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Feline Immunodeficiency Virus (FIV) Infection in Cats: A Possible Cause of Renal Pathological Changes

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1. Introduction

The feline immunodeficiency virus (FIV) is a lentivirus isolated from domestic cats with an acquired immunodeficiency syndrome-like condition, named feline AIDS (F-AIDS). The major immunological abnormalities observed in FIV-infected cats included a profound decline in the absolute number of the CD4⁺ T cells that caused the inversion of the CD4⁺/CD8⁺ T cell ratio and increased susceptibility to opportunistic infections and various clinic-pathological conditions [1]. FIV viruses encompass a large group of strains classified in subgroups from A to E, which are unevenly distributed geographically and have an inter-subtype diversity > 26% [2]. The isolates used in our study were Petaluma, of group A, and Pisa-M2, a local isolate belonging to group B, which encloses all isolates hitherto sequenced and circulating in Italy [3 4]. Serological screenings performed in the past demonstrated that the virus is distributed worldwide and incidence varies from 1 – 14% in healthy cats and up to 44% in sick cats. As other lentiviruses, FIV is a complex retrovirus with structural genes *gag*, *pol* and *env*, and a few accessory genes [5]; *gag* encodes the capsid protein p24, used in most diagnostic tests, and other inner structural proteins, *pol* encodes the enzymes necessary for viral replication and therefore targeted by most anti-lentiviral drugs, and *env* encode the outer glycoprotein (gp95) and trans-membrane protein (gp36) serving as viral receptor and, being constantly under immunological pressure, the less conserved proteins among the different subtypes. Like the human immunodeficiency virus (HIV), the gp95 is comprised of variable and conserved regions and binds the CD134 molecule, the FIV primary receptor [6]. Studies on HIV have shown that some conserved epitopes are accessible for neutralizing antibodies, while the co-receptor binding site is composed by interspersed domains. The binding site remains largely hidden and is therefore inaccessible for mentioned antibody. Whereas overall HIV and FIV Env structure is maintained [7], HIV uses various co-receptors, including a range transmembrane domain G-protein-coupled receptors. In

contrast,, all FIV strains tested so far use CXCR4 as a co-receptor [7]. Cats, once infected with FIV, remain infected life-long and in the face of strong humoral and cell-mediated immune responses that appear shortly after the initial viremic phase [2].

The acute phase of infection lasts a few days to a few weeks and is asymptomatic in a large proportion of cats. If clinically overt, it manifests with fever, lethargy and peripheral lymphadenomegaly with possible neutropenia. The acute phase eventually subsides and the infected cat enters in asymptomatic period that typically lasts 4 to 6 years or is life-long in some cats. In 30% cats and with percentages that greatly depend on cofactors and cat life-style [2], the infection proceeds to the last stage, the F-AIDS, that is characterized by profound immunodeficiency and, consequently, the presence of secondary infections sustained by viruses, bacteria, fungi, or protozoa, and various neoplastic diseases. Like HIV, FIV also infects and may damage the central nervous system as demonstrated in the past in experimental conditions [8-11]. As mentioned, clinical presentation and outcome of disease depend upon a combination of secondary factors and host immune responses. Immunodeficiency combined with immunostimulation by various factors most frequently results in the emergence of severe forms of gingivostomatitis, chronic rhinitis, lymphadenopathy, weight loss and immune-mediated glomerulonephritis [2]. Weaver and co-workers reported reproductive failure in FIV-infected cats. Viral DNA in placental and fetal tissues in affected cats was confirmed by PCR [12].

Despite detailed knowledge of most clinicopathological features during FIV infection, information on renal involvement is limited. Unspecified renal abnormalities were reported in some infected cats living in Australia [13] and in 5.5% of those living in New Zealand [14]. Ishida et al found that 9.3% of 700 Japanese FIV-infected cats presented clinical signs of renal diseases [15]. Most pathological findings observed in the kidney of naturally FIV-infected cats resemble those described in HIV-infected patients [16], but it is not clear whether FIV has a direct role in the induction of the renal damage or accelerates a phenomenon triggered by other factors. The fact that renal damage is mostly found in natural FIV infection supports the latter hypothesis [17]. However, since there are no detailed descriptions of the renal lesions found in experimentally FIV-infected cats this issue is still open. The aim of our study was to investigate the histological renal alterations caused by FIV in animals experimentally inoculated with FIV strains of different pathogenicity and at different times post-infection. Here, specific pathogen free (SPF) cats singly or doubly infected with Petaluma and Pisa-M2 were housed in germ-free conditions to exclude the influence of other pathogens. The pathological findings in these animals were compared with those found in naturally infected cats. These results were also compared to those found in HIV patients.

2. Material and methods

2.1. Cats

Ninety-nine naturally infected cats were collected from 1990 to 1993 and after diagnosis of FIV infection performed by western blot. Animals were referred to the Department of

Animal Pathology of the University of Pisa by veterinarian practitioners and owners from different part of Tuscany, Italy. All the subjects were submitted to an accurate clinical examination and classified according to a previously proposed classification [18]. Thirty-six of these subjects were sacrificed and immediately necropsied. There was no investigator bias in this sampling as the only criterion used was the owner's consent to post-mortem examination. Fifteen cats who tested positive for leukemia virus p27 antigen (CiteCombo FIV_FeLV, Agritech Systems, Portland, Me, USA) and feline infectious peritonitis antibodies (Diasystems Celisa FIP, Tech America, Omaha, NE, USA) were excluded from the study, as these viruses are known to be associated with renal alterations. The 21 selected cats were 16 males and 5 females, with a mean age of 96.0 months \pm 40.3 months, 10 cats were in the symptomatic phase of infection phase, while 11 had full-blown F-AIDS.

