical parameters such as hypergammaglobulinemia, CD4/CD8 ratio, proviral load and viremia have been previously reported in retroviral infected cats during rFeIFN-ω therapy (Domenech, et al., 2011). According to these authors, rFeIFN-ω is thought to act on innate immunity (Domenech, et al., 2011). More recently, another study reported that the rFeIFN-ω licensed protocol improves the clinical presentation and reduces concurrent viral excretion in naturally retroviral-infected cats living in catteries, suggesting its usefulness in multi-cat environments where viral-related disorders are often a clinical problem (Gil, et al., 2013). In that study, no significant abnormalities were observed in the hematology or biochemistry profiles during treatment (Gil, et al., 2013).

The rFeIFN-ω licensed protocol consists of 3 therapeutic cycles of 5 daily subcutaneous injections (1MU/kg/day), beginning respectively on days 0, 14 and 60. This protocol can be expensive and its cost may limit a more frequent use. To bypass this problem, alternative protocols such as oral ones have been suggested as an alternative use of rFeIFN-ω in certain situations (Addie, 2012; Bracklein, et al., 2006; Hennet, et al., 2011).

Some authors previously described the increased expression of Mx protein, a specific biomarker of an IFN-induced antiviral response, in specific-pathogen-free cats treated orally with various concentrations of rFeIFN-ω (Bracklein, et al., 2006). In that study, it was shown that a higher oral dose of rFeIFN-ω induced higher levels of Mx protein expression, confirming its activity in oral protocols (Bracklein, et al., 2006). Another recent study reported its successful use in a randomized double-blind study of FCV-positive, retrovirus negative cats with caudal stomatitis (Hennet, et al., 2011). The protocol consisted of daily oro-mucosal rFeIFN-ω administration (0.1MU/cat) for 90 days and was associated with a significant clinical improvement of lesions. Other authors describe its use in the non-effusive form of Feline Infectious Peritonitis (FIP) (50000U/cat PO daily) (Addie, 2012). Nevertheless, the beneficial effect of rFeIFN-ω for management of FIP is still not fully established (Ishida, et al., 2004; Ritz, et al., 2007; Addie, et al., 2009).

To date, little is known about the clinical benefits of rFeIFN-ω via the oral route in cats with retroviral infections. Similarly to what is observed with Human Interferon-Alpha (HIFN-α)

therapy, daily oral administration of rFeIFN-ω may provide effective immune modulation in FIV cats. To the authors' knowledge, only one trial has been performed, which used experimentally infected and asymptomatic FIV cats (Caney, 2003). It described the successful use of a daily oral rFeIFN-ω dose (0.1MU/cat) for 6 weeks, but was not intended to assess any clinical benefit. Although no significant changes were obtained in provirus loads or CD4:CD8 ratio, the animals increased their bodyweight and the oral protocol was well tolerated (Caney, 2003). However, no further studies were performed in order to evaluate whether oral administration of rFeIFN-ω is efficient in cats with naturally occurring retroviral infections or its use over longer periods in symptomatic cats.

In daily practice, there are a few clinical parameters that permit a direct or indirect assessment of the immune-stimulation induced by rFeIFN-ω. Among them, SPE and APPs are potential complementary exams to evaluate the immune system of treated cats. SPE is a laboratory test that allows the separation of serum proteins based on size and electrical charge. Serum proteins are therefore divided into different fractions (alpha, beta and gamma-globulins) whose increase or decrease can be interrelated (Taylor, et al., 2010). The gamma-globulin fraction has a special relevance in FIV cats. An increase in this fraction is associated with chronic antigenic stimulation and, according to previous studies, tends to occur in FIV positive cats (Gleich & Hartmann, 2009). This is mainly due to concurrent infections and a polyclonal B-cell activation which are a direct consequence of FIV infection and is seen even in apparently healthy FIV-positive cats (Ackley, et al., 1990; Flynn, et al., 1994; S. Gleich & Hartmann, 2009; Hartmann, 2011). In human medicine, especially in low resource areas, SPE has been used to monitor the response of anti-retroviral therapy in HIV patients (Sarro, et al., 2010). Therefore, SPE could be a promising complementary exam in FIV infected cats.

APPs have been recently measured in various feline diseases (Paltrinieri, 2008). They seem to modulate the innate immune response, reinforcing the body defenses during inflammation (Petersen, et al., 2004; Ceron, et al., 2005; Paltrinieri, 2008). Hence, APP serum levels seem to be indirect indicators of innate immune system stimulation. A recent study described the use APP levels to monitor the effect of the licensed rFeIFN-ω protocol on innate immunity in FIV-positive cats. All the treated cats increased AGP, SAA and CRP suggesting that these parameters may be reliable in the individual monitoring of rFeIFN-ω immune-modulation therapy (Leal, et al., 2014).

The present study aimed to evaluate the clinical improvement, laboratory findings (CBC, biochemistry and SPE), concurrent viral excretion and acute phase proteins (AGP, SAA and CRP) in naturally FIV-infected cats treated with an oral rFeIFN-ω protocol, in comparison to the licensed one.

Material and Methods

Animals and treatment protocols

11 FIV-positive cats were treated with oral rFeIFN-ω (PO Group) and the results were compared with data previously obtained from 7 FIV-positive cats treated with the licensed protocol (SC Group). The inclusion criteria used were based on previous publications (de Mari, et al., 2004; Gil, et al., 2013). No medications apart from rFeIFN-ω were allowed during the period of the study. Retroviral status was confirmed in all the animals by ELISA kits using serum or plasma samples from D0 (Viracheck/FIV and Viracheck/FeLV, Synbiotics). The results of the SC group were previously published as a single-arm trial which evaluated clinical improvement and concurrent viral excretion (Gil, et al., 2013). The data from this group were considered as a positive control for the current study. The PO Group consisted of 11 naturally FIV-infected cats referred to the Veterinary Teaching Hospital, which were treated, after obtaining the owner's consent, with 0.1MU/cat rFeIFN-ω orally, once a day for 90 consecutive days. 6/11 cats were single-housed or lived indoor with no more than one other cat while 5/11 cats were outdoor animals or came from a multi-cat environment. To obtain the correct dose, a vial containing the rFeIFN-ω freeze-dried pellet (10MU) was diluted in 25ml of sterile physiological saline. Single-doses were prepared using 1ml syringes containing 0.25ml each by one of the members of the research group and given to owners who were instructed how to administer the therapy. The syringes were kept frozen (-18 to -20ºC) after preparation and owners defrosted each single dose shortly before administration. All animals were submitted to full clinical evaluations on days 0 (before therapy), 10, 30 and 65. Animals in the PO group had an additional evaluation on day 90 (end of therapy).

Ethics

The study was approved by the Committee for Ethics and Animal Welfare of the Faculty of Veterinary Medicine – Technical University of Lisbon (CEBEA).

Clinical Evaluation

Clinical improvement was evaluated using a score-scale (Gil, et al., 2013) which included the most important parameters associated with retroviral infections namely: oral ulcers/gingivitis (score 0-2), caudal stomatitis/palatitis (score 0-2), ophthalmic abnormalities (score 0-2), lymphadenopathy (score 0-2), ocular and nasal discharge (score 0-2), mucous membrane color (score 0-2), coat appearance (score 0-1), body score (score 0-2), faecal appearance (score 0-1) and concurrent diseases/co-morbidities (score 0-2). At each time point, the total score for each cat was obtained by summing up all the corresponding clinical scores. These overall scores were then compared during the study period. Clinical improvement was classified as 'marked' (> 50% improvement of the initial score), 'mild' (up to 50%

improvement), ‘stable’ (same final and initial score) or ‘worse’ (final score more elevated than the initial).

Concurrent viral excretion assessment

Oral and Rectal swabs were collected at each time point to assess potential variations in concurrent excretion of FHV-1, FCV, FCoV and FPV. Swabs were processed for viral DNA/RNA extraction (QIAamp MinElute Virus Spin Kit, Qiagen, Portugal).

The determination and quantification of concurrent viral excretion was performed using the diagnostic procedures available in the Virology Laboratory of the Faculty of Veterinary Medicine – Technical University of Lisbon. In detail, screening and quantification of FHV-1 and FCoV was performed by RT-qPCR and FCV presence was assessed by conventional reverse transcriptase PCR. The methodology used was the same as previously published (Gil, et al., 2013). Due to a technical update, FPV was also assessed by RT-qPCR in the PO group. FPV primers and TaqMan® probes were calculated using the Primer designing tool of NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast/), based on the nucleotide sequence of the vp1 gene (AN: AB437433.1). Screening and quantification of FPV was assessed by RT-qPCR amplification (Applied 7300 instrument, Applied Biosystems), in a 20 µl reaction, using TaqMan® Gene Expression 2x Master Mix (Applied Biosystems), 0,9 µM of forward primer (5’ GGGCCTGGGAACAGTCTTGACC-3’), 0,9 µM of reverse primer (5’ACCAGAGCGAAGATAAGCAGCGT-3’) and 0,25 µM of TaqMan® probe (FAM 5’-CGCCGCTGCAAAAGAACACGACGAAGC- 3’ TAMRA) and 10ng of template. The cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 1 minute at 60°C. To estimate FPV copy number serial tenfold dilutions (10^{-1}–10^{-6}) of recombinant plasmid DNA were used to generate a standard curve with a correlation efficiency of r2 = 0.997, using the 7300 System SDS software.

Hematology and Biochemistry

Blood samples were collected by jugular venipuncture during each clinical evaluation. Complete blood-cell count (CBC), hepatic enzymes (alanine-transaminase, aspartate-transaminase) and renal function (serum creatinine and urea) were assessed. CBC and biochemistry were performed, respectively, on Cell-Dyn 3700 (Abbott diagnostics division) and Kone Optima 4.2 (Kemia Cientifica).

Acute Phase Proteins

SAA and CRP were measured by ELISA using previously validated kits (Phase SAA Multispecies/ Tridelta and Cat CRP ELISA/Kamiya Biomedical Company, respectively). AGP

was assessed by single radial immunodiffusion (Feline AGP, SRID, Tridelta). All the measurements were performed according to the manufacturer's instructions.

Statistical Analysis

For each clinical and immunological parameter of interest, the two groups were compared at the beginning and end of therapy using the Mann-Whitney-Wilcoxon test for independent samples. In each group, the comparison between measurements at the beginning and end of therapy was also carried out by the Mann-Whitney-Wilcoxon test but now for paired samples. In these tests we use their version for small sample sizes (e.g., with continuity correction). The significance level was fixed at 5%. In the PO group, we presented a simple descriptive statistical analysis for indoor and outdoor cats, where appropriate, due to their small sizes. Also for concurrent viral excretion, where results were marginal due to low initial excretion rates, a descriptive statistical analysis was only presented. All calculations were undertaken in the R statistical software (version 3.0, www.r-project.org).

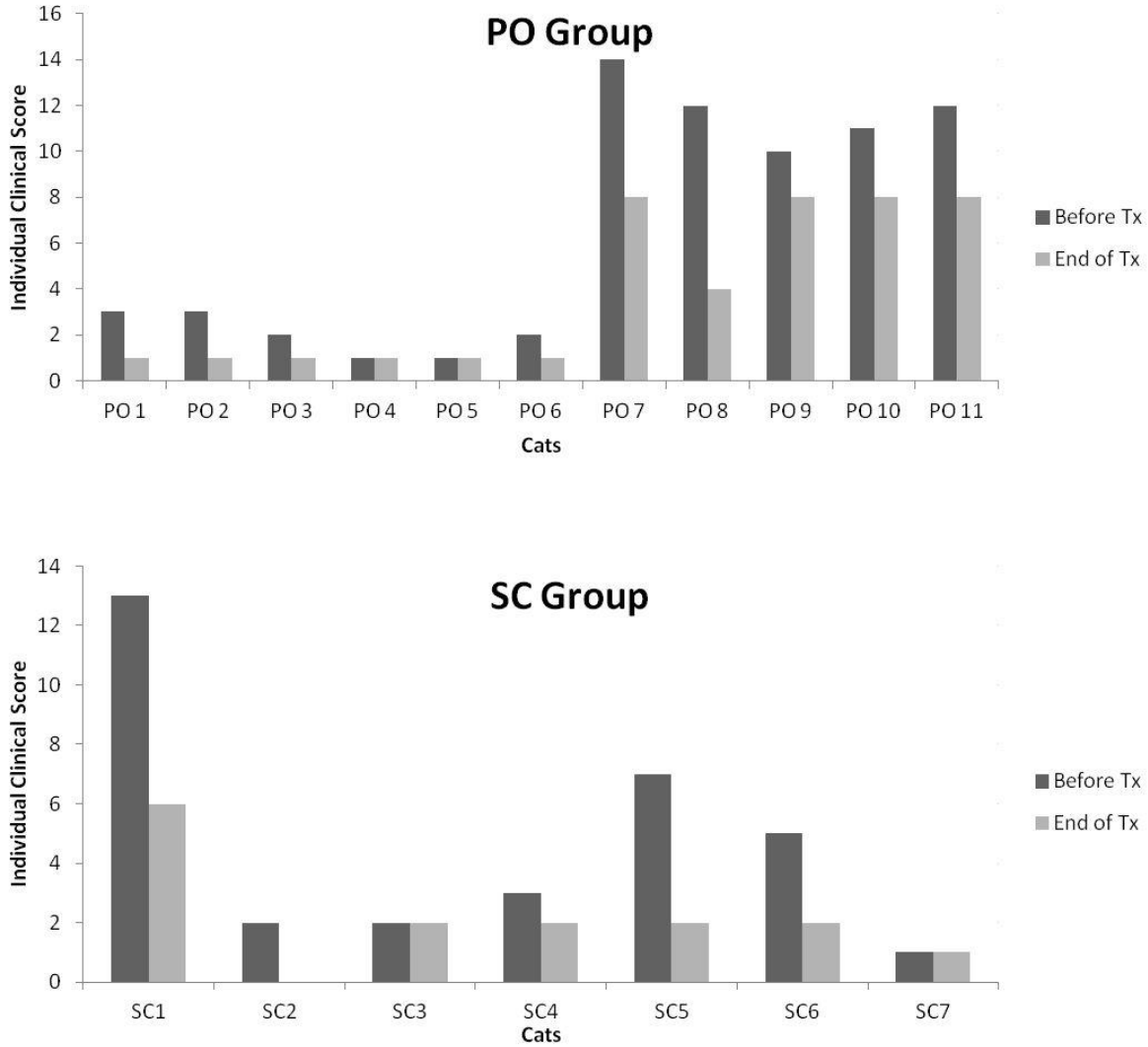
Results

Clinical Improvement

Regarding clinical scores, groups were indistinguishable at the beginning and at the end of therapy (p=0.71 and 0.74, respectively). Although not significant, the PO group revealed overall higher clinical scores than the SC one. Regarding clinical improvement, in the SC Group, 5/7 (71%) cats improved their overall score (p = 0.025). In particular: 4/7 (57%) had a marked improvement, 1/7 (14%) a mild improvement and 2/7 (29%) remained stable. No worsening was observed. Oral lesions were the most common clinical sign at D0. The individual clinical scores for each cat on D0 and D65 (end of therapy) are presented in figure 17. In the PO Group, 9/11 (82%) treated cats significantly improved their overall scores with therapy (p = 0,007). Specifically: 3/11 (27%) of treated cats showed a marked improvement, 6/11 (55%) revealed a mild improvement and 2/11 (18%) remained stable. Similarly to the SC group, no worsening was observed. The individual clinical scores for each cat on D0 and D90 (end of therapy) are presented in figure 17. Although some animals displayed only few clinical signs, an overall rapid improvement of cats was observed after the beginning of the study. Ten days after the onset of oral therapy, there was a significant reduction of the overall clinical score, which homogeneously dropped until the end of the study (data not shown). A very mild increase of the overall clinical score was observed from day 65 to day 90, due to specific worsening episodes of oral granulomas in one cat, and ocular and nasal discharge in another one (data not shown). At D0, the most significant clinical parameters affected were once again the oral lesions (oral ulcers and caudal stomatitis) and ocular discharge. With the exception of ocular and nasal discharge, which showed slight

fluctuations during therapy, all parameters revealed a homogenous improvement(data not shown). Coat appearance, body condition score and ocular discharge were the clinical parameters which showed the most remarkable improvement.

Figure 17: Individual clinical scores for each cat of each group, before and after rFeIFN-ω therapy. SC group refers to cats treated with the licensed sub-cutaneous protocol while the PO group refers to cats treated with oral protocol.



There was no statistical difference between groups in the proportion of cats showing an improvement (Pearson's Chi-square test with Yates continuity correction for small sample sizes; p= 0.95). Moreover, there were also no differences in the grade of clinical improvement (mild or marked) between groups (Pearson's Chi-square test; p= 0.23). The overall results of both groups are presented in Table 9.

Table 9: Overall Clinical Improvement of FIV positive cats treated with the rFeIFN-ω licensed protocol (SC Group) and rFeIFN-ω PO protocol. *Confidence intervals (shown in brackets)

were calculated at 95% and refer to the percentage of cats showing clinical improvement overall.

Clinical Improvement	SC Group		PO Group	
Improvement	5/7 (29.0-96.3)*	Marked: 4/7	9/11 (48.2-97.7)*	Marked 3/11
		Mild: 1/7		Mild 6/11
No Improvement	2/7	Stable: 2/7	2/11	Stable: 2/11
		Worsening: 0/7		Worsening: 0/11

Concurrent Viral Excretion

In the PO group, the concurrent viral excretion was very minor. None of the cats were positive for FHV-1 at the beginning of therapy. Only occasional excretion was detected and it was considered clinically irrelevant. At the end of therapy all the cats were FHV-1 negative. 5/11 (4 indoor and 1 outdoor) cats showed a very limited initial excretion of FPV which became negative. Also 5/11 cats (3 indoor and 2 outdoor) were residually excreting FCoV on D0. Despite some fluctuations, after the therapy two of them became negative, one increased the viral excretion and two reduced it. 7/11 (5/5 outdoors and 2/6 indoor cats) were positive for FCV on D0, retaining this status throughout the therapy. One indoor cat that was negative on D0 became positive at the end of therapy. In opposition, and as previously published, the SC group revealed a significant reduction of concurrent viral excretion (Gil, et al., 2013).

Hematology and Biochemistry

Concerning CBC results on D0, 3/11 cats (2 indoor and 1 outdoor cats) from the PO group revealed a mild to moderate leucopenia; 2/11 outdoor cats were slightly anemic and 3/11 (2 indoor and 1 outdoor cat) showed a clinically unremarkable erythrocytosis, clinically compatible with mild subclinical dehydration. During therapy, 2 out of 3 leucopenic cats normalized their leucocyte counts. One cat still had a persistent mild leucopenia at the end of the therapy, despite an improvement of the leucocyte count. The red-blood cell counts of the 2 slightly anemic cats fluctuated during therapy. At the end of the protocol, the hematocrit of one of the cats normalized whilst the other worsened, revealing at D90 a moderate non-regenerative normocytic and normochromic anemia. The differential leukogram of the PO group cats remained stable and unremarkable during therapy.

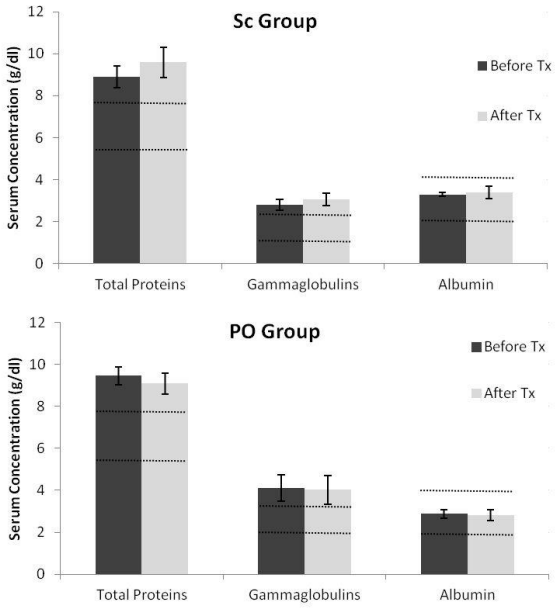
Urea and Creatinine serum levels remained within the reference interval before, during and after therapy. Regarding hepatic transaminases, 2 outdoor cats had a mild to moderately increased ALT and AST on D0. For one, this normalized by D10 and remained within the

reference interval until the end of study, whereas the other persisted for longer but normalized by D90.

Serum protein Electrophoresis

No significant changes in SPE were detected in either group during therapy (figure 18). Both groups initially presented with the same pattern on D0 with an increase in total proteins and a hypergammaglobulinemia. Also in both groups, albumin was within the normal range before and after the study. Despite being non statistically significant, the PO group experienced a slight decrease in total proteins while the SC group increased this parameter. Conversely, hypergammaglobulinemia remained stable in the PO group whilst in the SC group it increased with therapy. When considering the environment of the cats (Indoor/Outdoor) submitted to the PO protocol, outdoor cats had overall higher values of gamma-globulins and total proteins than indoor ones both before and after therapy.

Figure 18: Total Proteins, Gammaglobulins and Albumin serum levels of FIV positive Cats treated with two different protocols of rFeIFN-ω. (Mean values ± standard error). The SC group refers to cats treated with the licensed protocol while the PO group refers to cats treated with oral rFeIFN-ω. Horizontal lines (---) represent, respectively, the upper and lower limits of the normal range for each parameter.



Acute Phase Proteins

Regarding the APP profile on D0, the PO group showed significant higher serum levels of AGP, CRP and SAA than the SC one (p= 0,018, 0,02 and 0,00006 respectively). In detail,

the baseline values (D0) of AGP, CRP and SAA in the PO group were, respectively, three, nine and seven times higher than the SC one. Results are shown on table 10. As previously published, the APP levels increased in animals treated with the SC protocol (Leal, et al., 2014). In contrast, cats treated with the PO protocol did not demonstrate significant changes in APP serum levels after therapy (p =0.9; 0.4 and 0.9 for CRP, SAA and AGP, respectively).

Table 10: Mean values ± standard error of Serum Amyloid A (SAA), Alpha-1-Glycoprotein (AGP) and C-Reactive Protein (CRP) serum levels in FIV positive cats before and after therapy with licensed rFeIFN-ω (SC group) and oral (PO group) protocols.

	SAA µg/ml		**AGP µg/ml**		**CRP µg/ml**	
	Before Tx	After Tx	Before Tx	After Tx	Before Tx	After Tx
Licensed Protocol (SC group)	2.2 ± 0.2	2.8 ± 0.2	341.4 ± 56.8	544.3± 123.8	116.6± 17.7	215.4± 16.5
Oral Protocol (Po group)	15.9 ± 6.5	8.5 ± 1.9	929.1± 126.9	945.5± 157.3	1048.4± 68.4	985.8± 93.8

Discussion

This study showed that, independently of the protocol applied, rFeIFN-ω induces a significant clinical improvement of treated FIV-infected cats. Unexpectedly and although not statistically significant, the PO group had slightly higher overall clinical scores than the SC group. This can be due to the fact that the five outdoor cats revealed higher clinical scores than any cat from the SC group, counter-balancing the almost asymptomatic indoor cats. In fact, no significant differences were observed on clinical improvement between groups suggesting that, in a clinical setting where cost might be a limiting factor and subcutaneous administration might be problematic, rFeIFN-ω may be administered orally with success in FIV positive cats. However, despite the lack of statistical difference, the rFeIFN-ω licensed protocol (subcutaneous injections) appeared to induce a marked clinical improvement in a larger proportion of the cats (Gil, et al., 2013). This suggests that the licensed protocol seems to be a better choice in more symptomatic cats when an effective and marked clinical improvement is desired. Being a feline recombinant product, it does not induce neutralizing antibodies meaning that the high-dose protocol may be used safely and efficiently even if repeat administration is required. This is an important factor to consider in a condition where management will be life-long.

As expected, the concurrent viral excretion was minimal in the PO group mainly due to the fact that these animals were not living in a shelter condition, where opportunistic infections

are more difficult to control. Despite the apparent overall reduction in the viral loads of FHV-1, FCoV and FPV during oral treatment, these findings were considered to be without clinical significance taking into account the low initial viral loads in this group. Further studies are required to fully clarify the role of oral rFeIFN-ω in the reduction of concurrent viral excretion, particularly in shelter medicine. Regarding the FCV status, no changes were observed in positive animals during oral treatment, and one of the negative cats became positive during therapy. This had no relationship with the clinical presentation, as oral rFeIFN-ω induced a useful clinical improvement in the animals in spite of the fact that they remained FCV positive, and agrees with previous studies which describe the long-term carrier state of many cats (Coyne, Gaskell, Dawson, Porter, & Radford, 2007). According to these authors, truly persistent infection is relatively rare and most of the FCV-positive cats undergo cyclical reinfections. These results in the oral group are also somewhat in contrast to the group receiving higher doses by subcutaneous injection, where reduction of viral excretion was more marked. Nevertheless, this study reveals that beneficial immune-modulation can be obtained even with low oral doses of rFeIFN-ω. In a similar fashion to HIFN-α (Tompkins, 1999), it may induce a local stimulation of lymphoid tissues which results in a systemic modulation of the immune response. Studies regarding rFeIFN-ω pharmacokinetics are scarce (Ueda, et al., 1993). In contrast to HIFN-α, some authors report that rFeIFN-ω is acid resistant, which means that it may have a greater relative oral absorption or have activity on the gut-associated lymphoid tissue, thus better potentiating the overall immune system (Ueda, et al., 1993; Addie, 2012). In contrast to the licensed protocol (Gil et al., 2013), when the PO protocol was used the improvement of oral lesions (ulcers and caudal stomatitis) was milder. This is in contrast to the effects seen in a previous study where the same oral administration protocol induced significant improvements in cats with refractory caudal stomatitis (Hennet, et al., 2011). One major difference is that in that study all cats were retrovirus negative. This suggests that when treating retrovirus positive cats for caudal stomatitis, better results may be obtained with a protocol which, at least initially, uses higher injectable doses. Despite the more limited impact on caudal stomatitis and viral excretion, the oral protocol resulted in a significant improvement in other parameters such as body condition and coat appearance. Coat appearance is a non specific sign in cats but it is the authors' opinion that a good esthetical improvement in the animals might be a favorable point for improving the owners' compliance. Regarding the body condition, it was previously reported that rFeIFN-ω may be helpful in the initial resolution of anorexia in hyperthermic cats (Lutz TA, McGahie D, & Albout, 2011). Although there was no anorexia reported in this study, animals increased their weight which is in agreement with the previously cited trial using oral rFeIFN-ω in FIV-positive asymptomatic cats (Caney S., 2003). This is particularly important in thin and debilitated animals. As also described by some authors (Hennet, et al., 2011), the clinical improvement observed may be related to the relief of oral lesions, even if

only mild, which helps improve mastication and increase appetite. Animals with ocular discharge also showed a good improvement which is probably related to the control of opportunistic infections subsequent to immune modulation rather than a direct local antiviral effect.

Regarding hematology and biochemistry, despite mild fluctuations, no significant changes were observed in either group. The unremarkable erythrocytosis observed in 3 cats in the PO group on D0 was clinically compatible with mild subclinical dehydration. The cat that developed a moderate non-regenerative normocitic and normochromic anemia on D90 was submitted to a clinical workup. The anemia was considered to be resulted of chronic inflammation and concurrent respiratory tract disease. After the end of the study, this animal was treated with antibiotics (Cefovecine) which improved the respective blood results. Considering the two outdoor cats in the PO group that revealed transitory elevations of ALT and AST serum levels, a possible hepatic lipidosis secondary to an inappropriate food intake or a subclinical pancreatitis was considered. However, recognizing that both animals did not show any other clinical abnormalities, and that this increase was only analytical and values normalized within the period of study, the owners refused to perform the respective complementary exams suggested. Similarly to the licensed protocol, and as expected, oral rFeIFN-ω does not induce significant hematological or biochemical changes. The SPE results showed that all the animals of both groups presented an hypergammaglobulinemia and a concurrent hyperproteinemia at the beginning of the study. These findings corroborate the results of previous studies that describe an hypergammaglobulinemia in FIV positive cats, due to a concurrent opportunistic infections and polyclonal B-cell activation (S. Gleich & Hartmann, 2009; Hartmann, 2011). Considering that no significant changes were observed in either group, rFeIFN-ω does not seem to interfere with SPE, independently of the protocol administered. However, despite the lack of statistical significance, there appeared to be a tendency for the hypergammaglobulinemia to increase in the SC group, while concurrent viral excretion reduced (Gil, et al., 2013), suggesting that it could be related to a subtle but detectable immune stimulation increasing the activity of B-cells. In contrast, this was not observed in the PO group where the hypergammaglobulinemia and total proteins remained stable during therapy. As expected, outdoor cats showed a more evident hypergammaglobulinemia and raised total protein levels than indoor ones, which could simply be related to a higher level of antigen-exposure in different environments.

Considering APP profile, on D0 the PO group revealed higher AGP SAA and CRP serum levels when compared to the SC one. Previous results described an increase of APP levels in FIV-positive cats treated with the SC protocol (Leal, et al., 2014). In contrast, administration of oral rFeIFN-ω did not induce significant changes in CRP, SAA and AGP serum levels. This is in agreement with a previous study performed in dogs where APPs were higher in dogs from private householders in comparison with cleaned kennels

(Yamamoto, et al., 1994). Thus, one possible explanation relies on a wide exposure to different environmental factors. Although they were living in a shelter, cats from the SC group remained restricted to a particular area whilst cats from the PO group, even indoor ones, were probably in contact with a larger variety of different daily stimuli. However, there are some arguments which do not support this theory. In fact, cats from the animal shelter were positive to other concurrent viruses on D0 (Gil, et al., 2013), suggesting that environmental factors were less controlled in this group. Subsequently, as these cats improved their clinical conditions during rFeIFN-ω therapy, their APP levels increased and concurrent viral excretion decreased. Therefore, more than simply the environmental exposure to pathogens, these data support the hypothesis that the shelter cats had a more evident immune-suppressed basal health status. This can explain a poor innate response and subsequent lower levels of APP despite the clear evidence of opportunistic infections. Consequently, the shelter-housed SC group showed lower initial levels of APP, which increased with therapy suggesting a restoration of the immune competency. Oral rFeIFN-ω did not significantly change the APP profile meaning that, despite the chronic oral therapy, the observed clinical benefits do not seem to be related with an increase on APP profile in these animals. A suggested explanation for this relies on the fact that, a chronic oral therapy may induce an overall clinical improvement due to a local action directly into the mucosa and localized lymphoid areas, potentiating a local immune response rather than a systemic one. Therefore, while the SC protocol, based on pulsate cycles of higher doses of rFeIFN-ω seems to have a relevant systemic role potentiating the innate immunity, the oral protocol suggests to act differently and directed in the local immune response. Considering the higher initial APP levels in the PO group, this study alone is not sufficient to determine whether the licensed protocol is more potent in potentiating the innate-immune response or if this is simply the impact of the other factors such as living environment and better initial immune competence. Further studies namely the evaluation of cytokine profile expression namely pro-inflammatory ones (such as IL-6, IL-1 and TNF-α) and its relation with the APP profile would clarify these major differences between groups.

A limitation of this study is the use of cats living in different environments. It seems reasonable to assume that animals from a shelter tend to be more exposed to conditions of higher morbidity than housed cats and, therefore, marked clinical improvement may be more likely when immune-modulation therapy is performed. However, recognizing that both protocols have different durations and routes of administration, a blinded study seemed unreasonable. More than simply comparing protocols, this study allowed us to assess the clinical improvement of cats individually with the baseline data of each cat also providing useful comparative information. Despite the heterogeneity of environments, initial clinical scores did not differ between groups, which made the SC group a reliable positive control for clinical improvement assessment. Furthermore, having rFeIFN-ω treated FIV-infected cats

that had been previously studied in a single-arm trial permitted a reduction of the number of animals used for this research. However, in other parameters namely APPs, differences in groups on D0 are significant and reflect different basal immune status. Rather than household cats, it seems reasonable to assume that cats from an animal shelter and living in catteries have different extrinsic factors that can affect the immune response. Therefore, particularly in these parameters, the positive group control is less reliable. Even though, APPs are intrinsically variable even in healthy cats, reason why it is recommended that the animal should act as its own reference (Ceron, et al., 2005). Then, more than comparing both groups, this study evaluated APP's tendency after the oral protocol, having the baseline values as the intrinsic own reference. This analysis minimized the initial discrepancy observed between groups.

This is the first study describing the successful application of an oral rFeIFN-ω protocol in symptomatic FIV-infected cats, opening new insights into more detailed immunological studies. It is highly probable that the licensed protocol provides sufficient levels of interferon systemically to induce a direct antiviral stimulus, in contrast to the oral protocol where systemic absorption is relatively limited and the doses used are also significantly lower. This may explain the apparently greater benefit of the injectable protocol in cats with an initially higher clinical score and in cats with high initial levels of virus shedding, and suggests that this should be taken into account when choosing the protocol for an individual cat in a clinical setting. Although the laboratory changes are subtler than those observed in the SC protocol, oral rFeIFN-ω nevertheless resulted in a useful improvement of the animals' condition. Considering the significantly reduced cost of the product, it could be an interesting alternative for immune-modulation therapy of FIV-infected cats with a mild to moderate clinical presentation if the current licensed protocol is difficult to perform. Additionally, this may be an interesting option for treatment follow-up after the licensed protocol once the condition of the cat is better stabilized and secondary viral infections are better controlled.

PART II - Chapter IV:

Evaluation of Mx protein expression in naturally FIV-infected cats receiving oral Recombinant Feline Interferon Omega therapy

Chapter IV: Evaluation of Mx protein expression in naturally FIV-infected cats receiving oral Recombinant Feline Interferon Omega therapy

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Abstract

Recombinant feline interferon omega (rFeIFNω) is the first veterinary-licensed type I IFN, currently used in various viral diseases such as Feline Immunodeficiency virus (FIV). Despite the effectiveness of the licensed subcutaneous protocol, recent studies have suggested an alternative oral protocol (0.1MU/cat SID 90 days). This study assessed Mx protein expression as a molecular biomarker during oral rFeIFNω therapy in FIV- infected cats.

Mx expression was quantified by Real-Time PCR, using mRNA extracted from PBMCs of 7 client-owned naturally FIV-infected cats before (D0) and after (D90) treatment with oral rFeIFNω.

The mean Mx protein expression did not differ significantly after oral rFeIFNω therapy. On D0, expression was detected at low levels in the four more symptomatic cats with a slight decrease on D90. The other three tested negative on D0, but expression increased with therapy, resulting in a low quantity on D90. In symptomatic cats, oral rFeIFNω immune-modulation could decrease the production of pro-inflammatory cytokines leading to a concurrent paradoxical reduction of Mx protein, despite rFeIFNω therapy. In less symptomatic animals, the innate immunity seems to be initially less stimulated and oral rFeIFNω increases Mx protein expression.

This is the first pilot-study reporting Mx protein expression in Naturally FIV-infected cats under oral rFeIFNω therapy, opening new insights about its potential use in vivo as a biomarker of an innate immune-stimulation.

Introduction

From research to clinical practice, the therapeutic properties of IFN therapy have been considered in veterinary medicine. First cloned in 1992, recombinant feline IFN omega (rFeIFNω) is the first type I IFN licensed for use in veterinary practice (Nakamura, Sudo, Matsuda, & Yanai, 1992) and it is currently used in retroviral infected cats. Particularly in FIV-infected cats, little is known about the molecular action of rFeIFNω. Previous studies have shown that the licensed protocol (3 cycles of 5 daily SC injections 1MU/kg) has multiple clinical benefits such as an increase on the survival time, a clinical improvement or even a reduction of concurrent viral excretion (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013). However, it only induces slight changes on FIV virus and provirus loads *in* vivo (Domenech, et al., 2011). Recognizing that FIV-infected cats have a compromised innate immune-response (Dean, Bernales, & Pedersen, 1998; Dean, LaVoy, Yearley, & Stanton, 2006), the current authors have previously reported that the licensed protocol induces an increase of APP profile (Leal, et al., 2012) which, concurrently with a significant clinical improvement, seems to be beneficial (Gil, et al., 2013). This is in agreement with an eventual action of rFeIFNω, potentiating the innate immune-response in naturally retroviral infected cats. Despite its clinical benefit, the licensed protocol can be cost-limitative and other alternatives have being studied by authors (Gil, et al., 2014). After investigating other immune-modulation protocols for FIV-infected cats with this molecule, the authors have described an oral protocol (Gil, et al., 2014). This revealed a significant clinical improvement of treated cats but, in opposition to the licensed protocol, it failed to induce an increase of APPs (Gil, et al., 2014). This is in agreement with previous authors who described that oromucosal IFN therapy seems to have different mechanisms of action from the parenteral protocols (Tovey, 2002). Therefore, whilst in the licensed protocol the increased APP can reinforce a potentiated innate immune response, in the oral protocol these immunitary mechanisms beneath clinical improvement still remain unclear. Consequently, it is imperative to search for other parameters than APP which may clarify the effect of rFeIFNω on the innate immune system.

The innate immune system recognizes different invaders due to the presence of pathogen-associated molecular patterns (PAMPs). In viral infections, these are mainly nucleic acids such as double or single-stranded RNA (Akira, Uematsu, & Takeuchi, 2006). The detection of PAMPs by specific receptors such as Toll-like receptors (TLRs) activates a variety of signaling pathways and increases the expression of important cytokines such as type I interferons, considered to be particularly relevant for the antiviral innate response (Haller, et al., 2007; Schindler, Levy, & Decker, 2007; Sadler & Williams, 2008). This explains why type-I IFNs, such as rFeIFNω, have been an important therapeutic resource in viral infections.

Depending on the receptor complex to which the IFN binds (Sadler & Williams, 2008), three classes are distinguishable (type I, II and III). Type I IFNs bind to the IFNAR receptor complex, having an important role in the host response against viral infection (Haller, et al., 2007). Specifically, they act as positive feedback inducers, increasing the expression of TLRs and sensitizing cells to microbial recognition (Siren, et al., 2005). They establish an important link between innate and adaptive immunity, inducing the differentiation of several cell types (Colonna, et al., 2004). They are believed to not only block viral replication but also to slow the growth of infected cells making them more susceptible to apoptosis (Goodbourn, et al., 2000; Bracklein, et al., 2006). Type II IFNs bind to the receptor IFNγR and include only one gene product (IFN-γ). It is synthesized after the recognition of infected cells by natural killer cells and activated T lymphocytes (Goodbourn, et al., 2000). Less studied, type III IFN group includes IFN-λ, which binds to the receptors IFNLR1 and IL10-receptor 2. In a similar way to type I IFN, it seems to regulate the antiviral response (Haller, et al., 2007; Sadler & Williams, 2008).

