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EMERGING PATHOGENS AND CHRONIC KIDNEY DISEASE IN CATS

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ABBREVIATIONS

Ab:	Antibodies
ALB:	Albumin
ALKP:	Alkaline phosphatase
ALT:	Alanine aminotransferase
APP:	Acute phase proteins
AST:	Aspartate aminotransferase
ATL:	Animal Tissue Lysis
BCS:	Body condition score
BP:	Blood pressure
BUN:	Urea
CBC:	Complete Blood Count
CDV:	Canine distemper virus
CEUS:	Contrast-enhanced ultrasound examination
CHr:	Reticulocyte haemoglobing content
C.I:	Confidence interval
CKD:	Chronic kidney disease
CMV:	Cetacean morbillivirus
CPE:	Cytophatic effect
CRFK:	Crandell-Rees feline kidney
CRP:	C-reactive protein
Ct:	Cycle threshold
DAT:	Direct agglutination test
DLH:	Domestic longhair
DSH:	Domestic shorthair
ELFA:	Enzyme linked fluorescent assay
ELISA:	Enzymelinked immunosorbent assay
EPO:	Erythropoietin
FAM:	Carboxyfluorescein
FCoV:	Feline Coronavirus infection
FCS:	Fetal Calf Serum

FCV:	Feline Calicivirus
FEA:	Feline Embryonic Fibroblast
FeL:	Feline leishmaniosis
FeLV:	Feline Leukemia Virus infection
FIP:	Feline Infectious Peritonitis
FIV:	Feline Immunodeficiency Virus
FeMV：	Feline Morbillivirus
FNA:	Lymph node aspirates
GFR:	Glomerular filtration rate
GGT:	Gamma glutamyl transferase
GI:	Gastrointestinal
GLOB:	Globulins
HAC:	Hyperadrenocorticism
HCM:	Hypertrophic cardiomyopathy
HCT:	Hematocrit
Hgb:	Hemoglobin concentrations
HRE:	High resolution electrophoretic technique
iCa:	Active ionized calcium fraction
IFI:	Indirect immunofluorescence assay
IgG:	Immunoglobulin G
IRIS:	International Renal Interest Society
KD:	CKD group
LAI:	Leukocyte alterations suggestive of inflammation
LVH:	Left ventricular concentric hypertrophy
MAT:	Microscopic agglutination test
MCHC:	Mean corpuscolar hemoglobin concentration
MCS:	Muscle condition score
MCV:	Mean corpuscolar volume
MEM:	Minimum Essential Medium
MGG:	May Grünwald-Giemsa
MMP-9:	Matrix metalloproteinase-9
MV:	Measles virus

NI:	Normal intervals
NKD:	Not kidney diseases group
NP:	Non proteinuric
NRA:	Non regenerative anemia
OR:	Odds Ratio
PHA:	Hyperaldosteronism
PBS:	Phosphate buffered saline
PDV:	Phocine distemper virus
PHOS:	Phosphorus
PL:	Platelete
PPRV:	Peste-des-petits-ruminants virus
PTH:	Parathyroid hormone
qPCRFeMV:	Real-time RT-PCR for FeMV
RA:	Regenerative anemia
RAAS:	Renin –angiotensin–aldosterone system
RBC:	Red blood cell count
RI:	Arterial resistive index
RPV:	Rinderpest virus
RT-PCR:	Reverse transcriptase-PCR
SAA:	Serum amyloid A
SBP:	Systolic blood pressure
SCr:	Serum Creatinine
SDMA:	Serum symmetric dimethylarginine
SDS-AGE:	Sodium dodecyl sulfate-agarose gel electrophoresis
SDS-PAGE:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPE:	Serum protein electrophoresis
SSA:	Sulfosalicylic acid
TAMRA:	Tetramethylrhodamine
tCa:	Total calcium concentration
TGF-β1:	Transforming growth factor-β
TIN:	Tubulointerstitial nephritis
TOD:	Target organ damage

TP:	Total proteins
TPCK:	L-1Tosylamide-2-phenylethylchloromethylketone-treated
tT4:	Total thyroxine
UPC:	Urinary protein/creatinine ratio
USG:	Urine specific gravity
WB:	Western blotting
WBC:	Leukocyte

CHAPTER 1
GENERAL INTRODUCTION

Chronic kidney disease (CKD) is a common condition in cats defined as a multifactorial pathophysiologic process resulting in progressive loss of nephrons in both number and function, frequently culminating in end-stage renal disease (Khan and Khan et al., 2015). The term CKD is used to imply alterations in structure or function of the kidney that have occurred over a period of time, typically 3 months or more (Jepson, 2016). Prevalence of CKD in cats is high, with a 50% prevalence reported in a randomly selected population of cats (Marino et al., 2014). Median survival time recently reported was of 388 days, and previous studies reported median survival time from 21 to 1151 days depending on severity of renal dysfunction at diagnosis (Conroy et al., 2019). Causes of CKD are heterogeneus and most often not identified. Some risk factors such as genetic or acquired diseases can initiate renal damage (Reynolds and Lefebvre, 2013) and after the initial renal insult continues advancing with irreversible morphologic changes in renal parenchyma, including nephron loss and replacement by a selfperpetuating vicious cycle of fibrosis, lead to the progression of CKD (Khan and Khan et al., 2015; Chakrabarti et al., 2013). Considering the frequency and severity of this disease in cats, the evaluation of the possible influence that some risk factors and infectious diseases can have in the development of CKD is important. This awareness could be useful to avoid the development or at least slow progression of the disease. For this reason studies about epidemiology of infectious diseases and their clinical consequences as well as their relationship with CKD are constantly evolving.

1.1 NON-INFECTIOUS DISEASES AND CHRONIC KIDNEY DISEASE

Development of CKD is associated with some risk factors. Older cats seem to have more frequent renal disease (Trevejo et al., 2018) with an increased prevalence among cats older than 15 years (Marino et al., 2014) and a mortality rate greater in cats over

9 years old (Lawler et al., 2006). Cats can develop different age-related pathologies including renal inflammation and interstitial fibrosis, indeed, according to previous studies older cats were shown to have increased production of pro-inflammatory cytokines (Day, 2010). Moreover renal damage has a slow progression, with progressive and irreversible damage to kidneys (Reynolds and Lefebvre, 2013), and this could explain why CKD can be found more frequently in older cats rather than in young cats. A retrospective case-control study about association of gender and diagnosis of CKD in cats revealed that neutered males were 30% more likely than spayed females to develop CKD, probably because male cats tend to develop urethral obstruction and subsequent renal damage more easily than female cats (Greene et al., 2014). Breeds such as Siamese, Persian, Abyssinian, Himalayan, Maine Coon, Russian Blue, Burmese are reported to be more likely affected by CKD (Boyd et al., 2008; Trevejo et al., 2018; Conroy et al., 2019). Common congenital kidney disease, such as polycystic kidney disease (Persian and Persian-cross cats); familial secondary amyloidosis and glomerular disease (Abyssinian cats) (Lees, 1996; Bosje et al., 1998; Greco, 2001; White et al., 2008), juvenile renal dysplasia (Aresu et al., 2009; Greco, 2001) can be responsible for the development of CKD in pure breed individuals more frequently than in the general population.

Renal hypoperfusion, sympathetic nervous system activation and increased activity of the renin-angiotensin system observed in cats with hypertrophic cardiomyopathy (HCM) may lead to some degree of renal dysfunction. The prevalence of CKD reported in cats with HCM is 13% (Gouni et al., 2008) and the prevalence of left vetricular hypertrophy in cats with CKD has been estimated to be 46.6% (Taugner, 2001). However, further investigations are needed to document the pathophysiology of concomitant CKD and cardiac disease, mainly to understand if cardiovascular diseases are a consequence or a risk factor of CKD.

Primary hyperaldosteronism is considered a possible risk factor for the progression of CKD with kidney histopathological changes reported such as hyaline arteriolar sclerosis, glomerular sclerosis, tubular atrophy and interstitial fibrosis (Javadi et al., 2005).

Hyperthyroidism and CKD can occur concurrently in old cats (van Hoek et al., 2009). Thyroid hormones determine an increased renal blood flow because of an

increased cardiac output due to positive chronotropic and inotropic effects, decreased vascular resistance and also increased blood volume caused by renin-angiotensin-aldosterone system (RAAS) activation (van Hoek and Daminet, 2009). Consequences of hyperthyrodism are therefore increased glomerular filtration rate (GFR), decreased urine specific gravity (USG) and increased urinary protein/creatinine ratio (UPC) (Langston and Reine, 2006).

A positive association between urolithiasis and CKD in cats has been suggested, with a 56% prevalence of CKD among feline patients with urolithiasis. Nephrolithiasis and ureterolithiasis can potentially cause intermittent obstruction, which could lead to nephron damage and development of CKD, for this reason cats with urolithiasis should be evaluated for CKD (Cléroux et al., 2017). At the same time urolith formation could result from underlying CKD, leading to abnormal handling of certain minerals and metabolites (Cléroux et al., 2017; Kyles et al., 2005).

Kidney tumors may also cause CKD with 60% of cats with renal lymphoma found to have azotemia (Gabor et al., 2000). Lymphoma is the most common tumor in cats. Other primary renal tumors described in cats are carcinomas, transitional cell carcinomas, malignant nephroblastoma, haemangiosarcoma and adenoma (Henry et al., 1999).

Periodontal disease may contribute to the development of CKD (Finch et al., 2016; Greene et al., 2014) as a consequence of persisten-low grade insults. Production of inflammatory cytokines, endotoxemia and an immune response to bacteria may have a role in the development of CKD. However, also infectious agents associated with gingivitis, such as feline immunodeficiency virus (FIV) (Hosie et al., 2009), and management of dental disease (use of antibiotics, steroidal or nonsteroidal anti-inflammatory drugs, or general anesthesia required for dental procedures) may be confounders having a role in development of kidney disease (Finch et al., 2016).

Frequent or annual vaccinations are reported as potential risk factors for development of CKD (Finch et al., 2016). Vaccinal viruses feline herpesvirus 1, calicivirus and panleukopenia virus are cultured using Crandell-Rees feline kidney (CRFK) cells. CRFK proteins may become incorporated into vaccines during manufacture. In a previous study, parenteral administration of vaccines containing viruses likely grown on CRFK cells induced antibodies against CRFK cell and feline

renal cell lysates in cats, however hypersensitization with CRFK cell proteins did not result in renal disease in cats during the study. However in this study renal biopsies were collected just six weeks after the last vaccination or CRFK sensitization and a possible transient inflammation of renal tissues could not be excluded (Lappin et al., 2005). Moreover a later study reported lymphocytic-plasmacytic interstitial nephritis in a cat sensitized with CRFK lysate (Lappin et al., 2006).

Aminoglycosides, non-steroidal anti-inflammatory drugs, antineoplastic agents or exposure to toxins (lily or ethylen glycol poisoning are the most common) could cause acute kidney injury (AKI) with potential risk of development of CKD with time in cats overcoming the acute damage (Reynolds and Lefebvre, 2013). Anesthesia has also been considered a risk factor for AKI because of the risk of renal hypoperfusion particularly in cases of deep level of anesthesia and long lasting surgical procedures or concurrent risk factors such as dehydration, and use of amynoglycosides (Greene et al., 2014).

Concerning the role of diet type as a risk factor for CKD, in a recent study there was no difference in the rate of development of azotemia in cats fed standard adult diets compared to senior diets suggesting no effect of protein content on the development of azotemia (Finch et al., 2016). Also the moisture content of the diet fed (wet or dry diet) do not seem to be a significant risk factor (Finch et al., 2016; Greene et al., 2014). Ad libitum feeding and increased ash intake were found associated with increased risk of CKD (Hughes et al., 2002). The hypothesis that chronic increases in dietary salt intake could damage renal function in older cats was however not supported by a recent study where glomerular filtration rate (GFR), blood pressure, and other routine clinical pathological variables in healthy aged cats were not affected by dietary salt content (Reynolds et al., 2013).

1.2 INFECTIOUS AGENTS AND CHRONIC KIDNEY DISEASE

The relationship between some infectious agents and CKD in cats has already been documented, such as with feline immunodeficiency virus infection (FIV), feline leukemia virus infection (FeLV) and feline coronavirus (FCoV) infections (Rossi et al., 2019). Other infectious disesases that in dogs have already been linked to

development of CKD, in cats are currently being studied, with some cases reported as caused by *Leismania* or *Leptospira* spp infection. Moreover, recently, a relationship between the infection caused by a new feline paramyxovirus and development of CKD has been proposed and is the subject of study of numerous researches.

Feline morbillivirus (FeMV)

Paramyxoviruses are enveloped, negative-sense single-stranded RNA viruses which act as important pathogens for humans and animals. In 2012 a new paramyxovirus, feline morbillivirus (FeMV), was isolated for the first time from cats in Hong Kong and it was associated with tubulointerstitial nephritis (TIN) (Woo et al., 2012). After this discovery, different studies evaluated and reported its presence in Japan, Germany, Italy, USA, South America, Turkey and UK (Park et al., 2016; Koide et al., 2015; Sieg et al., 2015; Lorusso et al., 2015; Donato et al., 2018; Sharp et al., 2016; Darold et al., 2017; Yilmaz et al., 2017; McCallum et al., 2018) documenting that the virus can be found in both healthy and sick cats, with a prevalence ranging from 3% to 52.9% (Beatty et al., 2019). Feline morbillivirus is genetically related to viruses which belong to the genus morbillivirus, such as canine distemper virus (CDV), measles virus (MV), rinderpest virus (RPV), peste-des-petits-ruminants virus (PPRV), phocine distemper virus (PDV) and cetacean morbillivirus (CMV) (Park et al., 2016). Feline morbillivirus is characterized by genetic diversity among isolates (Sakaguchi et al., 2014; Sieg et al., 2015; Park et al., 2016). Stability at +4°C for at least 12 days is reported, freeze-and-thaw seems not to affect virus titers, while heat-treatment at +60°C and +70°C inactivated FeMV in 10 and 2 minutes, respectively (Koide et al., 2016).

Some studies reported the highest rate of positive reverse transcriptase-PCR (RT-PCR) tests from urine of cats coming from multicat environments (De Luca et al., 2017; Darold et al., 2017) suggesting that close contact favours transmission as expected for an enveloped virus which is easily inactivated in the environment. Cats from suburban/rural areas and those with outdoor access seem to be more exposed to FeMV infection (Donato et al., 2018; Yilmaz et al., 2017).

The relationship with CKD is controversial. Some studies reported in infected cats the development of an intestitial inflammatory infiltrate, tubular

degeneration/necrosis, glomerulosclerosis (Woo et al., 2012; Park et al., 2016; Yilmaz et al., 2017; Sutummaporn et al., 2019), and shedding of the virus in urine for up to 2 years (Sieg et al., 2019), therefore a chronic infection leading to CKD is supposed to affect cats (Lorusso et al., 2015; Sieg et al., 2015). However this relationship was not found in other studies which evaluated the presence of the infection in azotemic cats with confirmed CKD (Darold et al, 2017; Lo Russo et al., 2017; McCallum et al., 2018; Donato et al. 2018). A study which evaluated both clinico-pathological and histopatological abnormalities in FeMV RNA-positive cats found tubulointerstitial nephritis but also cholangiohepatitis and hepatic focal necrosis, however these abnormalities were similar to those observed in FeMV RNA-negative cats (Yilmaz et al., 2017). In addition to the evaluation of hepatic involvment of FeMV in cats, a recent study reported susceptibility of primary feline pulmonary epithelial cells and primary cells from the cerebrum and cerebellum, as well as immune cells in the blood, especially CD4+ T cells, CD20+ B cells and monocytes to *in vitro* experimental infection (Sieg et al., 2019) confirming that FeMV is able to infect different feline cell lines: epithelial, fibroblastic, lymphoid and glial cells. Moreover, the FeMV cell receptor is ubiquitously expressed in cats (Sakaguchi et al., 2014). In conclusion, the impact of feline morbilliviruses on cat health has to be confirmed by further research to understand their role in CKD development and potential damage to other organs.

Diagnosis of FeMV infection can be performed through molecular and serological investigations. Detection of virus RNA in urine (Woo et al., 2012; Lorusso et al., 2015; Sieg et al., 2015; Sharp et al., 2016; Darold et al., 2017; Yilmaz et al., 2017; McCallum et al., 2018), renal tissue (Park et al., 2016), blood and rectal swabs (Woo et al., 2012) with RT-PCR is reported. Anti-FeMV antibody detection by indirect immunofluorescence assay (IFI), (Park et al., 2016), enzymelinked immunosorbent assay (ELISA), (Arikawa et al., 2017) or western blotting (WB) are reported (Woo et al., 2012; McCallum et al., 2018). Histopathology, immunohistochemistry (Woo et al., 2012; Park et al., 2016; Yilmaz et al., 2017) and viral culture (Woo et al., 2012; Koide et al., 2015) are also available and useful for further investigations. A better understanding of the basic biology of the virus is required and could be performed through a cooperation of molecular virology and veterinary medicine (Beatty et al., 2019).

***Leptospira* spp.**

Leptospirosis is caused by an infection of spirochetal bacteria which belong to the genus *Leptospira.* They are Gram-negative, highly motile, elongated, helically coiled bacteria characterized by hook or question mark–shaped ends (Schuller et al., 2015). Taxonomy of the genus *Leptospira* is complex and leptospires are serologically classified by serovar (member of the genus *Leptospira*, which reacts with a specific monoclonal antiserum), serogroup (group of antigenically closely related leptospiral serovars) and strain (specific isolate of a defined leptospiral serovar) (Schuller et al., 2015). At present, there are over 250 pathogenic serovars (Hartmann et al., 2013a) which belong to 24 serogroups (Schuller et al., 2015).

Cats can be infected by feeding on animals harbouring leptospires or after exposure to infectious urine of cohabiting dogs (Hartmann et al., 2013a). Antibody prevalence was observed for cats with an outdoor lifestyle or those living in multicat households (Rodriguez et al., 2014). As observed in dogs, seasonality of leptospirosis was reported also for cats with a higher prevalence during most humid months (Rodriguez et al., 2014; Arbour et al., 2012). Serovars identified in cats include *L.* icterohaemorrhagiae, *L.* canicola, *L.* grippotyphosa, *L.* pomona, *L.* hardjo, *L.* autumnalis and *L.* ballum with a range of antileptospiral antibody prevalence between 0 and 35% reported (Hartmann et al., 2013a). In cats pathogenesis seems to be similar to that observed for dogs and humans, characterized by a systemic infection with invasion of many organs (kidneys, liver, spleen, central nervous system, eyes and genital tract) causing inflammation and tissue damage. Thanks to the adaptive humoral and cell-mediated immune response leptospires are removed from most organs except the kidneys, in which leptospires can persist with resultant chronic shedding (Hartmann et al., 2013a; Zuerner, 2015).

Clinical signs in infected cats seem to be rare, even if some have been reported manifesting in about 84 days in experimental studies or after few months in some reported cases after contact with possible animals harbouring leptospires or their urine (Arbour et al., 2012). Few studies have reported ascites, hepatomegaly (Agunloye and Nash, 1996) with severe centrilobular necrosis of the liver (Bryson and Ellis, 1976), vascular lesions in lung and brain with isolation of leptospires from thoracic fluid and

aqueous humour (Bryson and Ellis, 1976), ocular pathologies, particularly uveitis, and lameness (Arbour et al., 2012). Different studies reported that cats can shed leptospires in urine (Sprißler et al., 2019; Weis et al., 2017; Rodriguez et al., 2014), underlining their potential role as reservoirs or incidental hosts in transmission (Hartmann et al., 2013a). Chronic kidney disease in infected cats was also reported in some case reports (Arbour et al., 2012; Mason et al., 1972) with manifestation of polyuria and polydipsia (Arbour et al., 2012) and tubulointerstitial nephritis as histopathological findings (Arbour et al., 2012). However results from studies that evaluated serological evidence of exposure of cats to *Leptospira* spp., and kidney disease are conflicting (Shropshire et al., 2016: Rodriguez et al., 2014) and additional studies are needed to determine which is the real role of leptospirosis in the development of feline CKD.

Serological and molecular diagnosis can be respectively performed through microscopic agglutination test (MAT) or ELISA and PCR. Microscopic agglutination test is the most common diagnostic method used for antibody detection (Hartmann et al., 2013a) and it is based on determining the ability of serial dilutions of patient serum to agglutinate live leptospiral serovars in vitro. Positive MAT confirms the exposure to a serovar belonging to the corresponding serogroup (but not necessarily to the serovar tested) (Schuller et al., 2015). PCR targets the lipL32/hap1 gene or 23S rDNA, however a negative result on blood or urine does not rule out leptospirosis: leptospiraemia is transient (early stages of the disease), urinary shedding is delayed after acute infection and can be intermittent, moreover recent antibiotic treatment can cause negative results (Schuller et al., 2015). Other diagnostic methods are darkfield microscopy to identify entire leptospires in urine (poor sensitivity and specificity), and culture of biological samples (blood, urine, tissues) even if culturing leptospires is difficult and requiring up to six months of culture of fresh urine (Schuller et al., 2015).

Leishmania infantum

Feline leishmaniosis (FeL) is an emerging disease (Pennisi and Persichetti, 2018) determined by flagellated protozoan parasites of *Leishmania* genus (Pennisi et al., 2013a). In 1912 FeL was reported for the first time in Algeria (Soares et al., 2016) and since then it has been globally reported in endemic areas for canine leishmaniosis. *Leishmania infantum* is the most frequently detected species in both the New and Old

World but *Leishmania amazonensis, Leishmania braziliensis, Leishmania mexicana,* and *Leishmania venezuelensis* are also reported in the New World (Pennisi et al., 2013a; Pennisi et al., 2015; Soares et al., 2016; Pennisi and Persichetti, 2018) and infections by *Leishmania tropica* and *Leishmania major* were found in Turkey (Can et al., 2016; Paşa et al., 2015). Many studies investigated prevalence of anti-*L. infantum* antibodies or parasite DNA with ranges varying from 0 to 68.5% and from 0 and 60.7% respectively. This variability may be the consequence of different levels of endemicity in the investigated areas but, many factors may contribute such as the characteristics of the population under study or differences in diagnostic methodologies including the cut-off titres of antibody detection (Pennisi et al., 2015).

Transmission of *Leishmania* spp. was not investigated in cats but it is believed to occur by sand fly bites as for other hosts. In fact, it is well known that sand flies feed on cats and they were found infected after feeding on cats with FeL (Pennisi et al., 2015; Maroli et al., 2013). Blood transfusion could also be a source of infection in cats as it is in dogs and humans therefore feline blood donors should be tested in *L. infantum* endemic areas in order to exclude subclinical infections (Pennisi et al., 2015; Pennisi and Persichetti, 2018). The cat immune response to *L. infantum* infection was scarcely investigated. In dogs progressive infection and development of lesions and clinical signs are linked to an impaired Th1 immune response but a pan-T cell exhaustion is observed early in some dogs before the onset of clinical disease (Boggiatto et al., 2010; Esch et al., 2013). Cats from endemic areas produce IFNγ and are therefore able to activate a cell-mediated adaptive immune response against the parasite that is variably associated with antibody or blood PCR positivity (Priolo et al., 2019). However at present we do not have data about correlation between progression of the infection and characteristics of the feline immune response.

The most common clinical findings reported in FeL are skin or mucocutaneous lesions (ulcerative, crusty, nodular or scaly dermatitis, alopecia, poor coat condition), lymph node enlargment, ocular lesions (uveitis, nodular blepharitis and panophthalmitis), chronic gingivostomatis, hepatomegaly, spleen enlargment and non specific signs are also reported (weight loss, reduced appetite, dehydration, pale mucous membranes, fever, jaundice, polyuria/polydipsia, lethargy). Rare clinical manifestations include chronic nasal discharge and obstruptive upper respiratory tract

disease due to granulomatous rhinitis (Leal et al., 2018; Altuzarra et al., 2018). The more frequent clinicopathological abnormalities include mild to severe normocytic normochromic non-regenerative anemia, moderate to severe pancytopenia, lymphocytosis, hyperglobulinaemia and gammopathy, hypoalbuminemia, renal proteinuria, increased serum creatinine and increased serum alanine aminotransferase (ALT) (Pennisi et al., 2013a; Pennisi et al., 2015; Soares et al., 2016). Less information is available about renal involvement, even if in some clinical cases glomerular disease and chronic renal failure have been reported (Pennisi et al., 2013a). In a study conducted in 2004 four cats affected by FeL, followed up until their death or euthanasia, developed CKD progressively (Pennisi et al., 2004). In 2008 a case of *Leishmania* infection in a cat was reported with a positive PCR and isolation of *Leishmania* spp. from kidney (Caracappa et al., 2008). In 2010 histopathological lesions were investigated in 15 cats with leishmaniosis and one cat showed kidney moderate interstitial inflammatory infiltration composed predominantly of macrophages with some lymphocytes and plasma cells. Moderate interstitial fibrosis and tubular proteinuria were also present (Navarro et al., 2010). In 2016 in another study conducted in 14 followed up cats (four of them reported in the Pennisi et al., 2004 study) affected by FeL, 8 cats were affected by CKD with 3 cats in IRIS CKD stage 1, 4 cats in IRIS CKD stage 2 and one cat in IRIS CKD stage 4 (Pennisi et al., 2016). In another study diagnosis of FeL was performed by histopathology, immunohistochemistry and PCR and histopathology revealed a granulomatous nephritis with lymphoplasmacytic aggregates and macrophages containing some amastigotes (Puleio et al., 2011).

Diagnosis is performed by direct detection of *Leishmania* amastigotes in infected feline macrophages (rarely in circulating neutrophils) in smears from lymph nodes, bone marrow, skin, mucosal or eye lesions; histopathology with immunohistochemistry of lesions; molecular investigations of *Leishmania* DNA performed on EDTA-blood or other tissues (lymph node, bone marrow, skin) and non-invasive sampling (conjunctival or oral swabs), (Navarro et al., 2010; Pennisi and Persichetti, 2018; Pennisi et al., 2013a; Pennisi et al., 2015; Soares et al., 2016). Anti-*Leishmania* antibody detection is extensively used in cats, particularly IFI with a cut off established at 1:80 dilution (Pennisi et al., 2012), ELISA, direct agglutination test

(DAT) and WB techniques (Pennisi and Persichetti, 2018; Pennisi et al., 2013a; Pennisi et al., 2015; Soares et al., 2016) with WB offering the best sensitivity and specificity (Persichetti et al., 2017).

Feline immunodeficiency virus (FIV)

Feline immunodeficiency virus is an enveloped RNA virus, belonging to the family Retroviridae, subfamily Lentiviridae, group of viruses known to cause life-long infections with protracted incubation periods (Norris et al., 2007). Lentiviruses are complex retroviruses characterized by different genes, some of them responsible for their virulence and diversity: *gag* gene encodes the capsid protein p24 (important for diagnosis), *pol* gene encodes protease, integrase and reverse transcriptase proteins and enzymes determining the virulence of FIV, *env* gene encodes the viral glycoprotein (gp120) and the transmembrane protein (gp41) responsible for viral diversity among isolates. Five genetically distinct subtypes have been defined (A to E) and subtype A and B are the most frequently identified (Hosie et al., 2009).

Biting is the principal route of FIV transmission between cats and pregnant queens in the acute phase of infection may transmit virus to their offspring during the prenatal and postnatal periods. A potential way for transmission could be intravenous, subcutaneous or intraperitoneal inoculation of blood products from infected cats (Norris et al., 2007).

Initially, viraemia with non specific signs, peripheral lymphadenopathy is present (duration weeks to months), followed by an asymptomatic phase (duration many years), while the final phase is charatcterized by viral replication with clinical disease, in part due to a CD4+ lymphopenia and immunodeficiency syndrome (Westman et al., 2019). Clinical signs frequently reported in infected cats include periodontitis, gingivitis, stomatitis, rhinitis, anemia, lymphadenopathy, myeloproliferative disorders, diarrhea, dermatitis, emaciation, central nervous system involvment, neuropathy and reproductive failure (Poli et al., 1993; Hosie et al., 2009). Laboratory findings frequently observed in FIV infected cats are anemia, cytopenia with neutropenia and lymphopenia, thrombocytopenia and hypergammaglobulinemia (Gleich and Hartmann, 2009; Collado et al., 2012). A significant relationship exists between CKD and FIV infection (White et al., 2010). Infected cats often present with

hypergammaglobulinemia that is believed to be due to chronic polyclonal B cell activation, with production of auto-antibodies and immune complexes (Poli et al., 1993; Pennisi et al., 1994). In FIV-infected cats azotemia, proteinuria, low USG and renal ultrasonographic abnormalities such as hyperechoic cortices and renomegaly were reported (Poli et al., 1993; Poli et al., 1995; Asproni et al., 2013; Baxter et al., 2012; Taffin et al., 2017). Histopathology revealed segmental glomerulosclerosis, glomerular capillary collapse, increased mesangial matrix, tubular dilatation and amyloid deposits with both medullary and glomerular location (Poli et al., 1993; Poli et al., 1995; Poli et al., 2012; Asproni et al., 2013) and FIV antigen (protein 24) was detected within tubular, glomerular, or interstitial cells (Poli et al., 1995). Recently, diagnosis of immune-complex glomerulonephritis was found associated with FIV infection (Rossi et al., 2019).

Diagnosis of FIV infection is routinely performed by rapid tests (ELISA or immunochromatographic techniques) that detect antibodies against viral structural proteins (the capsid protein p24 and a gp41 peptide). Western blot analysis is however considered the 'gold standard' for anti-FIV antibody detection. Indeed, the diagnostic specificity of ELISA and immunochromatography tests is below 100%, therefore any positive result in a low-prevalence population (young, indoor, pedigree cats) must therefore be confirmed for example, by Western blot. A positive result in a cat from a high-risk group (free-roaming, aged, entire male) is likely to be correct, because the frequency of true positives will exceed that of false positives in this group. Proviral DNA can be evidenced through EDTA-blood PCR, however its performance may be inferior to serological tests, with sensitivities and specificities ranging from 40 to 100%. In infected cats staging of the level of immune dysfunction is determined by counting CD4+ and CD8+ blood lymphocyte subpopulations even if these last two methods are not used routinely (Hosie et al., 2009).

Feline leukemia virus (FeLV)

Feline leukemia virus belongs to the genus *Gammaretrovirus*. There are three major subgroups of FeLV: A, B and C and FeLV-A is the most abundant subtype, responsible for transmission of the virus between animals. Subgroups B and C arise in subgroup A infected cats following the establishment of viremia (Willet and Hosie, 2013).

Horizontal and vertical transmission can both occur (Willis, 2000). Oronasal exposure to virus-containing secretions is the primary way of transmission and high levels of FeLV are present in the saliva of viraemic cats. Intimate contact between animals during grooming, sharing feeding bowls or fightning are the most likely routes of transmission and young age, high population density, poor hygiene are important risk factors (Lutz et al., 2009; Willet and Hosie, 2013). In addition to saliva and nasal secretions viraemic cats can shed the virus also in faeces and milk. In pregnant queens, viraemia usually leads to embryonic death, stillbirth or viraemic kittens, which will fade rapidly. In latently infected (cats with proviral DNA in bone marrow stem cells, as described later) queens, virus is usually not transmitted to the fetuses, but transmission can take place from individual mammary glands, where sequestered virus remains latent until the mammary gland develops during the last period of pregnancy (Lutz et al., 2009).

Stages of FeLV infection are influenced by feline immune response. The infection is characterized by an initial replication of the virus in the local lymphoid tissue in the oropharyngeal area. In immunocompetent cats viral replication may be avoided by an effective immune response without development of viremia (FeLV antigen, viral RNA or proviral DNA cannot be detected) and these cats have just an abortive infection. After local initial infection, FeLV disseminates through infected mononuclear cells. Cats have positive results on tests that detect free antigen in blood and they shed the virus. This viremia can last for weeks or months (transient viremia) but these "regressor" cats do not always eliminate the virus with time from the body, and proviral DNA is present in bone marrow stem cells as latent infection, however they do not shed the virus. In the regressive stage of infection all tests that detect FeLV antigen are negative, however provirus can be detected in the blood by sensitive PCR methods and in these cats the virus can potentially be reactivated at some time of their life. In other cats FeLV infection is not contained and they remain persistently viremic and infectious to other cats. These cats with progressive infection develop FeLV-associated diseases. It is noteworthy that a persistent atypical local viral replication (e.g., in mammary glands, bladder, eyes) is related to intermittent or low-grade production of antigen from a focal site of infection (Hartmann, 2012). The diseases associated with persistent infection are primarily disorders of haematopoiesis, such as

lymphoma (thymic, multicentric or alimentary) and leukaemias, immune suppression and anemia (Willis, 2000; Willet and Hosie, 2013). A dysregulation of the immune system leads to immunosuppression and infected cats can develop immune-mediated diseases caused by an overactive immune response. The most common abnormality seen is hypergammaglobulinemia which is caused by an excessive antibody response against the chronic persistent infection that is not neutralizing and may lead to antigen antibody complex formation. These immune complexes can deposit, usually in narrow capillary beds, leading to glomerulonephritis, polyarthritis, uveitis, and vasculitis (Hartmann, 2012). Glomerulonephritis is caused by an immune complex dense deposits located on the subepithelial and subendothelial sides of the glomerular basement membrane, as well as in the mesangial regions in most cases (Glick et al., 1978). Recently, diagnosis of immune-complex glomerulonephritis was found associated with FeLV infection (Rossi et al., 2019).

The first laboratory test used in the diagnosis of FeLV infection was an indirect immunofluorescent antibody (IFI) assay to detect viral antigen in blood cell smears, but this test has been replaced by commercially available rapid ELISA or immunochromatographic kits (Parry et al., 1989). These tests detect p27 viral capsid antigen that is a viral protein most abundant in the plasma of viraemic cats. As reported above, detection of regressive infection relies on real-time PCR commercially available for the quantification of FeLV proviral DNA. This technique is useful to identify regressive infection targeting proviral DNA integrated into the genome of cells within the bone marrow or lymphoid tissues and to confirm progressive infection by detecting viral RNA in blood or secretions (Willet and Hosie, 2013).

Feline coronavirus (FCoV)

Feline coronavirus is a large, spherical, enveloped, positive-sense single-stranded RNA virus that belongs to the family Coronaviridae of the order Nidovirales (Addie et al., 2009). There are two serotypes of FCoV recognized: type 1, which represents the vast majority of field strains found in naturally infected cats and type 2, which arise following recombination events between type 1 FCoV and canine coronavirus (CCoV). The two FCoV serotypes are distinguished primarily by the genetic and serological differences in their transmembrane spike (S) gene and protein,

respectively. The S protein is important as it is the part of the FCoV that binds to the host (feline) receptor, mediating host cell entry (Herrewegh et al., 1998; Addie et al., 2003; Tasker, 2018). Feline coronavirus infection is very common in cats with 40% infected cats reported (90% in multi-cat households), however natural infections are transient (~70%) and less frequently persistent infection occurs (~13%) (Tasker, 2018). The main way of transmission of FCoV is oro-fecal, in fact the virus replicates in the intestinal epithelium sometimes causing a mild diarrhea and is shed with faeces. After the first infection a proportion of FCoV-infected cats develop a severe immune-mediated disease called feline infectious peritonitis (FIP) as peritonitis is the most frequent consequence of the effusive form of the disease (Addie et al., 2009). Factors that contribute to development of FIP are: viral factors (mutation in the S gene), host factors (impaired immune response, breed, genetic, young age especially <2 years old), environmental factors (history of stress, overcrowded household) (Addie et al., 2009; Tasker, 2018). Strength of the T cell-mediated response influences the outcome of the infection and the severity of the clinical disease. Conversely in the presence of high levels of anti-FCoV antibodies monocytes and macrophages remain infected and a quiescent infection state develops (Addie et al., 2009). Activation of monocytes and macrophages leads to the pathologic features of FIP, including vasculitis, body cavity effusions, and fibrinous and granulomatous inflammatory lesions. Vasculitis observed in FIP is a phlebitis, mediated and dominated by activated virus-infected monocytes (Kipar and Meli, 2014). In those cats in which FCoV is able to replicate freely within the monocytes, infected monocytes attach to the walls of small and medium sized veins, releasing matrix metalloproteinase-9 (MMP-9) which destroys the collagen of the basal lamina of affected vessels with extravasation of the monocytes. Monocytes, differentiate into macrophages, and this allows plasma to leak out of the vessels (Kipar et al., 2005). Only veins are affected, most frequently the small- and medium-sized veins with involvment of leptomeninges, renal cortex, and eyes and, less frequently, veins in lungs and liver (Kipar and Meli, 2014).

Effusive FIP (wet form) is more severe and it is a fatal polyserositis affecting mainly the peritoneal cavity. In non-effusive FIP (dry form) pyogranulomatous lesions develop in different organs (especially eye, brain, kidneys, omentum, and liver), but actually FIP has a dynamic clinical spectrum and “dry” and “wet” lesions can be

concurrently found or at different times in a cat (Addie et al., 2009; Kipar et al., 2005). This explains why clinical signs related to FIP are variable. Affected cats can be interested by non-specific signs due to severe inflammation (fever refractory to antibiotics, lethargy, anorexia, and weight loss) (Addie et al., 2009). In the effusive form protein-rich fluid is found in the pleural space, peritoneal cavity, pericardial space, vaginal tunic of testicles and the subcapsular space of the kidneys, which can cause a possible clinical emergency; respiratory failure (pleural effusion), cardiac tamponade (pericardial effusion), or paralytic ileus (fibrinous peritonitis). The non-effusive form can be associated with progressive multifocal neurologic signs, signs of uveitis or chorioretinitis, hepatitis or gastro-intestinal signs (Lewis and O'Brien, 2010). Kidneys can be interested by renomegaly, ultrasound abnormalities as hyperechoic appearance, hypoechoic subcapsular rim, hyperechoic and hypoechoic areas in the renal medulla. Histologically a pyogranulomatous interstitial nephritis and immune-complex glomerulonephritis is evidenced (Lewis and O'Brien, 2010; Rossi et al., 2019).

Suspicion of FIP is often supported by clinicopathological abnormalities mild to moderate non regenerative anaemia, lymphopenia, increase in serum protein concentration (Addie et al., 2009), high albumin/globulin ratio with a cut-off value of 0.8 established (Hartmann, 2005), increase of liver enzymes, bilirubin, urea and creatinine) depending on the degree of organ damage (Addie et al., 2009) but diagnosis can be definetly confirmed by histopathology of suggestive lesions and the detection of FCoV in macrophages by immunofluorescent or immunohistochemistry staining of biopsies or smears from effusions or cerebrospinal fluid (Hartmann, 2005). Effusion fluid is generally yellow and sticky and typically positive in a Rivalta test. Serum antibody titres and RT-PCR may contribute to diagnostic information. Serum FCoV antibody tests available are ELISA, IFI test or rapid immunomigration tests. A positive FCoV antibody test indicates that there was contact with FCoV and that the cat has developed antibodies. Cats with FIP tend to have higher FCoV antibody titres than cats without FIP however low antibody titres do not rule out FIP, and a significant proportion of cats manifesting FIP are seronegative. Concerning PCR, FCoV shows a high rate of errors during replication and any mutations at the site of primer and/or probe binding can result in loss of PCR assay efficiency, and ultimately sensitivity

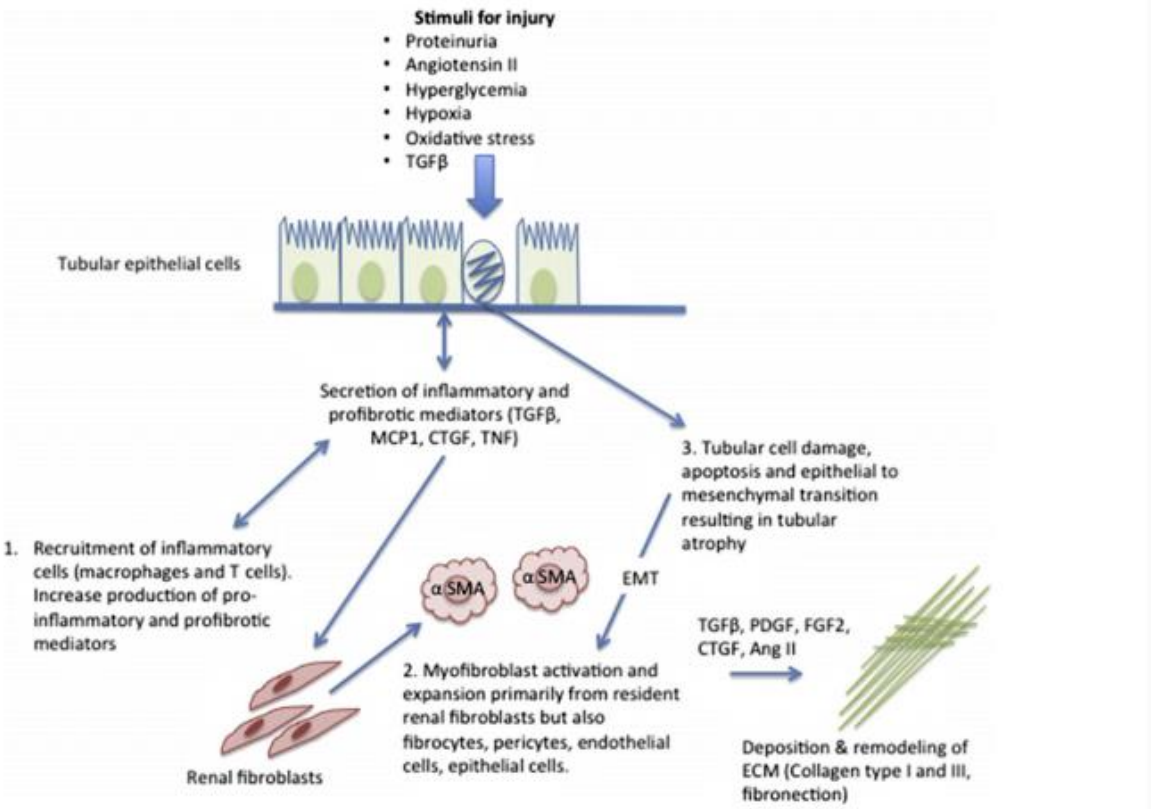
(Tasker, 2018). Both these factors compromise the ability of these diagnostic tests to provide a correct diagnosis.

1.3 PATHOGENESIS OF CHRONIC KIDNEY DISEASE

Development of CKD is characterized by an initiation phase and a progression phase. Indeed, primary renal disease initiates the damage to the kidneys with a variety of primary and secondary glomerulopathies, including immune complex glomerulonephritis, that act to initiate disease (Brown et al., 2016). Damage could be direct and or immune mediated and when injury is sustained and chronic follows the development of a chemotactic infiltration of inflammatory cells (neutrophils, platelets) that produce profibrotic cytokines (eg, transforming growth factor [TGF-β1]) which promote fibrogenesis by activation of matrix-producing cells (myofibroblasts, activated form of fibroblasts) (Reynolds and Lefebvre, 2013). Moreover, tubular epithelial cell cycle stops in response to toxic, obstructive, and ischemic injury with a lack of regeneration that may contribute to progression of disease and loss of nephrons (Jepson, 2016). In healthy cats, renal interstitium is composed of sparse cells (fibroblasts and dendritic cells) contained in an extracellular matrix composed of collagen, fibronectin, and glycoproteins. After an inciting injury, development of focal areas of inflammation and activation of mesenchymal cells begins. In CKD, fibrosis may represent a maladapted response of the kidney to injury with excessive fibrogenic response and expansion of extracellular matrix, which destroys the normal renal tissue (Jepson, 2016). Some pathophysiologic mechanisms can contribute to progression of CKD, aggravating the existing damage and reducing in some cases survival of cats. Vascular endothelial injury may lead to tissue hypoxia and ischemia, affecting renal parenchymal repair and contributing to renal damage and progression of CKD (Khan and Khan, 2015). In response to renal mass reduction, there are haemodynamic adataptions that lead to dilation of preglomerular afferent arterioles, increase in glomerular capillary pressure, and increased effective filtration pressure. Activation of the renin-angiotensin-aldosterone system contributes to kidney damage. Indeed, angiotensin II not only acts as a potent vasoconstrictor that contributes to the development of glomerular hypertension and hyperfiltration but also can modulate the

permeability of the glomerular filtration barrier, promoting proteinuria, and transcription and production of inflammatory and profibrogenic molecules. Aldosterone has also profibrotic effects contributing to the pathogenesis of CKD (Reynolds and Lefebvre, 2013; Jepson, 2016). Systemic hypertension may also affect renal function by inducing glomerular hypertension and proteinuria (Reynolds and Lefebvre, 2013). Proteinuria at the same time can contribute to kidney damage stimulating the inflammatory response. Even if in cats, the magnitude of proteinuria seems to be typically low compared to dogs, it has been significantly associated with the development of azotemia and reduced survival time. Finally, hyperphosphatemia can predispose to renal mineralization, with promotion of inflammation and fibrosis (Jepson, 2016). All these factors contribute to aggravate renal damage with development of subsequent clinical signs reported below.

(Jepson, 2016)



1.4 CLINICAL AND CLINICOPATHOLOGICAL FINDINGS OF CHRONIC KIDNEY DISEASE

Cats affected by CKD may have different complications that arise in advanced stages (Brown et al., 2016).

Polyuria and polydipsia are reported as the main clinical signs observed by the owners in the year that preceds CKD diagnosis (Bartlett et al., 2010). Polyuria may occur as a result of a decreased nephron mass and a consequent decrease of the urinary concentration ability. Polyuria is generally offset by polydipsia but dehydration may occur if water loss exceeds water intake (Bartges, 2012).

Urine specific gravity reflects the ability of tubules to concentrate and dilute urine to mantain fluid homeostasis. Healthy cats with the ability to concentrate urine have an USG ≥1.035. Some factors can minimally affect USG such as age, diet type, sex, fasting status, drinking avidity, refractometer type but generally older cats with USG <1.035 need further investigations (Rishniw and Bicalho, 2015). Some cats can retain their urine concentrating ability particularly in the early stages of CKD but with disease progression, USG usually gradually declines (Paepe and Daminet, 2013).

Decreased appetite is a common clinical finding of cats affected by CKD with a reported prevalence ranging from 21–92% (Freeman et al., 2016).

Cachexia and loss of muscle mass are common in companion animals with CKD (Freeman, 2012) and weight loss is also observed in cats affected by kidney disease with a prevalence range between 42 to 82%. Weight loss was related to a shorter survival time (Freeman et al., 2016). Multifactorial mechanisms are responsible for weight loss in CKD such as inflammation, malabsorption, increased energy requirements, and decreased appetite. Moreover cats with CKD and body weight ≤4 kg had a higher relative risk of death (Freeman et al., 2016).

Retention of uremic toxins (such as urea, creatinine, phosphates) is responsible for the uremic (Langston, 2003; McLeland et al., 2014) syndrome characterized by gastrointestinal manifestations such as reduced appetite and food intake, drooling and halitosis associated with, ulcerative stomatitis, vomiting, gastrointestinal hemorrhage, and diarrhea (Polzin, 2011). Gastric hyperacidity secondary to hypergastrinemia can contribute to some of the above manifestations. In fact, gastrin excretion is regulated

by kidneys therefore renal function decline is responsible for increase of gastrin levels and a consequent gastric hyperacidity (McLeland et al., 2014). Stomach histopathological lesions reported in cats affected by CKD include fibrosis and gastric mineralization, that can be a possible consequence of the dysregulation of the calcium-to-phosphorus ratio (McLeland et al., 2014).

Cats with CKD are affected by decreased production of erythropoietin (EPO) secondary to loss of functional renal mass. An altered/decreased iron metabolism can be seen as a consequence of chronic gastrointestinal hemorrhage and decreased intestinal absorption or intake (Gest et al., 2015; Javard et al., 2017). All these factors can be resposible for the development of anemia, and are poor prognostic indicators in terms of survival in animals affected by CKD. The anemia of CKD is usually non regenerative, normochromic normocytic, and its degree indicates the severity of loss of functional renal tissue (Elliot and Barber, 1998; Paepe and Daminet, 2013; Ettinger and Feldman, 2016). Cortical interstitial fibrosis is the renal lesion best correlated with the severity of anemia (Chakrabarti et al., 2013).

Progressive loss of functional nephrons leads to a decrease in the glomerular filtration rate that causes phosphorus retention, with hyperphosphatemia and an increased risk of development of secondary hyperparathyroidism. Hyperphosphatemia promotes parathyroid hormone (PTH) secretion that leads to phosphorus reabsorption in the renal tubules. However, as the glomerular filtration rate continues to decline, phosphorus retention becomes more severe with further secretion of PTH (Segev et al., 2016). At the same time calcitriol (active form of vitamin D) formed in the kidney, along with PTH is implicated in calcium homeostasis. In kidney disease there is a decrease in calcitriol concentration that decreases intestinal calcium absorption leading to hypocalcemia. The combination of low calcitriol and low calcium allows high concentration of PTH with an imbalance in the bone remodeling process and a consequent osteodystrophy (Segev et al., 2016; de Brito Galvao et al., 2013). Increases in plasma phosphate concentration can result in soft tissue mineralization especially in proton-secreting organs such as stomach or kidney where secretion of bicarbonates determines precipitation of calcium-phosphate crystals, however these mineralizations can also be seen in myocardium, lungs and liver (Chakrabarti et al., 2012; Ettinger and Feldman, 2016).

Total calcium (tCa) concentration includes hydrated free calcium ions (iCa), protein-bound calcium and a small portion of ionic complexes, such as calcium phosphate. Cats with CKD have increased risk of increased tCa. However, the biologically active iCa most accurately reflects true calcium status and measurement of iCa is necessary for an accurate assessment of calcium status in cats (van den Broek et al., 2017).

Chronic kidney disease can be responsible for increased blood pressure (BP) and increased blood pressure worsens the course of CKD. In cats increased BP can however be influenced by increased age, gender (with males and neutered cats having higher BP than females and intact animals), muscle condition score and skeletal muscle mass. Hypertension can be distinct in situational, idiopathic or secondary hypertension. Situational hypertension is caused by autonomic nervous system alterations that arise from the effects of excitement or anxiety on higher centers of the central nervous system. This can be minimized by measuring BP in a quiet area with the presence of the owner, away from other animals, before other procedures and only after the patients have been acclimated to their surroundings for 5-10 minutes. Idiopathic hypertension is suspected when there is a sustained increase in BP concurrent with normal complete blood count (CBC), serum biochemistry, and urinalysis results. Persistent, pathologically increased BP concurrent with a disease or condition known to cause hypertension is defined as secondary hypertension (Acierno et al., 2018). Chronic kidney disease, hyperthyroidism, primary hyperaldosteronism (PHA), hyperadrenocorticism (HAC) and phaeochromocytoma can be responsible for secondary hypertension (Taylor et al., 2017). Chronic kidney disease is the most common condition associated with feline hypertension. Azotaemia has been found in up to 74% of hypertensive cats, and conversely between 19% to 65% of cats with CKD have been found to be hypertensive (Taylor et al., 2017), moreover hypertension is associated with glomerulosclerosis and glomerular hypertension (Chakrabarti et al., 2013; Syme et al., 2006) and indirect blood pressure is correlated with the severity of CKD (Hori et al., 2018). Cats with CKD have to be monitored for hypertension because chronically sustained increases in BP cause injury to tissues with development of target organ damage (TOD). Eyes, brain, kidneys and myocardium are organs particularly vulnerable to injury. Indeed, hypertension has been associated with

proteinuria that can lead to a more rapid progression of renal disease, with ocular lesions (e.g. hypertensive retinopathy, choroidopathy, exudative retinal detachment, retinal hemorrhage, multifocal retinal edema, retinal vessel tortuosity, retinal perivascular edema, papilledema, vitreal hemorrhage, hyphema, secondary glaucoma, and retinal degeneration), with hypertensive encephalopathy (observed when systolic blood pressure exceeds 180 mm Hg) with lethargy, seizures, acute onset of altered mentation, altered behavior, disorientation, balance disturbances (e.g. vestibular signs, head tilt, and nystagmus), and focal neurologic defects because of stroke-associated ischemia. Hypertension appears to be a risk factor for ischemic myelopathy of the cranial cervical spinal cord, resulting in tetraparesis or tetraplegia with intact nociception in old cats. Cardiac abnormalities are common in hypertensive cats with cardiomegaly associated with left ventricular concentric hypertrophy (LVH). Epistaxis, aortic aneurysm and aortic dissection can be rare complications of hypertension (Acierno et al., 2018; Taylor et al., 2017).