Fifty-six specific pathogen free SPF cats, infected with Petaluma and/or Pisa-M2 isolates at six-ten months of age, were included in the study. All subjects were females that at time of analyses aged between 2 and 6 years (mean age of 47.8 months \pm 14.4 months). Seven-teen animals were inoculated with Petaluma (group 1), 28 with Pisa-M2 isolate (group 2), and 11 were first inoculated with Petaluma and, one year later, superinfected with Pisa-M2 (group 3). Petaluma isolate was obtained from supernatant of persistently infected FL4 cells [19], while Pisa-M2 was a local isolate propagated *in vivo* by monthly passages in SPF cats and never passaged *in vitro* [20]. Animals were inoculated intravenously with either 2 ml of freshly collected blood (Pisa-M2) or 20 cat infectious dose 50% of FL-4 supernatant (Petaluma). All cats seroconverted in 4-6 weeks and were characterized for a steady reduction in the number of circulating CD4+ lymphocyte that approximately halved in 1 year. Four FIV-negative, SPF cats were used as negative controls (group C). Infected and control cats were housed in biosafety hazard level 3 conditions at the Retrovirus Center of the University of Pisa, and were daily monitored for clinical conditions throughout the observation period. Physical examination was performed weekly for the first two months postinfection (pi) and then monthly. At 12, 24, 30, 36 and 48 months pi, randomly selected animals were deeply anesthetized, and euthanized for necropsy.

2.2. Biochemistry and urine analysis

Urine specimens were obtained by cystocentesis. After centrifugation, supernatants were used to determine protein and creatinine concentration using two commercial assays (BioRad, Richmond, Calif., USA, and Creatinine - Jaffe method, Verbena, Milano, Italy, respectively). In cats with marked proteinuria (>2 g/L), urine protein/urine creatinin ratio (UPC) was calculated using the following formula: $P(\text{g/L}) \times 100 / (\text{Cr mmol/L} / 0.0885)$. Protein qualitative analysis was performed with sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) according to Leamlli [21].

Blood samples for determination of biochemical profile were collected into serum separator tubes (Vacuette, Greiner Bio-One, Kremsmunster, Austria) and stood for 30 min at 4°C to clot, then centrifuged (1300 g for 10 min) to separate the serum. Serum samples were assayed for selected biochemical parameters including urea, creatinine, total protein,

albumin, using an automated analysis on a spectrophotometer (LKB Biochrom Ltd., Cambridge, UK). Serum albumin/globulin ratio was calculated.

2.3. FIV serology

All cats were screened for FIV antibodies and feline leukaemia virus antigen by a commercial ELISA (CITE COMBO FIV-FeLV; Agritech Systems, Portland, Maine) according to manufacturer's instructions.

FIV antibodies, detected with above rapid test, were confirmed by Western blot (WB). FIV antigen for WB analysis was produced from persistently FIV-infected FL4 T-cells. The virus was pelleted from tissue cultures medium, purified throughout a 10 to 50% continuous sucrose gradient and then disrupted by sonication followed by treatment with Triton X-100. Viral proteins were separated by electrophoresis with 12% SDS-PAGE and blotted onto nitrocellulose by standard methods. The nitrocellulose sheets were then blocked with 1% bovine serum albumin, cut into 0.5 cm wide strips, dried, and stored in the cold until use. Individual strips were incubated for two hours with serum samples diluted 1:100 in PBS-Tween 20 (0.05%), washed thoroughly, incubated with horseradish peroxidase-conjugated rabbit anti-cat immunoglobulin G (Bethyl Laboratories, Montgomery, Texas) for one hour. The strips were washed and incubated with 0.05% diaminobenzidine and 0.01% of H₂O₂ (what is this?) in 0.1M Tris pH 7.4 to visualize antigen-antibody binding. Strips were read by densitometric scanning (BioRad) immediately after the reaction was stopped with distilled water. The molecular weights of the reactive proteins were established by comparison with prestained low-molecular-weight markers (BioRad). Each WB contained positive and negative control sera. The sample was scored positively when at least two of p25, p31, gp40, gp65, and gp95 FIV-specific bands were clearly detectable.

2.4. PCR

Buffy coat of 2 ml whole blood samples was used to detect FIV DNA. FIV DNA extraction and amplification was performed as described [22]. All precautions were taken to avoid possible contamination and samples were examined at least twice in separate experiments. DNA from peripheral blood mononuclear cells (PBMC) of uninfected cats and from Petaluma-infected FL-4 cells were used as negative and positive controls, respectively.

2.5. Flow cytometry

T lymphocyte subsets were examined by flow cytometric analysis as described [23]. Flow cytometry analysis was performed by using fluorescein conjugated murine monoclonal antibodies to feline CD4 and CD8 T-cells surface markers (Southern, Biotech, Birmingham, AL, USA) and an Epics Elite cell analyzer (Coulter Electronics, Hialeah, Fla.).

2.6. Histology

Renal tissue samples were fixed in 10% buffered formalin solution and embedded in paraffin. 3µm thick sections from each specimen were stained with hematoxylin and eosin,

periodic-acidic Schiff and Jones periodic acid-silver methenamine. Sections with inflammatory lesions were stained with Ziehl-Neelsen acid-fast and Gram to exclude bacterial infections. Amyloid was demonstrated by the alkaline Congo red staining with polarization on 8 μm sections [24]. Differentiation between primary and secondary amyloidosis was based on staining by a modified Romhanyi method with pre treatment with potassium permanganate [25] and on immunohistochemistry (IHC) with antibodies against amyloid A protein.