From a molecular perspective, when type I IFN binds to the IFNAR receptor, it activates a signal transduction pathway that induces more than 300 IFN-stimulated genes (Der, et al., 1998). Consequently, they are involved in the synthesis of various enzymes, including the RNA-dependent protein kinase, 2´-5´ Oligoadenylate synthase (2-5 OAS) and the Mx protein GTPases (Samuel, 2001). In contrast to parenteral routes, oral administration of IFN does not result in detectable serum IFN levels. In this sense, the expression of these biomarkers can be useful to assess oral IFN activity (Gibson, Cotler, Spiegel, & Colburn, 1985; Fleischmann, Koren, & Fleischmann, 1992; Brod, Nelson, Jin, & Wolinsky, 1999). Then, some authors have developed different bioassays to evaluate interferon's biological activity, mainly based on these IFN-responsive genes in PBMCs and in urine (Schattner, et al., 1981; Cheng, Becker-Manley, Rucker, & Borden, 1988; Schiller, et al., 1990). The majority of these biomarkers assess IFN activity independently of the family, meaning that they are not useful to differentiate between type I or type II IFN actions. An exception is the Mx protein, which is a specific type I IFN biomarker (Bracklein, et al., 2006). With specific antiviral and GTPase properties, this compound is expressed in a variety of cells such as hepatocytes, endothelial cells and immune cells such as PBMCs, plasmacytoid dendritic cells and myeloid cells (Horisberger, Schrenk, Staiger, Leyvraz, & Martinod, 1990; Fernandez, et al., 1999; Sadler & Williams, 2008). Mx protein production is induced by type I IFN within 1-2 hours and it has a biological half-life of 2.5 days, reaching the maximum concentration around 36 hours after IFN induction (Ronni, Melen, Malygin, & Julkunen, 1993). Although the functions of Mx protein are not all completely understood, it is directly involved in viral recognition, it impairs viral transcription, it binds to essential viral components blocking their intracellular transport and it controls different processes such as exocytosis, preventing and avoiding viral

replication in an early phase (Turan, et al., 2004; Haller, et al., 2007; Sadler & Williams, 2008). Due to its relevance and anti-viral properties, Mx gene expression has been evaluated in several clinical conditions such as viral infections, autoimmune diseases and in specific cases of type I IFN therapy (Horisberger & De Staritzky, 1989; Horisberger, et al., 1990; von Wussow, et al., 1990; Bracklein, et al., 2006).

In veterinary medicine, similarly to murine models, the role of type I IFN and gene induction profiles have been explored (Horisberger, et al., 1990; Ueda, et al., 1993; Bracklein, et al., 2006; Robert-Tissot, et al., 2011). One study described a dose-dependent correlation between subcutaneous (SC) therapy with recombinant HuIFNα and Mx protein in cats, confirming that it is a stable marker to monitor IFN activity in this species (Horisberger, et al., 1990). With the release of rFeIFNω, further studies have been conducted to explore the impact of this molecule. One study reported that parenterally administered rFeIFNω modulates the activity of 2-5OAS (Ueda, et al., 1993). Another publication also reported that rFeIFNω induces the expression of Feline Interferon-Stimulated Gene 15 (FeISG15) in vitro (Tanabe, et al., 2008). Regarding Mx protein, some authors confirmed the activity of rFeIFNω after oral and ocular administration by measuring Mx protein expression by immunoblotting of white blood cells and immunostaining of conjunctival cells (Bracklein, et al., 2006). Specifically relating retroviruses and Mx expression, only one study have described that it increases in a similar way to other endogenous type I IFN genes in an early stage of in vitro infection of PBMCs with FIV (Robert-Tissot, et al., 2011).

To author's knowledge, there are no studies reporting Mx protein expression in naturally FIV-infected cats under immune-modulation therapy. However, even if APPs failed to prove it, the effect of oral rFeIFNω on other pathways of the innate-immunity such as Mx protein modulation should be considered. Therefore, this study aims to assess the role of Mx protein expression as a molecular biomarker of rFeIFNω therapy in naturally retroviral infected cats, submitted to an oral rFeIFNω protocol.

Material and Methods

The mRNA of PBMCs extracted from blood samples taken from 7 client-owned, naturally FIV-infected cats treated with oral rFeIFNω was used for this study. The animals had been referred/admitted to the Veterinary Teaching Hospital – University of Lisbon and enrolled for a previous study which described a clinical improvement induced by this protocol (Gil, et al., 2014). The study was approved by the local ethical committee (CEBEA-FMV-ULisboa).

After obtaining the owner´s informed consent, the animals were treated with oral rFeIFNω (Virbagen Omega®, Virbac, France) at 0.1MU/cat SID for 90 days. The daily doses were obtained by diluting a vial containing the freeze-dried pellet of rFeIFNω (10MU) in 25ml of

sterile saline. Single doses of 0.25ml (equivalent to 100kU per dose) were prepared in 1 ml syringes by one of the members of the team. Syringes were frozen (-18 to -20ºC) after preparation and until use. Doses were given to owners who were instructed to defrost each single dose shortly before use and to administer the solution directly into the oral cavity. No drugs other than rFeIFNω were permitted during the study.

The cats were clinically evaluated and blood was collected before (D0) and after therapy (D90). Being a prospective, single-arm trial, D0 was considered the basal value and the individual's own control for the measured gene expression.

Blood was collected using RNAprotect animal blood tubes (Qiagen). mRNA was extracted from whole blood using the RNeasy protect animal blood kit (Qiagen) according to the manufacturer instructions. cDNA was synthetized using the Transcriptor high fidelity cDNA synthesis kit (Roche) and thereafter used as a template for relative quantification of Mx Protein gene expression by Real-Time Polymerase Chain Reaction (Real-Time PCR).

Real-time PCR was performed using the Step One Plus analyser (Applied Biosystems). The cycling conditions comprised an initial denaturation step at 95°C for 10 minutes, followed by 50 cycles of 95°C for 15 seconds and 1 minute at 60°C.

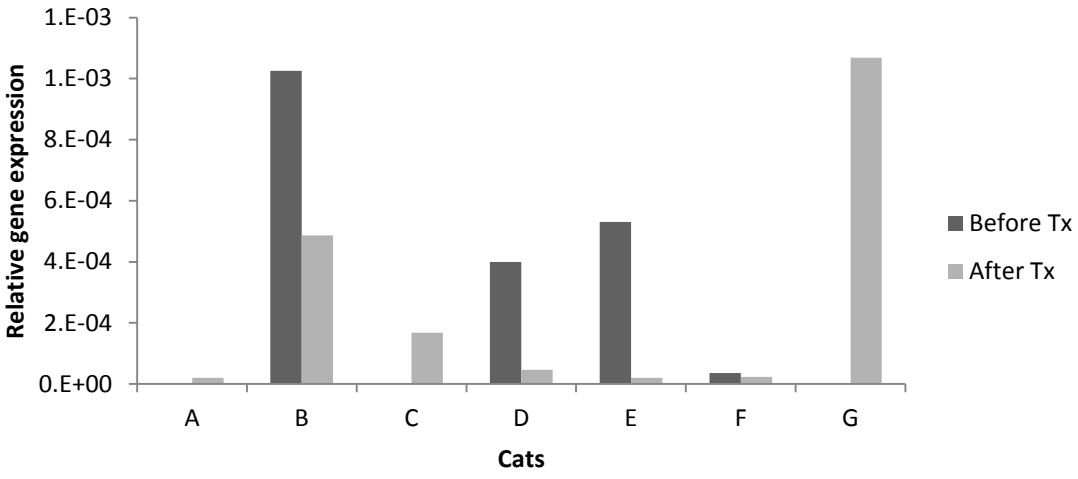
The primers and probe for Mx gene expression quantification have been previously published (Robert-Tissot, et al., 2011). Assays were performed using 2 µl of cDNA template in a total volume of 20µl per reaction using TaqMan® Gene Expression 2x Master Mix (Applied Biosystems). The following primers and probe concentrations were used: 900nM of forward primer (5´-ACCAGAGCTCGGGCAAGAG-3'), 900nM of reverse primer (5´-TTCAGCACCAGAGGACACCTT-3´) and 250nM of TaqMan Probe (FAM-5´-CCTTCCCAGAGGCAGCGGTATTGTC – 3´TAMRA). The recommended concentrations were tested and an efficiency of 0.88 was yielded in the authors' laboratory and conditions. For relative quantification, Mx gene expression was normalized using a reference gene – Beta Actin (housekeeping) for which the primers and probe were also already published (Scott, et al., 2011). Assay conditions were the same as those described for Mx gene quantification. The following concentrations and sequences of primers and probe were used: 900nM of forward primer (5´- GACTACCTCATGAAGATCCTCACG-3'), 900nM of reverse primer (5´- CCTTGATGTCACGCACAATTTCC-3´) and 250nM of TaqMan Probe (JOE-5´-CCTTGATGTCACGCACAATTTCC-3´TAMRA). In the authors' laboratory conditions, an efficiency of 0.85 was obtained for this gene. Relative gene quantification and normalization were assessed using Real Time Miner Software (http://miner.ewindup.info).

Mx expression was thereafter correlated with the previous published results of clinical improvement for each individual cat, where in order to assess clinical improvement, animals were evaluated using a previous validated score-scale system (Gil, et al., 2013).

Results

There was no statistical difference between mean Mx protein expression on D0 and D90 whether quantification of gene expression (p = 0.735 Wilcoxon Signed Rank Test for related samples) or the presence/absence of gene expression (p=0.250 McNemar Test for related samples) was considered. On D0, Mx protein expression was detected at low levels in 4 of the 7 cats which experienced a decrease by D90. After therapy, this gene was quantifiable in all of the cats (7/7) meaning that the 3 cats in which Mx gene expression was undetectable on D0 increased the expression by D90. Individual data are shown on figure 19.

Figure 19: Individual Mx gene expression of 7 naturally FIV-infected cats treated with oral rFeIFNω protocol. Presented values refer to quantification of Mx protein gene expression using beta-actin (the housekeeping gene) for normalization and relative quantification.



Correlating these findings with the previously described clinical improvement provided by this protocol (Gil, et al., 2014), the 4 cats which were positive on D0 for Mx expression had worse initial clinical conditions (mean clinical score 11.75/20 on D0) than the 3 cats which were negative (mean clinical score 4.7/20 on D0). All 4 of the cats with Mx gene expression on D0 improved their clinical signs (mean clinical score on D90 was 8/20), while Mx expression reduced. For the 3 cats which did not show Mx gene expression on D0, 2 remained clinically stable (cats A and G) while one improved its clinical score (cat C). The overall mean clinical score of these animals were 2/20 on D90. In all of them, Mx gene expression showed a tendency to increase.

Discussion

This study described the impact of oral rFeIFNω treatment on Mx gene expression in naturally FIV-infected cats. Although the truly function of Mx protein remains unclear (Sadler & Williams, 2008), it is believed to have anti-viral properties, being a reliable biomarker of type I IFN activity. Considering that, in vivo, rFeIFNω has shown a doubtful anti-viral activity against retroviruses at the normally used doses despite dramatic improvements in clinical scores (Domenech, et al., 2011), Mx gene expression measurement allowed us to investigate the systemic antiviral effect of oral rFeIFN therapyω. In this study, 4/7 cats had detectable Mx gene expression on D0 (before therapy) while 3/7 did not. This is in agreement with the previous literature which suggest that animals and humans may have basal values of interferon-induced genes, such as 2-5-OAS and Mx transcripts, even before type I IFN administration (Asada-Kubota, Ueda, Shimada, Takeda, & Sokawa, 1995). Studies in mice have demonstrated that endogenous IFN is continuously produced, possibly in order to provide a potential innate inhibition of viral activity (Galabru, Robert, Buffet-Janvresse, Riviere, & Hovanessian, 1985; Bocci, 1988).

In the present study, the 4/7 cats in which Mx gene expression was quantified on D0 had worse mean clinical conditions than the others 3/7 which tested negative. Being chronically infected with FIV, a possible explanation for the detection of Mx gene expression may be chronic stimulation of endogenous pro-inflammatory cytokines (such as IFN) in these animals. This can be induced and potentiated by FIV itself or other opportunistic subclinical infections which can contribute to a worse clinical condition. From the 3 cats which tested negative on D0, 2 showed an overall good clinical condition (with the exception of a discrete oral disease) and 1 was more symptomatic. These animals may therefore have had less stimulation of endogenous IFN due to opportunistic viral infections in the time shortly before the study.

After therapy, all the animals had detectable levels of Mx gene expression. In agreement with previous described studies, rFeIFNω seems to have an important clinical benefit. All of the 4 cats which were symptomatic and positive for Mx expression on D0 improved their clinical signs while Mx slightly decreased after therapy. As previously described, rFeIFNω can minimize opportunistic infections and improve health status of FIV-infected cats, either orally or subcutaneously (Gil, et al., 2013; Gil, et al., 2014). According to some authors, type I IFN was described to affect various pro-inflammatory cytokines such as IL-6, IL-1 and IFN-gamma (Taylor & Grossberg, 1998). Although only few interleukins are able to be Mx-homologous protein inducers, some of them can indirectly affect endogenous levels of endogenous type I IFNs and consequently alter Mx expression (von Wussow, et al., 1990). In symptomatic cats, potentiating the innate immune system, oral rFeIFNω can eventually

decrease the production of different pro-inflammatory mediators namely endogenous type I IFNs. Thus, it seems reasonable to say that the reduction of pro-inflammatory cytokines can lead to a concurrent paradoxical reduction of Mx protein, even if the animal is under rFeIFNω therapy. Consequently, although the counter-balance of an exogenous administration of rFeIFNω, type I IFN pathways are slightly decreased, leading to a concurrent decrease tendency of Mx protein in these cats.

In opposition, all of the 3 cats which tested negative on D0 became residually positive for Mx expression after therapy. Clinically, 2 of them remain stable while one improved his clinical condition. Recognizing that these animals were less symptomatic before therapy, the innate immunity can be less stimulated and the pro-inflammatory chronic mechanisms could be less relevant. Therefore, and in agreement with previous studies, the exogenous administration of rFeIFNω could residually activate type I IFN pathways leading to an increase of Mx protein expression.

Previous studies performed on specific pathogen free (SPF) cats using low-doses of rFeIFNω in a range of low doses from 200U to 20.000U/cat reported a dose-dependent increase of Mx-protein expression (Bracklein, et al., 2006). The current study was performed using a higher dose for a longer time of therapy (100.000U/cat during 90 days), previously discussed with the manufacturers of the rFeIFNω and formerly applied in other studies (Hennet, et al., 2011). Considering that Mx-protein did not seem to increase in treated cats, a possible inhibition of its expression with higher rFeIFNω protocols cannot be excluded. In murine models (Brod, et al., 1999) it was previously described that higher doses of ingested type I IFN may have different effects than lower doses. In divergence to other conclusions (von Wussow, et al., 1990; Bracklein, et al., 2006), authors defend that higher doses fail to induce Mx expression which can be due to various mechanisms namely: a downregulation of IFN receptors at higher doses, a cell-cell interaction induced by T-cells which have great affinity to type I-IFN and non-T cell populations with low affinity that are activated with higher doses and can block Mx gene expression or even the presence of different affinity-receptors meaning that with low doses only the highest affinity receptors are activated and induce IFN-gene transcription but in higher dose, that low affinity IFN receptors can transducer counter-regulatory signals which inhibits them.

Despite the decrease observed in 4/7 cats, all of FIV-treated cats (7/7) showed residual levels of Mx expression at the end of therapy. This corroborates with an eventual and discrete immune-modulation activity beneath the clinical improvement previously described. According to some authors, this can rely on a paracrine and local action in the gut-lymphoid tissues, which can spread to a regional and systemic response (Bocci, 1991; Ueda, et al., 1993).

The use of a single-arm trial to evaluate the effect of Mx expression can be considered a limitation of this study. However, to author's opinion, this can be considered a minor flaw. Firstly, the study uses naturally FIV infected cats where time of infection is unknown and clinical presentation is variable. To perform a reliable double-arm control trial, it would be necessary to have animals within the same biological conditions which, using naturally FIV infected cats, seems unreasonable. Secondly, this study only relies on the oral effect of rFeIFNω in a specific gene expression which, in particular, is considered a biological marker of type I IFN activity. Considering that each animal was monitored before therapy, the use of this individual time point allowed authors to establish a reliable endogenous control, assessing the direct effect of therapy in the basal gene expression.

Despite its proven effectiveness in vitro as a biomarker of the innate immune response in early stages of FIV infection (Robert-Tissot, et al., 2011), this study reveals that Mx protein expression showed a high variability pattern in FIV-infected cats under oral rFeIFNω therapy. This can be due to the low number of animals and the different clinical presentations. Correlating these data to other immunitary parameters such as APPs, it is observed that, in opposition to the licensed protocol in which APP increased with therapy, they did not change after oral rFeIFN therapy. In agreement with previous authors that describe different mechanisms of action according to the route administered (Tovey, 2002), it is possible that this oral protocol is not as effective as the licensed one in the innate immune-stimulation. In a similar way to APP, also Mx expression did not change after oral rFeIFN therapy meaning that, despite the observed clinical improvement, this protocol may only induce slight systemic changes on the innate-immunity which are not sufficiently evident to be measurable by these biomarkers. Even though, recognizing that all the animals expressed the Mx gene after therapy, it cannot be excluded that its expression can be slightly affected by exogenous type-I IFN. Although it could be related to clinical scores, the role of Mx protein expression was inconsistent and further studies are required in order to extend these results.

Besides speculations, this is the first pilot-study reporting Mx protein expression in Naturally FIV-infected cats under oral rFeIFNω therapy, opening new insights about its potential use in vivo as a biomarker of an innate immune-stimulation.

Part II - Chapter V:
Evaluation of viremia, proviral load and Cytokine profile in naturally FIV-infected cats treated with two different protocols of recombinant feline interferon Omega

Chapter V: Evaluation of viremia, proviral load and the cytokine profile in naturally FIV-infected cats treated with two different protocols of recombinant feline interferon omega

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Abstract

Recombinant Feline Interferon-Omega (rFeIFNω) is an immunomodulator licensed for use in feline retroviral infections using 3 cycles of 5 daily subcutaneous injections of 1MU/kg on Days (D)0, 14 and 60. This compound seems to act on the innate immunity and induces a clinical improvement, reduces concurrent viral excretion and increases acute phase proteins (APPs). Recently, an alternative oral rFeIFNω protocol (0.1MU/cat daily for 90 days) was successfully applied to FIV-infected cats. Despite the evident clinical improvement, APPs did not change significantly with oral therapy. Independently of the protocol, the action of rFeIFNω on the cytokine profile (namely Th1/Th2 subsets) is unclear.

This study aimed to evaluate the anti-viral and immunomodulation properties of rFeIFN-ω by monitoring changes in viremia, proviral load and blood cytokine profile (mRNA expression of Interleukin (IL)-1, IL-4, IL-6, IL-10, IL-12p40, Interferon (IFN)-γ and Tumor Necrosis Factor (TNF)-α and plasma levels of IL-6, IL-12p40 and IL-4) in naturally FIV-infected cats submitted to two distinct (oral and subcutaneous) rFeIFN-ω protocols.

18 naturally FIV-infected cats were enrolled: 7/18 received the licensed protocol (SC group) while 11/18 received the oral protocol (PO group). Animals were monitored before (D0) and after therapy (D65 and D90, respectively for SC and PO groups) and blood samples were collected. Blood cytokine expression, viremia and proviral load were assessed by Real-Time qPCR (RT-qPCR). Concurrent plasma levels of IL-6, IL-12p40 and IL-4 were measured with specific ELISA kits.

No significant changes were observed except for IL-6, a pro-inflammatory cytokine whose expression significantly decreased in the PO group (p=0.037). For plasma levels, IL-6 significantly decreased in the SC group (p =0.031) and no differences were observed in the other measured cytokines. Independently of the protocol, no significant changes were observed in viremia. Regarding proviral load, whilst it remained stable in the PO group, it significantly increased (p=0.031) in the SC cats. No correlation was obtained between provirus and viremia results in both groups.

While the high pulse scheme of the SC protocol leads to an important reduction of IL-6 plasma levels, the use of continuous lower doses with the oral protocol induces a decrease of IL-6 expression, albeit not sufficiently to be observed in its plasma levels. Despite its antiviral effect in FIV-cats, we did not observe an effect of rFeIFN-ω on the acquired immune-response (Th1/Th2 cytokine subsets) but it seemed to act on the innate immune response by reducing pro-inflammatory stimuli.

Introduction

Recombinant Feline Interferon Omega (rFeIFN-ω) is an immunomodulator commonly used in feline retroviral infections (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013). It is produced as a recombinant protein by means of a baculovirus expression vector which contains the feline interferon omega sequence (Ueda, et al., 1993). This baculovirus replicates in silkworms, permitting the production of the glycosylated molecule which, after purification, can be used therapeutically and which is currently licensed for use in all countries of Europe, in Australia and Asia (Ueda, et al., 1993).

The recommended protocol is based on 3 cycles of 5 daily subcutaneous administrations (1MU/kg), beginning respectively on days 0, 14 and 60. Following initial *in vitro* studies (Truyen U., 2002), several authors have been performing *in vivo* trials, in order to assess its clinical and immune properties. The action of rFeIFN-ω in cats naturally infected with FeLV and co-infected with FeLV and FIV has been described, showing that this compound induced an important clinical improvement and an increased survival time of treated cats (de Mari, et al., 2004). In agreement with the previous study, another group of authors (Domenech, et al., 2011) reported that rFeIFN-ω improved the clinical condition of retroviral infected cats, although minor changes were observed on other parameters such as hypergammaglobulinemia, CD4/CD8 ratio, proviral load and viremia. Thus, an overall improvement of innate-imunity was suspected (Domenech, et al., 2011). Recently, our own group has reported that, in addition to improving clinical signs, rFeIFN-ω also induces a reduction of concurrent viral excretion (namely herpesvirus, coronavirus, parvovirus and

calicivirus) which is particularly relevant in shelter medicine (Gil, et al., 2013). In an attempt to understand the immune pathways underlying the therapeutic action of rFeIFN-ω, we also evaluated the effect of this compound on acute phase proteins (APPs) during treatment of naturally retroviral infected cats (Leal, et al., 2014). Although they are not commonly measured in feline practice, APPs are one of the key components of the innate immune response and can be useful for monitoring its activity (Leal, et al., 2014). In agreement with the previous hypotheses, we concluded that rFeIFN-ω induced a significant increase of APPs in treated naturally retrovirally-infected cats, confirming that this compound potentiates the innate immune response (Leal, et al., 2014).

Despite the clinical benefit of the licensed protocol in naturally retrovirally-infected cats, it can be cost-limitative in some cases and alternative protocols have been investigated. After some trials describing the use of lower oral doses of rFeIFN-ω in various conditions such as chronic gingivostomatitis (Hennet, et al., 2011; Leal, et al., 2013), an oral protocol was recently proposed in naturally FIV-infected cats (Gil, et al., 2014). This was based on the daily oral administration of 0.1MU/cat during 90 consecutive days and, in a similar way to the licensed protocol, revealed a significant clinical improvement of treated cats without relevant changes on hematology, serum biochemistry or serum protein electrophoresis (Gil, et al., 2014). In contrast to the licensed protocol, it failed to induce an increase of APPs and considering that concurrent viral infections were low in the tested group at the start of the study, the reduction of opportunistic infections was irrelevant (Gil, et al., 2014). This apparent difference in the mechanism of action of each protocol is in agreement with previous authors who suggested that oromucosal IFN therapy seems to act by different mechanisms than parenteral protocols (Tovey, 2002). Therefore, whilst in the licensed protocol the increased APP and concurrent viral reduction seem to denote a potentiated innate immune response (Gil, et al., 2014; Leal, et al., 2014), in the oral protocol the immune mechanisms underlying the clinical improvement remain unclear.

Despite its complexity, the immune system can be divided into two general parts: the nonspecific (innate) response and the specific (acquired) immunity (Kennedy, 2010). Whilst the nonspecific response refers to innate mechanisms against pathogenic infections, the acquired immunity is mainly regulated by lymphocyte specific actions, with special relevance to the cellular and humoral response (Kennedy, 2010; Pedersen, et al., 1998). These specific cellular and humoral responses are mainly coordinated by distinct CD4+ T-helper subsets, respectively Th1 and Th2 (Pedersen, et al., 1998; Roitt & Delves, 2001; Tizard, 2009a, 2009b; Kennedy, 2010; Day, 2012). All of these components interact in order to maintain a competent immune system. This is achieved by the production and release of different cytokines which, being mediators of the immune response, have distinct functions such as potentiating cell growth, differentiation, migration, repair and activation of pro-inflammatory

and anti-inflammatory pathways (Pedersen, et al., 1998; Roitt & Delves, 2001; Tizard, 2009a, 2009b; Kennedy, 2010; Day, 2012).

Despite the fact that most cytokines are pleiotropic, each part of the immune system can be characterized by different cytokine patterns (Roitt & Delves, 2001). For instance, Interleukin-6 (IL-6), IL-1 and Tumor Necrosis Factor (TNF)-α are pro-inflammatory cytokines strongly involved on the innate immune response, potentiating nonspecific pathways such as acute phase response (APR) or fever (Ceron, et al., 2005; Paltrinieri, 2008; Tizard, 2009a). Concerning the cellular response (Th1 subset), IL-2, IL-12 and IFN-γ are strongly related to Th1 subset activation, mainly towards intracellular pathogens, leading to the stimulation of cytotoxic T-cell, Natural Killer (NK) and macrophage activity (Locksley & Scott, 1991; VanCott, et al., 1996; Pedersen, et al., 1998; Tizard, 2009b). On the other hand, the Th2 subset, when activated, induces a humoral antibody response mainly towards extracellular pathogens, based on IL-4, IL-5 and IL-10 production which consequently induces a B-cell differentiation and expansion (Romagnani, et al., 1994; Barnard, Mahon, Watkins, Redhead, & Mills, 1996; Osborne, Hunter, & Devaney, 1996; Pedersen, et al., 1998; Roitt & Delves, 2001).

In feline medicine, particularly in FIV, several studies have been performed mainly in cell cultures, in order to characterize cytokine profile after infection (Lawrence, et al., 1995; Dean, et al., 1998; Dean & Pedersen, 1998; Lerner, et al., 1998; Linenberger & Deng, 1999; Liang, et al., 2000; Ritchey, Levy, Bliss, Tompkins, & Tompkins, 2001; Kipar, et al., 2004). In detail, one study described the in vitro stimulation of peripheral blood mononuclear cells (PBMCs) from naturally and experimental infected cats, based on bioassays originally developed for quantification of cytokines in murine models (Lawrence, et al., 1995). These authors showed that PBMCs from naturally FIV-infected cats revealed a significant increase of IL-1, IL-6 and TNF-α production in response to mitogens, revealing that FIV-animals have a basal increase of pro-inflammatory pathways (Lawrence, et al., 1995). Another study evaluated the Th1/Th2 response in lymphoid tissues from experimentally FIV-infected cats, having shown that the cytokine response is heterogeneous during the early phase of FIV infection (Dean & Pedersen, 1998). Other authors reported that there is a decrease of two Th1 cytokines (IL-2 and IL-12) with a concurrent increase in IL-6 and IL-10 (Th2 subset) although IFN-γ, a Th1 cytokine, also increased (Tompkins & Tompkins, 2008). A recent study has also shown that plasma IL-12 was elevated in FIV-experimentally infected cats confirming that a Th1 response is present in the early phase of infection (Wood, et al., 2012). Various studies have confirmed that, despite the fact that there is no clear Th1 to Th2 shift in response to FIV infection, this retrovirus induces a cytokine dysregulation with a concurrent reduction in transcription levels of cytokines, leading to an inadequate innate and cell-mediated immune

response to other pathogens (Levy, et al., 1998; Kipar, et al., 2004; Tompkins & Tompkins, 2008).

As regards rFeIFN-ω therapy, to the authors' knowledge, there are no studies about the mechanism of action of this compound on the Th1/Th2 cytokine profile response of naturally FIV-infected cats. As noted earlier, only one study tried to assess the effect of this compound on the acquired immunity of naturally retrovirus-infected cats, evaluating variations in CD4/CD8 ratio, viremia and proviral load (Domenech, et al., 2011). Curiously, in contrast to *in vitro* studies involving different viruses (Truyen U., 2002), the anti-viral action of the rFeIFN-ω licensed protocol towards FIV and FeLV in vivo was negligible (Domenech, et al., 2011). Despite these results, the anti-viral potential of oral rFeIFN-ω protocol is still unknown and more studies are warranted to fully confirm that action.

This study aimed to evaluate the anti-viral and immunomodulation properties of rFeIFN-ω by monitoring changes on viremia, proviral load and blood cytokine profile (mRNA expression of IL-1, IL-4, IL-6, IL-10, IL-12p40, IFN-γ and TNF-α and plasma levels of IL-6, IL-12p40 and IL-4) in naturally FIV-infected cats receiving oral or subcutaneous rFeIFN-ω therapy.

Material and Methods

Animals and sample collection

The biological samples used in this study were collected from 18 naturally FIV-infected cats that had been previously enrolled in two past works from the group: 7/18 had received the licensed protocol (SC group) while 11/18 received the oral protocol (PO group) as described in the literature (Gil, et al., 2013; Gil, et al., 2014; Leal, et al., 2014).

The animals had been monitored and submitted to blood collections before (D0) and after therapy (D65 and D90, respectively for SC and PO groups). All the procedures were approved by the Committee for Ethics and Animal Welfare of the Faculty of Veterinary Medicine – University of Lisbon (CEBEA – FMV-ULisboa).

Similarly to studies previously published (Gil, et al., 2013; Leal, et al., 2014), a single-arm trial policy was applied in each group meaning that for each parameter, values on D0 was set as a baseline and were taken as the individual control for each cat.

Relative quantification of cytokine expression by Real-Time qPCR

At each specified time point, whole blood was collected in RNA protect tubes (RNAprotect Animal Blood Tubes, Qiagen) and, according to the manufacturer´s instruction, mRNA was extracted using specific kits (RNeasy protect animal blood kit, Qiagen). Thereafter, cDNA

was synthesized using Transcriptor High Fidelity (Roche) following the manufacturer's instructions and used as a template for Real-Time quantitative Polymerase Chain Reaction (qPCR).

The primers used for each gene were published in the literature and the respective authors and sequences are presented in table 11. Despite the DNAse step performed during the RNA extraction, in order to preclude genomic DNA amplification, primers covered putative exon-exon junctions. Optimization experiments and efficiency assessments for each amplification system were previously performed (data not shown). Primers were obtained from a commercial manufacturer (STAB Vida, Portugal). Relative expression of each cytokine was quantified using Miner software (http://www.miner.ewindup.info), following the computed algorithm for Quantitative Real-time PCR system (Zhao & Fernald, 2005). Beta-actin was set as the housekeeping/reference gene (table 11).

Table 11: Primers used to evaluate cytokine expression by Real-time qPCR in naturally FIV-infected cats treated with rFeIFN protocols.

Gene	Oligo	Sequence (5´-3´)	**Reference**
B – Actin	For	GACTACCTCATGAAGATCCTCACG	(Scott, et al., 2011)
	Rev	CCTTGATGTCACGCACAATTTCC	
IL-1β	For	ATTGTGGCTATGGAGAAACTGAAG	(Scott, et al., 2011)
	Rev	TCTTCTTCAAAGATGCAGCAAAAG	
IL-4	For	CCCCTAAGAACACAAGTGACAAG	(Taglinger, Van Nguyen, Helps, Day, & Foster, 2008)
	Rev	CCTTTGAGGAATTTGGTGGAG	
IL-6	For	GTGTGACAACTATAACAAATGTGAGG	(Scott, et al., 2011)
	Rev	GTCTCCTGATTGAACCCAGATTG	
IL-10	For	ACTTTCTTTCAAACCAAGGACGAG	(Scott, et al., 2011)
	Rev	GGCATCACCTCCTCCAAATAAAAC	
IL12p40	For	TGGCCTTCTGAAGCGTGTTG	(Scott, et al., 2011)
	Rev	GAAGTACACAGTGGAGTGTCAGG	
IFN-γ	For	TGCAAGTAATCCAGATGTAGCAG	(Taglinger, et al., 2008)
	Rev	GTTTTATCACTCTCCTCTTTCCAG	
TNF-α	For	CACATGGCCTGCAACTAATC	(Taglinger, et al., 2008)
	Rev	AGCTTCGGGGTTTGCTACTAC	

Real-time qPCR was performed using the StepOne Plus real-time analyser (Applied Biosystems). The PCR assays comprised, in each reaction, 2µl of each primer (final

concentration of 100nM), 2 µl of cDNA, 4µl of sterile water and 10 µl of SYBr (Applied Biosystems) in a total volume of 20 µl per reaction.

Thermocycling conditions consisted of an initial denaturation of 10min at 95ºC, followed by 50 cycles of amplification (95ºC for 15s and annealing at 60ºC for 1min). A final melting curve stage consisted of 95ºC for 15s, 60ºC for 1min followed by a ramp rate and heating of samples until 95ºC with a 0.3ºC/s ramp rate. The melting curves obtained after each PCR were used to verify the specificity of each amplicon.

Measurement of plasma levels of IL-6, IL-12p40 and IL-4 cytokines

At each time point, whole blood was also collected in EDTA tubes which were centrifuged (5000g for 10minutes) to obtain plasma which was subsequently frozen at -20ºC until use. Plasma levels of IL-6, IL-12p40 and IL-4 were measured by specific ELISA kits (SunRed Biotechnology Company).

Quantification of Provirus

In order to assess proviral load, DNA was extracted from whole blood using a specific kit (DNeasy Blood & Tissue, Qiagen) by following the manufacturer's instructions. DNA was stored at -20ºC until use as a template for proviral load quantification by Real-time PCR.

Taking into account the major prevalence of FIV-subtypes A and B in southern Europe (Duarte, et al., 2002; Duarte & Tavares, 2006), samples were screened for both subtypes. Primers used for FIV A subtype had been previously published and are presented in table 12. For FIV B subtype, the *gag* gene nucleotide sequences available through their Genebank accession number, were aligned for identification of conserved regions using specific software (CLC Main Workbench). Primers were chosen using Primer Express software (Applied Biosystems), after visual inspection of the multiple alignment.

Table 12: Real-time qPCR system to assess FIV provirus and viremia changes in naturally FIV-infected cats after rFEIFN therapy

Gene	Oligo	Sequence (5´-3´)	Reference
FIV-A subtype	For	GCC TTC TCT GCA AAT TTA ACA CCT	(Leutenegger, et al., 1999)
	Rev	GAT CAT ATT CTG CTG TCA ATT GCT TT	
	Probe	FAM* CATGGCCACATTAATAATGGCCGCA* TAMRA	
FIV-B subtype	For	AGACCGCTGCCCTATTTCACT	-
	Rev	TTCTGGCTGGTGCAAATCTG	
	Probe	FAM*TGCCTGTTGTTCTTGAGTTAATCCTATTCCCA*TAMRA	

Real-time qPCR was performed using StepOne Plus real-time analyser (Applied Biosystems). 50ng of DNA template was used in a total volume of 20 µl, comprising 10µl of TaqMan PCR Master Mix (Applied, Byosystem). Optimization of different primer and probe concentrations were performed. For the FIV-B system, a final concentration of 300nM for each primer and 250nM for the probe was used. For the FIV-A system, 900nM for the primers and 250nM for the probe was used.

Absolute quantification was assessed by real-time PCR using respective standard curves based on ten-fold dilutions of positive controls. For the FIV-B subtype, previously published plasmids (Duarte, et al., 2002) were used. For FIV-A, purified amplicons obtained from FIV-Pet cells were used.

For the FIV-A subtype, thermocycling conditions consisted of an initial denaturation (95ºC/3min) followed by five cycles of 95ºC/30sec and 60ºC/30sec and 40 cycles of 85ºC/30sec and 60ºC/60sec.

For the FIV-B subtype, thermocycling conditions began with an initial denaturation (95ºC/10min) followed by 50 cycles of 95ºC/15sec, 58ºC/20sec and 72ºC/20sec.

Quantification of Viremia

For viremia quantification, viral RNA was extracted from plasma samples using a specific kit (QIAmp Ultrasens Virus Kit). Plasma viral RNA was stored at -80ºC until use as a template on Real-time qPCR. Similarly to proviral load, the StepOne Plus Real-time analyser (applied Biosystems) was used.

A one-step Real-time qPCR was performed using 100ng of RNA in a total volume of 20µl of reaction using one-step PCR kit (MyTaq One-Step RT-PCR kit). Taking into account the provirus subtype's result, the respective system was applied to assess concurrent viremia levels. The same concentrations of primers and probe were used.

Thermocycling conditions used for one-step Real-Time qPCR were similar as the previously described for provirus, including in the beginning an initial step of reverse-transcription of 48ºC/15min.

Statistical analysis

For each measured parameter, the two groups were compared using the Mann-Whitney-Wilcoxon test for independent samples. The comparison between the end and the beginning of therapy in each group was carried out by the Mann-Whitney-Wilcoxon test for paired samples with appropriate small sample size correction. The significance level was set at 5%.

A descriptive statistical analysis was also performed when appropriate. In order to assess potential correlations between measured parameters, a spearman correlation was also performed when suitable. All the statistical analyses were carried out using R-software.

Results

Cytokine expression

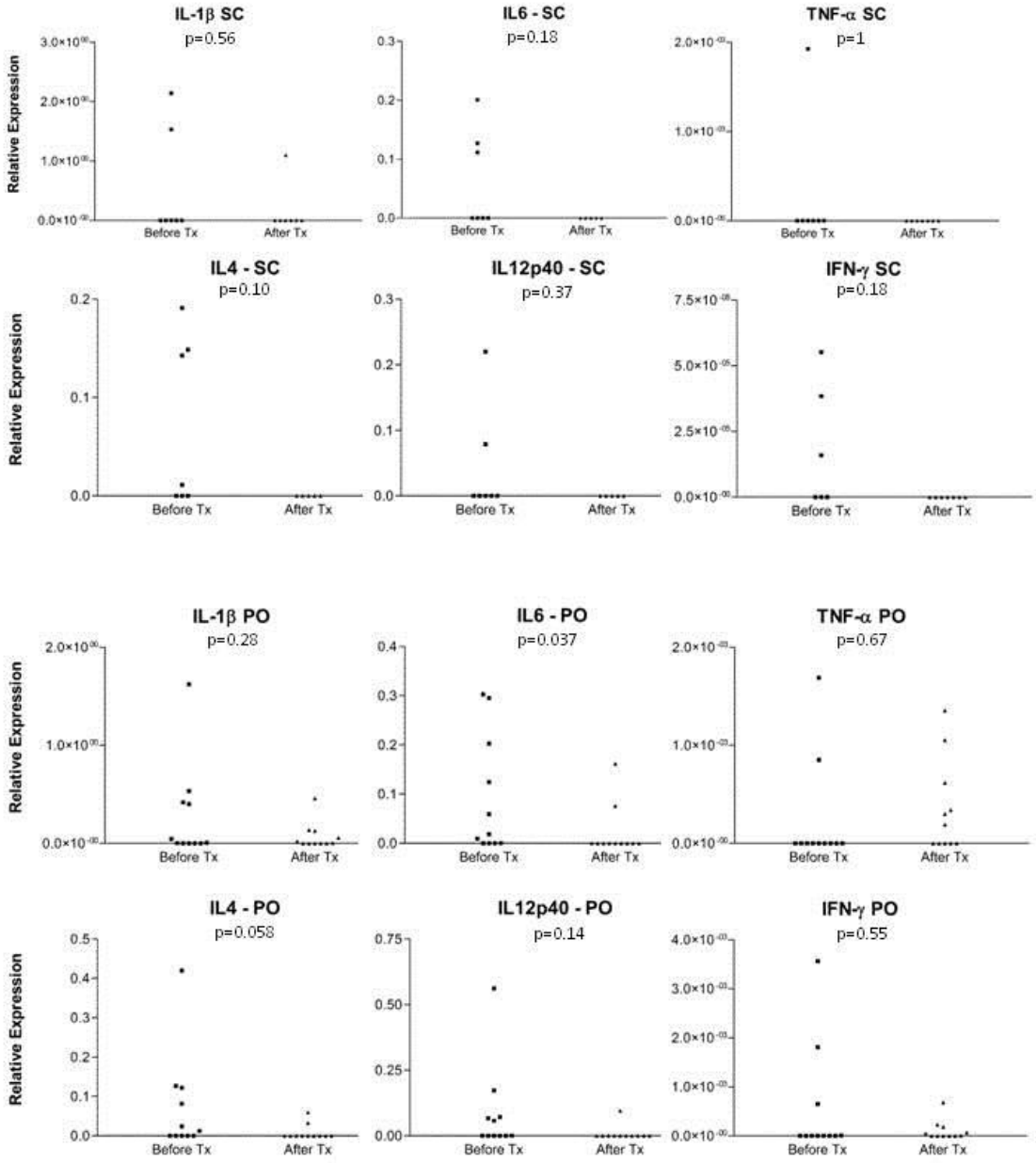
Relative quantification revealed very low levels in all the measured cytokines. In terms of mRNA expression, the groups were indistinguishable on D0 for all the evaluated cytokines (p=0.55, 0.71, 0.24, 0.26, 0.70, 0.51 and 1 for IL-1, IL-4, IL-6, IL-10, IL-12p40, IFN-γ and TNF-α, respectively).