Azotaemia is due to increased serum creatinine (sCr) and urea concentrations as a result of renal pathology (Paepe and Daminet, 2013). Serum creatinine is produced by muscle metabolism and is inversely related to glomerular filtration rate (GFR) (Sparkes et al., 2016; Yerramilli et al., 2016) with an exponential relationship with GFR, therefore early declines in GFR are characterized by small changes in creatinine (Sparkes et al., 2016). Significant changes in creatinine concentration occur when 60% to 70% of all nephrons are nonfunctional (Pressler, 2015). Plasma/serum creatinine values can be influenced by artifacts such as hemolysed samples with increased values or lipemic and icteric samples with lower estimates. Dehydration and muscle mass can also influence creatinine values, because depletion of body water leads to increases in creatinine concentration and muscle wasting leads to reduced creatinine values with understimation of kidney dysfunction if USG is not evaluated (Paepe and Daminet, 2013; Yerramilli et al., 2016). This aspect is of clinical relevance particularly in geriatric cats or during CKD progression where a tendence to muscle wasting is observed (Paepe and Daminet, 2013). Moreover some breeds tend to have higher levels of serum/plasma creatinine, such as Birman or Siberian cats and this should be considered when creatinine values are interpreted in these breeds (Paltrinieri et al., 2014; Reynolds et al., 2010).

Metabolic acidosis occurs commonly in cats with CKD as a consequence of retention of acids that are normally excreted through the kidneys (Bartges, 2012). Biochemical evidence of metabolic acidosis seems to occur with late stages of this disease syndrome (Elliot et al., 2003). Metabolic acidosis is characterized by an increased hydrogen ion concentration in blood that results in movement of hydrogen ions into cells in exchange for potassium ions that leave the cells and enter the circulation. Therefore potassium is excreted and this predispose to hypokalemia (Bartges, 2012).

The marked reduction in glomerular filtration rate of end-stage CKD tends to promote potassium retention and hyperkalemia (Polzin, 2011). In cats affected by CKD with hypokalemia, the muscle membrane becomes electrically hyperpolarized and refractory to stimulation for the action potentials. This explains manifestations of the kaliopenic polymyopathy/nephropathy syndrome characterized by a generalized appendicular muscle weakness, persistent ventroflexion of the neck, reluctance to walk and apparent muscular pain upon palpation. Moreover, in cats affected by hypokalemia reduction of renal function as well as anorexia are reported with an improvement of renal funtion when normokalemia is restored (Fettman, 1989; Ettinger and Feldman, 2016).

Proteinuria is a common laboratory finding in CKD, even if proteinuria in dogs seem to have a higher prevalence (90%) rather than proteinuria observed in cats (20%). This is compatible with published data, suggesting that the major disease process occurring in cats is chronic tubulointerstitial fibrosis rather than primary glomerular disease (Syme et al., 2006; Giraldi and Scarpa, 2018). However, a recent study found immune-complex glomerulonephritis in about half of examined tissue samples and this diagnosis was more frequently associated with FIV or FeLV infections, younger age and higher UPC values compared to non immune-complex renal disease (Rossi et al., 2019).

Physiologic or functional proteinuria is known and it can be distinguished from a pathologic proteinuria that is based on pre-renal, renal, or post-renal factors. Functional renal proteinuria is caused by heat, stress, seizure, venous congestion, fever and extreme muscle exercise and is generally of low grade and transient. Pre-renal proteinuria is caused by an overabundant filtered load of low molecular weight

proteins (hemoglobin, myoglobin, immunoglobulin light-chain monomers and dimers such as Bence Jones proteins from neoplastic plasma cells). Renal proteinuria is due to a defect in the glomerular filtration barrier, tubular reabsorption or interstitial damage. Post-renal proteinuria is due to protein that comes from any part of the urinary tract distal to the kidney (urinary tract infections, genital infections or inflammation) (Lees et al., 2005; Harley and Langston, 2012). Normally the majority of plasma albuminin is size excluded and charge excluded from the ultrafiltrate, but when glomerular damage occurs there is an increased passage of albumin into urine (Pressler, 2015). When albumin in the urine is in excess of 0.30 g/L the animal is affected by proteinuria, while albumin concentrations ≥0.01 and < 0.30 g/L are defined as microalbuminuria (Harley and Langston, 2012). Normally, proteins of molecular weight up to about 50 KD pass through the glomerular capillary wall and are reabsorbed by the proximal tubule but in CKD, changes in glomerular permeability can result in filtration of proteins with higher molecular weight and tubular damage reduces the reabsorption of low-molecular weight proteins. Proteinuria can also be affected by hemodynamic factors such as angiotensin II, local prostaglandins, endothelin, and other vasoactive mediators that can influence constriction or dilatation of afferent and efferent renal arterioles with development of glomerular hypertension (Harley and Langston, 2012). The level of proteinuria can be useful to evaluate the progression of CKD (Chakrabarti et al., 2012) and shorter survival times are associated with increased protein excretion (Syme et al., 2006) probably by invoking interstitial inflammation and fibrosis around renal tubules: filtered proteins may directly damage tubular cells or may activate complement and chemoattractants leading to the formation of inflammatory mediators which contribute to fibrosis (Harley and Langston, 2012).

Diagnostic methods for detection of proteinuria will be described below.

1.5 DIAGNOSIS AND STAGING OF CHRONIC KIDNEY DISEASE

Diagnosis of CKD in dogs and cats is based on evaluation of suggestive clinical signs, measurement of sCr, USG, proteinuria, and ultrasound evaluation of kidneys and urinary tract. Systolic blood pressure (SBP) measurement is required to substage

CKD in order to manage the risk for target organ damage related to hypertension. Moreover, as described later, novel biomarkers of CKD are currently being studied (Paepe and Daminet, 2013).

Diagnosis and staging of CKD in dogs and cats is at present based on internationally recognised guidelines established by a group of experts, the *International Renal Interest Society* (IRIS) (www.iris-kidney.com). Since 1998 IRIS objectives consisted in providing to practitioners guidelines for the diagnosis and treatment of renal disease in dogs and cats. Through the urine analyis and the measurement of creatinine, UPC, and blood pressure values, IRIS guidelines show how to confirm, stage and substage CKD. The staging system allows an accurate prognosis and more appropriate treatments to slow the progression of kideny damage, reduce the risk for complications and improve the clinical condition. Guidelines are regularly updated according to the most recent scientific evidence.

The staging system includes five stages including one stage for cats considered at risk for developing CKD and four stages (from stage 1 to stage 4) for grading the severity of renal damage in cats affected by CKD (table 1.a). Basically, staging is based on fasting blood creatinine, assessed on at least two occasions in the stable patient, but in non-azotemic individuals data from clinical examination and urine e analysis are also considered to exclude that they are “at risk” for CKD or are at “stage 1”.

Table 1.a IRIS staging system of CKD

STAGE	BLOOD CREATININE IN CATS (mg/dl)	COMMENTS
AT RISK	<1.6	**Increased risk of developing CKD** in the future because of different factors (e.g. exposure to nephrotoxic drugs, breed, high prevalence of infectious disease in the area, or old age).
1	<1.6	**Nonazotemic.** Presence of other renal abnormality (e.g. inadequate urinary concentrating ability without identifiable nonrenal cause, abnormal renal palpation or renal imaging findings, proteinuria of renal origin, abnormal renal biopsy results, increasing blood creatinine concentrations in samples collected serially)
2	1.6 – 2.8	**Mild renal azotemia.** Clinical signs usually mild or absent.
3	2.9 – 5.0	**Moderate renal azotemia.** Extrarenal signs may be present.
4	>5.0	**Severe azotemia.** Increasing risk of systemic clinical signs and uraemic crises

Modified from www.iris-kidney.com

As described previously, increased creatinine values are seen when a great portion of nephrons (70%) are no longer functional, moreover dehydration, muscle mass and breed can affect creatinine values. All these aspects can influence the correct staging of the disease as well as the choice of an adequate therapeutic plan. Therefore, different studies analyzed the role and utility of alternative biomarkers for CKD and the measurement of serum symmetric dimethylarginine (SDMA) has been recently proposed as a novel biomarker for CKD in dogs and cats (Hokamp and Nabity, 2016; Jepson et al., 2008; Braff et al. 2014; Hall et al., 2016; Hall et al., 2017, Peterson et al., 2018, Hall et al., 2014a). Symmetric dimethylarginine is produced in the nucleus of all nucleated cells after a post-translational modification and methylation of arginine residues of varius proteins and it is excreted by the kidney (Relford et al, 2016). According to recent studies, a SDMA value greater than 14 µg/dL shows a decrease in

GFR below 20% (Relford et al, 2016). This is because serum SDMA evaluation allows early detection of CKD in cats compared to serum creatinine measurement. Albeit increases of SDMA are found 17 months earlier than increases of creatinine in cats with CKD, SDMA higher sensitivity is associated with a lower specificity compared to creatinine (Hall et al., 2014a). The SDMA value correlates with plasma creatinine concentration (Jepson et al., 2008) and is inversely related to GFR (Hall et al., 2014a). Interestingly, it is not affected by lean body mass so it is a more sensitive marker for kidney disease in patients with muscle loss (Hall et al., 2014b; Relford et al, 2016). SDMA values may however be higher in kittens and in cats with kidney stones (Relford et al, 2016; Hall et al., 2017). In hyperthyroid cats, SDMA concentration together with the evaluation of USG were found useful to identify and predict hyperthyroid cats at risk to develop azotemia after treatment of hyperthyroidism (Peterson et al., 2018). Recently, SDMA values were found less frequently elevated than creatinine in apparently healthy Birman cats suggesting that the analysis of both creatinine and SDMA could be useful to prevent the over-diagnosis of CKD or errors in staging renal disease in this breed (Paltrinieri et al., 2018). Based on recent experimental and clinical data, SDMA measurement has been added to the IRIS staging system of CKD (table 1.b) (www.iris-kidney.com) as a persistent increase in SDMA above 14 µg/dL is considered suggestive of reduced renal function in adult cats. In the case of creatinine values <1.6 mg/dl, patients are now diagnosed with CKD at Stage 1. In the case of low body condition score (BCS) of patients diagnosed with CKD Stage 2, SDMA values ≥25 µg/dL are suggestive of more severe renal damage and treatment recommendations for CKD Stage 3 should be followed. Similarly, for Stage 3 patients with low BCS, SDMA values ≥45 µg/dl suggest following recommendations for IRIS CKD Stage 4 (www.iris-kidney.com).

Table 1.b IRIS staging system of CKD evaluating SDMA

	AT RISK	STAGE 1	STAGE 2	STAGE 3	STAGE 4
CREATININE VALUE (mg/dl)	**<1.6**	**<1.6**	**1.6 – 2.8**	**2.9 – 5.0**	**>5.0**
SDMA VALUE (µg/dl)	**>14**		**≥25**	**≥45**	
COMMENTS	In patients with low body condition score consider treatment recommendations listed under IRIS CKD Stage 1 for this patient.		In patients with low body condition score consider treatment recommendations listed under IRIS CKD Stage 3 for this patient.	In patients with low body condition score consider treatment recommendations listed under IRIS CKD Stage 4 for this patient	

Modified from www.iris-kidney.com

According to the IRIS guidelines, staging of cats with CKD is followed by substaging based on proteinuria and systolic hypertension detection. Concerning proteinuria, we have to consider that cats affected by CKD have lower levels of proteinuria than dogs (Harley and Langston, 2012). However, as mentioned before, shorter survival times have been associated with increased protein excretion in cats, underlining the importance of correct evaluation and monitoring of proteinuria in cats to avoid progression of the disease. Detection of proteinuria can be performed with different methods. The traditional reagent pad colorimetric method of "dipstick" devices is the first screening test used to detect albuminuria. However some artifacts can influence its performance, such as highly concentrated urine or pigmented urine that can give falsely elevated results. The sticks are designed for human urine which is rarely as concentrated as dog or cat urine. Moreover acidic urine can cause false negative and alkaline urine can cause false positive results (Harley and Langston, 2012). The sulfosalicylic acid (SSA) test can help to distinguish between a true and a false positive dipstick test. The sulfosalicylic acid test is a semi-quantitative test that can detect protein at >50 mg/dL. It is perfomed using equal parts urine supernatant to

5% SSA in a glass tube and grading the level of protein precipitation based on turbidity. False positive results may occur if the urine contains radiographic contrast agents, cephalosporins, penicillin, thymol, or sulfisoxazole while false negatives are rare. The UPC is considered the gold standard test for proteinuria as it closely correlate with the 24-hour urine protein quantification and it is indicated for IRIS substaging of patients with CKD (Harley and Langston, 2012). Obviously, substaging of proteinuria has to be performed once post-renal and pre-renal causes have been ruled out and UPC evaluation is based on at least two urine samples collected over a period of at least 2 weeks (www.iris-kidney.com).The IRIS substaging system for proteinuria divides feline proteinuria into 3 substages: non-proteinuric cats have UPC values <0.2, borderline proteinuric cats have UPC values between ≥0.2 and ≤0.4, and proteinuric cats have UPC values >0.4 (Table 1.c). A recent study that compared different tests for detection of albuminuria in cats revealed that in case of both positive urine dipstick (≥trace) and SSA, positive SSA alone or a highly positive urine dipstick (≥2+) alone are indicative of albuminuria and protein quantification would be warranted if these results are persistent (Hanzlicek et al., 2012). However, in this study an UPC value ≥0.2 was considered the single best test for the detection of albuminuria (Hanzlicek et al., 2012). Besides quantitative methods for the detection of proteinuria (UPC), electrophoresis allows fractionation and identification of the urine proteome offering more information on the area of the nephron involved. A recent study compared the urine proteome of healthy cats and cats with CKD. In healthy cats the most abundant protein was cauxin, produced by healthy tubular cells, excreted in urine and probably involved in the synthesis of feline pheromones; proteins involved in protein metabolism, immune response and transport (alpha-2-macroglobulin, albumin, transferrin, haemopexin, haptoglobin, uromodulin) were also found. In cats with CKD increased expression of retinol-binding protein, cystatin M and apolipoprotein-H associated with decreased expression of uromodulin and cauxin confirmed tubular damage (Ferlizza et al., 2015). Different methods of urinary electrophoresis have previously been assessed in dogs and cats and new alternative electrophoresis methods are the subject of a recent study. Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) is considered the gold standard electrophoretic method: all the proteins are denatured and negatively charged, and as a consequence, they

migrate in polyacrylamide gel electrophoresis based on their molecular weight, thus allowing the differentiation of glomerular versus tubular proteinuria or mixed proteinuria (Giori et al., 2011). Sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE) allows detection of urinary proteins that have a molecular mass ranging from 9 kd to 900 kd. After treatment with SDS that gives all proteins a negative charge proportional to their size, an electrophoretic separation of urinary proteins in an agarose gel matrix in accordance with mass is performed. SDS-AGE is preferred to SDS-PAGE for its superior ability to separate large proteins and for its lower toxicity (Zini et al., 2004). Recently, high-resolution electrophoresis (HRE) has been proposed as an alternative electrophoretic technique in dogs. HRE is a zonal electrophoresis in which proteins migrate based on charge, volume, and mass and differs from the standard AGE by the use of a more alkaline buffer (pH 8.6 vs. 8.5), a different barbital concentration (0.18% vs. 0.61%), and a different voltage per hour (40 V/hr vs. 36 V/hr) at 20°C. These different analytical conditions lead to a better separation of globulins compared with standard electrophoretic techniques. Bands on the gel are transformed by the gel scanner software into peaks corresponding to groups of proteins characterized by similar mass, volume, and weight. Results obtained from HRE and SDS-PAGE have been compared in dogs and in HRE the use of an alb/α 1 globulin ratio allowed differentiation between glomerular and tubular proteinuria: an increased alb/α1globulin ratio appeared useful to identify glomerular proteinuria, a decreased alb/α1 globulin ratio identified tubular proteinuria, and values between the 2 cut-offs (0.84-1.46) could suggest mixed proteinuria (Giori et al., 2011). Recently, HRE was also evaluated in cats with CKD and demonstrated greater variability in the electrophoretic profiles with increased concentration/density of the albumin band and of α, β and γ zones (Ferlizza et al., 2017).

Table 1.c IRIS substaging system of CKD based on proteinuria

UPC VALUE IN CATS	SUBSTAGE
< 0,2	Non-proteinuric
0,2-0,4	Borderline proteinuric
>0,4	Proteinuric

Modified from www.iris-kidney.com

UPC: urinary protein to urinary creatinine ratio

Substaging of cats according to SBP requires accurate measurements with non-invasive methods and high resolution oscillometric devices are considered the most appropriate in cats (Acierno et al., 2018). Hypertension in both dogs and cats is classified based on the risk of TOD as: normotensive cats with SBP <140 mm Hg are considered at minimal TOD risk; risk for TOD is respectively considered low, moderate or high in the case of prehypertensive (SBP 140-159 mm Hg), hypertensive (SBP 160-179 mm Hg), or severely hypertensive (SBP ≥180 mm Hg) conditions (Acierno et al., 2018). If there is no evidence of existing target organ damage, but there is persistence of increased SBP multiple measurements are needed. Therefore hypertension is considered if SBP is between 160 to 179 mm Hg measured over 1 to 2 months and classified as severe hypertension if it is ≥180 mm Hg measured over 1 to 2 weeks (www.iris-kidney.com). IRIS criteria used to substage CKD evaluating BP are described in table 1.d.

Table 1.d IRIS substaging system of CKD according to blood pressure measurement

SYSTOLIC BLOOD PRESSURE mm Hg	BLOOD PRESSURE SUBSTAGE	RISK OF FUTURE TARGET ORGAN DAMAGE
<140	Normotensive	Minimal
140 – 159	Prehypertensive	Low
160 – 179	Hypertensive	Moderate
≥180	Severely hypertensive	High

Modified from www.iris-kidney.com

1.6 ULTRASOUND EVALUATION

Ultrasonography represents one of the diagnostic methods for CKD diagnosis, but is also specifically useful to identify causes such as congenital abnormalities, urinary obstructions, neoplasia (Giraldi and Scarpa, 2018). However some pathological changes such as increased cortical echogenicity are significantly associated only with severe renal damage found in advanced stages of disease (Banzato et al., 2017).

Conversely in non-azotemic cats the presence of abnormalities are sometimes reported, therefore both sensitivity and specificity vary according to the change considered and in some cases the breed of cat (Paepe et al., 2013; Lamb et al., 2018). Ultrasonographic findings most frequently observed in cats with CKD are small and irregularly outlined kidneys, increased cortical and/or medullar echogenicity, loss of corticomedullary demarcation, areas of mineralization and poor visualisation of internal architecture, abnormal renal length, presence of pelvic dilatation and perinephric fluid (Paepe and Daminet, 2013; Lamb et al., 2018; Bragato et al., 2017). Arterial resistive index (RI) for feline kidneys should be approximately 29–51 mm (Lamb et al., 2018) but cats with CKD seem to have a higher RI. This index is calculated as follows: RI = (PSV – EDV)/PSV and it measures the arterial resistance in the peripheral vessels by calculating the ratio between the difference of peak systolic velocity (PSV) and end diastolic velocity (EDV) and PSV, which is independent from the angle and position of the transducer. Renal RI increases in the presence of higher vascular resistance, lower vascular compliance and renal tissue lesions (Matos et al., 2018). Contrast-enhanced ultrasound examination (CEUS) is a functional imaging technique allowing non-invasive assessment of tissue perfusion and it was recently used to evaluate renal perfusion in cats with CKD (Stock et al., 2018). Results reported in this study revealed a decrease in blood velocity for the renal cortex and an increased blood velocity in the renal medulla. Histopathological alterations (such as tubulointerstitial fibrosis) and the influence of the renin-angiotensin system with vasoconstriction determined by angiotensin II impair renal blood flow and increase renal vascular resistance. However vascular anatomy and physiology of the renal medulla differ substantially from those of the cortex and this can explain the locally increased blood velocity reported (Stock et al., 2018).

1.7 HISTOPATHOLOGICAL EVALUATION

Renal biopsies are needed to confirm a diagnosis of neoplasia but can also be considered to differentiate immune-mediated and non immune-mediated glomerulonephritis (Paepe and Daminet, 2013; Rossi et al., 2019). Histologic variables

that affect the interstitial, tubular, and glomerular compartments are significantly different among stages of CKD (McLeland et al., 2015). Earlier stages of CKD retained a greater proportion of normal parenchyma in comparison to later stages. Tubular degeneration, single epithelial cell necrosis, interstitial fibrosis and scarring, mineralization of Bowman's capsule and tubular basement membranes are significantly greater in later stages compared with earlier stages. Glomerulosclerosis progressively worsens with increasing CKD stage, while vascular lesions do not differ among stages. Lipids within the interstitium are frequently associated with interstitial inflammation and recent studies described interstitial lipid deposition as a pathological finding in cats with CKD (Schmiedt et al. 2016; Brown et al. 2016; Martino-Costa el at. 2017).

1.8 AIM OF THE THESIS

The epidemiology of CKD can be influenced by geographical factors that include different exposure of cats to potential risk factors. Moreover genetic factors could also be differently expressed in feline populations from different geographic areas.

As described above, exposure to some infectious agents is recognized as a risk factor for CKD but the role for some emerging pathogens is still unclear. Among them, we decided to focus on *Leishmania infantum*, FeMV, and *Leptospira* spp. and to evaluate the:

- Epidemiology of CKD in adult cats from the provinces of Reggio Calabria (Calabria) and Messina (Sicily) in Southern Italy, including the association with the pathogens under study.
- Epidemiology of *L. infantum*, *Leptospira* spp., FeMV and other better known viral infections (FIV, FeLV and FCoV) in cats from the same areas.

CHAPTER 2
MATERIALS AND METHODS

2.1 Cats, clinical examination and sampling

Cats were enrolled between October 2016 and April 2019 at four Veterinary Clinics located in Sicily (Veterinary Teaching Hospital, Università degli Studi di Messina, Messina; Ambulatorio Veterinario S. Lucia, Lipari-Messina) and Calabria (Clinica Veterinaria Camagna, Reggio Calabria; Ambulatorio Dr. Cardone, Gioia Tauro-Reggio Calabria). The study was approved by the Ethics and Welfare Commitee of the Department of Veterinary Medicine, University of Messina (018/2017; 024/2018). Inclusion criteria for enrollement were adult age ($\geq$ 6 months) but cats < 1 year of age were enrolled only if they were born not later than April, in order to enrol cats that had been exposed to vectors at least for one hot-season (April-October). Cats were mainly presented for an annual health check, medical problems, elective, minor or orthopedic surgeries and each cat was enrolled after their owner's signed consent was obtained.

Clinical history and physical examination findings were registered in a clinical form. Among data recorded the following information was analyzed: reason for consultation, age, sex and reproductive status, breed, husbandry, environment, geographic region, body condition score (BCS), muscle condition score (MCS), mucous membranes color, superficial lymph nodes enlargment, ocular, oral, dermatological, respiratory, gastrointestinal, or abdominal abnormalities. Body temperature was recorded but was not included in the set of data analyzed because about one third of cats were examined under chemical restraint (tiletamine hydrochloride in combination with zolazepam hydrochloride, 11.9 mg/kg IM) or general anesthesia (when needed for clinical reasons) and their values were therefore influenced by the drugs used. Complete ophthalmic and neurologic examinations were not routinely performed but, when detected, neurologic signs and ocular lesions were reported. Cats were classified as "junior" if they were less than one year old, "adult" if they were from one to eight years and senior if they were older than eight years of age. Body condition score was evaluated following a 5/5 scoring system and MCS was evaluated following a 4/4 scoring system (https://www.wsava.org/).

From each cat, about five milliliters of blood were taken from a jugular or brachial vein: one milliliter was placed into an EDTA tube and used within 24 hours for complete blood count (CBC); remaining blood (about four milliliters) was used to make blood smears and to obtain serum after clotting in a dry tube and centrfugation to obtain the serum. The EDTA was separated into four aliquots, one for feline virological investigations was stored at +4°C, while the other three were held at -20°C until analyzed. Aliquots of serum were stored at -20°C except the one stored at +4°C and used for FeMV virological investigations.

Urine samples were obtained by cystocentesis, free catch or catheterization (this latter only when an urinary catheter was in place for clinical reasons). Urinalysis was performed within 2 hours after collection. The supernatant was used for the evaluation of UPC within 24 hours after collection. An aliquot of urine was added to a minimum essential medium (MEM) (Sigma Aldrich, USA) with a 1:8 dilution and stored at +4°C for FeMV isolation and a minimum volume of one milliliter of urine was stored at +4°C for FeMV molecular investigations. *Leptospira* spp. investigation was performed from two milliliters of urine samples, stored at +4°C until processed within 24 hours.

Oral, conjunctival, auricolar swabs and swabs from any skin or mucosal lesions were collected using individual sterile cotton swabs rolled respectively on mucosal surface of fauces, lower conjunctival fornix and in the ear canal. Moreover, a hair tuft was taken from the back of the cat's head and saved in a microtube. Fine needle aspirates were taken from enlarged lymph nodes and cytology smears were prepared immediately. The syringe and the needle used for lymph node aspiration, swabs, and hair were aseptically saved at -20°C until analyzed for the detection of *L. infantum* DNA.

2.2 Complete blood count (CBC), biochemical profile, serum protein electrophoresis, total-T4, urinalysis, UPC, and ultrasonography

A complete blood count was performed within 24 hours using a laser haematology analyzer (IDEXX ProCyte Dx® Hematology Analyzer, Idexx Laboratories, Westbrook, Maine, USA). Reference intervals for the CBC are listed in Table 2.a.

Blood smears were stained by May Grünwald-Giemsa staining and examined for haematological abnormalities (Piaton et al., 2016). A biochemical profile was performed on a Catalyst Dx® Chemistry Analyzer (Idexx Laboratories, Westbrook, Maine, USA) while SDMA was measured with liquid chromatography-mass spectrometry (IDEXX Laboratories, Novara, Italia S.r.l).

Serum amyloid A (SAA) was evaluated at the Department of Veterinary Medicine at Cambridge University (UK) by a latex agglutination reaction on an automated analyser AU480 (Beckman Coulter, Brea, California). Total thyroxine (tT4) was evaluated at Biogene laboratory (Catania, Italy) with an enzyme linked fluorescent assay (ELFA). Reference intervals for the biochemical profile and tT4 are listed in Table 2b. Urinalysis was performed using Combur 9 Test strips (Roche Diagnostics, Indianapolis, Indiana, USA). Urine specific gravity (USG) was evaluated with a Vet 360 refractometer (Reichert, Seefeld, Germany) and microscopic evaluation of urine sediment was performed using the Kova glasstic slide (Kova International, Garden Grove, CA, USA). Urine protein to creatinine ratio was evaluated with the Catalyst Dx® Chemistry Analyzer (Idexx Laboratories, Westbrook, Maine, USA), and ≥0.2-0.4≤ was the range of border line proteinuria. Proteinuria was diagnosed with values >0.4 (http://www.iris-kidney.com/).

Serum protein electrophoresis (SPE) was performed at the Department of Veterinary Medicine at Cambridge University (UK) using pre cast gels Hydragel HR K20 (Sebia, EVRY Cedex, France). Briefly, serum was applyed into the applicator wells and then the applicator was loaded on the gel. After 1 minute, the gel was placed into an appropriate electrophoresis chamber filled with buffer and the voltage was set to 100 V. After migration (22 minutes) the gel was dried with hot air (+ 80°C), followed by immersion in the prepared staining solution for 6 minutes and dried at + 80°C. The gel was scanned using Sebia's Gel Scan software. Reference intervals for serum electrophoresis are listed in Table 2.b.

Ultrasound evaluation of kidneys and urinary tract were evaluated by B-mode and Doppler ultrasound, using Esaote Mylab 60 and Esaote Mylab 40 ultrasound systems. Kidneys were evaluated with sagittal, dorsal and transverse projections and kidney profile, cortical and medullary echogenicity, cortico-medullary ratio, presence of fibrosis or mineralization of renal pelvis, rim sign were evaluated in order to evaluate

the presence of glomerulonephritis. The urinary bladder was evaluated through a longitudinal projection evaluating wall appearance, alterations of the content, presence of stones.

Table 2.a CBC reference intervals

PARAMETER	RI
RBC (M/µL)	6.54-12.20
HCT (%)	30.3-52.3
HGB (g/dL)	9.8-16.2
MCV (fL)	35.9-53.1
MCH (pg)	11.8-17.3
MCHC (g/dL)	28.1-35.8
RDW (%)	15.0-27.0
RETIC (K/µL)	3.0-50.0
RETIC-HGB pg	13.2-20.8
WBC (K/µL)	2.87-17.02
NEU (K/µL)	2.30-10.29
LYM (K/µL)	0.92-6.88
MONO (K/µL)	0.05-0.67
EOS (K/µL)	0.17-1.57
BASO (K/µL)	0.01- 0.26
PLT (K/µL)	151-600

Legenda: RI: reference intervals, RBC: red blood cells, HCT: hematocrit, HGB: hemoglobin, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red blood cell distribution width, RETIC: reticulocytes, RETIC-HGB: reticulocyte hemoglobin content, WBC: white blood cells, NEU: neutrophils, LYM: lymphocytes, MONO: monocytes, EOS: eosinophils, BASO: basophils, PLT: platelets.

Table2.b Biochemical profile and serum protein electrophoresis reference intervals

PARAMETER	**RI**
SDMA (µg/dL)	≤ 14
sCr (mg/dL)	0.8-2.4
BUN (mg/dL)	16-36
PHOS (mg/dL)	3.1-7.5
ALT (U/L)	12-130
TP (g/dL)	5.7-8.9
ALB (g/dL)	2.3-3.9
GLOB (g/dL)	2.8-5.1
SAA (µg/mL)	< 0.5
AST (U/L)	14-71
GGT (U/L)	0-5
ALKP (U/L)	12-73
tT4 (µg/mL)	1-3
SPE	
ALBUMIN(g/L)	0-32
ALPHA-1 GLOBULIN(g/L)	1-2
ALPHA-2 GLOBULIN(g/L)	0-19
BETA-1 GLOBULIN(g/L)	1-2
BETA-2 GLOBULIN(g/L)	0-3
GAMMA-GLOBULIN(g/L)	6-9

Legenda: RI: reference intervals, SDMA: symmetric dimethylarginine, sCr: serum creatinine, BUN: blood urea nitrogen, PHOS: phosphorus, ALT: alanine amino transferase, TP: total proteins, ALB: albumin, GLOB: globulins, SAA: serum amyloid A, tT4: total thyroxine, AST: aspartate aminotransferase, GGT: gamma glutamyl transferase, ALKP: alkaline phosphatase, SPE: serum protein electrophoresis.

2.3 Antibody detection

2.3.1 Anti-*L. Infantum* immunoglobulinG (IgG) antibodies (Ab) were tested by indirect immunofluorescence (IFI) using *L. infantum* (strain MHOM/IT/80/IPT1) antigen slides produced by CReNal (Centro di Referenza Nazionale per la Leishmaniosi, Palermo, Italy). Fluoresceinated rabbit anti-cat IgG Ab (working anti-feline IgG (H+L)-FITC, Fuller Laboratories, Fullertone, CA, USA) was used. The manufacturer's protocol was followed and the end-point titer of positive samples was determined preparing PBS serial two-fold dilutions of serum starting from a dilution of 1:40. The cut off dilution value for positivity was set at 1:80 (Pennisi et al., 2012; Persichetti et al., 2017).

2.3.2 Serum samples were tested for the presence of Ab against FIV by enzyme - linked immunosorbent assay (ELISA) (Pet Chek FIV Antibody test kit; IDEXX Laboratory, Westbrook, ME, USA).

2.3.3 The presence of Ab against FeMV-N protein was investigated by IFI. Antigen slides of FeMV-N-expressing HeLa cells and rabbit polyclonal antibody against FeMV-N protein (kindly provided by Dr Shigeru Morikawa, National Institute of Infectious Diseases, Tokyo) were used for the IFI test. Serial PBS two-fold dilution of serum samples starting from a dilution of 1:40 were tested. After incubation of diluted serum on antigen slides for 1 h at 37 °C in a humidified chamber and washing in PBS, 1:32 diluted rabbit anti-cat-IgG FITC conjugated (Cappel) was applied and incubated as for the first step. Rabbit polyclonal antibody against FeMV-N protein and goat anti-rabbit-IgG (Sigma Aldrich) conjugated with FITC were used as the positive control. Uninfected cells were used as negative control. After final washing in PBS, the slides were examined for staining patterns under a Zeiss AxioVert.A1 Fluorescence Microscope and imaged using a Leica TCS SP5 II confocal laser scanning microscope. Sera showing Ab titers of less than 1:40 were considered negative.

2.3.4 Anti-*Leptospira* Ab were evaluated by MAT (Goris & Hartskeerl, 2014). Fourteen serovars (arborea, ballum, bratislava, canicola, copenhageni, grippotyphosa, icterohaemorragiae, hardjo, lora, mini, patoc, poi, pomona, tarassovi) belonging to 11 serogroups (Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Javanica, Mini, Pomona, Sejroe, Semaranga, Tarassovi) were used as antigens.

Leptospira species, serogroups, serovars and strains evaluated are described in table 2.c.

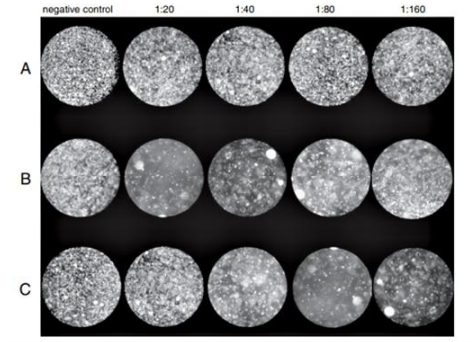
Table 2.c *Leptospira* species, serogroups, serovars and strains evaluated

SPECIES	SEROGROUP	SEROVAR	STRAIN
L. borgpetersenii	Ballum	ballum	Mus 127
L. interrogans	Canicola	canicola	Hond Utrecht IV
L. interrogans	Australis	bratislava	Jez Bratislava
L. kirschneri	Grippotyphosa	grippotyphosa	Duyster
L. interrogans	Icterohaemorragiae	icterohaemorragiae	Kantorowic
L. borgpetersenii	Javanica	poi	Poi
L. interrogans	Icterohaemorrhagiae	copenhageni	M20
L kirschneri	Grippotyphosa	grippotyphosa	Moskva V
L. interrogans	Pomona	pomona	Pomona
L. interrogans	Sejroe	hardjo	Hardjoprajitno
L. borgpetersenii	Tarassovi	tarassovi	Perepelitsin
L. interrogans	Australis	lora	Lora
L.borpetersenii	Ballum	arborea	Arborea
L.borpetersenii	Mini	mini	Sari
L.biflexa	Semeranga	patoc	Patoc I

Serial two-fold dilutions of serum were tested from 1:20 to 1:640. Inspection for agglutination, with a dark-field microscope using 80x to 120x total magnifying power was performed (fig 2.a).

Fig 2.a Example of agglutination with a dark-field microscope

(Goris and Hartskeerl, 2014)



Dark fiel microscopy (total magnifyin power 200x) pictures of agglutinations with various sera. (A) negative serum <1:20, (B) reactive serum, titre 1:80, (C) serum showing prozone effect, titre ≥1:160

To confirm positive results a droplet of the mixture in a wire loop was transfered onto a microscope slide and inspected for agglutination, prefarably at the border of the droplet with a dark-field microscope using 100-200x total magnifying power. The titre was recorded as the highest dilution of serum that agglutinated >50% of leptospires, compared to control suspension in the wells of the first column (which contains only *Leptospira* culture diluted in PBS without serum).

2.4 DNA extraction and *Leishmania* Real-Time PCR

DNA was extracted from blood EDTA, oral, conjunctival, auricolar swabs, swabs from lesions, lymph node aspirates (FNA), hair using the PureLink Genomic DNA kit (Invitrogen, California, USA) in different steps. According to the manufacturer's instructions, 200 µl of whole blood were employed for DNA extraction. Lymph node aspirates were resuspended in PBS (250 µl) and then homogenized. Swabs were individually placed for 1 minute in a sterile microcentrifuge tube with PBS solution (250 µl). Then, 200 µl (250 µl for swabs) of PureLink genomic lysis/binding buffer and 20 µl of Proteinase K were added to the suspension, followed by brief vortexing and incubated at 55°C for different times depending on the tissue sample to promote protein digestion. At the end of the extraction procedure, DNA was eluted in 100 µl of PureLink genomic elution buffer and stored at -20°C until used. The PCR test was targeted at the constant region in the minicircle Kinetoplast DNA (NCBI accession number AF291093). Real Time polymerase chain reaction of blood EDTA, swabs, lymph node was developed by the CFX96 Real-time System (Bio-Rad Laboratories s.r.l.) using TaqMan Master Mix (Applied Biosystems). A multiplex PCR was optimized including an internal DNA control with specific probe and primer according to the VIC internal PC Kit (Applied Biosystems). Real Time PCR was carried out in a final volume of 20 µl including a final concentration 1x of TaqMan Master Mix (Applied Biosystems), 0.3 µM of each primer (5'AAAATGGCATTTTCGGGCC-3' and 5'-GGCGTTCTGCGAAAACCG-3'), 0.25 µM of the fluorogenic probe (5'-FAM-TGGGTGCAGAAATCCCGTTCA3'-BHQ1) and 50 ng of DNA, 1x Exo IPC Mix, 1x Exo IPC DNA. The thermal cycle conditions consisted of 2 minutes initial incubation at 50°C and 10 minutes denaturation at 95°C, followed by 40 cycles at 95°C

for 15 s and at 60°C for 1 minute each. Samples were amplified in a single 96-well plate and in each one a positive control containing genomic *L. infantum* DNA and a negative control without DNA were included. Each standard, sample and negative control was analyzed in duplicate for each run. Cycle threshold (Ct) value was calculated for each sample by determining the point of the fluorescence value exceeding the threshold limit. The parasitic DNA load was defined in each examined sample by comparison of the data with a specific standard curve based on the number of *Leishmania* per ml of extracted volume. Standard curves were prepared for both the *Leishmania* gene target and the internal PC (IPC Applied Biosystems). A stock solution of *L. infantum* DNA was obtained by extraction from 10^9 promastigotes/ml. Ten fold serial dilutions of the DNA stock solution were performed to obtain the six points of the curve spanning from 10^6 to 10^1 DNA equivalent cells. The standard curve, calculated by independent experiments, was linear over at least 6 log ranges of DNA concentration points with an average correlation coefficient of 0.988. The difference for each point of the curve was one log factor (Vitale et al., 2004).

2.5 RNA extraction and PCR for FeMV and other viral pathogens

2.5.1 Nucleic acids were purified from EDTA-blood samples by means of the High Pure Viral Nucleic Acid Kit (Roche Life Science, Roche Diagnostics, Monza, Italy). According to the manufacturer's instructions, 200 µl of whole blood were used for the extraction. Viral nucleic acid was eluted in 50 µl of Elution Buffer and kept at -80°C until testing.

Nucleic acids were purified from urine by means of the QiAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the "spin protocol". Viral nucleic acid was eluted in 60 µl of Buffer AVE and kept at -80°C until testing for FeMV.

2.5.2 A real-time RT-PCR (qPCRFeMV) to detect and quantitate FeMV RNA in blood and urine was used (De Luca et al., 2018). Primers and probe were targeted to a conserved region of the FeMV P/V/C gene. Primer FeMVrt-F sequence was 5′- GGG ATCCAGAGGGTAACCT-3′ (position 2061-2079, KT825132), and primer FeMVrt-R sequence was 5′-CCGGCCATTAATCTCTGAA-3′ (position 2119-2137, KT825132). FeMVrt TaqMan probe (5′-TATTCGAA

AGCGATGATGATGAAAACCATTA-3′) was dual-labelled with 6-carboxyfluorescein (FAM) at the 5′ end and with tetramethylrhodamine (TAMRA) at the 3′ end (position 2088-2118, KT825132). Quantitect Probe RT-PCR Kit (Qiagen, Hilden, Germany) has been employed according to the manufacturer's instructions. The reaction was performed as follows: the 25 µl reaction volume contained 5 µl of purified RNA, 12.5 µl of 2× QuantiTect Probe RT-PCR Master Mix, 1 µl of Armored RNA West Nile Virus (HNY1999) (Asuragen) as non-competitive exogenous internal amplification control (EIAC), a final concentration of 200 nM for each EIAC primer (NS5-2-F, GAAGAGACCTGCGGCTC ATG; NS5-2-R, CGGTAGGGACCCAATTCACA), 160 nM for EIAC probe (NS5-2-P, CCAACGCCATTTGCTCCGCTG), 0.25 µL of QuantiTect RT Mix, a final concentration of 600 nM and 250 nM for each FeMV primer and probe, respectively, and nuclease-free water up to final volume. The assay was carried out on a 7900HT Fast Real Time System cycler (Applied Biosystem) using the following thermal profile: 1 cycle of reverse transcription at 50 °C for 30 min, 1 cycle of PCR initial activation step at 95 °C for 15 min followed by 45 cycles of 94 °C for 30 s and 55 °C for 1 min. The qPCRFeMV target sequence was generated sub-cloning the qPCRFeMV target sequence into a pGEM plasmid vector (pGEM®-T Easy Vector, Promega, Madison-WI, USA) according to the manufacturer's instructions. Ten-fold serial dilutions of RNA transcript which contained from 10^8 to 10° copies of cRNA μL^{-1} were used to plot the standard curve. The standard curve was linear with a wide dynamic range from 1×10^1 to 1×10^8 cRNA μL^{-1}. The LoQ (the lowest concentration of the analyte that could be quantified with acceptable accuracy) was defined as 10 cRNA copies μL^{-1}. (De Luca et al., 2018). Results were considered positive when Ct was < 45.

2.5.3 Blood samples were tested for FIV using a FIV Genesig real time PCR detection kit (PrimerDesign). Primers and probe were targeted on gag protein (gag) gene and the reaction was optimized including an endogenous control with specific probe and primers. The reaction was performed as follow: the 20 µl reaction volume contained 5 µl of purified RNA, 10 µl of PrecisionPLUS OneStep 2X RT-qPCR Master Mix, 1 µl of FIV primer/probe mix, 1 µl of Internal extraction control primer/probe mix, 3 µl of RNase/DNase free water. The assay was carried out on a 7900HT Fast Real Time

System cycler (Applied Biosystem) using the following thermal profile: 1 cycle of reverse transcription at 55°C for 10 minutes, 1 cycle of PCR initial activation step at 95 °C for 8 minutes, followed by 50 cycles of 95 °C for 10 seconds and 60°C for 1 minute. Results were considered positive when Ct was < 45.

2.5.4 Blood samples were tested for feline leukemia (FeLV) virus by using FeLV Genesig real time PCR detection kit (PrimerDesign). Primers and probe were targeted on the U3 region LTR (long terminal repeats) and the reaction was optimized including an endogenous control with specific probe and primers. The reaction was performed as follows: the 20 µl reaction volume contained 5 µl of purified RNA, 10 µl of PrecisionPLUS OneStep 2X RT-qPCR Master Mix, 1 µl of FIV primer/probe mix, 1 µl of Internal extraction control primer/probe mix, 3 µl of RNase/DNase free water. The assay was carried out on a 7900HT Fast Real Time System cycler (Applied Biosystem) using the following thermal profile: 1 cycle of reverse transcription at 55°C for 10 minutes, 1 cycle of PCR initial activation step at 95 °C for 8 minutes, followed by 50 cycles of 95 °C for 10 seconds and 60°C for 1 minute. Results were considered positive when Ct was < 45.

2.5.5 Blood samples were tested for feline coronavius (FCoV) virus by using TaqVet Feline Infectious Peritonitis-Dual IPC (LSI) kit (Thermo Fisher Scientific). The reaction was performed as follows: the 25 µl reaction volume contained 5 µl of purified RNA and 20 µl of Mix FIP. The assay was carried out on a 7900HT Fast Real Time System cycler (Applied Biosystem) using the following thermal profile: 1 cycle of reverse transcription at 45°C for 10 minutes, 1 cycle of PCR initial activation step at 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60°C for 45 seconds. Results were considered positive when Ct was < 45.

2.6 Isolation attempts from qPCR FeMV positive urine samples

Culture of urine samples diluted 1:8 with MEM and stored a 4°C was performed when qPCR was FeMV positive in urine samples. Briefly, 500 µl of diluted urine were centrifuged at 3000 rpm for 5 min to remove debris and filtered through 450 nm disc filters (Millipore). TPCK (L-1Tosylamide-2-phenylethylchloromethylketone-treated) trypsin (Sigma Aldrich, USA) was then added to a final concentration of 1 µg ml^{-1}.

Samples were incubated at 37 °C for 15 min. The mixture was then inoculated into feline embryonic fibroblast (FEA) cells in 24-well plates serum-free MEM Eagle (Sigma Aldrich, USA) supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}) (Invitrogen, California, USA). After 8 h, inocula were replaced with MEM supplemented by 3% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells were incubated at 37°C in a humidified atmosphere with 5% CO2 and observed daily for cytophatic effect (CPE) by microscopy. Each passage consisted of two weeks of incubation.

2.7 DNA extraction and PCR for *Leptospira* spp.

DNA was extracted from blood following procedure described in section 2.4.
Within 24 hours after collection, urine was centrifuged at 13,000 rpm for 15 minutes at room temperature. Supernatants were discarded and pellets were washed with phosphate buffered saline (PBS) and transferred into an Eppendorf tube (Eppendorf AG). After a second centrifugation step (13.000 rpm, room temperature, 15 minutes), the supernatant was discarded, and the pellet was resuspended in 180 µl animal tissue lysis (ATL) buffer (Qiagen GmbH, Hilden, Germany) and stored at -20°C until DNA extraction (Sprißler et al., 2019).
DNA was then extracted from urine using a Qiagen DNA Micro Extraction kit according to the tissue manufacturer's protocol, but with the lysis period reduced to 1 hour. To elute DNA, 54 µl AE buffer (Qiagen) was used.

Two different methods were used to perform PCR for *Leptospira* spp, (Stoddard et al. 2009; Ahmed and Goris personal communication, manuscript in preparation). With PCR described by Stoddard et al. (2009) primers (forward primer lip32-45F: 5′-AAGCATTACCGCTTGTGGTG-3′; reverse primer lip32-256R: 5′-GAACTCCCATTTCAGCGATT-3′) and TaqMan probe (lip32 189P: FAM-5′-AAAGCCAGGACAAGCGCCG3′-BHQ1) targeting the lipL32 gene of pathogenic *Leptospira* species, were used. Real-time PCR was performed using the Bio-Rad CFX96 system, C1000 cycler and TaqMan Universal PCR Master Mix (Thermo Fisher Scientific). Final reaction conditions were 12.5 µl of Master mix, 1.55 µl of each primer, 0.40 µl of the probe and 9 µl of DNA extract in a final volume of 25.0 µl. The

amplification protocol consisted of 2 mins at 50°C, 10 mins at 95°C and 45 cycles of amplification (95°C for 15 s and 60°C for 60 s), finishing with a cool cycle of 25°C for 10 mins. Each run included a single negative control containing PCR water and a single positive control containing leptospiral DNA. All samples as well as positive control and negative control were tested in duplicate. Results were considered positive if Ct values were recorded in at least one duplicate and were ≤ 40.

CHAPTER 3

PREVALENCE OF CHRONIC KIDNEY DISEASE AND ASSOCIATED RISK FACTORS IN CATS FROM PRIMARY CARE PRACTICES IN SOUTHERN ITALY

ABSTRACT

Chronic kidney disease (CKD) has high morbidity and mortality in feline patients. The study of prevalence and risk factors associated with this disease is of great interest. Among various risk factors associated with feline CKD, some emerging infectious pathogens were recently investigated. Serum creatinine (sCr) along with serum symmetric dimethylarginine (SDMA), urine protein to creatinine ratio (UPC) and blood pressure (BP) measurements are proposed by the *International Renal Interest Society* (IRIS) to stage and substage CKD in dogs and cats. The aims of the present study were to investigate the prevalence of CKD in cats attending primary care practices in Reggio Calabria (Calabria) and Messina (Sicily) provinces and to evaluate risk factors for CKD diagnosis. One hundred and eleven cats diagnosed with CKD according to IRIS guidelines (KD) and 104 healthy cats or cats with miscellaneous clinical abnormalities but with CKD ruled out by clinico-pathological investigation (NKD) were compared. Overall 30 variables were analyzed and, for the first time *Leishmania infantum*, *Leptospira* spp., and feline morbillivirus (FeMV) infections were investigated as risk factor for feline CKD. A high prevalence of CKD (52%) was observed, with higher prevalence in male cats (OR= 2.727) and cats with reduced body condition score (BCS) (OR= 2.117), pale mucous membranes (OR= 6.742), ocular (OR= 1.509) or oral (OR= 2.535) lesions, anemia (OR= 2.492), or increased urea values (OR=7.35). Moreover, increased urea values (OR= 55.8) and anemia (OR= 2.609) were associated with advanced CKD (IRIS stage 2-4) and prevalence of proteinuria (OR= 3.56) and inappropriate USG was higher in male cats (OR=2.481) with 42% of entire male cats affected by proteinuria (OR=2.79). No statistical differences were found in KD cats about age, breed, environment, poor muscle condition score

(MCS), increased phosphorus, increased total thyroxine (tT4), increased serum amyloid A (SAA) values and for the infectious pathogens investigated.

In conclusion, diagnosis of CKD has to be addressed in any adult cat, irrespective of age or reason for consultation but overestimation of proteinuria should be considered in entire male cats. A high prevalence of the investigated pathogens was found but this cross-sectional study did not show any associations between any of them and feline CKD.

3.1 INTRODUCTION

Chronic kidney disease (CKD) is defined as a prolonged process characterized by sustained alterations in structure or function of the kidney that has occurred over a period of time of more than 3 months (Jepson, 2016). In cats CKD has a high prevalence (Marino et al., 2014) and a slow progression (Polzin, 2011). Considering the important role of CKD in morbidity and mortality in cats (Finch et al., 2016), the study of prevalence and risk factors more frequently associated with development of this disease is a subject of great interest. Different risk factors have been found associated with feline CKD, age especially for old cats, sex with higher frequency in male cats, breed of pedigree cats (Reynold and Lefebvre, 2013), hyperthyroidism (van Hoek et al., 2009), and periodontal disease (Finch et al., 2016; Greene et al., 2014). Immune-complex deposition was demonstrated in glomeruli of FIP-affected cats (Jacobse-Geels et al., 1980) and there is some evidence that retroviral infections caused by feline immunodeficiency virus (FIV) or feline leukemia virurs (FeLV) have a causative role in CKD (Reynold and Lefebvre, 2013; Rossi et al., 2019). Other infectious agents are currently being studied such as *Leptospira* spp., feline morbillivirus (FeMV), and *Leishmania infantum* (Woo et al., 2012; Rodriguez et al., 2014; Pennisi et al., 2004, 2015).

Clinical features of CKD are widely reported (Reynold and Lefebvre, 2013) and some clinicopathological variables are associated with shorter survival time such as proteinuria (Syme et al., 2006) and increased serum creatinine (sCr) concentration (Boyd et al., 2008), therefore their evaluation and treatment is important to improve

prognosis. The *International Renal Interest Society* (IRIS) proposes a set of guidelines to diagnose and stage patients with CKD - basically by evaluating sCr - and to substage them by evaluating proteinuria through urine protein/creatinine ratio (UPC) and systolic blood pressure along with other clinicopathological signs and ultrasonographic findings (http://www.iris-kidney.com/). However, recently, different studies analyzed the utility of new biomarkers for CKD, such as serum symmetric dimethylarginine (SDMA) that is produced in the nucleus of all cells after a post-translational modification and methylation of arginine residues of various proteins and subsequent proteolysis. In fact SDMA is excreted by the kidney (Relford et al, 2016) and, compared to sCr value, is not affected by lean body mass and increases are detected 17 months earlier (Hall et al., 2014a). This means that SDMA is a more sensitive marker for kidney disease especially in patients with muscle mass loss (Hall et al., 2014b; Relford et al, 2016) and SDMA measurement has been introduced into the IRIS CKD staging system (http://www.iris-kidney.com/).

The aims of the present study were to investigate by a cross-sectional study the prevalence of CKD in cats attending primary care practice in provinces of Messina (Sicily) and Reggio Calabria (Calabria) and to evaluate risk factors associated with CKD diagnosis. Among risk factors, we evaluated infectious agents and for the first time emerging infectious pathogens such as FeMV, *Leptospira* spp., and *L. infantum* were concurrently investigated in the studied feline population.