2.7. Immunohistochemistry

The localization of IgG, IgA, IgM and C3 deposits was investigated by both indirect immunofluorescence method (IF) and streptavidin-biotin peroxidase method. IF was performed using as primary antibodies primary sheep monospecific antibodies to cat IgG, IgM, IgA and C3 (binding Site, Birmingham, UK) and a rabbit fluorescein anti-sheep IgG (vector Laboratorie, Burlingame, CA, USA), as previously described [16]. Control sections were incubated with normal sheep serum (Dako, Golstrup, Denmark) before treatment with the secondary antiserum. For IHC, sections were de-waxed in xylene, passed through a graded series of alcohols, and rehydrated in deionised water. For Ig and C3 localization, the tissues were digested with 0.5% protease (Protease XXIV; Sigma, Saint Louis, Mo, USA) in 0.05 M Tris-HCl, pH 7.6. Endogenous peroxidases were exhausted with 0.5% hydrogen peroxide for 30 minutes and after that, three washes were performed in 0.05% Tween Tris Buffered Saline solution (TBST) at pH 7.6. Normal serum from the host species of the secondary antibody diluted 1/10 in TBST was added to the sections and incubated for 30 minutes at room temperature. After three washes, the primary antibodies diluted in TBST were applied and incubated for 1 hour at RT. The antisera used included unlabeled sheep anti cat IgG, IgA, IgM and C3, an anti-AA (murine monoclonal mc4 against human AA protein, culture supernatant and anti-cat AA and AL (polyclonal antibody of rabbit origin; kind gift of R. P. Link, University of Munich, Germany). After three washes, secondary biotinylated antibody (Vectastain[®], Vector Labs Inc., Burlingame, CA, USA) was added and incubated for 30 minutes at RT. Peroxidase reaction was developed for 10 minutes using diaminobenzidine (DAB) (Impact DAB[®], Vector Labs inc., Burlingame, CA, USA) and blocked with deionised water. Negative controls were performed omitting the primary antibody and replacing the antibody with normal sheep or rabbit serum or murine subclass matched (IgG₁) unrelated primary monoclonal antibody.

2.8. Statistics

Statistical analysis was performed using the statistical package SPSS Advanced Statistics 13.0 (SPSS Inc., Chicago, IL, USA). Chi-square test was used to investigate the significance of the relationship between protein expression and individual variables. Statistical significance was based on a 5% (0.05) significance level.

3. Results

3.1. Immunological data

All FIV-seropositive cats, as determined by rapid test and then WB analysis, also scored positive for FIV DNA in whole blood samples (data not shown). The experimentally infected cats included in the study were routinely examined for CD4⁺ and CD8⁺ T lymphocyte subsets by flow cytometry and respective CD4⁺/CD8⁺ T cell ratio calculated. Inversion of the CD4⁺/CD8⁺ T cell ratio due to a selective decline in the absolute number of the CD4⁺ T cells was confirmed in 71.4% cats infected with FIV-Pisa M2, 76.5% of FIV-Petaluma infected cats and in all cats infected with both virus strains (Table 1). Although diminution of CD4⁺ T-cells and inversion CD4⁺/CD8⁺ correlated positively with duration of infection, no significant relationships were found between immunological data and renal alteration.

3.2. Clinical data

Total serum protein, albumin and globulins concentration values in experimentally infected cats were in the reference range, regardless infecting virus isolate and time post inoculation. None of the 56 SPF cats included in our study presented clinical signs of azotemia.

Serum creatinine concentration was measured in 37 experimentally infected cats and only one cat, infected with FIV isolate Pisa M2 had slightly increased concentration of serum creatinine (144 μ mol/L), mild proteinuria (3.9 g/L), and UPC ratio of 0.43, which is classified as stage 2 of chronic renal failure (CRF) [26].

Urine protein concentration was measured in all 56 experimentally and 21 naturally infected cats. Eighteen naturally infected cats (82%) had mild to severe proteinuria, mean value 26.46 \pm 22.41 (range 3.5 – 62 g/L). In experimentally infected cats, proteinuria (> 2mg/L) ranged from 18% in cats infected with both virus strains with mean value 14.3 \pm 22.73 (2.7-60 g/L), 32% in cats infected with strain Pisa M2 (mean value 11.78 \pm 22.80, ranging 2.2 – 100) and 58% in cats infected with Petaluma strain, in which the mean value of urine proteins concentration was the lowest 4.25 \pm 2.81 (2 - 8.25 g/L) (meaning not clear to me). UPC in proteinuric cats was calculated in order to confirm the renal proteinuria. UPC values were the highest in cats infected with Pisa M2 (mean 3.27 \pm 6.34; ranged 0.53-17.63), slightly lower in cats infected with both virus strains (mean 3.0 \pm 4.39; ranged 0.55-13.01) and the lowest in cats infected with Petaluma strain (mean 2.56 \pm 3.03; ranged 0.31 – 7.02).