When comparing cytokine mRNA expression before and after therapy in both groups, in spite of an overall decreasing tendency only IL-6 expression significantly decreased and only in the PO group (p=0.037). With the exception of this cytokine, no significant changes were observed in the cytokine profile of either group (D0 versus D65 for SC group: p=0.58, 0.10, 0.18, 1, 0.37, 0.18, 1 for IL-1, IL-4, IL-6, IL-10, IL-12p40, IFN-γ and TNF-α, respectively; and D0 versus D90 for PO group: p= 0.28, 0.058, 1, 0.14, 0.55, 0.67 for IL-1, IL-4, IL-10, IL-12p40, IFN-γ and TNF-α, respectively).

On D0, cats from both groups showed a minimal expression of IL-1, IL-4, IL-6, IL-12p40 and IFN-γ. TNF-α expression was only quantified in one cat from the SC group and in two from the PO group. At the end of therapy, no cytokine expression other than IL-1 (which was measured in one cat) was observed in the SC group. Therefore, cytokine expression was set as zero for all the quantified cytokines in this group. Conversely, in the PO group, minimal mRNA expression of IL-1, IL-4, IL-6, IL-12p40, IFN-γ and TNF-α could still be measured at the end of therapy.

IL-10 expression was negligible in both groups, and therefore these results were not charted. In detail, only two cats from the SC group and one cat from the PO group showed detectable mRNA expression on D0. On D90, only two other cats from the PO group, which tested negative before, expressed IL-10. The detailed results for the other cytokines are shown in Figure 20.

Figure 20: Detailed cytokine mRNA variation in naturally FIV-infected cats submitted to two different rFeIFNω protocols. SC refers to cats treated with subcutaneous rFeIFNω licensed protocol and PO to cats receiving the oral protocol. The values represent the expression of each cytokine using a housekeeping gene (beta-actin) for normalization and relative quantification. p values refers to statistical comparison between the end and the beginning of therapy for each cytokine.

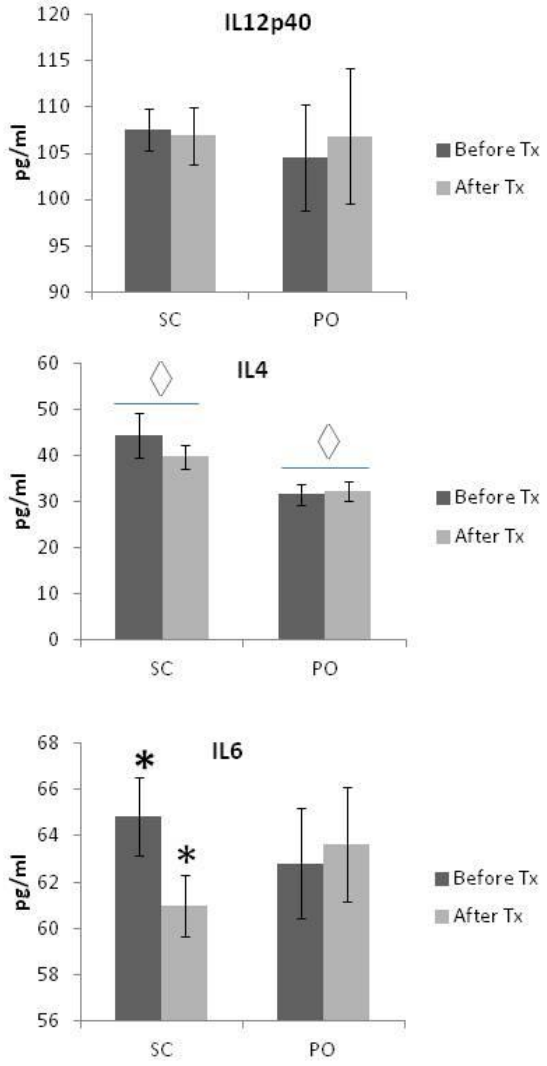


Plasma levels of IL-6, IL-12p40 and IL-4 cytokines

Concerning plasma levels of measured cytokines, the groups were similar on D0 for IL-12p40 and IL-6 (p=0.82 and p=0.22 respectively). For IL-4, plasma levels on D0 were significantly higher in the SC group than in the PO one (p=0.013).

Comparing the beginning and the end of therapy in the SC group, there was a significant decrease of IL-6 plasma levels (p=0.037). No statistical differences were observed for IL-12p40 and IL-4 plasma levels in this group (p=0.87 and p=0.24, respectively). In the PO group, no changes were observed in any of the measured plasmatic ILs (p=0.062, 0.248, 0.074 respectively for IL-4, IL-12p40 and IL-6). The detailed results are shown in figure 21.

Figure 21: Mean ± SE of plasma IL-12p40, IL-4 and IL-6 concentrations in naturally FIV-infected cats submitted to two different protocols of rFeIFNω. SC refers to cats treated with subcutaneous rFeIFNω licensed protocol and PO to cats receiving the oral protocol. The groups were statistically similar at baseline values except for IL-4 concentration which was higher in the SC group than in the PO group (◊- p=0.013 – comparison between groups). The SC group showed a statistically significant decrease of IL-6 concentration (*- p=0.037 - comparison between the end and beginning of therapy).

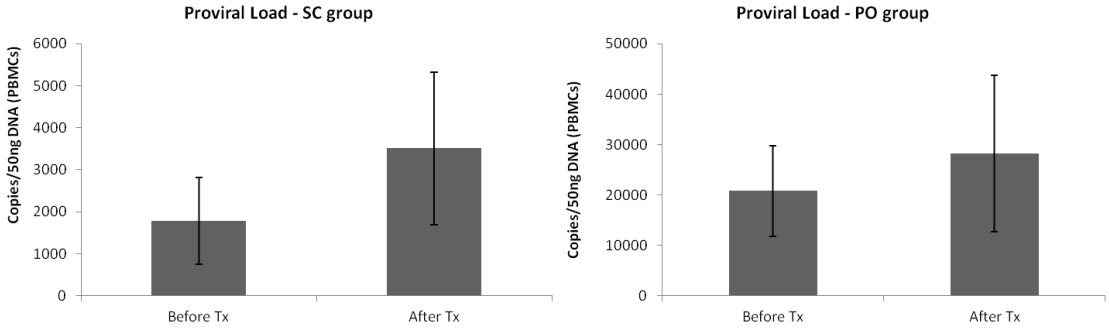


Quantification of Provirus

Regarding the primers and probes used, proviral load was quantified by the FIV-B system in 8/18 cats (3 from the SC group and 5 from the PO group), whilst the FIV-A system worked successfully in the other 10 cats (4 from the SC group and 6 from the PO group). To assess overall changes in the proviral load, the results of both subsystems were taken together and are shown in figure 22. There was no statistical difference between the groups at D0 before therapy (p=0.07). Also, at this time-point, a significant positive correlation could be observed in both groups between proviral loads and clinical condition (clinical scores previously published (Gil, et al., 2013)) (CC=88% and 61% for the SC and PO groups respectively).

After therapy there was a statistically significant increase in the SC group (p=0.031). In contrast, in the PO group, although the proviral load tended to increase it was not statistically significant (p=0.46).

Figure 22: Mean ± SE of proviral load of FIV in cats submitted to two different rFeIFNω protocols. SC refers to cats treated with subcutaneous rFeIFNω licensed protocol and PO to cats receiving the oral protocol.



Quantification of Viremia

There was a low level of viremia at both time points for both groups. On D0 the groups were similar (p=1).

In detail, only 7/18 cats (3/7 from the SC group and 4/11 from the PO group) showed detectable viremia on D0. Detailed individual values are presented in figure 23.

In the SC group, the four cats with undetectable viremia levels on D0 remained negative after therapy (D65). Of the three cats which had detectable viremia on D0, two became negative whilst one reduced it.

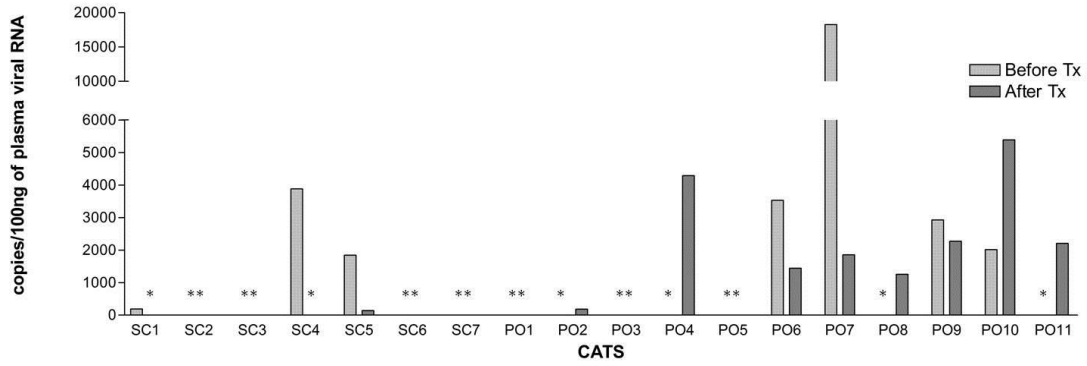
In the PO group, of the seven cats which had undetectable viremia on D0, three remained negative while four became positive after therapy (D90). Considering the four cats which

tested positive on D0, three of them reduced their viremia levels while one slightly increased it.

Despite this, when comparing the levels at the beginning and the end of therapy in each group, no significant differences were obtained for viremia measurements (p=0.52 and 0.18 for the PO and SC groups respectively).´

In contrast to what was seen with the provirus levels, no correlation was noted between the viremia levels and clinical health status (CC=0.57/p=0.18 for the SC group and CC=0.351/p=0.29 for the PO group on D0). Also no correlation was established between viremia and provirus at both time points (D0: CC=0.39/p=0.382 for the SC group and CC=0.50/p=0.11 for the PO group; after therapy: CC=0.41/p=0.36 for the SC group and CC=0.46/p=0.15 for the PO group).

Figure 23: Individual viremia changes of 18 FIV-infected cats submitted to two rFeIFNω protocols. 7/18 cats (SC group) received the subcutaneous licensed protocol while 11/18 cats were treated with oral rFeIFN-ω (PO group). * refers to time-points in which viremia was undetected.



Discussion

This study evaluated the effect of two distinct rFeIFN-ω protocols on blood cytokine profile, viremia and proviral load. It was previously reported that rFeIFN-ω (licensed protocol) induces minor changes in a variety of parameters suggesting that it may only act on the innate immunity (Domenech, et al., 2011). The confirmed increase of APP levels concurrently with clinical improvement reinforced this theory (Leal, et al., 2014). However, these data must be correlated to the cytokine profile for this to be confirmed. A clinical improvement was also described with oral use of rFeIFN-ω, although in this case the APP profile did not change (Gil, et al., 2014) leading to the conclusion that, similarly to what has been described for general IFN therapy (Tovey, 2002), these different administration routes

must result in different mechanisms of action. To date, no studies (*in vivo*) to assess the cytokine profiles in cats undergoing rFeIFN-ω therapy have been performed, meaning that these results are particularly important to assess the truly immunological pathways underlying the clinical improvement seen with both protocols.

In this study, mRNA expression of various cytokines was evaluated in blood samples. The cytokines were chosen taking into account their main functions on the immune system and were considered biomarkers of the innate or the acquired immune response. Recognizing that IL-1, IL-6 and TNF-α are three cytokines strongly involved in the innate immune response, they were considered as good biomarkers of the activation of the pro-inflammatory pathways. On the other hand, IL-12p40, IFN-γ, IL-4 and IL-10 are cytokines mainly involved in the acquired immune response. IL-12p40 and IFN-γ were chosen as good indicators of Th1 pathway activation. IL-12 is a heterodimeric cytokine composed of two chains (p40 and P35) (Trinchieri, Pflanz, & Kastelein, 2003). In this study, the measurement of the IL-12p40 subunit was chosen by the authors due to the fact that it was the only subunit available for complementary ELISA measurement. IL-4 and IL-10 are cytokines produced by Th2 cells, making these cytokines good indicators of a Th2 response.

The results have shown that, in both groups, although quantification was possible, mRNA expression was very low. The groups were indistinguishable on D0 for mRNA expression of all the measured cytokines, which made the SC group, submitted to the licensed protocol, a reliable positive control for potential comparisons between groups. Comparing the beginning and the end of therapy in both groups, there were no marked differences in mRNA expression for the majority of cytokines, although a decreasing tendency was observed. This decrease was only significant for IL-6 expression in FIV-infected cats after oral rFeIFN-ω therapy. It is well known that IL-6 is strongly involved in the acute phase response (Heinrich, Castell, & Andus, 1990). However, its pleiotropic action is not restricted to this function. Among its properties, IL-6 is also involved in hematopoiesis, as an endogenous pyrogen and even in the terminal differentiation of B-cells, cooperating in the development of a Th2 response. Despite its pleiotropism, it is consensually taken as a pro-inflammatory cytokine, mainly produced by monocytes/macrophages and dendritic cells (Heinrich, et al., 1990; Roitt & Delves, 2001). Therefore, this result can be considered a potential indicator of an overall reduction of pro-inflammatory pathways in the treated cats of this study. In fact, although not statistically significant, IL-1 and TNF-α also appear to decrease in these animals which corroborates with a potential anti-inflammatory action of rFeIFN-ω. Also in the SC group, the same tendency is observed for IL-1, TNF-α and IL-6 meaning that, also in the licensed protocol, the pro-inflammatory pathways of the innate immune response tend to be reduced with therapy.

In contrast to the oral protocol, which does not induce changes in APP levels (Gil, et al., 2014), subcutaneously administered rFeIFN-ω induces a clinical improvement with a concurrent increase of APPs (Leal, et al., 2014).Due to the fact that IL-6 is one of the cytokines involved in the stimulation of APP production, it was surprising that IL-6 expression appeared to decrease in these animals. Little is known about the feline acute phase response but although IL-6, IL-1 and TNF- α are thought to be the main inducers of this acute phase reaction (Martínez-Subiela S, 2001; Paltrinieri, 2008), it is important to state that they are not the only cytokines involved in this phenomenon. Several other pathways can lead to APP production. In fact, APPs have several protective functions (Steel & Whitehead, 1994; Hochepied, et al., 2003; Petersen, et al., 2004; Ceron, et al., 2005; Paltrinieri, 2008). Recognizing that APP levels increase concurrently with a clinical improvement in cats receiving subcutaneous rFeIFN-ω therapy, it seems reasonable to hypothesize that this increase may be stimulated by mediators other than IL-6, IL-1 or TNF-α such as IL-18 (Duan, Yarmush, Jayaraman, & Yarmush, 2004).

Concerning the mRNA levels of the Th1 measured cytokines (IL-12p40 and IFN-γ), no changes were noted in either group meaning that this pathway does not seem to be the way by which either SC or oral rFeIFN-ω therapy provides a benefit for FIV-infected cats. The same was observed for the Th2 quantified cytokines (IL-4 and IL-10). In particular, IL-10 was undetectable in the majority of animals which made the value of the overall results for this cytokine negligible by this method. Despite this, IL4 mRNA expression allowed a reasonable quantification of the level of Th2 activation. Although non-significant, a decreasing trend was noted in both protocols for IL-4 mRNA expression. This was closest to significance for the oral protocol ($p=0.058$), which, incidentally, may partially explain the benefit seen with oral rFeIFN-ω therapy in a recently published study on canine atopic dermatitis where a high IL-4/IFN-γ ratio is believed to contribute to the disorder (Litzlbauer, Weber, & Mueller, 2014).

Therefore, other than this potential trend for reduced IL-4 mRNA expression, our results did not demonstrate any significant impact of rFeIFN-ω therapy on the Th1 and Th2 responses in FIV-infected cats, independently of the chosen protocol. The overall decreasing tendency in the Th1 and Th2 cytokines observed visually in both groups can be potentially related to a reduction of exogenous stimuli such as concurrent/subclinical opportunistic infections. In fact, although the effect of the oral protocol on opportunistic infections was negligible (Gil, et al., 2014), the SC therapy has been related to a significant reduction of concurrent viral infections which can justify the slight reduction observed on mRNA expression of cytokines from Th1 and Th2 responses.

Concurrently to mRNA expression monitoring, the plasma levels of IL-6, IL-12p40 and IL-4 were also measured. Similarly to the basal values of cytokine expression, groups were

similar on D0 for IL-6 and IL-12p40. The exception was IL-4 which was significantly higher in animals from the SC group than in the PO group. This can be explained by different factors such as their environment (cats from the SC group were living in a cattery whereas cats from the PO group were mainly indoor animals with less exposure to other cats) and opportunistic infections (which were more evident in the SC group than in the PO one). In cats from animal shelters it is expected that the Th2 response will be increased due to the constant stimuli from concurrent infections and environmental challenges. However, as the effect of therapy was assessed by monitoring plasma levels of each cytokine before and after therapy and the cats did not alter their living conditions, it is not anticipated that this difference at baseline will have any relevant impact on the results. Nevertheless, the changes seen in IL-4 and IL-12p40 plasma levels were not statistically significant and the overall tendencies were considered negligible. They can probably be explained by a high individual variability which induced slight fluctuations on the overall results. Interestingly, IL-6 plasma levels significantly reduced in the SC group while it remained stable in cats treated with the oral protocol. Correlating these findings with the concurrent mRNA expression results, it is observed that in the SC group, although the decrease of IL-6 expression is not significant, rFeIFN-ω seems to induce an important reduction of IL-6 plasma levels. In contrast to these results, the significant decrease noted in IL-6 mRNA expression in cats submitted to oral therapy was not reflected in their plasma levels of this cytokine. Therefore, these results suggest that IL-6 cytokine production is affected in FIV-infected cats during rFeIFN-ω therapy, independently of the protocol applied, albeit with some differences depending on the route of administration used. It seems reasonable to state that higher pulsate subcutaneous doses seem to be more effective than lower continuous oral therapy for reducing pro-inflammatory stimuli in FIV-infected cats. However continuous oral therapy also altered IL-6 expression meaning that this immune modulation protocol retains some anti-inflammatory properties. These results reinforce the beneficial aspects of rFeIFN-ω as an immunomodulatory therapy, in light of the fact that basal levels of pro-inflammatory cytokines tend to be increased in FIV-infected cats (Lawrence, et al., 1995).

Regarding the proviral load, two systems were used taking into account the prevalence of FIV-A and FIV-B in Portugal. Previous epidemiological studies have shown that there is an increasing viral diversity among Portuguese FIV-infected cats and that FIV-B is predominant, although there is genetic complexity within each type (Duarte, et al., 2002; Duarte & Tavares, 2006). Although the characterization of subtypes was out of the scope of this work, all the cats were positive to one of the subtypes tested, which allowed proviral and viremia monitoring. Similarly to the other measured parameters, proviral load on D0 was similar in both groups and an expectable correlation was established between this parameter and clinical presentation, meaning that cats in worse condition showed higher levels of provirus.

As previously published, there was a significant clinical improvement of both groups after each protocol (Gil, et al., 2013; Gil, et al., 2014). However, proviral load did not concurrently decrease. Previous authors have reported that the licensed protocol does not induce significant changes in proviral load (Domenech, et al., 2011). In contrast to what was previously published, this study revealed that in the SC group, there was a significant increase of proviral load after therapy. Also in cats treated with the oral protocol, although not significant, an increasing tendency was noted. A possible explanation for this finding relies on the fact that, in both groups, lymphocyte numbers tend to increase with therapy. Although this increase is within the reference range and it seems to be clinically irrelevant (Gil, et al., 2014), it may explain the subsequent increase of proviral load. However, no correlation with viremia results was obtained in either group. Therefore, these changes cannot be explained by an activation of the acquired immune system. In fact, previous authors have even remarked that rFeIFN-ω does not induce changes in the CD4/CD8 ratio (Domenech, et al., 2011). Therefore, this relative increase in lymphocytes and concurrent increase of proviral load, both unrelated to the observed clinical improvement and not associated to an increased viremia, are unexpected findings and further studies are required to better characterize and understand this effect.

A previous study (Domenech, et al., 2011) also reported that rFeIFN-ω does not act on viremia. In our study, viremia was only detected in a small proportion of animals of both groups on D0. Although they were similar between groups, there was an observed fluctuation in the results of individual cats. Overall we found that, in agreement with previous authors (Domenech, et al., 2011), rFeIFN-ω does not change viremia levels, independently of the administered protocol. The low number of animals with detected viremia in both groups can be explained by virus latency . Previous studies have reported that plasma viral RNA is undetected in asymptomatic cats chronically infected with FIV (Tomonaga, Inoshima, Ikeda, & Mikami, 1995; Murphy, Vapniarsky, et al., 2012). Previous studies have also shown that, despite the fact that low copy numbers of viral RNA are intermittently identified in freshly isolated PBMCs, no viral RNA is detected at any time point after 44 weeks of infection (Murphy, Vapniarsky, et al., 2012). Considering that the present study involves naturally-infected cats with different clinical presentations, it was not unexpected to have found several cats in which viremia is below the detection range. Although no correlation was established between viremia and clinical scores, the majority of cats were in a reasonably healthy condition. The main clinical signs in the symptomatic animals were most likely due to opportunistic infections rather than directly induced by FIV replication. Therefore, the observed low levels of viremia were not surprising. In spite of these low results, it can be noted that the action of rFeIFN-ω on viremia levels was negligible, suggesting that its

beneficial effects in FIV-infected cats is unlikely to be related to a direct anti-viral action on the FIV virus in either protocol.

As for all clinical trials, there were a few limitations that must be stated. Even if the groups did not differ in the majority of measured variables, cats that received the SC group were living in a cattery/animal shelter whilst cats treated with oral rFeIFN-ω were mainly indoor/owned cats. Therefore, the groups were exposed to different environmental and human stimuli which cannot be precisely determined. However, recognizing that D0 results were set as the baseline value and the individual control for each cat, the overall tendency was analyzed for each group which minimized this limitation. Another point to consider is that cytokine expression results depended on blood mRNA collection and extraction. It is important to note that a reliable measurement of circulating mRNA is difficult as RNAases are present ubiquitously which degrade it (Etheridge, Gomes, Pereira, Galas, & Wang, 2013). Furthermore, mRNA extraction efficiency varies according to the method applied and can be affected by multiple external variables such as blood clots and sampling conditions which determine its quality (Wong, Lo, & Cheung, 2004).To the authors' knowledge, this is the first study reporting cytokine profiles based on blood mRNA measurements in naturally FIV-infected cats. Considering the low values obtained, it is reasonable to say that blood mRNA does not seem to be as effective as plasma levels or even in vitro studies for assessing this aspect of the immune response (Robert-Tissot, et al., 2011). A concurrent evaluation and stimulation of PBMCs from naturally-FIV infected cats would have been helpful in clarifying this data. Interestingly, a recent study validated a microsphere immunoassay for the detection of plasma IL12/23 (Wood, et al., 2012). Perhaps in the near future the evaluation of the cytokine profile of FIV cats will be easier and more helpful in the monitoring of infected cats and therapies.

Although the antiviral effect of rFeIFN-ω on FIV seems to be minor in FIV-infected cats, this study helped to enlarge our understanding of the role of this immunomodulator on the cytokine profile of these animals. Among the measured cytokines, this work revealed that IL-6 production was significantly affected in FIV-infected cats treated with subcutaneous or oral rFeIFN-ω protocols. While the high pulse scheme of the SC protocol leads to an important reduction on IL-6 plasma levels, the continuous lower doses of the oral protocol induces a decrease on IL-6 expression, although not sufficiently to be reflected in significant reductions of its plasma levels. In summary, the acquired immune-response, namely Th1/Th2 pathways, was not found to be the major means by which rFeIFN-ω acted in this study. Its main action seems to be on the innate immune response where it reduces the pro-inflammatory stimuli. This anti-inflammatory action can in part justify the observed clinical improvement induced by this immunomodulator.

Part III:

Extrapolating immune modulation properties: the use of oral recombinant feline interferon omega in other diseases than retroviral infections

Part III - Chapter I:

The use of oral recombinant feline interferon omega in two cats with type II diabetes mellitus and concurrent feline chronic gingivostomatitis syndrome

Chapter I: The use of oral recombinant feline interferon omega in two cats with type II diabetes mellitus and concurrent feline chronic gingivostomatitis syndrome

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Abstract

Feline Chronic Gingivostomatitis Syndrome (FCGS) is a common disease in clinical practice. Among the therapeutic options available, long-acting corticosteroids are frequently used due to their anti-inflammatory and immunosuppressive properties. Although they may improve the clinical symptoms, they can lead to a progressive form of the disease which becomes refractory to treatment. Furthermore, their direct relationship with type II diabetes mellitus (DM) is well known. Consequently, these drugs are controversial and not recommended for routine management of FCGS. Recombinant Feline Interferon-Omega (rFeIFN-ω) is an immune modulator compound. Recently, its daily oral administration has been shown to be successful in treating refractory cases of FCGS. This case study describes two clinical cases of type II DM complicated with FCGS. Both animals were calicivirus positive and they had been previously treated with long-acting corticosteroids which may have been the major cause of DM. The two cats were treated with glargine insulin (Lantus, starting dose 1IU/cat twice daily (BID)), achieving remission 10 and 18 weeks later respectively. Considering the difficulty to control FCGS in these animals, an oral daily dose of rFeIFN-ω was started as an alternative to long-acting corticosteroids. In both cats oral clinical signs gradually improved and 60 days after the start of therapy the owners reported a significant relief of pain during mastication. According to the authors' knowledge, this is the first case report that describes the successful use of rFeIFN-ω in the management of FCGS in type II diabetic cats, in which long-acting corticosteroids are contra-indicated.

Keywords: Interferon-Omega, Feline, Diabetes, Gingivostomatitis

Background

Feline Chronic Gingivostomatitis Syndrome (FCGS) is a multifactorial disease, very commonly seen in clinical practice (Pedersen, 1992). It is described as a severe oral inflammation (gingivitis, stomatitis and/or periodontitis) and may be secondary to various causes such as neoplasia, toxins or even metabolic disease (DeBowes, 2009). In some cases, a cause is not found but regarding the chronic inflammation observed in histopathology samples, an immune mediated etiology should always be considered (Tanney & Smith, 2010). Infectious viral diseases are also an important trigger of FCGS (Tenorio, et al., 1991; Pedersen, 1992). Retroviral infections may lead to FCGS due to an induced immune suppression and dysregulation (Sellon & Hartmann, 2012a, 2012b). Aditionally feline herpesvirus (FHV-1) and feline calicivirus (FCV) have been well-described as potential factors in the development of FCGS (Gaskell, Dawson, & Radford, 2012). In fact, a previous study showed that 88% of cats with chronic gingivostomatitis were excreting FHV-1 and FCV (Lommer & Verstraete, 2003). Animals with FCGS usually present with a poor body condition, dysphagia and mild to moderate anorexia (DeBowes, 2009). Most therapeutic approaches are not very effective and relapses are frequent (DeBowes, 2009). Among the available therapeutic options, dental extraction, antibiotics and corticosteroids or non-steroidal anti-inflammatory drugs (NSAID) are usually recommended (DeBowes, 2009; Sykes, 2009). Due to the doubtful efficacy of NSAIDs over the medium term, long-acting corticosteroids are more frequently used in first-opinion clinical practice (DeBowes, 2009; Sykes, 2009; Tanney & Smith, 2010). They are historically described as a good therapeutic approach to FCGS due to the fact that they reduce oral inflammation and immune-mediated causes, leading to a rapid improvement, an increased appetite and a relief of oral pain (DeBowes, 2009; Sykes, 2009; Tanney & Smith, 2010). In spite of their short-term efficacy at controlling the symptoms of the syndrome, long-acting corticosteroids are also a well-known cause of insulin-resistance (Rand & Marshall, 2005; Rand, 2012), by inducing chronic hyperglycemia which can lead to a glucotoxic beta-cell insufficiency (Rand, 2012). Therefore, being a potential cause of type II diabetes mellitus (DM) in cats, long-acting corticosteroid use is controversial in this species in general.

In refractory cases of FCGS, where dental extraction and antibiotics are not sufficient to induce remission of the lesions, there are few therapeutic alternatives to corticosteroids (Hennet, et al., 2011). Recombinant Feline Interferon Omega (rFeIFN-ω; Virbagen, Virbac) is an immune-modulator drug currently licensed in Europe for treatment of feline retroviral infections (de Mari, et al., 2004; Domenech, et al., 2011; Gil, et al., 2013). According to the manufacturer's instructions, the licensed protocol consists of three cycles of five daily administrations of 1MU/kg subcutaneously at Days 0, 14 and 60. However other dosages and routes have also been used in the management of other diseases. For instance,

alternative subcutaneous and topical protocols were tried in feline coronavirus and FHV-1 infection, respectively (Haid, et al., 2007; Ritz, et al., 2007). Also in cases of FCGS (some of them FCV positive), a study suggested the benefit of intra-lesional administration of rFeIFN-ω (Mihaljevic, 2003). More recently, its oral administration was documented in refractory FCGS (Hennet, et al., 2011; D. Addie, 2012) in an efficacy study which compared the use of an oral rFeIFN-ω protocol with the use of oral corticosteroids. This study concluded that oral rFeIFN-ω was associated with a significant clinical improvement of FCGS lesions (Hennet, et al., 2011). Furthermore, there was no difference between this protocol and corticosteroids except on pain control, where animals treated with rFeIFN-ω achieved a better pain relief (Hennet, et al., 2011). Therefore, as oral rFeIFN-ω is a useful alternative management option for refractory FCGS, it may also be of particular interest in cats where corticosteroid administration is contraindicated, such as those with DM. This report underlines the relevance of rFeIFN-ω by describing two clinical cases of diabetic cats in which it was successfully administered as an alternative therapy for concurrent FCGS.

Case Presentation

Case one: A 15 year-old castrated domestic short-hair (DSH) cat was presented to the endocrinology service of the Veterinary Teaching Hospital – Faculty of Veterinary Medicine, Techinical University of Lisbon (FMV-UTL) for polyuria/polidipsia (Pu/Pd), mild anorexia and weight loss. Prior to this consultation, the animal had been managed by the referring vet for severe dysphagia and weight loss, secondary to FCGS, diagnosed one year before. Considering the positive calicivirus status (assessed by PCR analysis of an oral swab), an infectious origin had been assumed. A partial exodontia had been performed without significant improvement. Furthermore, the cat had been recurrently treated with antibiotics (cefovecin; 8mg/kg SC every two weeks) and periodically with long-acting corticosteroids (methylprednisolone acetate; 10mg intramuscularly every four to six weeks). On clinical examination, the cat presented with a moderate gingivitis and caudal stomatitis which extended to the palatoglossal folds. After the initial workup (hematology, biochemistry, urine analysis and abdominal ultrasound), a type II DM complicated with ketoacidosis was diagnosed. After initial stabilization with intravenous fluids and a regular insulin protocol, the cat was progressively fed with a diabetic specific diet (Purina DM) and Insulin-Glargine (1IU/cat SC BID) was started. After three days of hospitalization and a good initial response to this insulin, the cat was discharged. The owners performed weekly home-made blood glucose curves (HMBG) and the insulin dose was adjusted according to the glycemia results. Ten weeks later, after a gradual decrease of insulin therapy, the cat went into remission of the DM and insulin therapy was stopped. However, after the remission of the diabetic clinical signs such as polyphagia, the gingivitis and caudal stomatitis got worse. Despite the good control of the DM and therapeutic trials with antibiotics (cefovecin; 8mg/kg SC every two

weeks), gastric protectants (sucralfate; 0.5g/cat per-os (PO) BID) and NSAID (meloxicam; 0.1mg/kg PO, SID), the FCGS became worse, with the cat developing a severe dysphagia with hypersalivation and weight loss. Due to the previous history of type II DM associated with long-acting corticosteroid therapy, an oral rFeIFN-ω protocol (0.1MU PO SID) was started, with the owner's informed consent. During the first two weeks of treatment, oral disinfectants (antiseptic oral solution: Collu-Hextril, Johnson & Johnson Lda; 1 diluted portion PO SID and enzymatic gel: Orozyme, Ceva; 1cm ointment PO SID) and an antibiotic (cefovecin; 8mg/kg SC administered once) were concurrently prescribed. After this initial therapeutic approach, only rFeIFN-ω was administered. The cat started to improve gradually, and 2 months later the owners described a significant improvement of mastication and reduced evidence of pain. The treatment was continued and animal was evaluated monthly. 6 months later, at the date of the last evaluation, the cat had only a mild gingivitis and stomatitis, without significant pain. The animal had been treated only with rFeIFN-ω, which was not discontinued due to the good clinical results obtained.

Case two: A 14 year-old castrated DSH cat was presented to the endocrinology service of the Veterinary Teaching Hospital FMV-UTL for DM monitoring. The animal had been diagnosed with DM 4 weeks prior to the consultation, following an acute onset of Pu/Pd and polyphagia. Apart from DM, it had been recurrently seen by the referring vet due to FCGS with concurrent documented calicivirus infection, diagnosed two years previously, based on a PCR analysis of an oral swab. The cat had been intermittently treated with antibiotics (potentiated amoxicillin 15-20mg/kg PO BID) and corticosteroids (prednisolone 0.5-1mg/kg PO SID intermittently for three-five days) until six weeks before the development of DM. The cat was first started on veterinary lente-insulin (0.5 IU/kg BID) and fed with an appropriate diet for DM (Purina DM). At clinical presentation, the animal had a significant alveolar and caudal mucositis, with concurrent inflammation of palatoglossal folds and severe pain on mouth manipulation. No other abnormalities were observed. The owners had made some HMBG curves that revealed inconstant values. Considering the difficult control of glycaemia and the apparent weak response to lente-insulin, the insulin was changed to insulin-glargine (1IU/cat SC BID). After three days of hospitalization, the animal was discharged and owners performed weekly HMBG curves. According to these measures, the insulin-glargine dose was adjusted weekly. After five weeks, the FCGS became worse with development of a severe dysphagia and hyper salivation. With the owner's informed consent, animal was started on the oral rFeIFN-ω protocol. The use of concurrent oral disinfectants was advised but was not regularly performed by the owner. Gradually, the FCGS started to improve and the insulin-glargine requirement decreased. Eighteen weeks after starting insulin therapy, the animal achieved clinical remission of the DM with no further requirement for insulin therapy. The oral rFeIFN-ω was continued and the animal was evaluated monthly. Despite the

persistence of gingivitis and caudal stomatitis, the owners reported a significant pain relief, more evident 60 days after the onset of therapy. The treatment was continued. Three months later, five months after the beginning of therapy, the cat presented with a good clinical condition with less pain on opening the mouth and a concurrent clinical improvement of the FCGS with less extensive lesions, a reduced hyper salivation and a more comfortable mastication.

Conclusions

This report describes two cases of clinical remission of DM in cats with FCGS under insulin-glargine and dietary management. Both cases had been previously treated with corticosteroids, which are considered a risk-factor for DM in cats (Rand, 2012). Although the corticosteroids were discontinued, these animals required insulin therapy and a concurrent suitable diet to control the DM. While one cat started insulin therapy with insulin-glargine, the other began the treatment with lente-insulin and later changed for insulin-glargine. Clinical remission was obtained 10 and 18 weeks after starting insulin therapy, respectively. This is in agreement with previous studies that describe a high-rate of clinical remission in feline DM managed with insulin-glargine and suitable diets (Bennett, et al., 2006; Marshall, Rand, & Morton, 2009; Roomp & Rand, 2009).

Concurrently, these animals were presenting with FCGS, and both cases were infected with calicivirus. This disease could have lead to a more difficult management of DM. In the first case the animal went into clinical DM remission and few days later the FCGS symptoms worsened. In the second one, the FCGS was a clinical problem during insulin therapy. Despite being associated with a previous good clinical improvement and a reduction of lesions, corticosteroids were contraindicated in both cases. Therefore, based on previous clinical trials (Hennet, et al., 2011), an oral protocol of rFeIFN-ω was successfully applied. In both cases, clinical improvement was gradually observed and was significantly marked (and noted by owners, who remarked that the animals started eating without discomfort and had reduced hypersalivation) around 60 days after the onset of therapy. This was particularly evident in the second case where rFeIFN-ω therapy was associated with a clinical improvement of oral lesions and a concurrent reduced insulin dose requirement which culminated in type II DM remission. This is in agreement with the previously cited work that describes an overall relief of pain in refractory cases of FCGS (Hennet, et al., 2011). It is also in agreement with multiple anecdotal reports that describe a rapid improvement in well-being in cats with FCGS during oral rFeIFN-ω treatment, but with a period of three to six months being necessary in some severe cases before the lesions are fully resolved, especially where there has been regular previous use of corticosteroids (McGahie – personal communication). Although there are no studies that clearly detail the immunomodulatory mechanisms of oral

rFeIFN-ω use, it has been proven that oral Human-Interferon alpha administration may potentiate a local T-helper 1 response (Th1) (Tompkins, 1999). In fact, Human-Interferon alpha seems to increase the expression of gamma-interferon, a Th1 cytokine inducer, while it reduces the Interleukin-4 production, responsible for a T-helper 2 response (Tompkins, 1999). Recognizing that the Th1 response is an important immunological pathway against viral infections (Tompkins, 1999), it seems reasonable that rFeIFN-ω and Human-Interferon alpha (both type-I interferons) may have a similar local action. Therefore, this Th1-enhancement may explain the clinical improvement observed in these calicivirus positive cats during rFeIFN-ω therapy. Further controlled prospective studies are needed to reinforce these clinical findings, correlating them with the local immune response. These two clinical cases describe the successful use of oral rFeIFN-ω in diabetic cats with FCGS as an appropriate alternative to corticosteroid treatment where its administration is contraindicated.

General Discussion and Conclusions

General discussion and conclusions

RFeIFN-ω therapy is common in retroviral infections, although there are few studies that support its use. In fact, only two studies have reported the clinical benefits of this compound in FIV and FeLV infected cats, before the publication of these results (de Mari, et al., 2004; Domenech, et al., 2011). In this work, the authors tried to extend the current knowledge about this compound, in order to support and clarify the evident clinical improvement previously described. The presented experimental work relied on two distinct clinical trials. One referred to 16 retroviral infected cats (7 FIV, 6 FeLV and 3 Co-infected cats) treated with rFeIFN-ω licensed subcutaneous protocol while the other described an alternative oral rFeIFN-ω protocol, administered to 11 FIV-infected cats. Detailing the experimental part, chapters I and II refers to the licensed protocol whilst chapter III regarded the oral one. In chapter IV, both protocols are compared in an immunological and virological perspective.