3.2 MATERIALS AND METHODS

3.2a Cats, clinical examination and sampling- see chapter 2, section 2.1

3.2b CBC, biochemical profile, total-T4 and urinalysis- see chapter 2, section 2.2
In this study, anemia (hemoglobin < 9.8 g/dL), serum amyloid A (SAA), total proteins (TP), albumin (ALB), globulins (GLOB), urea (BUN), phosphorus (PHOS), and total-thyroxine (tT4) values were analyzed as risk factors for CKD.

3.2c CKD diagnosis and staging

Enrolled cats were considered affected by CKD according to IRIS guidelines on the diagnosis and staging of CKD (http://www.iris-kidney.com/) through physical examination, evaluation of SDMA, sCr, urine specific gravity (USG), UPC (table 3.a)

and, when available, ultrasonographic evaluation. Therefore, two groups of cats were defined as follows:

- Cats not-affected by CKD (NKD): cats with SDMA, sCr, USG and UPC values within the reference range and without any clinical signs or ultrasonographic findings related to CKD;
- Cats affected by CKD (KD): cats with CKD diagnosed following IRIS guidelines and considering reference interval of the analyzer used. Staging and proteinuria substaging of KD cats was performed according to IRIS staging system (table 3.a) and proteinuria cut off was set at 0.2 for statistical analysis. Diagnosis of CKD was confirmed after stabilization in cases of critically ill cats but repeated evaluation of USG and UPC values was not possible in all enrolled cats. When SDMA values were not available but sCr was evaluated and it was over the reference range along with clinical signs compatible with CKD or ultrasonographic findings suggestive of CKD, the cat was included into KD. Moreover KD cats could have concomitant diseases.

Table 3.a: IRIS criteria used to include cats with CKD in one of the four stages of disease severity according to SDMA and sCr values, and to substage them according to proteinuria. USG cut off value for CKD was <1035.

STAGE	**SDMA**	**Creatinine**
1	> 14 and < 25 µg/dL	≤ 2.4 mg/dL
2	> 14 and < 25 µg/dL	2.5-2.8 mg/dL
3	≥ 25 and < 45 µg/dL	2.9-5 mg/dL
4	≥ 45 µg/dL	>5 mg/dL
SUBSTAGE	**UPC**	
NP	< 0.2	
BP	0.2-0.4	
P	> 0.4	

Legenda: NP= non proteinuric; BP= borderline proteinuric; P= proteinuric

3.2d Antibody detection- see chapter 2, section 2.3

3.2e DNA extraction and *Leishmania* Real-Time PCR- see chapter 2, section 2.4

3.2f RNA extraction and PCR for FeMV, FIV, FeLV, FCoV- see chapter 2, section 2.5

3.2g DNA extraction and PCR for *Leptospira* spp.- see chapter 2, section 2.7

3.2h Statistical analysis

Descriptive statistics of data collected about clinical history and physical examination findings was performed and normal distribution of values of continuous variables, such as age, USG, sCr, and SDMA was preliminarly assessed by D'Agostino & Pearson normality test.

The two study groups (NKD, KD) were compared by Chi-Square test, Fisher exact test or Mann-Whitney test when appropriate, examining categorical variables in order to indentify those associated with CKD status. Differences were considered significant if P value was ≤ 0.05 and a tendency to significance was considered when it was >0.05 and ≤ 0.06. Statistical results are reported as odds ratios (OR), 95% C.I. (confidence interval) and significance. Comparisons concerned overall 30 variables including geographic region (Calabria/Sicilia), demographic (male/female; domestic short-hair/domestic long-hair/pedigree; junior/adult/senior), husbandry and environment characteristics (indoor/ outdoor; indoor single-cat/indoor multi-cat household; rural/sub-urban/urban), reason for consultation, signs detected at physical examination (BCS, MCS, mucous membranes color, lymph node enlargment, ocular, oral, dermatological lesions, gastrointestinal and respiratory signs), presence of anemia, biochemical (SAA, BUN, TP, ALB, GLOB, PHOS) or tT4 alterations, positivity to *L. infantum*, FeMV, *Leptospira* spp., FIV, FeLV, and FCoV tests.

Obviously, values of SDMA, sCr, USG, and UPC or frequency of ultrasonographic alterations in cats of the two clinical groups were not individually compared because they were the inclusion criteria used to assign cats into the appropriate clinical group. However demographic (age, sex and reproductive status, breed), husbandry and environment characteristics, increased tT4 values, presence of ultrasonographic alterations compatible with CKD were individually compared with SDMA, sCr, USG and UPC values

Associations between CKD and infectious pathogens were evaluated considering positivity of serological and molecular analysis apart from or in combination with *L. infantum*, FeMV, and *Lesptospira* spp. In case of FIV serological and molecular results were analyzed in combination, while only molecular analysis was available for FCoV and FeLV.

Statistical analysis was performed using GraphPad Instat 7.00.

3.3 RESULTS

Among 279 cats evaluated in the study period, only 215 had measurement of SDMA, sCr, and USG values, needed to have a complete IRIS CKD staging, and were considered for statistical analysis. One hundred and four cats (48%) were included in the NKD group and 111 (52%) in the KD group. Cats with CKD were staged as follows: 89 (80%) were stage 1; three (3%) were stage 2; six (5%) were stage 3; 13 (12%) were stage 4. Substaging of proteinuria was as follows: 48 cats (58%) were NP, 17 (20%) were BP, and 18 (22%) P.

Almost all NKD cats had some clinical or clinico-pathological abnormalities (92%, 96/104) and a low number of them were healthy (8%, 8/104). Median USG value of NKD cats was 1.050 (25-75 percentile (25-75) = 1.041-1.058, range 1.035 to 1.060), mean SDMA was 10.8 ±2.1 μg/dL and mean ±SD sCr was 1.11±0.364 mg/dL. Median USG value of KD cats was 1.035 (25-75= 1.019-1.051, range 1.007 to 1.060), median SDMA was 16 μg/dL (25-75=12-21, range 7 to 100 μg/dL), and median sCr was 1.2 mg/dL (25-75=1-1.7, range 0.5 to 16.8 mg/dL).

3.3a Reasons for consultation

Reasons for consultations were classified into four main causes and their frequency in the two groups is reported in table 3.b. Medical problems causing consultation included weight loss (n= 36), dermatological (n= 29), gastrointestinal (n= 21) and respiratory (n=17) problems, CKD (n= 12), ocular problems (n=11), lower urinary tract disease (n=7), neurological (n=2) or endocrine disorders (n=2), poisoning (n= 3), polyuria/polydypsia (n=1). More than one of these problems was reported in some cats. Surgical procedures included osteosynthesis (n=3), and limb amputation (n= 2).

Cats in KD were more frequently admitted for medical problems than for routine health checks compared to NKD cats (P=0.0399, OR=2.093, 95% CI=1.101-3.884).

Table 3.b: reason for consultation of enrolled cats

REASON FOR CONSULTATION	NKD (%)	KD (%)	TOTAL (%)
Routine health check	34 (59)	24 (41)	58 (27)
Elective surgery	23 (53)	20 (47)	43 (20)
Medical problems*	44 (40)	65 (60)*	109 (51)
Surgical procedures	3 (60)	2 (40)	5 (2)

Legenda: *=significant difference

3.3b Geographic region, demographic, husbandry and environmental data

Demographic data of cats and information about their husbandry, environment and region of origin are summerized in table 3.c. Cats were aged between five and 216 months (median 36 months). Median age was the same in the two groups (36 months) and age range was between 6 and 180 months in NKD (25-75= 12-96) and between 6 and 216 months in KD (25-75= 12-108) cats. The frequency of male cats was significantly higher (P= 0.0004, OR= 2.727, 95% CI= 1.586-4.631) in KD but there was no difference between the prevalence of tomcats (58%, 41/71) and neutered individuals (42%, 30/71). There were only 14 pedigree cats with Maine Coon (n= 9), Persian (n= 2), Ragdoll (n= 1), British Short Hair (n= 1), and Chartreux (n= 1) breeds represented. Thirty four (33%) of the indoor cats were from single-cat households (NKD=44%; KD=56%) and 69 (67%) were from multi-cat households (NKD=48%; KD=52%).

Table 3.c: demographic, husbandry, environmental and geographic data analyzed as risk factors for CKD

PARAMETERS	NKD (%)	KD (%)	TOTAL (%)
AGE			
Junior	19 (18)	21 (19)	40 (19)
Adult	56 (54)	59 (53)	115 (53)
Senior	29 (28)	31 (28)	60 (28)
SEX*			
Female	63 (61)	40 (36)	103 (48)
Male	41 (39)	71 (64)*	112 (52)
BREED			
Domestic Short Hair	86 (82)	95 (85)	181 (84)
Domestic Long Hair	9 (9)	11 (10)	20 (9)
Pedigree	9 (9)	5 (5)	14 (7)
LIFESTYLE			
Indoor	48 (46)	55 (49)	103 (48)
Otdoor	56 (54)	56 (51)	112 (52)
ENVIRONMENT§			
Urban	63 (62)	74 (68)	137 (65)
Suburban	28 (27)	30 (27)	58 (27)
Rural	11 (11)	5 (5)	16 (8)
REGION			
Sicily	39 (38)	28 (25)	67 (31)
Calabria	65 (62)	83 (75)	147(69)

Legenda: §Information about environment was available for 211 cats. * significant difference

3.3c Physical examination

Clinical findings observed during physical examination and analyzed as risk factors are described in table 3.d.

Table3.d: Physical examination findings analyzed as risk factors for CKD

Physical examination findings	NKD (%)	KD (%)	TOTAL (%)
BCS*			
≥ 3/5	90 (87)	82 (75)	172 (81)
< 3/5	14 (13)	27(25)*	41 (19)
Missing data	0	2	2
MCS			
1/4	78 (79)	79 (75)	157 (77)
2-4/4	21 (21)	26 (25)	47 (23)
Missing data	5	6	11
MUCOUS MEMBRANES*			
No abnormality	100 (96)	89 (82)	189 (89)
Pale	3 (3)	18(16)*	21 (10)
Icteric	1 (1)	2 (2)	3 (1)
Missing data	0	2	2
LYMPH NODES			
No abnormality	77 (74)	70 (65)	147 (69)
Enlarged	27 (26)	38 (35)	65 (31)
Missing data	0	3	3
OCULAR LESIONS*			
Absent	101 (98)	97 (89)	198 (93)
Present	2 (2)	12(11)*	14 (7)
Missing data	1	2	3
ORAL LESIONS*			
Absent	75 (73)	56 (51)	131 (62)
Present	28 (27)	53(49)*	81 (38)
Missing data	1	2	3
RESPIRATORY SIGNS			
Absent	94 (91)	94 (86)	188 (89)
Present	9 (9)	15 (14)	24 (11)
Missing data	1	2	3

GASTROINTESTINAL SIGNS			
Absent	99 (96)	99 (91)	188 (89)
Present	4 (4)	10 (9)	14 (11)
Missing data	1	2	3
SKIN LESIONS			
Absent	86 (83)	87 (80)	173 (82)
Present	17 (16)	22 (20)	39 (18)
Missing data	1	2	3

Legenda: *significant difference

The observed ocular lesions were conjunctivitis (n=12) and keratoconjunctivitis (n=2), and the former was the only ocular abnormality observed in NKD cats. Oral lesions consisted of stomatitis (n=46), periodontitis (n=26), dental calculus (n=20), ulcers (n=10), drooling (n=9), faucitis (n=7), missing teeth (n=6), bleeding (n=1), cleft palate (n=1), neoplasia (n=1). Some cats had more than one of these abnormalities and the two most common lesions observed (stomatitis and periodonotitis) were similarly represented in the two groups.

Respiratory signs were related to upper (n=16) and lower respiratory tract (n=6) or both (n=2), and similarly represented in the two groups.

Gastrointestinal signs included diarrhea (KD=7; NKD=3), and vomiting (KD=3; NKD=1). Abdomen examination revealed hepatomegaly (KD= 7; NKD= 1), effusion (KD= 2; NKD=1), pain on palpation (KD= 1; NKD=1) or splenomegaly (KD=1) and some cats had more than one of these abnormalities.

Dermatological lesions included alopecia (n=24), crusty dermatitis (n=12), ulcers (n=5), abscess (n=5), nodular dermatitis (n=5), erythema (n=2), squamous dermatitis (n=1), pyoderma (n=1) and some cats had more than one of these lesions. The most frequent skin lesions were similarly represented in the two groups.

Reduced BCS (P=0.0388, OR=2.117, 95% CI=1.065-4.415), pale mucous membranes (P= 0.009, OR= 6.742, 95% CI= 2.073-22.08), ocular (P=0.017, OR= 6.247, 95% CI= 1.509-28.44) and oral lesions (P= 0.0018, OR= 2.536, 95% CI= 1.417-4.539) were significantly more frequent in KD cats.

3.3d Clinicopathological evaluation

Clinicopathological alterations evaluated as risk factors are listed in table 3.e. Frequency of proteinuria was significantly higher in male cats (74%, 26/35) compared to females (26%, 9/35) (P= 0.0022, OR= 3.56, 95% CI=1.573-8.599), and a tendency to significance was found for proteinuria in tomcats (69%, 18/26) compared to neutered males (31%, 8/26) (P= 0.0568, OR= 2.79, 95% CI= 1.086-7.681). Similarly inappropriate USG values were more frequently found in males (68%, 36/53) compared to females (32%, 17/53) (P=0.0068, OR 2.481, 95% CI= 1.303-4.906).

The presence of anemia (P= 0.0031, OR= 2.492, 95% CI=1.37-4.598) and increased urea values (P < 0.0001, OR= 7.35, 95% CI=2.867-18.17) were more frequent in KD with higher values of BUN observed in cats with CKD stage 2-4 (90%, 18/20) compared to cats in stage 1 (13%, 10/79) (P < 0.0001, OR= 55.8, 95% CI= 11.22-253.3). A tendency to significance was found in prevalence of anemia in CKD stages 2-4 (59%, 13/22) compared to stage 1 (36%, 31/87) (P= 0.0543, OR= 2.609, 95% CI= 1.025- 6.427).

Anemia affected one third of cats and it was non-regenerative in almost all cases (61/66). Non-regenerative anemia was seen in 67% of KD cats (41/61), and 33% (20/61) of NKD cats but the difference was non significant.

No other significant differences were found. All 10 cats with increased tT4 were non proteinuric.

Table3.e Clinicopathological variables analyzed as risk factors for CKD

VARIABLES (#)	NKD (%)	KD(%)	TOTAL(%)
SAA (114)			
Normal	43 (75)	37 (65)	80 (70)
Increased	14 (25)	20 (35)	34 (30)
BUN (193)*			
Normal	84 (90)	64 (65)	148 (77)
Increased	5 (5)	28 (28)*	33 (17)
Decreased	5 (5)	7 (7)	12 (6)
TP (161)			
Normal	73 (88)	66 (85)	139 (86)
Increased	10 (12)	9 (11)	19 (12)

Decreased	0	3 (4)	3 (2)
ALB (162)			
Normal	75 (91)	73 (91)	148 (91)
Decreased	7 (9)	7 (9)	14 (9)
GLOB (157)			
Normal	63 (78)	55 (72)	118 (75)
Increased	18 (22)	19 (25)	37 (24)
Decreased	0	2 (3)	2 (1)
PHOS (149)			
Normal	53 (68)	43 (60)	96 (64)
Increased	25 (32)	28 (40)	53 (36)
ANEMIA (212)*			
Absent	81 (79)	65 (60)	146 (69)
Present	22 (21)	44 (40)*	66 (31)
tT4 (39)§			
Normal	13 (76)	16 (73)	29 (74)
Increased	4 (24)	6 (27)	10 (26)

Legenda: (#): numebr of tested cats; §Total t4 was measured in cats aged $\geq$ 8 years; * significant difference.

3.3e Ultrasonographic examination

Ultrasonography was performed in 70 cats and 33 (47%) had renal abnormalities, distributed as follows: hyperecogenic kidney cortical (n=29), altered kidney profile (n=8), fibrosis or mineralization of renal pelvis (n=7), rim sign (n=5), altered cortico-medullary ratio (n=2), hyperecogenic kidney medullary (n=1), suspected neoplasia (n=1) and some cats had more than one of these findings. There were no differences in prevalence of ultrasound alterations compatible with CKD between cats with abnormal values of SDMA, sCr, USG or UPC and those with normal values. Of the 33 cats with renal abnormalities: 14 had a low USG, 12 increased SDMA, 12 proteinuria, 5 increased sCr and some of them had more than one of these abnormalities. However, 13 cats with ultrasonographic abnormalities compatible with CKD did not show any of the above clinicopathological abnormalities.

3.3f Infectious pathogens

A high prevalence of cats positive to FeMV (29%), *L. infantum* (24%), *Leptospira* spp. (22%), FIV (18%), and FCoV (17%) was found with a much higher prevalence of *Leptospira* spp. in the KD group (28%) compared to the other cats (16%), but no statistical differences were found in prevalence of investigated pathogens between the two clinical groups. Only five cats (2%, 5/209) were FeLV positive and just one in the KD was diagnosed with CKD IRIS stage 1.

Frequency of positivity to the investigated pathogens is summarized in table 3.f.

Table 3.f: Prevalence of pathogens

	NKD (%)	**KD(%)**	**TOTAL(%)**
***L. infantum* (IFAT and/or PCR)**			
Negative	79 (77)	79 (75)	158 (76)
Positive	23 (22)	26 (25)	49 (24)
FeMV (IFAT and/or b/u PCR)			
Negative	62 (72)	67 (71)	129 (71)
Positive	24 (28)	28 (29)	52 (29)
FIV (PCR)			
Negative	87 (85)	84 (79)	171 (82)
Positive	15 (15)	22 (21)	37 (18)
FCoV (PCR)			
Negative	67 (86)	67 (80)	134 (83)
Positive	11 (14)	17 (20)	28 (17)
***Leptospira* spp. (MAT and/or PCR)**			
Negative	47 (84)	39 (72)	86 (78)
Positive	9 (16)	15 (28)	24 (22)

Legenda: b/u= PCR positivity in blood and/or urine sample. Only overall prevalence is reported for pathogens investigated by both antibody detection and PCR.

3.4 DISCUSSION

This cross-sectional study provided for the first time information about the association between infections caused by three feline emerging pathogens and CKD and evaluated overall 30 variables as risk factors for chronic renal disease.

Despite a limitation of this study: lack of ultrasound examination of about two third of enrolled cats, a high prevalence of CKD (52%) was observed in cats from Messina (Sicily) and Reggio Calabria provinces (Calabria) similar to that reported in cats from the USA (Marino et al., 2014). Interestingly, CKD was diagnosed in 22% of cats admitted for annual health checks and in 18% of cats presented for elective surgery. This means that diagnosis was in some way unexpected to the owners. Surprisingly, frequency of CKD diagnosis was similar in the three age groups. This means that about half of the examined junior cats (< 1 year old) suffered from CKD therefore the annual health check should include urinalysis at any adult age, but at present it is included in the annual health check just of cats aged $\geq$ 7 years (International Cat Care). Old cats were reported to be more frequently affected by renal disease (Trevejo et al., 2018) with an increased prevalence among cats older than 15 years (Marino et al., 2014) and a mortality rate greater in cats over 9 years old (Lawler et al., 2006). However, Finch et al. (2016) did not find age as a predictor for azotemia in cats (Finch et al., 2016). However, one may take into account that some cat owners are unable to provide precise information about the age of their cats but rather give a rough estimate (Finch et al., 2016).

Findings significantly overrepresented among cats with CKD included male sex (OR=2.727), reduced BCS (OR=2.117), pale mucous membranes (OR=6.742), ocular (OR=6.247) and oral (OR=2.535) lesions, anemia (OR=2.492) and increased BUN (OR=7.35). Interestingly, proteinuria was significantly more frequent in males (OR=3.56) and in tomcats the higher frequence of proteinuria approached significance (OR=2.79). Additionally, inappropriate USG (OR=2.481) was significantly more frequent in males. Previous studies reported that males are more likely than females to develop CKD, with involvment of young and neutered male cats (Greene et al., 2014; White et al., 2006). The present study confirms that males are about three times more frequently affected by CKD and proteinuria than females. However, a recent study

reported that healthy entire male cats secrete cauxin into urine, this is a protein probably involved in the synthesis of feline pheromones and produced by healthy tubular cells that could be related to false positive proteinuria (Ferlizza et al., 2015). In fact, although we did not find any significant difference in CKD prevalence, proteinuria was marginally more frequent in entire males and they were almost three times more at risk for proteinuria compared to neuterd cats. Therefore, the presence of proteinuria in entire male cats has to be confirmed by more specific diagnostic techniques, especially for a qualitative and quantitative interpretation. Among these latter techniques, urine electrophoresis techniques such as sodium dodecyl sulphate-polyacrylamide gel glectrophoresis (SDS-PAGE) or sodium dodecyl sulfate-agarose gel electrophoresis (SDS-AGE) have been assessed in dogs and cats, and new alternative electrophoresis methods are the subject of recent studies , such as high-resolution electrophoresis (HRE) as their use allows fractionation and identification of the urine proteome offering more information on areas of the nephron involved. Moreover techinques such as mass spectrometry can be useful in protein identification (Zini et al., 2004, Giori et al., 2011, Ferlizza et al., 2015, Ferlizza et al., 2017). However, we think that an actual association between CKD and male sex was found in this study because males also had a 2.5 times increased risk for inappropriate USG. There is evidence that companion animals affected by CKD often have weight loss, or muscle wasting and cachexia (Freeman, 2012) which are related to a shorter survival time (Freeman et al., 2016). Decreased food consumption cannot be excluded as a cause of the weight loss (Greene et al., 2014) however metabolic changes are also involved. Reduction in body weight over the preceeding 6 to 12 months and a poor body condition at the time of study entry were associated with a diagnosis of CKD (Greene et al., 2014). In KD cats a higher prevalence of both low BCS (66%) and poor MCS (55%) compared to NKD was found and cats with CKD were two times more at risk of a decreased BCS.

Oral disease is very common in cats (Girard et al., 2009) and was the most frequent clinical finding among those examined in this study. Periodontal diseases may contribute to the development of CKD in cats as a result of persistent low-grade insults: production of inflammatory cytokines or endotoxemia and immune response to bacteria are mechanisms involved in periodontal diseases and can contribute to

increased risk of renal disease (Finch et al., 2016). Two previous studies reported a significant association between CKD and periodontal diseases, with 52% of cats affected and a risk factor for CKD that increased with severity of periodontal disease (Greene et al., 2014; Finch et al., 2016). A similar prevalence was found in the present study with a significant higher prevalence of oral lesions (65%) in KD cats which were twice as likely to have these lesions. However a limitation of our analysis is that not only periodontal disease but miscellaneous lesions were included in this category. Some of these oral lesions could be a consequence of CKD as uremic toxins are responsible for ulcerative stomatitis (Polzin, 2011) and this result does not demonstrate a causative role for the various lesions we observed. Moreover we had no information about the time of onset and the chronic course of inflammatory diseases is most importantly considered as risk factor for CKD. Therefore, evaluation and early control of oral disease is recommended in all cats as it is a frequent clinical abnormality as confirmed in the present study with 38% of cats affected.

Ocular lesions affected 86% of cats with CKD and consisted of conjunctivitis (10 KD cats and 2 NKD cats) and keratoconjunctivitis (2 KD cats). In cats affected by CKD ocular lesions are often reported as a cosequence of hypertension and are mainly fundoscopic abnormalities (Acierno et al., 2018; Taylor et al., 2017). In this study evaluation of blood pressure and ocular fundoscopy were not performed in all cats and this is a limitation, however we can say that cats with CKD were six times more at risk to have conjunctivitis or keratoconjunctivitis.

Shorter survival time of cats affected by CKD was found significantly associated with azotaemia including both increased serum creatinine and urea concentrations (Paepe and Daminet, 2013) or only high plasma concentrations of urea (King et al., 2007). In line with these latter studies, we found that about one third of cats affected by CKD had increased BUN values and KD cats were more than seven times at risk for this abnormality. As expected advanced renal disease (IRIS stage 2-4) carries a tremendous increased risk (more than 50 times) for high values of BUN compared to the early stage (IRIS stage 1).

Anemia is a well known complication of CKD, especially in end stage renal disease (Elliot and Barber, 1998). Low erythropoietin (EPO) is considered the main cause of CKD associated anemia (Chakrabarti et al., 2012) and anemic cats seem to have lower

EPO concentrations when compared with non-anemic cats with CKD and healthy cats (Javard et al., 2017). Anemia significantly affects the well-being of patients causing lethargy and anorexia, and anemic hypoxia impairs any organs (Ettinger and Feldman, 2016). Anemia can contribute to renal interstitial fibrosis (Chakrabarti et al., 2013) and it is a marker of poor prognosis in terms of survival (Elliot and Barber, 1998). In the present study anemia was a frequent abnormality affecting 31% of cats, but cats with pale mucous membranes or anemia were respectively more than six or two times at risk for CKD. A tendency for increased risk for anemia in advanced stages (59%, 13/22) of CKD compared to stage 1 (36%, 31/87) was also found confirming the prognostic value of this abnormality in cats with CKD. An anemia due to CKD is generally non-regenerative (Elliot and Barber, 1998; Paepe and Daminet, 2013; Ettinger and Feldman, 2016) but in both groups anemia was almost always non-regenerative. Apart from low EPO, chronic inflammation is a common cause of non regenerative anemia and this can explain the occurrence of this abnormality in NKD cats too. Clinicopathological abnormalities will however be analyzed in depth and discussed in chapter 5.

Recently, Lamb et al. (2018) reported some associations between ultrasonographic findings and clinicopathological abnormalities in CKD such as azotemia and lower USG (Lamb et al., 2018). In the present study kidney ultrasonographic changes did not differ in relation to SDMA, sCr, USG or UPC abnormalities. Importantly, this means that urinary ultrasound investigation can in some cases uncover renal disease earlier than clinicopathological investigations and it is recommended to perform ultrasound investigations to exclude the risk for renal disease in apparently healthy cats (www.iris-kidney.com). Indeed, in the present study, 39% (13/33) of renal abnormalities were found in cats with no changes in parameters related to altered renal function. Therefore, the lack of ultrasound examination in about two thirds of enrolled cats is a substantial limitation of this study because we could have missed some diagnoses in cats with stage 1 CKD. Ultrasonographic findings observed in KD cats with no other clinicopathological abnormalities compatible with CKD were similar to those of other KD cats.

No statistical differences in breed, environment and life style, increased SAA, phosphorus, or tT4 values and for the infectious pathogens investigated were found in KD cats. These findings are individually commented below.

Some breeds such as Siamese, Persian, Abyssinian, Himalayan, Maine Coon, Russian Blue, Burmese are reported to be more likely affected by CKD (Boyd et al., 2008; Trevejo et al., 2018; Conroy et al., 2019). We examined a very small number of cats of some of these breeds so we had to analyze the data as a single category of pedigree cats and this is clearly a limitation in the analysis of risk factor for any individual breeds. However, four out of nine examined Main Coon cats were affected by CKD (three of them in IRIS CKD stage 1 and one in IRIS CKD stage 3).

Lifestyle variables were evaluated as a risk factor for cat kidney disease but generally significant associations were not found. For instance, two studies did not find significative differences between cats from urban or semirural/rural environments (Finch et al., 2016; Hughes et al., 2002) and these results are confirmed in the present study. Moreover, we observed about the same prevalence of CKD in both indoor and outdoor cats.

Inflammatory cells are involved in the genesis of progressive renal fibrosis and are responsible for production of inflammatory mediators (Jepson, 2016). More advanced CKD seems to be associated with more severe inflammation and increased SAA values (Javard et al., 2017). Similar to a recent study evaluating a heterogeneous sample of cats from the same geographic area (Persichetti et al., 2018), we found that increased SAA values are frequently (30%) detected in cats. A higher prevalence (59%) of increased SAA values was found in KD cats, but the difference with NKD cats was not significant. After all this is a non specif marker of inflammation that therefore affected one fourth of cats in this latter group.

Hyperphosphatemia may reflect more advanced renal disease (Chakrabarti et al., 2012) and it is associated with more severe interstitial fibrosis (Chakrabarti et al., 2013). Moreover, increased PHOS levels predispose to renal mineralization, which subsequently promotes inflammation and fibrosis and it is therefore considered a risk

factor for the survival of cats with CKD (Jepson, 2016). We found a high prevalence (36%) of increased PHOS values in agreement with Persichetti et al. (2018). Despite most of KD cats were at stage 1 (80 %) of CKD when PHOS levels are usually within the reference range, we found a higher prevalence of hyperphosphatemia (53%) in cats from KD compared to NKD but the difference was not significant. Pathological increases of PHOS are most commonly caused by CKD but hemolysis with release of PHOS from red blood cells is a common cause of preanalytical error (Paltrinieri et al., 2010). We excluded from PHOS testing clearly hemolytic samples but a low grade of hemolysis could have been responsible for some borderline increased PHOS values that however added up to 44 % (11/25) of increased PHOS values in NKD and to 25% (7/28) in KD cats.

Hypethyroidism and CKD can occur concurrently in old cats (van Hoek et al., 2009) and prevalence of hyperthyroidism reported in a population of cats older than 9 years of age was 6% (Wakeling et al., 2011). Thyroid hormones cause increased renal blood flow because of an increased cardiac output due to positive chronotropic and inotropic effects, decreased vascular resistance and also increased blood volume caused by the renin-angiotensin-aldosterone system (RAAS) activation (van Hoek and Daminet, 2009). Consequences of hyperthyrodism are therefore increased glomerular filtration rate (GFR), decreased USG and increased UPC (Langston and Reine, 2006). However, in this study no association was found between increased tT4 values and CKD or individual abnormalities in USG, SDMA and sCr values. Moreover, all hyperthyroid cats were not proteinuric, but we do not know how long they had been suffering from hyperthyroidism. Interestingly, about one fourth of cats $\geq$ 8 years of age had increased tT4 levels and this prevalence is higher than reported in previous studies (Köhler et al., 2016; McLean et al., 2017).

Chronic renal failure is a severe consequence of disease progression and the main cause of mortality due to leishmaniosis in dogs. Renal involvment is mainly due to deposition of immune complexes with development of glomerulonephritis, tubulointerstitial nephritis, or occasionally renal amyloidosis. Therefore, progression of disease often manifests with proteinuria and leads to end stage renal disease

(Solano-Gallego et al., 2011; Baneth et al., 2018). Some studies reported the presence of glomerular disease and chronic renal failure also in cats infected by *L. infantum* (Pennisi et al., 2013a). In particular, some followed up cases of cats affected by feline leishmaniosis were diagnosed with renal disease at the time of diagnosis or progressively developed CKD (Pennisi et al., 2004; Pennisi et al., 2016). Moreover isolation of *Leishmania* spp. (Caracappa et al., 2008) and histopathological lesions in kidneys with a moderate interstitial inflammatory infiltrate, moderate interstitial fibrosis (Navarro et al., 2010) and granulomatous nephritis are reported (Puleio et al., 2011). We found a high prevalence of *L. infantum* exposure (24%) compared to other studies performed with the same IFI cut off in cats from the same area (Pennisi et al., 2012; Persichetti et al., 2018). Prevalence was similar in the two groups, as well as antibody titre, with 51% (18/35) and 49% (17/35) cats with border line ($\leq$160) antibody titres respectively in KD and NKD cats. However, the high prevalence of positive cats in the NKD group, where cats affected by a wide range of clinical manifestations were included, is compatible with the clinical characteristics of feline leishmaniosis (Pennisi et al., 2015). As reported above, in this cross-sectional study information about the onset or duration of a detected abnormality is lacking. In this case we do not know how long the cats of the two groups have been positive for *L. infantum* and, as far as we know for canine leishmaniosis, this is an important factor for development of renal disease.

Feline retroviruses are considered potentially responsible for CKD (Glick et al., 1978). As seen in previous studies, FeLV infection is rare in Sicily and Calabria (Persichetti et al., 2018) and in the present study only two percent of cats (5/209) were positive for FeLV with only one cat affected by renal disease with IRIS CKD stage 1. Conversely FIV is reported since 1991 (Buonavoglia et al., 1991) with positivity ranging between 7.6 % (Persichetti et al., 2018) and 16% (Bechtle et al., 1992). We found a high prevalence in the tested population (18%) with similar prevalences in the two groups which can be explained with the same comments we made above for *L. infantum.* A significant relationship is reported in some studies between feline CKD and FIV infection (White et al., 2010), due to deposition of immune complexes (Poli et al., 1993). Azotemia, proteinuria, low USG, renal ultrasonographical abnormalities and histopathological findings are reported in cats affected by FIV (Poli et al., 1993; Poli

et al., 1995; Asproni et al., 2013; Baxter et al., 2012; Taffin et al., 2017 Poli et al., 2012). However, similar to the present study, Baxter et al. (2012) and Gleich and Hartmann (2009) did not find associations between FIV infections and renal azotemia. Feline infectious peritonitis can involve kidneys in both effusive and non effusive forms, characterized by accumulation of protein-rich fluid in the subcapsular space of the kidneys, and pyogranulomatous inflammation respectively (Lewis and O'Brien, 2010). Renomegaly, altered echogenicity and histopathological alterations such as pyogranulomatous interstitial nephritis are reported in affected cats (Lewis and O'Brien, 2010). In this study we tested cats for FCoV infection by performing a blood PCR analysis and diagnosis of FIP was not our aim. Confirmatory tests for diagnosing FIP are based on histopathology from affected organs and cytological examination of effusions or biological fluids (liquor, aqueous humor) with the detection of intracellular FCoV by immunofluorescent or immunohistochemistry staining (Hartmann, 2005). We had a high prevalence (17%) of positive FCoV blood PCR in the 162 tested cats and no cat was clinically suspected of FIP but they were in most cases affected by other pathologies. Prevalence was higher in KD cats (61%) but the difference was not significant. However, a high viral load (Ct < 20) was detected in half (8/17) of FCoV positive cats in KD while the lowest Ct value was 21 in NKD cats and it was observed in only one individual. This observation confirms the low specificity of a positive blood PCR test to diagnose FIP and that FCoV quantitative estimations can be useful to evaluate the causative role of the infection in clinical settings (Kipar et al., 2006).

Chronic kidney disease in *Leptospira* spp. infected cats was reported in a few case reports (Arbour et al., 2012; Mason et al., 1972) with manifestation of polyuria and polydypsia and tubulointerstitial nephritis as histopathological finding (Arbour et al., 2012). However results from studies that evaluated serological evidence of exposure of cats to *Leptospira* spp. and kidney disease are conflicting (Shropshire et al., 2016; Rodriguez et al., 2014). In fact, Rodriguez et al. (2014) found a significant higher antibody prevalence against six serovars (from two serogroups of two *Leptospira* spp.) in azotemic cats, but no difference was found in urinary PCR positivity. Shropshire et al. (2016) did not find associations in aged cats between *Leptospira* spp. exposure measured by antibody detection against the same six serovars and increased values of

creatinine. Based on antibody detection and PCR we found 22% prevalence (see more details in chapter 4). Overall prevalence was higher in KD cats (63%) but the difference with NKD cats (37%) was not significant. Similarly significant differences were not found in antibody (KD=56%; NKD=44%) and DNA molecular analysis in blood and/or urine (KD=64%; NKD=36%) individually compared. However, we found a higher prevalence than the one observed in the two previous studies. In Shropshire et al., (2016) study, an antibody prevalence of 6% was reported among cats affected by renal disease and in Rodriguez et al. (2014) antibody prevalence was higher (14.9%) and similar to the one observed in the present study but molecular prevalence was as low as 5.3% of positive cats affected by renal disease. We measured antibody prevalence by detecting antibodies against 14 serovars belonging to 11 serogroups using two different molecular techniques for PCR and this has certainly increased the overall diagnostic sensitivity (see chapter 4).

Recently a new paramyxovirus, feline morbillivirus was isolated for the first time from cats in Hong Kong and associated with tubulointerstitial nephritis (TIN) (Woo et al., 2012). After this discovery, several studies evaluated the presence and the associations of this pathogen with CKD, but this relationship is controversial. Some studies reported intestitial inflammatory infiltrates, tubular degeneration/necrosis, and glomerulosclerosis in infected kidneys (Woo et al., 2012; Park et al., 2016; Yilmaz et al., 2017; Sutummaporn et al., 2019). However a causative relationship was not found in studies which evaluated the infection in azotemic CKD cats (Darold et al, 2017; Lo Russo et al., 2017; McCallum et al., 2018; Donato et al. 2018). As we observed in a preliminary investigation (Donato et al., 2018), FeMV infection is quite common in the studied area and prevalence of infection in cats with CKD is similar to that found in the control group (NKD). Therefore no association was observed analyzing data obtained individually by antibody detection and PCR performed in urine, combination of PCR results from blood and urine, and from the combination of all tests performed. Similarly to what we commented above concerning the role of *Leishmania* and FIV infections, NKD cats were affected by a wide range of clinical problems and this could be compatible with the clinical characteristics of FeMV.

3.5 CONCLUSION

We did not find significant associations between CKD and FeMV, *Leptospira* spp., or *L. infantum* infections - as well as with retroviral or FCoV infections - but a different methological approach is warranted to determine their role more accurately. This includes longitudinal studies with long term follow up of both positive and control negative individuals but these experimental conditons are not easily obtained in clinical field trials even when costs are not a limitation. In the absence of longitudinal studies, more extensive cross-sectional investigations with a clinically healthy control group can corroborate or refute the present results.

Among the information additionally provided in this chapter we think that the take-home-message includes:

- Diagnosis of CKD has to be addressed in any adult cat, irrespective of age or reason for consultation but overestimation of proteinuria should be considered in intact male cats
- To rule out CKD ultrasound evaluation of kidneys and urinary tract should be included.

CHAPTER 4

EPIDEMIOLOGY IN SOUTHERN ITALY OF INFECTIOUS AGENTS POTENTIALLY ASSOCIATED WITH CHRONIC KIDNEY DISEASE

ABSTRACT

The pathogenic role of feline emerging infectious pathogens, such as, *L. infantum*, feline morbillivirus (FeMV), *Leptospira* spp. has to be better defined. Aims of the present study were to evaluate the prevalence of *L. infantum*, *Leptospira* spp, and FeMV infections in a population of cats of Southern Italy and to investigate clinical and clinicopathological findings more frequently associated to them. We found a 20% antibody prevalence, 8% molecular prevalence and 23% overall prevalence of *L. infantum.* Sensitivity and specificity of *L. infantum* PCR tests performed with different tissues was compared to *L. infantum* antibody detection: PCR tests from enlarged lymph nodes provided poor sensitivity (12%, 95% CI 20-34%) and specificity (33%; 95% CI 17-88%). Performance of mucosal swabs, hair and whole blood was good for specificity (mucosal swabs=99.7%, 95% CI 98-99%; hair= 97.8%, 95% CI 94-99; blood= 97.6%; 95% CI 94.5-98.9%), but their sensitivity was poor (mucosal swabs= 10%, 95% CI 5.4-18.1%; hair= 0, 95% CI 0-12.; blood= 12.8%, 95 CI 5.9-25%). The best performance was obtained by PCR from skin lesions with fair (62.5%, 95% CI 30-86%) sensitivity and 100% specificity (95% CI 74-100%). Poor agreement between antibody detection and PCR tests results was found with the exception of PCR positive skin lesions usually found in antibody positive cats. Antibody prevalence of 14%, molecular prevalence in blood of 2% and of 16% in urine, overall molecular prevalence of 17% and overall antibody and molecular prevalence of 28% for FeMV was found. We followed up nine urine PCR positive cats for a variable time and shedding was in some cases documented after up to 12 months. Four out of eight followed up cats were also antibody positive and one seroconverted to negative status one month after the last detection of a positive urinary PCR. We obtained FeMV isolation in two urine samples out of the 14 PCR positive samples cultured (FeMV Tremedino/2018 Italy and FeMV Pepito 2018/Italy belonging to FeMV genotype 1). We found an antibody prevalence of 15%, molecular prevalence in blood of 3% and 9% in urine, an overall prevalence of 22% for *Leptospira* spp. and antibody positivity

against two serogroups of *L. interrogans*: Australis (serovar bratislava in five cats, and lora in one cat) never reported in cats before, and Pomona (serovar pomona in one cats). More frequent was positivity against *L. borgpetersenii* with serogroup Ballum (serovar ballum in one cat, serovar arborea in four cats), Javanica (serovar poi in nine cats), and Mini (serovar mini in one cat). A FIV prevalence of 16%, a FCoV prevalence of 18% and a FeLV prevalence of 2% was found. Coinfections between pathogens investigated were found: *L. infantum* was associated with FIV and FeMV. *Leptospira* spp. positivity was associated with FeMV and FIV positivity with FeMV. Significant associations were found between male gender and *L. infantum*, FIV and FCoV; adult age and FeMV, *L. infantum*; senior age and FeMV, *L. infantum* and FIV; outdoor husbandry and FCoV; rural environment and *L. infantum* and FCoV; suburban environment and FCoV; Sicily origin and FeMV and FCoV; single-cat household and FeMV; low BCS and *L. infantum*, FeMV, FIV, FCoV; poor muscle mass and *L. infantum*, FIV, FCoV; pale mucous membranes and FCoV; enlarged lymph nodes and FCoV; skin lesions and FCoV, *L. infantum*, FeMV, FIV; gastrointestinal signs and *L. infantum*, FIV; respiratory signs and FIV, FeMV; ophthalmic lesions and FIV, FeMV; oral lesions and FIV, FeMV; increased GLOB and *L. infantum*, FeMV, FIV; reduced ALB and FIV, *Leptospira* spp.; proteinuria, increased sCr, decreased TP and *L. infantum;* increased TP and FIV. Results of this study are of value from a clinical point of view and have a "One Health" relevance as *Leptospira* spp. and *L. infantum* are of zoonotic concern.

4.1 INTRODUCTION

Cats have been human companions for many years, sharing our environment and more and more frequently they are considered as “family members” within households. However cats, as well as dogs, still can be a source of human infection by various pathogens, including viruses, bacteria, parasites (Chomel, 2014, Baneth et al. 2016). Therefore the analysis of the prevalence of feline infectious diseases as well as the associated clinical and clinicopathological findings has to be perfomed in order to protect not only the health of pets but also the health of the public by reducing the risk of zoonotic parasite transmission. Many feline infectious diseases have been widely documented and their clinical and clinicopathological characteristics are widely reported, such as feline immunodeficiency virus (FIV), feline leukemia virus (FeLV), feline coronavirus (FCoV) and others (Lutz et al., 2009, Hosie et al., 2009, Addie et al., 2009). The role of infectious pathogens, such as, *L. infantum*, feline morbillivirus (FeMV), *Leptospira* spp. still needs to be better defined.

Dogs are considered the main domestic reservoir of *L. infantum* and play a key role in transmission to humans, however feline leishmaniosis (FeL) is also an emerging disease and different clinical and clinicopathological signs are already reported (Pennisi et al., 2015). Leptospirosis is a common zoonotic disease and is reported both in dogs and cats. However the role of *Leptospira* spp. in feline health is still unclear with subclinic disease often reported (Schuller et al., 2015). Recently, FeMV was isolated from cats and it was associated with tubulointerstitial nephritis (TIN) (Woo et al., 2012), but few studies evaluated whether this pathogen may be responsible for further clinical signs and complications in felines (Sakaguchi et al., 2014, Sieg et al., 2019). Moreover, many cats are often affected by more than one of these infectious pathogens, as reported for the relationship found between *L. infantum* and FIV or FeLV infection (Pennisi and Persichetti, 2018).

The aims of this study are to evaluate the prevalence of *L. infantum*, *Leptospira* spp, FeMV, FIV, FCoV infection in a population of cats of Southern Italy, to investigate clinical and clinicopathological findings more frequently found in cats affected by these pathogens and, considering the main purpose of this thesis and the role already reported for some of these pathogens in the development of feline kidney disease (Woo

et al., 2012; Park et al., 2016; Yilmaz et al., 2017; Sutummaporn et al., 2019, Sieg et al., 2019, Rodriguez et al., 2014, Pennisi et al., 2013a, Pennisi et al., 2004, Navarro et al., 2010, Poli et al., 1993; Poli et al., 1995; Asproni et al., 2013; Baxter et al., 2012; Taffin et al., 2017, Glick et al., 1978, Lewis and O'Brien, 2010), to evaluate whether changes in clinicopathological parameters related to feline renal function are associated with the pathogens investigated.

4.2 MATERIALS AND METHODS

4.2a Cats, clinical examination and sampling

See chapter 2, section 2.1

Nine cats were followed up for monitoring of FeMV positivity.

4.2b Biochemical profile and urinalysis- see chapter 2, section 2.2

In this study symmetric dimethylarginine (SDMA), serum creatinine (sCr), urea (BUN), total proteins (TP), albumin (ALB), total globulins (GLOB), phosphorus (PHOS), urine specific gravity (USG) and urine protein to creatinine ratio (UPC) values were evaluated as risk factors for the infectious pathogens analyzed.

Hepatic profile parameters: alkaline phosphatase (ALKP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamyl transferase (GGT), were only available for *Leptospira* spp. positive cats.

4.2c Antibody detection- see chapter 2, section 2.3

Antibody detection was evaluated in 269 cats for *L. infantum*, 258 for FIV, 191 for FeMV and 111 cats for *Lesptospira* spp.

4.2d DNA extraction and *Leishmania* Real-Time PCR- see chapter 2, section 2.4

Positivity to PCR was considered when the patient was positive for at least one examined sample, in a total number of 265 cats: conjunctival swabs (n=254), oral swabs (n=252), swabs from skin, mucosal lesions or ear cerumen swabs (n=162), needles from lymph nodes aspirates (n=20), EDTA blood (n=256), hair tuft (n= 164), other samples such as crusts, skin or mucosal biopsy, claw (n=10). Overall prevalence (combination of antibody and any PCR positivity) was considered in 263 cats. In cases of suspected *L. infantum* skin lesions histopathology and immunohistochemistry was performed (n=3).

4.2e RNA extraction and PCR for FeMV, FIV, FeLV, FCoV- see chapter 2, section 2.5

Feline morbillivirus molecular positivity was evaluated in a total number of 210 blood samples and 216 urine samples, and overall molecular prevalence (combination of blood and urine PCR positivity) was considered in 222 cats. Overall FeMV prevalence (combination of antibody and any PCR positivity) was considered in 191 cats.

Feline immunodeficiency virus positivity was based on positive antibody and/or molecular analysis in a total number of 261 cats. Feline leukemia virus was investigated in 262 cats and FCoV in 171 cats.

4.2f Culture for isolation of FeMV from qPCR positive urine samples- see chapter 2, section 2.6

4.2g DNA extraction and PCR for *Leptospira* spp. - see chapter 2, section 2.7

Lesptospira spp. molecular positivity was investigated in blood (109) and urine (111) samples, overall molecular positivity (based on positive blood and/or urine PCR tests), was investigated in 112 cats, and overall prevalence (combination of antibody and any PCR positivity) was considered in 111 cats.

4.2h Statistical analysis

Descriptive statistics of data collected about clinical history and physical examination findings were performed and normal distribution of values of continuous variables was preliminarly assessed by D'Agostino & Pearson normality test.

In order to indentify categorical variables associated with infectious pathogens examined, Chi-Square test, Fisher exact test or Mann-Whitney test were used when appropriate.

Positivity to the pathogens was evaluated as follows:

- *L. infantum*:
    - Serological: antibody detection by IFI with a titre $\geq$80
    - Molecular: DNA detected by PCR in at least one of the examined samples
    - Overall: positivity to serological and/or molecular investigations
- *Leptospira* spp.:
    - Serological: antibody detection by MAT with a titre $\geq$20

- Molecular: DNA detected by PCR in at least one of the examined samples
- Overall: positivity to serological and/or molecular investigations

- FeMV:
  - Serological: antibody detection by IFI and titre $\geq$40
  - Molecular: RNA detected by PCR in urine
  - Overall molecular: RNA detected by PCR in urine and/or EDTA blood
  - Overall: positivity to serological and/or molecular investigations
- FIV:
  - Overall: antibody detection (ELISA test) and/or RNA detected by PCR in EDTA blood
- FCoV:
  - Molecular: RNA detected by PCR in EDTA blood.

Differences were considered significant if P value was $\leq$0.05 and a tendency to significance was considered when it was >0.05 $\leq$ 0.06. Statistical results are reported as odds ratios (OR), 95% C.I. (confidence interval) and significance. Comparisons included the following 25 variables: geographic region (Calabria/Sicily), demographic (sex=male/female; breed=domestic short-hair (DSH)/domestic long-hair (DLH)/pedigree; age=junior/adult/senior), husbandry (indoor/ outdoor; indoor single-cat/indoor multi-cat household) and environment characteristics (rural/sub-urban/urban), signs detected at physical examination (BCS= $\geq$3/<3, MCS= 1/>1, mucous membranes color= pink/pale/jaundice/congested, lymph nodes enlargment= presence/absence, ocular lesions= presence/absence, oral lesions= presence/absence, dermatological lesions= presence/absence, gastrointestinal signs= presence/absence, and respiratory signs= presence/absence), values of SDMA (normal/increased), sCr (normal/increased), BUN (normal/decreased/increased, TP (normal/ decreased/increased), ALB (normal/decreased), GLOB (normal/decreased/increased), PHOS (normal/increased), USG (<1035/$\geq$1035), UPC (non proteinuric/borderline proteinuric/proteinuric). In case of null values statistical comparisons were not performed. The presence of coinfections was evaluated between pathogens examined. Statistical analysis was performed using GraphPad Instat 7.00.

4.3 RESULTS

L. infantum

Leishmania infantum antibody, molecular, and overall prevalence according to geographical, demographic, husbandry and environmental variables are reported in table 4.a.

Table 4.a *L. infantum* antibody, molecular, and overall prevalence according to geographical, demographic, husbandry and environmental variables

VARIABLE	IFI (%)		PCR (%)		OVERALL (%)	
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**
ORIGIN						
Sicily	20 (38)	64 (30)	7 (33)	78 (32)	23 (37)	60 (30)
Calabria	33 (62)	152 (70)	14 (67)	166 (68)	39 (63)	140 (70)
BREED						
DSH	47 (89)	181 (84)	18 (86)	206 (85)	53 (86)	170 (85)
DLH	5 (9)	20 (9)	2 (9)	23 (9)	7 (11)	17 (8)
Purebreed	1 (2)	15 (7)	1 (5)	15 (6)	2 (3)	14 (7)
SEX*						
Female	19 (36)	111 (51)	5 (24)	124 (51)	21 (34)	107 (53)
Male	34 (64)*	105 (49)	16 (76)*	120 (49)	41 (66)*	94 (47)
AGE*						
Junior	2 (4)	46 (21)	1 (5)	49 (20)	3 (5)	45 (22)
Adult	29 (55)*	119 (55)	13 (62)	132 (54)	35 (56)*	110 (55)
Senior	22 (41)*	51 (24)	7 (33)	63 (26)	24 (39)*	46 (23)
HUSBANDRY						
Indoor	18 (34)	101 (47)	8 (38)	112 (46)	23 (37)	95 (47)
Outdoor	35 (66)	115 (53)	13 (62)	132 (54)	39 (63)	106 (53)
HOUSEHOLD						
Single-cat	6 (33)	32 (32)	3 (37)	36 (32)	8 (35)	30 (32)
Multi-cat	12 (67)	69 (68)	5 (63)	76 (68)	15 (65)	65 (68)
ENVIRONMENT*						
Urban	26 (53)	127 (69)	9 (60)	141 (66)	30 (55)	119 (68)
Suburban	14 (29)	46 (25)	5 (33)	55 (25)	16 (30)	43 (25)
Rural	9 (18)*	12 (6)	1 (7)	19 (9)	8 (15)	12 (7)

Missing data	4	31	6	29	8	27

Legenda: DSH= domestic shorthair; DLH= domestic longhair *=significant difference

Serological evaluation: antibody prevalence was 20% with 53/269 positive cats. Titres ranged from 80 to 2560 as reported in table 4.b

Table 4.b *L. infantum* positive titres

Titre	Positive cats *(%)*
80	28 (53)
160	15 (28)
320	4 (7)
640	1 (2)
1280	3 (6)
2560	2 (4)

A significant association was found between antibody positivity and male sex (P= 0.0470, OR= 1.892, 95% CI= 1.011-3.423), adult (P= 0.0110, OR= 5.605, 95% CI= 1.441-24.54) or senior ages (P= 0.0003, OR= 9.922, 95% CI= 2.319-44.05) compared to junior age, rural habitat (P= 0.0162, OR= 3.663, 95% CI= 1.472-9.558) compared to urban habitat. No significant differences were found between urban and suburban habitat, suburban and rural habitat and between adult and senior age when compared.