In addition, electrophoresis of urine proteins was performed in an attempt to establish the localization and severity of renal alteration. 10/21 naturally infected cats had glomerulo non-selective proteinuria, 8/21 glomerulo non-selective and tubular, and only 3 of 21 cats had glomerulo selective proteinuria. Glomerulo selective proteinuria was confirmed only in 3 proteinuric cats, infected with Petaluma strain. In the others experimentally infected cats proteinuria glomerulo non-selective was most frequently found: 7/10, 7/9 and 1/2 cats infected with Petaluma, Pisa-M2 and both strains respectively. Remaining 2/9 (infected with

Pisa-M2) and 1/2 cats (infected with both strains) had glomerulo non-selective and tubular proteinuria (Table 1).

No clinical and selected laboratory parameters in SPF cats enclosed in the control group were found.

IRIS staging		Naturally FIV-infected cats	Experimentally FIV-infected cats		
			Petaluma	Pisa M2	Petaluma + Pisa M2
CD4+/CD8+	< 2.0	ND	13/17	20/28	11/11
serum creatinine (stage 1 - 4)	1 (< 140)	9/13	5/5	20/21	11/11
	2 (140-249)	3/13	0/5	1/21	0/11
	3 (250-439)	0/13	0/5	0/21	0/11
	4 (> 440)	1/13	0/5	0/21	0/11
urine protein concentration	< 2 g/L	3/21	5/17	10/28	5/11
	> 2 g/L	18/21	12/17	18/28	6/11
UPC (substaging)	< 0.2 (non proteinuric)	ND	0/12	0/18	0/6
	0.2-0.4 (borderline proteinuric)	ND	2/12	9/18	4/6
	> 0.4 (proteinuric)	ND	10/12	9/18	2/6
SDS-PAGE	GS	0/21	3/10	0/9	0/2
	GNS	10/21	7/10	7/9	1/2
	GNS+T	8/21	0/10	2/9	1/2

Legend: ND - not done; GS - glomerulo selective; GNS - glomerulo non-selective; GNS+T - glomerulo non-selective and tubular.

Table 1. Inversion of the CD4+/CD8+ T cell ratio, serum creatinine value, proteinuria, and urine protein/creatinine (UPC) ratio according to the IRIS classification.

3.3. Light microscopy

Table 2 summarizes the main histological glomerular alterations observed in naturally and experimentally FIV-infected cats. Glomerular changes were detected in 18 of 21 naturally infected subjects. Mild mesangial matrix increase with occasional segmental glomerulosclerosis was observed in 9 subjects (Figure 1A), while immune mediated glomerulonephritis of mesangioproliferative type was detected in 3 animals. In eight naturally FIV-infected cats, amyloid deposition was also detected (Figure 1E). These were segmental and focal in six cases and diffuse in two. In all cases the amyloid deposits were KMnO₄ sensitive. In experimentally FIV-infected animals, glomerular changes were detected in 9 of 17 Petaluma-infected cats, in 12 of 28 Pisa-M2-infected cats and in six of the 11 cats infected with both strain. In Petaluma-infected cats mesangial widening was observed in six subjects, while 3 showed a mesangioproliferative glomerulonephritis. In Pisa-M2-infected cats mesangial widening was detected in six subjects, five showed a

mesangioproliferative glomerulonephritis (Figure 1B), and one cats a membranoproliferative glomerulonephritis (Figure 1D). The exam of the kidneys from the eleven cats infected with both Petaluma and Pisa-M2 strains demonstrated the presence of mesangial widening in three subjects and mesangioproliferative glomerulonephritis in other three cats. Ten of the 16 SPF cats, sacrificed at 12 months post-infection showed no renal alteration (three and seven subjects infected with Petaluma and Pisa-M2, respectively), while 4 presented mesangial widening (two cats each infected with Petaluma and Pisa-M2) and two glomerulonephritis (one each infected with Petaluma and Pisa-M2). Of the 13 subjects sacrificed at 24 months post-infection, eight showed no alterations (4 each infected with Petaluma and Pisa-M2), while mesangial widening was observed in two cats (one each infected with Petaluma and Pisa-M2) and glomerulonephritis was detected in remaining three cats (two infected with Petaluma and one with Pisa-M2). Finally, 9 of the 27 cats sacrificed after 30 months post-infection, nine had mesangial widening (three each infected with Petaluma, Pisa-M2, and both Petaluma and Pisa-M2), while glomerulonephritis was detected in seven cats (four infected with Pisa-M2 and 3 with both Petaluma and Pisa-M2). Eleven of these cats, i.e. those sacrificed after 30 months post-infection, had no renal changes. In affected glomeruli of naturally and experimentally infected cats, dilatation of the Bowman's space was a frequent finding and, occasionally, protein droplets were present in prominent epithelial cells, particularly in subjects with an heavy proteinuria. The presence of mesangial widening and glomerulonephritis was detected both in experimentally (15/56 and 12/56, respectively) and naturally infected cats (8/21 and 3/21, respectively). Of note, naturally infected cats presented glomerular amyloid deposits that were never detected in the experimentally infected ones ($P < 0.001$).

Histopathological alterations	Control cats	Naturally FIV-infected cats	Experimentally FIV-infected cats		
			Petaluma	Pisa M2	Petaluma + Pisa M2
Glomerular					
Bowman's space dilatation	0/4	8/21	3/17	6/28	3/11
Mesangial widening	0/4	8/21	6/17	6/28	3/11
Mesangioproliferative glomerulonephritis	0/4	3/21	3/17	5/28	3/11
Membranoproliferative glomerulonephritis	0/4	0/21	0/17	1/28	0/11
Segmental glomerulosclerosis	0/4	1/21	0/17	0/28	0/11
Glomerular amyloidosis	0/4	8/21	0/17	0/28	0/11
No alterations	4/4	3/21	8/17	16/28	5/11

Table 2. Main histological glomerular findings in naturally and experimentally FIV-infected cats and uninfected controls.

