



# Alpha-enolase staining patterns in the renal tissues of cats with and without chronic kidney disease

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## ABSTRACT

Renal  $\alpha$ -enolase has variable expression in inflammatory and neoplastic diseases. Therefore, in order to define the distribution of  $\alpha$ -enolase in renal tissues of cats, an immunohistochemistry assay was validated and described here. Tissues from 29 cats with IRIS Stage 2–4 CKD, 8 control cats < 2 years of age, and 4 control cats > 10 years of age were assessed. Interstitial nephritis was the predominant histopathological finding in the CKD group. The control cats < 2 years of age had moderate  $\alpha$ -enolase immunoreactivity in tubular epithelium but staining was absent to mild in glomeruli. In contrast,  $\alpha$ -enolase was moderate to high in tubular epithelium and glomeruli in control cats > 10 years of age. In cats with CKD,  $\alpha$ -enolase was decreased in tubules that were degenerative or atrophic, similar to normal tubules in control groups, and moderate to high in glomeruli. When compared between the study groups, the results suggest that alpha-enolase decreases in damaged tubules and increases in the glomeruli of older cats prior to the development of detectable CKD. Further studies will be required to determine whether these findings relate to the pathogenesis or could be used in the diagnosis of feline CKD.

## 1. Introduction

The glycolytic enzyme, enolase, was discovered in 1934 during studies of the conversion of 3-phosphoglycerate to pyruvate in muscle extracts (Lohman and Meyerhof, 1934). The mammalian enolase enzyme family consists of 3 isoenzyme subunits: alpha, beta, and gamma; forming homo- or heterodimers (Fletcher et al., 1976a, b; Pancholi, 2001; Rider and Taylor, 1974, 1975). Alpha-enolase isoenzyme is ubiquitous and found in a variety of tissues with the highest concentration within the thymus and kidney (Pancholi, 2001; Sabbatini et al., 1997). Although originally characterized as a cytoplasmic, glycolytic enzyme, enolase has been shown to have multiple functions as well as variable cellular expression (Pancholi, 2001; Moscato et al., 2000, 2002; Wygrecka et al., 2009; Díaz-Ramos et al., 2012). Products of *eno1* gene can participate in transcriptional repression, cellular defense against hypoxia, and function as a plasminogen receptor (Aaronson et al., 1995; Graven et al., 1993; Miles et al., 1991; Redlitz et al., 1995; López-Alemay et al., 2003a, b). In limited studies in people, renal  $\alpha$ -enolase expression is localized to the proximal and distal tubules, and the collecting ducts. The enzyme is nearly undetectable in glomeruli and loop of Henle. (Migliorini et al., 2002; Haimoto et al., 1986)

Circulating  $\alpha$ -enolase antibodies have been repeatedly detected in serum of humans with autoimmune diseases such as but not limited to rheumatoid arthritis, systemic lupus erythematosus (SLE), and mixed cryoglobulinemia (Terrier et al., 2007; Lee et al., 2003, 2009; Pratesi et al., 2000; Hanrotel-Saliou et al., 2011). Approximately 70% of human SLE patients with detectable anti- $\alpha$ -enolase antibodies have active nephritis (Pratesi et al., 2000; Hanrotel-Saliou et al., 2011). In people with lupus nephritis,  $\alpha$ -enolase is overexpressed in tubules and glomeruli in comparison to healthy controls (Migliorini et al., 2002).

Chronic kidney disease (CKD) is one of the most common syndromes recognized in cats (Lulich et al., 1992; Finch et al., 2016). Most cats with CKD have interstitial nephritis characterized by infiltrates of lymphocytes and plasma cells (Lappin et al., 2006). In addition, antibodies against  $\alpha$ -enolase have been detected in the serum of cats spontaneously and after the administration of vaccines grown on cells (Whittemore et al., 2010). However, the distribution of  $\alpha$ -enolase in feline tissues has not been described. The objectives of this study were to utilize IHC to determine the location and immunoreactivity of  $\alpha$ -enolase within feline renal tissues and compare results amongst cats with and without CKD and within different stages of CKD.