Molecular evaluation: 8% (21/265) of cats were positive on PCR. Positivity and parasite loads of examined samples are reported in table 4.c

Table 4.c *L. infantum* molecular positivity and parasite loads in examined samples

SAMPLE (#)	Positive(%)	Parasite load*
EDTA blood (256)	11 (4)	5-81000
Lymph nodes fine needle aspirates (20)	4 (20)	5-4250
Skin lesions (biopsy or swab) (19)	5 (26)	10-100
Claw (1)	1	18500
Ear-cerumen swabs (151)	0	---
Hair (164)	3 (2)	3-270
Gingival lesion (biopsy) (1)	0	---

Nasal swab (1)	1	100
Conjunctival swabs (254)	6 (2)	10-2150
Oral swabs (n=252)	4 (2)	10-1500
Uterus (2)	1	9000

Legenda: *=parasite load is reported as number of amastigotes/DNA obtained from any sample or from 200 µL of EDTA blood.

A significantly higher prevalence of molecular positivity was found in males (P= 0.0220, OR= 3.307, 95% CI= 1.165-8.411).

We calculated sensitivity and specificity of PCR tests performed with different tissues compared to antibody detection (see table 4.d). The best performance was obtained by PCR from skin lesions as we obtained fair (62.5%, 95% CI 30-86%) sensitivity but 100% specificity (95% CI 74-100%). However, PCR tests from enlarged lymph nodes provided poor sensitivity (12%, 95% CI 20-34%) and specificity (33%; 95% CI 17-88%). Performance of mucosal swabs, hair and whole blood was good for specificity (mucosal swabs=99.7%, 95% CI 98-99%; hair= 97.8%, 95% CI 94-99; blood= 97.6%; 95% CI 94.5-98.9%), but their sensitivity was poor (mucosal swabs= 10%, 95% CI 5.4-18.1%; hair= 0, 95% CI 0-12.; blood= 12.8%, 95 CI 5.9-25%).

Table 4.d – IFI titre according to PCR positivity of different tissues tested

SAMPLE (#)	**IFI TITRE**						
EDTA blood (256)	**<80**	**80**	**160**	**320**	**640**	**1280**	**2560**
Positive (11)	5	1	1	1	0	2	1
Negative (245)	204	25	12	2	1	1	0
Lymph nodes fine needle aspirates (20)							
Positive (4)	2	0	1	0	0	1	0
Negative (16)	15	1	0	0	0	0	0
Skin lesions (biopsy or swab) (19)							
Positive (5)	0	1	2	0	0	2	0
Negative (14)	11	1	1	0	0	0	1
Claw (1)							
Positive (1)	0	0	0	0	0	1	0
Auricolar swabs (151)							
Negative (151)	121	15	10	2	1	2	0
Hair (164)							

Positive (3)	3	0	0	0	0	0	0
Negative (161)	134	15	9	1	0	2	0
Gingival lesion (biopsy) (1)							
Positive (1)	1	0	0	0	0	0	0
Nasal swab (1)							
Positive (1)	0	0	0	0	0	1	0
Conjunctival swabs (254)							
Positive (6)	0	0	2	0	0	2	2
Negative (248)	209	24	11	2	1	1	0
Oral swabs (n=252)							
Positive (4)	1	0	1	0	0	1	1
Negative (248)	207	23	13	2	1	2	0
Uterus (2)							
Positive (1)	0	0	0	0	0	1	0
Negative (1)	0	0	0	0	0	0	0

Overall prevalence: cats tested by both IFI and PCR techniques were 263 and overall prevalence was 24% (62/263). A statistically significant difference was found for male sex (P= 0.008, OR= 2.222, 95% CI= 1.226-4.027), adult (P=0.0060, OR= 4.773, 95% CI= 1.456-15.34) and senior ages (P= 0.0003, OR= 7.826, 95% CI= 2.348-25.72) compared to junior age. No significant difference was found between adult and senior ages.

Clinical findings and laboratory abnormalities: clinical findings according to antibody, molecular, and overall prevalence are described in table 4.e

Both antibody and molecular positivity were associated with reduced BCS (antibody positivity: P= 0.009, OR= 3.382, 95% CI= 1.636-6.62; molecular positivity: P= 0.0010, OR=4.862, 95% CI= 1.997-12.62), poor MCS (antibody positivity: P= 0.0133, OR= 2.516, 95% CI= 1.252-4.831; molecular positivity: P= 0.0110, OR= 4.531, 95% CI= 1.596-14.58), and presence of skin lesions (antibody positivity: P= 0.0070, OR= 2.741, 95% CI=1.371-5.437; molecular positivity: P= 0.0282, OR= 3.096, 95% CI= 1.199-7.977).

Similarly, overall positivity was associated with reduced BCS (P= 0.0032, OR=2.885, 95% CI= 1.404-5.626), poor MCS (P= 0.017, OR= 2.283, 95% CI= 1.147-4.546),

presence of skin lesions (P= 0.006, OR= 2.586, 95% CI=1.290-5.181) and additionally to the presence of gastrointestinal signs (P= 0.0325 OR= 3.203, 95% CI= 1.69-8.187).

Table 4.e *L. infantum* antibody, molecular and overall positivity according to clinical findings

VARIABLE	IFI (%)		PCR (%)		Overall (%)	
BCS*	POS	NEG	POS	NEG	POS	NEG
< 3/5	18 (35)*	30 (14)	9 (45)*	35 (14)	18 (30)*	26 (13)
≥3/5	33 (65)	186 (86)	11 (55)	208 (86)	42 (70)	175 (87)
MCS*						
≥2/4	19 (39)*	36 (20)	7 (54)*	43 (21)	18 (35)*	32 (19)
1/4	30 (61)	143 (80)	6 (46)	167 (79)	34 (65)	138 (81)
MUCOUS MEMBRANES						
Normal	44 (85)	195 (90)	15 (75)	221 (90)	51 (84)	183 (91)
Pale	7 (13)	18 (8)	4 (20)	20 (8)	9 (15)	15 (7)
Jaundice	1 (2)	2 (1)	1 (5)	2 (1)	1 (1%)	2 (1)
Congested	0	1 (1)	0	1 (1)	0	1 (1)
LYMPH NODES						
Normal	30 (59)	154 (71)	10 (53)	171 (70)	36 (60)	143 (71)
Enlarged	21 (41)	62 (29)	9 (47)	73 (20)	24 (40)	58 (29)
OCULAR LESIONS						
Presence	5 (10)	14 (6)	2 (10)	15 (6)	6 (10)	11 (5)
Absence	47 (90)	201 (94)	18 (90)	228 (94)	55 (90)	189 (95)
ORAL LESIONS						
Presence	24 (46)	70 (33)	10 (50)	80 (33)	26 (43)	63 (31)
Absence	28 (54)	145 (67)	10 (50)	163 (67)	35 (57)	137 (69)
RESPIRATORY SIGNS						
Presence	6 (11)	20 (9)	4 (20)	21 (9)	7 (11)	17 (8)
Absence	46 (89)	195 (91)	16 (80)	222 (91)	54 (89)	183 (92)
GI SIGNS*						
Presence	6 (12)	12 (6)	3 (15)	15 (6)	8 (13)*	9 (5)
Absence	46 (88)	203 (94)	17 (85)	228 (94)	53 (87)	191 (95)
SKIN LESIONS*						
Presence	16 (31)*	30 (14)	7 (35)*	36 (15)	17 (28)*	26 (13)
Absence	36 (69)	185 (86)	13 (65)	207 (85)	44 (72)	174 (87)

Legenda: GI= gastrointestinal. *= significant difference

Dermatological lesions and their distribution in positive (antibody and/or PCR) cats are described in table 4.f. Some cats had more than one of the dermatological lesions reported and positive PCR from skin lesions was found in one cat (crust: 100 L/s) that was high positive from a claw (18500 L/s). However one other cat with multifocal ulcers was positive at immunohistochemistry from a biopsed lesion but PCR was negative from crusts. In nine cats hair PCR was performed and two of them were found positive: both cats had crusts and hair parasite load was respectively 15 L/s and 270 L/s.

Table 4.f Dermatological lesions and their distribution in *L. infantum* antibody and/or PCR positive cats

DERMATOLOGICAL LESION in 17 cats	CAT # (%)	DISTRIBUTION (cat #)	FOCAL/ MULTIFOCAL (cat #)	SYMMETRICAL/ ASYMMETRICAL (cat#)
ALOPECIA	10/17 (59)	Ears (6)	1/9	5/4
		Head (3)		
		Neck (3)		
		Trunk (1)		
		Tail (1)		
		Limb (1)		
CRUSTS	7/17 (41)	Head (4)	6/1	0/1
		Neck (2)		
		Ears (2)		
		Limb (1)		
ULCERS	2/17 (12)	Limb (1)	1/1	1/0
		Perineum (1)		
ABSCESSES	2/17 (12)	Head (1)	2/0	
		Limb (1)		
SQUAMOUS DERMATITIS	3/17 (18)	Diffused (2)	---	---
		Head (1)	0/1	0/1

Twenty-six antibody and/or molecular positive cats were found affected by one or more than one of the following oral abnormalities: stomatitis (n=16, 61%), dental calculus (n=6, 23%), ulcers (n=4, 15%), periodontitis (n=3, 11%), drooling (n=3, 11%), faucitis (n=2, 8%), missing teeth (n=1, 4%), bleeding (n=1, 4%). Oral swabs were obtained in 23 of these cats and two were positive. One cat with high parasite

load (1500 L/s) had faucitis and the other one with low parasite load (10 L/s) had gingivitis. Similar oral abnormalities were found in 63 negative cats with stomatitis (n=26, 41%), periodontitis (n=21, 33%) and dental calculus (n=14, 22%) as the most frequent, but ulcers were not found.

Conjunctivitis was the only opthalmic abnormality found in antibody and/or PCR positive cats (n=6) as well as in negative cats (n=9). Conjunctival swabs were positive in just one cat (30-65 L/s)

Respiratory signs reported in seven antibody and/or PCR positive cats were from lower respiratory tract (n=5, 71%) or upper respiratory tract (n= 3, 43%) (fig 1) and one cat showed both of them. In negative cats most of the respiratory signs were related to upper respiratory tract (n=15, 88%) with only three cats affected by lower respiratory tract disease.

Fig 1 *L. infantum* (IFI and PCR), FeMV (IFI and urine PCR) positive cat affected by rhinitis

Diarrhea (n=6, 75%) and constipation (n=2, 25%) were the gastrointestinal signs reported in eight antibody and/or PCR positive cats. The nine negative cats were affected by diarrhea (n=5, 55%) and vomiting (n=4, 45%).

Abdomen examination revealed hepatomegaly in both antibody and/or PCR positive (five cats) and negative (3 cats). Similarly, pain on palpation was detected respectively

in one positive and two negative cats. Moreover, splenomegaly was detected in one positive cat and effusion in two negatives.

Biochemistry abnormalities evaluated according to antibody, molecular and overall positivity are described in table 4.g

Antibody positivity was associated with increased sCr (P= 0.0181, OR= 3.464, 95% CI= 1.386-9.296) and proteinuria (P) (P= 0.0151, OR=3.808, 95% CI= 1.494-10.12).

Molecular positivity was associated with reduced TP (P= 0.0253, OR= 16.13, 95% CI= 2.188-107.5), increased GLOB (P= 0.0062, OR= 7.419, 95% CI=1.943-27.81) and proteinuria (P) (P= 0.0032, OR=7.813, 95% CI=2.148-27.77).

Overall antibody and molecular positivity was associated with reduced TP (P= 0.0274, OR= 12.81, 95% CI= 1.802-167.3), proteinuria (P) (P= 0.0034, OR=4.421, 95% CI= 1.704- 12.2) and a tendency to significance was found with increased sCr (P= 0.0550, OR= 2.682, 95% CI= 1.092-7.052).

Table 4.g Biochemistry abnormalities according to *L. infantum* antibody, molecular and overall positivity

VARIABLE	IFI (%)		PCR (%)		Overall (%)	
	POS	NEG	POS	NEG	POS	NEG
SDMA						
Normal	25 (61)	110 (69)	7 (54)	128 (69)	28 (62)	106 (70)
Increased	16 (39)	49 (31)	6 (46)	57 (31)	17 (38)	46 (30)
Missing data	12	57	8	59	17	49
sCr*						
Normal	30 (67)	127 (75)	14 (88)	141 (72)	36 (70)	118 (74)
Increased	9 (20)*	11 (6)	1 (6)	19 (10)	9 (18)	11 (7)
Decreased	6 (13)	32 (19)	1 (6)	36 (18)	6 (12)	31 (19)
Missing data	8	46	5	48	11	41
BUN						
Normal	24 (67)	124 (79)	9 (56)	137 (79)	28 (67)	118 (80)
Increased	10 (28)	23 (15)	6 (38)	26 (15)	12 (28)	20 (13)
Decreased	2 (5)	10 (6)	1 (6)	11 (6)	2 (5)	10 (7)
Missing data	17	59	5	70	20	53
TP*						
Normal	24 (83)	113 (86)	8 (66)	129 (87)	26 (76)	111 (88)
Increased	4 (14)	16 (12)	2 (17)	17 (11)	5 (15)	14 (11)

Decreased	1 (3)	3 (2)	2 (17)*	2 (2)	3 (9)*	1 (1)
Missing data	24	84	9	96	28	72
ALB						
Normal	24 (83)	124 (93)	9 (82)	137 (92)	28 (85)	118 (93)
Decreased	5 (17)	9 (7)	2 (18)	12 (8)	5 (15)	9 (7)
Missing data	24	83	10	95	29	74
GLOB*						
Normal	18 (64)	100 (77)	3 (27)	115 (79)	19 (60)	99 (79)
Increased	10 (36)	28 (22)	6 (55)*	31 (21)	11 (34)*	26 (21)
Decreased	0	2 (1)	2 (18)	0	2 (6)	0
Missing data	25	86	10	98	30	76
PHOS						
Normal	16 (73)	80 (63)	6 (86)	89 (63)	18 (75)	77 (62)
Increased	6 (27)	48 (37)	1 (14)	53 (37)	6 (25)	48 (38)
Missing data	34	88	14	102	38	76
USG						
≥1035	27 (64)	151 (78)	12 (71)	164 (76)	36 (69)	139 (77)
<1035	15 (36)	43 (22)	5 (29)	52 (24)	16 (31)	41 (23)
Missing data	11	22	4	28	10	21
UPC*						
NP	20 (57)	110 (79)	5 (46)	125 (78)	22 (56)	107 (81)
BP	6 (17)	16 (12)	1 (9)	20 (12)	7 (18)	14 (11)
P	9 (26)*	13 (9)	5 (45)*	16 (10)	10 (26)*	11 (8)
Missing data	18	77	10	83	23	69

Legenda: *=significant difference. NP= non proteinuric, BP=borderline proteinuric, P=proteinuric.

FIV

In this study FIV infection was detected in 43/261 (16%) tested cats. Antibody positivity was 13% (36/258) and PCR positivity 10% (20/210). Fifty-five cats were tested only by antibody detection and three cats were tested only by PCR (one positive and two negatives). Twenty-five antibody positive cats were also tested by PCR and 12 of them were found negative.

A significant association was found with male sex (P= 0.006, OR= 2.624, 95% CI= 1.299-5.301), senior age (P= 0.0288, OR= 3.651, 95% CI= 1.202-10.46) compared to junior age.

FIV overall prevalence according to geographical, demographic, husbandry and environmental variables are reported in table 4.h

Table 4.h FIV overall prevalence according to geographical, demographic, husbandry and environmental variables

VARIABLE	FIV PCR and/or ELISA (%)	
	POS	**NEG**
ORIGIN		
Sicily	19 (44)	63 (29)
Calabria	24 (56)	155 (71)
BREED		
DSH	38 (88)	184 (84)
DLH	5 (12)	19 (9)
Purebreed	0	15 (7)
SEX*		
Male	30 (70)*	102 (47)
Female	13 (30)	116 (53)
AGE*		
Junior	4 (9)	43 (20)
Adult	21 (49)	122 (56)
Senior	18 (42)*	53 (24)
HUSBANDRY		
Indoor	14 (33)	101 (46)
Outdoor	29 (67)	117 (54)
HOUSEHOLD		
Single-cat	3 (21)	33 (33)
Multi-cat	11 (79)	68 (67)
ENVIRONMENT		
Urban	20 (49)	129 (68)
Suburban	15 (36)	45 (24)
Rural	6 (15)	15 (8)
Missing data	2	29

Legenda: *= significant difference

Prevalence according to clinical fidings is reported in table 4.i

A significant association was found between FIV positivity and reduced BCS (P= 0.0014, OR=3.481, 95% CI= 1.616-7.223) and poor MCS (P= 0.005, OR= 2.783, 95% CI= 1.337-5.790), presence of oral (P= 0.007, OR=2.467, 95% CI= 1.270-4.791) and cutaneuous lesions (P= 0.014, OR= 2.505, 95% CI= 1.182-5.310), respiratory (P= 0.000, OR= 5.613, 95% CI= 2.378-13.247) and gastrointestinal signs (P= 0.0055, OR=4.281, 95% CI= 1.595-11.55). Moreover, a tendency to significance was found for association with ocular lesions (P= 0.067, OR= 2.545, 95% CI= 0.910-7.119).

Table 4.i FIV prevalence according to clinical findings

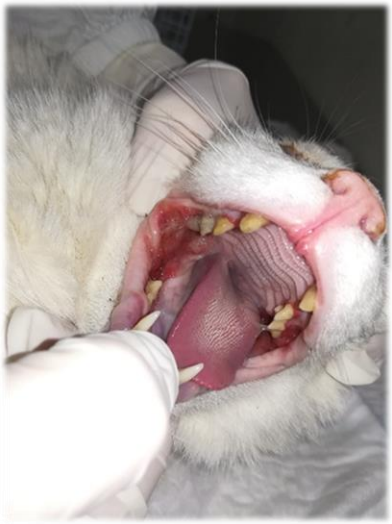
VARIABLE	PCR and/or ELISA (%)	
	POS	NEG
BCS*		
< 3/5	15 (36)*	30 (14)
≥3/5	27 (64)	188 (86)
MCS*		
≥2/4	16 (41)*	37 (20)
1/4	23 (59)	148 (80)
Missing data	4	33
MUCOUS MEMBRANES		
Normal	35 (81)	197 (90)
Pale	8 (19)	17 (8)
Jaundice	0	1 (1)
Congested	0	3 (1)
LYMPH NODES		
Normal	26 (62)	153 (70)
Enlarged	16 (38)	65 (30%)
Missing data	1	0
OCULAR LESIONS*		
Presence	6 (14)*	13 (6)
Absence	37 (86)	204 (94)
Missing data	0	1
ORAL LESIONS*		
Presence	23 (53)*	69 (32)
Absence	20(47)	148 (68)
Missing data	0	1

RESPIRATORY SIGNS*		
Presence	12 (28)*	14 (6)
Absence	31 (72)	203 (94)
Missing data	0	1
GI SIGNS*		
Presence	8 (19)*	11 (5)
Absence	35 (81)	206 (95)
Missing data	0	1
SKIN LESIONS*		
Presence	13 (30)*	32 (15)
Absence	30 (70)	185 (85)
Missing data	0	1

Legenda: *= significant difference.

Most of the cats were affected by more than one of the reported clinical abnormalities. The most common oral lesions (n=23, 53%) of FIV positive cats were stomatitis (n=16, 69%), dental calculus (n=7, 30%), ulcers (n=3, 13%), hypersalivation (n=3, 13%), faucitis (n=2, 9%), missing teeth (n=2, 9%), periodontitis (n=1, 4%), bleeding (n=1, 4%), fig 2. However similar lesions were found also in negative cats, as stomatitis (n=30, 43%), dental calculus (n=13, 19%), ulcers (n=7, 10%), drooling (n=6, 9%), faucitis (n=5, 7%), missing teeth (n=2, 3%), periodontitis (n=23, 33%), cleft palate (n=1, 1%), neoplasia (n=1, 1%).

Fig 2 FIV, *L. infantum* (IFI), FeMV (IFI and urine PCR) positive cat with stomatitis



Conjunctivitis (n=5, 83%) and keratoconjunctivitis (n=1, 17%) were detected as ocular lesions (n=6, 14%) and found also in negative cats (85%, 11/13 conjunctivitis, 23%, 3/13 keratoconjunctivitis). Dermatological lesions (n=13, 30%) observed in FIV positive cats were alopecia (n=6, 46%), crusty dermatitis (n=6, 46%), abscesses (n=2, 15%), ulcers (n=1, 8%) and nodular dermatitis (n=1, 8%). Skin lesions most frequently reported in negative cats were alopecia (n=21, 66%) and crusty dermatitis (n=9, 28%), followed by abscesses (n=3, 9%), ulcers (n=5, 16%), nodules (n=4, 13%), squamous dermatitis (n=1, 3%), pyoderma (n=1, 3%), erythema (n=3,9%).

All eight FIV positive cats with gastrointestinal signs had diarrhea. Negative cats also had diarrhea (n=5, 45%), vomiting (n=4,36%), constipation (n=2, 18%). Respiratory signs found in both FIV positive (10 related to upper and 4 to lower respiratory tract) and FIV negative cats (nine related with upper and 5 with lower respiratory tract involvment) were similar.

Clinicopathological alterations are listed in table 4.j.

Prevalence of increased TP was significantly higher in FIV positive cats (P=0.0038, OR=4.958, 95% CI= 1.67-12.92), as well as prevalence of reduced ALB (P= 0.031, OR= 3.472, 95% CI= 1.056-11.422) and increased GLOB (P= 0.0027, OR= 4.406, 95% CI= 1.78-11.21).

Table 4.j Clinicopathological variables according to FIV positivity

VARIABLE	FIV PCR/ELISA (%)	
	POS	NEG
SDMA		
Normal	20 (59)	114 (69)
Increased	14 (41)	51 (31)
Missing data	9	53
CREA		
Normal	32 (84)	160 (92)
Increased	6 (16)	14 (8)
Missing data	5	44
BUN		
Normal	19 (63)	126 (79)
Increased	9 (30)	23 (14)

Decreased	2 (7)	10 (6)
Missing data	13	59
TP*		
Normal	16 67)	119 (89)
Increased	8 (33)*	12 (9)
Decreased	0	3 (2)
Missing data	19	84
ALB*		
Normal	20 (80)	125 (93)
Decreased	5 (20)*	9 (7)
Missing data	18	84
GLOB*		
Normal	11(48)	105 (79)
Increased	12 (52)*	26 (20)
Decreased	0	1 (1)
Missing data	20	86
PHOS		
Normal	9 (50)	85 (65)
Increased	9 (50)	45 (35)
Missing data	25	88
USG		
≥1035	23 (70)	153 (77)
<1035	10 (30)	46 (23)
Missing data	10	19
UPC		
NP	17 (61)	111 (78)
BP	5 (18)	17 (12)
P	6 (21)	15 (10)
Missing data	15	75

Legenda: *= Significant difference. NP= non proteinuric, BP= borderline proteinuric, P= proteinuric.

FeMV

Antibody, molecular, and overall prevalence of FeMV according to geographical, demographic, husbandry and environmental variables are reported in table 4.k.

Serological evaluation: FeMV viral antibody prevalence was 14% with 26/191 positive cats and a significant higher prevalence (21%, 13/62) was found in cats from Sicily (P= 0.0455, OR= 2.367, 95% CI= 1.003-5.572).

Molecular evaluation: two percent of cats (4/210) were positive to FeMV PCR in blood and 16% (35/216) in urine. Overall molecular prevalence was of 17% (37/222). Threshold cycle (Ct) ranged from 34 to 37 (median 34.5, 25th percentile 34, 75th percentile 36.5) in blood and from 26 to 39 in urine samples (mean 32.51±3.081). A significant association was found between being a single-cat household individual and both positive urine PCR (P= 0.0168, OR= 4.02, 95% CI= 1.273-12.99) and overall molecular positive PCR (P= 0.0373, OR= 3.497, 95% CI= 1.171-9.527).

Overall prevalence: A 28% (53/191) overall prevalence was calculated. A significant association was found with adult (P= 0.0273, OR= 3.351, 95% CI= 1.102-9.372) and senior age (P= 0.0218, OR= 3.992, 95% CI= 1.191-11.73) compared to junior age for overall molecular positivity, and no significant difference was found between adult and senior age

Table 4.k FeMV antibody, molecular, and overall prevalence according to geographical, demographic, husbandry and environmental variables examined

VARIABLE	IFI (%)		urine PCR (%)		urine and/or blood PCR (%)		IFI and/or blood/urine PCR (%)	
	POS	NEG	POS	NEG	POS	NEG	POS	NEG
ORIGIN								
Sicily	13 (50)*	49 (30)	9 (26)	63 (35)	11 (30)	62 (33)	20 (38)	42 (30)
Calabria	13 (50)	116 (70)	26 (74)	118 (65)	26 (70)	123 (66)	33 (62)	96 (70)
SEX								
Male	14 (54)	79 (48)	20 (57)	87 (48)	22 (59)	88 (48)	28 (53)	65 (47)
Female	12 (46)	86 (52)	15 (43)	94 (52)	15 (41)	97 (52)	25 (47)	73 (53)
AGE								
Junior	2 (8)	33 (20)	2 (6)	35 (19)	2 (6)	37 (20)	4 (8)	31 (22)
Adult	15 (58)	91 (55)	22 (63)	96 (53)	23 (62)	98 (53)	32 (60)*	74 (54)
Senior	9 (34)	41 (25)	11 (31)	50 (28)	12 (32)	50 (27)	17 (32)*	33 (24)
HUSBANDRY								
Indoor	8 (31)	82 (50)	15 (43)	92 (51)	16 (43)	93 (50)	20 (38)	70 (51)
Outdoor	18 (69)	83 (50)	20 (57)	89 (49)	21 (57)	92 (50)	33 (62)	68 (49)
HOUSEHOLD*								
Single-cat	1 (12)	26 (32)	9 (60)*	25 (27)	9 (56)*	25 (27)	8 (40)	19 (27)
Multi-cat	7 (88)	56 (68)	6 (40)	67 (73)	7 (44)	68 (73)	12 (60)	51 (73)
HABITAT								

Urban	15 (58)	111 (67)	21 (60)	121 (67)	21 (57)	124 (67)	31 (59)	95 (69)
Suburban	10 (38)	43 (26)	10 (29)	50 (28)	12 (32)	50 (27)	17 (32)	36 (26)
Rural	1 (4)	11 (7)	4 (11)	10 (5)	4 (11)	11 (6)	5 (9)	7 (5)

Legenda: *= Significant difference

Clinical findings and laboratory abnormalities: frequency of clinical findings evaluated are described in table 4.l. Some clinical abnormalities were associated with FeMV positivity. Reduced BCS was associated with antibody positivity (P= 0.0372, OR=3.0, 95% CI=1.091-8.156). Oral lesions were more frequently detected in antibody (P= 0.008, OR=3.02, 95% CI= 1.292-7.112) and overall positive cats (P= 0.056, OR=1.86, 95% CI= 0.980-3.548). Dermatological lesions were more prevalent in both antibody (P= 0.004, OR= 3.5, 95% CI= 1.427-8.586) and overall (P= 0.027, OR= 2.33, 95% CI=1.086- 4.994) positive cats. Prevalence of ocular lesions was higher in cats with positive urine PCR (P= 0.007, OR= 4.29, 95% CI=1.387-13.285) and overall positive PCR (P= 0.009, OR= 4.11, 95% CI= 1.334-12.676). Respiratory signs were more frequent in overall molecular positive (P= 0.003, OR= 3.76, 95% CI= 1.488-9.527) and overall antibody and molecular positive cats (P= 0.049, OR= 2.44, 95% CI= 0.985-6.053).

Table 4.l FeMV antibody, molecular (urine, urine and/or blood) and overall positivity according to clinical findings

VARIABLE	**IFI (%)**		**urine PCR (%)**		**urine and/or blood PCR (%)**		**IFI and/or blood/urine PCR (%)**	
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**	**POS**	**NEG**
BCS*								
< 3/5	8 (31)*	26 (16)	5 (14)	30 (17)	5 (14)	32 (18)	12 (23)	22 (16)
≥3/5	18 (69)	139 (84)	30 (86)	145 (83)	32 (86)	147(82)	41 (77)	116 (84)
Missing data	0	0	0	6	0	6	0	0
MCS								
≥2/4	7 (30)	35 (21)	8 (23)	38 (22)	8 (22)	40 (23)	14 (29)	28 (20)
1/4	16 (70)	129 (79)	26 (77)	134 (78)	28 (78)	136(77)	35 (71)	110 (80)
Missing data	3	1	1	9	1	9	4	0

MUCOUS MEMBRANES								
Normal	21 (81)	149 (90)	30 (86)	156 (90)	32 (86)	159(89)	45 (85)	125 (90)
Pale	4 (15)	13 (8)	3 (8)	16 (8)	3 (9)	17 (9)	6 (11)	11 (8)
Jaundice	1 (4)	2 (1)	2 (6)	1 (1)	2 (5)	1 (1)	2 (4)	1 (1)
Congested	0	1 (1)	0	1 (1)	0	1 (1)	0	1 (1)
Missing data	0	0	0	7	0	7	0	0
LYMPH NODES								
Normal	17 (65)	117 (71)	25 (71)	124 (71)	27 (73)	126 71)	37 (70)	97 (70)
Enlarged	9 (35)	48 (29)	10 (29)	50 (29)	10 (27)	52 (29)	16 (30)	41 (30)
Missing data	0	0	0	7	0	7	0	0
OCULAR LESIONS*								
Presence	3 (11)	10 (6)	6 (17)*	8 (5)	6 (16)*	8 (4)	6 (11)	7 (5)
Absence	23 (89)	155 (94)	29 (83)	166 (95)	31 (84)	170(96)	47 (89)	131 (95)
Missing data	0	0	0	7	0	7	0	0
ORAL LESIONS*								
Presence	16 (61)*	57 (34)	15 (43)	63 (36)	17 (46)	63 (35)	26 (49)*	47 (34)
Absence	10 (39)	108 (66)	20 (57)	111 (64)	20 (54)	115(65)	27 (51)	91 (66)
Missing data	0	0	0	7	0	7	0	0
RESPIRATORY SIGNS*								
Presence	5 (19)	17 (10)	7 (20)	16 (9)	9 (24)*	14 (8)	10 (19)	12 (9)
Absence	21 (81)	148 (90)	28 (80)	158 (91)	28 (76)	164(92)	43 (81)	126 (91)
Missing data	0	0	0	7	1	7	0	0
GI SIGNS								
Presence	3 (12)	11 (7)	4 (11)	12 (7)	4 (11)	12 (7)	7 (13)	7 (5)
Absence	23 (88)	154 (93)	31 (89)	162 (12)	33 (89)	166(93)	46 (87)	131 (95)
Missing data	0	0	0	7	0	7	0	0
SKIN LESIONS*								
Presence	10 (38)*	25 (15)	9 (26)	28 (16)	10 (27)	28 (16)	15 (28)*	20 (14)
Absence	16 (62)	140 (85)	26 (74)	146 (84)	27 (73)	150(84)	38 (72)	118 (86)
Missing data	0	0	0	7	0	7	0	0

Legenda: GI= gastrointestinal. *= Significant difference

The analytical results of abnormalities obeserved in overall positive cats are as follows: ocular lesions were conjunctivitis (n=5, 83%) and keratoconjunctivitis (n=1, 17%); oral lesions included stomatitis (n=17, 65%), ulcers (n=5, 19%), periodontitis (n=4, 15%), dental calculus (n=4, 15%), faucitis (n=3, 12%) and missing teeth (n=3, 12%), drooling (n=1, 4%) and cleft palate (n=1, 4%); respiratory signs were suggestive

of upper (n=8, 80%) or lower (n=3, 20%) respiratory tract disease; gastrointestinal signs were diarrhea (n=5, 71%) and vomiting (n=2, 29%); dermatological lesions consisted of alopecia (n=11, 73%), ulcers (n=2, 13%), crusty dermatitis (n=1, 7%) and abscesses (n=2, 13%).

Frequency of biochemical abnormalities evaluated are described in table 4.m

Increased PHOS values (P= 0.0180, OR= 5.33, 95% CI= 1.341-24.1) were associated to antibody negativity. Increased GLOB values were associated with both antibody (P= 0.0073, OR= 4.72, 95% CI=1.437-13.24), urine molecular positivity (P= 0.0094, OR= 3.42, 95% CI=1.369-8.037), molecular positivity in urine and/or blood (P= 0.0233, OR=2.88, 95% CI= 1.193-7.166) and overall positivity (P= 0.0094, OR= 3.42, 95% CI=1.369-8.037).

Table 4.m Biochemistry abnormalities according to FeMV antibody, molecular (urine, urine and/or blood) and overall positivity.

VARIABLE	IFI (%)		urine PCR (%)		blood and/or urine PCR (%)		overall IFI and/or PCR (%)	
	POS	NEG	POS	NEG	POS	NEG	POS	NEG
SDMA								
Normal	12 (50)	108 (69)	26 (76)	106 (67)	27 (5)	107 (66)	33 (65)	87 (67)
Increased	12 (50)	48 (31)	8 (24)	54 (34)	9 (25)	55 (34)	18 (35)	42 (33)
Missing data	2	9	1	21	1	23	2	9
sCr								
Normal	20 (80)	145 (72)	33 (94)	150 (90)	34 (92)	152 (90)	46 (88)	119 (91)
Increased	5 (20)	13 (8)	2 (6)	17 (10)	3 (8)	17 (10)	6 (12)	12 (9)
Missing data	1	7	0	14	1	16	1	7
BUN								
Normal	14 (67)	110 (79)	25 (78)	117 (80)	26 (76)	116 (78)	35 (73)	89 (79)
Increased	6 (28)	20 (14)	5 (16)	21 (14)	6 (18)	22 (15)	10 (21)	16 (14)
Decreased	1 (5)	10 (7)	2 (6)	9 (6)	2 (6)	10 (7)	3 (6)	8 (7)
Missing data	5	25	3	34	3	37	5	25
TP								
Normal	12 (80)	106 (88)	21 (81)	110 (89)	23 (82)	110 (89)	30 (81)	88 (89)
Increased	3 (20)	13 (11)	5 (19)	11 (9)	5 (18)	12 (10)	7 (19)	9 (9)
Decreased	0	2 (1)	0	2 (2)	0	2 (1)	0	2 (2)
Missing data	11	44	9	58	9	61	16	39

ALB								
Normal	15 (94)	111 (92)	24 (86)	115 (93)	26 (87)	115 (93)	35 (90)	91 (93)
Decreased	1 (6)	10 (8)	4 (14)	8 (7)	4 (13)	9 (7)	4 (10)	7 (7)
Missing data	10	44	7	58	7	61	14	40
GLOB*								
Normal	7 (47)	95 (80)	15 (58)	98 (82)	17 (61)	98 (81)	19 (51)	83 (86)
Increased	8 (53)*	23 (19)	11 (42)*	21 (17)	11 (39)*	22 (18)	18 (49)*	13 (13)
Decreased	0	1 (1)	0	1 (1)	0	1 (1)	0	1 (1)
Missing data	11	46	9	61	9	64	16	41
PHOS*								
Normal	16 (89)	69 (60)	16 (70)	77 (63)	17 (71)	77 (63)	29 (76)	56 (59)
Increased	2 (11)	46 (40)*	7 (30)	45 (37)	7 (29)	46 (37)	9 (24)	39 (41)
Missing data	8	50	12	99	13	62	18	43
USG								
≥1035	18 (72)	117 (74)	25 (71)	128 (74)	25 (69)	129 (74)	37 (71)	98 (74)
<1035	7 (28)	42 (26)	10 (29)	46 (26)	11 (31)	46 (26)	15 (29)	34 (26)
Missing data	1	6	0	7	1	10	1	6
UPC								
NP	11 (79)	94 (75)	22 (76)	99 (75)	22 (73)	99 (76)	29 (80)	76 (74)
BP	1 (7)	16 (13)	2 (7)	17 (13)	2 (7)	17 (13)	2 (6)	15 (14)
P	2 (14)	15 (12)	5 (17)	16 (12)	6 (20)	15 (11)	5 (14)	12 (12)
Missing data	12	40	6	49	7	54	17	35

Legenda: *=Significant difference. NP= non proteinuric, BP= borderline proteinuric, P=proteinuric.

FeMV culture was performed in urine of 14 cats that tested positive to urine PCR and in two cases FeMV strains were isolated and sequenced (Donato et al., 2019).

Follow up: nine cats found PCR positive in urine were periodically re-evaluated for monitoring viral urinary shedding by PCR, USG, FeMV PCR in EDTA blood and anti-FeMV antibodies (IFI) (tables 4.n1 and 4.n2). Two of the four antibody positive cats and one of the antibody negative cats were retested respectively after four, 10 and 11 months after the first evaluation (table 4.n2).

Table 4.n1: urinary follow up of nine urinary PCR positive cats

CAT	FOLLOW-UP TIME	SHEDDING TIME	TYPE OF SHEDDING	USG FIRST EVALUATION	USG LAST EVALUATION
CAT 1	24 D	24 D	C	1047	1045

CAT 2	2 M	2 M	C	1033	1033
CAT 3	3 M	3 M	C	1047	1045
CAT 4	3 M	3 M	C	1044	1048
CAT 5	7 M	3 M	C	1025	1014
CAT 6	10 M	10 M	C	1053	1055
CAT 7	11 M	9 M	C	1055	1025
CAT 8	12 M	12 M	C	1051	1052
CAT 9	18 M	12 M	I	1038	1021

Legenda: D= days; M= months; C= continuous; I= intermittent

Table 4.n2: blood PCR and IFI follow up of the nine cats of table 4.n1

CAT	EDTA	FOLLOW UP	SERUM	FOLLOW UP
CAT 1	NEG	4 M	POS	OE
CAT 2	NEG	OE	NEG	OE
CAT 3	NEG	3 M	POS	OE
CAT 4	NEG	OE	-	-
CAT 5	NEG	6 M	POS[1] NEG[2]	4 M
CAT 6	NEG	OE	NEG	OE
CAT 7	NEG	11 M	NEG[1] NEG[2]	11 M
CAT 8	NEG	OE	NEG	OE
CAT 9	NEG	18 M	POS[1] NEG[2]	10 M

Legenda: M= months; OE= one evaluation; [1]= first evaluation; [2]= second evaluation

***Leptospira* spp.**

Serological evaluation: antibody prevalence was 15% with 17/111 positive cats. Number of antibody positive cats and antibody titres according to *Leptospira* species, serogroups, serovars, and strain tested are described in table 4.o

Table 4.o Number of antibody positive cats and antibody titres according to *Leptospira* species, serogroups, serovars, and strain tested

SPECIES	SEROGROUP	SEROVAR	STRAIN	Cat number (titre)
L. borgpetersenii	Ballum	ballum	Mus 127	1 (80)
L. interrogans	Canicola	canicola	Hond Utrecht IV	0
L. interrogans	Australis	bratislava	Jez Bratislava	3 (20)
				1 (40)
				1 (320)
L. kirschneri	Grippotyphosa	grippotyphosa	Duyster	0

L. interrogans	Icterohaemorragiae	icterohaemorragiae	Kantorowic	0
L. borgpetersenii	Javanica	poi	Poi	8 (20)
				1 (40)
L. interrogans	Icterohaemorrhagiae	copenhageni	M20	0
L kirschneri	Grippotyphosa	grippotyphosa	Moskva V	0
L. interrogans	Pomona	pomona	Pomona	1 (20)
L. interrogans	Sejroe	hardjo	Hardjoprajitno	0
L. borgpetersenii	Tarassovi	tarassovi	Perepelitsin	0
L. interrogans	Australis	lora	Lora	1 (80)
L.borpetersenii	Ballum	arborea	Arborea	1 (20)
				1 (40)
				1 (80)
L.borpetersenii	Mini	mini	Sari	1 (80)
L.biflexa	Semeranga	patoc	Patoc I	0

Two cats tested positive to more than one strain: one cat was positive to Jez Bratislava (titre 20) and Poi (titre 20) strains belonging to different serogroups; another cat was positive to Jez Bratislava (titre 320) and Lora (titre 80) strains belonging to the same serogroup (Australis), and to Sari (titre 80) and Arborea (titre 40) strains belonging to two more serogroups.

No associations were found between antibody positivity and investigated variables.

Molecular evaluation: all blood samples tested negative to PCR performed according to Stoddard et al. (2009) and just one (1%) cat was positive in urine. The positive sample was confirmed by PCR performed according to Ahmed and Goris (unpublished personal communication). Positive blood (3%, 4/109) and urine samples (9%, 10/111) were detected with Ahmed and Goris PCR technique. Overall molecular prevalence was 12% with 14/112 positive cats and no cats were simultaneously positive in blood and urine.

Overall prevalence: an overall prevalence of 22% (25/111) for *Leptospira* spp. was found and the only significant association was with reduced ALB values (P= 0.020, OR= 4.471, 95% CI= 1.163-17.185).

Leptospira spp. antibody, molecular, and overall prevalence according to geographical, demographic, husbandry and environmental variables are reported in table 4.p

Table 4.p *Leptospira* spp. antibody, molecular, overall prevalence according to geographical, demographic, husbandry and environmental variables

VARIABLE	MAT		Urine or blood PCR (%)		Overall (%)	
	POS	NEG	POS	NEG	POS	NEG
ORIGIN						
Sicily	4 (24)	21 (22)	4 (29)	22 (22)	6 (24)	19 (22)
Calabria	13 (76)	73 (78)	10 (71)	76 (78)	19 (76)	67 (78)
SEX						
Male	8 (47)	42 (45)	4 (29)	47 (48)	10 (40)	40 (46)
Female	9 (53)	52 (55)	10 (71)	51 (52)	15 (60)	46 (54)
AGE						
Junior	4 (24)	24 (26)	3 (22)	25 (25)	5 (20)	23 (27)
Adult	8 (47)	52 (55)	9 (64)	52 (53)	15 (60)	45 (52)
Senior	5 (29)	18 (19)	2 (14)	21 (22)	5 (20)	18 (21)
HUSBANDRY						
Indoor	9 (53)	52 (55)	7 (50)	55 (56)	11 (44)	50 (58)
Outdoor	8 (47)	42 (45)	7 (50)	43 (44)	14 (56)	36 (42)
HABITAT						
Urban	12 (71)	72 (77)	11 (79)	73 (74)	19 (76)	65 (76)
Suburban	5 (29)	20 (21)	3 (21)	23 (24)	6 (24)	19 (22)
Rural	0	2 (2)	0	2 (2)	0	2 (2)
HOUSEHOLDS						
Single-cat	2 (22)	15 (29)	2 (29)	15 (27)	3 (27)	14 (28)
Multi-cat	7 (78)	37 (71)	5 (71)	40 (73)	8 (73)	36 (72)

Leptospira spp. antibody, molecular, overall prevalence according to clinical and clinicopathological abnormalities are reported respectively in table 4.q and 4.r

Table 4.q *Leptospira* spp. antibody, molecular, overall prevalence according to clinical abnormalities.

VARIABLE	MAT (%)		urine or blood PCR (%)		Overall (%)	
	POS	NEG	POS	NEG	POS	NEG
BCS						
<3/5	4 (24)	7 (7)	2 (14)	9 (9)	4 (16)	7 (8)

≥3/5	13 (76)	87 (93)	12 (86)	89 (91)	21 (84)	79 (92)
MCS						
>2/4	4 (23)	11 (12)	1 (7)	14 (14)	4 (16)	11 (13)
1/4	13 (77)	83 (88)	13 (93)	84 (86)	21 (84)	75 (87)
MUCOUS MEMBRANES						
Pale	0	5 (5)	0	5 (5)	0	5 (6)
Normal	17	88 (94)	14	92 (94)	25	80 (93)
Jaundice	0	1 (1)	0	1 (1)	0	1 (1)
Congested	0	0	0	0	0	0
LYMPH NODES						
Enlarged	4 (23)	22 (23)	5 (36)	21 (21)	8 (32)	18 (21)
Normal	13 (77)	72 (77)	9 (64)	77 (79)	17 (68)	68 (79)
OCULAR LESIONS						
Presence	2 (12)	4 (4)	1 (7)	5 (5)	2 (8)	4 (5)
Absence	15 (88)	90 (96)	13 (93)	93 (95)	23 (92)	82 (95
ORAL LESIONS						
Presence	4 (23)	29 (31)	5 (36)	28 (29)	8 (32)	25 (29)
Absence	13 (77)	65 (69)	9 (64)	70 (71)	17 (68)	61 (71)
RESPIRATORY SIGNS						
Presence	0	10 (11)	1 (7)	9 (9)	1 (4)	9 (10)
Absence	17	84 (89)	13 (93)	89 (91)	24 (96)	77 (90)
GI SIGNS						
Presence	0	4 (4)	1 (7)	3 (3)	1 (4)	3 (3)
Absence	17	90 (96)	13 (93)	95 (97)	24 (96)	83 (97)
SKIN LESIONS						
Presence	3 (18)	11 (12)	3 (21)	11 (11)	4 (16)	10 (12)
Absence	14 (82)	83 (88)	11 (79)	87 (89)	21 (84)	76 (88)

Legenda: GI= gastrointestinal

Table 4.r *Leptospira* spp. antibody, molecular, overall prevalence according to clinicopathological abnormalities.

VARIABLE	MAT (%)		Urine or blood PCR (%)		Overall (%)	
	POS	NEG	POS	NEG	POS	NEG
SDMA						
Increased	3 (18)	23 (25)	5 (36)	21 (22)	7 (28)	19 (22)
Normal	14 (82)	70 (75)	9 (64)	76 (78)	18 (72)	66 (78)
Missing data	0	1	0	1	0	1
sCr						

Increased	1 (6)	5 (5)	1 (7)	5 (5)	2 (8)	4 (5)
Normal	16 (94)	89 (95)	13 (93)	93(95)	23 (92)	72 (95)
BUN						
Increased	1 (6)	10 (11)	1 (7)	10 (11)	2 (8)	9 (11)
Decreased	1 (6)	3 (3)	0	4 (4)	1 (4)	3 (4)
Normal	14 (88)	78 (86)	13 (93)	80 (85)	21 (88)	71 (85)
Missing data	1	3	0	4	1	3
TOTAL PROTEIN						
Increased	1 (7)	9 (10)	0	10 (11)	1 (5)	9 (11)
Normal	13 (93)	78 (90)	13	79 (89)	20 (95)	71 (89)
Missing data	3	7	1	9	4	6
ALBUMIN*						
Decreased	2 (14)	8 (9)	3 (21)	7 (8)	5 (23)*	5 (6)
Normal	12 (86)	81 (91)	11 (79)	83 (92)	17 (77)	76 (94)
Missing data	3	5	0	8	3	5
GLOBULINS						
Increased	5 (36)	16 (18)	4 (31)	17 (19)	7 (33)	14 (17)
Normal	9 (64)	71 (82)	9 (69)	72 (81)	14 (67)	66 (83)
Missing data	2	7	1	9	4	6
PHOSPHORUS						
Increased	5 (38)	38 (44)	6 (50)	37 (42)	9 (45)	34 (42)
Normal	8 (62)	49 (56)	6 (50)	52 (58)	11 (55)	46 (58)
Missing data	4	7	2	9	5	6
USG						
<1035	5 (29)	19 (20)	2 (14)	22 (22)	6 (24)	18 (21)
≥1035	12 (71)	75 (80)	12 (86)	76 (78)	19 (76)	68 (79)
UPC						
NP	12 (80)	74 (84)	12 (92)	75 (82)	19 (86)	67 (84)
BP	2 (13)	7 (8)	1 (8)	8 (9)	2 (9)	7 (8)
P	1 (7)	7 (8)	0	8 (9)	1 (5)	7 (8)
Missing data	2	6	1	7	3	5

Legnda: *= Significant difference. NP= non proteinuric, BP= borderline proteinuric, P= proteinuric

Measuerement of hepatic profile parameters (ALKP, ALT, AST, GGT), were available only for 23 *Leptospira* spp. positive cats and 13% (3/23) had increased ALKP, 9% (2/22) increased ALT, 10% (2/21) increased AST, while increased GGT

was not observed in 18 tested cats. Decreased ALB was found in 23% of these cats (5/22).

FCoV

A prevalence of 18 % (31/171) was observed for FCoV infection.

FCoV molecular prevalence according to geographical, demographic, husbandry and environmental variables are reported in table 4.s. A higher prevalence was seen in cats from Sicily (P= 0.0066, OR= 3.036, 95% CI= 1.325-6.617), male cats (P= 0.053, OR= 2.224, 95% CI= 0.977-5.062), outdoor husbandry (P= 0.014, OR= 2.875, 95% CI= 1.204-6.863) as well as rural environment compared to urban (P= 0.0001, OR=12.06, 95% CI=3.647-38.77) and suburban environment (P= 0.013, OR= 5.92, 95% CI= 1.613-21.26).

Table 4.s FCoV molecular prevalence according to geographical, demographic, husbandry and environmental variables.

VARIABLE	Blood PCR (%)	
ORIGIN*	**POS**	**NEG**
Sicily	17 (55)*	40 (29)
Calabria	14 (45)	100 (71)
AGE		
Junior	1 (3)	22 (16)
Adult	19 (61)	79 (56)
Senior	11 (36)	39 (28)
SEX*		
Male	21 (68)*	68 (49)
Female	10 (32)	72 (51)
HUSBANDRY*		
Indoor	8 (26)	70 (50)
Outdoor	23 (74)*	70 (50)
ENVIRONMENT*		
Urban	13 (42)	98 (70)
Suburban	10 (32)	37 (26)
Rural	8 (26)*	5 (4)
HOUSEHOLDS		
Single-cat	2 (25)	21 (30)

Multi-cat	6 (75)	49 (70)

Legenda: *= Significant difference

Clinical signs according to FCoV positivity is reported in table 4.t. Positive cats were affected more frequently by reduced BCS (P=0.0212, OR= 2.95, 95% CI=1.267-6.904) and poor MCS (P= 0.000, OR= 8.933, 95% CI= 3.774-21.143), pale mucous membranes (P= 0.0077, OR=4.198, 95% CI=1.464-10.77), lymph node enlargment (P= 0.033, OR= 2.344, 95% CI= 1.059-5.186) and dermatological lesions (P= 0.012, OR= 2.950, 95% CI= 1.242-7.007). Dermatological lesions reported in FCoV positive cats were alopecia (n=8, 73%), crusty dermatitis (n=4, 36%), abscess (n=1, 9%), nodular dermatitis (n=1, 9%), erythema (n=1, 9%), however similar lesions were also seen in negative cats with alopecia (n=15, 68%), crusty dermatitis (n=4, 18%), nodular dermatitis (n=2, 9%), abscess (n=1, 5%) pyoderma (n=1, 5%).

Table 4.t Clinical signs according to FCoV blood PCR positivity

VARIABLE	**Blood PCR (%)**	
	POS	**NEG**
BCS*		
<3/5	11 (35)*	22 (16)
≥3/5	20 (65)	118 (84)
REDUCED MCS*		
1/4	11 (35)	113 (83)
≥2/4	20 (65)*	23 (17)
Missing data	0	14
MUCOUS MEMBRANES*		
Normal	22 (71)	127 (91)
Pale	8 (26)*	11 (8)
Jaundice	0	2 (1)
Congested	1 (3)	0
LYMPH NODES*		
Normal	16 (52)	100 (71)
Enlarged	15 (48)*	40 (29)
OCULAR LESIONS		
Absent	27 (87)	132 (94)
Present	4 (13)	8 (6)
ORAL LESIONS		

Absent	17 (55)	90 (64)
Present	14 (45)	50 (36)
GI SIGNS		
Absent	27 (87)	130 (93)
Present	4 (13)	10 (7)
RESPIRATORY SIGNS		
Absent	26 (84)	124 (89)
Present	5 (16)	16 (11)
SKIN LESIONS*		
Absent	20 (65)	118 (84)
Present	11 (35)*	22 (16)

Legenda: *=Significant difference

Clinicopathological abnormalities according to FCoV positivity are reported in table 4.u. and no significant differences were found.