In chapter I, authors evaluated the effect of rFeIFN-ω licensed protocol in cats living in a animal shelter, assessing clinical improvement and monitoring concurrent viral excretion (namely herpesvirus, calicivirus and coronavirus). To author's knowledge, no studies had been previously performed neither on the use of rFeIFN-ω in shelter medicine nor on the effect of this compound in opportunistic infections. Only in cats treated with HuIFN-α, a previous study documented that the induced clinical improvement could be potentially related with a recovery of serious opportunistic infections (Pedretti, et al., 2006). In this chapter, authors developed and validated a score-scale system that allowed the evaluation of clinical improvement and monitored different hematology and biochemistry parameters in treated cats. In agreement with the two previous referred studies, authors reported a significant clinical improvement in treated animals, without relevant changes on hematology and biochemistry profiles. Furthermore, a significant reduction of concurrent viral infections was also stated. Therefore, with this study authors proved that rFeIFN-ω must be considered as an effective immune-modulator therapy for use in shelter medicine, particularly in animals where opportunistic infections are a real problem.

In chapter II, authors tried to deep-in on the influence of rFeIFN-ω in the acute phase reaction. Following the previous conclusions suggesting that this compound acts on the innate response rather than on the acquired immune system, authors evaluated its effect on APPs. Taking into account that APPs are one of the key components of the innate immune system, they seemed reasonable predictors of an innate immune-stimulation. In this chapter, authors showed that APPs significantly increased in cats treated with the licensed rFeIFN-ω protocol. Despite the fact that APPs usually increase in different situations such as chronic infection and severe inflammation (Ceron, et al., 2005; Paltrinieri, 2008), in this study, their increment is concomitant with the described clinical improvement and reduction of opportunistic infections. This part of the work demonstrates that APP's increment may be

beneficial in retroviral infected cats, confirming that they can be a reasonable indicator of a potentiated innate immune response. Being the first study documenting the effect of rFeIFN-ω on APP profile, this chapter reinforces the action of this compound on the innate immune system helping to clarify the mechanisms of action of the licensed protocol.

Recognizing that in clinical practice rFeIFN-ω is very often a cost-limitative therapy, in chapter III, authors documented the use of an alternative oral protocol in FIV-infected cats. In fact, different authors had documented the clinical benefits of oral low dose HuIFN-α protocols (Pedretti, et al., 2006; Tompkins, 1999). After the release of rFeIFN-ω, several clinical trials have also been conducted in order to study the effect of oral rFeIFN-ω protocols (Bracklein, et al., 2006; Hennet, et al., 2011). However, no studies had documented its use in retroviral infected cats namely in FIV-infected ones. In this sense, authors developed and administered an oral rFeIFN-ω protocol to 11 client-owned FIV-infected cats. The dose applied was based on the referred previous studies and was decided according to the manufacturer's previous trials. The oral protocol was 10-40 fold lower than the licensed subcutaneous protocol, it was given orally instead of subcutaneously and for a longer period of 90 continuous days. Following the same methodology applied on chapters I and II, authors evaluated the clinical improvement, concurrent viral excretion, APPs profile and different hematology and biochemistry parameters in FIV-infected cats treated with the oral protocol. Similarly to what was observed for the licensed protocol, cats treated with oral rFeIFN-ω showed a significant clinical improvement, without remarked changes on hematology and biochemistry profiles. Conversely, mainly due to the fact that client-owned cats were less prone to opportunistic infections, concurrent viral excretion was very low and did not change with the applied protocol. Also APPs profile did not change in cats treated with oral rFeIFN-ω meaning that the two distinct protocols have distinct mechanisms of action. Taking APPs as biomarkers of the innate immune response, it seems reasonable to say that the innate immune reaction is not potentiated at the same way as the observed in the licensed protocol. Even though, results documented on chapter III reinforced the potential extra-label use of rFeIFN-ω in an oral continuous low-dose protocol.

In order to deepen the immune modulation properties of oral rFeIFN-ω, once APPs did not change, authors tried to assess other innate immunity biomarker in treated animals, namely Mx protein. Although its use in feline medicine is scarce, Mx protein is a specific type I IFN biomarker (Bracklein, et al., 2006; Robert-Tissot, et al., 2011) hence its production is directly related with the activation of a type I-IFN signal transduction pathway. In this sense, to author's point of view, Mx protein seemed a reasonable biomarker of immune modulation induced by rFeIFN-ω therapy. In chapter IV, authors evaluated Mx protein expression in FIV-infected cats treated with oral rFeIFN-ω. Although the low number of tested animals and the inconsistency variation of the results all the animals revealed a detectable Mx expression

after therapy. This corroborates with an eventual and discrete immune-stimulation of type-I IFN pathways by oral rFeIFN-ω. However, despite the fact that Mx protein is a strong *in vitro* biomarker of the innate immune response (Robert-Tissot, et al., 2011), to author's point of view it was not a reliable parameter for naturally FIV-infected cats under oral rFeIFN-ω therapy. Even though, this chapter opened new insights about the *in vivo* use of this parameter as a biomarker of the feline innate immune response.

In chapter V, authors evaluated the effect of both protocols of rFeIFN-ω on cytokine profile, viremia and proviral load. Only one study had previously reported that the licensed protocol does not change viremia or proviral load in treated FIV-infected cats (Domenech, et al., 2011) suggesting that this compound may not act on acquired immunity. In agreement with these results, authors concluded that viremia did not change in the group of FIV-infected cats treated with the licensed protocol. However, in opposition to what was previously described, a significant increase on proviral load was reported and correlated to a relative increase of lymphocytes cell count (even within the normal range). In this chapter, authors also evaluated viremia and proviral load changes in FIV-infected cats treated with oral rFeIFN-ω. As expected, no changes were obtained in both parameters which reinforce the previous suggestion that, independently of the administered protocol, the rFeIFN-ω's anti-viral effect *in vivo* for FIV is negligible. Nonetheless, in the author's point of view, to state that rFeIFN-ω does not act on the acquired immune system, only based on viremia and proviral load changes is overspeculative and an evaluation of cytokine profiles in these animals are essential to fully understand it. To author's knowledge, there are no previous studies about the effect of rFeIFN-ω on cytokine profile. In order to evaluate whether the clinical benefits of rFeIFN-ω were due to the activation of the acquired immune system of FIV-infected cats, authors monitored mRNA expression and concurrent plasma levels of various cytokines using biological samples from the two groups of FIV-infected cats treated with either subcutaneous or oral rFEIFN-ω protocols. Despite its pleiotropic effect, authors assessed variations of Th-1 and Th-2 responses based on the different cytokines profiles measured. Results showed that Th-1 and Th-2 responses did not significantly change in both protocols, which supported the previous suggestions that rFeIFN-ω does not strongly affect the acquired immune system. Among the measured cytokines, only IL-6 (a pro-inflammatory cytokine involved in different immune pathways and particularly in the innate immune response) significantly changed in both groups. In fact, in cats treated with the licensed protocol, IL-6 plasma levels significantly reduced whilst its respective mRNA expression showed a decreasing tendency, not statistically significant. On the other hand, in cats treated with oral rFEIFN-ω, IL-6 plasma levels did not change but the concurrent mRNA expression significantly decreased. All in all, authors documented that IL-6 production is affected in both protocols meaning that rFeIFN-ω have anti-inflammatory properties. Moreover, considering

that plasma changes were only significant in cats treated with the licensed protocol, it seems reasonable to state that this higher pulsate therapeutic scheme is more efficient reducing the pro-inflammatory stimuli than the continuous low dose therapy.

On part III authors described two clinical cases, beyond the scope of retroviral infections, of type II diabetes mellitus with concurrent FCGS, in which rFEIFN-ω was used as an alternative to steroid therapy. One study had previously compared the benefits of rFEIFN-ω versus steroids on refractory cases of FCGS (Hennet, et al., 2011). To author's knowledge, this is the first successful report of its use as an alternative therapy in cats with type II diabetes mellitus in which steroid therapy is not recommended. As reported, these two cats had a severe oral disease which was complicating the type II-DM. After oral rFEIFN-ω therapy, both cats showed an important clinical improvement of FCGS and a concurrent remission of DM. Although this chapter is unrelated to the rest of the work, which focused on interferon effects in retroviral infection, it was included to illustrate the individual extrapolation of rFEIFN-ω oral protocol potential to treat other diseases.

Recognizing that this work is mainly based on clinical trials, several limitations must be considered. The main discussable limitation is the use of single arm trials instead of double-arm studies with placebo or control group. In fact, although it seemed reasonable when dealing with SPF cats, it is unreal to perform double arm trials when the main scope is naturally retroviral infected animals. To be rigorous, a double-arm trial would involve animals with the same clinical scores, proviral loads and which have been infected with the same FIV subtype at the same time point. It seemed unreasonable in this scenario where author's tried to study what was the main action of two protocols of rFeIFN-ω in naturally infected animals. Consequently, and to bypass this problem, the time point before therapy, set as day zero (D0) was considered the respective individual own control for each animal. Moreover, considering that the subcutaneous protocol is the licensed one, results obtained on chapter I and II were considered a reliable positive control for the further works respectively developed on chapters III and IV. When authors developed the oral rFeIFN-ω protocol, the comparison between both therapeutic schemes was then possible. Interestingly, in spite of the different conditions and environments, groups were even indistinguishable on day zero in the majority of the variables studied which reinforced the result's reliability. Despite these findings, the fact that treated groups came from two different environments must be considered. Both in the animal shelter and client-owned cats (which were mainly indoor-animals), there were several variables such as house-conditions, outdoor exposure, compliance or feeding intake, which could not be controlled. For instance, while cats from animal shelter were treated by two members of the research team, in the client-owned cats treated with oral therapy, owners were instructed to administer the compound. Although owners seemed comfortable with the administration, authors could not certify whether therapy was correctly administered

or not. Independently of these limitations, owners often remarked that the administration was easy and without troubles, which helped to minimize possible complications. To author's point of view, this is an inevitable critical point in clinical trials involving owners and care takers.

Another limitation that should be considered is the low number of animals per group, which consequently influence the reliability of statistical results. In chapter I and II, authors followed 16 naturally retroviral infected cats which were living in an animal shelter. As in all the animal shelters, there are also human and environmental conditions that are difficult to control. Among the 16 naturally retroviral infected cats, 7 were FIV-positive, 6 were FeLV- positive and 3 were co-infected FIV/FeLV. Ideally, study would be stronger if the 16 treated cats were all exclusively FIV-positive. However, taking into account that the subcutaneous protocol is also licensed for use in FeLV cats, authors decided to treat all the retroviral infected cats of the shelter, which were living in distinct wards (FIV-cats were isolated from FeLV and Co-infected ones) but at the same environment. Moreover, it seemed unreasonable and unethical to treat only the FIV-positive cats in an animal shelter that have more retroviral infected cats and where collaborators are made aware for retroviral infections, taking an important daily care of them. Therefore, beyond the scientific purpose, authors decided to extend the treatment to the other retroviral infected cats of the shelter, helping to improve their quality of life and overall clinical health status. Considering that all the results and tendencies were similar among FIV, FeLV or even FIV/FeLV co-infected treated cats, in chapters I and II, the whole group of naturally retroviral infected animals were considered. Although only 7 FIV-infected cats were used for further comparisons of the work, in these first two chapters, the overall group of 16 naturally retroviral infected cats represented a better basis to extend the clinical benefits of the previous described rFeIFN-ω licensed protocol. Also for the study of oral rFeIFN-ω protocol, the use of client-owned cats limited the work. In fact, 22 cats were initially enrolled in this part of the work but only 11 completed the 90 days of therapy. In detail, 3 cats received corticosteroids therapy by the assistant vet during the oral rFeIFN-ω protocol (which was an exclusion criteria), 2 cats died from suspected effusive-FIP, 1 cat developed intestinal lymphoma 60 days after therapy, 2 cats underwent surgery during therapy, 1 cat was taking nature extracts that were considered an immune modulation therapy, 1 cat run away from home and another one developed fibrosarcoma during the study. These occurrences reduced the number of animals per group and limited major conclusions, impairing the correlation among variables and weakening the power of the results. However, to author's point of view, the number was reasonable for this work in which to mimic the real clinical cases conditions was intended.

As previously stated on chapter V, the use of mRNA from circulating blood to assess immune parameters such as cytokine profile can also be considered a limitation of this work. Indeed,

the quality of nucleic-acid's extraction depends on various factors such as sample conditions and the presence of blood clots (Wong, et al., 2004). Collecting blood from animals living in an animal shelter and which were not familiarized with human contact was particularly complicated and limitative of a better quality samples. Also in client-owned cats, despite the fact that the study only required a total of five blood collections in distinct time-points, blood sampling was not an easy process and clots formation also limited the quality of samples. More than in the oral group, sample quality of the subcutaneous group was critical mainly due to the blood sample collection in the field. Although the relative quantification of cytokine profile was achieved, there were not enough samples for Mx-protein quantification which limited the results of this parameter in this group. In order to bypass this problem and reinforce these results, it would have been interesting to isolate and purify circulating PBMCs at different time-points of therapy. These cells would thereafter be stimulated by immune response modifiers and according to the response, the respective activation/suppression of Th1 or Th2 pathways would be assessed. Although this seems to be a reasonable way to bypass this problem, it would have increased considerably research's costs. Following the same research line, it would be interesting to complement these conclusions with a direct evaluation of lymphoid tissue samples such as mucosal-oral lymphoid tissue. In a similar way to what is documented for other type-I IFNs (Tompkins, 1999), a direct assessment of cytokine expression on the lymphoid tissue would help to evaluate Th1 or Th2 pathways.

To author's knowledge, these were the first studies exploring the effect of rFeIFN-ω in different biomarkers of the immune system. However, there are still many points to investigate in order to explore the whole potential of this compound in retroviral infections. In FIV-infected cats, only two protocols were suggested. Following the same research line, it would be interesting to reinforce these conclusions with pharmacokinetic and pharmacodynamic studies. In the subcutaneous protocol, it would be useful to document the effect of lower doses but in longer therapy cycles and vice-versa, in order to relate the therapy requirements with clinical and virological conditions. For the oral group, it would also be interesting to assess the effect of intermediate higher doses for shorter periods and also to extend the protocol to FeLV-infected cats. Taking always into account the different physiopathology and the aggressive clinical portrait of FeLV infections, it would be interesting to assess the effect of the suggested oral rFeIFN-ω in these animals. According to author's point of view, it is unlikely that such lower oral dose will have the same effect on clinical improvement of FeLV-infected cats, since these animals are usually more symptomatic and in worse overall clinical condition than FIV-infected animals. Consequently, the higher subcutaneous protocol would always be preferable in these cats.

More than exploring the full potential of each route and dose, it would be interesting to study a combination of protocols to be used according to different clinical presentations. Although

further studies would be useful to reinforce a possible association between protocols, it can be individually performed since both therapies are currently well-documented and do not show important adverse effects. For instance, in symptomatic animals, an initial subcutaneous protocol followed by an oral continuous lower-dose therapy can now be recommended. To the author's point of view, this seems a reasonable approach that sooner or later can be considered in routine clinical practice. However, more studies can still be performed to fully clarify these possible associations.

Further studies regarding the follow-up and life-span after each protocol are warranted. In fact, authors tried to perform monthly to trimester follow ups after the end of therapy. In both groups it was unsuccessful. In the animal shelter, clinical evaluations after therapy were unreasonable since the income and outcome of cats (blocked during the study but allowed after the last day of it) impaired correct conclusions. Regarding client-owned cats, it was only correctly achieved in a small percentage of treated animals. In fact, the individual oral therapy for each cat was free of charge and comprised a total of 90 daily doses that were partially given to the owners at each evaluation time-point. After the end of therapy, the monitoring was unpractical once the majority of owners were unavailable to pursuit with monthly follow-ups. Consequently, a structured prospective study would be interesting in order to evaluate, not only the long-term benefits of therapy, but also any effect on the mean life-span of FIV-infected cats.

This work innovated in the extension of the main therapeutic properties of the licensed rFeIFN-ω protocol in naturally retroviral infected cats. It is now documented that it must be used not only in symptomatic cats but also in animals living in catteries or shelters where opportunistic infections are problematic. Although without a direct effect on Th1/Th2 acquired immunity, this compound potentiates the innate immunity , reducing the pro-inflammatory stimuli. Particularly for FIV-infected cats, this work presents a new oral rFeIFN-ω protocol which was successfully tested and validated. Although inducing a significant clinical improvement, its overall action as immune modulator seems to be less relevant than the subcutaneous protocol. In fact, it slightly decreases the pro-inflammatory stimuli without affecting the acquired immunity or even other parameters of the innate response such as acute phase proteins. Therefore, whilst the high pulsate subcutaneous protocol is strongly recommended for symptomatic FIV-infected cats, this lower continuous oral protocol can be a good alternative for less symptomatic FIV infected animals, in cases where there might be financial constrains to the use of the licensed protocol or even for cats which previously received subcutaneous rFeIFN-ω and require a continuous immune modulation therapy. More than contributing for a better knowledge of rFeIFN-ω, this work explored its immune modulation properties and validated a new oral protocol which can be included on future FIV-guidelines.

References

References

Abramo, F., Bo, S., Canese, M. G., & Poli, A. (1995). Regional distribution of lesions in the central nervous system of cats infected with feline immunodeficiency virus. *AIDS Res Hum Retroviruses, 11*(10), 1247-1253.

Ackley, C. D., Yamamoto, J. K., Levy, N., Pedersen, N. C., & Cooper, M. D. (1990). Immunologic abnormalities in pathogen-free cats experimentally infected with feline immunodeficiency virus. *J Virol, 64*(11), 5652-5655.

Addie, D. (2012). Feline Coronavirus Infections. In C. Green (Ed.), *Infectious Diseases of the Dog and Cat* (Elsevier ed., pp. 92-108). Missouri: Elsevier.

Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., et al. (2009). Feline infectious peritonitis. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 594-604.

Addie, D. D., Dennis, J. M., Toth, S., Callanan, J. J., Reid, S., & Jarrett, O. (2000). Long-term impact on a closed household of pet cats of natural infection with feline coronavirus, feline leukaemia virus and feline immunodeficiency virus. *Vet Rec, 146*(15), 419-424.

Akira, S., Uematsu, S., & Takeuchi, O. (2006). Pathogen recognition and innate immunity. *Cell, 124*(4), 783-801.

Alimonti, J. B., Ball, T. B., & Fowke, K. R. (2003). Mechanisms of CD4+ T lymphocyte cell death in human immunodeficiency virus infection and AIDS. *J Gen Virol, 84*(Pt 7), 1649-1661.

Allison, R. W., & Hoover, E. A. (2003). Feline immunodeficiency virus is concentrated in milk early in lactation. *AIDS Res Hum Retroviruses, 19*(3), 245-253.

Anderson, L. J., & Jarrett, W. F. (1971). Membranous glomerulonephritis associated with leukaemia in cats. *Res Vet Sci, 12*(2), 179-180.

Anderson, M. M., Lauring, A. S., Burns, C. C., & Overbaugh, J. (2000). Identification of a cellular cofactor required for infection by feline leukemia virus. *Science, 287*(5459), 1828-1830.

Anderson, M. M., Lauring, A. S., Robertson, S., Dirks, C., & Overbaugh, J. (2001). Feline Pit2 functions as a receptor for subgroup B feline leukemia viruses. *J Virol, 75*(22), 10563-10572.

Arai, M., Darman, J., Lewis, A., & Yamamoto, J. K. (2000). The use of human hematopoietic growth factors (rhGM-CSF and rhEPO) as a supportive therapy for FIV-infected cats. *Vet Immunol Immunopathol, 77*(1-2), 71-92.

Arai, M., Earl, D. D., & Yamamoto, J. K. (2002). Is AZT/3TC therapy effective against FIV infection or immunopathogenesis? *Vet Immunol Immunopathol, 85*(3-4), 189-204.

Arjona, A., Barquero, N., Domenech, A., Tejerizo, G., Collado, V. M., Toural, C., et al. (2007). Evaluation of a novel nested PCR for the routine diagnosis of feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV). *J Feline Med Surg, 9*(1), 14-22.

Arjona, A., Escolar, E., Soto, I., Barquero, N., Martin, D., & Gomez-Lucia, E. (2000). Seroepidemiological survey of infection by feline leukemia virus and immunodeficiency virus in Madrid and correlation with some clinical aspects. *J Clin Microbiol, 38*(9), 3448-3449.

Asada-Kubota, M., Ueda, T., Shimada, M., Takeda, K., & Sokawa, Y. (1995). Distribution of immunoreactive 2',5'-oligoadenylate synthetase in mouse digestive tract. *J Interferon Cytokine Res, 15*(10), 863-867.

Babyak, S. D., Groves, M. G., Dimski, D. S., & Taboada, J. (1996). Evaluation of a saliva test kit for feline leukemia virus antigen. *J Am Anim Hosp Assoc, 32*(5), 397-400.

Bachmann, M. H., Mathiason-Dubard, C., Learn, G. H., Rodrigo, A. G., Sodora, D. L., Mazzetti, P., et al. (1997). Genetic diversity of feline immunodeficiency virus: dual infection, recombination, and distinct evolutionary rates among envelope sequence clades. *J Virol, 71*(6), 4241-4253.

Bandecchi, P., Dell'Omodarme, M., Magi, M., Palamidessi, A., & Prati, M. C. (2006). Feline leukaemia virus (FeLV) and feline immunodeficiency virus infections in cats in the Pisa district of Tuscany, and attempts to control FeLV infection in a colony of domestic cats by vaccination. *Vet Rec, 158*(16), 555-557.

Bandecchi, P., Matteucci, D., Baldinotti, F., Guidi, G., Abramo, F., Tozzini, F., et al. (1992). Prevalence of feline immunodeficiency virus and other retroviral infections in sick cats in Italy. *Vet Immunol Immunopathol, 31*(3-4), 337-345.

Barbai, V. H., Ujhelyi, E., Szlavik, J., Vietorisz, I., Varga, L., Fey, E., et al. (2010). Changes in the levels of some acute-phase proteins in human immunodeficiency virus-1 infected patients, following interleukin-2 treatment. *Clin Exp Immunol, 161*(1), 134-141.

Barnard, A., Mahon, B. P., Watkins, J., Redhead, K., & Mills, K. H. (1996). Th1/Th2 cell dichotomy in acquired immunity to Bordetella pertussis: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. *Immunology, 87*(3), 372-380.

Barnett, A. L., Wensel, D. L., Li, W., Fass, D., & Cunningham, J. M. (2003). Structure and mechanism of a coreceptor for infection by a pathogenic feline retrovirus. *J Virol, 77*(4), 2717-2729.

Barr, M. C. (1996). FIV, FeLV, and FIPV: interpretation and misinterpretation of serological test results. *Semin Vet Med Surg (Small Anim), 11*(3), 144-153.

Barrs, V. R., Martin, P., Nicoll, R. G., Beatty, J. A., & Malik, R. (2000). Pulmonary cryptococcosis and Capillaria aerophila infection in an FIV-positive cat. *Aust Vet J, 78*(3), 154-158.

Bauer, N., Balzer, H. J., Thure, S., & Moritz, A. (2008). Prevalence of feline haemotropic mycoplasmas in convenience samples of cats in Germany. *J Feline Med Surg, 10*(3), 252-258.

Baumann, H., & Gauldie, J. (1994). The acute phase response. *Immunol Today, 15*(2), 74-80.

Baxter, K. J., Levy, J. K., Edinboro, C. H., Vaden, S. L., & Tompkins, M. B. (2012). Renal disease in cats infected with feline immunodeficiency virus. *J Vet Intern Med, 26*(2), 238-243.

Beatty, J. A., Callanan, J. J., Terry, A., Jarrett, O., & Neil, J. C. (1998). Molecular and immunophenotypical characterization of a feline immunodeficiency virus (FIV)-associated lymphoma: a direct role for FIV in B-lymphocyte transformation? *J Virol, 72*(1), 767-771.

Beatty, J. A., Lawrence, C. E., Callanan, J. J., Grant, C. K., Gault, E. A., Neil, J. C., et al. (1998). Feline immunodeficiency virus (FIV)-associated lymphoma: a potential role for immune dysfunction in tumourigenesis. *Vet Immunol Immunopathol, 65*(2-4), 309-322.

Beatty, J. A., Willett, B. J., Gault, E. A., & Jarrett, O. (1996). A longitudinal study of feline immunodeficiency virus-specific cytotoxic T lymphocytes in experimentally infected cats, using antigen-specific induction. *J Virol, 70*(9), 6199-6206.

Beebe, A. M., Dua, N., Faith, T. G., Moore, P. F., Pedersen, N. C., & Dandekar, S. (1994). Primary stage of feline immunodeficiency virus infection: viral dissemination and cellular targets. *J Virol, 68*(5), 3080-3091.

Bendinelli, M., Pistello, M., Lombardi, S., Poli, A., Garzelli, C., Matteucci, D., et al. (1995). Feline immunodeficiency virus: an interesting model for AIDS studies and an important cat pathogen. *Clin Microbiol Rev, 8*(1), 87-112.

Bennett, N., Greco, D. S., Peterson, M. E., Kirk, C., Mathes, M., & Fettman, M. J. (2006). Comparison of a low carbohydrate-low fiber diet and a moderate carbohydrate-high fiber diet in the management of feline diabetes mellitus. *J Feline Med Surg, 8*(2), 73-84.

Bienzle, D., Reggeti, F., Wen, X., Little, S., Hobson, J., & Kruth, S. (2004). The variability of serological and molecular diagnosis of feline immunodeficiency virus infection. *Can Vet J, 45*(9), 753-757.

Bishop, S. A., Gruffydd-Jones, T. J., Harbour, D. A., & Stokes, C. R. (1993). Programmed cell death (apoptosis) as a mechanism of cell death in peripheral blood mononuclear cells from cats infected with feline immunodeficiency virus (FIV). *Clin Exp Immunol, 93*(1), 65-71.

Blair, A., & Hayes, H. M., Jr. (1980). Cancer and other causes of death among U.S. veterinarians, 1966-1977. *Int J Cancer, 25*(2), 181-185.

Bocci, V. (1988). Roles of interferon produced in physiological conditions. A speculative review. *Immunology, 64*(1), 1-9.

Bocci, V. (1991). Absorption of cytokines via oropharyngeal-associated lymphoid tissues. Does an unorthodox route improve the therapeutic index of interferon? *Clin Pharmacokinet, 21*(6), 411-417.

Bracklein, T., Theise, S., Metzler, A., Spiess, B. M., & Richter, M. (2006). Activity of feline interferon-omega after ocular or oral administration in cats as indicated by Mx protein expression in conjunctival and white blood cells. *Am J Vet Res, 67*(6), 1025-1032.

Bragg, D. C., Boles, J. C., & Meeker, R. B. (2002). Destabilization of neuronal calcium homeostasis by factors secreted from choroid plexus macrophage cultures in response to feline immunodeficiency virus. *Neurobiol Dis, 9*(2), 173-186.

Brightman, A. H., 2nd, Ogilvie, G. K., & Tompkins, M. (1991). Ocular disease in FeLV-positive cats: 11 cases (1981-1986). *J Am Vet Med Assoc, 198*(6), 1049-1051.

Broche-Pierre, S., Richardson, J., Moraillon, A., & Sonigo, P. (2005). Evaluation of live feline immunodeficiency virus vaccines with modified antigenic properties. *J Gen Virol, 86*(Pt 9), 2495-2506.

Brod, S. A., Nelson, L., Jin, R., & Wolinsky, J. S. (1999). Ingested interferon alpha induces Mx mRNA. *Cytokine, 11*(7), 492-499.

Bross, I. D., & Gibson, R. (1970). Cats and childhood leukemia. *J Med, 1*(3), 180-187.

Bucci, J. G., English, R. V., Jordan, H. L., Childers, T. A., Tompkins, M. B., & Tompkins, W. A. (1998). Mucosally transmitted feline immunodeficiency virus induces a CD8+ antiviral response that correlates with reduction of cell-associated virus. *J Infect Dis, 177*(1), 18-25.

Bull, M. E., Kennedy-Stoskopf, S., Levine, J. F., Loomis, M., Gebhard, D. G., & Tompkins, W. A. (2003). Evaluation of T lymphocytes in captive african lions (Panthera leo) infected with feline immunodeficiency virus. *Am J Vet Res, 64*(10), 1293-1300.

Burkhard, M. J., & Dean, G. A. (2003). Transmission and immunopathogenesis of FIV in cats as a model for HIV. *Curr HIV Res, 1*(1), 15-29.

Butera, S. T., Brown, J., Callahan, M. E., Owen, S. M., Matthews, A. L., Weigner, D. D., et al. (2000). Survey of veterinary conference attendees for evidence of zoonotic infection by feline retroviruses. *J Am Vet Med Assoc, 217*(10), 1475-1479.

Callanan, J. J., Jones, B. A., Irvine, J., Willett, B. J., McCandlish, I. A., & Jarrett, O. (1996). Histologic classification and immunophenotype of lymphosarcomas in cats with naturally and experimentally acquired feline immunodeficiency virus infections. *Vet Pathol, 33*(3), 264-272.

Caney S., H. C., Finerty S., Tasker S., Gruffydd-Jones TJ. (2003). *Treatment of asymptomatic chroniically FIV-infected cats with recombinant feline interferon omega.* Paper presented at the Proceedings of the annual conference of the American College of Veterinary Internal Medicine, Charlotte, USA.

Cantell, K., & Pyhala, L. (1973). Circulating interferon in rabbits after administration of human interferon by different routes. *J Gen Virol, 20*(1), 97-104.

Carmichael, K. P., Bienzle, D., & McDonnell, J. J. (2002). Feline leukemia virus-associated myelopathy in cats. *Vet Pathol, 39*(5), 536-545.

Carpenter, M. A., Brown, E. W., MacDonald, D. W., & O'Brien S, J. (1998). Phylogeographic patterns of feline immunodeficiency virus genetic diversity in the domestic cat. *Virology, 251*(2), 234-243.

Carroll, E. E., Dubielzig, R. R., & Schultz, R. D. (2002). Cats differ from mink and ferrets in their response to commercial vaccines: a histologic comparison of early vaccine reactions. *Vet Pathol, 39*(2), 216-227.

Cattori, V., & Hofmann-Lehmann, R. (2008). Absolute quantitation of feline leukemia virus proviral DNA and viral RNA loads by TaqMan real-time PCR and RT-PCR. *Methods Mol Biol, 429*, 73-87.

Cattori, V., Tandon, R., Riond, B., Pepin, A. C., Lutz, H., & Hofmann-Lehmann, R. (2009). The kinetics of feline leukaemia virus shedding in experimentally infected cats are associated with infection outcome. *Vet Microbiol, 133*(3), 292-296.

Caxito, F. A., Coelho, F. M., Oliveira, M. E., & Resende, M. (2006). Feline immunodeficiency virus subtype B in domestic cats in Minas Gerais, Brazil. *Vet Res Commun, 30*(8), 953-956.

Ceciliani, F., Giordano, A., & Spagnolo, V. (2002). The systemic reaction during inflammation: the acute-phase proteins. *Protein Pept Lett, 9*(3), 211-223.

Ceron, J. J., Eckersall, P. D., & Martynez-Subiela, S. (2005). Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol, 34*(2), 85-99.

Cheng, Y. S., Becker-Manley, M. F., Rucker, R. G., & Borden, E. C. (1988). Accumulation of guanylate binding proteins in patients treated with interferons. *J Interferon Res, 8*(3), 385-391.

Choi, I. S., Yoo, H. S., & Collisson, E. W. (2000). Evaluation of expression patterns of feline CD28 and CTLA-4 in feline immunodeficiency virus (FIV)-infected and FIV antigen-induced PBMC. *J Vet Sci, 1*(2), 97-103.

Clegg, S. R., Coyne, K. P., Dawson, S., Spibey, N., Gaskell, R. M., & Radford, A. D. (2011). Canine parvovirus in asymptomatic feline carriers. *Vet Microbiol*.

Collado, V. M., Doménech, A., Gómez-Lucía, E., Tejerizo, G., Miró, G. (2006). Usos de interferón en la clínica de pequeños animales. *Pequeños Animales, 63*, 68-75.

Collado, V. M., Gomez-Lucia, E., Tejerizo, G., Miro, G., Escolar, E., Martin, S., et al. (2007). Effect of type I interferons on the expression of feline leukaemia virus. *Vet Microbiol, 123*(1-3), 180-186.

Colonna, M., Trinchieri, G., & Liu, Y. J. (2004). Plasmacytoid dendritic cells in immunity. *Nat Immunol, 5*(12), 1219-1226.

Copelan, E. A., Rinehart, J. J., Lewis, M., Mathes, L., Olsen, R., & Sagone, A. (1983). The mechanism of retrovirus suppression of human T cell proliferation in vitro. *J Immunol, 131*(4), 2017-2020.

Cotter, S. M. (1991). Management of healthy feline leukemia virus-positive cats. *J Am Vet Med Assoc, 199*(10), 1470-1473.

Cotter, S. M., Hardy, W. D., Jr., & Essex, M. (1975). Association of feline leukemia virus with lymphosarcoma and other disorders in the cat. *J Am Vet Med Assoc, 166*(5), 449-454.

Coyne, K. P., Gaskell, R. M., Dawson, S., Porter, C. J., & Radford, A. D. (2007). Evolutionary mechanisms of persistence and diversification of a calicivirus within endemically infected natural host populations. *J Virol, 81*(4), 1961-1971.

Crawford, P. C., & Levy, J. K. (2007). New challenges for the diagnosis of feline immunodeficiency virus infection. *Vet Clin North Am Small Anim Pract, 37*(2), 335-350, vii.

Crawford, P. C., Papadi, G. P., Levy, J. K., Benson, N. A., Mergia, A., & Johnson, C. M. (2001). Tissue dynamics of CD8 lymphocytes that suppress viral replication in cats infected neonatally with feline immunodeficiency virus. *J Infect Dis, 184*(6), 671-681.

Cummins, J. M., Tompkins, M. B., Olsen, R. G., Tompkins, W. A., & Lewis, M. G. (1988). Oral use of human alpha interferon in cats. *J Biol Response Mod, 7*(5), 513-523.

Dandekar, S., Beebe, A. M., Barlough, J., Phillips, T., Elder, J., Torten, M., et al. (1992). Detection of feline immunodeficiency virus (FIV) nucleic acids in FIV-seronegative cats. *J Virol, 66*(7), 4040-4049.

Davidson, M. G., Rottman, J. B., English, R. V., Lappin, M. R., & Tompkins, M. B. (1993). Feline immunodeficiency virus predisposes cats to acute generalized toxoplasmosis. *Am J Pathol, 143*(5), 1486-1497.

Dawson, S., Smyth, N. R., Bennett, M., Gaskell, R. M., McCracken, C. M., Brown, A., et al. (1991). Effect of primary-stage feline immunodeficiency virus infection on subsequent feline calicivirus vaccination and challenge in cats. *AIDS, 5*(6), 747-750.

Day, M. J. (2012). Basic Immunology. In M. J. Day (Ed.), *Clinical Immunology of the Dog and Cat* (Second Edition ed., pp. 11-61). London Uk: Manson Publishing.

Day, N. K., O'Reilly-Felice, C., Hardy, W. D., Jr., Good, R. A., & Witkin, S. S. (1980). Circulating immune complexes associated with naturally occurring lymphosarcoma in pet cats. *J Immunol, 125*(6), 2363-2366.

de Mari, K., Maynard, L., Sanquer, A., Lebreux, B., & Eun, H. M. (2004). Therapeutic effects of recombinant feline interferon-omega on feline leukemia virus (FeLV)-infected and

FeLV/feline immunodeficiency virus (FIV)-coinfected symptomatic cats. *J Vet Intern Med, 18*(4), 477-482.

de Parseval, A., Chatterji, U., Sun, P., & Elder, J. H. (2004). Feline immunodeficiency virus targets activated CD4+ T cells by using CD134 as a binding receptor. *Proc Natl Acad Sci U S A, 101*(35), 13044-13049.

Dean, G. A., Bernales, J. A., & Pedersen, N. C. (1998). Effect of feline immunodeficiency virus on cytokine response to Listeria monocytogenes in vivo. *Vet Immunol Immunopathol, 65*(2-4), 125-138.

Dean, G. A., LaVoy, A., Yearley, J., & Stanton, C. (2006). Cytokine modulation of the innate immune response in feline immunodeficiency virus-infected cats. *J Infect Dis, 193*(11), 1520-1527.

Dean, G. A., & Pedersen, N. C. (1998). Cytokine response in multiple lymphoid tissues during the primary phase of feline immunodeficiency virus infection. *J Virol, 72*(12), 9436-9440.

DeBowes, L. (2009). Feline Caudal Stomatitis. In J. Bonagura & D. Twedt (Eds.), *Kirk's Current Veterinary Therapy XIV* (Vol. XIV, pp. 476-479). Missouri: Saunders Elsevier.

Del Mauro, D., Matteucci, D., Giannecchini, S., Maggi, F., Pistello, M., & Bendinelli, M. (1998). Autologous and heterologous neutralization analyses of primary feline immunodeficiency virus isolates. *J Virol, 72*(3), 2199-2207.

Der, S. D., Zhou, A., Williams, B. R., & Silverman, R. H. (1998). Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A, 95*(26), 15623-15628.

Desario, C., Decaro, N., Campolo, M., Cavalli, A., Cirone, F., Elia, G., et al. (2005). Canine parvovirus infection: which diagnostic test for virus? *J Virol Methods, 126*(1-2), 179-185.

Diehl, L. J., & Hoover, E. A. (1992). Early and progressive helper T-cell dysfunction in feline leukemia virus-induced immunodeficiency. *J Acquir Immune Defic Syndr, 5*(12), 1188-1194.

Diehl, L. J., Mathiason-DuBard, C. K., O'Neil, L. L., & Hoover, E. A. (1995). Longitudinal assessment of feline immunodeficiency virus kinetics in plasma by use of a quantitative competitive reverse transcriptase PCR. *J Virol, 69*(4), 2328-2332.

Diehl, L. J., Mathiason-Dubard, C. K., O'Neil, L. L., & Hoover, E. A. (1996). Plasma viral RNA load predicts disease progression in accelerated feline immunodeficiency virus infection. *J Virol, 70*(4), 2503-2507.

Domenech, A., Miro, G., Collado, V. M., Ballesteros, N., Sanjose, L., Escolar, E., et al. (2011). Use of recombinant interferon omega in feline retrovirosis: from theory to practice. *Vet Immunol Immunopathol, 143*(3-4), 301-306.

Donzella, G. A., Schols, D., Lin, S. W., Este, J. A., Nagashima, K. A., Maddon, P. J., et al. (1998). AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med, 4*(1), 72-77.

Dorny, P., Speybroeck, N., Verstraete, S., Baeke, M., De Becker, A., Berkvens, D., et al. (2002). Serological survey of Toxoplasma gondii, feline immunodeficiency virus and feline leukaemia virus in urban stray cats in Belgium. *Vet Rec, 151*(21), 626-629.

Dow, S. W., Dreitz, M. J., & Hoover, E. A. (1992). Feline immunodeficiency virus neurotropism: evidence that astrocytes and microglia are the primary target cells. *Vet Immunol Immunopathol, 35*(1-2), 23-35.

Dow, S. W., Poss, M. L., & Hoover, E. A. (1990). Feline immunodeficiency virus: a neurotropic lentivirus. *J Acquir Immune Defic Syndr, 3*(7), 658-668.