Abbreviations: CKD, chronic kidney disease; IHC, immunohistochemistry

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## 2. Materials and methods

### 2.1. Tissue samples

This study was approved by the Institutional Animal Care and Use Committee at Colorado State University. The tissues from cats with CKD were collected after the cats were presented Colorado State University Diagnostic Medical Center for necropsy. The tissues from cats without CKD were collected after euthanasia for unrelated reasons at a contract research facility or a local animal shelter. Based on previous studies, thymic tissue from a young cat (< 2 years of age) was collected and used for primary antibody optimization and assay validation (Sabbatini et al., 1997). All tissues were fixed in 10% neutral buffered formalin solution, paraffin embedded and sectioned at 5  $\mu$ m.

Using the International Renal Interest Society (IRIS) staging scheme for CKD, the cats were assigned into a stage between 2 and 4 based on repeated elevated serum creatinine concentration, a urine specific gravity < 1.035, and clinical history consistent with CKD (Anon, 2013). Stages 2, 3, and 4 were defined by serum creatinine concentrations measuring 1.6–2.8 mg/dl, 2.9–5.0 mg/dl and > 5.0 mg/dl, respectively. No cats with Stage I were included. Cats were classified as not having CKD (controls) if there was no clinical evidence or history of renal disease, the serum creatinine concentration was  $\leq$  1.6 mg/dl, and the USG was > 1.035.

### 2.2. Immunohistochemistry

Immunohistochemistry to detect  $\alpha$ -enolase was performed on an autostainer<sup>3</sup> according to the established manufacturer's protocol. Tissue sections were deparaffinized and rehydrated. Antigen retrieval consisted of heating slides for 20 min with a commercially available retrieval solution.<sup>4</sup> Endogenous peroxidase activity was blocked with 3% hydrogen peroxide solution. Slides were incubated with mouse monoclonal anti-enolase antibody<sup>5</sup> for 15 min at room temperature. The primary antibody was replaced with a commercially available mouse negative antibody<sup>6</sup> on the negative control tissues. Antibody detection was accomplished by applying 3,3'-diaminobenzidine chromagen and hematoxylin counterstain.

The optimal primary antibody concentration for appropriate signal intensity was determined by applying serial dilutions (1:500, 1:1000, 1:2000) of primary  $\alpha$ -enolase antibody to thymic tissue. The 1:2000 concentration of  $\alpha$ -enolase antibody provided the highest intensity with the least background staining and was used in all IHC as described above with thymus as a positive control. The assay was validated by scoring thymic tissue at the optimal antibody concentration on 3 separate occasions. The interassay variation was estimated by calculating the quotient of the standard deviations and mean (i.e. coefficient of variation).

A grid was utilized to randomly select no more than 2 tubules and 2 glomeruli per high powered field (40X) within the renal cortex for scoring based on signal intensity. Scoring was defined as 0 = no staining; 1 = light brown/tan, 2 = moderate brown staining; and 3 = dark brown which may obscure the nucleus. A total of 20 cortical tubules from controls or 10 injured and 10 normal tubules from CKD cats, and 20 non-sclerotic glomeruli from each cat were scored and a mean score per cat for each structure was determined by the same investigator (SM) who was initially blinded to the clinical information. Injured tubules were defined as those that were degenerative or

atrophic. Sections from bilateral kidneys were evaluated separately and compared to determine if there was variability between kidneys in individual cats with CKD (n = 4) and cats without CKD (n = 2).

### 2.3. Statistical evaluations

The  $\alpha$ -enolase immunoreactivity scores for normal renal cortical tubules and glomeruli were compared between the two groups of control cats and CKD cats, and amongst IRIS Stages 2, 3 and 4 within CKD cats, by non-parametric one-way analysis of variance (Kruskal-Wallis) with Dunn's post-hoc comparison. The mean  $\alpha$ -enolase immunoreactivity scores for normal and injured tubules of CKD cats were compared with a paired *t*-test. The mean  $\alpha$ -enolase immunoreactivity scores for tubules and glomeruli from bilateral kidneys of CKD cats and control cats were compared by the Wilcoxon matched pairs signed rank test. Statistical significance for all analyses were set at *p* < 0.05 and performed in GraphPad Prism version 7.02 for Windows (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)).