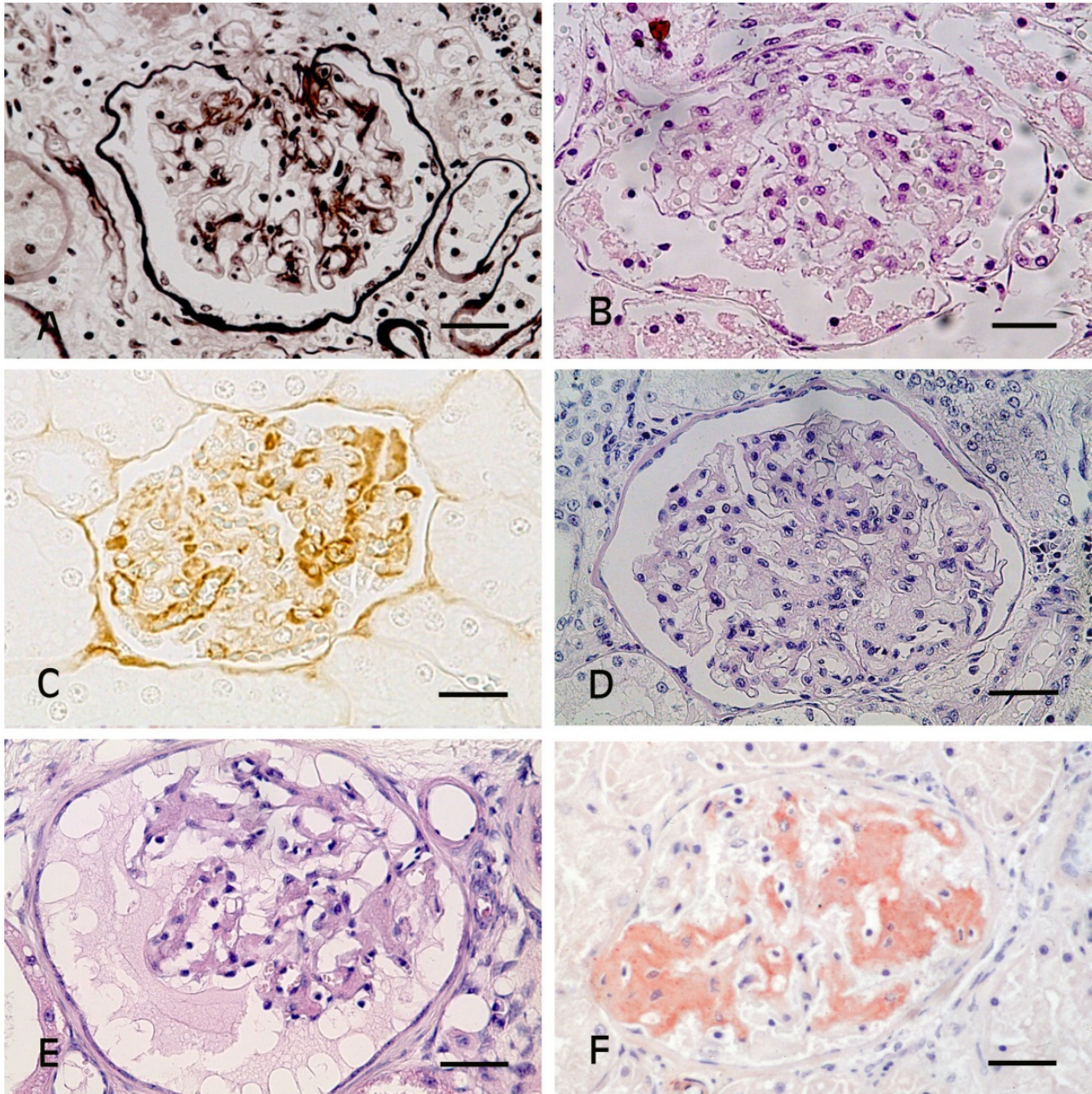


Figure 1. A. Naturally FIV-infected cat. Mesangial widening. Mild increase in mesangial matrix with a minimal increase in intraglomerular cellularity. Jones' periodic acid-silver methenamine stain. (Bar = 80 μ m) B. Experimentally Pisa-M2-infected cat. Mesangioproliferative glomerulonephritis. Focal mild proliferation of mesangial cells. Hematoxylin-Eosin stain. (Bar = 80 μ m) C. Experimentally Pisa-M2-infected cat. Mesangioproliferative glomerulonephritis. Segmental deposition of IgG. Strepavidin biotin peroxidase complex method, Mayer's hematoxylin counterstain. (Bar = 80 μ m) D. Experimentally Pisa-M2-infected cat. Membranoproliferative glomerulonephritis. Mesangial enlargement and thickening of capillary walls. Hematoxylin-Eosin stain. (Bar = 80 μ m) E. Naturally FIV-infected cat. Mild amyloid deposition (pale structureless material), occupying the slightly expanded mesangial areas. H-E stain. (Bar = 80 μ m) F. Naturally FIV-infected cat. The amyloid is immunoreactive with the antiserum to feline AA amyloid. Strepavidin biotin peroxidase complex method, Mayer's hematoxylin counterstain. (Bar = 80 μ m).