Table 4.u Clinicopathological abnormalities according to FCoV positivity

	Blood PCR (%)	
	POS	**NEG**
SDMA		
Normal	16 (57)	88 (67)
Increased	12 (43)	44 (33)
Missing data	3	18
sCr		
Normal	24 (83)	122 (90)
Increased	5 (17)	13 (10)
Missing data	2	15
BUN		
Normal	19 (73)	87 (75)
Increased	7 (37)	19 (16)
Decreased	0	10 (9)
Missing data	5	34
TP		
Normal	15 (79)	87 (90)
Increased	3 (16)	9 (9)
Decreased	1 (5)	1 (1)
Missing data	12	53

ALB		
Normal	19	88 (91)
Decreased	0	9 (9)
Missing data	12	53
GLOB		
Normal	12 (63)	73 (77)
Increased	6 (32)	22 (23)
Decreased	1 (5)	0
Missing data	12	55
PHOS		
Normal	14 (78)	62 (65)
Increased	4 (22)	33 (35)
Missing data	13	55
USG		
≥1035	18 (60)	98 (73)
<1035	12 (40)	36 (27)
Missing data	1	16
UPC		
NP	15 (79)	75 (72)
BP	0	14 (14)
P	4 (21)	14 (14)
Missing data	12	47

FeLV

Two percent of cats were FeLV positive (5/262). Because of the low number of positive cats no statistical analysis was performed and geographical, demographic, husbandry, environmental characteristics; clinical and clinicopathological abnormalities and positivity to other investigated pathogens of the five positive cats are summarized in table 4.v

Table 4.v Geographical, demographic, husbandry, environmental characteristics; clinical and clinicopathological abnormalities and positivity to other investigated pathogens of the five FeLV positive cats

VARIABLE	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5
ORIGIN	Sicily	Calabria	Calabria	Sicily	Calabria
SEX	Male	Male	Female	Male	Female
BREED	DSH	DSH	DSH	DSH	DSH
AGE	Adult	Adult	Adult	Adult	Adult
HUSBANDRY	Outdoor	Indoor	Indoor	Indoor	Outdoor
HOUSEHOLD	na	Single-cat	Multi-cat	Single-cat	na
ENVIRONMENT	Rural	Urban	Suburban	Urban	Urban
CLINICAL ABNORMALITY	Enlarged Lymph nodes	Periodontitis, hepatic lipidosis	Rhinitis	NCS	CKD
CLINICOPATHOLOGICAL ABNORMALITIES	Eosinopenia	Increased GLOB, monocytosis, eosinopenia	Increased TP and GLOB, neutrohpilia with left shift, monocytosis	Neutropenia	increased SAA, SDMA, PHOS; Anemia, neutrophilia with left shift, thrombocytopenia
CONCURRENT POSITIVITY	*L. infantum*	*L. infantum*	FeMV, FIV	FIV	*Leptospira* spp.

Legenda: na= non applicable; NCS= no clinical signs

Coinfections

Combinations of positivity between investigated pathogens were found with two to four positivities concurrently found in a single cat and few cats were found positive to just one single tested pathogen. The number of cats with single positivities (based on evaluation of the overall positivity) is reported in table 4.w according to the pathogens evaluated.

Table 4.w: number of cats found positive (overall positivity) only to one single pathogen based on the tested pathogens.

Pathogens tested (# cat)	*L. infantum*	*Leptospira*	FeMV	FIV	FCoV	FeLV
***Li, Lepto,* FeMV, FIV, FCoV, FeLV (68)**	1	8	9	2	3	0
***Li,* FeMV, FIV, FCoV, FeLV (151)**	12	--	25	5	10	1
***Li,* FeMV, FIV, FeLV (185)**	20	--	31	9	--	1

***Li*, FIV, FeLV (254)**	39	--	--	18	--	1

Legenda: Li= *L. infantum*; Lepto= *Leptospira* spp

Exposure to or coinfection (overall positivity) with investigated pathogens according to *L. infantum*, FeMV, and *Leptospira* spp. positivity are reported in Tables 4.x, 4.y, and 4.z. Combination of FIV and FCoV positivities is in table 4.z1
A coinfection between *L. infantum* and FeLV was found in two out of five FeLV positive cats: one was antibody positive and the other one was antibody and molecular positive to *L. infantum*. One FeLV positive cat was molecular (urine) positive to *Leptospira* spp. and one other to FIV. Finally one of the FeLV positive cats was concurrently positive to FIV and FeMV (urine PCR).
Some significant associations of positivity were found among investigated pathogens. Antibody (P= 0.002, OR= 4.205, 95% CI= 2.111-8.718) and overall (P= 0.000, OR= 3.840, 95% CI= 1.892-7.792) positivity to *L. infantum* was associated with FIV infection. Molecular positivity to *L. infantum* was associated with FeMV infection or exposure (P= 0.0109, OR= 5.172, 95% CI= 1.527-16.22) and a significant association was found between *L. infantum* and FeMV PCR positive cats (P= 0.044, OR= 2.229, 95% CI= 1.007-4.933). *Leptospira* spp. positivity was associated to FeMV antibody positivity (P= 0.018, OR= 10.143, 95% CI= 1.002-102.690) and overall FeMV positivity (P= 0.041, OR= 3.378, 95% CI= 1.004-11.367). FIV positivity was found more frequently in cats positive to FeMV urine PCR (P= 0.041, OR= 2.455, 95% CI= 1.014-5.939).

Table 4.x FeMV (overall), *Leptospira* spp. (overall), FIV and FCoV positivity according to *L. infantum* positivity

PATHOGEN (#)	***L. infantum IFI (%)***		***L. infantum PCR (%)***		***L. infantum IFI and/or PCR (%)***	
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**
FeMV*	Tot 179		Tot 189		Tot 188	
Negative	26 (70)	111 (73)	4 (36)	133 (75)	27 (66)	109 (74)
Positive	11 (30)	42 (27)	7 (64)*****	45 (25)	14 (34)	38 (26)
Leptospira spp.	Tot 111		Tot 110		Tot 110	
Negative	10 (77)	76 (78)	2 (67)	83 (78)	11 (79)	74 (77)
Positive	3 (23)	22 (22)	1 (33)	24 (22)	3 (21)	22 (23)

FIV*	Tot 260		Tot 256		Tot 255	
Negative	33 (65)	185 (89)	13 (68)	202 (85)	41 (68)	174 (89)
Positive	18 (35)****	24 (11)	6 (32)	35 (15)	19 (32)****	21 (11)
FCoV	Tot 170		Tot 168		Tot 167	
Negative	27 (77)	112 (83)	7 (64)	132 (84)	28 (74)	110 (85)
Positive	8 (23)	23 (17)	4 (36)	25 (16)	10 (26)	19 (15)

*: significant difference

Table 4.y *L. infantum* (overall), *Leptospira* spp. (overall), FIV (overall) and FCoV (molecular) positivity according to FeMV positivity.

PATHOGEN (#)	**FeMV IFI (%)**		**FeMV urine PCR (%)**		**FeMV blood and/or urine PCR (%)**		**FeMV overall IFI and/or PCR (%)**	
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**	**POS**	**NEG**
L. infantum*	Tot 188		Tot 203		Tot 209		Tot 188	
Negative	18 (72)	129 (79)	22 (67)	138 (81)	23 (66)	141 (81)	38 (73)	109 (80)
Positive	7 (28)	34 (21)	11 (33)	32 (19)	12 (34)*	33 (19)	14 (27)	27 (20)
Leptospira spp.*	Tot 96		Tot 110		Tot 110		Tot 96	
Negative	1 (25)	71 (77)	15 (71)	70 (79)	15 (71)	70 (79)	14 (61)	58 (79)
Positive	3 (75)*	21 (23)	6 (29)	19 (21)	6 (29)	19 (21)	9 (39)*	15 (21)
FIV*	Tot 189		Tot 206		Tot 212		Tot 189	
Negative	22 (85)	138 (85)	25 (74)	150 (87)	27 (75)	151 (86)	43 (81)	117 (86)
Positive	4 (15)	25 (15)	9 (26)*	22 (13)	9 (25)	25 (14)	10 (19)	19 (14)
FCoV	Tot 154		Tot 167		Tot 171		Tot 154	
Negative	22 (88)	107 (83)	24 (86)	112 (81)	25 (83)	115 (82)	40 (85)	89 (83)
Positive	3 (12)	22 (17)	4 (14)	27 (19)	5 (17)	26 (18)	7 (15)	18 (17)

*: significant difference

Table 4.z *L. infantum* (overall), FeMV (overall), FIV (overall) and FCoV (molecular) positivity according to *Leptospira* spp. positivity

PATHOGEN (#)	**MAT (%)**		**urine/blood PCR (%)**		**MAT/PCR (%)**	
	POS	**NEG**	**POS**	**NEG**	**POS**	**NEG**
FeMV*	Tot 96		Tot 97		Tot 96	
Negative	10 (63)	63 (79)	7 (54)	67 (80)	15 (63)	58 (81)
Positive	6 (37)	17 (21)	6 (46)	17 (20)	9 (37)*	14 (19)
L. infantum	Tot 110		Tot 111		Tot 110	

Negative	14 (82)	82 (88)	13 (93)	84 (87)	22 (88)	74 (87)
Positive	3 (18)	11 (12)	1 (7)	13 (13)	3 (12)	11 (13)
FIV	Tot 111		Tot 111		Tot 111	
Negative	14 (82)	83 (88)	12 (86)	85 (88)	21 (84)	76 (88)
Positive	3 (18)	11 (12)	2 (14)	12 (12)	4 (16)	10 (12)
FCoV	Tot 77		Tot 77		Tot 77	
Negative	12 (92)	57 (89)	10	59 (88)	17 (94)	52 (88)
Positive	1 (8)	7 (11)	0	8 (12)	1 (6)	7 (12)

*: significant difference

Table 4.z1 FIV and FCoV positivity relationship

	FcoV	
FIV (171)		
Negative	25 (81)	117 (84)
Positive	6 (19)	23 (16)

4.4 DISCUSSION

We investigated for the first time the epidemiology of a viral (FeMV), a bacterial (*Leptospira* spp.), and a protozoal (*L. infantum*) feline emerging pathogen with the aim of documenting their prevalence in the provinces of Messina (Sicily) and Reggio Calabria (Calabria) but also to increase current knowledge about their pathogenic role. To better understand this role, investigations of feline viral pathogens known to be able to reduce immunocompetence (FIV, FeLV) or to give rise to chronic infections (FCoV) were also performed.

A preliminary methodological consideration has to be done. This concerns the possibility to diagnose pathogen infections by antibody and/or molecular techniques. Generally speaking, detection of a given pathogen by culture from appropriate tissues, secretions or other biological samples confirms infection of the host. However this is not always feasible or easy enough to be used in clinical practice and in field investigations involving a large number of tested individuals.

Antibody detection is used to diagnose infections by proving an adaptive humoral immune response. However in some cases antibody positivity is associated with persistent infection and this is the case for FIV, FCoV and *L. infantum* (at least in dogs) (Addie and Jarret, 2001; Hosie et al., 2009; Gharbi et al. 2015). In other cases infections can be cleared and antibodies can wane with variable time as occurs for feline calicivirus (FCV) infections (Radford et al.,2007), therefore a cross sectional study based on just a single evaluation is not able to differentiate cats that are infected from cats that have cleared the infection. In the cases of FeMV and *Leptospira* spp. infections we do not actually know if cats clear the infection or not and how long the infection would last. Therefore it is appropriate to consider antibody positive cats as "exposed" rather than infected. Additionally, in some cases adaptive cell mediate immune response is elicited after infection and antibody production is low and undetectable. This typically occurs in dogs after *L. infantum* infection and it is a marker of a good prognosis about progression of infection and development of clinical signs. This could be the same in feline leishmaniosis but information is currently limited (Priolo et al., 2019).

Conversely, detection of pathogen nucleic acid (DNA or RNA) demonstrates that the pathogen is actually found in the investigated sample but not always we can state that it is vital and therefore infectiousness is not demonstrated as it is by cultivation. Detection of FeLV RNA (Tandon et al., 2005) is for instance confirmatory for current progressive infection and infectiousness of the cat but we do not have similar data about FeMV and *Leptospira* spp. Moreover, sensitivity and specificity of PCR vary according to the tissue investigated and the techniques used and primers and we decided to test at least two different samples (blood and urine, or blood and mucosal swabs) to detect the investigated feline emerging pathogens in enrolled cats. A limitation of this study is that, apart from the two isolates of FeMV, we did not sequenced positive PCR samples yet.

Moreover, based on the above considerations we combined antibody and molecular techniques to investigate FeMV, *L. infantum* and *Leptospira* spp. circulation in the feline population from primary care practices in two areas of Southern Italy. Comments about these individual pathogens and coinfections follow below.

L. infantum

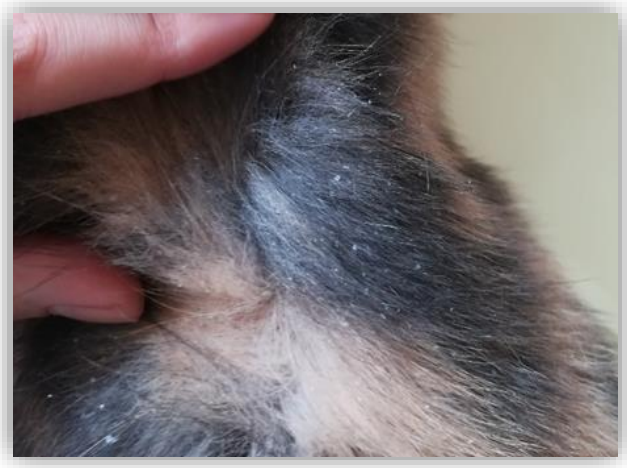
In the present study we found a 20% antibody prevalence, 8% molecular prevalence and 23% overall prevalence of *L. infantum* in cats from two provinces in Sicily (Messina) and Calabria (Reggio Calabria) in Southern Italy. These prevalences are however lower than those recently observed in dogs in Messina province (Ippolito et al., 2017; Otranto et al., 2017) and this is commonly observed when comparing dogs and cats in endemic areas.

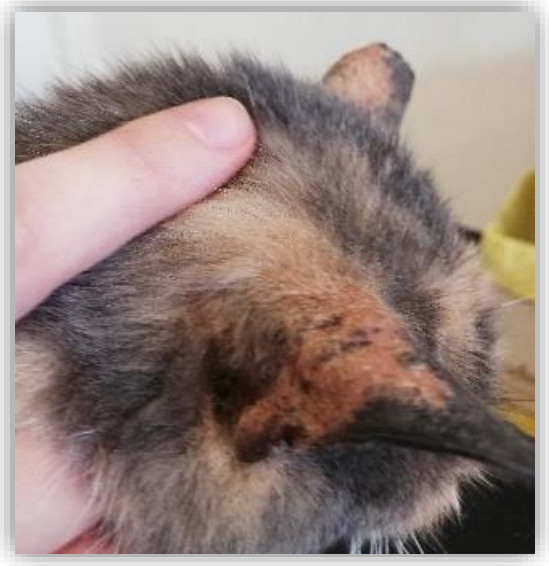
Some studies in Southern Italy investigated the prevalence of anti-*L. infantum* antibodies or parasite DNA in blood with ranges varying from 2.4 to 59% and from 7.1 to 61% respectively (Pennisi et al., 1998; Pennisi et al., 2000; Pennisi et al., 2012; Persichetti et al., 2016; Otranto et al., 2017). This variability can be the consequence of different levels of endemicity in the investigated areas but many factors can contribute such as the characteristics of the population under study or differences in diagnostic methodologies including the cut-off titres of antibody detection (Pennisi et al., 2015) or different PCR assays. There is a study that compared different serological techniques for anti- *L. infantum* antibody detection in cats (Persichetti et al., 2017) confirming the good performance of IFI with 1:80 cut off dilution, but there are no studies comparing different PCR assays in cats. Conventional, nested, and real-time PCR techniques are used for the detection of *L. infantum* DNA in diagnostic samples, usually targeting kinetoplast DNA, and in dogs real-time PCR is considered more sensitive (Solano-Gallego et al., 2017). However, sensitivity of PCR tests strongly relies on the type and number of different tissues evaluated (Solano-Gallego et al., 2017). Bone marrow, lymph node, spleen, skin and conjunctival swabs are considered more sensitive in dogs compared to whole blood or urine samples (Solano-Gallego et al., 2017), but epidemiological investigations must rely on minimally invasive (whole blood) or non invasive (swabs, hair) samples and conjunctival swabs are considered more sensitive in dogs (Solano-Gallego et al., 2017). Few studies used in cats conjunctival (Pennisi et al., 2012; Chatzis et al., 2014; Otranto et al., 2017; Persichetti et al., 2018), and oral swabs or urine (Persichetti et al., 2018). For the first time we examined cat ear-cerumen swabs and hair that are both more easily obtained from cats during physical examination compared to conjunctival and oral swabs. Unfortunately, ear-cerumen was never found positive and hair was positive in 2% of tested samples

similar to conjunctival and oral swabs respectively. Interestingly, the three hair positive cats were antibody negative and PCR negative in blood and swabs so hair was the only tissue confirming exposure to *L. infantum*. Conversely, hair PCR was negative in cats with high antibody titres or parasite loads in other tissues. Cerumen and hair were both proposed as non-invasive samples for detection of *L. infantum* infection in dogs in Brazil (Belinchòn-Lorenzo et al., 2016, 2019; de Sousa Gonçalves et al., 2016) and hair PCR was used for testing leporidae in Spain (Ortega et al., 2017) but we did not have promising results in cats in our study. Positivity of mucosal swabs was similar to previous studies (Chatzis et al., 2014; Otranto et al., 2017; Persichetti et al., 2018) but lower than conjunctival swab positivity found in the same area by Pennisi et al. (2012). Whole blood provided a higher percentage of positivity (4%) compared to swabs. Obviously, positivity was much more prevalent from clinical samples such as puncture of enlarged lymph nodes (20%), and samples from skin lesions (26%). However, sensitivity and specificity of PCR tests performed with different tissues compared to antibody detection showed that non invasive samplings are very specific but they have very poor sensitivity. Among clinical samples the best performance was obtained by PCR from skin lesions with fair (62.5%, 95% CI 30-86%) sensitivity and 100% specificity (95% CI 74-100%). The PCR test from enlarged lymph nodes provided poor sensitivity (12%, 95% CI 20-34%) and specificity (33%; 95% CI 17-88%). These data are not conclusive but in any case they confirm the poor agreement between antibody detection and PCR test results (Pennisi et al., 2015; Persichetti et al., 2017) with the exception of PCR positive skin lesions that are usually found in antibody positive cats. This information shows that antibody positivity is a marker of skin lesions associated with *L. infantum* infection in cats.

We tested other tissues (skin scales, claw, nasal swab, uterus) in a cat with confirmed diagnosis of FeL and they were all found positive with high parasite loads (see table 4.c and figures 3 and 4). This is the first time that uterus, claw and scales were successfully used to detect *L. infantum* DNA in a cat with FeL. This finding confirms the wide dissemination of *L. infantum* infection in cats with FeL.

Fig.3, 4 Scales and alopecia of a cat with confirmed diagnosis of FeL (IFI and PCR)





A recent nationwide survey of feline *L. infantum* infection in Italy reported a lower prevalence compared to the one obtained in the present study with an anti-*L. infantum* antibodies prevalence of 9.6%, a blood PCR prevalence of 1.7% and an overall prevalence of 10.5% in cats from Southern Italy (Iatta et al., 2019). However, most of the cats that we enrolled were from Reggio Calabria province that was not evaluated by Iatta et al. (2019) and we obtained a higher positivity from this province compared to Messina, with both antibody and molecular investigations although the difference was not significant. A recent study in cats enrolled at the same clinics in Reggio Calabria and Messina provinces found a lower antibody (9.6%), molecular (6.6%), and overall (14.7%) prevalence compared to the present study (Persichetti et al., 2018).

The two studies shared analytical features and laboratories where tests were performed, but the two tested cat populations differed in some characteristics and age is probably the most important one. In fact in the previous study median age of cats was 2 years (25th percentile 1 year, 75th percentile 5 years) and they were therefore younger compared to cats we tested (median age 3 years, 25th percentile 1 year, 75th percentile 8 years). Age is reported to be a significant risk factor for feline *L. infantum* infection (Pennisi et al., 2000; Cardoso et al., 2010; Pennisi et al.,2012; Iatta et al., 2019). We found an increased risk for antibody and overall positivity respectively of 5.6 and 4.7 times in adults and 9.9 and 7.8 times in senior cats. This is in line with a vector-borne transmission of *L. infantum* infection where cumulative time of exposure to the vector increases year by year and with an infection that is not rapidly cleared in the infected individual.

Male cats were also more frequently antibody (OR= 1.892), PCR (OR= 3.307) positive and had a higher *L. infantum* overall prevalence (OR= 2.222) than female cats, in agreement with previous studies (Cardoso et al., 2010; Sobrinho et al., 2012) but there is no apparent reason for this occurrence. However, in our study *L. infantum* and FIV shared some demographic risk factors being male sex (OR= 2.624) and senior age (OR= 3.651) confirming that FIV risk factors are similar to those for *L. infantum* infection. Moreover, an association between FIV positivity and *L. infantum* antibody (OR=4.205) and overall (OR= 3.84) prevalence was found and this can be just due to endemicity of both infections in the investigated areas, but interactions between FIV and *L. infantum* infections can exist due to impaired immunocompetence caused by FIV and the virus could play a causative role in *L. infantum* infections. Cats from rural environments were also four time more at risk to be found antibody positive to *L. infantum*, as reported in a previous study (Cardoso et al., 2010), possibly because of a higher prevalence of sand flies in investigated rural areas.

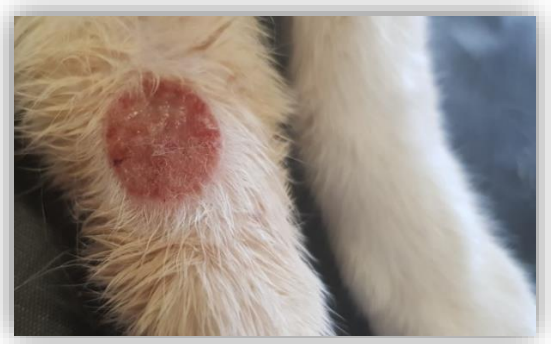
Interestingly, molecular positivity to *L. infantum* was associated with a five times higher risk for FeMV infection or exposure and this is the first time that this association was investigated in cats.

Clinical findings significantly associated with *L. infantum* posititivity included some abnormalities considered suggestive of FeL (Pennisi et al., 2015; Pennisi and Persichetti, 2018). Among them low BCS, poor MCS, skin lesions and proteinuria

were risk factors for antibody (low BCS OR= 3.38; poor MCS OR= 2.5; skin lesions OR= 2.7; proteinuria OR= 3.8), PCR (low BCS OR= 4.86; poor MCS OR= 4.5; skin lesions OR= 3.09; proteinuria OR= 7.8), and overall positivity (low BCS OR= 2.88; poor MCS OR= 2.28; skin lesions OR= 2.58; proteinuria OR= 4.4). Additionally, an increased sCr value was a risk factor for antibody (OR=3.46) and overall positivity (OR=3.09) while decreased TP and increased GLOB were risk factors for PCR (decreased TP OR= 16.1; increased GLOB OR= 7.4) and overall positivity (decreased TP OR= 12.8; increased GLOB OR= 7.4). Gastrointestinal signs were associated with overall positivity (OR=3.02) and diarrhea was the most frequently observed sign. Diarrhea is reported in less than 10% of cats with FeL (Pennisi et al., 2015). In dogs leishmanial enteritis is reported with amastigotes in the mucosa, submucosa and muscle layers of the small and large intestines, chronic infiltration of mononuclear cells (macrophages, lymphocytes and plasma cells) and rarely erosive and ulcerative colitis and hemorrhagic diarrhea (Pinto et al., 2011, Silva et al., 2016). However, gastrointestinal signs were also significantly associated with FIV infection, therefore this coinfection could concur.

In the present study some (ulcers and crusts, squamous dermatitis) dermatological lesions suggestive of FeL were found but nodules were not observed (Pennisi et al., 2015). Alopecia was the most frequent (59%) skin abnormality but this is a non specific skin lesion. In most cases, suspected *L. infantum* dermatological lesions were evaluated by PCR or histopathology and immunohistochemistry and 26% were found positive. The highest parasite load was found in a cat with multifocal crusty dermatitis on limbs, and crust was PCR positive with 100 *L*/sp. Interestingly, a much higher parasite load was found in DNA from a claw of a cat (18500 *L*/sp) with no clinical abnormality. Histopathology with *L. infantum* immunohistochemistry (IHC) was obtained in a single case of multifocal ulcerations on limbs and chronic inflammation with widespread monocytic infiltrates and positive IHC, fig 5.

Fig 5,6,7 Ulcer, histopathology and immunohistochemistry results of a *L. infantum* positive cat



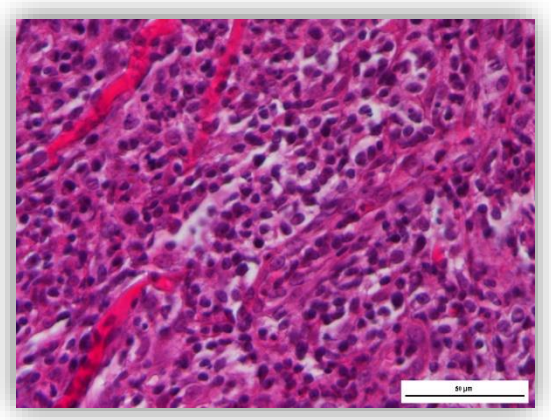


Fig 6 Courtesy of Dr. Puleio, histopathology with haematoxylin and eosin stain (40x), scale bar represents 50 µm

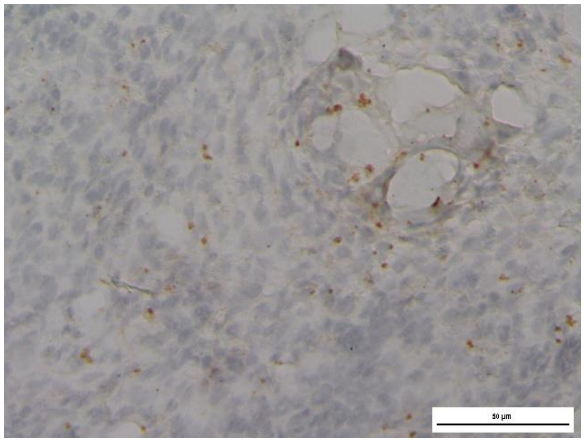


Fig 7 Courtesy of Dr. Puleio, immunohistochemistry (40x, scale bar represents 50 µm) shows dark brown amastigotes higlighted by a chromogen reaction using canine antiserum as the primary antibody (streptavidin–peroxidase method, 3-3' diamminobenzidine tetrahydrochloride chromogen, heterologous immune serum from dogs naturally infected with *L. infantum* diluted 1:100 in 0.01 M PBS, used as primary antibody) (Migliazzo et all., 2015; Tafuri et all, 2004).

In a field study performed with 197 cats enrolled at the same practices as the present study (Persichetti et al., 2018) significant associations of *L. infantum* positivity with higher values of sCr, and with inappropriate USG evaluated in 127 samples, were not found. However prevalence of *L. infantum* positivity was lower in cats from that study compared to the present investigation and this could have influenced the analysis of the data. Renal proteinuria and increased sCr values were reported in some FeL cases at diagnosis or during follow-up (Pennisi et al., 2015). Moreover, granulomatous nephritis, lymphoplasmacytic aggregates and macrophages containing some amastigotes were reported in one case of FeL (Puleio et al., 2011). Moderate interstitial inflammatory infiltration composed predominantly of macrophages with some lymphocytes and plasma cells, moderate interstitial fibrosis and tubular proteinuria were reported in another case (Navarro et al., 2010).

In a recent study that evaluated the role of SDMA in dogs with leishmaniosis, SDMA was not useful as an early marker of CKD when compared to creatinine and UPC values (Torrent et al., 2018). This result is similar to the one observed in our study where no significant association was found between increased SDMA and *L. infantum* positivity, but we found this association with increased sCr and UPC values. Significant association between *L. infantum* and FeMV can contribute to the renal damage in coinfected cats.

Hyperglobulinemia and polyclonal gammopathy are widely reported in cats (Pennisi and Perischetti, 2018; Pennisi et al., 2015; Soares et al., 2016) as well as in dogs (Solano-Gallego et al., 2011) with leishmaniosis. Hyperproteinemia is also frequently found in FeL cases (Pennisi et al., 2015), but unexpectedly we found a significant association with hypoproteinemia. However hypoproteinemia was mild (5.3-5.6 g/dL) and not associated with hypoalbuminemia in *L. infantum* positive cats.

Lymph node enlargement was frequent but similar reported in both *L. infantum* positive and negative cats in this study, therefore we were not able to find an association with *L. infantum* as reported in a previous study (Pennisi et al., 2000), although this is a very common abnormality in FeL cases (Pennisi et al., 2015). Similarly, oral lesions in this study were frequently detected but irrespective of *L. infantum* positivity.

Ophthalmic abnormalities are a frequent finding in FeL cases (Pennisi et al. 2015) but we cannot comment our findings because they are biased by the lack of a complete ophthalmic examination.

In conclusion, we found a high antibody (20%), molecular (8%) and overall (23%) prevalence of *L. infantum* in cats from Reggio Calabria and Messina provinces, and particularly in adult and old cats. Males and cats from rural areas were also more frequently positive. *Leishmania infantum* infection disseminates in cats as the protozoal DNA was found in multiple investigated tissues. Cats with *L. infantum* infection were at risk for wasting disease, skin lesions, renal damage, dysproteinemia and diarrhea. The significant association found with FIV is interesting because impaired immunocompetence can influence outcome of FeL and because it can be responsible for the male sex risk to be *L. infantum* positive. For the first time association with FeMV was demonstrated and this is very important when considering the higher risk for renal damage detected in *L. infantum* positive cats.

We think that these findings are of value from a clinical point of view and we intend to make a more robust multivariate analysis of significant variables.

FeMV

After it's discovery in Hong Kong (Woo et al., 2012) FeMV infection was investigated and reported in different countries such as Japan, Germany, Italy, USA, South America, Turkey and UK (Park et al., 2016; Koide et al., 2015; Sieg et al., 2015; Lorusso et al., 2015; Donato et al., 2018; Sharp et al., 2016; Darold et al., 2017; Yilmaz et al., 2017; McCallum et al., 2018) documenting that the virus is spread worldwide and is commonly found in both healthy and sick cats. In fact, antibody prevalence reported in previous studies range from 21.2% to 66.7% and molecular prevalence from 4.2% to 52.9% (Beatty et al., 2019). Two recent studies evaluated prevalence of FeMV in cats in Italy. De Luca et al., (2018) found a low molecular prevalence in blood (0.4%) with only one positive cat out of 264 and a higher molecular prevalence in urine (15%) with 40 positive cats out of 264, while Stranieri et al. (2019) found a low molecular prevalence in Northern Italy (1.23%) with only one positive cat out of 81. These previous studies differ in numbers and demographic characteristics of the

cats enrolled, as well as in methods and tissues used for molecular diagnosis and these factors may have influenced results obtained.

In the present study we found an antibody prevalence of 14% (26/191), a molecular prevalence in blood of 2% (4/210) and of 16% in urine (35/216) and an overall molecular prevalence of 17% (37/222). The overall antibody and molecular prevalence was 28% (53/191).

Some previous studies evaluated the persistence of virus shedding in urine. Lorusso et al. (2015), found urine shedding for at least 14 days in one cat, Sieg et al. (2015) for several weeks in three cats (4-11 weeks) and Sharp et al. (2016) for 15 months in one cat. Persistence or randomness of urine shedding as well as the duration of systemic infection are still not clear (Lorusso et al., 2015, Sharp et al., 2016, Sieg et al., 2015). We followed up nine urine PCR positive cats for a variable time and shedding was documented after up to 12 months in some cases (tables 4q.1 and 4q.2). Daily shedding evaluation was not performed, but eight cats were always positive in several urine samples during the follow up period. However five of them lived in a multi-cat household, two cats were outdoors and only two cats were hospitalized without direct contact with other cats during follow up, therefore we cannot state that shedding was actually continuous or just very frequent or in some cases there was a re-infection. Moreover, in cat 9 urine shedding was not continuous and this cat was from a multi-cat household therefore this could support the hypothesis of a re-infection.

We found that four out of eight followed up cats were also antibody positive, but positive blood PCR was not seen at any time in these cats. Unfortunately we were able to retest by IFI only three cats (5, 7, and 9) and cat 5 seroconverted to negative status one month after the last detection of a positive urinary PCR. This is the first time that seroconversion to a negative status after stopping urinary shedding is documented in a field study and this is suggestive of spontaneous clearance of FeMV infection in this cat.

Few studies evaluated risk factors of FeMV infection, and they reported a higher prevalence in multicat environments (De Luca et al., 2017; Darold et al., 2017), outdoor husbandry, or suburban/rural environment (Yilmaz et al., 2017; Donato et al., 2018).

We found a higher prevalence of antibody positive cats in Sicily (21%; OR=2.37%) compared to Calabria (10%). Unexpectedly we found that single-cat household cats were more frequently positive (urine PCR, OR= 4.02; overall PCR, OR= 3.49). This is in contrast with the higher rate of positivity reported in multi-cat environment by previous studies (De Luca et al., 2017; Darold et al., 2017). We investigated the past history of all these cats and they were adult or senior outdoor cats before being adopted some time before enrollement in the study. This means that they probably had a chronic long lasting infection transmitted before adoption. Additionally, adult (OR=3.35) and senior (OR= 3.99) ages were both a risk factor for overall positivity supporting the hypothesis of a chronic infection caused by FeMV (Lorusso et al., 2015; Sieg et al., 2015).

Most of the studies focused on the association between FeMV and feline CKD and there are few data about other abnormalities associated with FeMV. However, a study which evaluated both clinico-pathological and histopathological abnormalities in FeMV RNA-positive cats found lower median red blood cell counts, haemoglobin, ALB, ALB/GLOB and urobilinogen and higher ALT, alkaline phosphatase and bilirubin in positive cats (3/68) compared with non-infected cats. Histopahology revealed tubulointerstitial nephritis, cholangiohepatitis and hepatic focal necrosis even if these findings were similar to those observed in negative cats (Yilmaz et al., 2017). Moreover based on *in vitro* studies, FeMV is able to infect feline epithelial, fibroblastic, lymphoid and glial cells (Sakaguchi et al., 2014). Recently, a new genotype of feline morbillivirus, tentatively named feline morbillivirus genotype 2 (FeMV-GT2) was described, showing 78% nucleotide homology to known feline morbilliviruses (Sieg et al., 2019). Infection of primary feline pulmonary epithelial cells and primary cells from the cerebrum and cerebellum, as well as blood CD4+ T cells, and CD20+ B cells, was obtained through experimental infection. This means that there is a diversity of feline paramyxoviruses (Sieg et al., 2019), and that both have receptors ubiquitously expressed in cats (Sakaguchi et al., 2014). Therefore other tissues than kidney can be involved causing other clinical abnormalities. Experimental susceptibility of lymphocytes and monocytes means that impairment of immune response could occur in infected cats but this was not explored. In this regard the significant association we found between FeMV and other tested pathogens (FIV, *L.*

infantum, and *Leptospira* spp.) could be of some relevance and could be better investigated by multivariate analysis.

From a clinical point of view, we found associations between FeMV positivity and reduced BCS (antibody positivity, OR= 3.0), oral disease (antibody positivity, OR= 3.02; overall antibody and/or PCR positivity, OR= 1.86), ophthalmic abnormalities (urine PCR positivity, OR=4.29; overall PCR positivity, OR= 4.11), skin lesions (antibody positivity, OR= 3.5; overall antibody and/or PCR positivity, OR= 2.33), and respiratory signs (overall PCR positivity, OR=3.76; overall antibody and/or PCR positivity, OR= 2.44). Increased globulin values were associated with antibody (OR= 4.72), urinary PCR (OR= 4.72), overall PCR (OR= 2.88) and overall antibody and/or PCR (OR= 3.42) positivity. These associations are probably influenced by the significant occurrence of coinfections that we found in FeMV positive cats. In fact increased globulins values were found associated, as it may be expected, with both *L. infantum* and FIV infections. Moreover, FIV infected cats were as expected at higher risk for various clinical abnormalities (see table 4.j).

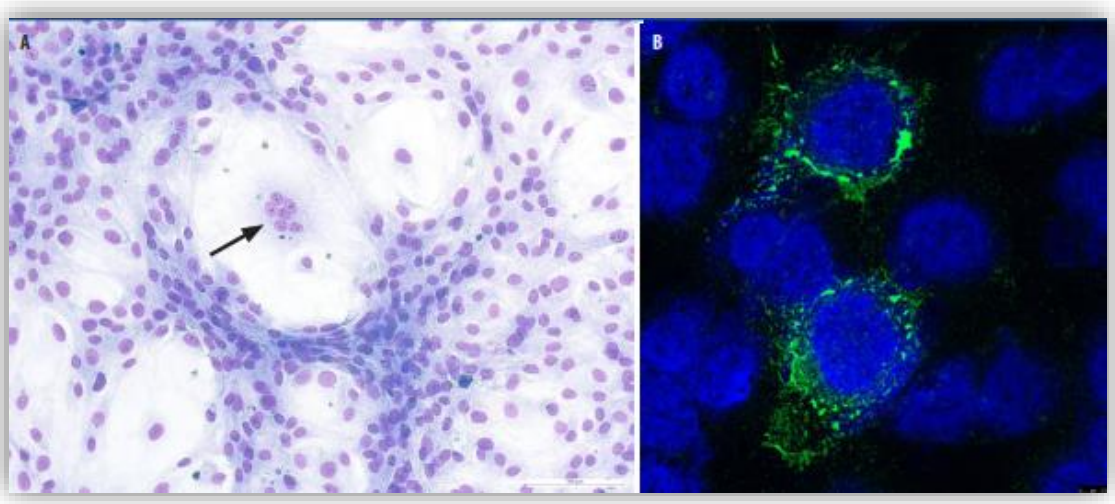
The association between FeMV (antibody positivity, OR= 10.143) and *Leptospira* spp. positivity could also be clinically relevant as both pathogens are found associated with feline kidney diseases. However, in the present study we did not find associations between either of them and changes in markers of CKD. Moreover in the controlled study of chapter 3, where prevalence of these pathogens was investigated in cats affected by CKD, no significant association was found. The same observation was reported in most of previous studies that investigated this association (Shropshire et al., 2016, Darold et al, 2017; Lo Russo et al., 2017; McCallum et al., 2018; Donato et al. 2018) and there is no evidence for a role of FeMV in feline CKD.

Finally, we isolated FeMV from two urine samples out of the 14 PCR positive samples cultured (FeMV Tremedino/2018 Italy and FeMV Pepito 2018/Italy belonging to FeMV genotype 1, fig 6) (Donato et al., 2019). These two cats, were from the same clinical practice in Reggio Calabria province and they did not show clinical and laboratory signs of renal damage but were affected by other clinical signs. Briefly, Tremedino showed stomatitis and enlarged poplyteal and submandibular lymph nodes, whereas Pepito was overweight and presented for stomatitis and bilateral otitis.

In conclusion, we found that almost one third of cats adimtted to primary care practices in the provinces of Reggio Calabria and Messina are exposed to FeMV and we obtainded isolation of two strains. Continous or frequent urinary shedding or re-infection were documented in some followed up cases after up to 12 months and we postulated spontaneous clearence in one cat.

Significant associations with FIV, *Leptospira* spp. and *L. infantum* positivity was recorded for the first time in FeMV exposed cats and risk factors evaluation should therefore be performed by multivariable analysis. However, in the present study we did not find associations between FeMV infection or exposure and abnormality of CKD markers.

Fig 8,9 FEA cells infected by FeMV Pepito2018/Italy.



(Donato et al., 2019) Multinucleated syncytium was observed by May Grünwald-Giemsa staining (A); strong and specific cytoplasmic fluorescence (green), nuclei are stained with DAPI (blue) (B). Scale bar = 100 µm (A), 75 µm (B).

Leptospira spp.

Leptospirosis is a zoonotic disease with a worldwide distribution, affecting both dogs and cats. Generally, clinical leptospirosis is common in dogs but appears to be rare in cats, however both species can shed leptospires in urine (Schuller et al., 2015).

Prevalence of serovars differs significantly between geographical regions (Hartmann et al., 2013a), and distribution is reported in table 4.z2

Table 4.z2 Prevalence of serovars detected in different countries

COUNTRY	SEROVARS	PREVALENCE (%)	TITRES	REFERENCES
Thailand	anhoa autumnalis celledon copenhageni djasiman icterohaemorrhagiae patoc	14/260 (5.4)	20-160	Sprißler et al., 2019
Germany	australis autumnalis bratislava copenhageni grippotyphosa pomona saxkoebing	35/195 (17.9)	100-6400	Weis et al., 2017
Greece	ballum bataviae bratislava canicola panama rachmati salinen	33/99 (33.3)	50-400	Mylonakis et al., 2005a
Australia	copenhageni grippothyphosa pomona tarassovi zanoni	10/59 (17)	50-3250	Dickenson and Love, 1993
Scotland	autumnalis hardjo icterohemorrhagiae	8/87 (9.2)	30-300	Agunloye and Nash, 1996
USA	bratislava canicola icterohaemorrhagiae	12/141 (8.5)	100-200	Shropshire et al., 2016
Taiwan	australis icterohaemorrhagiae javanica pyrogenes	21/225 (9.3)	100-400	Chan et al., 2014

	shermani			
Québec	bratislava grippothyphosa icterohaemorragiae pomona	26/239 (10.9)	100-12800	Rodriguez et al., 2014
Québec	autmnalis bratislava	10/40 (25)	≥100	Lapointe et al., 2013
Massachusetts	autumnalis bratislava icterohemorrhagiae pomona	3/63 (4.8)	200-12800	Markovich et al., 2012

We found anti-*Leptospira* antibodies in 15% (17/111) of cats with titres ranging from 20 to 320. Antibody levels are usually low in cats and often lower than those in other host species (Agunloye and Nash, 1996). The reason for low titres in cats could be due to potential cross-reactivity or paradoxical reactions (i.e. in which the highest titre are to a serogroup unrelated to the infecting one) with serovars not tested for, or simply low antibody titres are able to control the infection in cats (Rodriguez et al., 2014, Ahmad et al., 2005). Additionally titres could rapidly decrease due to quick clearence of infection in cats.

In an experimental study, 11 cats were inoculated with *Leptospira interrogans* and five of them were inoculated subcutaneously with serovar icterohaemorrhagiae and the other five with serovar canicola. No clinical or laboratorial alterations were found in these animals. Antileptospiral agglutinins were detected in 90% of the infected cats, shortly after the 1st week post inoculation. The leptospiral agglutinins were detected for 8 to 12 weeks and the elimination of leptospires through urine was observed only in animals infected with serovar canicola, beginning 2 to 4 weeks after inoculation and lasting for 2 to 8 weeks (Larsson et al., 1985).

We found antibody positivity against two serogroups of *L. interrogans*: Australis (serovar bratislava in five cats, and lora in one cat) whic has never been reported in cats before, and Pomona (serovar pomona in one cat). More frequent was positivity against *L. borgpetersenii* with serogroup Ballum (serovar ballum in one cat, serovar arborea in four cats), Javanica (serovar poi in nine cats), and Mini (serovar mini in one

cat). Antibodies against serovars poi, arborea, and mini were never reported before in cats.

The most frequent seroreactivity involved serovars previously described in other host species in Italy. In two large studies on human clinical leptospirosis conducted between 1986 to 1993 with 312 cases and between 1994 to 1996 with 222 cases, the majority of cases was observed in northern regions and serovars icterohaemorrhagiae, poi, copenhageni, bratislava were the most frequently found (Ciceroni et al., 1995, Ciceroni et al., 2000a).

Leptospira bratislava was reported in wild ruminants (red deer) in the Central Italian Alps (Andreoli et al., 2014), in kenneled dogs in Milan (Scanziani et al., 2002) and in a case of human leptospirosis reported in Modica (Sicily) (Cinco et al., 1989).

Leptospira poi was reported in sheep and goats in Alto Adige-South Tyrol (Ciceroni et al., 2000b) and in another study of human leptospirosis in Vicenza (Veneto region) performed between 1990 and 2003 it was the most frequent serovar reported (Conti et al., 2005). *Leptospira* arborea was isolated for the first time in 1955 from a wood mouse (*Apodemus sylvaticus*), captured at Arborea (Sardegna, Italy) (Babudieri and Moscovici,1955). Similarly seroreactivity against the other serovars was found in Italy. Tagliabue et al. (2016) in a serological surveillance of leptospirosis evaluated data obtained between 2010-2011, collecting 43935 animal specimens (bovine, swine, ovine and goat, dog, and wild boar) and testing a panel of 8 serogroups as antigens (Australis, Ballum, Canicola, Grippotyphosa, Icterohaemorrhagiae, Pomona, Sejroe, Tarassovi). In this study, the most common antibody positivity was against the following serogroups: Australis (dogs, wild boars, horses, hares, swine, foxes, and rodents), Sejroe (cattle, sheep, goats, and buffaloes), Icterohaemorrhagiae (dogs, goats, and foxes), Pomona (swine, cattle, and wild species), Grippotyphosa (hares).

Another study evaluated seroprevalence of *Leptospira* spp. in healthy horses living in central Italy reporting positivity for the serovars icterohaemorrhagiae, bratislava, and pomona (Ebani et al., 2012). In Ancona (Marche region) eighteen animals were found positive to leptospirosis and the highest titers of agglutinins were recorded against australis, bratislava, and lora (Lupidi et al., 1991).

Interestingly we found the highest titre (320) in a cat PCR positive in urine and it was against serovar Bratislava (strain Jez Bratislava) with titre 80 against a serovar of the

same serogroup (Lora). But positivity was also found in this cat to other serogroups (Mini, serovar mini: titre 80; Bratislava, serovar arborea: titre 40). In this case a recent, active infection or a re-infection can be suspected (Rodriguez et al., 2014) and a mixed infection as well. This cat was mildly anemic, with neutrophilia and monocytosis, increased SAA and GLOB and a borderline proteinuria. Coinfection with FeMV (urine PCR) and FIV positivity was reported.

Antibody positivity was observed in cats with outdoor lifestyles or living in multicat households (Rodriguez et al., 2014). In the present study no association with outdoor lifestyle, multicat household or the type of environment was found, however the 78% (7/9) antibody positive cats were from multicat households and a low number of cats (22%, 2/9) from a singlecat household. It is suggested that infection of cats is due to predation of mammals harbouring leptospires or after exposure to infectious urine of cohabiting dogs (Hartmann et al., 2013a), but intraspecies transmission can be possible as most antibody positive cats were from multicat households.

In many countries DNA of pathogenic *Leptospira* was detected in feline urine with variable prevalence reported. The DNA of pathogenic *Leptospira* spp. was detected in feline urine in 67.8% of samples by conventional PCR in Taiwan (Chan et al., 2014), in 5.3% of cats with CKD and in 1.6% in a healthy group tested by a conventional PCR in Canada (Rodriguez et al., 2014), in 3.3% in Germany (Weis et al., 2017) and in 0.8% in Thailand (Sprißler et al., 2019) in both cases by qPCR. Conversely, a study did not find positivity in Algiers in urine tested by qPCR (Zaidi et al., 2018).

In the present study *Leptospira* DNA was detected in 3% (4/109) blood and in 9% (10/111) feline urine samples. These results suggest that cats may have a role in the transmission of leptospirosis, as a reservoir or as an accidental host.

A positive PCR in blood together with consistent clinical signs is higly suggestive of acute leptospirosis, while a positive PCR in urine is a marker for urinary shedding, which can occur in both acutely infected animals and chronic renal carriers. However PCR negative results do not rule out leptospirosis cosidering that leptospiraemia is transient and can be found in early stages of the disease and that urinary shedding is delayed after acute infection and can be intermittent, moreover negative results can also be due to recent antibiotic treatments (Schuller et al., 2015). Few studies investigated by PCR urinary *Leptospira* shedding in cats (Hartmann et al. 2013a).

However, MAT is considered the preferred confirmatory test for leptospirosis in dogs and positive PCR is associated with the antibody detection (Schuller et al., 2015). In the present study we found four cats positive to MAT and shedding leptospires in urine and two cats positive to MAT and blood PCR, 11 cats were positive only to MAT, and 8 cats were positive only by PCR. Interestingly, for the first time we compared two different PCR assays in cats and we obtained a tremendous increase in sensitivity by using a more specific and sensitive PCR, based on the use of hydrolysis probes (dual-labelled oligonucleotides), of an internal control and a different amplification procedure (Ahmed and Goris manuscript in preparation) compared to Stoddard et al. (2009) technique.

Clinical signs in infected cats seem to be rare (Arbour et al., 2012). In a study the major clinical signs reported were ascites and hepatomegaly (Agunloye and Nash, 1996). Liver centrilobular necrosis (Bryson and Ellis, 1976) and hepatic amyloidosis (Mason et al., 1972) were diagnosed postmortem in two cats after histopathological examination (Bryson and Ellis, 1976). Subpleural and intra alveolar haemorrhage of lungs and perivascular haemorrhage in brain with isolation of leptospires from thoracic fluid and aqueous humour was also observed in a cat (Bryson and Ellis, 1976). Ocular pathologies (particularly uveitis), and lameness (Arbour et al., 2012) were described in a cat.

Chronic kidney disease was associated with infection in four cats (Arbour et al., 2012; Mason et al., 1972). Two of them were presented for polyuria and polydipsia: one cat was positive to *L.* pomona (12800), *L.* grippothyphosa *L.* icterohemorragiae and *L.* hardjo (titre 200), *L.* canicola and *L.* bratislava (titre <100) and had low USG, mild increased BUN and sCr within the normal reference interval, however clinical improvement was observed after the therapy. One cat with increased sCr and BUN and several clinical signs was positive to *Leptospira* spp. diagnosed by PCR and MAT (*L.* pomona and *L.* icterohemorragiae 3200 titre, *L.* bratislava, *L.* autumnalis 600 titre, *L.* grippothyphosa 100 titre, *L.* canicola <100 titre) and was euthanized because of prognosis and a diagnosis of tubulointerstitial nephritis was made postmortem (Arbour et al., 2012).

In the other study focal interstitial nephritis and fatty degenerative tubular nephrosis were diagnosed in two positive cats, one high titre (1000) positive to *L*. pomona and one diagnosed through silver impregnation staining (Mason et al., 1972).

We did not find associations between markers of CKD and *Leptospira* spp. overall positivity and no association with ocular or respiratory tract disease was found. We found a significant association with decreased ALB values that is compatible, among other causes, with both chronic liver or kidney insufficiency. Lower BUN was however rare and not associated with *Leptospira* spp. positivity. Unfortunately, we were not able to evaluate the association of positivity with markers of hepatitis or cholangiohepatitis as we could only test *Leptospira* spp. positive cats. A few cats had increased ALKP (13%), ALT (9%) and AST (10%). Among these cats: two cats with reduced ALB were also positive to FeMV and one also to FIV, another cat with increased AST and ALT was also positive to FeMV and FIV, one cat with increased ALKP was also positive to FeMV and one cat with increased ALT was also positive to *L. infantum* and FCoV.

As reported above, we found a significant association of molecular positivity with FeMV (OR= 3.378) and this finding is interesting considering that in both infections the pathogen is shed with urine (Hartmann et al., 2013a; Zuerner, 2015, Sieg et al., 2019). However, in the present study we did not find an association between these pathogens and CKD as reported in previous studies (Shropshire et al., 2016, Darold et al, 2017; Lo Russo et al., 2017; McCallum et al., 2018; Donato et al. 2018).

In conclusion, we found that cats are frequently infected by or exposed to *Leptospira* spp. and that cat infection is caused by the same serovars found in other animal species. This means that they can have a role in the epidemiology of leptospirosis, as an additional reservoir or just sentinels for a risk of infection. Additionally, cat-to-cat transmission can be considered and this could be a serious risk for indoor multicat household cats. A significant association with FeMV exposure was found but no associations were detected with investigated clinical abnormalities.

FIV

In this study FIV infection was evaluated by antibody detection which is the gold standard technique for diagnosis (Hosie et al., 2009). However, blood PCR was also

performed for most of cats and 12 out of 25 antibody positive cats were found negative by blood PCR test.