Dowers, K. L., Hawley, J. R., Brewer, M. M., Morris, A. K., Radecki, S. V., & Lappin, M. R. (2010). Association of Bartonella species, feline calicivirus, and feline herpesvirus 1 infection with gingivostomatitis in cats. *J Feline Med Surg, 12*(4), 314-321.

Duan, X., Yarmush, D. M., Jayaraman, A., & Yarmush, M. L. (2004). Dispensable role for interferon-gamma in the burn-induced acute phase response: a proteomic analysis. *Proteomics, 4*(6), 1830-1839.

Duarte, A., Gil, S., Leal, R. O., & Tavares, L. (2012). Vírus da Imunodeficiência Felina (VIF): Da etiologia às novas abordagens terapêuticas. *Medicina Veterinária - Revista da Associação de Estudantes da Faculdade de Medicina Veterinária*(66), 37-43.

Duarte, A., Marques, M. I., Tavares, L., & Fevereiro, M. (2002). Phylogenetic analysis of five Portuguese strains of FIV. *Arch Virol, 147*(5), 1061-1070.

Duarte, A., & Tavares, L. (2006). Phylogenetic analysis of Portuguese Feline Immunodeficiency Virus sequences reveals high genetic diversity. *Vet Microbiol, 114*(1-2), 25-33.

Duarte, A., Veiga, I., & Tavares, L. (2009). Genetic diversity and phylogenetic analysis of Feline Coronavirus sequences from Portugal. *Vet Microbiol, 138*(1-2), 163-168.

Dunham, S. P. (2006). Lessons from the cat: development of vaccines against lentiviruses. *Vet Immunol Immunopathol, 112*(1-2), 67-77.

Dunham, S. P., Bruce, J., Klein, D., Flynn, J. N., Golder, M. C., MacDonald, S., et al. (2006). Prime-boost vaccination using DNA and whole inactivated virus vaccines provides limited protection against virulent feline immunodeficiency virus. *Vaccine, 24*(49-50), 7095-7108.

Dunham, S. P., & Graham, E. (2008). Retroviral infections of small animals. *Vet Clin North Am Small Anim Pract, 38*(4), 879-901, ix.

Dupuis, S., Jouanguy, E., Al-Hajjar, S., Fieschi, C., Al-Mohsen, I. Z., Al-Jumaah, S., et al. (2003). Impaired response to interferon-alpha/beta and lethal viral disease in human STAT1 deficiency. *Nat Genet, 33*(3), 388-391.

Duthie, S., Eckersall, P. D., Addie, D. D., Lawrence, C. E., & Jarrett, O. (1997). Value of alpha 1-acid glycoprotein in the diagnosis of feline infectious peritonitis. *Vet Rec, 141*(12), 299-303.

Eckersall, P. D., & Bell, R. (2010). Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. *Vet J, 185*(1), 23-27.

Egberink, H. F., De Clercq, E., Van Vliet, A. L., Balzarini, J., Bridger, G. J., Henson, G., et al. (1999). Bicyclams, selective antagonists of the human chemokine receptor CXCR4, potently inhibit feline immunodeficiency virus replication. *J Virol, 73*(8), 6346-6352.

Elder, J. H., Lin, Y. C., Fink, E., & Grant, C. K. (2010). Feline immunodeficiency virus (FIV) as a model for study of lentivirus infections: parallels with HIV. *Curr HIV Res, 8*(1), 73-80.

Elder, J. H., McGee, J. S., Munson, M., Houghten, R. A., Kloetzer, W., Bittle, J. L., et al. (1987). Localization of neutralizing regions of the envelope gene of feline leukemia virus by using anti-synthetic peptide antibodies. *J Virol, 61*(1), 8-15.

Ellis, J. A., Jackson, M. L., Bartsch, R. C., McGill, L. G., Martin, K. M., Trask, B. R., et al. (1996). Use of immunohistochemistry and polymerase chain reaction for detection of oncornaviruses in formalin-fixed, paraffin-embedded fibrosarcomas from cats. *J Am Vet Med Assoc, 209*(4), 767-771.

Endo, Y., Cho, K. W., Nishigaki, K., Momoi, Y., Nishimura, Y., Mizuno, T., et al. (1997). Molecular characteristics of malignant lymphomas in cats naturally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol, 57*(3-4), 153-167.

English, R. V., Davidson, M. G., Nasisse, M. P., Jamieson, V. E., & Lappin, M. R. (1990). Intraocular disease associated with feline immunodeficiency virus infection in cats. *J Am Vet Med Assoc, 196*(7), 1116-1119.

English, R. V., Nelson, P., Johnson, C. M., Nasisse, M., Tompkins, W. A., & Tompkins, M. B. (1994). Development of clinical disease in cats experimentally infected with feline immunodeficiency virus. *J Infect Dis, 170*(3), 543-552.

Essex, M., Cotter, S. M., Hardy, W. D., Jr., Hess, P., Jarrett, W., Jarrett, O., et al. (1975). Feline oncornavirus-associated cell membrane antigen. IV. Antibody titers in cats with naturally occurring leukemia, lymphoma, and other diseases. *J Natl Cancer Inst, 55*(2), 463-467.

Etheridge, A., Gomes, C. P., Pereira, R. W., Galas, D., & Wang, K. (2013). The complexity, function and applications of RNA in circulation. *Front Genet, 4*, 115.

Evermann, J. F., Sellon, R., & Sykes, J. E. (2012). Chapter 1: Laboratory Diagnosis of Viral and Rickettsial Infections and Clinical Epidemiology of Infectious Disease (Fourth Edition). In C. Greene (Ed.), *Infectious Diseases of the Dog and Cat* (Fourth ed.). Missouri: Saunders Elsevier.

Fails, A. D., Mitchell, T. W., Rojko, J. L., & Whalen, L. R. (1997). An oligopeptide of the feline leukemia virus envelope glycoprotein is associated with morphological changes and calcium dysregulation in neuronal growth cones. *J Neurovirol, 3*(3), 179-191.

Fernandez, M., Quiroga, J. A., Martin, J., Herrero, M., Pardo, M., Horisberger, M. A., et al. (1999). In vivo and in vitro induction of MxA protein in peripheral blood mononuclear cells from patients chronically infected with hepatitis C virus. *J Infect Dis, 180*(2), 262-267.

Fevereiro, M., Roneker, C., Laufs, A., Tavares, L., & de Noronha, F. (1991). Characterization of two monoclonal antibodies against feline immunodeficiency virus gag gene products and their application in an assay to evaluate neutralizing antibody activity. *J Gen Virol, 72 (Pt 3)*, 617-622.

Fleischmann, W. R., Jr., Koren, S., & Fleischmann, C. M. (1992). Orally administered interferons exert their white blood cell suppressive effects via a novel mechanism. *Proc Soc Exp Biol Med, 201*(2), 200-207.

Fletcher, N. F., Meeker, R. B., Hudson, L. C., & Callanan, J. J. (2011). The neuropathogenesis of feline immunodeficiency virus infection: barriers to overcome. *Vet J, 188*(3), 260-269.

Flynn, J. N., Cannon, C. A., Lawrence, C. E., & Jarrett, O. (1994). Polyclonal B-cell activation in cats infected with feline immunodeficiency virus. *Immunology, 81*(4), 626-630.

Flynn, J. N., Dunham, S., Mueller, A., Cannon, C., & Jarrett, O. (2002). Involvement of cytolytic and non-cytolytic T cells in the control of feline immunodeficiency virus infection. *Vet Immunol Immunopathol, 85*(3-4), 159-170.

Flynn, J. N., Dunham, S. P., Watson, V., & Jarrett, O. (2002). Longitudinal analysis of feline leukemia virus-specific cytotoxic T lymphocytes: correlation with recovery from infection. *J Virol, 76*(5), 2306-2315.

Flynn, J. N., Hanlon, L., & Jarrett, O. (2000). Feline leukaemia virus: protective immunity is mediated by virus-specific cytotoxic T lymphocytes. *Immunology, 101*(1), 120-125.

Fogle, J. E., Mexas, A. M., Tompkins, W. A., & Tompkins, M. B. (2010). CD4(+)CD25(+) T regulatory cells inhibit CD8(+) IFN-gamma production during acute and chronic FIV infection utilizing a membrane TGF-beta-dependent mechanism. *AIDS Res Hum Retroviruses, 26*(2), 201-216.

Fogle, J. E., Tompkins, W. A., Campbell, B., Sumner, D., & Tompkins, M. B. (2011). Fozivudine tidoxil as single-agent therapy decreases plasma and cell-associated viremia during acute feline immunodeficiency virus infection. *J Vet Intern Med, 25*(3), 413-418.

Foley, J. E., Poland, A., Carlson, J., & Pedersen, N. C. (1997). Patterns of feline coronavirus infection and fecal shedding from cats in multiple-cat environments. *J Am Vet Med Assoc, 210*(9), 1307-1312.

Forman, L. W., Pal-Ghosh, R., Spanjaard, R. A., Faller, D. V., & Ghosh, S. K. (2009). Identification of LTR-specific small non-coding RNA in FeLV infected cells. *FEBS Lett, 583*(8), 1386-1390.

Freer, G., Matteucci, D., Mazzetti, P., Tarabella, F., Catalucci, V., Ricci, E., et al. (2008). Evaluation of feline monocyte-derived dendritic cells loaded with internally inactivated virus as a vaccine against feline immunodeficiency virus. *Clin Vaccine Immunol, 15*(3), 452-459.

Fujino, Y., Horiuchi, H., Mizukoshi, F., Baba, K., Goto-Koshino, Y., Ohno, K., et al. (2009). Prevalence of hematological abnormalities and detection of infected bone marrow cells in asymptomatic cats with feline immunodeficiency virus infection. *Vet Microbiol, 136*(3-4), 217-225.

Fujino, Y., Liao, C. P., Zhao, Y. S., Pan, J., Mathes, L. E., Hayes, K. A., et al. (2009). Identification of a novel common proviral integration site, flit-1, in feline leukemia virus induced thymic lymphoma. *Virology, 386*(1), 16-22.

Fulton, R. W., & Burge, L. J. (1985). Susceptibility of feline herpesvirus 1 and a feline calicivirus to feline interferon and recombinant human leukocyte interferons. *Antimicrob Agents Chemother, 28*(5), 698-699.

Gabor, L. J., Love, D. N., Malik, R., & Canfield, P. J. (2001). Feline immunodeficiency virus status of Australian cats with lymphosarcoma. *Aust Vet J, 79*(8), 540-545.

Galabru, J., Robert, N., Buffet-Janvresse, C., Riviere, Y., & Hovanessian, A. G. (1985). Continuous production of interferon in normal mice: effect of anti-interferon globulin, sex, age, strain and environment on the levels of 2-5A synthetase and p67K kinase. *J Gen Virol, 66 (Pt 4)*, 711-718.

Gaskell, R., Dawson, S., & Radford, A. (2012). Feline Respiratory Disease. In C. Greene (Ed.), *Infectious Diseases of the Dog and Cat* (Fourth ed., pp. 151-162). Missouri: Saunders Elsevier.

George, J. W., Rideout, B. A., Griffey, S. M., & Pedersen, N. C. (2002). Effect of preexisting FeLV infection or FeLV and feline immunodeficiency virus coinfection on pathogenicity of the small variant of Haemobartonella felis in cats. *Am J Vet Res, 63*(8), 1172-1178.

Gerlach, N., Gibbert, K., Alter, C., Nair, S., Zelinskyy, G., James, C. M., et al. (2009). Anti-retroviral effects of type I IFN subtypes in vivo. *Eur J Immunol, 39*(1), 136-146.

Gerlach, N., Schimmer, S., Weiss, S., Kalinke, U., & Dittmer, U. (2006). Effects of type I interferons on Friend retrovirus infection. *J Virol, 80*(7), 3438-3444.

Gibson, D. M., Cotler, S., Spiegel, H. E., & Colburn, W. A. (1985). Pharmacokinetics of recombinant leukocyte A interferon following various routes and modes of administration to the dog. *J Interferon Res, 5*(3), 403-408.

Gil, S., Leal, R. O., Duarte, A., McGahie, D., Sepulveda, N., Siborro, I., et al. (2013). Relevance of feline interferon omega for clinical improvement and reduction of concurrent viral excretion in retrovirus infected cats from a rescue shelter. *Res Vet Sci, 94*(3), 753-763.

Gil, S., Leal, R. O., McGahie, D., Sepulveda, N., Duarte, A., Niza, M. M., et al. (2014). Oral Recombinant Feline Interferon-Omega as an alternative immune modulation therapy in FIV positive cats: Clinical and laboratory evaluation. *Res Vet Sci, 96*(1), 79-85.

Gil, S., Spagnuolo-Weaver, M., Canals, A., Sepulveda, N., Oliveira, J., Aleixo, A., et al. (2003). Expression at mRNA level of cytokines and A238L gene in porcine blood-derived macrophages infected in vitro with African swine fever virus (ASFV) isolates of different virulence. *Arch Virol, 148*(11), 2077-2097.

Giordano, A., Spagnolo, V., Colombo, A., & Paltrinieri, S. (2004). Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. *Vet J, 167*(1), 38-44.

Gleich, S., & Hartmann, K. (2009). Hematology and serum biochemistry of feline immunodeficiency virus-infected and feline leukemia virus-infected cats. *J Vet Intern Med, 23*(3), 552-558.

Gleich, S. E., Krieger, S., & Hartmann, K. (2009). Prevalence of feline immunodeficiency virus and feline leukaemia virus among client-owned cats and risk factors for infection in Germany. *J Feline Med Surg, 11*(12), 985-992.

Goff, S. (2007). Chapter 55: Retroviridae - The Retroviruses and their replication. In D. Knipe & P. Howley (Eds.), *Fields in Veterinary Virology* (pp. 1999-2070): Lippincott Williams & Wilkins Publishers.

Goldkamp, C. E., Levy, J. K., Edinboro, C. H., & Lachtara, J. L. (2008). Seroprevalences of feline leukemia virus and feline immunodeficiency virus in cats with abscesses or bite wounds and rate of veterinarian compliance with current guidelines for retrovirus testing. *J Am Vet Med Assoc, 232*(8), 1152-1158.

Gomes-Keller, M. A., Gonczi, E., Grenacher, B., Tandon, R., Hofman-Lehmann, R., & Lutz, H. (2009). Fecal shedding of infectious feline leukemia virus and its nucleic acids: a transmission potential. *Vet Microbiol, 134*(3-4), 208-217.

Gomes-Keller, M. A., Gonczi, E., Tandon, R., Riondato, F., Hofmann-Lehmann, R., Meli, M. L., et al. (2006). Detection of feline leukemia virus RNA in saliva from naturally infected cats and correlation of PCR results with those of current diagnostic methods. *J Clin Microbiol, 44*(3), 916-922.

Gomes-Keller, M. A., Tandon, R., Gonczi, E., Meli, M. L., Hofmann-Lehmann, R., & Lutz, H. (2006). Shedding of feline leukemia virus RNA in saliva is a consistent feature in viremic cats. *Vet Microbiol, 112*(1), 11-21.

Goodbourn, S., Didcock, L., & Randall, R. E. (2000). Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. *J Gen Virol, 81*(Pt 10), 2341-2364.

Goto, Y., Nishimura, Y., Mizuno, T., Endo, Y., Baba, K., Momoi, Y., et al. (2000). Quantification of viral ribonucleic acid in plasma of cats naturally infected with feline immunodeficiency virus. *Am J Vet Res, 61*(12), 1609-1613.

Grant, C. K., Fink, E. A., Sundstrom, M., Torbett, B. E., & Elder, J. H. (2009). Improved health and survival of FIV-infected cats is associated with the presence of autoantibodies to the primary receptor, CD134. *Proc Natl Acad Sci U S A, 106*(47), 19980-19985.

Guiot, A. L., Rigal, D., & Chappuis, G. (1997). Spontaneous programmed cell death (PCD) process of lymphocytes of FIV-infected cats: cellular targets and modulation. *Vet Immunol Immunopathol, 58*(2), 93-106.

Gunn-Moore, D. A., Pearson, G. R., Harbour, D. A., & Whiting, C. V. (1996). Encephalitis associated with giant cells in a cat with naturally occurring feline immunodeficiency virus infection demonstrated by in situ hybridization. *Vet Pathol, 33*(6), 699-703.

Haid, C., Kaps, S., Gonczi, E., Hassig, M., Metzler, A., Spiess, B. M., et al. (2007). Pretreatment with feline interferon omega and the course of subsequent infection with feline herpesvirus in cats. *Vet Ophthalmol, 10*(5), 278-284.

Haller, O., Kochs, G., & Weber, F. (2007). Interferon, Mx, and viral countermeasures. *Cytokine Growth Factor Rev, 18*(5-6), 425-433.

Hanlon, L., Argyle, D., Bain, D., Nicolson, L., Dunham, S., Golder, M. C., et al. (2001). Feline leukemia virus DNA vaccine efficacy is enhanced by coadministration with interleukin-12 (IL-12) and IL-18 expression vectors. *J Virol, 75*(18), 8424-8433.

Hanlon, M. A., Marr, J. M., Hayes, K. A., Mathes, L. E., Stromberg, P. C., Ringler, S., et al. (1993). Loss of neutrophil and natural killer cell function following feline immunodeficiency virus infection. *Viral Immunol, 6*(2), 119-124.

Hardy, W. D., Jr., Hess, P. W., Essex, M., Cotter, S., McClelland, A. J., & MacEwen, G. (1975). Horizontal transmission of feline leukemia virus in cats. *Bibl Haematol*(40), 67-74.

Hardy, W. D., Jr., McClelland, A. J., Zuckerman, E. E., Snyder, H. W., Jr., MacEwen, E. G., Francis, D., et al. (1980). Development of virus non-producer lymphosarcomas in pet cats exposed to FeLv. *Nature, 288*(5786), 90-92.

Hardy, W. D., Jr., & Zuckerman, E. E. (1991). Ten-year study comparing enzyme-linked immunosorbent assay with the immunofluorescent antibody test for detection of feline leukemia virus infection in cats. *J Am Vet Med Assoc, 199*(10), 1365-1373.

Hardy, W. D., Jr., Zuckerman, E. E., MacEwen, E. G., Hayes, A. A., & Essex, M. (1977). A feline leukaemia virus- and sarcoma virus-induced tumour-specific antigen. *Nature, 270*(5634), 249-251.

Harrus, S., Klement, E., Aroch, I., Stein, T., Bark, H., Lavy, E., et al. (2002). Retrospective study of 46 cases of feline haemobartonellosis in Israel and their relationships with FeLV and FIV infections. *Vet Rec, 151*(3), 82-85.

Hart, S. W., & Nolte, I. (1994). Hemostatic disorders in feline immunodeficiency virus-seropositive cats. *J Vet Intern Med, 8*(5), 355-362.

Hartmann, K. (1995a). AZT in the treatment of feline immunodeficiency virus infection: part 1. . *Feline Pract, 23(5)*, 16-20.

Hartmann, K. (1995b). AZT in the treatment of feline immunodeficiency virus infection: part 2. *Feline Pract, 23(6)*, 13-20.

Hartmann, K. (2009). Chapter 281: Feline Leukemia Virus and Feline Immunodeficiency Virus. In J. Bonagura & D. Twedt (Eds.), *Kirk's Current Veterinary Therapy XIV* (Vol. XIV, pp. 1278-1284). Missouri: Saunders Elsevier.

Hartmann, K. (2011). Clinical aspects of feline immunodeficiency and feline leukemia virus infection. *Vet Immunol Immunopathol, 143*(3-4), 190-201.

Hartmann, K. (2012a). Chapter 2:Antiviral and Immmunomodulatory Chemotherapy. In C. Greene (Ed.), *Infectious Diseases of the Dog and Cat* (Elsevier ed., pp. 10-25). Missouri: Elsevier.

Hartmann, K. (2012b). Chapter 11: Feline Leukemia Virus Infection. In C. Greene (Ed.), *Infectious Diseases of the Dog and Cat* (Fourth Edition ed., pp. 106-136). Missouri: Elsevier.

Hartmann, K. (2012c). Clinical aspects of feline retroviruses: a review. *Viruses, 4*(11), 2684-2710.

Hartmann, K., Donath, A., Beer, B., Egberink, H. F., Horzinek, M. C., Lutz, H., et al. (1992). Use of two virustatica (AZT, PMEA) in the treatment of FIV and of FeLV seropositive cats with clinical symptoms. *Vet Immunol Immunopathol, 35*(1-2), 167-175.

Hartmann, K., Ferk, G., North, T. W., & Pedersen, N. C. (1997). Toxicity associated with high dosage 9-[(2R,5R-2,5-dihydro-5-phosphonomethoxy)-2-furanyl]adenine therapy off attempts to abort early FIV infection. *Antiviral Res, 36*(1), 11-25.

Hartmann, K., Griessmayr, P., Schulz, B., Greene, C. E., Vidyashankar, A. N., Jarrett, O., et al. (2007). Quality of different in-clinic test systems for feline immunodeficiency virus and feline leukaemia virus infection. *J Feline Med Surg, 9*(6), 439-445.

Hartmann, K., Stengel, C., Klein, D., Egberink, H., & Balzarini, J. (2012). Efficacy and adverse effects of the antiviral compound plerixafor in feline immunodeficiency virus-infected cats. *J Vet Intern Med, 26*(3), 483-490.

Hartmann, K., Werner, R. M., Egberink, H., & Jarrett, O. (2001). Comparison of six in-house tests for the rapid diagnosis of feline immunodeficiency and feline leukaemia virus infections. *Vet Rec, 149*(11), 317-320.

Hawkins, E. C. (1991). Saliva and tear tests for feline leukemia virus. *J Am Vet Med Assoc, 199*(10), 1382-1385.

Hawks, D. M., Legendre, A. M., Rohrbach, B. W., Sebring, R., Chavez, L., Chu, H. J., et al. (1991). Antibody response of kittens after vaccination followed by exposure to feline leukemia virus-infected cats. *J Am Vet Med Assoc, 199*(10), 1463-1469.

Hayes, K. A., Phipps, A. J., Francke, S., & Mathes, L. E. (2000). Antiviral therapy reduces viral burden but does not prevent thymic involution in young cats infected with feline immunodeficiency virus. *Antimicrob Agents Chemother, 44*(9), 2399-2405.

Hayes, K. A., Rojko, J. L., Tarr, M. J., Polas, P. J., Olsen, R. G., & Mathes, L. E. (1989). Atypical localised viral expression in a cat with feline leukaemia. *Vet Rec, 124*(13), 344-346.

Hayes, K. A., Wilkinson, J. G., Frick, R., Francke, S., & Mathes, L. E. (1995). Early suppression of viremia by ZDV does not alter the spread of feline immunodeficiency virus infection in cats. *J Acquir Immune Defic Syndr Hum Retrovirol, 9*(2), 114-122.

Hayward, J. J., & Rodrigo, A. G. (2010). Molecular epidemiology of feline immunodeficiency virus in the domestic cat (Felis catus). *Vet Immunol Immunopathol, 134*(1-2), 68-74.

Hayward, J. J., Taylor, J., & Rodrigo, A. G. (2007). Phylogenetic analysis of feline immunodeficiency virus in feral and companion domestic cats of New Zealand. *J Virol, 81*(6), 2999-3004.

He, R., Shepard, L. W., Chen, J., Pan, Z. K., & Ye, R. D. (2006). Serum amyloid A is an endogenous ligand that differentially induces IL-12 and IL-23. *J Immunol, 177*(6), 4072-4079.

Heinrich, P. C., Castell, J. V., & Andus, T. (1990). Interleukin-6 and the acute phase response. *Biochem J, 265*(3), 621-636.

Heit, B., Jones, G., Knight, D., Antony, J. M., Gill, M. J., Brown, C., et al. (2006). HIV and other lentiviral infections cause defects in neutrophil chemotaxis, recruitment, and cell structure: immunorestorative effects of granulocyte-macrophage colony-stimulating factor. *J Immunol, 177*(9), 6405-6414.

Hennet, P., Boucraut-Baralon C. (2005). *Relationship between oral calicivirus and herpesvirus carriage and palatoglossitis.* Paper presented at the Proceedings of the 19th annual Veterinary Dental Forum, Orlando, USA.

Hennet, P. R., Camy, G. A., McGahie, D. M., & Albouy, M. V. (2011). Comparative efficacy of a recombinant feline interferon omega in refractory cases of calicivirus-positive cats with caudal stomatitis: a randomised, multi-centre, controlled, double-blind study in 39 cats. *J Feline Med Surg, 13*(8), 577-587.

Herrewegh, A. A., de Groot, R. J., Cepica, A., Egberink, H. F., Horzinek, M. C., & Rottier, P. J. (1995). Detection of feline coronavirus RNA in feces, tissues, and body fluids of naturally infected cats by reverse transcriptase PCR. *J Clin Microbiol, 33*(3), 684-689.

Hisasue, M., Nagashima, N., Nishigaki, K., Fukuzawa, I., Ura, S., Katae, H., et al. (2009). Myelodysplastic syndromes and acute myeloid leukemia in cats infected with feline leukemia virus clone33 containing a unique long terminal repeat. *Int J Cancer, 124*(5), 1133-1141.

Hochepied, T., Berger, F. G., Baumann, H., & Libert, C. (2003). Alpha(1)-acid glycoprotein: an acute phase protein with inflammatory and immunomodulating properties. *Cytokine Growth Factor Rev, 14*(1), 25-34.

Hoffmann-Fezer, G., Mortelbauer, W., Hartmann, K., Mysliwietz, J., Thefeld, S., Beer, B., et al. (1996). Comparison of T-cell subpopulations in cats naturally infected with feline leukaemia virus or feline immunodeficiency virus. *Res Vet Sci, 61*(3), 222-226.

Hoffmann-Fezer, G., Thum, J., Ackley, C., Herbold, M., Mysliwietz, J., Thefeld, S., et al. (1992). Decline in CD4+ cell numbers in cats with naturally acquired feline immunodeficiency virus infection. *J Virol, 66*(3), 1484-1488.

Hofmann-Lehmann, R., Cattori, V., Tandon, R., Boretti, F. S., Meli, M. L., Riond, B., et al. (2008). How molecular methods change our views of FeLV infection and vaccination. *Vet Immunol Immunopathol, 123*(1-2), 119-123.

Hofmann-Lehmann, R., Cattori, V., Tandon, R., Boretti, F. S., Meli, M. L., Riond, B., et al. (2007). Vaccination against the feline leukaemia virus: outcome and response categories and long-term follow-up. *Vaccine, 25*(30), 5531-5539.

Hofmann-Lehmann, R., Holznagel, E., Ossent, P., & Lutz, H. (1997). Parameters of disease progression in long-term experimental feline retrovirus (feline immunodeficiency virus and feline leukemia virus) infections: hematology, clinical chemistry, and lymphocyte subsets. *Clin Diagn Lab Immunol, 4*(1), 33-42.

Hofmann-Lehmann, R., Huder, J. B., Gruber, S., Boretti, F., Sigrist, B., & Lutz, H. (2001). Feline leukaemia provirus load during the course of experimental infection and in naturally infected cats. *J Gen Virol, 82*(Pt 7), 1589-1596.

Hofmann-Lehmann, R., Tandon, R., Boretti, F. S., Meli, M. L., Willi, B., Cattori, V., et al. (2006). Reassessment of feline leukaemia virus (FeLV) vaccines with novel sensitive molecular assays. *Vaccine, 24*(8), 1087-1094.

Hohdatsu, T., Nakanishi, T., Saito, I., & Koyama, H. (2005). Ability of CD8+ T cell anti-feline immunodeficiency virus (FIV) activity and FIV proviral DNA load in mononuclear cells in FIV-infected cats. *J Vet Med Sci, 67*(1), 129-131.

Hohdatsu, T., Yamazaki, A., Yamada, M., Kusuhara, H., Kaneshima, T., & Koyama, H. (2003). Ability of CD8(+) T cell anti-feline immunodeficiency virus activity correlated with peripheral CD4(+) T cell counts and plasma viremia. *Microbiol Immunol, 47*(10), 765-773.

Holznagel, E., Hofmann-Lehmann, R., Leutenegger, C. M., Allenspach, K., Huettner, S., Forster, U., et al. (1998). The role of in vitro-induced lymphocyte apoptosis in feline immunodeficiency virus infection: correlation with different markers of disease progression. *J Virol, 72*(11), 9025-9033.

Hoover, E. A., Zeidner, N. S., Perigo, N. A., Quackenbush, S. L., Strobel, J. D., Hill, D. L., et al. (1989). Feline leukemia virus-induced immunodeficiency syndrome in cats as a model for evaluation of antiretroviral therapy. *Intervirology, 30 Suppl 1*, 12-25.

Horisberger, M. A., & De Staritzky, K. (1989). Expression and stability of the Mx protein in different tissues of mice, in response to interferon inducers or to influenza virus infection. *J Interferon Res, 9*(5), 583-590.

Horisberger, M. A., Schrenk, R., Staiger, S., Leyvraz, A. R., & Martinod, S. (1990). Induction of Mx-related protein in cat peripheral blood mononuclear cells after administration of recombinant human interferon hybrid. *Antiviral Res, 13*(2), 53-59.

Hosie, M. J., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline immunodeficiency. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 575-584.

Hosie, M. J., Broere, N., Hesselgesser, J., Turner, J. D., Hoxie, J. A., Neil, J. C., et al. (1998). Modulation of feline immunodeficiency virus infection by stromal cell-derived factor. *J Virol, 72*(3), 2097-2104.

Hosie, M. J., Robertson, C., & Jarrett, O. (1989). Prevalence of feline leukaemia virus and antibodies to feline immunodeficiency virus in cats in the United Kingdom. *Vet Rec, 125*(11), 293-297.

Hosie, M. J., Willett, B. J., Dunsford, T. H., Jarrett, O., & Neil, J. C. (1993). A monoclonal antibody which blocks infection with feline immunodeficiency virus identifies a possible non-CD4 receptor. *J Virol, 67*(3), 1667-1671.

Huang, C., Conlee, D., Gill, M., & Chu, H. J. (2010). Dual-subtype feline immunodeficiency virus vaccine provides 12 months of protective immunity against heterologous challenge. *J Feline Med Surg, 12*(6), 451-457.

Huang, C., Conlee, D., Loop, J., Champ, D., Gill, M., & Chu, H. J. (2004). Efficacy and safety of a feline immunodeficiency virus vaccine. *Anim Health Res Rev, 5*(2), 295-300.

Huisman, W., Martina, B. E., Rimmelzwaan, G. F., Gruters, R. A., & Osterhaus, A. D. (2009). Vaccine-induced enhancement of viral infections. *Vaccine, 27*(4), 505-512.

Hutson, C. A., Rideout, B. A., & Pedersen, N. C. (1991). Neoplasia associated with feline immunodeficiency virus infection in cats of southern California. *J Am Vet Med Assoc, 199*(10), 1357-1362.

Inoshima, Y., Miyazawa, T., Kohmoto, M., Ikeda, Y., Sato, E., Hohdatsu, T., et al. (1998). Cross virus neutralizing antibodies against feline immunodeficiency virus genotypes A, B, C, D and E. *Arch Virol, 143*(1), 157-162.

Ishida, T., Shibanai, A., Tanaka, S., Uchida, K., & Mochizuki, M. (2004). Use of recombinant feline interferon and glucocorticoid in the treatment of feline infectious peritonitis. *J Feline Med Surg, 6*(2), 107-109.

Ishida, T., Washizu, T., Toriyabe, K., Motoyoshi, S., Tomoda, I., & Pedersen, N. C. (1989). Feline immunodeficiency virus infection in cats of Japan. *J Am Vet Med Assoc, 194*(2), 221-225.

Ishiwata, K., Minagawa, T., & Kajimoto, T. (1998). Clinical effects of the recombinant feline interferon-omega on experimental parvovirus infection in beagle dogs. *J Vet Med Sci, 60*(8), 911-917.

Jackson, M. L., Haines, D. M., Meric, S. M., & Misra, V. (1993). Feline leukemia virus detection by immunohistochemistry and polymerase chain reaction in formalin-fixed, paraffin-embedded tumor tissue from cats with lymphosarcoma. *Can J Vet Res, 57*(4), 269-276.

Jackson, M. L., Haines, D. M., Taylor, S. M., & Misra, V. (1996). Feline leukemia virus detection by ELISA and PCR in peripheral blood from 68 cats with high, moderate, or low suspicion of having FeLV-related disease. *J Vet Diagn Invest, 8*(1), 25-30.

Jahoor, F., Gazzard, B., Phillips, G., Sharpstone, D., Delrosario, M., Frazer, M. E., et al. (1999). The acute-phase protein response to human immunodeficiency virus infection in human subjects. *Am J Physiol, 276*(6 Pt 1), E1092-1098.

Jarrett, O. (1999). Strategies of retrovirus survival in the cat. *Vet Microbiol, 69*(1-2), 99-107.

Jarrett, O., Golder, M. C., & Stewart, M. F. (1982). Detection of transient and persistent feline leukaemia virus infections. *Vet Rec, 110*(10), 225-228.

Jarrett, O., Laird, H. M., & Hay, D. (1973). Determinants of the host range of feline leukaemia viruses. *J Gen Virol, 20*(2), 169-175.

Jarrett, O., Laird, H. M., Hay, D., & Crighton, G. W. (1968). Replication of cat leukemia virus in cell cultures. *Nature, 219*(5153), 521-522.

Jarrett, O., Pacitti, A. M., Hosie, M. J., & Reid, G. (1991). Comparison of diagnostic methods for feline leukemia virus and feline immunodeficiency virus. *J Am Vet Med Assoc, 199*(10), 1362-1364.

Jarrett, O., Russell, P. H., & Stewart, M. F. (1977). Protection of kittens from feline leukaemia virus infection by maternally-derived antibody. *Vet Rec, 101*(15), 304-305.

Jarrett, W. F., Crawford, E. M., Martin, W. B., & Davie, F. (1964). A VIRUS-LIKE PARTICLE ASSOCIATED WITH LEUKEMIA (LYMPHOSARCOMA). *Nature, 202*, 567-569.

Jirjis, F. F., Davis, T., Lane, J., Carritt, K., Sweeney, D., Williams, J., et al. (2010). Protection against feline leukemia virus challenge for at least 2 years after vaccination with an inactivated feline leukemia virus vaccine. *Vet Ther, 11*(2), E1-6.

Johnston, J. B., Olson, M. E., Rud, E. W., & Power, C. (2001). Xenoinfection of nonhuman primates by feline immunodeficiency virus. *Curr Biol, 11*(14), 1109-1113.

Johnston, J. B., Silva, C., & Power, C. (2002). Envelope gene-mediated neurovirulence in feline immunodeficiency virus infection: induction of matrix metalloproteinases and neuronal injury. *J Virol, 76*(6), 2622-2633.

Jordan, H. L., Howard, J., Barr, M. C., Kennedy-Stoskopf, S., Levy, J. K., & Tompkins, W. A. (1998). Feline immunodeficiency virus is shed in semen from experimentally and naturally infected cats. *AIDS Res Hum Retroviruses, 14*(12), 1087-1092.

Jordan, H. L., Howard, J., Sellon, R. K., Wildt, D. E., Tompkins, W. A., & Kennedy-Stoskopf, S. (1996). Transmission of feline immunodeficiency virus in domestic cats via artificial insemination. *J Virol, 70*(11), 8224-8228.

Joshi, A., Vahlenkamp, T. W., Garg, H., Tompkins, W. A., & Tompkins, M. B. (2004). Preferential replication of FIV in activated CD4(+)CD25(+)T cells independent of cellular proliferation. *Virology, 321*(2), 307-322.

Kajikawa, T., Furuta, A., Onishi, T., Tajima, T., & Sugii, S. (1999). Changes in concentrations of serum amyloid A protein, alpha 1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. *Vet Immunol Immunopathol, 68*(1), 91-98.

Kann, R., Seddon, J., Kyaw-Tanner, M., & Meers, J. (2007). Co-infection with different subtypes of feline immunodeficiency virus can complicate subtype assignment by phylogenetic analysis. *Arch Virol, 152*(6), 1187-1193.

Kann, R., Seddon, J., Kyaw-Tanner, M., Schoeman, J. P., Schoeman, T., & Meers, J. (2006). Phylogenetic analysis to define feline immunodeficiency virus subtypes in 31 domestic cats in South Africa. *J S Afr Vet Assoc, 77*(3), 108-113.

Kann, R. K., Kyaw-Tanner, M. T., Seddon, J. M., Lehrbach, P. R., Zwijnenberg, R. J., & Meers, J. (2006). Molecular subtyping of feline immunodeficiency virus from domestic cats in Australia. *Aust Vet J, 84*(4), 112-116.

Kennedy, M. A. (2010). A brief review of the basics of immunology: the innate and adaptive response. *Vet Clin North Am Small Anim Pract, 40*(3), 369-379.

Kipar, A., Boretti, F. S., Meli, M. M., Failing, K., Reinacher, M., & Lutz, H. (2004). Reduced constitutive cytokine transcription in isolated monocytes of clinically healthy cats, infected with an FIV strain of low pathogenicity. *Vet Immunol Immunopathol, 98*(3-4), 215-221.

Kirpensteijn, J. (2006). Feline injection site-associated sarcoma: Is it a reason to critically evaluate our vaccination policies? *Vet Microbiol, 117*(1), 59-65.

Kohmoto, M., Miyazawa, T., Sato, E., Uetsuka, K., Nishimura, Y., Ikeda, Y., et al. (1998). Cats are protected against feline immunodeficiency virus infection following vaccination with a homologous AP-1 binding site-deleted mutant. *Arch Virol, 143*(9), 1839-1845.

Kohn, B., Weingart, C., Eckmann, V., Ottenjann, M., & Leibold, W. (2006). Primary immune-mediated hemolytic anemia in 19 cats: diagnosis, therapy, and outcome (1998-2004). *J Vet Intern Med, 20*(1), 159-166.

Kolenda-Roberts, H. M., Kuhnt, L. A., Jennings, R. N., Mergia, A., Gengozian, N., & Johnson, C. M. (2007). Immunopathogenesis of feline immunodeficiency virus infection in the fetal and neonatal cat. *Front Biosci, 12*, 3668-3682.

Korman, R. M., Ceron, J. J., Knowles, T. G., Barker, E. N., Eckersall, P. D., & Tasker, S. (2012). Acute phase response to Mycoplasma haemofelis and 'Candidatus Mycoplasma haemominutum' infection in FIV-infected and non-FIV-infected cats. *Vet J, 193*(2), 433-438.

Kraase, M., Sloan, R., Klein, D., Logan, N., McMonagle, L., Biek, R., et al. (2010). Feline immunodeficiency virus env gene evolution in experimentally infected cats. *Vet Immunol Immunopathol, 134*(1-2), 96-106.

Kraft, W., & Kuffer, M. (1995). [Treatment of severe neutropenias in dogs and cats with filgrastim]. *Tierarztl Prax, 23*(6), 609-613.

Kubes, P., Heit, B., van Marle, G., Johnston, J. B., Knight, D., Khan, A., et al. (2003). In vivo impairment of neutrophil recruitment during lentivirus infection. *J Immunol, 171*(9), 4801-4808.

Kusuhara, H., Hohdatsu, T., Okumura, M., Sato, K., Suzuki, Y., Motokawa, K., et al. (2005). Dual-subtype vaccine (Fel-O-Vax FIV) protects cats against contact challenge with heterologous subtype B FIV infected cats. *Vet Microbiol, 108*(3-4), 155-165.

Kusuhara, H., Hohdatsu, T., Seta, T., Nemoto, K., Motokawa, K., Gemma, T., et al. (2007). Serological differentiation of FIV-infected cats from dual-subtype feline immunodeficiency virus vaccine (Fel-O-Vax FIV) inoculated cats. *Vet Microbiol, 120*(3-4), 217-225.