Tubulointerstitial alterations were frequently detected in naturally and experimentally infected cats. In the former, degeneration of tubular epithelial cells was observed in ten cats, tubular microcysts in eight, and giant protein tubular casts in four subjects (Table 3). Only six Petaluma-infected cats showed degenerative changes of tubular epithelial cells, while no alteration were detected in the other eleven animals. In Pisa-M2-infected cats, degenerative changes were observed in eleven subjects, tubular microcysts in two and giant protein casts in only one subject, while 17 cats presented no tubular alterations. In cats infected with both strains, degenerative changes of tubular epithelial cells were detected in three cats, tubular microcysts in two and giant protein tubular casts in one subjects. Eight cats showed no alterations. Interstitial alterations were commonly observed in naturally infected cats (Table 4) and consisted of interstitial infiltration by lymphocytes and plasmacells. This was scanty periglomerular (eight subjects), diffuse without fibrosis (six subjects) and diffuse with interstitial fibrosis (three subjects). Interstitial amyloidosis was detected in seven subjects, while no interstitial alterations were detected in four naturally infected cats. Also in these cases the amyloid deposits were KMnO₄ sensitive. Experimentally infected cats had seldom interstitial alterations. Scanty periglomerular infiltrates were detected in 1/12 Petaluma-infected subjects sacrificed at 24 months post-infection, 3/17 cats infected with Pisa-M2 (one sacrificed at 24 months and two sacrificed \geq 30 months post-infection) and 2/6 cats infected with both viruses and sacrificed \geq 30 months post-infection.

Histopathological alterations	Control cats	Naturally FIV-infected cats	Experimentally FIV -infected cats		
			Petaluma	Pisa M2	Petaluma + Pisa M2
Tubular					
Degeneration of tubular epithelial cells	0/4	10/21	6/17	11/28	3/11
Tubular microcysts	0/4	8/21	0/17	2/28	2/11
Giant protein tubular casts	0/4	4/21	0/17	1/28	1/11
No alterations	4/4	3/21	11/17	17/28	8/11
Interstitial					
Scanty periglomerular infiltrates	0/4	8/21	1/17	3/28	2/11
Diffuse interstitial infiltrates without fibrosis	0/4	6/21	0/17	0/28	0/11
Diffuse interstitial infiltrates with fibrosis	0/4	3/21	0/17	0/28	0/11
Interstitial amyloidosis	0/4	7/21	0/17	0/28	0/11
No alterations	4/4	4/21	16/17	25/28	9/11

Table 3. Main histological tubular and interstitial findings in naturally and experimentally FIV-infected cats and uninfected controls.

Tubular alterations were more frequently detected in naturally infected cats (18/21) than in experimentally ones (22/56). Particularly, the presence of giant protein tubular cats (4/21 *vs* 2/56; $P<0.05$) and tubular microcysts (8/21 *vs* 4/56; $P<0.05$) was more frequently detected in naturally than experimentally infected subjects. Interstitial alterations were also markedly more frequently in naturally infected cats (17/21) compared to experimentally infected cats (6/56; $P<0.001$), particularly the presence of diffuse interstitial infiltrates and interstitial amyloidosis was never detected in the latter group.

In Table 4, the renal alterations according virus strain inoculated and time post-inoculation are presented. 14/29 cats without any histological renal alteration observed had the lowest mean value of urine protein concentration (uP) 3.95 ± 1.37 g/L (2.5 – 7.05) and expected the lowest UPC mean value of 0.45 ± 0.12 (0.25-0.67). The mean values of uP and UPC in 10 proteinuric cats with mesangial widening was slightly higher: 3.54 ± 1.52 g/L (2.0 – 5.4) and 0.81 ± 0.44 (0.53 – 1.94), respectively. Five cats with similar mesangial alteration had uP concentration lowest then 2.0 g/L. Significantly higher mean values were established in 12 cats with glomerular alteration. Mean value of uP in this group was 21.53 ± 29.39 g/L (2.8 – 100.0) and UPC ratio was 4.8 ± 5.77 (0.90 – 17.63). All 20 cats with tubular alteration had glomerulonephritis (9/20) or mesangial widening (11/20) too. Three of them were unproteinuric. Only four of six cats with interstitial infiltrates were proteinuric, two 24 months pi and 4 after more then 30 months pi.

Lasting	Virus inoculated	Renal changes				
		No alterations	Mesangial widening	Glomerulonephritis	Tubular alterations	Interstitial infiltrates
12 months pi	Petaluma (n=6)	3	2	1	2	0
	Pisa M2 (n=10)	7	2	1	2	0
24 months pi	Petaluma (n=7)	4	1	2	1	1
	Pisa M2 (n=6)	4	1	1	2	1
> 30 months pi	Petaluma (n=4)	1	3	0	3	0
	Pisa M2 (n=12)	5	3	4	7	2
	Petaluma + Pisa M2 (n=11)	5	3	3	3	2

Table 4. Renal alterations detected in FIV experimentally infected cats sacrificed with different lasting.

3.4. Immunohistochemistry

The results of immunohistochemical investigations are summarized in table 5. By IF and IHC positive specimens showed segmental, predominantly granular mesangial deposits of IgG, IgM and C3, while rarely scattered granular deposits were detected along the capillary loops (Figure 1C). IgA staining was never observed. Cellular infiltrates were characterized by the presence of IgG secreting plasma cells and scattered IgM. Large proteinaceous casts were positive for IgG and weakly for IgA. Amyloid deposits were always positive for the mouse monoclonal anti-human AA and the rabbit polyclonal against the feline AA amyloid (Figure 1F), while were always negative for the rabbit polyclonal anti-AL amyloid.