In the present study 16% of cats were FIV positive, similarly to prevalence reported in a past investigation from Sicily and Calabria (Pennisi and Bo, 1994). However, this is a higher prevalence when compared to a study by Persichetti et al. (2018) (7.6%) who tested cats enrolled at the same veterinary practices. As we commented above about differences in *L. infantum* prevalence between the two studies, the younger age of cats of the previous study influenced results as FIV positivity is age related with higher prevalences found in older cats (Hosie et al., 2009). In the case of FIV, senior cats were 3.65 times more at risk to be FIV infected. This is because FIV is mainly trasmitted by aggressive interactions (biting) typically occurring between adult cats and the infection lasts for a lifetime (Hosie et al., 2009). Aggressive interactions occur more frequently between males so, as expected, we found a higher risk for FIV infection (OR= 2.624) in males (20% positivity) compared to females (11% positivity). Many clinical abormalities were significantly associated with FIV positivity with higher risk for respiratory signs (OR= 5.6), gastrointestinal signs (OR= 4.281), low BCS (OR= 3.481), poor MCS (OR= 2.783), and ophthalmic (OR= 2.545), skin (OR= 2.505), and oral (OR= 2.467) lesions. This means that the FIV positive cats were probably in an advanced stage of infection when immunodeficieny and opportunistic infections occur (Hosie et al., 2009). Dysproteinemia with increased TP (OR= 4.958) and GLOB (OR= 4.406) and reduced ALB (OR= 3.472) values was significantly associated with FIV infection. Hyperglobulinemia and polyclonal hypergammaglobulinemia are widely reported in cats affected by FIV (Sparkes et al., 1993; Hosie et al., 2009) as an aberrant polyclonal B-cell hyperactivity causes hypergammaglobulinemia because of production of antibodies against both virus-specific and non-virus specific antigens (Pennisi et al., 1994; Gleich and Hartmann, 2009). Albumin is a negative acute-phase protein (Chikazawa and Dunning, 2016), and hypoalbuminemia in these FIV positive cats can be the consequence of their concurrent infectious diseases or immune-mediated reactions. However, albumin homeostatis is complex and the serum albumin level is the end result of synthesis (involving hepatic, nutritional, and endocrine factors), catabolism, renal and

gastrointestinal excretions, and distribution, therefore other mechanisms were probably responsible for hypoalbuminemia (Conner, 2017).

Interestingly, *L. infantum* antibody (OR= 4.205) and overall positivity (OR= 3.84) were significantly associated with FIV positivity and most of the investigated risk factors were shared by the two pathogens. This retroviral infection is the most frequent immunesuppressive factor detected in cats affected by FeL and suspected to facilitate progressive *L. infantum* infection in cats (Pennisi and Persichetti, 2018). For the first time an association between FIV positivity and FeMV urine PCR positivity is reported.

In conclusion, we confirmed endemicity of FIV in investigated areas where it was reported for the first time in Italy in 1989 (Pennisi, 1989). Risk factors were typical for this retroviral infection with the most interesting the association with *L. infantum* because of the potential causative role for retroviral immunesuppression upon development of clinical FeL and the overlapping of some clinical features associated with both pathogens.

FCoV

Feline coronavirus infection is very common in cats with 40% infected cats reported (90% in multi-cat households). Natural infections are transient (~70%) and mostly asymptomatic but less frequently persistent infection occurs (~13%) (Tasker, 2018) and a proportion of FCoV-infected cats develops a severe immunemediated disease called feline infectious peritonitis (FIP) (Addie et al., 2009). In the present study we found a 18% FCoV prevalence (31/171) evaluated by blood PCR but none of these cats were clinically suspected of FIP after physical examination and a higher prevalence was found in cats from Messina province (OR= 3.036).

Systemic infection was considered a feature of FIP, but it is now known that it is not always associated with the development of the disease and can occur in healthy individuals (Fish et al., 2018). In fact nine healthy shelter cats were blood PCR positive (4.4% of tested population) and one had FCoV replicating in blood, but did not develop signs compatible with FIP within six months of testing. This percentage is however lower compared to fecal positivity of healthy cats but in any case FCoV blood PCR investigation has no diagnostic or prognostic value (Addie et al., 2009; Fish et al.,

2018). The significance of extraintestinal detection of FCoV is not fully understood as FCoV infection of monocytes was considered a key event in FIP pathogenesis.

Feline infectious peritonitis includes a wide spectrum of various clinical signs and diagnosis is definetly confirmed by histopathology/cytology of compatible lesions and the detection of FCoV in macrophages by immunofluorescent or immunohistochemical/imunocytochemical staining of biopsies or smears from effusions or cerebrospinal fluid (Hartmann et al., 2005). Diagnosis of FIP was not an objective of this study and it was not needed for clinical reasons in the investigated cats.

Among risk factors associated with FCoV positivity we did not find the most commonly reported, such as indoor lifestyle in multicat household. Surprisingly, outdoor cats (OR= 2.875) and cats from rural areas compared to urban (OR= 12.06) or suburban areas (OR= 5.96) were more frequently infected in this study as well as males (OR= 2.224). Males are often found more frequently affected by FIP but the same risk is not reported for FCoV infection (Addie et al., 2009).

Clinical findings most frequently associated to FCoV infection included signs compatible with a wasting chronic disease because of poor MCS (OR= 8.9) and low BCS (OR= 2.95). Additionally pale mucous membranes (OR=4.198), enlarged lymph nodes (P=2.344) and skin lesions (OR= 2.95) were found but no association was detected with investigated biochemical parameters and other pathogens under study.

In conclusion, FCoV viremia is frequent in cats admitted to the investigated primary practices as we found about one cat out of five examined was positive, but it is not associated with exposure to the other investigated pathogens.

FeLV

In the present study, a small number of cats was blood PCR positive to FeLV (2%, 5/262) and this precluded statistical analysis. This data confirms previous investigations performed in the same area by detection of p27 antigenemia (Pennisi e Bo, 1994; Persichetti et al., 2018). Particularly, Persichetti et al. (2018) found positive p27 antigenemia in 1% (2/197) of cats admitted to the same four practices we investigated in this study.

Interestingly, coinfections were detected in all these cats: *L. infantum* (n=2), FIV (n=2), *Leptospira* spp. (n=1) and FeMV (n=1) with one cat affected by both FeMV and FIV.

In conclusion FeLV infection is very rare in cats admitted to the study primary practices and confirmatory tests should be performed on positive samples because of the positive predictive value of any positive test when prevalence of pathogen is very low.

Coinfections

We found that coinfections were very frequent and the higher the number of pathogens tested, the more it was rare to dected cats with one single pathogen . Additionally, we have to consider that we could not include in this study the investigation of pathogens causing frequent chronic infections such as hemoplasmas and *Bartonella* spp. detected with a high prevalence by Persichetti et al. (2018) in cats admitted to the same primary care practices in Messina and Reggio Calabria provinces.

There is increasing interest in coinfections in dogs and cats and the severity of leishmaniosis was higher in dogs coinfected by other vecor-borne pathogens (Baxarias et al., 2018). However in some cases VB coinfections does not seem to worsen clinical manifestations of canine monocytic ehrlichiosis in dogs (Mylonakis et al., 2005b).

We have abundant information about coinfections in cats infected by retroviruses (Hosie et al., 2009; Lutz et al., 2009) and recently studies investigated vector-borne coinfections in cats including *L. infantum* infection in endemic areas (Maia et al., 2014; Attipa et al., 2017; Persichetti et al., 2018). However this is the first time that FeMV and *Leptospira* spp. were investigated with *L. infantum,* FIV and FeLV infections in cats and no data are available to make comparisons with previous studies.

We investigated 269 cats for *L. infantum* but a lower number of cats was tested for the other pathogens; with 191 tested for FeMV and 111 for *Leptospira* spp. This limitation reduced the number of cats evaluated for coinfections, however some interesting results were obtained. The significant association of *L. infantum* and FIV is debated (Pennisi et al., 2013; Pennisi and Persichetti, 2018) but it is confirmed in the present study. However, significant associations with FIV, *Leptospira* spp. and *L. infantum* was recorded for the first time in FeMV exposed cats and risk factors evaluation should

therefore be performed by multivariable analysis to have more robust information from data analysis.

CONCLUSIONS

This epidemiological study was based on detection of both antibodies against the emerging pathogens investigated and of the pathogen nucleic acids in blood and other tissues with the aim to increase diagnostic sensitivity. The prevalence of exposure was over 20% for most of them with 22%, 24% and 28% overall prevalence of *Leptospira* spp., *L. infantum* and FeMV respectively. The other viral pathogens investigated (FIV= 16% and FCoV= 18%) were frequently detected but with a lower prevalence. This means that few cats (32 in total) were negative to any pathogens and conversely coinfections with two to four pathogens were commonly found. Blood PCR positivities were obtained and it gives rise to the question of the risk for blood tranfusion transmission of all investigated pathogens.

Some pathogens were significantly associated with each other and multiple reasons for and consequences of this occurrence can be presumed but this observational controlled study cannot show causative relationships. Longitudinal controlled studies are needed to demonstrate a causative effect but such studies are very difficult to perform, not only because of the economic burden but also because owners are not readily compliant with long term follow up required to demonstrate associations of persistent infections with development of chronic diseases. Moreover, during follow up monitoring cats included in longitudinal studies are continuously exposed to infectious and non infectious disorders that can influence their clinical status and have to be carefully considered. These are the main reasons that make observational studies valuable, especially when they are performed on an appropriate number of cats and it is also useful that many studies are performed with similar methods to confirm the results obtained.

We think that the findings of this study are of value from a clinical point of view but also have a “One Health” relevance as *Leptospira* spp. and *L. infantum* are of zoonotic concern and we intend to make a more robust multivariate analysis of significant associations.

CHAPTER 5

CELL BLOOD COUNT AND INFLAMMATION MARKERS IN CATS EXPOSED TO SOME EMERGING PATHOGENS IN SOUTHERN ITALY

ABSTRACT

Several factors can determine complete blood count (CBC) abnormalities and changes in acute phase markers. The purposes of this study were to evaluate for the first time both CBC abnormalities and acute phase markers in a population of cats of Southern Italy exposed to some feline emerging pathogens and to detect risk factors for clinical findings and pathogen positivity. We studied the presence of anemia, leukocytes, platelets abnormalities, leukocyte alterations suggestive of inflammation (LAI), serum amyloid A (SAA), total gobulins increase (GLOB), albumin (ALB) decrease, reticulocyte haemoglobing content decrease, serum protein electrophoresis abnormalities. Several associations were found: outdoor husbandry was a risk factor for anemia (OR=3.089), monocytosis (OR=2.115) and neutrophilia (OR=2.707); adult age for anemia (OR=3.842), neutrophilia (OR=3.429), eosinophilia (OR=7.105), eosinopenia (OR=9) and increased total gobulins (OR=10.97); senior age for anemia (OR=4.667), neutrophilia (OR=4.488), eosinopenia (OR=8.1), increased total globulins (OR=18.87); Calabria origin (OR=3.706) and urban environment (OR=3.443) for thrombocytopenia; suburban environment for neutropenia (OR=15.32); reduced body condition score (BCS) for anemia (OR=6.682), neutrophilia (OR=6.914), lymphopenia (OR=3.596), monocytosis (OR=3.42), thrombocytosis (OR=4.094), LAI (OR=2.767), increased SAA (OR=4.444), decreased ALB (OR=6.583); poor muscle condition score (MCS) for anemia (OR=4.836), neutrophilia (OR=5.826), monocytosis (OR=3.404), thrombocytosis (OR=7.568), LAI (OR=3.112), increased SAA (OR=3.61); pale mucous membranes for anemia (OR=15.23), neutrophilia (OR=3.632), lymphopenia (OR=10), eosinopenia (OR=3.37), monocytosis (OR=5.515), thrombocytopenia (OR=4.542), LAI (OR=3.242), increased SAA (OR=7.966); gastrointestinal signs for neutrophilia (OR=3.694), lymphopenia (OR=4.54), monocytosis (OR=2.984) and LAI (OR=7.65); respiratory signs for neutrophilia (OR=3.577), monocytosis (OR=7.123), thrombocytosis (OR=4.947), LAI (OR=6.9); skin lesions for neutrophilia (OR=3.295),

monocytosis (OR=2.295); chronic kidney disease for anemia (OR=2.492), neutrophilia (OR=4.603); oral lesions for lymphopenia (OR=3.087), eosinopenia (OR=2.541); enlarged lymph nodes for neutrophilia (OR=2.069). Concerning pathogens investigated: *L. infantum* was a risk factor for anemia (OR=1.897), monocytosis (molecular positivity: OR=5.382, overall positivity: OR=2.308), thrombocytosis (molecular positivity: OR=9.65, overall positivity: OR=5.688), eosinopenia (OR=3.848), increased total globulins (OR=6.452); *Leptospira* spp. for neutrophilia (molecular positivity: OR=6.533, overall positivity: OR=3.974) and monocytosis (antibody positivity: OR=3.769, molecular positivity: OR=4.821, overall positivity: OR=4.267); feline morbillivirus for neutrophilia (OR=2.287), monocytosis (OR=2.614), thrombocytosis (OR=13.72), increased α-globulins (OR=5.067), increased total globulins (antibody positivity: OR=4.969, urine molecular positivity: OR=3.422, overall molecular positivity: OR=2.882, overall antibody and molecular positivity: OR=6.049); feline immunodeficiency virus for LAI (OR=3.15) and increased total globulins (OR=4.406).

The risk factors detected in the population under study for cat inflammatory conditions and CBC abnormalities should be carefully considered when cats are examined in veterinary pratice.

5.1 INTRODUCTION

Anemia and other cell blood count (CBC) abnormalities are commonly detected in small animal practice and arise from a number of different causes (Paltrinieri et al., 2010). Inflammation is one frequent cause for CBC abnormalities and some serum confirmatory markers are currently available in cats (Eckersall and Bell, 2010, von Roedern et al., 2017).

Anemia is defined as a decrease in hematocrit (HCT), red blood cell count (RBC) or hemoglobin concentrations (Hgb), due to haemorrhage, haemolysis, decreased bone marrow red blood cell production (Korman et al., 2013). Evaluation of mean corpuscolar volume (MCV), mean corpuscolar hemoglobin concentration (MCHC) and reticulocyte count provide information about pathomechanisms causing anemia (Furman et al., 2014) (table 5.a).

Table 5.a Pathomechanisms causing anemia

(Paltrinieri et al., 2010)

CLASSIFICATION OF ANEMIA	PATHOMECHANISMS
Hypochromic microcytic (↓MCV, ↓MCHC)	Iron deficiency in the advanced phase
Normochromic microcytic (↓MCV, n MCHC)	Chronic flogosis
Hypochromic normocytic (n MCV, ↓ MCHC)	Iron deficiency in the initial phase
Normochromic normocytic (n MCV, n MCHC)	Reduced erythrocyte production; Initial phase of anemia from reduced life cycle of erythrocytes; Iron deficiency in the initial phase
Normochromic macrocytic (↑ MCV, n MCHC)	FeLV anemia; Conservation artifact
Hypochromic macrocytic (↑ MCV, ↓ MCHC)	Anemia from reduced life cycle of erythrocytes; Vitamin B12 deficiency
Macro-Normo-Microcytic hyperchromic (↑/n/↑ MCV, ↑ MCHC)	Artifact (hemolysis, Heinz bodies)

Legenda: ↑=increased, ↓=decreased, n= normal

Changes in leukocyte (WBC) number are caused by alterations in bone marrow production, distribution between different pools and half-life in blood. Inflammatory and immune-mediated processes, ormonal stimulations, neoplastic and non-neoplastic bone marrow diseases cause and influence, even in combination, white cell populations count (table 5.b).

Table 5.b Pathomechanisms causing leukocytes count abnormalities

(Paltrinieri et al., 2010; Gough and Murphy, 2015)

LEUKOCYTES	PATHOMECHANISMS
NEUTROPHILIA	**Physiologic**
	digestion
	physical exercise
	pregnancy
	excitement
	Without left shift
	inflammatory leukogram (acute, chronic)
	corticosteroids (stress / hypercortisolism/iatrogenic)
	With regenerative left shift
	inflammatory acute leukogram
	With degenerative left shift
	severe acute inflammatory leukogram
	Paraneoplastic neutrophilia
	cytokine production by cancer cells
	Neoplastic neutrophilia
	myeloid leukemias of the granulocyte series
NEUTROPENIA	**Physiologic**
	normal in some animals
	Consumption neutropenia (with degenerative left shift)
	severe inflammatory process
	Reduced bone marrow production
	toxic / drugs
	acute viral infections
	cachexia
	myelodysplasia / neoplastic substitution / myeloftysis
	Destruction of neutrophils

	autoimmune/ immune-mediated disease
EOSINOPHILIA	**Tissue pathologies**
	phlogosis
	allergic disease
	parassitic diseases
	mast cell tumor
	Production of eosinopoietic cytokynes
	paraneoplastic eosinophilia
	hypereosinophilic syndrome
	Neoplastic eosinophilia
	eosinophilic leukemia
EOSINOPENIA	**Decreased medullary production**
	toxic / drugs
	myelodysplasia / neoplastic substitution / myeloftysis
	corticosteroids (stress / hypercortisolism/iatrogenic)
LYMPHOCYTOSIS	**Physiologic**
	catecholamines
	Increased production
	vaccinated
	chronic inflammation
	Neoplastic lymphocytosis
	acute/chronic lymphoid leukemia
LYMPHOPENIA	**Physiologic**
	young animals
	Sequestration and apoptosis
	corticosterois (stress, hyperadrenocorticism, iatrogenic)
	Reduced production
	acute viral infections;
	chemotherapy treatments;
	neoplastic replacement of lymphoid tissue in lymphoma
	peripheral loss
	chylous effusions
MONOCYTOSIS	chronic inflammation
	corticosterois (stress, hyperadrenocorticism, iatrogenic)
	tissue necrosis
	monocytic leukemia
BASOPHILIA	allergic disease
	basophilic leukemia

Reduced production, increased consumption or destruction, sequestration and severe bleeding are responsible for reduction of platelets (PL) numbers (Paltrinieri et al., 2010). Thrombocytosis is associated with inflammatory conditions, neoplastic (essential thrombocythemia), splenic contraction or splenectomy, action of corticosteroids, and iron deficiency (Hogan et al., 1999, Rizzo et al., 2007, Paltrinieri et al., 2010).

Serum amyloid A (SAA) is one of the major acute phase proteins (APP) in cats and its evaluation is useful to detect inflammation but increasing is found also in neoplastic diseases, diabetes mellitus, and hyperthyrodism (Tamamoto et al., 2013). Recently, reticulocyte haemoglobing content (CHr) has been proposed as a sensitive indicator of iron deficiency and inflammatory process in dogs (Melendez-Lazo et al., 2015) as already seen in humans. Evaluation of reticulocyte indices has been available since last years by some in-house veterinary laser hemocytometers, but we are aware of just one study about cat reticulocytes and evaluating CHr values to diagnose iron deficiency (Prins et al., 2009).

Increase in serum globulins (GLOB) is compatible with inflammatory or neoplastic disorders particularly when associated with antibody production, but a pseudo-increase is produced by dehydration (Paltrinieri et al., 2010). A serum proteins fractionation can be performed through electrophoresis which separates them on the basis of their molecular weight and electrical charge, allowing to separate proteins in four main fractions: albumin, α-globulins (increased during acute inflammation and hyperlipidemia), β-globulins (increased during acute and cronic inflammation and neoplasia such as multiple myeloma, lymphoma and some leukemias) , γ-globulins (increased in chronic antigenic stimulation and neoplastic disorders such as multiple myeloma, plasmacytoma, lymphomas and lymphoid leukemias) (Taylor et al., 2010, Paltrinieri et al., 2010).

Some infectious pathogens are widely reported to be responsible for CBC abnormalities such as feline immunodeficiency virus (FIV) (Hosie et al., 2009), feline leukemia virus (FeLV) (Lutz et al., 2009), feline coronavirus (FCoV) (Addie et al., 2009), but few information is available for feline emerging pathogens, such as *Leishmania infantum* (Pennisi et al., 2013a, Pennisi et al., 2015, Pennisi and Persichetti, 2018) or *Leptospira* spp. (Hartmann et al., 2013a, Schuller et al., 2015)

and even more limited are for a new feline paramyxovirus recently reported, feline morbillivirus (FeMV) (Sieg et al., 2019). Investigations on infectious causes of APP increases were performed in cats with FIP (Hazuchova et al., 2017), pyometra (Vilhena et al., 2018a), sepsis (Troìa et al., 2017), hemotropic mycoplasmas (Vilhena et al., 2018b) or *Hepatozoon felis* and *Babesia vogeli* (Vilhena et al., 2017) infections, or exposed to *Dirofilaria immitis* (Silvestre-Ferreira et al., 2017).

The aims of this study are to evaluate for the first time both CBC abnormalities and acute phase markers in a population of cats of Southern Italy exposed to some feline emerging pathogens and to detect risk factors for clinical findings and pathogen positivity.

5.2 MATERIALS AND METHODS

5.2b Cats, clinical examination and sampling - see chapter 2, section 2.1

5.2c Cell blood count, serum amyloid A, total globulins, albumin, and serum protein electrophoresis- see chapter 2, section 2.2

Presence of anemia was defined as the reduction of Hgb concentration <9.8 g/dL. Reticulocyte hemoglobin content was considered decreased when it was <13.2 pg. Anemia was considered regenerative (RA) or non regenerative (NRA) when reticulocyte count was, respectively, higher or ≤ 50.0 K/μL. Anemia severity was evaluated considering HCT values, and anemia was mild when HCT was ≥20%, moderate when HCT was 14-19.9%, severe when HCT was ≤13.9%. Anemia was categorized as macrocytic when MCV was >53.1 fL, normocytic when MCV was 35.9-53.1 fL, and microcytic when MCV was < 35.9 fL. Additionally, it was classified as normochromic when MCHC was 28.1-35.8 g/dL and hypochromic when MCHC was <28.1 g/dL.

Abnormalities related to WBC and PL counts were recorded as “increased” or “decreased” and all low PL counts as well as any “smart flag” messages reported by the analyzer about WBC or PL counts (i.e. the analyzer was not able to make the count or accuracy of the analyzer count was low) was respectively confirmed or settled by the microscopic examination of May Grünwald-Giemsa (MGG) stained blood smears. Blood smears were also examined for morfological alterations of cells but 183 smears

were considered technically assessable and therefore morfological observations as well as WBC differential counts, basophil counts, and thrombocytopenia did not concern all enrolled cats.

Stress leukogram was diagnosed when lymphopenia and/or eosinopenia were detected. Stress leukogram was however excluded when lymphocyte count was severe (<0.46 K/μL) and in case of neutropenia or monocytopenia.

We examined as markers of inflammation the following abnormalities: leukocyte alterations suggestive of inflammation (LAI) (i.e. neutrophil left shift, toxic neutrophils or reactive lymphocytes), increased total globulins (GLOB) (evaluated by spectrophotometric method after evaluation of the difference between TP and ALB) and serum amyloid A (SAA), and decreased albumin (ALB) values. Hypoalbuminemia was included in statistical analysis when urea (BUN), urine protein to creatinine ratio (UPC) were within the normal interval and cats were not affected by diarrhea in order to exclude a possibile decrease of ALB values due to renal, hepatic or gastrointestinal problems. Fractionated globulins were evaluated through serum protein electrophoresis (SPE) and reference intervals are described in chapter 2 (table 2.b). Abnormalities related to their values were recorded as "increased" or "decreased".

5.2e Antibody detection- see chapter 2, section 2.3

5.2f DNA extraction and *Leishmania* Real-Time PCR- see chapter 2, section 2.4

5.2g RNA extraction and PCR for FeMV, FIV, FeLV, FCoV- see chapter 2, section 2.5

5.2h DNA extraction and PCR for *Leptospira* spp.- see chapter 2, section 2.7

5.2i CKD diagnosis and staging- see chapter 3, section 3.2d

5.2l Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.00. Data about clinical history and physical examination findings were analyzed using descriptive statistics. Statistical differences were tested by Chi-square test or Fisher exact test, examining categorical variables potentially associated with abnormalities in CBC or markers of inflammation considered.

Statistical elaboration concerned overall 22 variables including geographic region (Calabria/Sicilia), demographic (male/female; domestic shorthair/domestic long-

hair/pedigree; junior/adult/senior), husbandry and environment characteristics (indoor/ outdoor; indoor single-cat/indoor multi-cat household; rural/sub-urban/urban), reason for consultation, signs detected at physical examination (body condition score (BCS), muscle condition score (MCS), mucous membranes color, lymph nodes enlargment, ocular, oral, dermatological lesions, gastrointestinal and respiratory signs), positivity to *L. infantum*, FeMV, *Leptospira* spp., FIV, FeLV, and FCoV tests.

Positivity to the pathogens was evaluated as follows:

L. infantum:

- Serological: antibody detection by IFI and titre ≥80
- Molecular: DNA detected by PCR in at least one of the examined samples
- Overall: positivity to serological and/or molecular investigations

Leptospira spp.:

- Serological: antibody detection by MAT and titre ≥20
- Molecular: DNA detected by PCR in at least one of the examined samples
- Overall: positivity to serological and/or molecular investigations

FeMV:

- Serological: antibody detection by IFI and titre ≥40
- Molecular: RNA detected by PCR in urine
- Overall molecular: RNA detected by PCR in urine and/or EDTA blood
- Overall: positivity to serological and/or molecular investigations

FIV:

- Overall: antibody detection (ELISA test) and/or RNA detected by PCR in EDTA blood

FCoV:

- Molecular: RNA detected by PCR in EDTA blood.

Statistical analysis of β-globulins values and basophil counts were not performed. In fact, few cats had normal β-globulin (beta1=16, beta2=8) values, increased β1 or β2 globulin values were found in almost all tested sera (74/75) and reduced β-globulin values were never found. Conversely, basophilia was found in only three cats. In case

of γ-globulins, decreased values were not found, therefore analysis concerned just their increases.

Differences were considered significant if P value was ≤0.05 and odds ratios (OR), 95% C.I. (confidence interval) and significance were reported. In case of null values statistical comparisons were not performed.

In each table, statistical analysis and percentages of overall positivity of emerging pathogens are always represented. Statistical analysis and percentages of antibody or molecular positivity (individually evaluated) are reported if a significant difference was found.

5.3 RESULTS

5.3a Cats data

Between October 2016 and April 2019, 269 were evaluated and blood samples were collected. Blood smears were evaluated in 183 cats.

Reasons for consultations were classified into four main causes and their frequency is reported in table 5.c. Medical problems causing consultation included dermatological (n= 30) and respiratory (n=19) disorders, weakness (n=17), gastrointestinal signs (n= 16), CKD (n= 13), ocular problems (n= 13), weight loss (n= 8), lower urinary tract disease (n= 8), neurological (n= 5) or endocrine disorders (n= 2), poisonings (n= 3), polyuria/polydpsia (n= 2). More than one of these problems was reported in some cats. Surgery included osteosynthesis (n= 4), limb amputation (n= 3), dental extraction (n=1), rectal prolapse correction (n=1).

Table 5.c Main reason for consultation

REASON FOR CONSULTATION	CATS (%)
Routine health check	71 (26)
Elective surgery	65 (24)
Medical problems	124 (46)
Minor surgeries or orthopedic problems	9 (4)

Demographic data of cats and information about their husbandry, environment and region of origin are summerized in table 5.d. Pedigree cats were only 17 with Maine Coon (n= 10), Persian (n= 3), Ragdoll (n= 1), British Short Hair (n= 1), and Chartreux (n= 2) breeds represented. The 33% of indoor cats (40/120) were from a single-cat household and the 67% (80/120) were from a multi-cat household.

Table 5.d Demographic, husbandry, environmental and geographic data analyzed

VARIABLE	CATS (%)
AGE	
Junior	49 (18)
Adult	149 (56)
Senior	71 (26)
SEX	
Female	129 (48)
Male	140 (52)
BREED	
DSH	226 (84)
DLH	26 (10)
Pure-Breed	17 (6)
HUSBANDRY	
Indoor	120 (45)
Otdoor	149 (55)
ENVIRONMENT	
Urban	154 (66)
Suburban	59 (25)
Rural	21 (9)
Missing data	35
ORIGIN	
Sicily	83 (31)
Calabria	186 (69)

Clinical findings observed during physical examination are described in table 5.e

Table 5.e Physical examination data

VARIABLE	CATS (%)
BCS	
≥3	219 (82)
<3	47 (18)
Missing data	3
MCS	
1	173 (76)
>1	54 (24)
Missing data	42
MUCOUS MEMBRANES	
Normal	238 (89)
Pale	25 (9)
Jaundice	3 (1)
Congested	1 (1)
Missing data	2
LYMPH NODES	
Normal	184 (69)
Enlarged	82 (31)
Missing data	3
OCULAR LESIONS	
Absent	247 (93)
Present	19 (7)
Missing data	3
ORAL LESIONS	
Absent	173 (65)
Present	93 (35)
Missing data	3
RESPIRATORY SIGNS	
Absent	241 (91)
Present	25 (9)
Missing data	3
GI SIGNS	
Absent	248 (93)
Present	18 (7)
Missing data	3

SKIN LESIONS	
Absent	222 (83)
Present	44 (17)
Missing data	3

The observed ocular lesions were conjunctivitis (n= 16/19) and keratoconjunctivitis (n=4/19). Oral lesions consisted in stomatitis (n=46/93), periodontitis (n= 25/93), dental calculus (n= 19/93), ulcers (n= 10/93), drooling (n= 9/93), faucitis (n= 7/93), missing teeth (n= 5/93), bleeding (n= 1/93), cleft palate (n= 1/93), neoplasia (n= 1/93) and some cats had more than one of these abnormalities. Respiratory signs were related to upper (n= 16/25) and lower respiratory tract (n= 7/25) or both (n=2). Gastrointestinal signs included diarrhea (n= 13/18), vomit (n= 4/18), constipation (n=1/18). Abdomen examination revealed hepatomegaly (n=8), effusion (n=2), pain on palpation (n= 3) or splenomegaly (n= 1). Skin lesions were alopecia (n= 27/44), crusty dermatitis (n= 14/44), ulcers (n= 6/44), abscess (n= 5/44), nodular dermatitis (n= 5/44), erythema (n= 3/44), squamous dermatitis (n=1/44), pyoderma (n=1/44). Some cats had more than one of these lesions.

5.3b Anemia

Blood cell count was performed in 269 cats and anemia was detected in 86 of them (32%): 68 (79%) cats had a mild anemia, 14 (16%) cats a moderate anemia and four (5%) cats a severe anemia. Regenerative anemia was observed in seven (8%) cats, while the others had a non regenerative anemia (92%). Anemia was classified as microcytic in 29 cats (34%), normocytic in 56 cats (65%), macrocytic in one cat (1%). Normochromic anemia was seen in 57 cats (66%) and hypochromic in 4 cats (5%). In 25 cats MCHC was increased therefore classification of anemia was not possible considering that this is due to artifacts (e.g. hemolysis or lipemia). Anemia classification considering regeneration, eritrocytic indices and severity are described in table 5.f

Table 5.f Classification of anemia according to regeneration, eritrocytic indices and severity

CLASSIFICATION	CATS (%)
REGENERATIVE ANEMIA	7 (8)
SEVERITY	
Mild	4 (57)
Moderate	2 (29)
Severe	1 (14)
ERITROCYTIC INDICES	
Normocytic normochromic	3 (43)
Normocytic*	2 (29)
Macrocytic hypochromic	1 (14)
Microcytic normochromic	1 (14)
NON REGENERATIVE ANEMIA	79 (92)
SEVERITY	
Mild	64 (81)
Moderate	12 (15)
Severe	3 (4)
ERITROCYTIC INDICES	
Normocytic normochromic	38 (48)
Normocytic hypochromic	3 (4)
Normocytic*	10 (13)
Microcytic normochromic	15 (19)
Microcytic*	13 (16)

Legenda: *= increased MCHC value

Erythrocytes morfological alterations were observed in 18 cats. Type and frequence of erythrocytes morfological alterations in anemic and non anemic cats is described in table 5.g.

Table 5.g Erythrocytes morfological alterations in anemic and non anemic cats

	ANEMIC	NON ANEMIC
ACANTHOCYTES	2	1
SCHISTOCYTES AND KERATOCYTES	1	1
HEINZ BODIES	0	1

HOWELL JOLLY BODIES	0	1
ERYTHROBLASTS	6	4
INCLUSION BODIES COMPATIBLE WITH HEMOPLASMAS	0	1

A higher prevalence of anemia was found in adult (P=0.0021, OR= 3.842, 95% CI= 1.597-9.113) or senior (P= 0.0017, OR= 4.667, 95% CI= 1.813-12.07) cats compared to junior cats, and outdoor cats (P < 0.0001, OR= 3.089, 95% CI= 1.741-5.485) compared to indoors. Demographic, husbandry, environmental and geographic data analyzed as risk factors for anemia are described 5.h

Table 5.h Demographic, husbandry, environmental and geographic data analyzed as risk factors for anemia

VARIABLE	**ANEMIA (%)**	
ORIGIN	**PRESENT**	**ABSENT**
Sicily	23 (27)	60 (33)
Calabria	63 (73)	123 (67)
SEX		
Female	43 (50)	86 (47)
Male	43 (50)	97 (53)
BREED		
DSH and or/DLH	84 (98)	168 (92)
Pure-Breed	2 (2)	15 (8)
AGE*		
Junior	6 (7)	43 (23)
Adult	52 (61)*	97 (53)
Senior	28 (32)*	43 (24)
HUSBANDRY*		
Indoor	23 (27)	97 (53)
Outdoor	63 (73%)*	86 (47%)
ENVIRONMENT		
Urban	54 (73)	100 (62)
Suburban	14 (19)	45 (29)
Rural	6 (8)	15 (9)
Missing data	12	23

Legenda: *= significant difference

Prevalence of anemia was significantly higher in cats with reduced BCS (P < 0.0001, OR= 6.682, 95% CI= 3.345-13.13) and poor MCS (P < 0.0001, OR= 4.836, 95% CI= 2-553-9.041), pale mucous membranes (P < 0.0001, OR= 15.23, 95 % CI= 5.119-41.97), CKD (P=0.0031, OR= 2.492, 95% CI= 1.37-4.598). Moreover, prevalence of moderate/severe anemia was higher in cats with low BCS (P=0.004, OR= 5.76, 95% CI= 1.88-16.29), pale mucous membranes (P <0.0001; OR=25.78; 95%CI= 5.603-92.5) and a high prevalence of normocytic anemia was observed in cats with low BCS (P= 0.0328, OR=3.178, 95% CI=1.165-9.119) (table 5.k).

Clinical variables analyzed as risk factors for anemia are described in table 5.i

Table 5.i Clinical variables analyzed as risk factors for anemia

VARIABLE	**ANEMIA (%)**	
BCS*	**PRESENT**	**ABSENT**
≥3	53 (62)	166 (92)
<3	32 (38)*	15 (8)
Missing data	1	2
MCS*		
1	40 (56)	133 (86)
>1	32 (44)*	22 (14)
Missing data	14	28
MUCOUS MEMBRANES*		
Normal	61 (72)	177 (97)
Pale	21 (25)*	4 (2)
Jaundiced	3 (3)	0
Cogested	0	1 (1)
Missing data	1	1
LYMPH NODES		
Normal	53 (63)	131 (72)
Enlarged	31 (37)	51 (28)
Missing data	2	1
OCULAR LESIONS		
Absent	75 (88)	172 (95)
Present	10 (12)	9 (5)
Missing data	1	2
ORAL LESIONS		

Absent	53 (62)	120 (66)
Present	32 (38)	61 (34)
Missing data	1	2
GI SIGNS		
Absent	76 (89)	172 (95)
Present	9 (11)	9 (5)
Missing data	1	2
RESPIRATORY SIGNS		
Absent	75 (88)	166 (92)
Present	10 (12)	15 (8)
Missing data	1	2
SKIN SIGNS		
Absent	67 (79)	155 (86)
Present	18 (21)	26 (14)
Missing data	1	2
CKD *		
Absent	22 (33)	81 (55)
Present	44 (67)*	65 (35)
Missing data	20	37

Legenda: *= significant difference, GI= gatrointestinal signs

A significantly higher prevalence of anemia was detected in cats with *L. infantum* overall antibody and molecular positivity (P= 0.0406, OR= 1.897, 95% CI= 1.022-3.429) with a higher prevalence of moderate/severe anemia (38%) (P= 0.0213, OR= 3.75, 95% CI=1.279-10.25) compared to negative cats (14%) (table 5.k).
Infectious pathogens analyzed as risk factors for anemia are described in table 5.j

Table 5.j Infectious pathogens analyzed as risk factors for anemia

VARIABLE	**ANEMIA (%)**	
	PRESENT	**ABSENT**
***L. infantum* (IFI and/or PCR) ***	**Tot 260**	
Negative	56 (68)	143 (80)
Positive	26 (32)*	35 (20)
FeMV (IFI and/or PCR)	**Tot 189**	
Negative	40 (64)	96 (76)
Positive	22 (36)	31 (24)

***Leptospira spp.* (MAT and/or PCR)**	**Tot 111**	
Negative	20 (69)	66 (80)
Positive	9 (31)	16 (20)
***FIV* (ELISA and/or PCR)**	**Tot 258**	
Negative	68 (82)	149 (85)
Positive	15 (18)	26 (15)
FCoV (PCR)	**Tot 170**	
Negative	43 (75)	96 (85)
Positive	14 (25)	17 (15)

Legenda: *= significant difference

Table 5.k Diagnostic characteristics of anemia according to investigated risk factors

VARIABLE	**DEGREE**		**REGENERATION**		**MCV**			**MCHC**	
AGE	**MILD**	**MOD./SEV.**	**RA**	**NRA**	**MICRO.**	**NORM.**	**MACRO.**	**IPO.**	**NORM.**
Junior	5 (7)	1 (6)	0	6(8)	3(10)	3(5)	0	0	3(5)
Adult/Senior	63(93)	17 (94)	7	73(92)	26(90)	53(95)	1	4	54(95)
HUSBANDRY									
Indoor	18(26)	5 (28)	1(14)	22(28)	9(31)	14(25)	0	1(25)	13(23)
Outdoor	50(74)	13 (72)	6(86)	57(72)	20(69)	42(75)	1	3(75)	44(77)
BCS*									
≥3	48(71)	5 (29)	3(43)	50(64)	22(79)	30(54)	1	3(75)	34(60)
<3	20(29)	12 (71)*	4(57)	28(36)	6(21)	26(46)*	0	1(25)	23(40)
Missing data	0	1	0	1	1	0	0	0	0
MCS									
1	35(61)	5 (33)	3(43)	37(57)	16(70)	23(48)	1	3	24(52)
>1	22(39)	10 (67)	4(57)	28(43)	7(30)	25(52)	0	0	22(48)
Missing data	11	3	0	14	6	8	0	1	11
MUCOUS MEMBRANES									
Normal	58(87)	3(20)	4(67)	57(75)	21(78)	39(72)	1	4	42
Pale	9(13)	12(80)*	2(33)	19(25)	6(22)	15(28)	0	14	0
Missing data	1	3	1	2	2	2	0	0	0
GROUP									
Health/Other	18(35)	4(27)	2(40)	20(33)	6(29)	16(36)	0	2(67)	16(36)

CKD	33(65)	11(73)	3(60)	41(67)	15(71)	28(64)	1	1(33)	29(64)
Missing data	17	3	2	18	8	12	0	1	2
***L. infantum* (IFI and/or PCR) ***									
Negative	48(75)	8(44)	5(71)	51(68)	23(79)	32(62)	1	3(75)	35(64)
Positive	16(25)	10 (56)*	2(29)	24(32)	6(21)	20(38)	0	1(25)	20(36)
Missing data	4	0	0	4	0	6	0	0	2

5.3c Leukocytes

Leukocytes estimation was evaluated in 240 cats and distribution is listed in table 5.l

Table 5.l Cats with increased or decreased leukocyte counts

LEUKOCYTES COUNT	NORMAL (%)	INCREASED (%)	REDUCED (%)
NEUTROPHILS	165 (68)	64 (27)	11 (5)
LYMPHOCYTES	211 (88)	14 (6)	15 (6)
MONOCYTES	194 (81)	43 (18)	3 (1)
EOSINOPHILS	190 (79)	21 (9)	29 (12)
BASOPHILS	214 (89)	3 (1)	23 (10)

Demographic, husbandry, environmental and geographic data analyzed as risk factors for neutrophils, lymphocytes, eosinophils and monocytes abnormalities are described in table 5.m

Prevalence of neutrophilia was higher in adult (P=0.0145, OR=3.429, 95% CI= 1.334-8.525) and senior (P=0.0038, OR=4.488, 95% CI= 1.525-11.55) cats compared to junior cats, as well as in outdoor cats (P=0.0018, OR=2.707, 95% CI= 1.473-4.877) compared to indoors. Prevalence of neutropenia was higher in cats living in suburban area (P <0.0001, OR= 15.32, 95% CI=5.106-43.23) compared to those living in urban area.

Prevalence of monocytosis was higher in outdoor cats (P= 0.0423, OR= 2.115, 95%CI= 1.038-4.304) compared to indoors.

Prevalence of eosinophilia was higher in adult cats (P= 0.0402, OR= 7.105, 95% CI=1.102-76.67) compared to junior cats.

Prevalence of eosinopenia was higher in males (P=0.0092, OR= 3.108, 95% CI= 1.312-6.979) compared to females, in adult (P=0.0150, OR= 9, 95% CI= 1.479-95.94) and senior (P=0.0401, OR= 8.1, 95% CI= 1.202-90.65) cats compared to junior cats.

Table 5.m Demographic, husbandry, environmental and geographic data analyzed as risk factors for neutrophil, lymphocyte, eosinophil and monocyte count abnormalities

VARIABLE	↑ NEU (%)	↓NEU (%)	nNEU (%)	↑ LYM (%)	↓ LYM (%)	nLYM (%)	↑ MON (%)	nMON (%)	↑ EOS (%)	↓EOS (%)	nEOS (%)
ORIGIN											
Siciliy	20 (31)	5 (45)	51 (31)	4 (29)	5 (33)	67 (32)	11 (25)	65(34)	5 (24)	8 (28)	63(33)
Calabria	44 (69)	6 (55)	114 (69)	10 (71)	10 (67)	144(68)	32 (75)	129(66)	16 (76)	21 (72)	127(67)
AGE*											
Junior	5 (8)	2 (18)	40 (24)	3 (21)	1 (7)	43 (20)	10 (23)	37 (19)	1 (5)	1 (3)	45 (24)
Adult	36 (56)*	9 (82)	84 (51)	8 (58)	9 (60)	112(53)	23 (54)	104(54)	15 71)*	19 (66)*	95 (50)
Senior	23 (36)*	0	41 (25)	3 (21)	5 (33)	56 (27)	10 (23)	53 (27)	5 (24)	9 (31)*	50 (26)
SEX*											
Male	33 (52)	5 (45)	78 (47)	5 (36)	10 (67)	101(48)	21 (49)	92 (47)	8 (38)	21 (72)*	87 (46)
Female	31 (48)	6 (55)	87 (53)	9 (64)	5 (33)	110(52)	22 (51)	102(53)	13 (62)	8 (28)	103(54)
BREED											
DSH/DLH	61 (95)	9 (82)	153 (93)	13 (93)	12 (80)	198(94)	43	177(91)	20 (95)	27 (93)	176(93)
Pure- Breed	3 (5)	2 (18)	12 (7)	1 (7)	3 (20)	13 (6)	0	17 (9)	1 (5)	2 (7)	14 (7)
HUSBANDRY*											
Indoor	19 (30)	5 (45)	88 (53)	6 (43)	4 (27)	102(48)	14 (33)	98 (51)	5 (24)	12 (41)	95 (50)
Outdoor	45 (70)*	6 (55)	77 (47)	8 (57)	11 (73)	109(52)	29 (67)*	96 (49)	16 (76)	17 (59)	95 (50)
HOUEHOLDS											
Single-cat	6 (32)	2 (40)	29 (33)	3 (50)	3 (75)	31 (30)	5 (36)	32 (33)	2 (40)	5 (42)	30 (32)

Multi-cat	13 (68)	3 (60)	59 (67)	3 (50)	1 (25)	71 (70)	9 (64)	66 (67)	3 (60)	7 (58)	65 (68)
HABITAT*											
Urban	35 (60)	4 (44)	100 (70)	8 (73)	10 (84)	121(65)	25 (64)	114(67)	12 (75)	18 (69)	109(65)
Suburban	19 (33)	5 (56)*	31 (22)	2 (18)	1 (8)	52 (28)	12 (31)	43 (25)	3 (19)	7 (27)	45 (27)
Rural	4 (7)	0	11 (8)	1 (9)	1 (8)	13 (7)	2 (5)	13 (8)	1 (6)	1 (4)	13 (8)
Missing data	6	2	23	3	3	25	4	24	5	3	23

Legenda: *=significant difference; n= normal count; ↑=increased count, ↓=decreased count

Clinical variables evaluated as risk factors for neutrophils, lymphocytes, eosinophils and monocytes abnormalities are described in table 5.n

Prevalence of neutrophilia was significantly higher in cats with reduced BCS (P< 0.0001, OR=6.914, 95% CI= 3.525-13.84) and poor muscle mass (P< 0.0001, OR=5.826, 95% CI= 2.822-11.88), pale mucous membranes (P= 0.0117, OR=3.632, 95% CI= 1.292-9.631), enlarged lymph nodes (P= 0.0212, OR=2.069, 95% CI= 1.141-3.761), respiratory signs (P= 0.0057, OR=3.577, 95% CI= 1.532-8.879), gastrointestinal signs (P=0.0172,OR= 3.694,95%CI= 1.37-9.61), dermatological lesions (P= 0.0015, OR=3.295, 95% CI= 1.593-6.838) and CKD (P<0.001, OR=4.603, 95%CI=2.14-9.82).

Prevalence of lymphopenia was higher in cats with reduced BCS (P= 0.0273, OR= 3.596, 95% CI=1.164-10.56), pale mucous membranes (P= 0.0005, OR= 10, 95% CI=2.836-29.12), oral lesions (P= 0.0467, OR= 3.087, 95% CI=1.097-9.052), and a tendency to significance was found for gastrointestinal signs (P= 0.055, OR= 4.54, 95% CI=1.21-16.39).

Prevalence of monocytosis was higher in cats with reduced BCS (P= 0.0024, OR= 3.42, 95%CI= 1.636-7.212) and poor muscle mass (P= 0.0021, OR= 3.404, 95%CI= 1.557-7.122), pale mucous membranes (P= 0.0012, OR= 5.515, 95%CI= 2.127-14.15), gastrointestinal signs (P=0.0483, OR= 2.98, 95%CI= 1-8.09), repiratory signs (P <0.0001, OR= 7.123, 95%CI= 2.909-18.55), skin lesions (P= 0.042, OR= 2.295, 95%CI= 1.035-4.817).

Prevalence of eosinopenia was higher in cats with pale mucouse membranes (P=0.0299, OR= 3.37, 95% CI=1.152-9.669) and oral lesions (P= 0.0225, OR= 2.541, 95% CI= 1.115-5.556).

table 5.n Clinical variables evaluated as risk factors for neutrophil, lymphocyte, eosinophil and monocyte count abnormalities

VARIABLE	↑ NEU (%)	↓NEU (%)	nNEU (%)	↑ LYM (%)	↓ LYM (%)	nLYM (%)	↑MON (%)	nMON (%)	↑ EOS (%)	↓EOS (%)	nEOS (%)
BCS*											
≥3	39 (61)	10 (91)	151(92)	13 (93)	9 (60)	178(84)	29(67)	170(88)	18 (86)	20 (69)	162(85)
<3	25 (39)*	1 (9)	14 (8)	1 (7)	6 (40)*	33 (16)	14(33)*	24 (12)	3 (14)	9 (31)	28 (15)
MCS*											
1	30 (54)	9	121(87)	9 (90)	7 (58)	144(79)	23 (59)	137(83)	14 (93)	15 (63)	131(79)
>1	26 (46)*	0	18 (13)	1 (10)	5 (42)	38 (21)	16(41)*	28 (17)	1 (7)	9 (37)	34 (21)
Missing data	8	2	26	4	3	29	4	29	6	5	25
MUCOUS MEMBRANES*											
Pale	10 (16)*	2 (18)	8 (5)	1 (7)	6 (40)*	13 (6)	9 (21)*	9 (4)	0	6 (21)*	14 (7)
Normal	53 (83)	9 (82)	154(93)	12 (86)	9 (60)	195(92)	33 (77)	182(94)	21	22 (76)	173(91)
Jaundice	1 (1)	0	2 (1)	1 (7)	0	2 (1)	1 (2)	2 (1)	0	1 (3)	2 (1)
Congested	0	0	1 (1)	0	0	1 (1)	0	1 (1)	0	0	1 (1)
LYMPH NODES *											
Enlarged	23 (36)*	3 (27)	48 (29)	3 (21)	2 (13)	69 (33)	13 (30)	60 (31)	8 (38)	11 (38)	55 (29)
Normal	41 (64)	8 (73)	117(71)	11 (79)	13 (87)	142(67)	30 (70)	134(69)	13 (62)	18 (62)	135(71)
GI SIGNS*											
Present	9 (14)*	0	7 (4)	2 (14)	3 (20)*	11 (5)	6 (14)*	10 (5)	1 (5)	1 (3)	14 (7)
Absent	55 (86)	11	158(96)	12 (86)	12 (80)	200(95)	37 (86)	184(95)	20 (95)	28 (97)	176(93)

RESPIRATORY SIGNS*											
Present	12 (19)*	0	10 (6)	2 (14)	2 (13)	18 (9)	12(28)*	10 (5)	4 (19)	5 (17)	13 (7)
Absent	52 (81)	11	155(94)	12 (86)	13 (87)	193(91)	31 (72)	184(95)	17 (81)	24 (83)	177(93)
ORAL LESIONS*											
Present	29 (45)	2 (18)	52 (32)	5 (36)	9 (60)*	69 (33)	17 (40)	64 (33)	5 (24)	16(55)*	62 (33)
Absent	35 (55)	9 (82)	113(68)	9 (64)	6 (40)	142(67)	26 (60)	130(67)	16 (76)	13 (45)	128(67)
SKIN LESIONS*											
Present	20 (31)*	1 (9)	20 (12)	1 (7)	3 (20)	37 (18)	12(28)*	28 (14)	2 (10)	6 (21)	33 (17)
Absent	44 (69)	10 (91)	145(88)	13 (93)	12 (80)	174(82)	31 (72)	166(86)	19 (90)	23 (79)	157(83)
GROUP*											
Healthy/Other	21 (42)	5 (56)	70 (53)	6 (67)	3 (23)	87 (52)	17 (50)	78 (51)	9 (69)	9 (38)	78 (51)
CKD	29 (58)*	4 (44)	61 (47)	3 (33)	10 (77)	81 (48)	17 (50)	76 (39)	4 (31)	15 (62)	75 (49)
Missing data	14	2	34	5	2	43	9	40	8	5	37

Legenda: *=significant difference; n= normal count;↑=increased count; ↓=decreased count

Infectious pathogens evaluated as risk factors for development of neutrophils, lymphocytes, eosinophils and monocytes abnormalities are described in table 5.o

We found a higher prevalence of neutrophilia in antibody and/or molecular FeMV positive cats (P=0.0366, OR= 2.287, 95% CI= 1.137-4.616). In case of *Leptospira* spp. association of neutrophilia was for both overall urine/blood PCR (P=0.0047, OR= 6.533, 95% CI= 1.822-21.46) and overall antibody and/or molecular positivity (P=0.013, OR= 3.974, 95% CI= 1.474-10.64).

A higher prevalence of monocytosis was found in any of the three pathogens. In case of *L. infantum* association was with both PCR (P=0.0025, OR= 5.382, 95% CI=1.824-15.72) and overall positivity (P=0.038, OR= 2.308, 95% CI=1.092-4.809), *Leptospira* spp. positive cats both with MAT (P=0.0319, OR= 3.769, 95% CI= 1.061-11.48), overall urine/blood PCR (P= 0.024, OR= 4.821, 95%CI=1.392-18.18) and overall antibody and/or molecular diagnosis (P= 0.0155, OR= 4.267, 95%CI= 1.385-13.47), FeMV PCR positivity (P= 0.0329, OR= 2.614, 95%CI= 1.086-5.98).

A higher prevalence of eosinopenia was found among *L. infantum* PCR positive cats (P=0.0309, OR= 3.848, 95%CI= 1.357-11.91).