## 3. Results and discussion

The optimized  $\alpha$ -enolase IHC had a coefficient of variation of 8.2%. Pale to light staining was present in the cytoplasm of most thymic epithelial cells with a median immunoreactivity score of 1. Alpha-enolase immunoreactivity scores compared between bilateral kidneys were not different for cats with CKD or control cats. Thus, results from a single kidney from each cat was used for the study.

Renal tissues were collected from 29 cats with IRIS Stage 2–4 CKD and 12 control cats. In the CKD group, there were 9 Stage 2 cats, 8 Stage 3 cats, and 12 Stage 4 cats. The mean age of cats with CKD was 14.7 years (range 8–20.5 years) and included 14 castrated male and 15 spayed female cats. Domestic shorthair cats were most prevalent (n = 17) followed by domestic longhair cats (n = 6), Siamese cats (n = 3), Tonkinese (1 cat), and Ragdoll (1 cat). The control cats without CKD were subdivided into those < 2 years of age (n = 8 cats) and > 10 years of age (n = 4 cats). The control cats < 2 years of age included 6 spayed females and 2 castrated males, and all were domestic shorthairs. The exact birth dates were not available for all cats in this group and so a mean age was not calculated. The mean age of control cats > 10 years of age was 12.3 years (range 10–15 years), included 3 spayed females and one castrated male, and consisted of 2 domestic shorthairs, 1 domestic longhair and 1 Siamese. Interstitial nephritis was the predominant histopathological finding in the CKD group and was minimal to absent in the control cats.

Most sections from control cats showed moderate, monochromatic, cytoplasmic  $\alpha$ -enolase staining of tubular epithelial cells with only a few tubules that stained intensely (Fig. 1). There was no significant difference in  $\alpha$ -enolase immunoreactivity scores amongst normal tubules of control cats < 2 years of age (median score = 2; range 2–3), of control cats that were > 10 years of age (median score = 2; range 1.95–2.05), or of CKD cats (median score = 2; range 1–3). In cats with CKD,  $\alpha$ -enolase staining was decreased in injured tubules that were degenerative or atrophic (median score = 1; range 0–3) (Fig. 2). Injured tubules of cats at any stage of CKD (mean 1.4  $\pm$  0.4) had significantly less  $\alpha$ -enolase immunoreactivity when compared to normal tubules at any IRIS stage (mean 2.2  $\pm$  0.3) (*p* < 0.0001) (Fig. 3). However, there were no differences in  $\alpha$ -enolase immunoreactivity amongst normal or injured tubules when compared between IRIS stages.

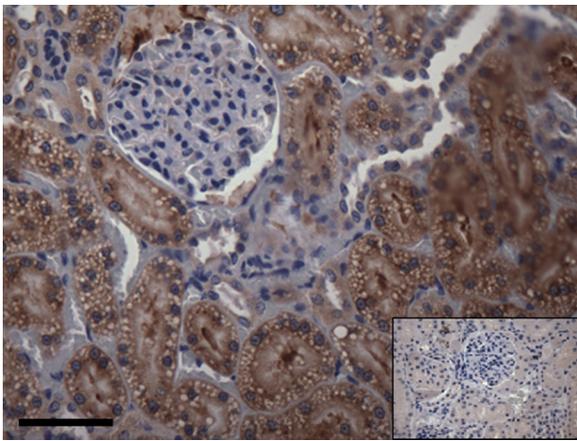
In glomeruli,  $\alpha$ -enolase immunoreactivity was present within the cytoplasm of glomerular epithelial cells (visceral and parietal) and less frequently mesangial cells. In the control cats < 2 years of age,  $\alpha$ -enolase immunoreactivity was low in glomeruli (median score = 1; range 0–2) (Fig. 1). In contrast,  $\alpha$ -enolase immunoreactivity was significantly higher in glomeruli of control cats that were > 10 years of age (median score = 2; range 1.8–2.6; *p* = 0.04), and cats with CKD (median

<sup>3</sup> Leica Bond-Max Leica Microsystems, Buffalo Grove, IL.