Immunohistochemistry	Control cats	Naturally FIV-infected cats	Experimentally FIV-infected cats		
			Petaluma	Pisa M2	Petaluma + Pisa M2
IgG deposits in mesangium	0/4	3/21	3/17	5/28	3/11
IgG deposits in capillary loops	0/4	1/21	1/17	2/28	2/11
IgM deposits	0/4	14/21	6/17	6/28	3/11
IgA deposits	0/4	0/21	0/17	0/28	0/11
C3 deposits	0/4	14/21	6/17	6/28	3/11

Table 5. Main immunohistochemical findings in naturally and experimentally FIV-infected cats and uninfected controls.

4. Discussion

The results of our investigation clearly demonstrate that FIV infection induce tissue alterations in kidneys. The study on naturally infected subjects confirmed the presence of renal alterations in a high percentage of the examined animals (18/21). Eleven cases presented glomerular changes (three glomerulonephritis and 8 glomerular amyloidosis) and tubulointerstitial lesions, and were therefore considered severe alterations. The other seven subjects presented milder glomerular changes that consisted in mesangial-matrix expansion with or without segmental glomerulosclerosis.

Previous studies with naturally FIV-infected cats suggested significantly higher rates of renal dysfunction and histological changes compared to FIV seronegative ones. Examinations of 326 sick cats from Australia demonstrated a significant association between FIV infection and azotemia and palpably small kidneys [27]. Concerning small kidneys, as noted by abdominal palpation, they were also reported by Brown and colleagues [28]. Nonspecific renal abnormalities were also reported by several studies [13,29]. The percentage of FIV infected cats with similar renal alteration ranged from 5.5% in New Zealand [14] to 9.3% (from a survey of 700 cats) in Japan [15]. In 76 cats from three regions in Italy (Piemonte, Liguria and Val d'Aosta) 9% were affected by renal disease [30]. Previous histopathological and ultrastructural

investigations described kidney abnormalities in 12 of 15 cats [16] and 10 of 14 cats [17] with naturally acquired FIV infection. Six of the twelve subjects of the first study presented lesions that caused a marked increase in serum BUN and creatinine concentration and heavy glomerular non-selective proteinuria; the other nine cats with renal abnormalities, the urine protein content was higher than normal range ($>0,2\text{g/l}$) [16]. Results obtained in the present study showed similar findings. 18 of 21 (82%) of naturally infected cats had mild to severe proteinuria (mean value of 26.46 ± 22.41 (ranging from 3.5 – 62 g/L). 10/21 cats had glomerulo non-selective proteinuria, 8/21 of them combined with tubular proteinuria.

The investigations carried out on experimentally FIV-infected SPF cats demonstrated renal alterations partially similar to those detected in naturally FIV-infected ones. Particularly, mesangial widening with or without segmental glomerulosclerosis and immune mediated glomerulonephritis were observed in these subjects, no matter that they were infected with different FIV isolates, maintained in isolation units, and sacrificed at different times post-infection.

Mesangial widening with segmental to diffuse glomerulosclerosis [16], nephrosclerosis [29] and thickened Bowman's membrane [28] have been previously described in naturally FIV-infected cats. These alterations represent glomerular reactions common to many apparently unrelated, clinical entities that are currently believed to result from intraglomerular hemodynamic alterations [31]. In FIV-infected cats hemodynamic alterations might be mediated by sustained production of lymphokines and/or factors of mesangial proliferation with activity on glomerular capillary permeability as a consequence of the chronic systemic viral infection. Although controversial, increasing evidence supports a direct effect of the virus on renal cells either as a result of exposure to viral proteins or direct renal parenchyma infection. The use of a HIV-1 transgenic mouse model demonstrated a direct etiologic link between HIV-1 expression in kidney and the development of segmental glomerulosclerosis in HIV associated nephropathy (HIVAN) with unique viral-host interactions, which depends on inherent features of the virus and, at the same time, host response [32]. In FIV infection the direct role of the virus in the pathogenesis of renal alterations is postulated by the presence of p24 viral antigen in tubular epithelial cells as well as scattered interstitial inflammatory and glomerular cells and by detection of FIV *gag* DNA and RNA sequences in these subjects [17,33].

Even if FIV-infected cats often present hypergammaglobulinemia, which is believed to be triggered by chronic polyclonal B cell activation [34], which, in turn, can lead to the production of immune complexes [34,35] and auto-antibodies [36], immune complex glomerulonephritis are infrequent. In a previous study on 15 naturally FIV-infected cats only one subjects showed IgG deposits in mesangial areas [16]. In this study IgG deposits were detected in 3/21 naturally and in 11/56 experimentally FIV-infected cats, associated with segmental and focal mesangioproliferative glomerulonephritis in 13 cases and only with a membranoproliferative glomerulonephritis. Even if, as mentioned, immune mediated glomerulonephritis seem uncommon in FIV-infected cats, previous studies demonstrated that the mean concentration of circulating IgG immune complex (CIC) in FIV-infected cats were significantly higher than in control cats, while IgM levels increased only slightly. The

immunoglobulin fractions from 10/15 renal tissue samples were analysed, found to be polyclonal, and only partly specific for FIV antigens. All these results, including hypergammaglobulinemia and high levels of CIC, together suggest that IC might play a role in the pathogenesis of the renal alteration observed in FIV-infected animals [34]. The evidence of focal and granular deposits of IgM and C3 in mesangium and sclerotic loops of the other subjects is likely the result from nonspecific trapping of serum proteins rather than from immune complex deposition [16].