Table 5.o Infectious pathogens evaluated as risk factors for development of neutrophil, lymphocyte, eosinophil and monocyte count abnormalities

VARIABLE	↑ NEU (%)	↓ NEU (%)	nNEU (%)	↑LYM (%)	↓LYM (%)	nLYM (%)	↑MON (%)	nMON (%)	↑ EOS (%)	↓ EOS (%)	nEOS (%)
L. infantum* PCR											
Positive	6 (10)	0	11 (7)	1 (7)	2 (14)	14 (7)	8 (19) *	8 (4)	2 (10)	5 (18)*	10 (5)
Negative	54 (90)	11	154(93)	13 (93)	12 (86)	194(93)	34(81)	183(96)	19 (90)	23 (82)	177(95)
Missing data	4	0	0	0	1	13	1	3	0	1	3
***L. infantum* PCR/ IFI**											
Positive	15 (25)	1 (9)	38 (23)	2 (15)	3 (21)	49 (24)	15(37)*	38 (20)	6 (29)	11 (39)	37 (20)
Negative	45 (75)	10 (91)	125(77)	11 (85)	11 (79)	158(76)	26 (63)	152(80)	15 (71)	17 (61)	148(80)
Missing data	4	0	2	1	1	4	2	4	0	1	5
Leptospira* spp. PCR/MAT											
Positive	10 (45)*	0	13 (17)	1 (20)	1 (33)	21 (22)	8 (47)*	15 (17)	1 (25)	2 (18)	20 (22)
Negative	12 (55)	7	62 (83)	4 (80)	2 (67)	75 (78)	9 (53)	72 (83)	3 (75)	9 (82)	69 (78)
Missing data	42	4	90	9	12	115	26	107	17	18	101
FeMV* blood and/or urine PCR											

Positive	12 (24)	1 (11)	19 (14)	1 (10)	3 (27)	28 (16)	10 (29)	22 (14)	1 (8)	5 (22)	26 (16)
Negative	38 (76)	8 (89)	116(86)	9 (90)	8 (73)	144(84)	24 (71)	138(86)	12 (92)	18 (78)	132(84)
Missing data	14	2	30	4	4	39	9	34	8	6	32
FeMV											
PCR/IFI*											
Positive	19 (41)*	0	28 (24)	2 (22)	3 (27)	42 (28)	13 (39)	34 (24)	2 (17)	5 (26)	40 (28)
Negative	27 (59)	7	91 (76)	7 (78)	8 (73)	110(72)	20 (61)	105(76)	10 (83)	14 (74)	101(72)
Missing data	18	0	46	5	4	59	9	55	9	10	49
FIV											
Positive	13 (21)	3 (30)	17 (11)	0	5 (33)	28 (14)	9 (21)	24 (13)	0	8 (28)	25 (14)
Negative	50 (79)	7 (70)	143(89)	13	10 (67)	177(86)	34 (79)	163(87)	21	21 (72)	158(86)
Missing data	1	1	5	1	0	6	0	7	0	0	7
FCoV											
Positive	6 (14)	0	18 (17)	1 (17)	0	23 (17)	5 (17)	19 (15)	1 (10)	1 (5)	22 (18)
Negative	37 (86)	4	87 (83)	5 (83)	10	113(83)	24 (83)	104(85)	9 (90)	18 (95)	101(82)
Missing data	21	7	60	8	5	75	14	71	11	10	67

Legenda: *=significant difference, n=normal count, ↑=increased count, ↓=decreased count

After examination of MGG-stained blood smears, some morfological alterations of leukocytes were found: neutrophils right shift (n=1), band neutrophils (n=25), myelocytes and metamyelocytes (n=1), cytoplasmic toxicity of neutrophils (n=20), reactive lymphocytes (n=10), monocytes cytoplasmic foaming (n=12), neutrophil cytoplasmic inclusion body morula-like (n=1), monocyte cytoplasmic inclusion body morula-like (n=1), band eosinophils and metamyelocytes and myelocytes (n=1).
Stress leukogram was investigated in 240 cats and found in 33 cats.

5.3d Platelets

Platelet count was evaluated in 250 cats (209 normal, 9 increased, 32 decreased).
Significantly higher prevalence of thrombocytopenia was detected in cats from Calabria (P=0.0134,OR=3.706, 95%CI=1.336-10.11) and in those living in urban environment (P=0.0297,OR=3.443, 95%CI=1.19-9.503) compared to cats from suburban or rural areas. Demographic, husbandry, environmental and geographic data analyzed as risk factors for platelets abnormalities are described in table 5.p

Table 5.p Demographic, husbandry, environmental and geographic data analyzed as risk factors for platelet count abnormalities

VARIABLE	↓ PLATELETS	↑PLATELETS	NORMAL PLATELETS
ORIGIN*			
Sicily	4 (13)	4 (44)	72 (35)
Calabria	28 (87)*	5 (56)	137 (65)
SEX			
Male	17 (53)	6 (67)	101 (48)
Female	15 (47)	3 (33)	108 (52)
BREED			
DSH and/orDLH	29 (91)	9	195 (93)
Pure-Breed	3 (9)	0	14 (7)
AGE			
Junior	7 (22)	0	38 (18)
Adult	17 (53)	8 (89)	113 (54)
Senior	8 (25)	1 (11)	58 (28)

HUSBANDRY			
Indoor	17 (53)	1 (11)	94 (45)
Outdoor	15 (47)	8 (89)	115 (55)
HABITAT *			
Urban	24 (86)*	6 (76)	115 (64)
Suburban	4 (14)	1 (12)	49 (27)
Rural	0	1 (12)	17 (9)
Missing data	4	1	28
HOUSEHOLDS			
Single-cat	5 (29)	1	31 (33)
Multi-cat	12 (71)	0	63 (67)

Legenda: *=significant difference, ↑=increased, ↓=decreased

Significantly higher prevalence of thrombocytopenia was found in cats with pale mucous membranes (P=0.0062, OR= 4.542, 95%CI= 1.737-12.53). Prevalence of thrombocytosis was higher in cats with reduced BCS (P=0.0525, OR=4.094, 95%CI=1.195-14.56), poor muscle mass (P=0.0054, OR=7.568, 95%CI=1.982-28.24) and respiratory signs (P=0.0516, OR= 4.947, 95% CI= 1.259-18.16). Clinical variables evaluated as risk factors for platelet count abnormalities are described in table 5.q

Table 5.q Clinical variables evaluated as risk factors for platelet count abnormalities

VARIABLE	↓ PLATELETS	↑PLATELETS	NORMAL PLATELETS
MUCOUS MEMBRANES*			
Pale	7 (22)*	1 (11)	13 (6)
Normal	23 (72)	7 (78)	194 (93)
Jaundice	1 (3)	1 (11)	1 (1)
Congested	1 (3)	0	0
Missing data	0	0	1
BCS*			
<3	4 (13)	4 (44)*	34 (16)
≥3	28 (87)	5 (56)	174 (84)
Missing data	0	0	1
MCS*			
>1	6 (21)	4 (57)*	37 (21)
1	22 (79)	3 (43)	140 (79)

Missing data	4	2	32
LYMPH NODES			
Normal	24 (75)	6 (67)	143 (69)
Enlarged	8 (25)	3 (33)	64 (31)
Missing data	0	0	2
OCULAR SIGNS			
Present	0	0	18 (9)
Absent	32	9	189 (91)
Missing data	0	0	2
GI SIGNS			
Present	1 (3)	2 (22)	14 (7)
Absent	31 (97)	7 (78)	193 (93)
Missing data	0	0	2
RESPIRATORY SIGNS*			
Present	2 (6)	3 (33)*	19 (9)
Absent	30 (94)	6 (67)	188 (91)
Missing data	0	0	2
SKIN LESIONS			
Present	6 (19)	3 (33)	32 (15)
Absent	26 (81)	6 (67)	175 (85)
Missing data	0	0	2
GROUP			
Healthy/Other	11 (39)	4 (57)	83 (52)
CKD	17 (61)	3 (43)	78 (48)
Missing data	4	2	46

Legenda: *=significant difference, ↑=increased count, ↓=decreased count

Prevalence of thrombocytosis was significantly higher in *L. infantum* PCR positive cats (P=0.012,OR=9.65, 95%CI=2.283-44.29) and antibody and molecular positive cats (P=0.0214, OR=5.688, 95%CI= 1.415-21.9) and in FeMV overall positive cats (P=0.008,OR=13.72, 95%CI=1.748-162). Infectious pathogens evaluated as risk factors for platelet count abnormalities are described in table 5.r

Table 5.r Infectious pathogens evaluated as risk factors for platelet count abnormalities

VARIABLE	↓ PLATELETS	↑PLATELETS	NORMAL PLATELETS
L. infantum* PCR			
Positive	4 (13)	3 (38)*	12 (6)
Negative	28 (87)	5 (62)	193 (94)
Missing data	0	1	4
L. infantum* PCR and/ or IFI			
Positive	5 (16)	5 (62)*	46 (23)
Negative	27 (84)	3 (38)	157 (77)
Missing data	0	1	6
FeMV PCR and/ or IFI*			
Positive	4 (16)	5 (83)*	39 (27)
Negative	21 (84)	1 (17)	107 (73)
Missing data	7	3	63
FIV			
Positive	2 (6)	3 (33)	31 (16)
Negative	30 (94)	6 (67)	168 (84)
Missing data	0	0	10
FCoV			
Positive	3 (14)	2 (50)	23 (17)
Negative	19 (86)	2 (50)	109 (83)
Missing data	10	5	77
***Leptospira* spp. (MAT and /or PCR)**			
Negative	15 (79)	3	61 (76)
Positive	4 (21)	0	19 (24)
Missing data	13	6	129

Legenda: *=significant difference, ↑=increased count; ↓=decreased count

5.3e Markers of inflammation

Presence of LAI was evaluated in 183 cats and it was observed in 39 cats. Significantly higher prevalence was found in cats with reduced BCS (P=0.0352, OR=2.767, 95% CI=1.114-6.698) and poor muscle mass (P=0.019, OR=3.11, 95% CI= 1.27-7.81), pale mucous membranes (P=0.043, OR=3.24, 95% CI= 1.00-9.84), presence of gastrointestinal (P= 0.0021, OR=7.65, 95% CI= 2.2-21.13) and respiratory (P=0.0007, OR=6.9, 95% CI= 2.29-21.32) signs and in FIV positive cats (P=0.0299,OR=3.15,95%CI=1.287-8.041).

Serum amyloid A was evaluated in 115 cats and it was increased in 35 cats. Higher prevalence was detected in cats with reduced BCS (P= 0.020, OR= 4.44, 95% CI= 1.44-13.08) and poor muscle mass (P= 0.0212, OR= 3.61, 95% CI= 1.21-9.75), pale mucous membranes (P=0.001, OR=7.966, 95% CI= 1.82-39.74). In 87 cats SAA concentrations along with presence of LAI through blood smear observation were evaluated. Three cats with SAA within the normal interval showed LAI, but 10 cats had both increased concentration of SAA and LAI.

Total globulins were evaluated in 159 cats (normal 119, increased 38, reduced 2). Prevalence of hyperglobulinemia was significantly higher in adult (P= 0.004, OR= 10.97, 95% CI= 1.77-117) and senior cats (P= 0.0004, OR= 18.87, 95% CI= 2.95-205.7) compared to junior cats. Hyperglobulinemia was associated with *L. infantum* PCR positivity (P= 0.0107, OR= 6.45, 95% CI= 1.69-24.23), FeMV positivity (antibody: P= 0.0057, OR= 4.96, 95% CI= 1.51-13.91; urine PCR: P= 0.009, OR= 3.42, 95% CI= 1.36-8.03; overall PCR: P= 0.0233, OR= 2.882, 95% CI= 1.193-7.166; overall positivity: P<0.001, OR= 6.04, 95% CI= 2.556-13.86) and FIV positivity (P= 0.0027, OR= 4.40, 95% CI= 1.78-11.21).

Serum protein electrophoresis was performed in 75 cats and in no cases all globulin fractions were within reference limits. Apart from α2 increases detected in just 7% of samples (5/75), all globulin fraction increases were largely found and respectively: in 95% (71/75) of γ-globulins (95%, 71/75), in 89% (67/75) of β2-globulins, 79% (59/75) of β1-globulins and 64% (48 /75) α1 globulins. The increases of γ-globulins were always polyclonal.

Prevalence of increased α-globulins was significantly higher in FeMV positive cats (P= 0.014, OR= 5.06, 95% CI= 1.38-17.65). No significant associations were found between increased γ- globulins and origin, sex, age, husbandry, MCS, oral lesions, *L. infantum*, FIV, FCoV, *Leptospira* spp. Other statistical comparisons were not performed because of null values.

Albumin was evaluated in 94 cats and decreased values were observed in 9 of them. Low albumin concentrations were seen more frequently in cats with reduced BCS (33%) (P= 0.0387, OR= 6.583, 95% CI= 1.461-34.38) compared to cats with a normal one (7%).

Evaluation of CHr was performed in 119 cats and decreased values were found in six (5%, 6/119) cats: three cats were non anemic and three cats were affected by a mild normochromic microcytic anemia. Behaviour of the markers of inflammation in the six cats with reduced CHr is described in table 5.s.

Table 5.s: Behaviour of markers of inflammation in the six cats with reduced CHr

ANEMIA	SAA	TOT. GLOB	α1 GLOB	α2 GLOB	β1 GLOB	β2 GLOB	γ GLOB	LAI	ALB
absent	na	normal	na	na	na	na	na	absent	normal
present⁂	normal	normal	normal	normal	increased	increased	normal	absent	normal
present⁂	increased	normal	normal	normal	increased	increased	increased	present	na
present⁂	increased	increased	increased	normal	increased	normal	increased	na	normal
absent	normal	normal	increased	normal	normal	increased	normal	na	normal
absent	normal	normal	increased	normal	increased	normal	increased	na	normal

⁂ non regenerative mild normochromic microcytic anemia, na=non available

Demographic, husbandry, environmental, geographic and clinical data, infectious pathogens analyzed as risk factors for changes in markers of inflammation are described in table 5.t

Table 5.t Demographic, husbandry, environmental, geographic and clinical data, infectious pathogens analyzed as risk factors for changes in markers of inflammation

VARIABLE	**LAI**		**SAA**		***α GLOB***		***γ GLOB***		**TOT GLOB**		**ALBUMIN**	
AGE*	PRESENT	ABSENT	↑	NORMAL	↑	NORMAL	↑	NORMAL	↑	NORMAL	↓	NORMAL
Junior	7 (18)	31 (22)	5 (14)	23 (29)	8 (16)	7 (28)	14 (20)	1 (25)	1 (3)	31 (26)	1 (11)	21 (25)
Adult	19 (49)	81 (56)	21 (60)	41 (51)	33 (68)	10 (40)	42 (59)	2 (50)	23 (60)*	65 (55)	6 (67)	45 (53)
Senior	13 (33)	32 (22)	9 (26)	16 (20)	8 (16)	8 (32)	15 (21)	1 (25)	14 (37)*	23 (19)	2 (22)	19 (22)
ORIGIN												
Sicily	10 (26)	45 (31)	4 (11)	23 (29)	10 (20)	4 (16)	14 (20)	1 (25)	10 (26)	34 (29)	1 (11)	29 (34)
Calabria	29 (74)	99 (69)	31 (89)	57 (71)	39 (80)	21 (84)	57 (80)	3 (75)	28 (74)	85 (71)	8 (89)	56 (66)
SEX												
Male	16 (41)	68 (47)	20 (57)	31 (39)	22 (45)	11 (44)	31 (44)	3 (75)	21 (55)	53 (45)	2 (22)	35 (41)
Female	23 (59)	76 (53)	15 (43)	49 (61)	27 (55)	14 (56)	40 (56)	1 (25)	17 (45)	66 (55)	7 (78)	50 (59)
BREED												
DSH/DLH	37 (95)	131 (91)	35	69 (86)	46 (94)	21 (84)	64 (90)	4	36 (95)	108 (91)	8 (89)	75 (88)
Pedigree	2 (5)	13 (9)	0	11 (14)	3 (6)	4 (16)	7 (10)	0	2 (5)	11 (9)	1 (11)	10 (12)
HUSBANDRY												
Indoor	17 (44)	72 (50)	15 (43)	50 (63)	23 (47)	17 (68)	37 (52)	3 (75)	18 (47)	61 (51)	3 (33)	50 (59)
Outdoor	22 (56)	72 (50)	20 (57)	30 (37)	26 (53)	8 (32)	34 (48)	1 (25)	20 (53)	58 (49)	6 (67)	35 (41)
HOUSEHOLDS												
Single	4 (24)	27 (37)	4 (27)	15 (30)	8 (35)	3 (18)	8 (22)	3	6 (33)	19 (31)	2 (67)	13 (26)

Multi	13 (76)	45 (63)	11 (73)	35 (70)	15 (65)	14 (82)	29 (78)	0	12 (67)	42 (69)	1 (33)	37 (74)
ENVIRONMENT												
Urban	19 (59)	85 (70)	28 (80)	56 (70)	32 (65)	20 (80)	49 (69)	4	24 (67)	80 (68)	8 (89)	55 (65)
Suburban	11 (34)	32 (26)	5 (14)	22 (28)	15 (31)	5 (20)	20 (28)	0	9 (25)	31 (27)	1 (11)	24 (28)
Rural	2 (7)	4 (4)	2 (6)	2 (2)	2 (4)	0	2 (3)	0	3 (8)	6 (5)	0	6 (7)
Missing data	7	23	0	0	0	0	0	0	2	2	0	0
BCS*												
Normal/Increased	27 (73)	127 (88)	27 (77)	75 (94)	41 (84)	24 (96)	61 (86)	4	27 (71)	100 (84)	6 (67)	79 (93)
Reduced	10 (27)*	17 (12)	8 (23)*	5 (6)	8 (16)	1 (4)	10 (14)	0	11 (29)	19 (16)	3 (33)*	6 (7)
Missing data	2	0	0	0	0	0	0	0	0	0	0	0
MCS*												
Normal	21 (66)	101 (86)	26 (74)	73 (91)	40 (82)	23(92)	61 (86)	2 (50)	20 (57)	98 (84)	6 (67)	76 (93)
Reduced	11 (34)*	17 (14)	9 (26)*	7 (9)	9 (18)	2 (8)	10 (14)	2 (50)	15 (43)	18 (16)	3 (33)	9 (7)
Missing data	7	26	0	0	0	0	0	0	3	3	0	0
MUCOUS MEMBRANES*												
Normal	31 (80)	134 (93)	29 (83)	77 (96)	44 (90)	25	65 (91)	4	29 (76)	109 (92)	9	82 (96)
Pale	6 (15)*	8 (5)	6 (17)*	2 (3)	4 (8)	0	5 (7)	0	7 (19)	10 (8)	0	3 (4)
Jaundice	2 (5)	1 (1)	0	1 (1)	1 (2)	0	1 (2)	0	2 (5)	0	0	0
Cogestion	0	1 (1)	0	0	0	0	0	0	0	0	0	0
LYMPH NODES												
Normal	28 (72)	103 (72)	25 (74)	62 (78)	34 (69)	21 (84)	51 (73)	4	27 (73)	84 (71)	7 (78)	64 (75)
Enlarged	11 (28)	41 (28)	9 (26)	18 (22)	15 (31)	4 (16)	19 (27)	0	10 (27)	35 (29)	2 (22)	21 (25)

Missing data	0	0	1	0	0	0	1	0	1	0	0	0
OCULAR L.												
Absent	35 (90)	133 (92)	33 (94)	75 (94)	45 (92)	24 (96)	66 (93)	4	33 (89)	112 (94)	9	80 (94)
Present	4 (10)	11 (8)	2 (6)	5 (6)	4 (8)	1 (4)	5 (7)	0	4 (11)	7 (6)	0	5 (6)
Missing data	0	0	0	0	0	0	0	0	1	0	0	0
ORAL L.												
Absent	28 (72)	95 (66)	23 (66)	55 (69)	35 (71)	17 (68)	49 (69)	3 (75)	20 (54)	78 (66)	6 (67)	61 (72)
Present	11 (28)	49 (34)	12 (34)	25 (31)	14 (29)	8 (32)	22 (31)	1 (25)	17 (46)	41 (34)	3 (33)	24 (28)
Missing data	0	0	0	0	0	0	9	0	1	0	0	0
GI SIGNS*												
Absent	32 (82)	140 (97)	33 (94)	77 (96)	44 (90)	25	66 (93)	4	33 (89)	115 (97)	9	84 (99)
Present	7 (18)*	4 (3)	2 (6)	3 (4)	5 (10)	0	5 (7)	0	4 (11)	4 (3)	0	1 (1)
Missing data	0	0	0	0	0	0	0	0	1	0	0	0
RESPIRATORY SIGNS*												
Absent	30 (77)	138 (96)	31 (89)	74 (93)	42 (86)	25	64 (90)	4	32 (86)	109 (92)	9	79 (93)
Present	9 (23)*	6 (4)	4 (11)	6 (7)	7 (14)	0	7 (10)	0	5 (14)	10 (8)	0	6 (7)
Missing data	0	0	0	0	0	0	0	0	1	0	0	0
SKIN SIGNS												
Absent	33 (85)	121 (84)	31 (89)	69 (86)	42 (86)	23 (92)	62 (87)	4	28 (76)	103 (87)	9	71 (84)
Present	6 (15)	23 (16)	4 (11)	11 (14)	7 (14)	2 (8)	9 (13)	0	9 (24)	16 (13)	0	14 (16)
Missing data	0	0	0	0	0	0	0	0	1	0	0	0
CKD												

Absence	14 (45)	61 (55)	14 (41)	43 (54)	27 (5)	8 (33)	33 (46)	2 (67)	18 (49)	63 (53)	6 (67)	53 (62)
Presence	17 (55)	49 (45)	20 (59)	37 (46)	22 (45)	16 (67)	38 (54)	1 (33)	19 (51)	55 (47)	3 (33)	32 (38)
Missing data	8	34	1	0	0	1	0	1	1	1	0	0
Leishmania* spp. PCR												
Negative	34 (87)	133 (94)	31 (91)	78 (98)	46 (94)	25	67 (94)	4	31 (84)	115 (97)	9	82 (96)
Positive	5 (13)	9 (6)	3 (9)	2 (2)	3 (6)	0	4 (6)	0	6 (16)*	3 (3)	0	3 (4)
Missing data	0	2	1	0	0	0	0	0	1	1	0	0
***Leishmania* spp. IFI and/or PCR**												
Negative	30 (79)	116 (82)	25 (74)	71 (89)	42 (86)	20 (80)	59 (83)	3 (75)	26 (70)	99 (84)	7 (78)	73 (86)
Positive	8 (21)	25 (18)	9 (26)	9 (11)	7 (14)	5 (20)	12 (17)	1 (25)	11 (30)	19 (16)	2 (22)	12 (14)
Missing data	1	3	1	0	0	0	0	0	1	1	0	0
FeMV IFI and/or PCR*												
Negative	18 (62)	80 (78)	22 (71)	52 (76)	25 (57)	20 (87)	41 (65)	4	13 (42)	83 (81)	5 (71)	58 (76)
Positive	11 (38)	22 (22)	9 (29)	16 (24)	19 (43)*	3 (13)	22 (35)	0	18 (58)*	19 (19)	2 (29)	18 (24)
Missing data	10	42	4	12	5	2	8	0	7	17	2	9
FIV*												
Negative	30 (77)	126 (91)	27 (77)	71 (89)	38 (78)	24 (96)	59 (83)	3 (75)	26 (68)	105 (91)	7 (78)	75 (90)
Positive	9 (23)*	12 (9)	8 (23)	9 (11)	11 (22)	1 (4)	12 (17)	1 (25)	12 (32)*	11 (9)	2 (22)	8 (10)
Missing data	0	6	0	0	0	0	0	0	0	3	0	2
FCoV												

Negative	24 (89)	67 (82)	22 (88)	49 (91)	32 (86)	18 (95)	48 (91)	2 (67)	22 (79)	73 (86)	5	54 (87)
Positive	3 (11)	15 (18)	3 (12)	5 (9)	5 (14)	1 (5)	5 (9)	1 (33)	6 (21)	12 (14)	0	8 (13)
Missing data	2	62	0	26	12	6	18	1	10	34	4	23
***Leptospira* spp. MAT and/or PCR**												
Negative	9 (69)	57 (79)	24 (75)	59 (78)	33 (72)	16 (67)	47 (70)	2 (67)	14 (67)	66 (83)	5 (63)	56 (79)
Positive	4 (31)	15 (21)	8 (25)	17 (22)	13 (28)	8 (33)	20 (30)	1 (33)	7 (33)	14 (17)	3 (37)	15 (21)
Missing data	26	72	3	4	3	1	4	1	17	39	1	14

Legenda: *=significant difference, ↑= increased, ↓=decreased count

Estimation of globulin fraction values obtained with SPE are listed in table 5.u

Table 5.u Estimation of globulin fraction values obtained with SPE

PARAMETER	NORMAL VALUES	INCREASED VALUES	REDUCED VALUES
α1 GLOBULINS	26	48	1
α2 GLOBULINS	69	5	1
TOTAL αGLOBULINS	25	49	1
β1 GLOBULINS	16	59	0
β2 GLOBULINS	8	67	0
TOTAL β GLOBULINS	1	74	0
γ GLOBULINS	4	71	0

Significant associations of abnormalities and variables evaluated in this study are listed with P-value, OR and 95% CI in table 5.v

Table 5.v Significant associations of abnormalities and variables evaluated in this study with their P-value, OR and 95% CI

VARIABLE	ABNORMALITIES	P-value	OR	95% CI
HUSBANDRY	Anemia	< 0.0001	3.089	1.741-5.485
Indoor vs Outdoor	Monocytosis	0.0423	2.115	1.038-4.304
	Neutrophilia	0.0018	2.707	1.473-4.877
AGE	Anemia	0.0021	3.842	1.597-9.113
Junior vs Adult	Neutrophilia	0.0145	3.429	1.334-8.525
	Eosinophilia	0.0402	7.105	1.102-76.67
	Eosinopenia	0.0150	9	1.479-95.94
	↑ total glob	0.004	10.97	1.771-117
	Anemia	0.0017	4.667	1.813-12.07
Junior vs Senior	Neutrophilia	0.0038	4.488	1.525-11.55
	Eosinopenia	0.0401	8.1	1.202-90.65
	↑ total glob	0.0004	18.87	2.954-205.7
ORIGIN				
Sicily vs Calabria	Trombocytopenia	0.0134	3.706	1.336-10.11
ENVIRONMENT				

Urban vs Suburban/Rural	Trombocytopenia	0.0297	3.443	1.19-9.503
Urban vs Suburban	Neutropenia	< 0.0001	15.32	5.106-43.23
BCS	Anemia	< 0.0001	6.682	3.345-13.13
<3 vs ≥3	Neutrophilia	< 0.0001	6.914	3.252-13.84
	Lymphopenia	0.0273	3.596	1.164-10.56
	Monocytosis	0.0024	3.42	1.636-7.212
	Thrombocytosis	0.0525	4.094	1.195-14.56
	LAI	0.0352	2.767	1.114-6.698
	↑ SAA	0.0207	4.444	1.443-13.08
	↓ ALB	0.0387	6.583	1.461-34.38
MCS	Anemia	< 0.0001	4.836	2.553-9.041
1 vs >1	Neutrophilia	< 0.0001	5.826	2.822-11.88
	Monocytosis	0.0021	3.404	1.557-7.122
	Thrombocytosis	0.0054	7.568	1.982-28.24
	LAI	0.019	3.112	1.279-7.818
	↑ SAA	0.0212	3.61	1.219-9.758
MUCOUS	Anemia	< 0.0001	15.23	5.119-41.97
MEMBRANES	Neutrophilia	0.0117	3.632	1.292-9.631
Normal vs Pale	Lymphopenia	0.0005	10	2.836-29.12
	Eosinopenia	0.0299	3.37	1.152-9.669
	Monocytosis	0.0012	5.515	2.127-14.15
	Thrombocytopenia	0.0062	4.542	1.737-12.53
	LAI	0.0436	3.242	1.005-9.843
	↑ SAA	0.0101	7.966	1.826-39.74
GI SIGNS	Neutrophilia	0.0172	3.694	1.37-9.61
Presence vs Absence	Lymphopenia	0.055	4.54	1.21-16.39
	Monocytosis	0.0483	2.984	1-8.099
	LAI	0.0021	7.65	2.12-21.13
RESPIRATORY SIGNS	Neutrophilia	0.0057	3.577	1.532-8.879
Presence vs Absence	Monocytosis	< 0.0001	7.123	2.909-18.55
	Thrombocytosis	0.0516	4.947	1.259-18.16
	LAI	0.0007	6.9	2.29-21.32
SKIN LESIONS	Neutrophilia	0.0015	3.295	1.593-6.838
Presence vs Absence	Monocytosis	0.042	2.295	1.035-4.817
CKD	Anemia	0.031	2.492	1.37-4.598
Presence vs Absence	Neutrophilia	<0.0001	4.603	2.148-9.829

ORAL LESIONS	Lymphopenia	0.0467	3.087	1.097-9.052
Presence vs Absence	Eosinopenia	0.0225	2.541	1.115-5.556
LYMPH NODES	Neutrophilia	0.0212	2.069	1.141-3.761
Normal vs Enlarged				
***L. infantum* (PCR)**	Monocytosis	0.0025	5.382	1.824-15.72
Negative vs Positive	Thrombocytosis	0.012	9.65	2.283-4.29
	↑ tot glob	0.0107	6.452	1.692-24.23
	Eosinopenia	0.0309	3.848	1.357-11.91
***L. infantum*(serology/PCR)**	Anemia	0.0406	1.897	1.022-3.429
Negative vs Positive	Monocytosis	0.038	2.308	1.092-4.809
	Thrombocytosis	0.0214	5.688	1.415-21.9
***Leptospira* spp. (serology)**	Monocytosis	0.0319	3.769	1.061-11.48
Negative vs Positive				
***Leptospira* spp. (urine/blood PCR)**	Neutrophilia	0.0047	6.533	1.822-21.46
Negative vs Positive	Monocytosis	0.024	4.821	1.392-18.18
***Leptospira* spp. (serology/PCR)**	Neutrophilia	0.013	3.974	1.474-10.64
Negative vs Positive	Monocytosis	0.0115	4.267	1.385-13.47
FeMV (serology)	↑ tot glob	0.0057	4.969	1.515-13.91
Negative vs Positive				
FeMV (urine PCR)	↑ tot glob	0.0094	3.422	1.369-8.037
Negative vs Positive				
FeMV (urine/blood PCR)	↑ tot glob	0.0233	2.882	1.193-7.166
	Monocytosis	0.0329	2.614	1.086-5.98
Negative vs Positive				
FeMV (serology/PCR)	Neutrophilia	0.0366	2.287	1.137-4.616
Negative vs Positive	Thrombocytosis	0.008	13.72	1.748-162.8
	↑ α-glob	0.0146	5.067	1.383-17.65
	↑ tot glob	< 0.0001	6.049	2.556-13.86
FIV (serology/PCR)	LAI	0.0299	3.15	1.287-8.041
Negative vs Positive	↑ tot glob	0.0027	4.406	1.78-11.21

Legenda: ↑=increased, ↓=decreased count

DISCUSSION

In the present study we reported the prevalence of CBC abnormalities and markers of inflammations observed in a population of cats of Southern Italy exposed to feline emerging pathogens (*L. infantum*, FeMV, and *Leptospira* spp.) and other viral infections (FIV, FeLV, FCoV).

Anemia was observed in 32% of studied cats and it was the most common CBC abnormality. In most cases anemia was NRA (92%, 79/86) and severity was mild or moderate with only 4 cats (5%, 4/86) affected by severe anemia.

Normocytic normochromic anemia was the type of anemia more frequently observed both in RA (43%, 3/7) and NRA (48%,38/79), followed by microcytic normochromic anemia observed in 14% of RA and in 19% of NRA. Tipically, macrocytic hypochromic anemia was observed just in RA (14%, 17/) and normocytic hypochromic anemia just in NRA (4%, 3/79). A previous study investigated the prevalence of anemia in a large number of feline blood samples submitted by first opinion veterinarians in and surrounding Vienna (Austria) and a prevalence of 3.6% of anemia found using as cut off values Ht ≤27%, RBC ≤5.5 x 10^6/μL and Hb ≤9 g/dL. Moreover, 57.7% of these anemic samples were classified as NRA, while 42.3% as RA (Furman et al., 2014). In another study that evaluated a population of 180 anemic cats admitted to The Feline Centre of the University of Bristol, 35.2% were affected by a NRA (Korman et al., 2013). Prevalence of anemia found in our study is quite different compared to the ones reported by the two previous studies. Contrarly, our result is similar to the one observed in a population of cats of South Italy where prevalence of anemia reported was of 34.5% (Persichetti et al., 2018). However difference in reference intervals, as well as in risk factors for anemia that can change depending on the assessed geographical area or type of feline population evaluated (such as outdoor or indoor cats, cats from urban or rural environment) may affect the results.

Frequency of neutrophilia, monocytosis and eosinopenia in cats evaluated was of 27% (64/240), 18% (43/240) and 12% (27/235) respectively and they were more frequently observed compared to eosinophilia (9%, 21/235), lymphocytosis (6%, 14/240),

neutropenia (5%, 11/240) and basophilia (1%, 3/219). Thrombocytopenia (13%, 32/250) was observed more frequently than thrombocytosis (4%, 9/250).
Our results are quite similar to the one reported by Persichetti et al. (2018), where neutrophilia (28.9%) and monocytosis (23.9%) were reported more frequently than eosinophilia (9.1%), lymphocytosis (5.6%), and neutropenia (2%).

In inflammatory and neoplastic diseases as well as in other conditions such as diabetes mellitus or hyperthyrodism, SAA tends to be increased, moreover it seems to be a significant prognostic factor affecting survival of feline patient (Tamamoto et al., 2013). In the feline population examined, increased SAA was found in about one third of cats (35/115) but a slightly lower prevalence was found for LAI (21%, 39/183). As reported before, leukocyte differential counts are influenced by multiple factors (see table 5.b) that can act simultaneously with different effects and this can explain a lower sensitivity of LAI compared to SAA as a marker of inflammation.
A previous study that evaluated SPE abnormalities found in 155 clinical cases examined at University of Bristol, reported a prevalence of 87.7% and polyclonal gamma globulin increase was the most common finding, related to infectious/inflammatory diseases in 58.8% of cats (Taylor et al., 2010). Methods for SPE and inclusion criteria of examined cats were not the same as in our study, however we similarly found an extremely high prevalence of increased globulin fractions with β2-globulins (89%%, 67/75) and γ- globulins (95%, 71/75) as the most prevalent.
Reticulocyte haemoglobin content is a sensitive marker of iron deficiency. Reticulocytes have a short life span in the circulation and reduction of CHr happens more rapidly than development of anemia and of reductions of RBC hemoglobin content during iron deficiency anemia (Melendez-Lazo et al., 2015). Moreover, high C-reactive protein (CRP) was associated to low CHr in humans and dogs, and this is suggestive of an inflammatory process (Melendez-Lazo et al., 2015). In this study CHr was evaluated in 119 cats and in just 6 cats the value was reduced (5%). Of the six cats with reduced CHr, three were not anemic and had normal SAA values so they potentially had iron deficiency, but three cats were anemic with normochromic microcytic mild anemia and two of them had increased SAA therefore suggesting the occurrence of a chronic inflammation.

We found significant associations between clinicopathological abnormalities reported and geographic origin, husbandry, demographic or environmental characteristics, and clinical examinations findings.

Cats living in Calabria were more frequently thrombocytopenic compared to cats from Sicily (OR= 3.706) as well as cats living in urban areas compared to the ones living in suburban or rural areas (OR= 3.443). Interestingly, a higher prevalence of cats living in urban area was found in Calabria (81% 125/155) rather than in Sicily (37%, 29/79) with a significant difference (P<0.0001, OR= 7.184, 95%CI= 3.956-13.15) and this can explain the different prevalence of thrombocytopenia in the two regions. Thrombocytopenia is often reported in FeLV and FIV infections (Lutz et al., 2009, Gleich and Hartmann, 2009; Collado et al., 2012), feline leishmaniosis (FeL) (Marcos et al., 2009) and in cats affected by *Mycoplasma haemofelis* (Raimundo et al., 2016) or *Bartonella* spp. (Bergmann et al., 2017) infection. A recent study evaluated outdoor cats exposed to ectoparasites and associated risk for vector-borne infections, in southern Italy, investingating *Bartonella henselae, Rickettsia conorii, Ehrlichia canis, Anaplasma phagocytophilum, Babesia microti, Leishmania infantum, Mycoplasma* spp, *Hepatozoon felis* and reporting a very high antibody (87.8%) and PCR (40.1%) positivity to at least one pathogen in outdoor cats. Among outdoor cats prevalence of thrombocytopenia reported was of 3.7%, but no significant association was found with outdoor lifestyle as in the present study (Persichetti et a., 2018). We found that just eight out of 28 cats from Calabria with low PL counts tested positive to an investigated pathogens (two cats were FIV positive, one cat FeLV positive and 5 cats were *L. infantum* positive). Cats from urban areas could be exposed to other infectious or non infectious agents that we did not investigate. In fact, short term and generally mild or moderate thrombocytopenias are found in various infections in dogs and cats (Lutz et al., 2009, Gleich and Hartmann, 2009; Collado et al., 2012, Marcos et al., 2009, Bergmann et al., 2017, Antognoni et al., 2014, Paltrinieri et al., 2016, Shapiro et al., 2017).

Outdoor husbandry was a risk factor for abnormalities other than thrombocytopenia, such as anemia (OR= 3.089), neutrophilia (OR= 2.707), and monocytosis (OR= 2.115). As we found almost exclusively mild or moderate non regenerative anemias in investigated cats the above data are in line with a chronic inflammation found in outdoor cats. Persichetti et al. (2018) found that outdoor cats non protected against ectoparasites are more exposed to several vector borne pathogens (VBP) and, similarly to our study, they found a higher risk for anemia, neutrophilia, and monocytosis associated with cat outdoor access. Moreover, outdoor cats are more exposed to cat-to-cat transmissions of infectious pathogens such as FIV or FeLV that can cause anemia (Lutz et al., 2009, Hosie et al., 2009, Korman et al., 2013).

In the present study adult (OR= 3.842) and senior (OR= 4.667) cats were affected more frequently by anemia when compared with junior cats (7%, 6/86). Age-related changes can affect the immune system (Campbell et al., 2004) and physiology of the digestive tract or kidneys function of senior cats, moreover development of osteoarthritis or endocrine disorders such as hyperthyroidism, diabetes mellitus can be seen more frequently in older cats (Beale, 2005, Laflamme, 2005, Vaske et al., 2016, Sparkes et al., 2015). Some factors such as physical inactivity, increased cytokine production, decreased protein synthesis potentially affecting old cats can be responsible for sarcopenia manifested as loss of lean muscle mass (Freeman, 2012).
Anemia in old cats can be related to several factors described above such as chronic kidney disease (Gest et al., 2015; Javard et al., 2017) or osteoarthritis that determine inflammatory conditions with possible development of anemia.
Neutrophilia and eosinopenia were seen more frequently in adult (OR= 3.429; OR= 8.047) and senior (OR= 4.488; OR= 7.92) cats rather than junior cats. Conversely, eosinophilia was more frequently found in adults than in junior cats (OR= 7.105). These relationship can be determined by a greater likelihood of the adult and senior cats to contract infectious or inflammatory pathologies responsible for both neutrophilia and eosinopenia rather than junior cats due to a longer exposure time in older cats. Furthermore, both of these abnormalities are a marker of stress which could affect older cats due to a greater likelihood of a compromised health status. Concerning the greater risk for eosinophilia of adult cats allergic diseases (feline asthma, flea

allergy, food allergies) can be responsible as these conditions are more likely to be found in an adult rather than a junior cat.

Adult (OR=10.97) and senior cats (OR=18.87) had a higher risk for total globulins values increases when compared with junior cats.

In a previous study age-related differences in leukocytes and acute phase proteins were evaluated comparing two groups of cats: adult (age 2-5 years) and senior cats (10-14 years). Number of lymphocytes and eosinophils were significantly lower in the senior animals while relative percentage of neutrophils was significantly higher in older animals. Moreover no significant difference was found in concentrations of acute phase proteins evaluated (SAA and haptoglobin) (Campbell et al., 2004). In the present study we found changes both in adult and senior cats but age groups were differently defined in our study. In accordance with the result of the previous study we did not find a significant association with changes in SAA concentration, as well as other markers of inflammation apart from the observed increased values of total globulins in adult (OR= 10.97) or senior cats (OR= 18.87) rather than junior cats. Therefore, in these cats ageing is not clearly associated with inflammageing as reported by Day M. (2010).

Interestingly, we found that reduced BCS and poor MCS were both risk factors for anemia (BCS: OR= 6.682; MCS: OR= 4.836), as well as neutrophilia (BCS: OR= 6.914; MCS:OR= 5.826), monocytosis (BCS: OR= 3.42; MCS:OR= 3.404), thrombocytosis (BCS:OR=4.094;MCS: OR=7.568), LAI (BCS: OR= 2.767; MCS: OR=. 3.11) and increased SAA values (BCS: OR= 4.44; MCS: OR= 3.61). Reduced BCS was also risk factor for lymphopenia (OR= 3.596) and decreased ALB values (OR= 6.583). A similar observation concerned pale mucous membranes that was risk factor for anemia (OR= 15.23), neutrophilia (OR=3.632), monocytosis (OR=5.515), lymphopenia (OR=10), eosinopenia (OR=3.37), thrombocytopenia (OR=4.542), increased SAA (OR=7.966) and LAI (OR=3.24). As noted above, anemia detected in the studied cats was mild, non regenerative and mostly normocytic and normochromic so we can conclude that occurrence of reduced BCS, poor MCS and pale mucous membranes at physical examination was probably associated with inflammation in

these cats. In fact, cachexia is seen in chronic conditions that influence concentrations of different mediators such as inflammatory cytokines (Freeman, 2012).

In the present study few cats showed gastrointestinal (7%, 18/266) or respiratory signs (9%, 25/266) but in both cases we found CBC abnormalities (monocytosis, neutrophilia and LAI) compatible with inflammation. Segev et al. (2006) evaluated the clinical meaning of toxic neutrophils in cat CBC and, among other things, they were associated with diarrhea and vomit or pneumonia. Moreover they were a negative prognostic factor as were associated with a longer duration of hospitalization.

Dermatological lesions were found in 14% (44/266) of cats. Neutrophilia (OR= 3.295) and monocytosis (OR= 2.295) were significantly associated with skin disease. Relationship between infectious pathogens under study and detected dermatological abnormalities is described in chapter 4. Briefly, some infectious pathogens such as FIV impaire feline immune system with a higher occurrence of secondary or opportunistic infections (Hosie et al., 2009). Moreover coinfection between FIV and FeL is widely reported and *L. infantum* is known to be responsible of various dermatological signs in cats (Pennisi and Persichetti, 2018). Positivity to these infectious pathogens can be partly responsible for the association found, however dermatological lesions can be at the same time associated with a more or less severe inflammation.
In the present study 35% (93/266) of cats were affected by oral diseases and stomatitis (49%, 46/93) and periodontitis (27%, 25/93), were the more frequently observed. Feline chronic gingivostomatitis (FCG) is a common feline disease and several infectious agents, such as FCV, FHV, FeLV and FIV, along with a wide variety of bacteria, have been investigated as responsible (Rolim et al., 2017). Moreover, production of inflammatory cytokines or endotoxemia and immune response to bacteria are mechanisms involved in feline periodontal diseases (Finch et al., 2016). Unexpectedly, we did not find CBC abnormalities suggestive of inflammation as well as changes in markers of inflammations in cats with oral disease despite half of them were affected by stomatitis. In the present study cats with oral lesions had lymphopenia (11%) or eosinopenia (21%). These clinicopathological abnormalities are compatible with viral infections usually associated to oral disease such as FCV or

immunesuppression associated with FeLV and FIV infections (Gleich and Hartmann, 2009; Collado et al., 2012).

In the present study lymphadenopathy affected 31% (82/266) of cats and it was associated only with neutrophilia (OR= 2.069). Many different causes such as infectious, inflammatory or neoplastic disorders can be responsible for enlargment of superficial lymph nodes and apart from skin disease, regional or systemic pathologies are also involved and make this sign very common as seen also by Persichetti et al. (2018).

Anemia (OR= 2.492) and neutrophilia (OR= 4.603) were significantly associated with CKD diagnosis. Anemia is a well known complication of CKD, especially in end stage renal disease (Elliot and Barber, 1998). Erythropoietin (EPO) deficiency is considered the main cause of CKD associated anemia (Chakrabarti et al., 2012) and anemic cats seem to have lower EPO concentrations when compared with non-anemic cats with CKD and healthy cats (Javard et al., 2017). Moreover other factors can influence the development of anemia in cats affected by kidney disease such as gastrointestinal hemorrhages as a possible consequence of the uremic syndrome (Polzin, 2011) or inhibition of erythropoiesis due to secondary renal hyperparathyroidism. Shortened lifespan of red blood cells and iron deficiences are also reported (Ettinger and Feldman, 2016). Anemia due to CKD is generally non-regenerative, normochromic normocytic, and its degree indicates the severity of loss of functional renal tissue (Elliot and Barber, 1998; Paepe and Daminet, 2013; Ettinger and Feldman, 2016). In the present study cats with CKD had mostly a non regenerative anemia (93%), and normochromic (66%) as well as normocytic (64%) anemia were the most frequently seen. Most of the cats affected by CKD had a mild anemia (75%) and a lower number of cats a moderate/severe anemia (25%). Few studies evaluated the association between CKD and leukocytes abonormalities, but several disorders seen in cats affected by kidney disfunction can be responsible for the association found with neutrophilia in the present study. In fact, chronic kidney disease is associated with oral and gastrointestinal disorders, mostly related to development of uremia (Langston, 2003; McLeland et al., 2014). The syndrome is characterized by different

gastrointestinal manifestations and drooling and halitosis associated with, ulcerative stomatitis, vomiting, gastrointestinal hemorrhage, and diarrhea are seen (Polzin, 2011) and these signs can be associated with an increased inflammatory response. Moreover neutrophilia can be seen also in stressed animals (Paltrinieri et al., 2010) and cats affected by chronic disease, such as CKD, can be chronically stressed.

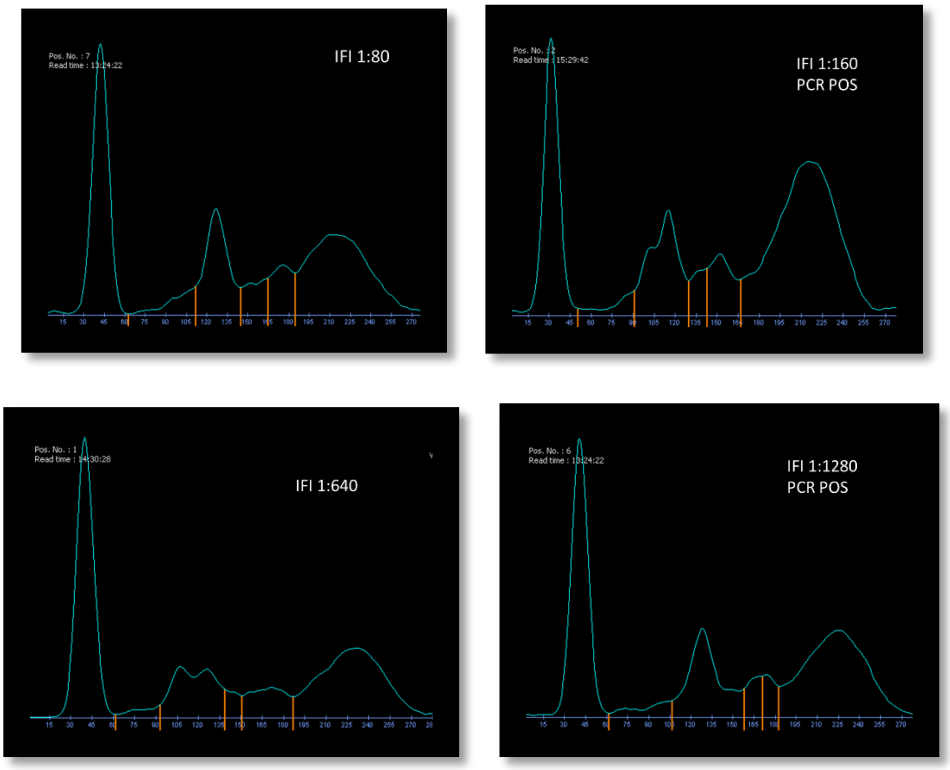
Different clinicopathological abnormalities can be seen in *L. infantum* positive animals. A mild to severe normocytic normochromic non-regenerative anemia is reported both in dogs and cats (Paltrinieri et al., 2016, Pennisi et al., 2013a, Pennisi et al., 2015, Pennisi and Persichetti, 2018). Several mechanisms can be responsible of its development such as reduced erythropoietin synthesis due to renal failure, possibile hemolysis suggested by some positive Coombs test in dogs with canine leishmaniosis (Paltrinieri et al., 2016), and anti-RBC antibodies and hemophagocytosis in dog bone marrow or spleen (Goto et al., 2017). In the present study cats positive to *L. infantum* were slightly more at risk for anemia (OR= 1.897) but this risk was higher when we considered association only with moderate or severe anemias (OR= 3.75). As in *Leishmania* negative cats anemia was more frequently non regenerative, normocytic and normochromic.

Pancytopenia, hyperglobulinemia, leukocytosis/leukopenia, thrombocytopenia are also reported in some cases of feline leishmaniosis (Pennisi et al., 2013a, Pennisi et al., 2015, Pennisi and Persichetti, 2018). In the present study, white blood cells and platelets abnormalities significantly associated with FeL were monocytosis (PCR positivity: OR= 5.382, overall positivity: OR= 2.308), eosinopenia (PCR positivity: OR= 3.848) and thrombocytosis (PCR positivity: OR=9.65 and overall positivity: OR=5.688).

Generally, thrombocytosis as well as monocytosis can be seen in case of chronic inflammatory stimuli and this could be in line with *L. infantum* infection that is not rapidly cleared in the infected individual. Moreover monocytosis as well as eosinopenia can develop in stressed animals, and chronic diseases can be responsible for an increase in stressful condition. Considering the intense inflammatory response, both leishmaniotic dogs and cats can be affected by increased total proteins and total globulins, while albumin is generally decreased because it is a negative acute phase

proteins (Paltrinieri et al., 2016, Pennisi et al., 2015) and can be lost due to proteinuric nephropathy (Paltrinieri et al., 2016). Generally leishmaniotic dogs with overt clinical signs displays hypoalbuminemia, a moderate increase of α2-globulins (which include most of the positive APP), a marked increase of γ-globulins (due to the high levels of circulating antibodies) and possible peaks of β- globulins (including IgM and some APP) at SPE. The gammopathy is typically polyclonal but sometimes the peak may appear narrower (i.e., oligoclonal), biclonal or monoclonal. However, the presence of monoclonal peaks should also include consideration of concurrent diseases (Paltrinieri et al., 2016). In our study a significant association was found in PCR positive cats with increased total globulins (OR= 6.45). Increases of α- globulins or γ-globulins were seen (fig 5.a) but no significant association was found. However, as reported in chapter 4, in our study a low number of cats was negative to all pathogens investigated and this among with different other non infectious diseases may have resulted in increase of these globulins fractions.

Fig 5.a *L. infantum* positive cats SPE



1)cat positive 1:80 (IFI) with negative PCR, 2) cat positive 1:160 (IFI), positive to conjunctival swabs and EDTA blood PCR, also positive to FIV, 3) cat positive 1:640 (IFI) with negative PCR, also positive

to FeMV and FIV, 4) cat positive 1:1280 (IFI), positive to conjunctival, nasal swabs, EDTA blood, skin biopsy, claw PCR, also positive to FeMV.

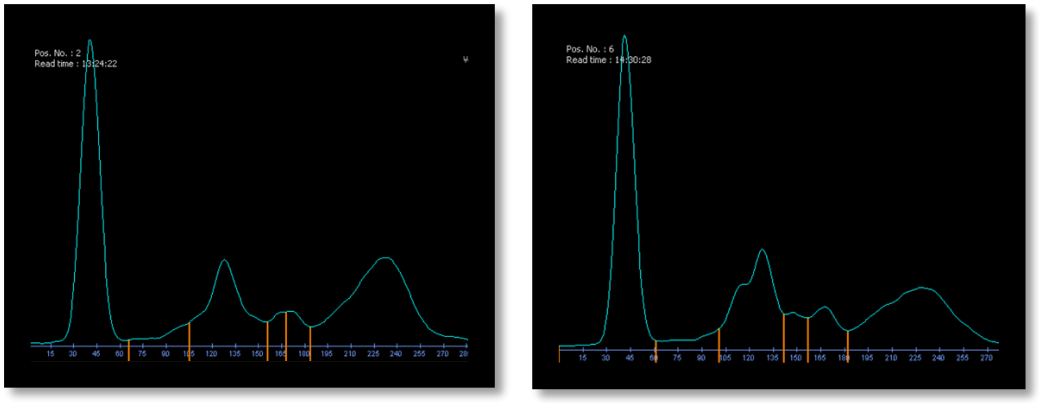
In leishmaniotic dogs increased CRP, haptoglobin (Hp), ceruloplasmin (Cp), serum amyloid A (SAA), and ferritin are reported (Paltrinieri et al., 2016). The feline APP partly differe from dogs, and we investigated SAA, with 26% of FeL positive cats detected with increased SAA concentrations, however no significant association was found. Similarly we did not find associations with any other marker of inflammation evaluated in this study. It will be probably useful to make this analysis including just cats with high titers suggestive of progression of infection.