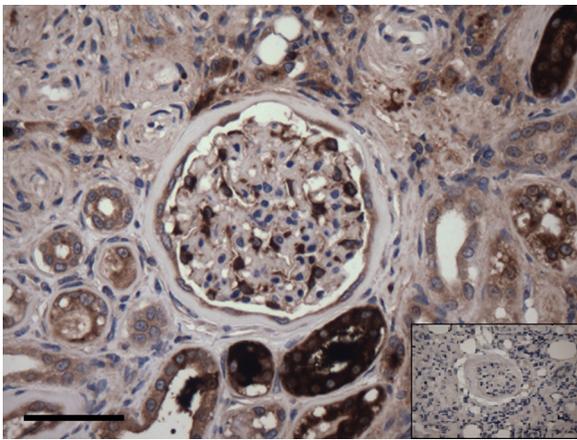
<sup>4</sup> Bond Epitope Retrieval Solution 1 (pH 6), Leica Microsystems, Buffalo Grove, IL.

<sup>5</sup> anti-ENO1 antibody, Abnova Taiwan Corp., Taipei, Taiwan.

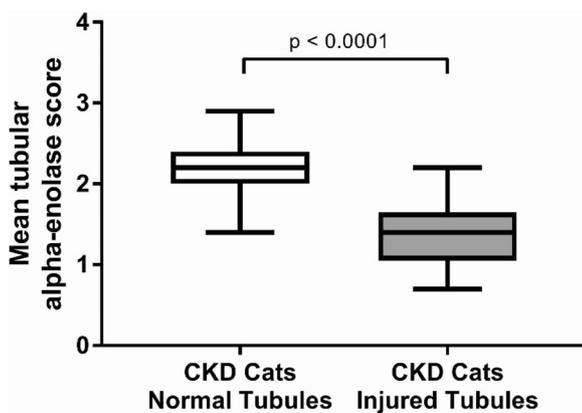
<sup>6</sup> Bond Ready-to-Use Mouse Negative Control antibody, Leica Microsystems, Buffalo Grove, IL.



**Fig. 1.** Alpha-enolase immunohistochemistry of the kidney of a control cat without CKD that was < 2 years of age. There is minimal staining in glomeruli with moderate, monochromatic staining of tubules. Black bar = 50 μm. Inset: negative control.

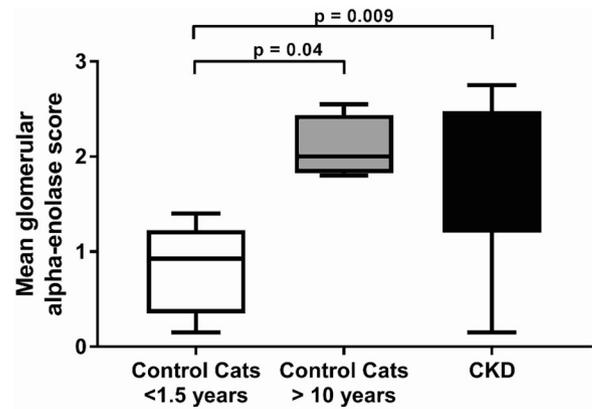


**Fig. 2.** Alpha-enolase immunohistochemistry of the kidney in a CKD cat. There is moderate to marked glomerular staining in epithelial and few mesangial cells. Tubules have variable staining intensities. Black bar = 50 μm. Inset: negative control.