Tubulo-interstitial lesions consisting of interstitial infiltration by lymphocytes and plasma cells, as well as fibrosis and tubular degenerative changes have been detected in a high proportion of naturally FIV-infected cats [16,28,29,37], but rarely in experimentally infected ones (17/21 vs 6/56, respectively).

Our study confirmed that glomerular and interstitial amyloidosis can be observed in the kidney of naturally FIV-infected animals as previously reported [17,37,38], but amyloid deposits were not detected in the renal tissues of the 56 experimentally infected cats examined. Histochemical and immunohistochemical studies demonstrated that amyloid deposits were consistent with secondary amyloidosis, associated with chronic infections. Previous unpublished data, demonstrated that naturally FIV-infected cats had a higher prevalence of renal amyloidosis compared to uninfected subjects, 12/34 naturally FIV-infected cats vs 1/30 age-matched control cats. The data of this study show that FIV infection alone is not sufficient for the development of amyloid deposition, as demonstrated by the absence of amyloidosis in the 56 experimentally FIV-infected cats and that other concurrent factors are needed.

In cats naturally and experimentally infected with FIV have been reported the presence of renal lymphoid tumours [39,40]. In our series we have no cases of lymphosarcoma, but the lack of these neoplastic alterations could be related to the reduced number of subjects examined.

Clinicopathological studies revealed the relatively high possibility of mild to severe renal proteinuria without clinical signs of azotemia. None of 56 SPF cats included in our study presented any clinical signs of azotemia except one cat that was infected by Pisa-M2, sacrificed one year post-infection and that showed serum creatinine concentration (144 $\mu\text{mol/L}$) with mild proteinuria (3.9 g/L and UPC 0.43) (CRF stage 2) [26]. Renal proteinuria, established by UPC was present in 10 of 17 cats infected with Petaluma, 9 of 28 cats, infected with Pisa-M2 and 2 of 11 in cats infected with both virus isolates, regardless the time from infection. However, the most severe renal proteinuria was observed in cats infected with Pisa-M2 (mean 3.27 ± 6.34 ; ranged 0.53-17.63), slightly milder in cats infected with both virus strains (mean 3.0 ± 4.39 ; ranged 0.55-13.01) and the less severe in cats infected with Petaluma (mean 2.56 ± 3.03 ; ranged 0.31 – 7.02). Fourteen of 29 cats without any histological renal alteration observed had the lowest mean value of urine protein concentration (uP) 3.95 ± 1.37 g/L (2.5 – 7.05) and the lowest UPC mean value of 0.45 ± 0.12 (0.25-0.67). The mean values of uP and UPC in 10 proteinuric cats with mesangial widening was slightly higher 3.54 ± 1.52 g/L (2.0 – 5.4) and 0.81 ± 0.44 (0.53 – 1.94), respectively. Five cats with similar mesangial

alteration were aproteinuric. (>2.0 g/L). Significantly higher mean values were established in 12 cats with glomerular alteration; mean value of uP in this group was 21.53 ± 29.39 g/L (2.8 – 100.0) and UPC ration was 4.8 ± 5.77 (0.90 – 17.63). All 20 cats with tubular alteration had glomerulonephritis (9/20) or mesangial widening (11/20). Three of them were aproteinuric. Only four of six cats, with interstitial infiltrates were proteinuric, two 24 months post-infection and 4 after over 30 months post inoculation. In addition, electrophoresis of urine proteins confirmed the correlation between proteins excreted in the urine and the histological alterations found in observed cats; three of 21 cats had glomerulo selective proteinuria, 15 glomerulo non-selective, three glomerulo non-selective and tubular proteinuria.

The overwhelming majority of experimentally infected cats (71.4 to 100%) had inverted CD4+/CD8+ T cell ratio that depended on infecting viral isolate and, albeit with low or no statistical significance, time of infection. Furthermore, no correlation between CD4+/CD8+ T cell ratio and renal alteration was found. As well as clinical and laboratory findings, as azotemia and proteinuria, renal disease in HIV positive patients seem to be similar in FIV infected, which do not correlate with CD4+ T-cell count or CD4+/CD8+ T cell ratio [28].

There are many reports about various organ system involvements, including renal in HIV-infected people [41]. Patient with HIVAN may develop a spectrum of renal pathology that most likely manifests with an acute rapidly progressive loss of renal function, characterised by proteinuria, nephrotic syndrome and azotemia [27]. Typical histological features consist of focal sclerosing glomerulopathy and microcystic tubular dilatation. Some of the patients have mesangial proliferation [27,41,42]. The pathogenesis of HIVAN is still unclear, although presence of viral proteins in glomerular and tubular epithelium cells suggested an important role of HIV in the initiation or progression of HIVAN [27]. Glomerular manifestations include antigen-antibody complex and nonimmune-complex-mediated pathology [43,44].

5. Conclusion

In conclusion, our study confirmed that renal involvement occurs in a high proportion of naturally FIV-infected cats and that these alterations can, in part, be detected in experimentally infected subjects. In all, these results suggest a causative relationship between FIV infection and renal abnormalities. This damage seems to consist of mesangial increase, sometimes accompanied by mesangial cell proliferation and glomerulosclerosis, a lower percentage of immune mediate glomerulonephritis and, in naturally infected subjects' glomerular and interstitial amyloidosis. Besides, these alteration are partially similar to those detected in HIV-infected patients, FIV-infected cats might represent an interesting natural animal model for the study of the pathogenesis of HIV-associated nephropathy and renal alterations associated with chronic viral infection.

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6. References

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