Clinicopathological abnormalities more frequently reported in *Leptospira* spp. positive dogs are neutrophilia (sometimes with a left shift), lymphopenia, monocytosis, mild-to-severe thrombocytopenia and mild to moderate anemia (Schuller et al., 2015). Neutrophilia with or without left shift and toxic changes, lymphopenia, monocytosis, moderate thrombocytopenia and hyperproteinemia were also reported in *Leptospira* spp. positive cats (Arbour et al., 2012, Ojeda et al., 2018) and similarly we found in the present study an association with monocytosis (antibody positivity: OR= 3.769; PCR positivity: OR= 4.821; overall PCR: OR=4.267) and neutrophilia (PCR positivity: OR=6.533; overall PCR: OR=3.974).

FeMV was isolated for the first time from cats in Hong Kong and it was initially associated with tubulointerstitial nephritis (TIN) (Woo et al., 2012). However FeMV role on feline health is still not clear. A recent study suggested that the virus is able to infect lymphoid cells (Sieg et al., 2019). In the present study we evaluated for the first time CBC abornormalities and markers of inflammation in cats exposed to FeMV and we found a significant association with neutrophilia (OR=2.287), monocytosis (OR=2.614) and thrombocytosis (OR=13.72). These CBC changes are compatible with inflammation. Moreover, significant increases of total globulins (antibody positive: OR=4.969; urine PCR positive: OR= 3.422; overall PCR positive: OR=2.882; , overall positive: OR=6.049) and α-globulins (OR=5.067) (fig 5.b) support this hypothesis. Moreover in chapter 4, we described as FeMV exposure can be associated to several clinical manifestations, such as oral, dermatological and ocular

lesions, as well as respiratory signs and these may have contributed to the development of the clinicopathological abnormalities found.

Fig 5.b FeMV positive cats SPE



Both cats were positive exclusively to FeMV among infectious pathogens investigated and both in urine PCR.

Hyperglobulinemia and policlonal hypergammaglobulinemia are widely reported in cats affected by FIV (Sparkes et al., 1993; Hosie et al., 2009). This clincopathological finding could be determined both by presence of concurrent infections and B-cell hyperactivation. During FIV infection, a generalized immune hyperactivation of both B-cells and T-cells through the course of infection can occur. In FIV infected cats, both with experimental and natural infection, an aberrant polyclonal B-cell hyperactivity results in expanded lymphatic B-cell areas, hypergammaglobulinemia and production of antibodies against virus-specific and non-virus-specific antigens (Gleich and Hartmann, 2009). In the present study we found that the 32% (12/38) of FIV positive cats were affected by hyperglobulinemia and, as in FeMV positive cats, a significant association was found (OR= 4.4). Similarly to previous studies (Segev et al., 2006) we also found a significant association with presence of LAI (OR= 3.15).

In conclusion, although with the limitation of an univariate analysis, several variables were found associated with CBC abnormalities and changes of markers of inflammation in the population under study and in some cases the investigated pathogens were also involved. They include outdoor husbandry as risk factor for anemia, monocytosis and neutrophilia; adult age for anemia, neutrophilia,

eosinophilia, eosinopenia and increased total gobulins; senior age for anemia, neutrophilia, eosinopenia, increased total globulins; Calabria origin and urban environment for thrombocytopenia; suburban environment for neutropenia; reduced BCS for anemia, neutrophilia, lymphopenia, monocytosis, thrombocytosis, LAI, increased SAA, decreased ALB; poor MCS for anemia, neutrophilia, monocytosis, thrombocytosis, LAI, increased SAA; pale mucous membranes for anemia, neutrophilia, lymphopenia, eosinopenia, monocytosis, thrombocytopenia, LAI, increased SAA; gastrointestinal signs for neutrophilia, lymphopenia, monocytosis and LAI; respiratory signs for neutrophilia, monocytosis, thrombocytosis, LAI; skin lesions for neutrophilia, monocytosis; chronic kidney disease for anemia, neutrophilia; oral lesions for lymphopenia, eosinopenia; enlarged lymph nodes for neutrophilia. Concerning pathogens investigated: *L. infantum* was a risk factor for anemia, monocytosis, thrombocytosis, eosinopenia, increased total globulins; *Leptospira* spp. for neutrophilia and monocytosis; feline morbillivirus for neutrophilia, monocytosis, thrombocytosis, increased α-globulins, increased total globulins; feline immunodeficiency virus for LAI and increased total globulins.

The risk factors detected in the population under study for cat inflammatory conditions and CBC abnormalities should be carefully considered when cats are examined in veterinary practice.

CHAPTER 6
GENERAL DISCUSSION AND CONCLUSION

Chronic kidney disease (CKD) is a common condition in cats, with a high prevalence reported (Marino et al., 2014). Different risk factors have been found associated to feline CKD, such as age especially for old cats, sex with higher frequency in male cats, breed of pedigree cats (Reynold and Lefebvre, 2013), hypethyroidism (van Hoek et al., 2009), and periodontal disease (Finch et al., 2016; Greene et al., 2014). Immune-complex deposition was demonstrated in glomeruli of feline infectious peritonitis (FIP) (JacobseGeels et al., 1980), feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) (Rossi et al., 2019) affected cats. However the role of *Leptospira* spp., feline morbillivirus (FeMV), and *Leishmania infantum* in feline CKD (Woo et al., 2012; Rodriguez et al., 2014; Pennisi et al., 2004, 2015) still needs to be understood. Moreover, for some infectious diseases evaluated in the present study such as FIV, FeLV, feline coronavirus (FCoV) there are several informations about their clinical and clinicopathological characteristics, but the role of some feline emerging pathogens such as *L. infantum*, feline morbillivirus (FeMV), *Leptospira* spp. in feline health status has to be better defined.

The results obtained in this thesis provided the prevalence of renal disease in the investigated population, risk factors and clinicopathological features more frequently associated with CKD.

For the first time cat concurrent exposure to *L. infantum*, FeMV and *Leptospira* spp. is investigated and associated clinical and clinicopathological features are reported with a focus on CKD.

Additionally common viral infections (FIV, FeLV, and FCoV) affecting the cat immune system were evaluated and their association with the emerging pathogen under study was investigated for the first time.

- In the first study the high prevalence of CKD found confirms as this disease is quite common in cats. In a large recent study that investigated risk factors and survival of cats diagnosed with CKD in UK primary care practices prevalence found was of 1.2%, and in most of the cats CKD diagnosis was performed when cats showed two or more

clinical signs and this imply that at least the 75 % of kidney function was already lost and that those cats with subclinical disease may be missed (Conroy et al., 2019). We diagnosed CKD in 22% of cats admitted for annual health check and in 18% of cats presented for elective surgery. This means that diagnosis was in some way unexpected to the owners. Moreover, frequency of CKD diagnosis was quite the same in the three age groups unlike what was reported in previous studies (Trevejo et al., 2018, Marino et al., 2014). This means that about half of the examined junior cats (< 1 year old) suffered from CKD, therefore, the annual health check should include urinalysis at any adult age. As previously reported, in our study male cats seem to be more frequently affected by CKD. Moreover inappropriate USG was found more frequently in male compared with female cats as well as higher proteinuria values, affecting mainly entire male cats. Moreover, we found an association with oral lesions, generally reported as a possible risk factor for CKD development (Finch et al., 2016; Greene et al., 2014). However we did not know the time of onset of these lesions, therefore we cannot exclude that they were a consequence of CKD rather than a risk factor related to its development. Considering the high prevalence of CKD as well as the high morbidity and mortality in cats affected (Finch et al., 2016), an early diagnosis of the disease both using markers able to perform an early diagnosis and considering risk factors that predispose to development of CKD is required. We did not find significant associations between CKD and FeMV, *Leptospira* spp., or *L. infantum* infections, as well as with retroviral or FCoV infections. However a different methodological approach, such as longitudinal studies more extensive cross-sectional investigations with a clinically healthy control group, could confirm or not our results.

- In the second study we investigated and reported for the first time the prevalence of FeMV and *Leptospira* spp. in cats from Southern Italy, detecting antibodies against several *Leptospira* serovars (ballum, bratislava, poi, pomona, lora, aroborea, mini) previously found in other host species in Italy. Moreover we documented prevalence of *L. infantum,* FIV, FeLV, and FCoV in cats from Southern Italy, and these data support what was already reported in the literature. This is the first time that coinfections between these pathogens are documented and according to our results few cats (32 in total) were negative to any investigated pathogens while coinfections with two to four pathogens were commonly found. Interestingly most of these pathogens

shared similar clinical or clinicopathological signs. The role of FIV in acquired immunedeficiency is widely known (Hosie et al., 2009), and this predisposes cats to the development of further non infectious and infectious pathologies, such as feline leishmaniosis (Pennisi and Persichetti, 2018). Less is known about the relationship between FeMV infection/exposure and feline immune system. Our results suggest that this virus can compromise feline health determining several clinical signs, therefore further studies could help to understand pathomechanisms responsible of the associations found. Concerning the pathogenic role of *Leptospira* spp. in cats, our results are in line with previous studies where clinical signs were rarely reported in this host species (Schuller et al., 2015). However, the high prevalence found of cat exposure to the same serovars reported in other host species in Italy, suggests to evaluate the cat role in transmission of this zoonotic pathogen. Conversely, we found that *L. infantum* exposure or infection are a risk factor for increased serum creatinine values as well as proteinuria. This is the first time that this relationship is reported. A study performed with the same IFI cut off in cats from the same area did not find these associations (Persichetti et al., 2018). However we enrolled cats with older age, and as confirmed by our results, antibody positivity seems to increase in older cats and this may have determine our results.

-The results obtained in the third study, revealed as changes in complete blood count (CBC) or markers of inflammation were associated to some risk factors. We found a significant association between Calabria origin or urban environment and thrombocytopenia, as well as between outdoor husbandry and anemia, neutrophilia, and monocytosis. Husbandry and lifestyle influence exposure to infectious pathogens as outdoor cats could be more exposed to contact with other cats. Adult or senior cats were affected by several abnormalities in CBC and markers of inflammation evaluated, probably for a reduction in immune defenses and a greater predisposition to age-related diseases. This is the first time that CBC abnormalities and markers of inflammation were examined in relation to *Leptospira* spp. or FeMV. Several abnormalities were associated with *L. infantum*, *Leptospira* spp., FeMV and FIV and in most cases changes of parameters suggestive of inflammation has been observed.

In conclusion, prevalence of CKD in cats is high and should be evaluated routinely and at any age. The prevalence of the three emerging pathogens analyzed: *L. infantum*,

Leptospira spp. and FeMV in Southern Italy is not negligible and considering the impairment on feline health status reported in the present study, their investigation in cats affected by clinical or clinicopathological signs compatible with infection/exposure is required.

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PUBLISHED ARTICLES AND POSTERS

Isolation and genome sequences of two Feline Morbillivirus genotype 1 strains from Italy

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Feline morbillivirus, Isolation, Sequencing.

Summary
Feline morbillivirus (FeMV) is a novel viral paramyxovirus detected in cats. FeMV is suspected to be associated to tubulointerstitial nephritis, but its pathogenic role is far to be clearly understood. In this short communication, we report the whole genome coding sequences of the first two FeMV strains isolated in Italy.

Isolamento e sequenziamento del genoma di due ceppi di Feline Morbillivirus genotipo 1

Parole chiave
Feline morbillivirus, Isolamento, Sequenziamento.

Riassunto
Il morbillivirus felino (FeMV) è un nuovo paramyxovirus rilevato nei gatti. FeMV è sospettato di essere associato a nefrite tubulointerstiziale, ma il suo ruolo patogenetico non è stato ancora ben compreso. In questa breve comunicazione sono riportate le sequenze dell'intero genoma dei primi due ceppi FeMV isolati in Italia.

The genus *Morbillivirus* includes several enveloped negative-sense single-stranded RNA viruses infecting humans and animals. Feline morbillivirus (FeMV) is a novel morbillivirus infecting cats and first described in stray cats from Hong Kong nearly ten years ago (Woo *et al.* 2012). Soon after, FeMV circulation was detected worldwide (Furuya *et al.* 2014, Park *et al.* 2014, Sakaguchi *et al.* 2014, Lorusso *et al.* 2015, Sieg *et al.* 2015, Sharp *et al.* 2016, Yilmaz *et al.* 2017, Darold *et al.* 2017).

FeMV isolation on cell culture has been described to be difficult and time consuming (Sakaguchi *et al.* 2014) and a limited number of viral isolates and related whole genome sequences are, indeed, publicly available. Here, we describe the complete genome coding sequences of two FeMV isolates from Italy. Urine samples were taken in March 2018 from two male cats (Tremedino and Pepito, 1 and 7 year old, respectively), living in Reggio Calabria (Calabria region, Southern Italy). The two cats did not show clinical and laboratory signs of renal damage (Donato, manuscript in preparation). Briefly, the first cat (Tremedino, one year old, domestic short-air, male cat) showed stomatitis and an enlargement of popliteal and submandibular lymph nodes, whereas the second cat (Pepito, 7 year old, domestic long-hair male cat) was overweight and presented for stomatitis and bilateral otitis. In both cats, no abnormalities suggestive of renal disease

were recorded at history or physical examination. In the first cat, the haemato-biochemical profile indicated mild eosinophilia [2.85 K/µL, reference range (RR) = 0.17-1.57 K/µL], and severe thrombocytopenia (platelet count 20 K/µL, RR = 300-700 K/µL; low platelet concentration after blood smear examination). Creatinine was 1.1 mg/dl (RR = 0.8-2.4 mg/dL) with serum symmetric dimethylarginine (SDMA) within normal limits [10 µg/dL, reference value (RV) = ≤ 14 µg/dL]. Urine specific gravity (USG) was 1,056 (RV = > 1,035), with a Urine Protein to Creatinine Ratio (UPCR) of 0.09 (RV > 0.4); struvite crystals were also observed. In the second cat, haemato-biochemical profile and urinalysis were unremarkable with serum creatinine (1.0 mg/dL) SDMA (8 µg/dL) and USG (1,038) within normal limits. When urine was collected, an aliquot was immediately 1:8 diluted with MEM for virus isolation. RNA was purified from 280 µL of undiluted urine samples (Biosprint 96 One-For-All-Vet Kit) and tested by a FeMV specific real time RT-PCR (De Luca *et al.* 2018). Resulting C_q values were 35 and 32, for Tremedino and Pepito, respectively. As for virus isolation, five hundred µL of diluted FeMV RNA-positive urine was centrifuged at 3,000 rpm for 5 min to remove debris and filtered through 450 nm disc filters (Millipore). TPCK trypsin (Sigma-Aldrich, Zwijndrecht, The Netherlands) was then added to a final concentration of 0.1 µg ml^{-1}. Samples were incubated at 37 °C for 15 minutes. The mixture was then inoculated into feline embryonic fibroblast (FEA) cells in 24-well plates serum-free Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, Zwijndrecht, The Netherlands) supplemented with penicillin (100 units ml^{-1}) and streptomycin (100 µg ml^{-1}) (Invitrogen). After 8 hours, inocula were replaced with MEM (total volume 1 ml) supplemented by 3% heat inactivated fetal calf serum and antibiotics. Cells were incubated at 37 °C in a humidified atmosphere with 5% of CO_2 and observed daily for cytophatic effect by microscopy. At the 1st cell passage, syncytia were evident at day 8. Cells were stained by May Grünwald-Giemsa (Figure 1a). RNA was purified (QIAamp® Viral RNA minikit, Qiagen, Germantown, MD, USA) from 140 µL of cell culture supernatants and tested by real time RT-PCR for FeMV (C_q 26 and 23 for Tremedino and Pepito, respectively).

FEA cells that tested positive by real time RT-PCR were also fixed in chilled acetone at - 20 °C for 20 min. Fixed cells were incubated with 1:100 dilution of rabbit polyclonal antibody against the N protein of FeMV (kindly provided by Dr Shigeru Morikawa, National Institute of Infectious Diseases, Tokyo), followed by incubation with a FITC-goat anti-rabbit IgG (Sigma-Aldrich, Zwijndrecht, The Netherlands) 1:32 diluted. Cells were then examined under a fluorescence microscope and imaged using the Leica TCS SP5 II confocal laser scanning microscope. Uninfected FEA cells were used as negative control. Infected cells tested positive for FeMV (Figure 1b).

Isolates were named FeMV Tremedino/2018 Italy and FeMV Pepito 2018/Italy, further passaged and stored at - 80°. Total RNA was purified from 300 µL of supernatant of the first passage by using the QIAamp® viral RNA minikit (Qiagen, Germantown, MD, USA). Sequencing was performed by using a combination of sequence-independent/single-primer amplification (SISPA) and next generation sequencing (NGS) as previously described (Marcacci *et al.* 2015). Library preparation was carried out by using the Nextera XT Library Prep kit (Illumina Inc., San Diego, CA) according to the manufacturer's protocol. Sequencing was

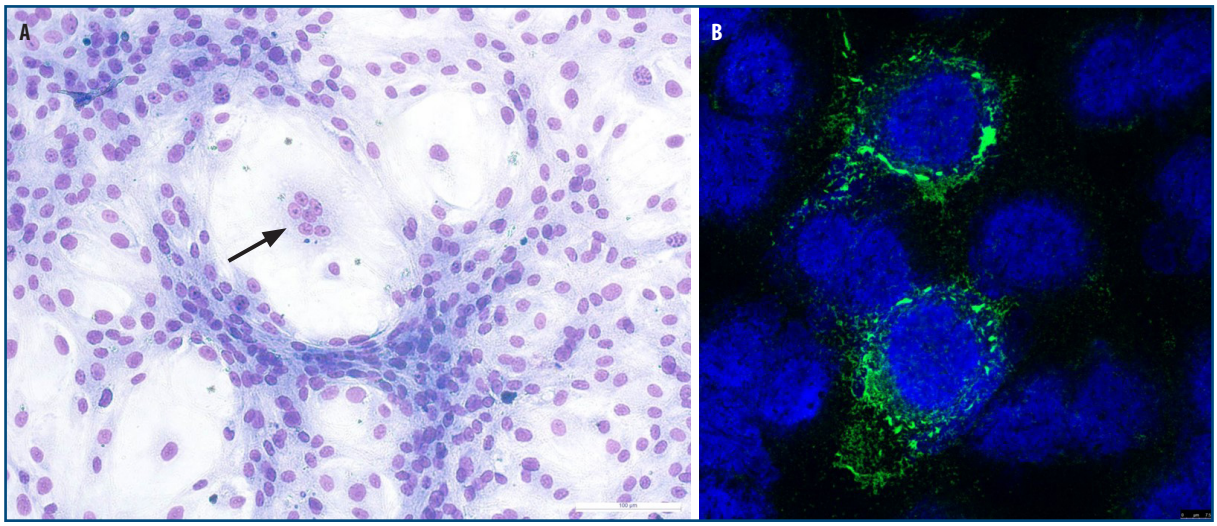

Figure 1. *FEA cells infected by FeMV Pepito2018/Italy.* Multinucleated syncytium was observed by May Grünwald-Giemsa staining (**A**); strong and specific cytoplasmic fluorescence (green color), nuclei are stained with DAPI (blue) (**B**). Scale bar = 100 µm (A), 75 µm (B).

performed on the NextSeq 500 (Illumina Inc., San Diego, CA) using the NextSeq 500/550 Mid Output Reagent Cartridge v2, 300 cycles and standard 150 bp paired-end reads. The resulting 3,539,382 and 1,421,904 reads for Tremedino and Pepito, respectively, were *de novo* assembled by SPAdes v3.8.0. A total number of 266,976 and 388,432 reads mapped on a reference FeMV sequence (GenBank accession number AB924120, strain OtJP001). The length of the final *de novo* assemblies were of 16,027 and 15,946 bp for FeMV Tremedino2018/Italy and FeMV Pepito2018/Italy, respectively. The obtained nucleotide (nt) genome sequences were compared to those of extant FeMVs available online and genetic distances were calculated by using MegAlign (Lasergene 15.0, Madison, WI, USA). The genome sequences of Tremedino2018/Italy and Pepito2018/Italy were found to be nearly identical as they share the 99.2% of nt identity. Nt identity between sequences obtained in this study and extant whole FeMV genome sequences ranges from 98.7% to 78.1%. Tremedino2018/Italy and Pepito2018/Italy showed the highest % of nt sequence identity with the Japanese strains SS1 (98.7%, AB910309) and OtJP001 (98.5%, AB924120); nt identity was lower with the early FeMV strains 761U and 776U (87.8%, JQ411014 and JQ411015) isolated in Hong Kong (Woo *et al.* 2012) and with strain US1 from USA (87.9%-87.8%, KR014147). Tremedino2018/Italy and Pepito2018/Italy strains share the 88.1% of nt identity with Piuma/2015, the first FeMV strain described in Italy in 2015 (Lorusso *et al.* 2015). Very recently, a new genotype of FeMV, tentatively named FeMV genotype 2 (FeMVGT2), was described in Germany (Sieg *et al.* 2019). FeMV sequences obtained in this study share the 78.1% of nt identity with FeMVGT2 sequences. Overall, our results confirm the viral heterogeneity existing between FeMV circulating strains and that the strains described in this study belong to the FeMV genotype 1. Further molecular analysis of FeMV strains circulating in Southern Italy is currently underway (Donato, manuscript in preparation) as well as the assessment of a serum-neutralization assay to quantify specific FeMV antibodies in cat serum.

Nucleotide sequence accession numbers

Nucleotide sequences of Tremedino2018/Italy and Pepito2018/Italy have been deposited in GenBank with accession numbers MK088516 and MK088517, respectively.

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MORBILLIVIRUS RNA AND ANTIBODY PREVALENCE IN CATS WITH RENAL DISEASE INVESTIGATED BY MEASURING SERUM SDMA

Giulia Donato, Eliana De Luca, Federica Pizzurro, Marisa Masucci, Alessio Lorusso, Maria Grazia Pennisi

ISFM European Feline Congress, Sorrento (Italy), June 27th-July 1st 2018, 140

SUMMARY

Feline morbillivirus (FeMV) infection was investigated in 61 cats from Southern Italy (Calabria and Sicily regions). Data from signalment, history, physical examination, biochemical profile and urinalysis were collected and FeMV infection was evaluated by a specific qPCR on nucleic acids purified from urine and blood samples. Antibody detection was performed by IFAT. In total, nine urine and 2 blood samples were RT-PCR positive. Anti-FeMV antibodies were detected in 17 cats. Viral shedding with urine was detected in four cats over a 4-to-29 week period. In this study we describe for the first time FeMV in cats from Southern Italy and based upon the obtained results, a higher number of qPCR positive cats was revealed in Sicily. The number of qPCR positive cats was significantly higher in suburban/rural areas compared to urban areas.Cats with CKD were not more frequently associated with FeMV infection. SDMA evaluation changed the number of CKD diagnoses and CKD stage in some examined cats.

INTRODUCTION

Feline morbillivirus (FeMV) is an enveloped, negative-sense single stranded RNA virus belonging to the family Paramyxoviridae and recently associated with tubule-interstitial nephritis in cats. It was isolated for the first time in Hong Kong (2012) and subsequent studies found it in Japan, Germany, Italy (Abruzzo, Piedmont and Lombardia regions), USA, South America, Turkey and United Kingdom.

The aim of this study was: to investigate FeMV prevalence in cats from the South of Italy, to evaluate association between FeMV and clinical data, including diagnosis of chronic kidney disease (CKD) according to the International Renal Interest Society (IRIS), to explore the influence of symmetric dimethylarginine (SDMA) measurement in staging of CKD.

MATERIALS AND METHODS

Cats: 61 cats admitted in 2017 for health problems (50 cats) or for annual check (11 cats) in Southern Italy (29 cats from Calabria and 32 from Sicily regions). Clinical data were collected (tables 1 and 2).

Diagnosis of CKD: history, physical examination, urinalysis, serum creatinine (0,8-2,4 mg/dL) and SDMA (>14 mcg/dL) measuring. CKD staging was done according to IRIS guidelines and when SDMA was > 14, ≥ 25 or 45 µg/dL, stage 1, 3 and 4 were respectively assigned.

FeMV RNA assay: purified RNAs from urine and EDTA-blood of cats were tested for feline morbillivirus (FeMV) by qPCR (De Luca et al., 2018).

FeMV antibody detection: anti-FeMV antibodies were tested by IFAT (cut off: 1:40) in 57 cats.

Virus isolation: isolation was attempted from qPCR-positive urine sample onto FEA cells.

Statistical analysis: Fisher's exact test was used to investigate associations between qPCR or IFAT positivity and several variables (tables 1 and 2), diagnosis of CKD, IRIS staging of CKD (including or not SDMA evaluation).

Table 1: cat data (number of cats)
BREED domestic shorthair (53) domestic longhair (8)
SEX males (32) ; females (29)
AGE (range 6-204 months) < 84 months (32); ≥84 months (29)
BODY CONDITION SCORE (5/5) <3 (19) ; 3 (27); >3 (15)
MUSCLE CONDITION SCORE (4/4) 1 (35); 2 (7); 3 (10); 4 (6) Unknown (3)
LIFESTYLE Outdoors (43); Indoors (18) Indoors in multi-cat household (10) Indoors in single-cat household (8)
REGION Calabria (29) Sicily (32)
ENVIRONMENT Urban (31) Suburban/rural (30)

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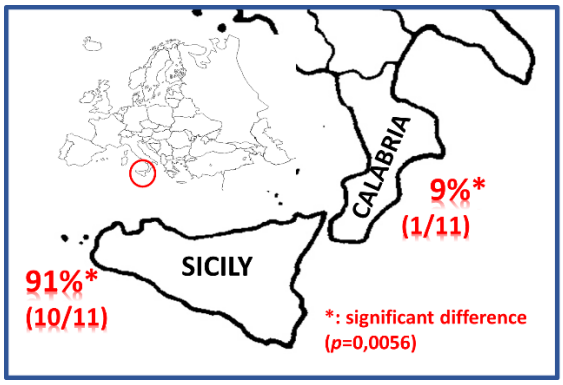
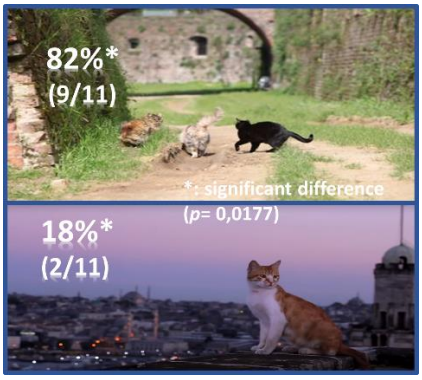
RESULTS

qPCR: urine (nine cats) and blood (two cats) samples were demonstrated to be positive for FeMV by qPCR (overall 18% qPCR positivity). Persistence of viral shedding was confirmed in 4 qPCR-positive cats over a 4-to-29 week period in the follow up of urine samples.

Isolation: FeMV (FeMV TE6073/2018) was successful isolated at passage 3 onto FEA cells from the urine sample of one cat (figure 1).

Significant difference in RT-PCR positivity according to region (Sicily vs. Calabria) (figure 2) and environment (urban vs. suburban or rural areas) (figure 3).





IFAT: anti-FeMV antibodies were detected in 17 cats (29.8%) and five of them were also qPCR positive (3 in the urine and 2 in blood samples).

Table 3: CKD diagnosis and IRIS staging based on creatinine values and FeMV antibody or PCR (urine or blood) positivity

CKD diagnosis and staging	Number of CKD diagnosis based on creatinine values	Number of PCR positive cats	Number of IFAT positive cats
No CKD	27	4	8
AT RISK	13	3	2
STAGE 1	10	2	2
STAGE 2	2	1	1
STAGE 3	3	0	2
STAGE 4	6	1	2

Table 4: CKD diagnosis and IRIS staging based on SDMA values and FeMV antibody or PCR (urine or blood) positivity

CKD diagnosis and staging	Number of CKD diagnosis based on SDMA values	Number of PCR positive cats	Number of IFAT positive cats
No CKD	16	3	3
AT RISK	9	2	1
STAGE 1	23	3	7
STAGE 2	3	2	2
STAGE 3	2	0	1
STAGE 4	7	1	3

DISCUSSION

The overall prevalence of FeMV was 18%, which was similar to the prevalence documented in Japan (6%-23%), but higher than that found in Hong Kong (12,3%), UK (13%), Germany (6,7%), Turkey (5,4%), USA (3%).

As previously reported, FeMV was detected in cat urine and some cats were qPCR positive in the urine up to 29 weeks. FeMV RNA was also detected in blood (two cats).

Cats from suburban/rural areas were more exposed to FeMV compared to cats from urban areas. Cats with CKD were not more frequently FeMV positive as previously observed in studies performed in Brazil and UK.

Increased SDMA values were found in cats without urinary or serum abnormalities suggestive of CKD and we will further evaluate and follow up these cats.

We reasonably acknowledge that the number of enrolled cats represents a limit of this study. Therefore, a larger epidemiological survey is currently ongoing to better understand the role of FeMV in feline medicine.

EFFECT OF STORAGE TIME AND TEMPERATURE IN THE CLASSIFICATION OF CAT URINE PROTEIN-TO-CREATINE RATIO

Giulia Donato, Cyndi Mangano, Maria Flaminia Persichetti, Vito Priolo, Maria Grazia Pennisi

ISFM European Feline Congress, Sorrento (Italy), June 27th-July 1st 2018, 140

SUMMARY

We evaluated the effect of storage time and temperature in urine protein-to-creatinine ratio (UPC) examining 38 feline urine samples. Urinalysis and UPC were performed within 2 hours after collection (T0) and UPC was re-evaluated after 12, 24 or 48 hours of storage of samples at room temperature or +4°C and after 7 or 90 days of storage at -20°C.The UPC values of samples stored at +4°C for 48 hours and at -20°C (both 7 and 90 days) were significantly different from those found at T0. Five samples with UPC ratios close to the threshold values between consecutive IRIS proteinuria substages were misclassified in stored samples. Preanalytical variability due to storage of urine samples may significantly change UPC values and clinical classification of proteinuria.

INTRODUCTION

Urine protein-to-creatinine ratio (UPC) is the most used method to quantify proteinuria in dogs and cats and it was included by the International Renal Interest Society (IRIS) in the substaging system for chronic kidney disease to classify cats as proteinuric (UPC >0.4) border-line proteinuric (UPC 0.2-0.4) non-proteinuric (UPC <0.2). Usually, practitioners have to deliver to external laboratories urine samples intended for UPC ratio evaluations. On the other hand, especially during research activity, samples may have to be stored for some time before performing UPC ratio measurement and freezing may be needed.

AIM

Evaluate the effect of urine storage time and temperature in the evaluation of UPC ratio in cats.

MATERIAL

URINE SAMPLES- at least 6 ml obtained by cystocentesis or voided samples from 38 cats.

URINE TEST STRIPS- Combur 9® (Roche Diagnostics, Swiss).

REFRACTOMETER- Reichert VET360 (Reichert Technologies, NY- USA).

CHAMBERED MICROSCOPE SLIDES- Kova Glasstic slide 10 (Kova Int., CA-USA).

CHEMISTRY ANALYZER- Catalyst DX® and slides urine P:C ratio, IDEXX laboratories (Hoofddorp, The Netherlands).

METHODS

Urinalysis and UPC were performed within 2 hours after collection (T0) and different aliquots of the urine supernatants were used to evaluate UPC ratio after storage at: room temperature (RT) for 12 (30 samples), 24 (32 samples) or 48 (25 samples) hours; +4°C for 12 (28 samples), 24 (34 samples) or 48 (30 samples) hours; -20°C for 7 (35 samples) or 90 (29 samples) days. The complete set of eight reruns was obtained for 20 samples only.

STATISTICAL ANALYSIS

Friedman test was performed to compare results obtained at different times for each storage condition (RT, +4°C, -20°C). Afterwards, significant comparisons were explored by Wilcoxon matched-pairs signed rank test.

RESULTS

URINARY PROTEINS (fig.1) - significant difference between values determined at T0 and after storage: at room-temperature for: 12 hours ($p=0.0083$), 24 hours ($p=0.0223$), 48 hours ($p=0.0356$); at +4°C for: 12 hours ($p=0.0001$), 24 hours ($p=0.0069$), 48 hours ($p=0.0002$); at -20°C for: 7 days ($p <0.0001$) or 90 days ($p=0.0014$).

URINARY CREATININE- no significant difference between values at T0 and concentrations measured at the following evaluations of the stored samples.

UPC RATIO (fig.1) - significant difference between values obtained at T0 and after storage for: 48 hours at +4°C ($p= 0.0007$); 7 days ($p= 0.0019$) or 90 days ($p=0.0025$) at -20°C. Storage at -20°C for 90 days yielded up to 50% decreases of UPC values in four highly proteinuric samples (UPC range: 1.11-8.0). Changes of UPC values close to the threshold between consecutive IRIS proteinuria substages led to misclassification of five urine samples (table 1).

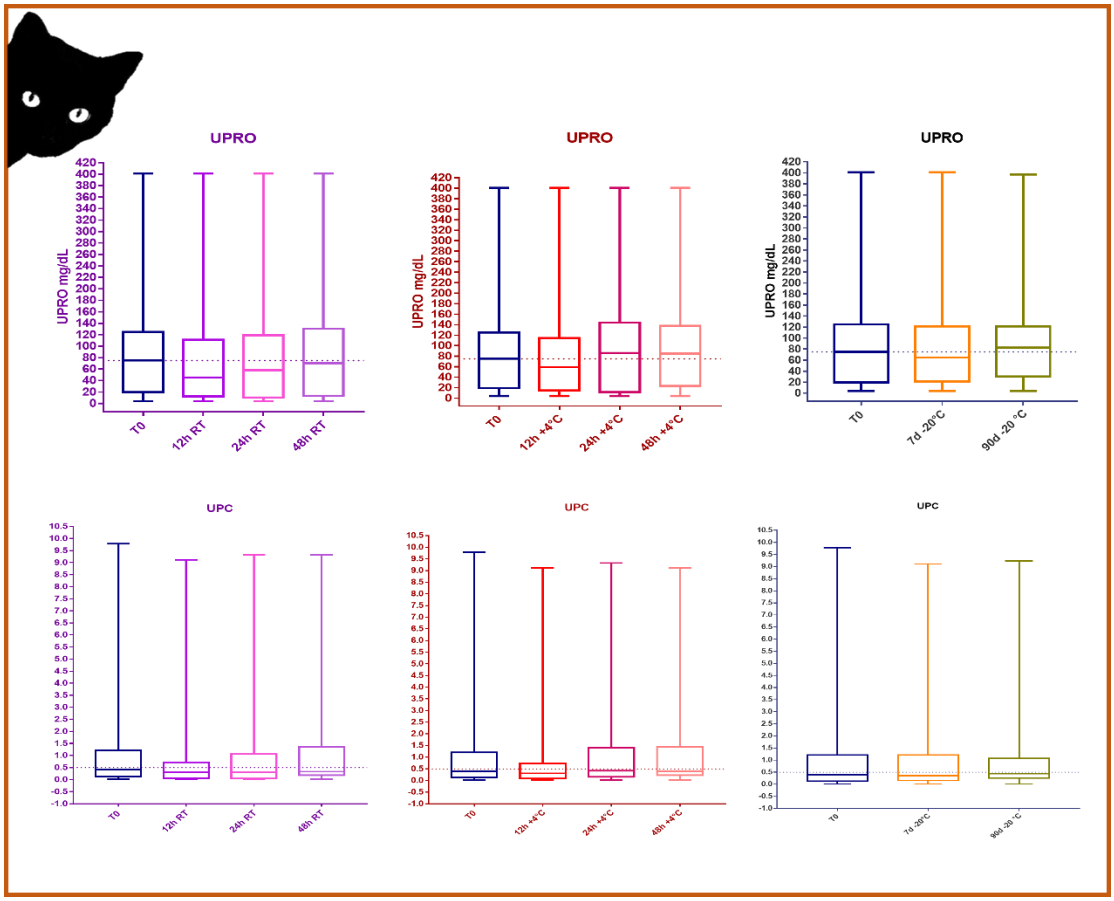


Figure 1: box and whiskers graphs representing the distribution of urine protein concentrations (upper), and UPC ratios (lower) determined at the time of sample collection (T0) and after 12,24,48 hours of storage at room temperature (RT) (left) and at +4°C (middle); 7 and 90 days of storage at -20°C (right).

Storage	Non-proteinuric cats	Border-line proteinurics	Proteinurics cats
T0 : ≤ 2 hours at RT)	3	8	9
12 hours at RT	3	7	10
24 hours at RT	4	6	10
48 hours at RT	3	7	10
12 hours at +4°C	3	7	10
24 hours at +4°C	4	6	10
48 hours at +4°C	3	8	9
7 days at -20°C	4	7	9
90 days at -20°C	4	7	9

Table 1: Number of cats (out of 20 with a complete set of eight reruns) classified as proteinuric, border-line proteinuric or non-proteinuric according to storage conditions of their urine sample

DISCUSSION

Preanalytical variability due to storage of feline urine samples at RT, +4°C or -20°C can significantly change UPC values from a diagnostic and prognostic standpoint. In fact, five samples with UPC ratios close to the threshold values between consecutive IRIS proteinuria substages were misclassified in stored samples.

This source of error should be considered when interpreting UPC data obtained from samples shipped to external laboratories or from freezed samples analysed in retrospective studies.

COMORBIDITIES AND COINFECTIONS IN A CAT AFFECTED BY LEISHMANIOSIS

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Congresso SCIVAC: Quali novità nella diagnosi e nella terapia della leishmaniosi, Ferrara, 5-7 aprile 2019.

INTRODUCTION

Feline leishmaniosis (FeL) manifests with skin and mucosal lesions, lymph node enlargement and less frequently with many other signs. However, coinfections or comorbidities concur to clinical manifestations and should be considered. Among them, Chronic Kidney Disease (CKD) is mostly important for prognosis and can be the consequence of immunopathogenesis of FeL. We report a case of FeL in a cat affected by CKD, hepatobiliary abnormalities, rhinosinusitis, coinfections by Feline Immunodeficiency Virus (FIV), Feline Morbillivirus (FeMV), and infestations by *Toxocara cati* and *Aelurostrongylus abstrusus*.

NOVEMBER 27, 2017- Molly, 9 years of age, DSH (domestic short hair), spayed female, foundling cat, housed in a rescue cattery where health care is poor was hospitalized for severe weight loss and large bowel diarrhea but multiple clinical signs (Table 1) and clinico-pathological abnormalities were observed (Tables 2 and 3; figures 1, 2 and 3). Retroviral testing was positive for FIV; coprological examinations was positive for *Toxocara cati* eggs and *Aelurostrongylus abstrusus* larvae. Abdominal ultrasonography found mild peritoneal effusion, biliary sludge, inhomogeneous avascular content with normal gallbladder wall, 3 focal hyperechoic hepatic lesions (~3 mm of diameter) (fig. 5), and slightly increased renal cortical echogenicity. Thoracic Xray was negative for lungworm disease.

List of problems: normotensive (136 mmHg systolic blood pressure), proteinuric IRIS (International Renal Interest Society) stage 1 CKD in a FIV positive cat with severe regenerative anemia, liver focal lesions and increased ALT (alanine aminotransferase) with concurrent *T. cati* and *A. abstrusus* infestations.

Renal diet, fluid and doxycycline (10 mg/kg PO q24h) therapy were started while virological, hemoplasma, *Babesia* and *L. infantum* (*Li*) investigations were pending. Moxidectin&imidacloprid spot-on was administered.

DECEMBER 2017- CBC (complete blood count) rapidly improved but neutrophilic lymphocytic monocytic leukocytosis recurred. Biochemical profile was within reference range, apart from symmetric dimethylarginine (SDMA) concentration (tables 2 and 3). Anti-*Li* antibodies and *Li* DNA were detected (table 4) but amastigotes were not seen in the reactive lymph nodes (fig. 7). FeMV infection was confirmed by antibody detection and positive PCR in urine samples. *Mycoplasma hemofelis* and *Babesia* spp. PCR were negative and doxycycline was stopped after 10 days. Rhinosinusitis was confirmed by X-ray evaluation and amoxycillin/clavulanate (8,75 mg/kg SC SID for 5 weeks) was given according to antibiotic susceptibility testing. Allopurinol was started on December 21 to treat FeL (table 4).

JANUARY-FEBRUARY 2018- overall clinical improvement was observed (tables 1 and 2), but neutrophilic lymphocytic monocytic leukocytosis recurred and SDMA values increased moving CKD IRIS stage to 3 (table 3). Allopurinol was therefore switched with spiramycin plus metronidazole (table 4) and fluid therapy and telmisartan (1 mg/kg PO q24h) were started. Positive FeMV PCR was confirmed in urine samples up to end of January but no longer during following months.

MARCH 2018- IRIS stage of CKD was back to 2 but a progressive clinical worsening occurred with anorexia, weight loss, non-regenerative anemia (tables 1-3) and this was considered a side effect of telmisartan. The drug was stopped and allopurinol was restarted. Marbofloxacyn was also administered because of recurrence of mucopurulent nasal discharge, neutrophilia and monocytosis (tables 1-4).

APRIL-JUNE 2018- CKD stage fluctuated between stage 3 in April when proteinuria peaked and stage 2 in May when body weight further increased, and nasal discharge, anemia, neutrophilia and monocytosis were cured (tables 1-3). Contrast-enhanced abdominal ultrasonography was performed and normal vascularization of gallbladder wall without enhancement of the intraluminal material was observed in addition to normal enhancement of cortex and medulla of left kidney (fig. 4 and 6).

Molly was back from foster care to the shelter at the end of May and adherence to prescribed therapy was no longer provided. Clinical worsening was seen at last check (tables 1-4) and death caused by a trauma was reported in July.

Table 1: Body weight and abnormalities reported at clinical evaluations

CLINICAL EVALUATION	NOV 27	DEC 19	JAN 9	FEB 6	MAR 12	APR 18	MAY 7	JUN 6
Weight (kg)	2.5	2.75	2.95	3.15	3.25	3.5	3.84	3.5
BCS (9/9)	3	3	3	4	4	4	5	4
MCS (4/4)	4	4	4	3	3	3	3	2
Pale (P) Icteric (I) mucous membranes	(P) (I)	(P)	-	-	-	(P)	-	(P)
Enlarged lymph nodes (+/+++)								
- Mandibular	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++	R:+++ L:+++
- Prescapular	R: ++ L: ++	R: ++ L: ++	R: ++ L: ++	R: ++ L: +	R: ++ L: +	R: ++ L: +	R: + L: +	R: + L: +
- Axillary	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +
- Popliteal	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +	R: + L: +
Polyuria/polydipsia (+/-)	+	+	+	+	+	+	+	+
Polyphagia (+/-)	+	+	+	+	-	-	-	-
Fecal score (7/7)	6	3	2	3	2	2	2	Not evaluable
Dehydration (%)	5	5	3	5	5	3	3	3
Mucopurulent nasal discharge (+/+++)	+++	+++	++	++	+	-	-	-
Skin abnormalities (+/+++)								
- Poor coat condition	+++	+++	++	++	++	+	-	-
- Diffuse scaling	+++	+++	++	++	++	+	-	+
- Chronic otitis	+++	+++	++	++	++	++	+	++
- Indolent ulcer	+++	+++	++	+	-	-	-	-

Legenda: R: right; L: left

Tab. 2: Complete Blood Count (CBC) results.

CBC	REFEENCE RANGE	NOV 27	DEC 5	DEC 29	JAN 18	FEB 6	MARCH 29	MAY 7
RBC (M/µL)	6.54-12.20	4.48	5.79	8.44	8.37	8.61	5.88	7.11
HCT (%)	30.3-52.3	16.4	20.2	38.2	36.3	38.2	26.8	29.7
HGB (g/dL)	9.8-16.2	6.6	7.8	10.9	10.7	11.4	8.2	9.4
MCV (fL)	35.9-53.1	36.6	34.9	45.3	43.4	44.4	45.6	41.8
MCH (pg)	11.8-17.3	14.7	13.5	12.9	12.8	13.2	13.9	13.2
MCHC (g/dL)	28.1-35.8	40.2	38.6	28.5	29.5	29.8	30.6	31.6
RDW (%)	15.0-27.0	40.3	31.9	31.3	29.9	30.7	27.1	28.8
RETIC (k/µL)	3.0-50.0 (*)	209.2	74.7	59.9	25.1	55.1	36.5	45.5
WBC (k/µL)	2.87-17.02	56.85	15.44	21.14	16.83	29.18	24.59	12.88
NEU (k/µL)	2.30-10.29	41.1	11.69	11.35	8.96	17.83	18.98	7.74
BAND (k/µl)		0.56	_	_	_	0.29	_	_
LYM (k/µL)	0.92-6.88	9.18	2.32	8.01	5.79	8.11	3.71	4.13
MONO (k/µL)	0.05-0.67	5.39	1.13	1.16	0.95	1.12	1.67	0.52
EOS (k/µL)	0.17-1.57	1.09	0.28	0.59	1.07	1.87	0.15	0.43
BASO (k/µL)	0.01-0.26	0.09	0.02	0.03	0.06	_	0.08	0.06
PLT (k/µL)	151-600	458	548	686	570	414	353	580

Legenda: in red and blue color values respectively > or < reference range. (*): reticulocyte count was considered as marker of non regenerative anemia when < 50 k/µL

Tab. 3: Blood serum biochemical profile, urine specific gravity (USG) and urine proteine to creatinine ratio (UPC) during follow up.

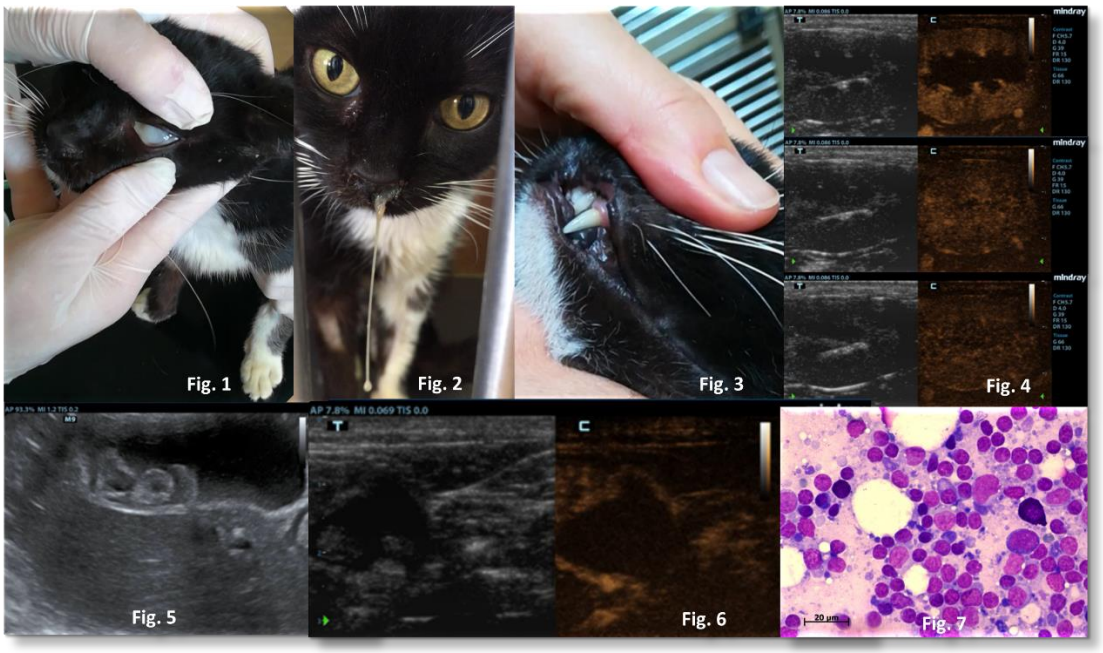
PARAMETER	REFERENCE RANGE	NOV 30	DEC 19	JAN 29	MAR 12	APR 18	MAY 7
GLUCOSE	77-159 (mg/dL)	87	96	93	113	_	51
BUN	16-36 (mg/dL)	35	29	38	23	_	39
CREATININE	0.8-2.4 (mg/dL)	1.5	1.4	1.7	2.1	2,3	2
SDMA	≤14 (µg/dL)	_	22	31	22	26	24
PHOSPHORUS	3.1-7.5 (mg/dL)	_	_	8.4	7.6	_	_
USG		1015-1025	_	1013	1013	1017	_
UPC	<0.4	1.85-2.7	_	1.55	2.78	>4.65	_
TOTAL PROTEIN	5.7-8.9 (g/dL)	7.3	6.9	7.9	8.8	_	7.7
ALBUMIN	2.3-3.9 (g/dL)	2.3	2.2	2.6	2.8	_	2.6
GLOBULINS	2.8-5.1 (g/dL)	5	4.7	5.3	6	_	5.1
ALB/GLOB	_	0.5	0.5	0.5	0.5	_	0.5
ALT	12-130 (U/L)	167	109	95	69	_	105
ALKP	14-111 (U/L)	27	28	40	39	_	56

Legenda: BUN: blood urea nitrogen; SDMA: symmetric d-methyl arginine; ALB/GLOB: albumin/globulins ratio; ALT: alanine aminotransferase; ALKP: alkaline phosphatase. In red or blue color values respectively > or < reference range.

Tab. 4: Anti-Leishmania therapy, IFAT titre, qPCR (Leishmania amastigotes/ml) in blood, conjunctival, oral, and auricolar swabs and lymph node fine needle infission during follow up.

	Nov 27	Dec 29	Jan 18	Feb 6	Mar 29	Apr 18	May 7	Jun 18
Therapy	No	Allopurinol (*)	Allopurinol (*)	Allopurinol (**)	Spyr+Metr (**)	Allopurinol (***)	Allopurinol (***)	Allopurinol
IFAT	1280	5120	5120	10240	10240	2560	5120	1280
qPCR								
EDTA Blood	210	Negative	ND	Negative	Negative	Negative	Negative	ND
Swabs:								
- Conjunctival R/L	1400/520	460/Negative	ND	ND	ND	ND	Negative	ND
- Oral	1500	25	ND	ND	ND	ND	Negative	ND
- Auricular	ND	15	ND	ND	ND	ND	Negative	ND
Lymph node:								
- Mandibular R/L	ND	2340/4320	ND	ND	ND	ND	ND	ND
- Prescapular R/L	ND	ND	ND/30	ND	ND	ND	ND	ND
- Popliteal R/L	ND	ND	ND	ND/35	ND	ND	ND	ND

Legenda: R: right; L: left; ND: not done. Spyr+Metr: Spyramycine and Metronidazole oral tablet.
(*) First course of allopurinol from December 21 to February 12.
(**) Spyr+Metr from February 12 to March 13.
(***) Second course of Allopurinol from March 30



DISCUSSION

Viral, bacterial and nematode infections were diagnosed in a cat affected by FeL who suffered from acute hemolityc anemia, CKD, and persistent hepatobiliary lesions. This latter finding was unfortunately not specifically investigated but it seemed to be not clinically relevant and it was not modified by anti-*Leishmania* and antibiotic treatments. Creatinine evaluation failed to detect CKD, and SDMA measurements, urynalisis and UPC were used to follow up renal disease. Hemolysis detected at admission was doxycycline-responsive but hemoplasmosis was not confirmed. Bacterial chronic rhinosinusitis recurred after antibiotic treatments and impaired immunocompetence due to FIV could contribute to this. The causative role of viral pathogens (FIV, FeMV) and *Li* (individually or by interactions between them) in the multiple manifestations reported and in CKD development cannot be concluded. Interestingly, anemia, BCS and MCS were rapidly improved by both courses of allopurinol therapy despite ongoing CKD and FIV infection.

In conclusion, disease management of cats with FeL complicated by CKD and coinfections is challenging but relevant clinical improvement can be obtained with allopurinol, other required therapies and good nursing care.

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