Lafrado, L. J., Lewis, M. G., Mathes, L. E., & Olsen, R. G. (1987). Suppression of in vitro neutrophil function by feline leukaemia virus (FeLV) and purified FeLV-p15E. *J Gen Virol, 68 (Pt 2)*, 507-513.

Langhammer, S., Hubner, J., Kurth, R., & Denner, J. (2006). Antibodies neutralizing feline leukaemia virus (FeLV) in cats immunized with the transmembrane envelope protein p15E. *Immunology, 117*(2), 229-237.

Lappin, M. R., Marks, A., Greene, C. E., Collins, J. K., Carman, J., Reif, J. S., et al. (1992). Serologic prevalence of selected infectious diseases in cats with uveitis. *J Am Vet Med Assoc, 201*(7), 1005-1009.

Lauring, A. S., Anderson, M. M., & Overbaugh, J. (2001). Specificity in receptor usage by T-cell-tropic feline leukemia viruses: implications for the in vivo tropism of immunodeficiency-inducing variants. *J Virol, 75*(19), 8888-8898.

Lauring, A. S., Cheng, H. H., Eiden, M. V., & Overbaugh, J. (2002). Genetic and biochemical analyses of receptor and cofactor determinants for T-cell-tropic feline leukemia virus infection. *J Virol, 76*(16), 8069-8078.

Lawrence, C. E., Callanan, J. J., Willett, B. J., & Jarrett, O. (1995). Cytokine production by cats infected with feline immunodeficiency virus: a longitudinal study. *Immunology, 85*(4), 568-574.

Leal, R. O., Gil, S., Brito, M. T., McGahie, D., Niza, M. M., & Tavares, L. (2013). The use of oral recombinant feline interferon omega in two cats with type II diabetes mellitus and concurrent feline chronic gingivostomatitis syndrome. *Ir Vet J, 66*(1), 19.

Leal, R. O., Gil, S., Sepulveda, N., McGahie, D., Duarte, A., MMRE, N., et al. (2012). *Acute Phase Proteins: Potential Predictors of an Immune-modulation in natural retroviral infected cats receiving recombinant Interferon-omega therapy.* Paper presented at the Proceedings of the annual conference of the European College of Veterinary Internal Medicine - Companion Animals, Maastricht, Netherlands.

Leal, R. O., Gil, S., Sepulveda, N., McGahie, D., Duarte, A., Niza, M. M., et al. (2014). Monitoring acute phase proteins in retrovirus infected cats undergoing feline interferon-omega therapy. *J Small Anim Pract, 55*(1), 39-45.

Lee, I. T., Levy, J. K., Gorman, S. P., Crawford, P. C., & Slater, M. R. (2002). Prevalence of feline leukemia virus infection and serum antibodies against feline immunodeficiency virus in unowned free-roaming cats. *J Am Vet Med Assoc, 220*(5), 620-622.

Lehman, T. L., O'Halloran, K. P., Fallon, S. A., Habermann, L. M., Campbell, J. A., Nordone, S., et al. (2009). Altered bone marrow dendritic cell cytokine production to toll-like receptor and CD40 ligation during chronic feline immunodeficiency virus infection. *Immunology, 126*(3), 405-412.

Lehmann, R., von Beust, B., Niederer, E., Condrau, M. A., Fierz, W., Aubert, A., et al. (1992). Immunization-induced decrease of the CD4+:CD8+ ratio in cats experimentally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol, 35*(1-2), 199-214.

Lerner, D. L., Grant, C. K., de Parseval, A., & Elder, J. H. (1998). FIV infection of IL-2-dependent and -independent feline lymphocyte lines: host cells range distinctions and specific cytokine upregulation. *Vet Immunol Immunopathol, 65*(2-4), 277-297.

Leutenegger, C. M., Klein, D., Hofmann-Lehmann, R., Mislin, C., Hummel, U., Böni, J., Lutz, H. (1999). Rapid feline immunodeficiency virus provirus quantitation by polymerase chain reaction using the TaqMan fluorogenic real-time detection system. *Journal of Virological Methods*, *78*(1-2), 105–116.

Levy, J., Crawford, C., Hartmann, K., Hofmann-Lehmann, R., Little, S., Sundahl, E., et al. (2008). 2008 American Association of Feline Practitioners' feline retrovirus management guidelines. *J Feline Med Surg, 10*(3), 300-316.

Levy, J. K., Ritchey, J. W., Rottman, J. B., Davidson, M. G., Liang, Y. H., Jordan, H. L., et al. (1998). Elevated interleukin-10-to-interleukin-12 ratio in feline immunodeficiency virus-infected cats predicts loss of type 1 immunity to Toxoplasma gondii. *J Infect Dis, 178*(2), 503-511.

Levy, J. K., Scott, H. M., Lachtara, J. L., & Crawford, P. C. (2006). Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in North America and risk factors for seropositivity. *J Am Vet Med Assoc, 228*(3), 371-376.

Liang, Y., Hudson, L. C., Levy, J. K., Ritchey, J. W., Tompkins, W. A., & Tompkins, M. B. (2000). T cells overexpressing interferon-gamma and interleukin-10 are found in both the thymus and secondary lymphoid tissues of feline immunodeficiency virus-infected cats. *J Infect Dis, 181*(2), 564-575.

Linenberger, M. L., & Abkowitz, J. L. (1995). Haematological disorders associated with feline retrovirus infections. *Baillieres Clin Haematol, 8*(1), 73-112.

Linenberger, M. L., & Deng, T. (1999). The effects of feline retroviruses on cytokine expression. *Vet Immunol Immunopathol, 72*(3-4), 343-368.

Linenberger, M. L., Shelton, G. H., Persik, M. T., & Abkowitz, J. L. (1991). Hematopoiesis in asymptomatic cats infected with feline immunodeficiency virus. *Blood, 78*(8), 1963-1968.

Little, S., Sears, W., Lachtara, J., & Bienzle, D. (2009). Seroprevalence of feline leukemia virus and feline immunodeficiency virus infection among cats in Canada. *Can Vet J, 50*(6), 644-648.

Little, S. E. (2005). Feline immunodeficiency virus testing in stray, feral, and client-owned cats of Ottawa. *Can Vet J, 46*(10), 898-901.

Litzlbauer, P., Weber, K., & Mueller, R. S. (2014). Oral and subcutaneous therapy of canine atopic dermatitis with recombinant feline interferon omega. *Cytokine, 66*(1), 54-59.

Lockridge, K. M., Chien, M., Dean, G. A., Stefano Cole, K., Montelaro, R. C., Luciw, P. A., et al. (2000). Protective immunity against feline immunodeficiency virus induced by inoculation with vif-deleted proviral DNA. *Virology, 273*(1), 67-79.

Locksley, R. M., & Scott, P. (1991). Helper T-cell subsets in mouse leishmaniasis: induction, expansion and effector function. *Immunol Today, 12*(3), A58-61.

Lommer, M. J., & Verstraete, F. J. (2003). Concurrent oral shedding of feline calicivirus and feline herpesvirus 1 in cats with chronic gingivostomatitis. *Oral Microbiol Immunol, 18*(2), 131-134.

Lopes, A. P., Cardoso, L., & Rodrigues, M. (2008). Serological survey of Toxoplasma gondii infection in domestic cats from northeastern Portugal. *Vet Parasitol, 155*(3-4), 184-189.

Louwerens, M., London, C. A., Pedersen, N. C., & Lyons, L. A. (2005). Feline lymphoma in the post-feline leukemia virus era. *J Vet Intern Med, 19*(3), 329-335.

Lubkin, S. R., Romatowski, J., Zhu, M., Kulesa, P. M., & White, K. A. (1996). Evaluation of feline leukemia virus control measures. *J Theor Biol, 178*(1), 53-60.

Lutz, H., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline leukaemia. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 565-574.

Lutz, H., Castelli, I., Ehrensperger, F., Pospischil, A., Rosskopf, M., Siegl, G., et al. (1995). Panleukopenia-like syndrome of FeLV caused by co-infection with FeLV and feline panleukopenia virus. *Vet Immunol Immunopathol, 46*(1-2), 21-33.

Lutz TA, McGahie D, & Albouy M. (2011). *Impact of treatment with recombinant feline interferon omega on resolution of anorexia of unknown origin in cats.* Paper presented at the European College of Veterinary Internal Medicine Congress, Sevilla.

Macieira, D. B., de Menezes Rde, C., Damico, C. B., Almosny, N. R., McLane, H. L., Daggy, J. K., et al. (2008). Prevalence and risk factors for hemoplasmas in domestic cats naturally infected with feline immunodeficiency virus and/or feline leukemia virus in Rio de Janeiro--Brazil. *J Feline Med Surg, 10*(2), 120-129.

MacLachland, N., & Dubovi, E. (2011). Chapter 14: Retroviridae. In Elsevier (Ed.), *Fenner's Veterinary Virology* (Fourth Edition ed.). San Diego: Elsevier.

Major, A., Cattori, V., Boenzli, E., Riond, B., Ossent, P., Meli, M. L., et al. (2010). Exposure of cats to low doses of FeLV: seroconversion as the sole parameter of infection. *Vet Res, 41*(2), 17.

Mancianti, F., Giannelli, C., Bendinelli, M., & Poli, A. (1992). Mycological findings in feline immunodeficiency virus-infected cats. *J Med Vet Mycol, 30*(3), 257-259.

Marshall, R. D., Rand, J. S., & Morton, J. M. (2009). Treatment of newly diagnosed diabetic cats with glargine insulin improves glycaemic control and results in higher probability of remission than protamine zinc and lente insulins. *J Feline Med Surg, 11*(8), 683-691.

Martínez-Subiela S, T. V. F., Parra Muñoz MD, Cerón JJM;. (2001). Proteínas de fase aguda: conceptos básicos y principales aplicaciones clínicas en medicina veterinaria/ Acute phase proteins: general concepts and main clinical applications in veterinary medicine. *Anales de Veterinaria de Murcia, 17*, 97-113.

Martins, A. N., Medeiros, S. O., Simonetti, J. P., Schatzmayr, H. G., Tanuri, A., & Brindeiro, R. M. (2008). Phylogenetic and genetic analysis of feline immunodeficiency virus gag, pol, and env genes from domestic cats undergoing nucleoside reverse transcriptase inhibitor treatment or treatment-naive cats in Rio de Janeiro, Brazil. *J Virol, 82*(16), 7863-7874.

Massi, C., Lombardi, S., Indino, E., Matteucci, D., La Rosa, C., Esposito, F., et al. (1997). Most potential linear B cell epitopes of Env glycoproteins of feline immunodeficiency virus are immunogenically silent in infected cats. *AIDS Res Hum Retroviruses, 13*(13), 1121-1129.

Matsumoto, H., Takemura, N., Sako, T., Koyama, H., Motoyoshi, S., & Inada, Y. (1997). Serum concentration of circulating immune complexes in cats infected with feline immunodeficiency virus detected by immune adherence hemagglutination method. *J Vet Med Sci, 59*(5), 395-396.

Matsumoto, Y., Momoi, Y., Watari, T., Goitsuka, R., Tsujimoto, H., & Hasegawa, A. (1992). Detection of enhancer repeats in the long terminal repeats of feline leukemia viruses from cats with spontaneous neoplastic and nonneoplastic diseases. *Virology, 189*(2), 745-749.

Matteucci, D., Baldinotti, F., Mazzetti, P., Pistello, M., Bandecchi, P., Ghilarducci, R., et al. (1993). Detection of feline immunodeficiency virus in saliva and plasma by cultivation and polymerase chain reaction. *J Clin Microbiol, 31*(3), 494-501.

Mazzetti, P., Giannecchini, S., Del Mauro, D., Matteucci, D., Portincasa, P., Merico, A., et al. (1999). AIDS vaccination studies using an ex vivo feline immunodeficiency virus model: detailed analysis of the humoral immune response to a protective vaccine. *J Virol, 73*(1), 1-10.

McCaw, D. L., Boon, G. D., Jergens, A. E., Kern, M. R., Bowles, M. H., & Johnson, J. C. (2001). Immunomodulation therapy for feline leukemia virus infection. *J Am Anim Hosp Assoc, 37*(4), 356-363.

McCrackin Stevenson, M. A., & McBroom, D. G. (2001). In vitro characterization of FIV-pPPR, a pathogenic molecular clone of feline immunodeficiency virus, and two drug-resistant pol gene mutants. *Am J Vet Res, 62*(4), 588-594.

McDonnel, S. J., Sparger, E. E., Luciw, P. A., & Murphy, B. G. (2012). Transcriptional regulation of latent feline immunodeficiency virus in peripheral CD4+ T-lymphocytes. *Viruses, 4*(5), 878-888.

McDonnel, S. J., Sparger, E. E., & Murphy, B. G. (2013). Feline immunodeficiency virus latency. *Retrovirology, 10*, 69.

Medeiros Sde, O., Martins, A. N., Dias, C. G., Tanuri, A., & Brindeiro Rde, M. (2012). Natural transmission of feline immunodeficiency virus from infected queen to kitten. *Virol J, 9*, 99.

Meeker, R. B. (2007). Feline immunodeficiency virus neuropathogenesis: from cats to calcium. *J Neuroimmune Pharmacol, 2*(2), 154-170.

Meers, J., del Fierro, G. M., Cope, R. B., Park, H. S., Greene, W. K., & Robinson, W. F. (1993). Feline immunodeficiency virus infection: plasma, but not peripheral blood

mononuclear cell virus titer is influenced by zidovudine and cyclosporine. *Arch Virol, 132*(1-2), 67-81.

Meli, M., Kipar, A., Muller, C., Jenal, K., Gonczi, E., Borel, N., et al. (2004). High viral loads despite absence of clinical and pathological findings in cats experimentally infected with feline coronavirus (FCoV) type I and in naturally FCoV-infected cats. *J Feline Med Surg, 6*(2), 69-81.

Mendoza, R., Anderson, M. M., & Overbaugh, J. (2006). A putative thiamine transport protein is a receptor for feline leukemia virus subgroup A. *J Virol, 80*(7), 3378-3385.

Meuer, S. C., Dumann, H., Meyer zum Buschenfelde, K. H., & Kohler, H. (1989). Low-dose interleukin-2 induces systemic immune responses against HBsAg in immunodeficient non-responders to hepatitis B vaccination. *Lancet, 1*(8628), 15-18.

Mexas, A. M., Fogle, J. E., Tompkins, W. A., & Tompkins, M. B. (2008). CD4+CD25+ regulatory T cells are infected and activated during acute FIV infection. *Vet Immunol Immunopathol, 126*(3-4), 263-272.

Mihaljevic, S. (2003). First clinical experiences with omega-interferon in the treatment of chronic gingivitis-stomatitis-oropharyngitis of cats. *Der Prakt Tierarzt, 84*, 350-361.

Miro, G., Domenech, A., Escolar, E., Collado, V. M., Tejerizo, G., De Las Heras, A., et al. (2007). Plasma electrophoretogram in feline immunodeficiency virus (FIV) and/or feline leukaemia virus (FeLV) infections. *J Vet Med A Physiol Pathol Clin Med, 54*(4), 203-209.

Mitchell, T. W., Rojko, J. L., Hartke, J. R., Mihajlov, A. R., Kasameyer, G. A., Gasper, P. W., et al. (1997). FeLV envelope protein (gp70) variable region 5 causes alterations in calcium homeostasis and toxicity of neurons. *J Acquir Immune Defic Syndr Hum Retrovirol, 14*(4), 307-320.

Mizukoshi, F., Baba, K., Goto, Y., Setoguchi, A., Fujino, Y., Ohno, K., et al. (2009). Antiviral activity of membrane fusion inhibitors that target gp40 of the feline immunodeficiency virus envelope protein. *Vet Microbiol, 136*(1-2), 155-159.

Mizuno, T., Goto, Y., Baba, K., Masuda, K., Ohno, K., & Tsujimoto, H. (2001). TNF-alpha-induced cell death in feline immunodeficiency virus-infected cells is mediated by the caspase cascade. *Virology, 287*(2), 446-455.

Mochizuki, M., Nakatani, H., & Yoshida, M. (1994). Inhibitory effects of recombinant feline interferon on the replication of feline enteropathogenic viruses in vitro. *Vet Microbiol, 39*(1-2), 145-152.

Moench, T. R., Whaley, K. J., Mandrell, T. D., Bishop, B. D., Witt, C. J., & Cone, R. A. (1993). The cat/feline immunodeficiency virus model for transmucosal transmission of AIDS: nonoxynol-9 contraceptive jelly blocks transmission by an infected cell inoculum. *AIDS, 7*(6), 797-802.

Morgan, R. A., Dornsife, R. E., Anderson, W. F., & Hoover, E. A. (1993). In vitro infection of human bone marrow by feline leukemia viruses. *Virology, 193*(1), 439-442.

Muirden, A. (2002). Prevalence of feline leukaemia virus and antibodies to feline immunodeficiency virus and feline coronavirus in stray cats sent to an RSPCA hospital. *Vet Rec, 150*(20), 621-625.

Muller, D. (2002). Interferon therapy in dogs and cats. *Kleintiermedizin 8*, 334-337.

Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M., et al. (1994). Functional role of type I and type II interferons in antiviral defense. *Science, 264*(5167), 1918-1921.

Murphy, B., Hillman, C., Mok, M., & Vapniarsky, N. (2012). Lentiviral latency in peripheral CD4+ T cells isolated from feline immunodeficiency virus-infected cats during the asymptomatic phase is not associated with hypermethylation of the proviral promoter. *Virus Res, 169*(1), 117-126.

Murphy, B., Vapniarsky, N., Hillman, C., Castillo, D., McDonnel, S., Moore, P., et al. (2012). FIV establishes a latent infection in feline peripheral blood CD4+ T lymphocytes in vivo during the asymptomatic phase of infection. *Retrovirology, 9*, 12.

Murphy, F., Gibbs, E., Horzineck, M., & Studdert, M. (1999a). Chapter 3. Viral Replication *Veterinary Virology*. San Diego: Elsevier.

Murphy, F., Gibbs, E., Horzineck, M., & Studdert, M. (1999b). Chapter 23. Retroviridae *Veterinary Virology* (Third ed.). San Diego: Elsevier.

Murray, J. K., Roberts, M. A., Skillings, E., Morrow, L. D., & Gruffydd-Jones, T. J. (2009). Risk factors for feline immunodeficiency virus antibody test status in Cats Protection adoption centres (2004). *J Feline Med Surg, 11*(6), 467-473.

Nakamura, N., Sudo, T., Matsuda, S., & Yanai, A. (1992). Molecular cloning of feline interferon cDNA by direct expression. *Biosci Biotechnol Biochem, 56*(2), 211-214.

Nakamura, Y., Ura, A., Hirata, M., Sakuma, M., Sakata, Y., Nishigaki, K., et al. (2010). An updated nation-wide epidemiological survey of feline immunodeficiency virus (FIV) infection in Japan. *J Vet Med Sci, 72*(8), 1051-1056.

Nick, S., Klaws, J., Friebel, K., Birr, C., Hunsmann, G., & Bayer, H. (1990). Virus neutralizing and enhancing epitopes characterized by synthetic oligopeptides derived from the feline leukaemia virus glycoprotein sequence. *J Gen Virol, 71 (Pt 1)*, 77-83.

Nishigaki, K., Okuda, M., Endo, Y., Watari, T., Tsujimoto, H., & Hasegawa, A. (1997). Structure and function of the long terminal repeats of feline leukemia viruses derived from naturally occurring acute myeloid leukemias in cats. *J Virol, 71*(12), 9823-9827.

Nishimura, Y., Goto, Y., Pang, H., Endo, Y., Mizuno, T., Momoi, Y., et al. (1998). Genetic heterogeneity of env gene of feline immunodeficiency virus obtained from multiple districts in Japan. *Virus Res, 57*(1), 101-112.

Nishimura, Y., Goto, Y., Yoneda, K., Endo, Y., Mizuno, T., Hamachi, M., et al. (1999). Interspecies transmission of feline immunodeficiency virus from the domestic cat to the Tsushima cat (Felis bengalensis euptilura) in the wild. *J Virol, 73*(9), 7916-7921.

Nishimura, Y., Shimojima, M., Sato, E., Izumiya, Y., Tohya, Y., Mikami, T., et al. (2004). Downmodulation of CD3epsilon expression in CD8alpha+beta- T cells of feline immunodeficiency virus-infected cats. *J Gen Virol, 85*(Pt 9), 2585-2589.

Norris, J. M., Bell, E. T., Hales, L., Toribio, J. A., White, J. D., Wigney, D. I., et al. (2007). Prevalence of feline immunodeficiency virus infection in domesticated and feral cats in eastern Australia. *J Feline Med Surg, 9*(4), 300-308.

Nowotny, N., Uthman, A., Haas, O. A., Borkhardt, A., Lechner, K., Egberink, H. F., et al. (1995). Is it possible to catch leukemia from a cat? *Lancet, 346*(8969), 252-253.

O'Neil, L. L., Burkhard, M. J., Diehl, L. J., & Hoover, E. A. (1995). Vertical transmission of feline immunodeficiency virus. *AIDS Res Hum Retroviruses, 11*(1), 171-182.

O'Neil, L. L., Burkhard, M. J., & Hoover, E. A. (1996). Frequent perinatal transmission of feline immunodeficiency virus by chronically infected cats. *J Virol, 70*(5), 2894-2901.

Ogilvie, G. K. (1995). Hematopoietic growth factors: frontiers for cure. *Vet Clin North Am Small Anim Pract, 25*(6), 1441-1456.

Ohno, K., Nakano, T., Matsumoto, Y., Watari, T., Goitsuka, R., Nakayama, H., et al. (1993). Apoptosis induced by tumor necrosis factor in cells chronically infected with feline immunodeficiency virus. *J Virol, 67*(5), 2429-2433.

Ohno, K., Okamoto, Y., Miyazawa, T., Mikami, T., Watari, T., Goitsuka, R., et al. (1994). Induction of apoptosis in a T lymphoblastoid cell line infected with feline immunodeficiency virus. *Arch Virol, 135*(1-2), 153-158.

Ohno, K., Watari, T., Goitsuka, R., Tsujimoto, H., & Hasegawa, A. (1992). Altered surface antigen expression on peripheral blood mononuclear cells in cats infected with feline immunodeficiency virus. *J Vet Med Sci, 54*(3), 517-522.

Orandle, M. S., Crawford, P. C., Levy, J. K., Udoji, R., Papadi, G. P., Ciccarone, T., et al. (2000). CD8+ thymic lymphocytes express reduced levels of CD8beta and increased interferon gamma in cats perinatally infected with the JSY3 molecular clone of feline immunodeficiency virus. *AIDS Res Hum Retroviruses, 16*(15), 1559-1571.

Osborne, J., Hunter, S. J., & Devaney, E. (1996). Anti-interleukin-4 modulation of the Th2 polarized response to the parasitic nematode Brugia pahangi. *Infect Immun, 64*(9), 3461-3466.

Osterhaus, A., Weijer, K., Uytdehaag, F., Jarrett, O., Sundquist, B., & Morein, B. (1985). Induction of protective immune response in cats by vaccination with feline leukemia virus iscom. *J Immunol, 135*(1), 591-596.

Pacitti, A. M., Jarrett, O., & Hay, D. (1986). Transmission of feline leukaemia virus in the milk of a non-viraemic cat. *Vet Rec, 118*(14), 381-384.

Paltrinieri, S. (2008). The feline acute phase reaction. *Vet J, 177*(1), 26-35.

Paltrinieri, S., Crippa, A., Comerio, T., Angioletti, A., & Roccabianca, P. (2007). Evaluation of inflammation and immunity in cats with spontaneous parvovirus infection: consequences of recombinant feline interferon-omega administration. *Vet Immunol Immunopathol, 118*(1-2), 68-74.

Paltrinieri, S., Giordano, A., Ceciliani, F., & Sironi, G. (2004). Tissue distribution of a feline AGP related protein (fAGPrP) in cats with feline infectious peritonitis (FIP). *J Feline Med Surg, 6*(2), 99-105.

Paltrinieri, S., Giordano, A., Tranquillo, V., & Guazzetti, S. (2007). Critical assessment of the diagnostic value of feline alpha1-acid glycoprotein for feline infectious peritonitis using the likelihood ratios approach. *J Vet Diagn Invest, 19*(3), 266-272.

Paltrinieri, S., Metzger, C., Battilani, M., Pocacqua, V., Gelain, M. E., & Giordano, A. (2007). Serum alpha1-acid glycoprotein (AGP) concentration in non-symptomatic cats with feline coronavirus (FCoV) infection. *J Feline Med Surg, 9*(4), 271-277.

Pancino, G., Castelot, S., & Sonigo, P. (1995). Differences in feline immunodeficiency virus host cell range correlate with envelope fusogenic properties. *Virology, 206*(2), 796-806.

Pancino, G., Fossati, I., Chappey, C., Castelot, S., Hurtrel, B., Moraillon, A., et al. (1993). Structure and variations of feline immunodeficiency virus envelope glycoproteins. *Virology, 192*(2), 659-662.

Papasouliotis, K., Gruffydd-Jones, T. J., Werrett, G., Brown, P. J., Hopper, C. D., Stokes, C. R., et al. (1998). Assessment of intestinal function in cats with chronic diarrhea after infection with feline immunodeficiency virus. *Am J Vet Res, 59*(5), 569-574.

Pardi, D., Hoover, E. A., Quackenbush, S. L., Mullins, J. I., & Callahan, G. N. (1991). Selective impairment of humoral immunity in feline leukemia virus-induced immunodeficiency. *Vet Immunol Immunopathol, 28*(3-4), 183-200.

Park, H. S., Kyaw-Tanner, M., Thomas, J., & Robinson, W. F. (1995). Feline immunodeficiency virus replicates in salivary gland ductular epithelium during the initial phase of infection. *Vet Microbiol, 46*(1-3), 257-267.

Pecoraro, M. R., Tomonaga, K., Miyazawa, T., Kawaguchi, Y., Sugita, S., Tohya, Y., et al. (1996). Genetic diversity of Argentine isolates of feline immunodeficiency virus. *J Gen Virol, 77 (Pt 9)*, 2031-2035.

Pedersen, N. C. (1992). Inflammatory oral cavity diseases of the cat. *Vet Clin North Am Small Anim Pract, 22*(6), 1323-1345.

Pedersen, N. C. (1993). Immunogenicity and efficacy of a commercial feline leukemia virus vaccine. *J Vet Intern Med, 7*(1), 34-39.

Pedersen, N. C., Dean, G. A., Bernales, J., Sukura, A., & Higgins, J. (1998). Listeria monocytogenes and Serratia marcescens infections as models for Th1/Th2 immunity in laboratory cats. *Vet Immunol Immunopathol, 63*(1-2), 83-103.

Pedersen, N. C., Ho, E. W., Brown, M. L., & Yamamoto, J. K. (1987). Isolation of a T-lymphotropic virus from domestic cats with an immunodeficiency-like syndrome. *Science, 235*(4790), 790-793.

Pedersen, N. C., Leutenegger, C. M., Woo, J., & Higgins, J. (2001). Virulence differences between two field isolates of feline immunodeficiency virus (FIV-APetaluma and FIV-CPGammar) in young adult specific pathogen free cats. *Vet Immunol Immunopathol, 79*(1-2), 53-67.

Pedretti, E., Passeri, B., Amadori, M., Isola, P., Di Pede, P., Telera, A., et al. (2006). Low-dose interferon-alpha treatment for feline immunodeficiency virus infection. *Vet Immunol Immunopathol, 109*(3-4), 245-254.

Peri, E. V., Ponti, W., Dall'ara, P., Rocchi, M., Zecconi, A., & Bonizzi, L. (1994). Seroepidemiological and clinical survey of feline immunodeficiency virus infection in northern Italy. *Vet Immunol Immunopathol, 40*(4), 285-297.

Pestka, S., Krause, C. D., & Walter, M. R. (2004). Interferons, interferon-like cytokines, and their receptors. *Immunol Rev, 202*, 8-32.

Petersen, H. H., Nielsen, J. P., & Heegaard, P. M. (2004). Application of acute phase protein measurements in veterinary clinical chemistry. *Vet Res, 35*(2), 163-187.

Petty, C. S., Tompkins, M. B., & Tompkins, W. A. (2008). Transforming growth factor-beta/transforming growth factor-betaRII signaling may regulate CD4+CD25+ T-

regulatory cell homeostasis and suppressor function in feline AIDS lentivirus infection. *J Acquir Immune Defic Syndr, 47*(2), 148-160.

Pinches, M. D., Diesel, G., Helps, C. R., Tasker, S., Egan, K., & Gruffydd-Jones, T. J. (2007). An update on FIV and FeLV test performance using a Bayesian statistical approach. *Vet Clin Pathol, 36*(2), 141-147.

Pistello, M., Bonci, F., Isola, P., Mazzetti, P., Merico, A., Zaccaro, L., et al. (2005). Evaluation of feline immunodeficiency virus ORF-A mutants as candidate attenuated vaccine. *Virology, 332*(2), 676-690.

Pistello, M., Cammarota, G., Nicoletti, E., Matteucci, D., Curcio, M., Del Mauro, D., et al. (1997). Analysis of the genetic diversity and phylogenetic relationship of Italian isolates of feline immunodeficiency virus indicates a high prevalence and heterogeneity of subtype B. *J Gen Virol, 78 (Pt 9)*, 2247-2257.

Polas, P. J., Swenson, C. L., Sams, R., Cheney, C. M., Hayes, K. A., Tarr, M. J., et al. (1990). In vitro and in vivo evidence that the antiviral activity of 2',3'-dideoxycytidine is target cell dependent in a feline retrovirus animal model. *Antimicrob Agents Chemother, 34*(7), 1414-1421.

Poli, A., Abramo, F., Baldinotti, F., Pistello, M., Da Prato, L., & Bendinelli, M. (1994). Malignant lymphoma associated with experimentally induced feline immunodeficiency virus infection. *J Comp Pathol, 110*(4), 319-328.

Poli, A., Abramo, F., Taccini, E., Guidi, G., Barsotti, P., Bendinelli, M., et al. (1993). Renal involvement in feline immunodeficiency virus infection: a clinicopathological study. *Nephron, 64*(2), 282-288.

Poli, A., Falcone, M. L., Bigalli, L., Massi, C., Hofmann-Lehmann, R., Lombardi, S., et al. (1995). Circulating immune complexes and analysis of renal immune deposits in feline immunodeficiency virus-infected cats. *Clin Exp Immunol, 101*(2), 254-258.

Priosoeryanto, B. P., Tateyama, S., Yamaguchi, R., & Uchida, K. (1995). Antiproliferation and colony-forming inhibition activities of recombinant feline interferon (rFeIFN) on various cells in vitro. *Can J Vet Res, 59*(1), 67-69.

Prospero-Garcia, O., Herold, N., Phillips, T. R., Elder, J. H., Bloom, F. E., & Henriksen, S. J. (1994). Sleep patterns are disturbed in cats infected with feline immunodeficiency virus. *Proc Natl Acad Sci U S A, 91*(26), 12947-12951.

Pu, R., Coleman, J., Coisman, J., Sato, E., Tanabe, T., Arai, M., et al. (2005). Dual-subtype FIV vaccine (Fel-O-Vax FIV) protection against a heterologous subtype B FIV isolate. *J Feline Med Surg, 7*(1), 65-70.

Pu, R., Coleman, J., Omori, M., Arai, M., Hohdatsu, T., Huang, C., et al. (2001). Dual-subtype FIV vaccine protects cats against in vivo swarms of both homologous and heterologous subtype FIV isolates. *AIDS, 15*(10), 1225-1237.

Quackenbush, S. L., Donahue, P. R., Dean, G. A., Myles, M. H., Ackley, C. D., Cooper, M. D., et al. (1990). Lymphocyte subset alterations and viral determinants of immunodeficiency disease induction by the feline leukemia virus FeLV-FAIDS. *J Virol, 64*(11), 5465-5474.

Quigley, J. G., Burns, C. C., Anderson, M. M., Lynch, E. D., Sabo, K. M., Overbaugh, J., et al. (2000). Cloning of the cellular receptor for feline leukemia virus subgroup C (FeLV-C), a retrovirus that induces red cell aplasia. *Blood, 95*(3), 1093-1099.

Radford, A. D., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline calicivirus infection. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 556-564.

Rand, J. (2012). Feline diabetes mellitus. In C. Mooney & M. Peterson (Eds.), *BSAVA Manual of Canine and Feline Endocrinology* (Fourth ed., pp. 133-147). Gloucester: BSAVA.

Rand, J. S., & Marshall, R. D. (2005). Diabetes mellitus in cats. *Vet Clin North Am Small Anim Pract, 35*(1), 211-224.

Ravi, M., Wobeser, G. A., Taylor, S. M., & Jackson, M. L. (2010). Naturally acquired feline immunodeficiency virus (FIV) infection in cats from western Canada: Prevalence, disease associations, and survival analysis. *Can Vet J, 51*(3), 271-276.

Reche, A., Jr., Daniel, A. G., Lazaro Strauss, T. C., Taborda, C. P., Vieira Marques, S. A., Haipek, K., et al. (2010). Cutaneous mycoflora and CD4:CD8 ratio of cats infected with feline immunodeficiency virus. *J Feline Med Surg, 12*(4), 355-358.

Reggeti, F., Ackerley, C., & Bienzle, D. (2008). CD134 and CXCR4 expression corresponds to feline immunodeficiency virus infection of lymphocytes, macrophages and dendritic cells. *J Gen Virol, 89*(Pt 1), 277-287.

Reggeti, F., & Bienzle, D. (2004). Feline immunodeficiency virus subtypes A, B and C and intersubtype recombinants in Ontario, Canada. *J Gen Virol, 85*(Pt 7), 1843-1852.

Reubel, G. H., Dean, G. A., George, J. W., Barlough, J. E., & Pedersen, N. C. (1994). Effects of incidental infections and immune activation on disease progression in experimentally feline immunodeficiency virus-infected cats. *J Acquir Immune Defic Syndr, 7*(10), 1003-1015.

Reubel, G. H., George, J. W., Higgins, J., & Pedersen, N. C. (1994). Effect of chronic feline immunodeficiency virus infection on experimental feline calicivirus-induced disease. *Vet Microbiol, 39*(3-4), 335-351.

Rey, M. A., Prasad, R., & Tailor, C. S. (2008). The C domain in the surface envelope glycoprotein of subgroup C feline leukemia virus is a second receptor-binding domain. *Virology, 370*(2), 273-284.

Richards, J. R., Elston, T. H., Ford, R. B., Gaskell, R. M., Hartmann, K., Hurley, K. F., et al. (2006). The 2006 American Association of Feline Practitioners Feline Vaccine Advisory Panel report. *J Am Vet Med Assoc, 229*(9), 1405-1441.

Rideout, B. A., Moore, P. F., & Pedersen, N. C. (1992). Persistent upregulation of MHC class II antigen expression on T-lymphocytes from cats experimentally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol, 35*(1-2), 71-81.

Rigby, M. A., Hosie, M. J., Willett, B. J., Mackay, N., McDonald, M., Cannon, C., et al. (1997). Comparative efficiency of feline immunodeficiency virus infection by DNA inoculation. *AIDS Res Hum Retroviruses, 13*(5), 405-412.

Ritchey, J. W., Levy, J. K., Bliss, S. K., Tompkins, W. A., & Tompkins, M. B. (2001). Constitutive expression of types 1 and 2 cytokines by alveolar macrophages from feline immunodeficiency virus-infected cats. *Vet Immunol Immunopathol, 79*(1-2), 83-100.

Ritz, S., Egberink, H., & Hartmann, K. (2007). Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. *J Vet Intern Med, 21*(6), 1193-1197.

Robert-Tissot, C., Ruegger, V. L., Cattori, V., Meli, M. L., Riond, B., Gomes-Keller, M. A., et al. (2011). The innate antiviral immune system of the cat: molecular tools for the measurement of its state of activation. *Vet Immunol Immunopathol, 143*(3-4), 269-281.

Robinson, A., DeCann, K., Aitken, E., Gruffydd-Jones, T. J., Sparkes, A. H., Werret, G., et al. (1998). Comparison of a rapid immunomigration test and ELISA for FIV antibody and FeLV antigen testing in cats. *Vet Rec, 142*(18), 491-492.

Rogers, A. B., & Hoover, E. A. (1998). Maternal-fetal feline immunodeficiency virus transmission: timing and tissue tropisms. *J Infect Dis, 178*(4), 960-967.

Rogers, A. B., Mathiason, C. K., & Hoover, E. A. (2002). Immunohistochemical localization of feline immunodeficiency virus using native species antibodies. *Am J Pathol, 161*(4), 1143-1151.

Rogers, R., Merigan, T. C., Hardy, W. D., Jr., Old, L. J., & Kassel, R. (1972). Cat interferon inhibits feline leukaemia virus infection in cell culture. *Nat New Biol, 237*(78), 270-271.

Roitt, I. M., & Delves, P. J. (2001). 10.The production of effectors. In I. M. Roitt & P. J. Delves (Eds.), *Essential Immunology* (Tenth Edition ed., pp. 147-164). Massachussets: Blackwell Publishing.

Rojko, J. L., Hoover, E. A., Quackenbush, S. L., & Olsen, R. G. (1982). Reactivation of latent feline leukaemia virus infection. *Nature, 298*(5872), 385-388.

Romagnani, S., Del Prete, G., Manetti, R., Ravina, A., Annunziato, F., De Carli, M., et al. (1994). Role of TH1/TH2 cytokines in HIV infection. *Immunol Rev, 140*, 73-92.

Ronni, T., Melen, K., Malygin, A., & Julkunen, I. (1993). Control of IFN-inducible MxA gene expression in human cells. *J Immunol, 150*(5), 1715-1726.

Roomp, K., & Rand, J. (2009). Intensive blood glucose control is safe and effective in diabetic cats using home monitoring and treatment with glargine. *J Feline Med Surg, 11*(8), 668-682.

Rosati, S., Profiti, M., Lorenzetti, R., Bandecchi, P., Mannelli, A., Ortoffi, M., et al. (2004). Development of recombinant capsid antigen/transmembrane epitope fusion proteins for serological diagnosis of animal lentivirus infections. *J Virol Methods, 121*(1), 73-78.

Roy-Burman, P. (1995). Endogenous env elements: partners in generation of pathogenic feline leukemia viruses. *Virus Genes, 11*(2-3), 147-161.

Russell, P. H., & Jarrett, O. (1978). The occurrence of feline leukaemia virus neutralizing antibodies in cats. *Int J Cancer, 22*(3), 351-357.

Sadler, A. J., & Williams, B. R. (2008). Interferon-inducible antiviral effectors. *Nat Rev Immunol, 8*(7), 559-568.

Samuel, C. E. (2001). Antiviral actions of interferons. *Clin Microbiol Rev, 14*(4), 778-809, table of contents.

Sand, C., Englert, T., Egberink, H., Lutz, H., & Hartmann, K. (2010). Evaluation of a new in-clinic test system to detect feline immunodeficiency virus and feline leukemia virus infection. *Vet Clin Pathol, 39*(2), 210-214.

Sandy, J. R., Robinson, W. F., Bredhauer, B., Kyaw-Tanner, M., & Howlett, C. R. (2002). Productive infection of the bone marrow cells in feline immunodeficiency virus infected cats. *Arch Virol, 147*(5), 1053-1059.