**Fig. 3.** Mean alpha-enolase immunoreactivity scores in normal and injured tubules from cats with CKD. Injured tubules of CKD cats had significantly less α-enolase immunoreactivity when compared to normal tubules of CKD cats ( $p < 0.0001$ ).

score = 2; range 0–3;  $p = 0.009$ ) in comparison to control cats < 2 years of age (Fig. 4). However, there was no significant difference in α-enolase immunoreactivity amongst glomeruli when compared between



**Fig. 4.** Mean alpha-enolase immunoreactivity scores in glomeruli of control cats and cats with CKD. Mean α-enolase immunoreactivity was significantly higher in glomeruli of control cats that were > 10 years of age ( $p = 0.04$ ), and cats with CKD ( $p = 0.009$ ) in comparison to cats < 2 years of age.

IRIS stages.

Alpha-enolase is considered a ubiquitous protein within mammalian cells with the thymus and kidney containing greater amounts of protein than other cells from other tissues (Pancholi, 2001; Sabbatini et al., 1997). In this study, α-enolase immunoreactivity appeared lower in cells from the thymus than normal renal tubules. This finding varies from a previous study using rabbit thymic tissue extracts evaluated in an immunoblot assay where the α-enolase immunoreactivity was intense (Sabbatini et al., 1997). The significance of this finding is unknown and the results cannot be directly compared as different anti-enolase antibodies were used in the 2 studies.

In the control cats without CKD, regardless of age, renal α-enolase immunoreactivity was greatest in renal cortical tubules. In contrast, tubular α-enolase immunoreactivity was decreased in injured tubules of cats with IRIS Stages 2, 3, and 4 CKD suggesting an association between this pathology and decreased protein expression. In humans with SLE nephritis, one of the few diseases for which tubular α-enolase expression is described, α-enolase expression is increased in comparison to healthy controls (Migliorini et al., 2002). However, presence of tubular injury and/or interstitial nephritis was not specified in this study (Migliorini et al., 2002). As SLE nephritis is a disease that is morphologically and pathophysiologically distinct from feline CKD, it is challenging to compare enolase immunoreactivity patterns. In injured feline renal tubules, production of α-enolase may merely decrease as a result of tubular degeneration and atrophy, common histological features of feline CKD (Lucke, 1968; McLeland et al., 2015). Another potential cause for decreased α-enolase immunoreactivity in injured tubules could be the presence of nephritogenic autoantibodies that are bound to tubular α-enolase and thus blocking available epitopes for binding of monoclonal antibody in the immunohistochemistry assay. However, this was not a reported interference for protein detection in similar assays with known nephritogenic autoantibodies in human patients (Migliorini et al., 2002).

When compared to control cats < 2 years of age, α-enolase immunoreactivity in the glomeruli was increased in both the control cats > 10 years of age and all 3 IRIS stages of CKD studied. These findings suggest an age-related change in production of α-enolase within glomeruli. Increases in α-enolase immunoreactivity was present in the cytoplasm of mesangial cells and both visceral and parietal epithelium similar to the pattern described in patients with SLE nephritis (Migliorini et al., 2002). The mechanism for increased expression of renal epithelial α-enolase in patients with SLE nephritis have not been definitively determined, but possibly include changes within normal glycolytic and non-glycolytic pathways in response to hypoxic conditions and increased anaerobic metabolism (Pancholi, 2001; Aaronson et al., 1995; Graven et al., 1993; Zimmerman et al., 1991).

In cats, administration of vaccines grown on cell lines containing  $\alpha$ -enolase have been shown to induce strong serum  $\alpha$ -enolase responses (Whittemore et al., 2010). In addition, one of the risk factors associated with CKD in a study of cats was increased vaccination frequency (Finch et al., 2016). In future studies, serum  $\alpha$ -enolase antibodies and quantitative tissue levels of  $\alpha$ -enolase antibodies and enolase mRNA will be studied concurrently with  $\alpha$ -enolase IHC in cats with and without CKD from which the complete vaccination history is known. Additionally, further studies will be required to determine whether the differences in  $\alpha$ -enolase immunoreactivity amongst groups of cats described here relate to the pathogenesis of CKD or could be used to aid the early diagnosis of CKD.

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### Conflicts of interest

The authors have no conflicts of interest to disclose. The work was presented in part as a poster at the American College of Veterinary Internal Medicine Annual Forum, June 2017.

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