Sarli, G., Della Salda, L., Zaccaro, L., Bendinelli, M., Piedimonte, G., & Marcato, P. S. (1998). Apoptotic fraction in lymphoid tissue of FIV-infected SPF cats. *Vet Immunol Immunopathol, 64*(1), 33-44.

Sarro, Y. S., Tounkara, A., Tangara, E., Guindo, O., White, H. L., Chamot, E., et al. (2010). Serum protein electrophoresis: any role in monitoring for antiretroviral therapy? *Afr Health Sci, 10*(2), 138-143.

Sasaki, K., Ma, Z., Khatlani, T. S., Okuda, M., Inokuma, H., & Onishi, T. (2003). Evaluation of feline serum amyloid A (SAA) as an inflammatory marker. *J Vet Med Sci, 65*(4), 545-548.

Sasaki, K., Ma, Z., Okazaki, K., Khatlani, T. S., Okuda, M., Kajikawa, T., et al. (2001). Characterization of monoclonal antibodies specific for feline serum amyloid (SAA) protein. *Hybridoma, 20*(2), 103-108.

Schattner, A., Wallach, D., Merlin, G., Hahn, T., Levin, S., & Revel, M. (1981). Assay of an interferon-induced enzyme in white blood cells as a diagnostic aid in viral diseases. *Lancet, 2*(8245), 497-500.

Schellekens, H., Geelen, G., Meritet, J. F., Maury, C., & Tovey, M. G. (2001). Oromucosal interferon therapy: relationship between antiviral activity and viral load. *J Interferon Cytokine Res, 21*(8), 575-581.

Scherk, M. A., Ford, R. B., Gaskell, R. M., Hartmann, K., Hurley, K. F., Lappin, M. R., et al. (2013). 2013 AAFP Feline Vaccination Advisory Panel Report. *J Feline Med Surg, 15*(9), 785-808.

Schiller, J. H., Horisberger, M. A., Bittner, G., Carlin, J. M., Storer, B., Byrne, G. I., et al. (1990). Effects of combinations of interferon-beta ser and interferon-gamma on interferon-inducible proteins and on the cell cycle. *J Biol Response Mod, 9*(4), 368-377.

Schindler, C., Levy, D. E., & Decker, T. (2007). JAK-STAT signaling: from interferons to cytokines. *J Biol Chem, 282*(28), 20059-20063.

Schols, D., Struyf, S., Van Damme, J., Este, J. A., Henson, G., & De Clercq, E. (1997). Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J Exp Med, 186*(8), 1383-1388.

Schrenzel, M. D., Higgins, R. J., Hinrichs, S. H., Smith, M. O., & Torten, M. (1990). Type C retroviral expression in spontaneous feline olfactory neuroblastomas. *Acta Neuropathol, 80*(5), 547-553.

Schultz, D. R., & Arnold, P. I. (1990). Properties of four acute phase proteins: C-reactive protein, serum amyloid a protein, α1-acid glycoprotein, and fibrinogen. [doi: 10.1016/0049-0172(90)90055-K]. *Seminars in Arthritis and Rheumatism, 20*(3), 129-147.

Scott, V. L., Shack, L. A., Eells, J. B., Ryan, P. L., Donaldson, J. R., & Coats, K. S. (2011). Immunomodulator expression in trophoblasts from the feline immunodeficiency virus (FIV)-infected cat. *Virol J, 8*, 336.

Sellon, R., & Hartmann, K. (2012a). Chapter 12: Feline Immunodeficiency Virus. In C. Greene (Ed.), *Infectious Diseases of the Dog and Cat* (Fourth ed., pp. 136-149). Missouri: Saunders Elsevier.

Sellon, R., & Hartmann, K. (2012b). Feline Immunodeficiency Virus Infection. In C. Greene (Ed.), *Infectious diseases of the dog and cat* (Fourth ed., pp. 136-149). Missouri: Saunders Elsevier.

Sellon, R. K., Jordan, H. L., Kennedy-Stoskopf, S., Tompkins, M. B., & Tompkins, W. A. (1994). Feline immunodeficiency virus can be experimentally transmitted via milk during acute maternal infection. *J Virol, 68*(5), 3380-3385.

Selting, K. A., Ogilvie, G. K., Lana, S. E., Fettman, M. J., Mitchener, K. L., Hansen, R. A., et al. (2000). Serum alhpa 1-acid glycoprotein concentrations in healthy and tumor-bearing cats. *J Vet Intern Med, 14*(5), 503-506.

Shalev, Z., Duffy, S. P., Adema, K. W., Prasad, R., Hussain, N., Willett, B. J., et al. (2009). Identification of a feline leukemia virus variant that can use THTR1, FLVCR1, and FLVCR2 for infection. *J Virol, 83*(13), 6706-6716.

Shaw, S. C., Kent, M. S., Gordon, I. K., Collins, C. J., Greasby, T. A., Beckett, L. A., et al. (2009). Temporal changes in characteristics of injection-site sarcomas in cats: 392 cases (1990-2006). *J Am Vet Med Assoc, 234*(3), 376-380.

Shelton, G. H., Grant, C. K., Cotter, S. M., Gardner, M. B., Hardy, W. D., Jr., & DiGiacomo, R. F. (1990). Feline immunodeficiency virus and feline leukemia virus infections and their relationships to lymphoid malignancies in cats: a retrospective study (1968-1988). *J Acquir Immune Defic Syndr, 3*(6), 623-630.

Shelton, G. H., & Linenberger, M. L. (1995). Hematologic abnormalities associated with retroviral infections in the cat. *Semin Vet Med Surg (Small Anim), 10*(4), 220-233.

Shelton, G. H., Linenberger, M. L., Persik, M. T., & Abkowitz, J. L. (1995). Prospective hematologic and clinicopathologic study of asymptomatic cats with naturally acquired feline immunodeficiency virus infection. *J Vet Intern Med, 9*(3), 133-140.

Shibagaki, Y., & Chow, S. A. (1997). Central core domain of retroviral integrase is responsible for target site selection. *J Biol Chem, 272*(13), 8361-8369.

Shibagaki, Y., Holmes, M. L., Appa, R. S., & Chow, S. A. (1997). Characterization of feline immunodeficiency virus integrase and analysis of functional domains. *Virology, 230*(1), 1-10.

Shimojima, M., Miyazawa, T., Ikeda, Y., McMonagle, E. L., Haining, H., Akashi, H., et al. (2004). Use of CD134 as a primary receptor by the feline immunodeficiency virus. *Science, 303*(5661), 1192-1195.

Shojima, T., Nakata, R., & Miyazawa, T. (2006). Host cell range of T-lymphotropic feline leukemia virus in vitro. *Biochem Biophys Res Commun, 345*(4), 1466-1470.

Siebeck, N., Hurley, D. J., Garcia, M., Greene, C. E., Kostlin, R. G., Moore, P. A., et al. (2006). Effects of human recombinant alpha-2b interferon and feline recombinant omega interferon on in vitro replication of feline herpesvirus-1. *Am J Vet Res, 67*(8), 1406-1411.

Sierra, P., Guillot, J., Jacob, H., Bussieras, S., & Chermette, R. (2000). Fungal flora on cutaneous and mucosal surfaces of cats infected with feline immunodeficiency virus or feline leukemia virus. *Am J Vet Res, 61*(2), 158-161.

Siren, J., Pirhonen, J., Julkunen, I., & Matikainen, S. (2005). IFN-alpha regulates TLR-dependent gene expression of IFN-alpha, IFN-beta, IL-28, and IL-29. *J Immunol, 174*(4), 1932-1937.

Smith, R. A., Remington, K. M., Preston, B. D., Schinazi, R. F., & North, T. W. (1998). A novel point mutation at position 156 of reverse transcriptase from feline

immunodeficiency virus confers resistance to the combination of (-)-beta-2',3'-dideoxy-3'-thiacytidine and 3'-azido-3'-deoxythymidine. *J Virol, 72*(3), 2335-2340.

Smyth, N. R., Bennett, M., Gaskell, R. M., McCracken, C. M., Hart, C. A., & Howe, J. L. (1994). Effect of 3'azido-2',3'-deoxythymidine (AZT) on experimental feline immunodeficiency virus infection in domestic cats. *Res Vet Sci, 57*(2), 220-224.

Sodora, D. L., Shpaer, E. G., Kitchell, B. E., Dow, S. W., Hoover, E. A., & Mullins, J. I. (1994). Identification of three feline immunodeficiency virus (FIV) env gene subtypes and comparison of the FIV and human immunodeficiency virus type 1 evolutionary patterns. *J Virol, 68*(4), 2230-2238.

Solano-Gallego, L., Hegarty, B., Espada, Y., Llull, J., & Breitschwerdt, E. (2006). Serological and molecular evidence of exposure to arthropod-borne organisms in cats from northeastern Spain. *Vet Microbiol, 118*(3-4), 274-277.

Sparger, E. E., Louie, H., Ziomeck, A. M., & Luciw, P. A. (1997). Infection of cats by injection with DNA of a feline immunodeficiency virus molecular clone. *Virology, 238*(1), 157-160.

Sprague, W. S., Robbiani, M., Avery, P. R., O'Halloran, K. P., & Hoover, E. A. (2008). Feline immunodeficiency virus dendritic cell infection and transfer. *J Gen Virol, 89*(Pt 3), 709-715.

Steel, D. M., & Whitehead, A. S. (1994). The major acute phase reactants: C-reactive protein, serum amyloid P component and serum amyloid A protein. *Immunol Today, 15*(2), 81-88.

Steigerwald, E. S., Sarter, M., March, P., & Podell, M. (1999). Effects of feline immunodeficiency virus on cognition and behavioral function in cats. *J Acquir Immune Defic Syndr Hum Retrovirol, 20*(5), 411-419.

Steinrigl, A., & Klein, D. (2003). Phylogenetic analysis of feline immunodeficiency virus in Central Europe: a prerequisite for vaccination and molecular diagnostics. *J Gen Virol, 84*(Pt 5), 1301-1307.

Sun, J. C., & Lanier, L. L. (2009). Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity? *Eur J Immunol, 39*(8), 2059-2064.

Suntz, M., Failing, K., Hecht, W., Schwartz, D., & Reinacher, M. (2010). High prevalence of non-productive FeLV infection in necropsied cats and significant association with pathological findings. *Vet Immunol Immunopathol, 136*(1-2), 71-80.

Sykes, J. (2009). Feline Calicivirus Infection. In J. BONAGURA & D. TWEDT (Eds.), *Kirk's Current Veterinary Therapy* (Vol. XIV, pp. 1284-1288). Missouri: Saunders Elsevier.

Sykes, J. E., Drazenovich, N. L., Ball, L. M., & Leutenegger, C. M. (2007). Use of conventional and real-time polymerase chain reaction to determine the epidemiology of hemoplasma infections in anemic and nonanemic cats. *J Vet Intern Med, 21*(4), 685-693.

Taglinger, K., Van Nguyen, N., Helps, C. R., Day, M. J., & Foster, A. P. (2008). Quantitative real-time RT-PCR measurement of cytokine mRNA expression in the skin of normal cats and cats with allergic skin disease. *Vet Immunol Immunopathol, 122*(3-4), 216-230.

Tailor, C. S., Willett, B. J., & Kabat, D. (1999). A putative cell surface receptor for anemia-inducing feline leukemia virus subgroup C is a member of a transporter superfamily. *J Virol, 73*(8), 6500-6505.

Tamamoto, T., Ohno, K., Ohmi, A., Goto-Koshino, Y., & Tsujimoto, H. (2008). Verification of measurement of the feline serum amyloid A (SAA) concentration by human SAA turbidimetric immunoassay and its clinical application. *J Vet Med Sci, 70*(11), 1247-1252.

Tamamoto, T., Ohno, K., Ohmi, A., Seki, I., & Tsujimoto, H. (2009). Time-course monitoring of serum amyloid A in a cat with pancreatitis. *Vet Clin Pathol, 38*(1), 83-86.

Tanabe, T., Shimoda, M., Soeno, T., Suzuki, M., Tajima, M., & Sato, H. (2008). Molecular cloning and sequence analysis of feline interferon-stimulated gene 15. *Vet Immunol Immunopathol, 126*(1-2), 20-26.

Tanney, K., & Smith, M. (2010). Oral and Salivary Gland Disorders. In S. Ettinger & E. Feldman (Eds.), *Textbook of Veterinary Internal Medicine: Diseases of the dog and cat* (Seventh ed., Vol. 2). Missouri: Saunders Elsevier.

Tasker, S., Murray, J. K., Knowles, T. G., & Day, M. J. (2010). Coombs', haemoplasma and retrovirus testing in feline anaemia. *J Small Anim Pract, 51*(4), 192-199.

Tateyama, S., Priosoeryanto, B. P., Yamaguchi, R., Uchida, K., Ogiwara, K., & Suchiya, A. T. (1995). In vitro growth inhibition activities of recombinant feline interferon on all lines derived from canine tumours. *Res Vet Sci, 59*(3), 275-277.

Tavares, L., Roneker, C., Postie, L., & de Noronha, F. (1989). Testing of nucleoside analogues in cats infected with feline leukemia virus: a model. *Intervirology, 30 Suppl 1*, 26-35.

Taylor, J. L., & Grossberg, S. E. (1998). The effects of interferon-alpha on the production and action of other cytokines. *Semin Oncol, 25*(1 Suppl 1), 23-29.

Taylor, S. S., Goodfellow, M. R., Browne, W. J., Walding, B., Murphy, S., Tzannes, S., et al. (2009). Feline extranodal lymphoma: response to chemotherapy and survival in 110 cats. *J Small Anim Pract, 50*(11), 584-592.

Taylor, S. S., Tappin, S. W., Dodkin, S. J., Papasouliotis, K., Casamian-Sorrosal, D., & Tasker, S. (2010). Serum protein electrophoresis in 155 cats. *J Feline Med Surg, 12*(8), 643-653.

Tenorio, A. P., Franti, C. E., Madewell, B. R., & Pedersen, N. C. (1991). Chronic oral infections of cats and their relationship to persistent oral carriage of feline calici-, immunodeficiency, or leukemia viruses. *Vet Immunol Immunopathol, 29*(1-2), 1-14.

Terry, A., Callanan, J. J., Fulton, R., Jarrett, O., & Neil, J. C. (1995). Molecular analysis of tumours from feline immunodeficiency virus (FIV)-infected cats: an indirect role for FIV? *Int J Cancer, 61*(2), 227-232.

Thiry, E., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline herpesvirus infection. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 547-555.

Tizard, I. R. (2009a). Cell signaling: cytokines and their receptors. In I. R. Tizard (Ed.), *Veterinary Immunology: an introduction* (Eighth Edition ed., pp. 70-81). Missouri: Saunders Elsevier.

Tizard, I. R. (2009b). Helper T Cells and their response to antigen. In I. R. Tizard (Ed.), *Veterinary Immunology: an introduction* (Eighth Edition ed., pp. 139-152). Missouri: Saunders Elsevier.

Tomonaga, K., Inoshima, Y., Ikeda, Y., & Mikami, T. (1995). Temporal patterns of feline immunodeficiency virus transcripts in peripheral blood cells during the latent stage of infection. *J Gen Virol, 76 (Pt 9)*, 2193-2204.

Tompkins, M. B., Bull, M. E., Dow, J. L., Ball, J. M., Collisson, E. W., Winslow, B. J., et al. (2002). Feline immunodeficiency virus infection is characterized by B7+CTLA4+ T cell apoptosis. *J Infect Dis, 185*(8), 1077-1093.

Tompkins, M. B., Nelson, P. D., English, R. V., & Novotney, C. (1991). Early events in the immunopathogenesis of feline retrovirus infections. *J Am Vet Med Assoc, 199*(10), 1311-1315.

Tompkins, M. B., & Tompkins, W. A. (2008). Lentivirus-induced immune dysregulation. *Vet Immunol Immunopathol, 123*(1-2), 45-55.

Tompkins, W. A. (1999). Immunomodulation and therapeutic effects of the oral use of interferon-alpha: mechanism of action. *J Interferon Cytokine Res, 19*(8), 817-828.

Torres, A. N., Mathiason, C. K., & Hoover, E. A. (2005). Re-examination of feline leukemia virus: host relationships using real-time PCR. *Virology, 332*(1), 272-283.

Tovey, M. G. (2002). Oromucosal cytokine therapy: mechanism(s) of action. *Taehan Kan Hakhoe Chi, 8*(2), 125-131.

Toyosaki, T., Miyazawa, T., Furuya, T., Tomonaga, K., Shin, Y. S., Okita, M., et al. (1993). Localization of the viral antigen of feline immunodeficiency virus in the lymph nodes of cats at the early stage of infection. *Arch Virol, 131*(3-4), 335-347.

Treitinger, A., Spada, C., da Silva, L. M., Hermes, E. M., Amaral, J. A., & Abdalla, D. S. (2001). Lipid and acute-phase protein alterations in HIV-1 infected patients in the early stages of infection: correlation with CD4+ lymphocytes. *Braz J Infect Dis, 5*(4), 192-199.

Trinchieri, G., Pflanz, S., & Kastelein, R. A. (2003). The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity, 19*(5), 641-644.

Troth, S. P., Dean, A. D., & Hoover, E. A. (2008). In vivo CXCR4 expression, lymphoid cell phenotype, and feline immunodeficiency virus infection. *Vet Immunol Immunopathol, 123*(1-2), 97-105.

Troyer, R. M., Thompson, J., Elder, J. H., & VandeWoude, S. (2013). Accessory genes confer a high replication rate to virulent feline immunodeficiency virus. *J Virol, 87*(14), 7940-7951.

Truyen, U., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., et al. (2009). Feline panleukopenia. ABCD guidelines on prevention and management. *J Feline Med Surg, 11*(7), 538-546.

Truyen U., B. S., Schultheiss U. (2002). A study of the anti-viral activity of interferon omega against selected canine and feline viruses. *Journal of Modern Veterinary Medicine, 10*, 862-864.

Tsatsanis, C., Fulton, R., Nishigaki, K., Tsujimoto, H., Levy, L., Terry, A., et al. (1994). Genetic determinants of feline leukemia virus-induced lymphoid tumors: patterns of proviral insertion and gene rearrangement. *J Virol, 68*(12), 8296-8303.

Tuomari, D. L., Olsen, R. G., Singh, V. K., & Kraut, E. H. (1984). Detection of circulating immune complexes by a Clq/protein A-ELISA during the preneoplastic stages of feline leukemia virus infection. *Vet Immunol Immunopathol, 7*(3-4), 227-238.

Turan, K., Mibayashi, M., Sugiyama, K., Saito, S., Numajiri, A., & Nagata, K. (2004). Nuclear MxA proteins form a complex with influenza virus NP and inhibit the transcription of the engineered influenza virus genome. *Nucleic Acids Res, 32*(2), 643-652.

Ueda, Y., Sakurai, T., Kasama, K., Satoh, Y., Atsumi, K., Hanawa, S., et al. (1993). Pharmacokinetic properties of recombinant feline interferon and its stimulatory effect on 2',5'-oligoadenylate synthetase activity in the cat. *J Vet Med Sci, 55*(1), 1-6.

Uhl, E. W., Heaton-Jones, T. G., Pu, R., & Yamamoto, J. K. (2002). FIV vaccine development and its importance to veterinary and human medicine: a review FIV vaccine 2002 update and review. *Vet Immunol Immunopathol, 90*(3-4), 113-132.

Uhlar, C. M., & Whitehead, A. S. (1999). Serum amyloid A, the major vertebrate acute-phase reactant. *Eur J Biochem, 265*(2), 501-523.

VanCott, J. L., Staats, H. F., Pascual, D. W., Roberts, M., Chatfield, S. N., Yamamoto, M., et al. (1996). Regulation of mucosal and systemic antibody responses by T helper cell subsets, macrophages, and derived cytokines following oral immunization with live recombinant Salmonella. *J Immunol, 156*(4), 1504-1514.

VandeWoude, S., Hageman, C. A., O'Brien, S. J., & Hoover, E. A. (2002). Nonpathogenic lion and puma lentiviruses impart resistance to superinfection by virulent feline immunodeficiency virus. *J Acquir Immune Defic Syndr, 29*(1), 1-10.

VandeWoude, S., Hageman, C. L., & Hoover, E. A. (2003). Domestic cats infected with lion or puma lentivirus develop anti-feline immunodeficiency virus immune responses. *J Acquir Immune Defic Syndr, 34*(1), 20-31.

Verschoor, E. J., Boven, L. A., Blaak, H., van Vliet, A. L., Horzinek, M. C., & de Ronde, A. (1995). A single mutation within the V3 envelope neutralization domain of feline immunodeficiency virus determines its tropism for CRFK cells. *J Virol, 69*(8), 4752-4757.

Vobis, M., D'Haese, J., Mehlhorn, H., & Mencke, N. (2003). Evidence of horizontal transmission of feline leukemia virus by the cat flea (Ctenocephalides felis). *Parasitol Res, 91*(6), 467-470.

von Wussow, P., Jakschies, D., Hochkeppel, H. K., Fibich, C., Penner, L., & Deicher, H. (1990). The human intracellular Mx-homologous protein is specifically induced by type I interferons. *Eur J Immunol, 20*(9), 2015-2019.

Walker, C., Canfield, P. J., & Love, D. N. (1994). Analysis of leucocytes and lymphocyte subsets for different clinical stages of naturally acquired feline immunodeficiency virus infection. *Vet Immunol Immunopathol, 44*(1), 1-12.

Walker, C., Malik, R., & Canfield, P. J. (1995). Analysis of leucocytes and lymphocyte subsets in cats with naturally-occurring cryptococcosis but differing feline immunodeficiency virus status. *Aust Vet J, 72*(3), 93-97.

Wasmoen, T., Armiger-Luhman, S., Egan, C., Hall, V., Chu, H. J., Chavez, L., et al. (1992). Transmission of feline immunodeficiency virus from infected queens to kittens. *Vet Immunol Immunopathol, 35*(1-2), 83-93.

Wasunna, K. M., Raynes, J. G., Were, J. B., Muigai, R., Sherwood, J., Gachihi, G., et al. (1995). Acute phase protein concentrations predict parasite clearance rate during therapy for visceral leishmaniasis. *Trans R Soc Trop Med Hyg, 89*(6), 678-681.

Weaver, E. A. (2010). A detailed phylogenetic analysis of FIV in the United States. *PLoS One, 5*(8), e12004.

Weaver, E. A., Collisson, E. W., Slater, M., & Zhu, G. (2004). Phylogenetic analyses of Texas isolates indicate an evolving subtype of the clade B feline immunodeficiency viruses. *J Virol, 78*(4), 2158-2163.

Webb, C. B., Lehman, T. L., & McCord, K. W. (2008). Effects of an oral superoxide dismutase enzyme supplementation on indices of oxidative stress, proviral load, and CD4:CD8 ratios in asymptomatic FIV-infected cats. *J Feline Med Surg, 10*(5), 423-430.

Weiss, R. C., Cummins, J. M., & Richards, A. B. (1991). Low-dose orally administered alpha interferon treatment for feline leukemia virus infection. *J Am Vet Med Assoc, 199*(10), 1477-1481.

Weiss, R. C., & Oostrom-Ram, T. (1989). Inhibitory effects of ribavirin alone or combined with human alpha interferon on feline infectious peritonitis virus replication in vitro. *Vet Microbiol, 20*(3), 255-265.

Wilhelm, S., & Truyen, U. (2006). Real-time reverse transcription polymerase chain reaction assay to detect a broad range of feline calicivirus isolates. *J Virol Methods, 133*(1), 105-108.

Willett, B. J., Cannon, C. A., & Hosie, M. J. (2003). Expression of CXCR4 on feline peripheral blood mononuclear cells: effect of feline immunodeficiency virus infection. *J Virol, 77*(1), 709-712.

Willett, B. J., & Hosie, M. J. (1999). The role of the chemokine receptor CXCR4 in infection with feline immunodeficiency virus. *Mol Membr Biol, 16*(1), 67-72.

Willett, B. J., Hosie, M. J., Callanan, J. J., Neil, J. C., & Jarrett, O. (1993). Infection with feline immunodeficiency virus is followed by the rapid expansion of a CD8+ lymphocyte subset. *Immunology, 78*(1), 1-6.

Willett, B. J., Hosie, M. J., Neil, J. C., Turner, J. D., & Hoxie, J. A. (1997). Common mechanism of infection by lentiviruses. *Nature, 385*(6617), 587.

Willett, B. J., McMonagle, E. L., Logan, N., Spiller, O. B., Schneider, P., & Hosie, M. J. (2007). Probing the interaction between feline immunodeficiency virus and CD134 by using the novel monoclonal antibody 7D6 and the CD134 (Ox40) ligand. *J Virol, 81*(18), 9665-9679.

Willis, A. M. (2000). Feline leukemia virus and feline immunodeficiency virus. *Vet Clin North Am Small Anim Pract, 30*(5), 971-986.

Wong, S. C., Lo, E. S., & Cheung, M. T. (2004). An optimised protocol for the extraction of non-viral mRNA from human plasma frozen for three years. *J Clin Pathol, 57*(7), 766-768.

Woo, J. C., Dean, G. A., Lavoy, A., Clark, R., & Moore, P. F. (1999). Investigation of recombinant human insulin-like growth factor type I in thymus regeneration in the acute stage of experimental FIV infection in juvenile cats. *AIDS Res Hum Retroviruses, 15*(15), 1377-1388.

Wood, B. A., Troyer, R. M., Terwee, J. A., & Vandewoude, S. (2012). Microsphere immunoassay for the detection of cytokines in domestic cat (Felis catus) plasma: elevated IL-12/23 in acute feline immunodeficiency virus infections. *Vet Immunol Immunopathol, 145*(3-4), 604-610.

Yamamoto, J. K., Sanou, M. P., Abbott, J. R., & Coleman, J. K. (2010). Feline immunodeficiency virus model for designing HIV/AIDS vaccines. *Curr HIV Res, 8*(1), 14-25.

Yamamoto, S., Shida, T., Okimura, T., Otabe, K., Honda, M., Ashida, Y., et al. (1994). Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. *Vet Q, 16*(2), 74-77.

Zaccaro, L., Falcone, M. L., Silva, S., Bigalli, L., Cecchettini, A., Giorgi, F., et al. (1995). Defective natural killer cell cytotoxic activity in feline immunodeficiency virus-infected cats. *AIDS Res Hum Retroviruses, 11*(6), 747-752.

Zeidner, N. S., Myles, M. H., Mathiason-DuBard, C. K., Dreitz, M. J., Mullins, J. I., & Hoover, E. A. (1990). Alpha interferon (2b) in combination with zidovudine for the treatment of presymptomatic feline leukemia virus-induced immunodeficiency syndrome. *Antimicrob Agents Chemother, 34*(9), 1749-1756.

Zhao, S., & Fernald, R. D. (2005). Comprehensive algorithm for quantitative real-time polymerase chain reaction. *J Comput Biol*,12(8), 1047–1064.

Zhao, Y., Gebhard, D., English, R., Sellon, R., Tompkins, M., & Tompkins, W. (1995). Enhanced expression of novel CD57+CD8+ LAK cells from cats infected with feline immunodeficiency virus. *J Leukoc Biol, 58*(4), 423-431.

Annexes

Annexe I: Abstract of the oral communication (in portuguese) presented on the 20º Congresso Nacional da Associação Portuguesa de Médicos Veterinários Especialistas em Animais de Companhia (APMVEAC), Oeiras, Junho 2011

RESUMO: INTERFERÃO OMEGA FELINO (FE-IFN_): A PROPÓSITO DA AVALIAÇÃO 1 CLÍNICA DE GATOS NATURALMENTE INFECTADOS COM OS VÍRUS DA IMUNODEFICIÊNCIA (FIV) E LEUCEMIA FELINAS (FELV)

R. Leal, S. Gil, D. Mcgahie, A. Duarte, MMRE. Niza, L.Tavares

No tratamento de animais infectados com retrovirus, o recurso a imunomoduladores como o Interferão (IFN), constitui uma opção terapêutica viável e actual em medicina veterinária. Hoje em dia existem disponíveis o IFNα Humano (recombinante) e o IFNω Felino (recombinante) (reFelIFNω). Embora o IFNα humano seja frequentemente utilizado, a sua administração leva ao desenvolvimento de anticorpos, prejudicando a manutenção do tratamento. Recentemente, foi desenvolvido o reFelIFNω, o qual demonstrou uma acção *in vitro* contra alguns vírus felinos (nomeadamente o herpesvirus, calicivirus e peritonite infecciosa). Outros estudos pioneiros na área (De Mari et al., 2004) revelam que o reFelIFNω pode ser um candidato promissor no tratamento de gatos infectados com retrovírus. Neste contexto, este estudo visa avaliar a resposta clínica de gatos infectados com FIV e/ou FeLV, sob tratamento com reFelIFNω.

Um grupo de 18 gatos (10 machos e 8 fêmeas), de raça Europeu comum, alojados em gatil e indicados como positivos em kit rápido (FIV/FeLV), foram re-testados por ELISA para a presença dos mesmos. 2/18 gatos revelaram-se negativos tendo sido considerados 16 animais para estudo (7/16 FIV, 6/16 FeLV e 3/16 co-infectados). Estes animais foram submetidos a tratamento com reFelIFNω (Virbagen®), realizado em 3 ciclos de cinco injecções 1MU/kg SC, aos dias 0, 14 e 60, de acordo com o licenciado para o produto. Ao longo do tratamento (dias 0, 10, 30 e 65) os animais foram submetidos a exames clínicos completos. Estes foram efectuados de forma independente e compreenderam a avaliação de diferentes sinais clínicos relacionados com infecções virais bem como indicadores de estado geral e condição corporal dos animais. Os sinais clínicos foram pontuados numa auto-escala de 0 (sinal clínico ausente/favorável) a +2 (sinal clínico muito evidente/desfavorável) obtendo-se assim um valor final para cada avaliação. Estes exames clínicos puderam, portanto, ser comparáveis ao longo do estudo. 8/16 gatos (3/7 FIV, 3/6 FeLV, 2/3 Co-infectados) obtiveram uma melhoria clínica muito significativa, 4/16 gatos (2/7 FIV, 2/7 FeLV) uma melhoria moderada e apenas 4/16 animais (2/7 FIV 1/7 FeLV e 1/3 Co-infectado) não evidenciaram alterações significativas do seu estado geral ao longo do tratamento.

Em conclusão, este estudo demonstra que o tratamento com reFelIFNω (Virbagen®) induz uma melhoria clínica relevante em gatos infectados com retrovírus.

Annexe II: Abstract of the poster presented on the 21st European College of Veterinary Internal Medicine – Companion Animals Congress, Seville, September 2011

ABSTRACT: COMPLETE BLOOD COUNT (CBC), BIOCHEMISTRY AND SERUM PROTEIN PROFILE EVALUATION IN FELINE IMMUNODEFICIENCY VIRUS (FIV), FELINE LEUKEMIA VIRUS (FELV) AND CO-INFECTED FELV/FIV CATS SUBMITTED TO FELINE IFN ω (FE-IFN ω) THERAPY

Gil, S., Leal, R., Duarte A., Sepúlveda N, McGahie D., Siborro I., Cravo J.,Cartaxeiro C., Niza MMRE, Tavares L.

Retroviruses can induce immunodeficiency syndromes by distinct mechanisms leading to impairment of the immune system or to persistent chronic infection.

In order to evaluate how Fe-IFNω alters the course of disease, 16 naturally infected retroviral cats (7 FIV, 6 FeLV and 3 co-infected) housed in a Lisbon Animal Shelter were followed during Fe-IFNω therapy: 3 cycles of 5 injections at 1MU/kg SID SC (D0, D14 and D60). Clinical evaluation was assessed and blood samples were collected (D0, D10, D30, D65) to monitor cell line variations (CBC), hepatic enzymes (Alanine-transaminase, Aspartate-transaminase), renal function (Creatinine, Urea) and serum protein profile.

All cats had normal analytical profiles when beginning treatment. Red-blood cell counts did not change significantly in the majority of cats (15/16) during therapy. 14/16 cats maintained normal white-blood cell values while 2/16 cats presented a mild leukocytosis at D65. No changes of hepatic and renal function were observed during therapy.

Despite some mild variations, results revealed that Fe-IFNω therapy does not seem to alter CBC values or the measured hepatic and renal parameters.

At D0, protein profile analysis revealed that FeLV and Co-infected cats had increased total proteins (TPs) with normal albuminemia, α2-hyperglobulinemia, α1- and β-hypoglobulinemia and a normal to increased gamma profile. FIV cats had a hyperproteinemia with normal albuminemia, α2-hyperglobulinemia, α1- and β-hypoglobulinemia and a consistent γ-hyperglobulinemia.

Kruskall-Wallis analysis revealed statistically significant differences that were discriminated by pairwise comparison. The α1-globulins were significantly lower in FIV cats at D0 in comparison to FeLV and co-infected cats ($p<0,05$). Most relevantly, γ-globulins and TPs decreased in most FeLV and co-infected cats during Fe-IFNω therapy (D30 and D65). FIV cats, by comparison, presented higher levels at the end of the treatment than at the beginning.

Differences in the initial and final levels of γ-globulins in FeLV and FIV cats are relevant. Enhancement of γ-globulins is described in retrovirus infections. Actually, increased levels of γ-globulins in FIV infected cats after Fe-IFNω therapy could be due to the induction of neutralizing antibodies which are found to be effective in the cat immune response against FIV. Conversely reduction of γ- globulins observed in FeLV cats could be a strong indicator of the benefits of Fe-IFNω therapy. In order to deepen this knowledge, cellular and humoral related cytokines need to be quantified and correlated to globulin levels.

Complete Blood Count (CBC), Biochemistry and Serum Protein Profile evaluation in Feline immunodeficiency virus (FIV), Feline leukemia virus (FeLV) and co-infected FeLV/FIV cats submitted to Feline-Interferon ω (rFE-IFNω) therapy

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Introduction

Retroviruses can induce immunodeficiency syndromes by distinct mechanisms leading to impairment of the immune system or to persistent chronic infection[1,2].

The aim of this study is to evaluate how recombinant feline interferon ω (rFe-IFNω) therapy affects complete blood count (CBC), biochemistry and serum protein profile in naturally infected retroviral cats.

Material and Methods

In order to evaluate how rFe-IFNω alters the course of disease, 16 cats naturally infected with retroviruses (7 FIV, 6 FeLV and 3 co-infected) housed in a Lisbon Animal Shelter were followed during reFe-IFNω therapy (Virbagen Omega®, Virbac, France) : 3 cycles of 5 injections at 1MU/kg SID SC (D0, D14 and D60)[2].

Clinical evaluation was assessed and blood samples were collected (D0, D10, D30, D65) to monitor cell line variations, hepatic enzymes (Alanine-transaminase, Aspartate-transaminase), renal function (Creatinine, Urea) and serum protein profile (electrophoresis).

Results

All cats had normal analytical profiles when treatment began. Red-blood cell counts did not change significantly in the majority of cats (15/16) during therapy. 14/16 cats maintained normal white-blood cell values while 2/16 cats presented a mild leukocytosis at D65. Average values (AVG) ± Standard Error (SE) for RBC and WBC are presented in Figures 1 and 2.

No changes of hepatic and renal function were observed during therapy.

At D0, protein profile analysis revealed that FeLV and Co-infected cats had increased total proteins (TPs) with normal albuminemia, α2-hyperglobulinemia, α1- and β-hypoglobulinemia and a normal to increased gamma profile. FIV cats had a hyperproteinemia with normal albuminemia, α2 hyperglobulinemia, α1- and β-hypoglobulinemia and a consistent γ- hyperglobulinemia.

Kruskall-Wallis analysis revealed statistically significant differences that were discriminated by pairwise comparison. The α1-globulins were significantly lower in FIV cats at D0 in comparison to FeLV ($p = 0,047$) and co-infected cats ($p=0,008$). Most relevantly, γ-globulins and TPs decreased in most FeLV and co-infected cats during rFe-IFNω therapy (D30 and D65). FIV cats, by comparison, presented higher levels at the end of the treatment than at the beginning (Figures 3-5).

Fig.1: Red Blood Cells variation of FIV, FeLV and Co-infected cats under treatment with rFe-IFN ω (reference range 5,0-10,0x10⁶/µl).

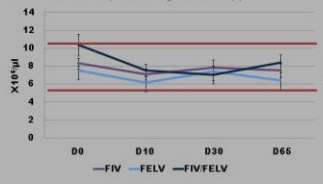


Fig.2: White Blood Cells variation of FIV, FeLV and Co-infected cats under treatment with rFe-IFN ω (reference range 5,5-19,5x10³/µl).

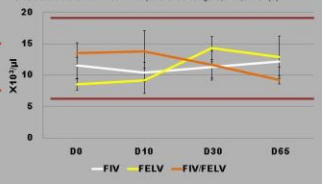


Fig.3: Serum Protein Profile Reference Range (g/dl)

Parameter	Reference Range (g/dl)
Alb	2,1-4,0
α1	0,2-1,1
α2	0,4-0,9
β	0,9-1,9
γ	1,3-2,2
A/G Ratio	0,45-1,3
TP	5,7-7,9

Fig.4: AVG ± SE of Serum protein profile in FIV cats under treatment with rFe-IFNω

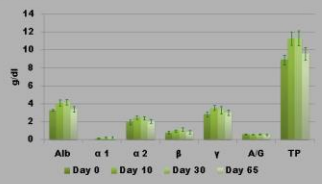


Fig.5: AVG ± SE of Serum protein profile in FeLV cats under treatment with rFe-IFNω

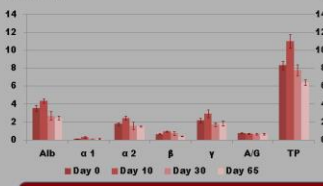
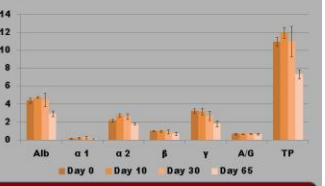


Fig.6: AVG ± SE of Serum protein profile in Co-infected cats under treatment with rFe-IFNω



Discussion

Despite some mild variations, rFe-IFNω therapy does not seem to alter CBC values or the measured hepatic and renal parameters.

Differences in the initial and final levels of γ-globulins in FeLV and FIV cats are relevant. Enhancement of γ-globulins is described in retrovirus infections[1]. Actually, increased levels of γ-globulins in FIV infected cats after Fe-IFNω therapy could be due to the induction of neutralizing antibodies which are found to be effective in the cat immune response against FIV. Conversely reduction of γ- globulins observed in FeLV cats could be a strong indicator of the benefits of rFe-IFNω therapy. In order to deepen this knowledge, cellular and humoral related cytokines need to be quantified and correlated to globulin levels.

Acknowledgments and Support

This work was supported by the project CIISA 50, by the pHD fellowship FCT SFRH / BD / 62917 / 2009 and by Virbac Portugal;
The authors would like to thank União Zoófila (Lisboa).

References

1. GLEICH S., HARTMANN K. (2009) Hematology and Serum Biochemistry of Feline Immunodeficiency Virus- Infected and Feline Leukemia Virus-Infected Cats. J Vet Intern Med 2009; 23: 552-558.
2. DE MARI K, MAYNARD L, SANQUER A, LEBREUX B, EUN HM (2004) Therapeutic effects of recombinant feline interferon-omega on feline leukemia virus (FeLV)-infected and FeLV/feline immunodeficency virus (FIV) co-infected symptomatic cats. J Vet Intern Med. 2004 Jul-Aug; 18(4): 477-82.

21 st ECVIM-CA Congress
8-11 September 2011
Sevilla, Spain

Annexe III: Abstract of the poster presented on the Southern European Veterinary Conference, Barcelona, October 2011

ABSTRACT: INTERFERON-ω THERAPY ON FELINE IMMUNODEFICIENCY AND LEUKEMIA INFECTED STRAY CATS: CLINICAL IMPROVEMENT AND CONTROL OF CONCOMITANT VIRAL EXCRETION

Solange Gil, Rodolfo Leal, Ana Duarte, David McGahie, Ines Siborro, Joana Cravo, Clara Cartaxeiro Maria MRE Niza, Luis Tavares

Objetives of the Study:

FIV and FeLV viruses are common infectious agents in stray cats and shelter environments. In Veterinary Medicine, therapy is based on immunostimulating drugs such as IFN inductors or IFNs (Human IFNα/Feline IFN ω) (1). Although Human IFNα is currently used, its frequent administration leads to development of antibodies, undermining continuous administration (2).

Recently developed Fe-IFNω has shown an antiviral action "in vitro" not only against FIV and FeLV but also against feline herpesvirus (3) and calicivirus (4). In order to clarify the improvement of clinical signs in cats naturally infected or co-infected with FIV/FeLV and to correlate this improvement with reduced excretion of concomitant virus, cats naturally infected with FIV/FeLV were followed during Fe-IFNω therapy.

Materials and Methods:

Eighteen stray cats, housed in a Lisbon Animal Shelter and indicated as retroviral infected were tested for FIV and FeLV (ELISA). Positive cats (16/18: 7/16 FIV, 6/16 FeLV and 3/16 co-infected) were tested for Herpesvirus (QRT-PCR), Calicivirus (PCR) (5) and Coronavirus (QRT-PCR). 2/18 cats were negative for retrovirus and excluded from therapy. Cats received Fe-IFNω using the licensed protocol (6): 5 injections,1MU/kg SC SID (Day 0, Day 14, Day 60). At D0, D10, D30 and D65 all cats were submitted to regular clinical evaluations and samples collected for quantification of concomitant virus excretion. At D65, all cats were re-tested for FIV and FeLV.

Results:

ELISA re-testing showed that all FIV cats remained positive while 1 FeLV cat tested negative. Calicivirus prevalence was 14/16 (5/7 FIV, 6/6 FeLV and 3/3 Co-infected) at D0 and from D10 to D65 all the animals tested negative. Coronavirus viral loads at D0 showed 11/16 positives (5/7 FIV, 4/6 FeLV and 2/3 Coinfected) which decreased in 4/5 FIV cats, 3/4 FeLV cats and in all Co-infected cats. 11/16 were Herpesvirus positive at D0 (4/7 FIV, 5/6 FeLV and 2/3 Co-infected). Its viral excretion during therapy is under current analysis.

Clinical evaluation revealed that 10/16 cats improved their clinical signs with Fe-IFNω therapy (5/7 FIV, 3/6 FeLV and 2/3 co-infected) while 6/16 remained stable during treatment.

Conclusions:

In conclusion, Fe-IFNω therapy is helpful in the improvement of clinical signs in FIV and FeLV Co-infected cats and it is particularly relevant in the control and decrease of concurrent viral excretion namely Calicivirus and Coronavirus.

Bibliography:

(1) Hosie, M.J., et al., Feline immunodeficiency. ABCD guidelines on prevention and management. J Feline Med Surg, 2009. 11(7): p. 575-84.

(2) Zeidner, N.S., et al., Alpha interferon (2b) in combination with zidovudine for thetreatment of presymptomatic feline leukemia virus-induced immunodeficiency syndrome. Antimicrob Agents Chemother, 1990. 34(9): p. 1749-56.

(3) Bracklein, T., et al., Activity of feline interferon-omega after ocular or oral administration in cats as indicated by Mx protein expression in conjunctival and white blood cells. Am J Vet Res, 2006. 67(6): p. 1025-32.

(4) Radford, A. D., Addie, D., Belak, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K.,. Hosie, M.J., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M. G., Thiry, E., Truyen, U. and Horzinek, M. C.. Feline calicivirus infection. ABCD guidelines on prevention and management. Journal of Feline Medicine & Surgery Volume 11, Issue 7, July 2009, Pages 556-564

(5) Wilhelm, S. and U. Truyen, Real-time reverse transcription polymerase chain reaction assay to detect a broad range of feline calicivirus isolates. Journal of Virological Methods, 2006. 133(1): p. 105-108.

(6) de Mari, K., et al., Therapeutic effects of recombinant feline interferon-omega on feline leukemia virus (FeLV)-infected and FeLV/feline immunodeficiency virus (FIV)-coinfected symptomatic cats. J Vet Intern Med, 2004. 18(4): p. 477-82.

INTERFERON-ω THERAPY ON FELINE IMMUNODEFICIENCY AND LEUKEMIA INFECTED STRAY CATS: CLINICAL IMPROVEMENT AND CONTROL OF CONCOMITANT VIRAL EXCRETION

Gil S.[1], Oliveira Leal R. [1], Duarte A. [1], McGahie D. [2], Siborro I. [1], Cravo J. [1], Cartaxeiro C. [1], Niza MMRE. [1], Tavares L. [1]

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Objectives of the Study

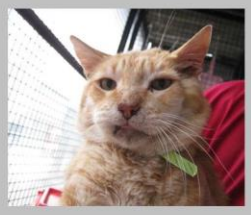
FIV and FeLV viruses are common infectious agents in stray cats and shelter environments. In Veterinary Medicine, therapy is based on immunostimulating drugs such as interferon (IFN) inductors or directly with IFNs (Human IFNα/Feline IFNω)[1]. Although Human IFNα is currently used, its frequent administration leads to development of antibodies, undermining continuous administration[2]. Recently developed recombinant feline interferon omega (rFe-IFNω; Virbac, Carros, France) has shown an antiviral action in vitro not only against FIV and FeLV but also against feline herpesvirus[3] and calicivirus[4].

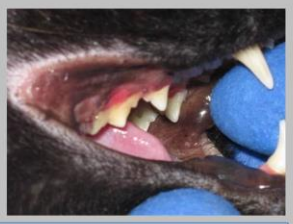
AIMS OF THE STUDY:

-to clarify the improvement of clinical signs in FIV, FeLV and Co-infected FIV/FeLV naturally infected cats under rFe-IFNω therapy;

-to correlate this improvement with reduced excretion of concomitant viruses;

Material and Methods





18 stray cats, housed in a Lisbon Animal Shelter and noted as retroviral infected were tested for FIV and FeLV (ELISA).

16/18 cats were positive:
7/16 FIV
6/16 FeLV
3/16 Co-infected

2/18 cats were negative for retroviruses and excluded

Positive cats were tested for Herpesvirus (QRT-PCR) Calicivirus[5] (PCR) and Coronavirus (QRT-PCR).

Cats received rFe-IFNω using the licensed protocol[6]:
3 courses of 5 injections at 1MU/kg SC SID (Day 0, Day 14, Day 60).

At D0, D10, D30 and D65
- all cats were submitted to full clinical evaluations (score analysis).
– samples were collected for quantification of concomitant virus excretion

At D65
all cats were re-tested for FIV and FeLV by ELISA

Results

- Clinical evaluation revealed that 10/16 cats improved their clinical signs with rFe-IFNω therapy (5/7 FIV, 3/6 FeLV and 2/3 co-infected) while 6/16 remained stable during therapy.
- ELISA re-testing on day 65 showed that all FIV cats remained positive while 1 FeLV cat tested negative.
- Calicivirus prevalence was 14/16 (5/7 FIV, 6/6 FeLV and 3/3 Co-infected) at D0. From D10 to D65 all the animals tested negative.
- Coronavirus viral loads (Fig.1) at D0 showed 11/16 positive cats (5/7 FIV, 4/6 FeLV and 2/3 Co-infected) which decreased in 9 of those cats (4/5 FIV cats, 3/4 FeLV cats and in all Co-infected cats).
- 10/16 were Herpesvirus excretors at D0 (4/7 FIV, 4/6 FeLV and 2/3 Co-infected). 6 of them reduced their viral excretion during therapy (3/4 FIV, 2/4 FeLV and 1/2 Co-infected) and 4 cats tested negative for viral excretion at D65 (Fig.2).

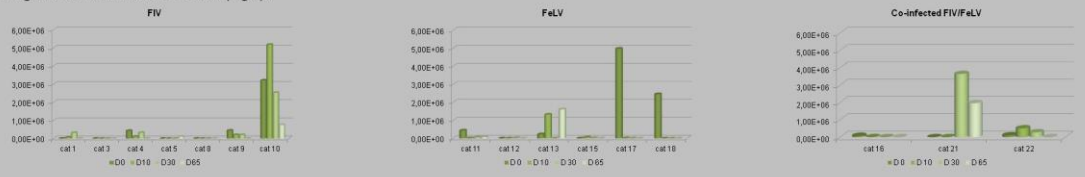


Fig. 1: Real- Time viral load quantification (ng/µl) of coronavirus excretion in FIV, FeLV and co-infected cats under rFe-IFNω therapy (D0, D10, D30, D65).

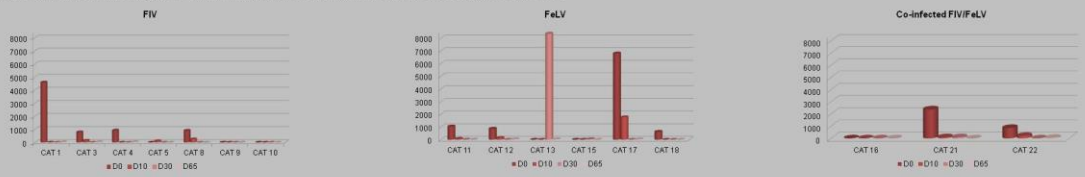


Fig. 2: Real- Time viral load quantification (ng/µl) of herpesvirus excretion in FIV, FeLV and co-infected cats under rFe-IFNω therapy (D0, D10, D30, D65).

Conclusion

In conclusion, rFe-IFNω therapy is helpful for the improvement of clinical signs in FIV and FeLV and Co-infected cats and it is particularly relevant in the control and decrease of concurrent viral excretion namely Calicivirus, Herpesvirus and Coronavirus.

References

1. Hosie, M.J., et al., Feline immunodeficiency ABCD guidelines on prevention and management. J Feline Med Surg, 2009. 11(7): p. 575-84. 2. Zeidner, N.S., et al., Alpha interferon (2b) in combination with zidovudine for thetreatment of presymptomatic feline leukemia virus-induced immunodeficiency syndrome. Antimicrob Agents Chemother, 1990. 34(9): p. 1749-56. 3. Bracklein, T., et al., Activity of feline interferon-omega after ocular or oral administration in cats as indicated by Mx protein expression in conjunctival and white blood cells. Am J Vet Res, 2006. 67(6): p. 1025-32. 4. Radford, A. D., Addie, D., Bel´ák, S., Boucraut-Baralon, C., Egberink, H., Frymus, T., Gruffydd-Jones, T., Hartmann, K., Hosie, M.J., Lloret, A., Lutz, H., Marsilio, F., Pennisi, M. G., Thiry, E., Truyen, U. and Horzinek, M. C.. Feline calicivirus infection. ABCD guidelines on prevention and management. Journal of Feline Medicine & Surgery Volume 11, Issue 7, July 2009, Pages 556-564 5. Wilhelm, S. and U. Truyen, Real-time reverse transcription polymerase chain reaction assay to detect a broad range of feline calicivirus isolates. Journal of Virological Methods, 2006. 133(1): p. 105-108. 6. de Mari, K., et al., Therapeutic effects of recombinant feline interferon-omega on feline leukemia virus (FeLV)-infected and FeLV/feline immunodeficiency virus (FIV)-coinfected symptomatic cats. J Vet Intern Med, 2004. 18(4): p. 477-82.

Acknowledgements and Support

This work was supported by the project CIISA 50, by the pHD fellowship FCT SFRH / BD / 62917 / 2009 and by Virbac Portugal;
The authors would like to thank União Zoófila (Lisboa).

Annexe IV: Abstract of the poster presented on the 21st European College of Veterinary Internal Medicine – Companion Animals Congress, Seville, September 2011

ABSTRACT: C-REACTIVE PROTEIN (CRP) QUANTIFICATION IN FIV (FELINE IMMUNODEFICIENCY VIRUS) AND FELV (FELINE LEUKEMIA VIRUS) POSITIVE CATS UNDER TREATMENT WITH FELINE INTERFERON OMEGA (FE-IFNO): A BIOMARKER OF INFLAMMATORY RESPONSE?

Leal RO, Gil S, Duarte A, Félix N, McGahie D, Cartaxeiro C, Niza MMRE, Tavares L.

CRP is an acute phase protein with an important relevance in human medicine due to the fact that it has been used as a marker for multiple clinical and sub-clinical inflammatory disorders. In Veterinary Medicine, CRP has been recently studied, particularly in dogs, where it is considered a biomarker for numerous inflammatory diseases. In cats, its interpretation is less understood and still under evaluation. Some authors consider that CRP may not be directly involved in acute inflammatory responses. Nevertheless, quantification of acute phase proteins has been demonstrated to be helpful in the diagnosis of some viral diseases (such as Alpha-1-acid-glycoprotein in feline infectious peritonitis).

IFNω is an immuno-modulator drug that seems to be a promising therapeutic candidate in retroviral infections. However, little is known about how the induction or use of IFNs in FIV infection relates to the expected immune benefits of IFN therapy using the recent licensed protocols. To the authors' knowledge, there are no studies about the effect of IFNω on CRP values in cats. To investigate this, serum CRP concentrations were monitored in FIV/ FeLV positive cats under Fe-IFNω treatment.

Sixteen retroviral infected cats (7 FIV, 6 FeLV and 3 FeLV/FIV stray cats), housed in a Lisbon Animal Shelter, were treated with Fe-IFNω using the licensed protocol: 5 injections, 1MU/kg SC SID (D0, D14 and D60). Blood samples were collected at days 0, 10, 30 and 65 after treatment started and Feline CRP was quantified by ELISA (Kamiya Biomedical Company).10/16 animals (5/7 FIV, 3/6 FeLV and 2/3 Co-infected cats) showed normal CRP values at the beginning of therapy while 6/16 cats showed slightly altered values (3/6 FeLV, 1/3 Co-infected had an increased CRP at D0 and 2/7 FIV had a decreased value at D0). Although some variation was evident in the measurements at D10 and D30, all the animals revealed an important increase of CRP values at the end of the treatment (D65), when compared with the baseline value (D0).

This study describes for the first time CRP concentrations in retroviral infected cats under treatment with Fe-IFNω. In conclusion, CRP increased in all retroviral infected cats under this therapy. A possible explanation is that Fe-IFNω stimulates multiple immunological pathways to improve the animal's inflammatory response. As CRP is an unspecific biomarker of the inflammatory response, further studies are needed to correlate these results with other acute phase proteins and cytokine expression.

C-Reactive Protein Quantification in Feline Immunodeficiency Virus (FIV) and Feline Leukemia virus (FeLV) positive cats under treatment with Feline Interferon –ω : a biomarker of inflammatory response?

Leal R.[1], Gil S. [1], Duarte A. [1], Félix N. [1], McGahie D. [2], Cartaxeiro C. [1], Niza M. [1], Tavares L.[1]

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Introduction

C-Reactive Protein (CRP) is an acute phase protein with an important relevance in human medicine due to the fact that it has been used as a marker for multiple clinical and sub-clinical inflammatory disorders [1,2]. In Veterinary Medicine, CRP has been recently studied, particularly in dogs, where it is considered a biomarker for numerous inflammatory diseases. In cats, its interpretation is less understood and still under evaluation[2]. Some authors consider that CRP may not be directly involved in acute inflammatory responses[1,2]. Nevertheless, quantification of acute phase proteins has been demonstrated to be helpful in the diagnosis of some viral diseases (such as Alpha-1-acid-glycoprotein in feline infectious peritonitis)[3].

Recombinant Feline Interferon- ω (rFe-IFN-ω; Virbagen Omega®, Virbac, France) is an immune-modulator drug that seems to be a promising therapeutic candidate in retroviral infections[4]. However, little is known about how the induction or use of IFNs in FIV infection relates to the expected immune benefits of IFN therapy using the recent licensed protocols. To the authors' knowledge, there are no studies about the effect of IFNω on CRP values in cats.

Aim of the Study: To monitor serum CRP concentrations in FIV, FeLV and co-infected cats under Fe-IFNω treatment.

Material and Methods

Sixteen cats naturally infected with retroviruses (7 FIV, 6 FeLV and 3 co-infected FeLV/FIV stray cats), housed in a Lisbon Animal Shelter, were treated with rFe-IFNω (Virbagen Omega®, Virbac, France) using the licensed protocol: 5 injections, 1MU/kg SC SID (D0, D14 and D60)[4].

Blood samples were collected at days 0, 10, 30 and 65 after treatment started (Fig.1).

Feline CRP was quantified by ELISA (Kamiya Biomedical Company).

Fig.1: Treatment protocol and blood samples timeline.

Results

10/16 animals (5/7 FIV, 3/6 FeLV and 2/3 Co-infected cats) showed normal CRP values at the beginning of therapy while 6/16 cats showed slightly altered values (3/6 FeLV, 1/3 Co-infected had an increased CRP at D0 and 2/7 FIV had a decreased value at D0). Although some individual variation was evident, all the animals revealed an increase of CRP values at the end of the treatment (D65), when compared with the baseline values (D0). Results are detailed below (Fig. 2-4).

Statistical analysis (by Kruskall Wallis and Pairwise comparison) showed similar results for the 3 groups at D0, D10 and D30 ($p > 0,05$). At D65, Co-infected cats presented a statistically significant increased CRP concentration when compared with FIV cats ($p = 0,008$).

Fig.2: CRP concentration in FIV cats under rFe-IFNω treatment.

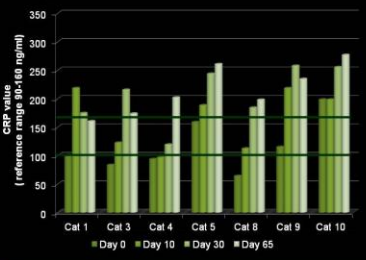


Fig.3: CRP concentration in FeLV cats under rFe-IFNω treatment.

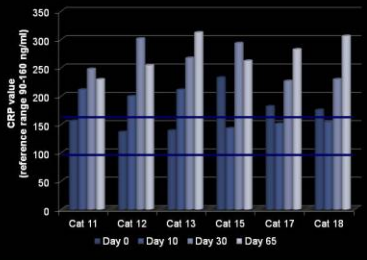
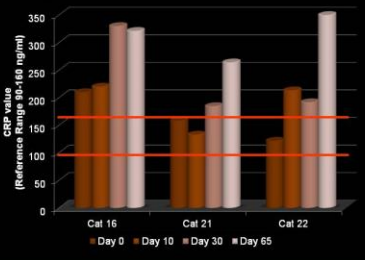


Fig.4: CRP concentration in co-infected cats under rFe-IFNω treatment.



Discussion

This study describes for the first time CRP concentrations in retroviral infected cats under treatment with rFe-IFNω. In conclusion, CRP increased in all retroviral infected cats under this therapy. A possible explanation is that rFe-IFNω stimulates multiple immunological pathways to improve the animal's inflammatory response. As CRP is a non-specific biomarker of the inflammatory response, further studies are needed to correlate these results with other acute phase proteins and cytokine expression.

Acknowledgment and Support

This work was supported by the project CIISA 50, by the pHD fellowship FCT SFRH / BD / 62517 / 2009 and by Virbac Portugal;
The authors would like to thank União Zoófila (Lisboa).

References

1. CERON JJ, ECKERSALL PD, MARTYNEZ-SUBIELA S. (2005) Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet. Clin. Pathol. June 2005; 34 (2): 85-99.
2. ECKERSALL PD, BELL R (2010) Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. Vet. J. July 2010; 185 (1): 23-7
3. DUTHIE S, ECKERSALL PD, ADDIE DD et al. (1997) Value of α1- acid glycoprotein in the diagnosis of feline infectious peritonitis. Vet Rec. 1997; 141; 299-303.
4. DE MARI K, MAYNARD L, SANQUER A, LEBREUX B, EUN HM (2004) Therapeutic effects of recombinant feline interferon-omega on feline leukemia virus (FeLV)-infected and FeLV/feline immunodeficency virus (FIV) co-infected symptomatic cats. J Vet Intern Med. 2004 Jul-Aug; 18(4): 477-82.

21 st ECVIM-CA Congress
8-11 September 2011

Annexe V: Abstract of the oral communication presented on the 11st International Feline Retrovirus Research Symposium, Leipzig, August 2012

ABSTRACT: RECOMBINANT FELINE INTERFERON-ω IN NATURALLY RETROVIRAL INFECTED CATS: THE RELEVANCE OF ACUTE PHASE PROTEINS IN THE FOLLOW UP OF AN IMMUNE-STIMULATION THERAPY

Leal R., Gil S., Sepúlveda N., McGahie D., Duarte A., Niza MMRE, Tavares L.

Acute Phase Proteins (APP) are key components of the Acute Phase Reaction. This is a nonspecific response of the innate immune system which consists of the production and release of various mediators such as pro-inflammatory cytokines during the early stages of inflammation. These mediators act in synergy to modulate the protein synthesis, leading to an increase or decrease of these specific proteins, named Positive APP or Negative APP respectively. In cats, the most relevant positive APPs are Alpha-1-Glycoprotein (AGP) and Serum-Amyloid-A (SAA). Although a major positive protein in many species, C-Reactive Protein (CRP) is not well-studied in feline medicine.

Recombinant Feline Interferon-ω (rFeIFNω) is an immune-modulator commonly used in naturally retroviral infected cats which seems to potentiate the innate immune response. Our group has found that the above mentioned positive APPs (AGP, SAA and CRP) increase in FIV/FeLV cats during rFeIFNω therapy (unpublished data).As follow-up, this study aims to identify the most appropriate APP to predict a potentiated innate immune response.

Sixteen naturally retroviral infected cats (7 FIV, 6 FeLV and 3 Co-infected) were submitted to the licensed protocol of rFeIFNω therapy. A single-arm study was performed and blood samples were collected before (D0), during (D10, D30) and after therapy (D65, D200). D0 was considered the baseline value for each animal. APPs were measured at each time-point by specific methods (SAA: Phase SAA Multispecies/ Tridelta; CRP: Cat CRP ELISA/Kamiya Biomedical Company; AGP: Feline AGP, SRID, Tridelta).

From D0 to D65, serum levels of all the APPs increased (Friedman test $p < 0.05$). Five months after rFeIFNω therapy (D200), SAA and CRP values remained significantly higher than baseline (Friedman test $p1 < 0.05$). In marked contrast, AGP concentration increased with therapy dropping to the same level as baseline (D0) after five months of its withdrawal (D200) (Friedman test $p \approx 1$).

This study suggests that AGP seems to be the most appropriate APP to predict the innate-immune stimulation observed in naturally retroviral infected cats under rFeIFNω therapy.

Annexe VI: Abstract of the oral communication presented on the 22nd European European College of Veterinary Internal Medicine – Companion Animals Congress, Maastricht, September 2012

ABSTRACT: ACUTE PHASE PROTEINS: POTENTIAL PREDICTORS OF AN IMMUNE-MODULATION IN NATURAL RETROVIRAL-INFECTED CATS RECEIVING RECOMBINANT INTERFERON-OMEGA THERAPY. *Abstract published on Journal of Veterinary Internal Medicine, first published online : 20 NOV 2012, DOI: 10.1111/jvim.12000*

Leal R., Gil S., Sepúlveda N., McGahie D., Duarte A., Niza MMRE, Tavares L.

Acute phase proteins (APP) are considered one of the hallmarks of the inflammatory response. Among their major functions, APPs seem to modulate innate immune system efficiency. In cats, serum amyloid A (SAA) and α1- glycoprotein (AGP) are two major positive APPs that are increased during inflammation. This rise is presumed to be secondary to various cytokines that are involved in the innate inflammatory response. Recombinant Feline Interferon-ω (rFeIFN-ω) is an immune-modulator drug that is commonly used in cats naturally infected with retroviruses, namely feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV). Several studies have been performed to clarify the clinical benefits of rFeIFN-ω therapy in naturally infected FIV and/or FeLV cats. Our group has previously described that C-reactive Protein (CRP) increased in naturally retroviral-infected cats under rFeIFN-ω therapy. However, the role of APPs such as SAA, AGP and CRP in the innate immune-response, remains unknown. The aim of this study was to evaluate SAA, AGP and CRP serum levels in naturally retroviral-infected cats under rFeIFN-ω therapy.

Sixteen naturally retroviral infected cats (7 FIV, 6 FeLV and 3 co-infected FIV/FeLV stray cats) housed in a Lisbon Animal Rescue Shelter were submitted to rFeIFN-ω therapy. The licensed protocol was used: 3 courses of 1MU/kg SC administered once daily for 5 days, beginning on days 0, 14 and 60. Blood samples were collected for SAA, AGP and CRP quantification before, during and after treatment (at D0, 10, 30, 65). SAA was quantified by ELISA (Phase SAA, Tridelta) and AGP was determined by single radial immunodifusion (AGP,Tridelta). Feline CRP was quantified by ELISA (Kamiya Biomedical Company). APP serum levels were compared before and after rFeIFN-ω therapy. A statistically significant increase of SAA and AGP ($p=0.0005$ and $p=0.012$ respectively - Friedman test) was observed at D65 in comparison to D0. These findings corroborate the significant increase of CRP serum levels previously described ($p <0.0001$– Friedman test).

All the APPs tested behaved similarly, showing an evident increase in their serum values after rFeIFN-ω therapy. These results suggest a possible immune modulation effect induced by rFeIFN-ω which seems to maximize the efficiency of innate immune response. Further studies correlating these findings with the cytokine profile will extend our knowledge about the efficiency of rFeIFN-ω therapy in naturally retroviral infected cats.

Annexe VII: Abstract of the oral communication (in portuguese) presented on the III Encontro de Formação da Ordem dos Médicos Veterinários, Lisbon, October 2012

RESUMO: O PAPEL DO INTERFERÃO ÓMEGA FELINO (rFEIFNω) NO TRATAMENTO DE INFEÇÕES RETROVIRAIS: DA IMUNIDADE INATA À IMUNIDADE ADQUIRIDA.

R. LEAL, S. GIL, D. MCGAHIE, A. DUARTE, MMRE. NIZA, L. TAVARES

O rFeIFNω é um imunomodulador correntemente utilizado em medicina felina como coadjuvante terapêutico em infeções retrovirais nomeadamente pelo vírus da imunodeficiência (VIF) e/ou leucemia felina (VLF). Ao potenciar a imunidade inata, este fármaco possibilita uma maximização do sistema imunitário que se traduz num melhor controlo das infeções secundárias e na melhoria clínica dos animais.

Tendo como base as conclusões preliminares obtidas, este estudo visa correlacionar os efeitos clínicos do rFeIFNω com resultados laboratoriais, permitindo clarificar o seu efeito não só na imunidade inata como também na imunidade adquirida.

16 gatos infetados por retrovírus (7 FIV, 6 FeLV e 3 Co-infetados) foram monitorizados clinica e laboratorialmente antes, durante e após a terapêutica com rFeIFNω (D0, D10, D30, D65, D200).

Concluiu-se previamente que os animais melhoraram os sinais clinicos, tendo reduzido a excreção de vírus concomitantes aquando da terapêutica. Constatou-se ainda que os animais FIV positivos exibiram uma melhor e mais duradoura resposta clinica. De forma similar, os animais apresentaram um aumento dos níveis basais de diferentes proteínas de fase aguda, as quais são indicadores indiretos de estimulação da imunidade inata. Quanto ao proteinograma, enquanto os gatos FIV positivos revelaram um aumento das gama-globulinas, estas decresceram nos gatos FeLV.

Correlacionando os resultados preliminares obtidos, verifica-se que a melhoria observada dos sinais clínicos se associa com um aumento das proteínas de fase aguda e, no caso particular dos gatos FIV positivos, com uma hipergamaglobulinemia. Assim, além de maximizar a imunidade inata, o rFEIFNω poderá também potenciar a imunidade adquirida nestes animais, o que reforça o seu efeito benéfico em infeções por retrovirus. A correlação futura com o perfil de citocinas nestes animais permitirá aferir os mecanismos específicos de imunomodulação subjacentes ao tratamento com rFEIFNω.

Annexe VIII: Abstract of the oral communication presented on the 23rd European College of Veterinary Internal Medicine – Companion Animals Congress, Liverpool, September 2013

ABSTRACT: THE USE OF ORAL RECOMBINANT FELINE INTERFERON-OMEGA IN NATURALLY FELINE IMMUNODEFICIENCY VIRUS INFECTED CATS: NEW INSIGHTS INTO AN ALTERNATIVE IMMUNOMODULATION THERAPY *Abstract published on Journal of Veterinary Internal Medicine, first published online : 26 DEC 2013, DOI: 10.1111/jvim.12278*

Rodolfo Oliveira Leal, Solange Gil, David McGahie, Nuno Sepúlveda, Ana Duarte, Manuela Rodeia Niza, Luís Tavares

Recombinant-Feline Interferon-Omega is an immunomodulator drug often used in feline medicine. Although alternative oral trials have been successfully applied in some viral infections, only the licensed protocol has been recommended to Feline Immunodeficiency Virus (FIV) infected cats. This protocol has been shown to improve clinical signs, reduce concurrent viral excretion and increase levels of Acute Phase Proteins (APP). Despite these effects, its cost can be limiting and alternative protocols are required. This study aimed to evaluate the clinical improvement of naturally FIV-infected cats treated with an oral rFeIFN-ω protocol (0.1MU/cat per os daily for 90 days) in comparison to the licensed one (3 cycles of 5 subcutaneous daily injections at Day (D) 0, 14 and 60).

11 FIV naturally-infected cats were treated with oral rFeIFN-ω protocol (PO Group). 6 cats were indoor single-housed animals and 5 lived in a multi-cat/outdoor environment. Clinical signs were monitored at D0 (before therapy), 10, 30, 65 and 90 (end of therapy) using a previously validated score scale which included the most relevant signs for FIV infection. Results were compared to previous clinical data of 7 naturally FIV-infected cats, living in an animal shelter and treated with the licensed rFeIFN-ω protocol (SC Group). According to EMEA-guidelines, this group was considered an external positive control. For both groups, clinical improvement was classified as 'marked' (> 50% improvement of the initial score), 'mild' (up to 50% improvement), 'stable' (same final and initial score) or 'worse' (final score higher than the initial).

There was no difference between groups in the proportion of cats showing improvement (Pearson's Chi-square test adjusted for small samples; p= 0.95) or in the grade of clinical improvement (Pearson's Chi-square test; p= 0.23). In detail, 9/11 (82%) cats improved their overall scores with oral rFeIFN-ω therapy. Specifically: 3/11 (27%) showed a marked improvement, 6/11 (55%) a mild improvement and 2/11 (18%) remained stable. In the SC Group, 5/7 (71%) cats improved their overall score. In particular: 4/7 (57%) had marked improvement, 1/7 (14%) mild improvement and 2/7 (29%) remained stable. No worsening was observed in both groups.

Independently of the protocol applied, this study showed that rFeIFN-ω induced an overall significant clinical improvement of treated cats, supporting a potential immune-stimulation. Although the licensed protocol is better recommended in more symptomatic animals, in cases where cost might be an issue, oral rFeIFN-ω may be considered as an alternative therapy in the management of FIV-infected cats.

Annexe IX: Abstract of the oral communication (in portuguese) presented on the IV Encontro de Formação da Ordem dos Médicos Veterinários, Lisbon, October 2013

RESUMO: AVALIAÇÃO DA EXPRESSÃO DA PROTEÍNA MX EM GATOS NATURALMENTE INFECTADOS COM O VIRUS DA IMUNODEFICIÊNCIA FELINA E TRATADOS COM INTERFERÃO OMEGA ORAL

Rodolfo Oliveira Leal, Solange Gil, David Mcgahie, Ana Duarte, Manuela Rodeia, Luís Tavares

O Interferão omega felino (rFeIFNω) é o primeiro interferão (IFN) do tipo I licenciado para uso médico veterinário, sendo correntemente utilizado no tratamento de gatos infectados pelo vírus da imunodeficiência felina (FIV). Apesar dos benefícios clínicos induzidos pelo protocolo subcutâneo licenciado, o seu custo elevado é frequentemente limitativo. Recentemente alguns estudos sugerem a utilização de um protocolo oral alternativo (0.1MU/gato SID 90 dias). A administração oral de rFeIFNω não permite a sua detecção sérica pelo que todos os estudos de eficácia deste composto se baseiam na detecção de biomarcadores. Sendo a proteína Mx um biomarcador específico da acção de IFNs do tipo I, este estudo baseia-se na avaliação da sua expressão em gatos FIV-positivos tratados com rFeIFNω por via oral.

Sete gatos naturalmente infectados com FIV foram tratados com rFeIFNω oral. A expressão da Mx foi avaliada por quantificação relativa por PCR em tempo real a partir de sangue total colhido previamente (D0) e após o tratamento (D90).

Não se registaram alterações significativas ao nível da expressão da Mx em gatos tratados com rFeIFNω oral. Ao D0, a expressão da Mx foi residual em 4/7 gatos (os mais sintomáticos) tendo decrescido com o tratamento. Os restantes 3/7 gatos (menos sintomáticos) não revelaram expressão basal de Mx, tendo expresso valores residuais ao D90. Sendo um imunomodulador, a administração oral de rFeIFNω em gatos sintomáticos poderá reduzir a produção de citoquinas pró-inflamatórias, nomeadamente de IFN endógenos do tipo I, o que paradoxalmente resulta num decréscimo da expressão da Mx. Nos animais menos sintomáticos com estímulo pró-inflamatório menos exuberante, a terapêutica com rFeIFNω poderá induzir um aumento residual da expressão de Mx.

Este é o primeiro estudo que reporta a avaliação da expressão da proteína Mx em gatos naturalmente infectados com FIV e tratados com rFeIFNω oral.

Annexe X: Abstract of the oral communication presented on the British Small Animal Veterinary Association (BSAVA) Congress, Birmingham, April 2014

ABSTRACT: EVALUATION OF THE CYTOKINE PROFILE IN NATURALLY FELINE IMMUNODEFICIENCY VIRUS-INFECTED CATS TREATED WITH TWO DIFFERENT PROTOCOLS OF RECOMBINANT FELINE INTERFERON OMEGA

Rodolfo Oliveira Leal, Solange Gil, David Mcgahie, Nuno Sepúlveda, Ana Duarte, Manuela Rodeia, Luís Tavares

Recombinant Feline Interferon-Omega (rFeIFNω) is an immunomodulator licensed for Feline Immunodeficiency Virus (FIV) infections using 3 cycles of 5 daily subcutaneous injections of 1MU/kg on D0, 14 and 60. This compound seems to act on innate immunity as it induces a clinical improvement, reduces concurrent viral excretion and increases acute phase proteins without affecting parameters such as viremia or CD4/CD8 ratio. Recently, an alternative oral rFeIFNω protocol (0.1MU/cat daily for 90 days) was successfully applied to FIV-infected cats. However, studies relating these rFeIFNω protocols to cytokine profile variations are lacking. This study aimed to evaluate the mRNA expression of several cytokines (Interleukin (IL)-1, IL-4, IL-6, IL-10, IL-12p40, Interferon-gamma and Tumor-necrosis factor-α) and plasma levels of IL-6, IL-12p40 and IL-4 in naturally FIV-infected cats treated with the two rFeIFNω protocols.

18 naturally FIV-infected cats were enrolled: 7/18 received the licensed protocol (SC group) while 11/18 received the oral protocol (PO group). Animals were monitored before (D0) and after therapy (D65 and D90, respectively for SC and PO groups). Cytokine expression was assessed by relative quantification using Real-Time PCR. Plasma levels of IL-6, IL-12p40 and IL-4 were measured with specific ELISA kits.

Cytokine expression was low, and similar in both groups, on D0. Despite an overall decreasing tendency, no significant changes were observed except for IL-6, a pro-inflammatory cytokine that significantly decreased in the PO group ($p=0.037$). Regarding plasma levels, circulating IL6 remained stable on the PO group ($p=0.087$) but significantly decreased in the SC group ($p =0.031$). No statistical differences were noted for the plasma IL-12p40 and IL-4 after either protocols. These results showed that serum IL6 levels decreased in the SC group, even if its expression was not apparently affected. This may be due to the higher pulsate doses which seem to be quickly effective for reducing pro-inflammatory stimuli. However, in the PO group, IL-6 expression significantly decreased but this was not reflected in the plasma levels. This suggests that an oral rFeIFN protocol, based on continuous low doses for a longer period, may also act on the inflammatory cascade, decreasing IL6 expression but not sufficiently to produce a statistically significant decrease in its plasma concentration.

As IL-6 is a pro-inflammatory cytokine involved in the innate response, this study corroborates with previous suggestions that rFeIFNω therapy seems to primarily act on the innate immune response.

Annexe XI: Abstract of the oral communication (in portuguese) presented on the IX Congresso Hospital Veterinário Montenegro, Oporto, February 2013

RESUMO: O USO DE INTERFERÃO ÓMEGA FELINO NO MANEIO DE GENGIVOESTOMATITE CRÓNICA EM GATOS DIABÉTICOS: A PROPÓSITO DE 2 CASOS CLÍNICOS

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O interferão ómega felino (rFeIFN-ω) é um fármaco frequentemente utilizado devido às suas propriedades anti-virais e imuno-moduladoras. Em medicina felina, são vários os estudos que descrevem o seu uso em diferentes protocolos, nomeadamente em infeções por retrovirus, herpesvirus e calicivirus. As gengivoestomatites secundárias a calicivirus são frequentes em gatos. Ainda que controverso, o tratamento com corticosteroides permite melhorias temporárias das lesões, pelo que o seu uso é frequente na prática clínica. No entanto, a sua administração é um dos fatores predisponentes ao desenvolvimento de *diabetes mellitus* (DM).

Dois gatos com 14 e 15 anos foram avaliados pelo serviço de Endocrinologia para monitorização de DM. Ambos apresentavam como história pregressa gengivoestomatite crónica, secundária a calicivirus e à subsequente administração de corticosteroides por períodos intermitentes. A insulina glargina foi instituída no momento do diagnóstico da DM em um dos gatos. Neste animal, o tratamento da gengivoestomatite foi iniciado com antibioterapia (cefovecina) e com anti-inflamatórios não esteroides aquando das agudizações. Após uma monitorização estrita semanal, o gato entrou em remissão clínica da DM nas 10 semanas subsequentes. Tendo em conta a persistência de gengivoestomatite, foi iniciado rFeIFN-ω (0.1MU/gato PO SID), o qual até à última avaliação (6 meses depois) permitiu uma melhoria clinica da gengivoestomatite sem necessidade de terapêutica concomitante. No outro gato, a DM tinha sido diagnosticada há 4 semanas, data em que se iniciou insulinoterapia (insulina lenta). Devido à persistência de hiperglicemia e difícil monitorização no primeiro mês de tratamento, substitui-se por insulina glargina. Para controlar a gengivoestomatite persistente neste animal, foi iniciado rFeIFN-ω (0.1MU/gato PO SID). Após 4 semanas, apesar de não ter existido remissão, o animal apresentava uma melhoria significativa da gengivoestomatite assim como dos níveis de glicémia.

Reconhecendo o seu efeito benéfico como imuno-modulador e os resultados anteriormente descritos, o rFeIFN-ω deverá ser considerado uma alternativa terapêutica no maneio da gengivoestomatite crónica em gatos diabéticos, situação em que os corticoesteroides estão contra-indicados.