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Correlation of histopathological features and renal impairment in autosomal dominant Alport syndrome in Bull terriers

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Abstract

Background. Bull terrier hereditary nephritis represents a model for autosomal dominant Alport syndrome, as affected dogs have the characteristically lamellated glomerular basement membrane and demonstrate vertical male-to-male disease transmission.

Methods. This study compared the histopathological features in kidneys from affected Bull terrier neonates, puppies, and adult dogs with normal or impaired renal function, with the histopathological appearance of kidneys from age- and size-matched normal dogs.

Results. There were fewer glomeruli per unit area of cortex in kidneys from affected neonatal kidneys ($P < 0.05$), increased numbers of fetal glomeruli in affected puppy kidneys ($P < 0.05$), and a separate population of glomeruli with larger renal corpuscles and glomerular tufts in kidneys from affected adult dogs with normal renal function (both $P < 0.0001$) compared with normal dogs. Other histological features that are characteristic of human X-linked and autosomal recessive Alport syndrome and that were present included hypercellular glomeruli, occasional crescents, segmental and global glomerular sclerosis, periglomerular fibrosis, interstitial fibrosis without significant cellular infiltrates and cystic dilatation of Bowman's capsular space and tubules. In dogs with renal impairment, the tubular index was the best predictor of increased urinary protein : creatinine ($r = 0.92$) compared with glomerular, interstitial and vascular indices ($r = 0.77$, 0.88 and 0.81), and medullary fibrosis correlated best with serum creatinine ($r = 0.72$, $P = 0.0002$).

Conclusions. The demonstration in Bull terrier kidneys of fewer nephrons in neonates increased fetal glomeruli, and a separate population of glomeruli with larger corpuscles and tufts reflects the effects of the underlying genetic mutation that are first manifest antenatally. The major determinant of renal impairment in adult affected Bull terriers is, however, progressive tubulointerstitial damage after birth.

Keywords: animal model; autosomal dominant Alport syndrome; glomerular basement membrane; renal failure

Introduction

Most individuals with Alport syndrome have X-linked or autosomal recessive disease, which is characterized clinically by haematuria, progressive renal failure, hearing loss, lenticonus and dot-and-fleck retinopathy [1]. In X-linked Alport syndrome, all mutations affect the COL4A5 gene [2], resulting in the loss of the corresponding α5(IV) collagen chain together with the α3(IV) and α4(IV) chains from the glomerular (GBM) and other basement membranes [3]. In the less common autosomal recessive disease, mutations affect the COL4A3 or COL4A4 genes [4]. These encode the α3(IV) and α4(IV) collagen chains, which together with the α5(IV) chain are again usually absent from the affected glomerular membrane [5].

Autosomal dominant Alport syndrome is rare, and the clinical phenotype differs from X-linked and autosomal recessive disease. Haematuria may be associated with normal renal function or with progressive renal impairment, hearing loss is common, but ocular abnormalities do not occur [6,7]. Again the GBM is lamellated but the α3(IV) – α5(IV) collagen chains are present in the affected GBM [8]. Mutations again affect the COL4A3 or COL4A4 genes [6,7]. Autosomal dominant Alport syndrome has to be distinguished

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from autosomal dominant hereditary nephritis with haematological abnormalities caused by mutations in the MYH9 gene, which encodes a non-muscle myosin heavy chain [9].

The diagnosis of all forms of Alport syndrome usually depends on the presence of the typical clinical features together with a family history of the disease, or on the ultrastructural demonstration of a lamellated GBM [10]. The light microscopic appearance of the kidney in Alport syndrome has been best described in X-linked disease and is not diagnostic [11–14]. The appearance is usually normal at birth, and the earliest abnormalities include slight mesangial hypercellularity and expansion, and focal capillary wall thickening and wrinkling. Fetal glomeruli are often present and persist into adult life. More advanced glomerular lesions include diffuse mesangial proliferation, segmental or global sclerosis, periglomerular fibrosis, and occasional crescents. The appearance of segmental glomerular lesions signals the initiation of rapidly progressive obliteration of the glomeruli, which in turn leads to interstitial inflammation and fibrosis. The appearance in end-stage kidneys is of segmental glomerular sclerosis and glomerular obsolescence, with a corresponding degree of tubular atrophy, interstitial inflammation and fibrosis, and interstitial foam cells.

There have been no detailed light microscopic studies of the kidney in autosomal dominant Alport syndrome both because the condition is rare and the mode of inheritance is often unrecognized. Bull terrier hereditary nephritis represents a model for autosomal dominant Alport syndrome as affected dogs have a lamellated GBM and their pedigrees demonstrate vertical father-to-son disease transmission [15,16]. In addition, their GBM contains all type IV collagen chains [16]. Affected animals have haematuria and progressive renal failure, and although they are not deaf this is still consistent with autosomal dominant Alport syndrome, as clinical features in humans are variably penetrant, and deafness is absent from other canine models of Alport disease [17,18]. All affected Bull terriers have a common progenitor and disease caused by a single mutation presumably affecting the COL4A3 or COL4A4 genes.

Bull terrier hereditary nephritis provides us with the opportunity to describe the renal histopathology in autosomal dominant Alport syndrome, and to determine the parameters that correlate best with the development of renal failure.

Animals and methods

Dogs

Affected puppies and dogs were descendants of an affected male Bull terrier with hereditary nephritis who was imported into Australia for breeding purposes.

Affected Bull terrier puppies were neonates, aged 4 days or less, or older puppies, who were still aged <12 weeks. All of these had a lamellated GBM on ultrastructural examination at post-mortem or on biopsy.

Affected adult Bull terriers had a urinary protein : creatinine (UPC) of $\geqslant 0.3$, which correlates with ultrastructural evidence of Bull terrier hereditary nephritis in adults [19], or had a lamellated GBM on ultrastructural examination at post-mortem. (UPC was not measured in puppies because they often have incidental proteinuria.) Affected Bull terriers with 'normal renal function' had a serum creatinine <132 μmol/l, and those with 'impaired renal function' function had a serum creatinine >132 μmol/l.

Normal dogs were breeds other than Bull terriers, and were clinically healthy with a UPC <0.3. They were age- and size-matched for the Bull terriers. 'Unaffected' Bull terriers were not used because hereditary nephritis is widespread among Australian dogs and animals with subclinical disease might have been examined inadvertently.

Serum and urinary creatinine, and urinary protein levels were measured on a Cobas Mira Autoanalyser (Hoffman-LaRoche). Renal tissue was obtained when dogs were euthanazed, or from ultrasound-guided Trucut biopsies performed under general anaesthetic. Tissue for the electron microscopic diagnosis of Bull terrier hereditary nephritis was fixed and processed as described previously [20].

Light microscope examination

Kidneys were fixed in 10% neutral-buffered formalin, and processed for light microscopy by standard methods. Sections (4 μm) were stained with haematoxylin and eosin, Masson's trichrome, Jones' silver stain, periodic acid Schiff, alcian blue, haematoxylin acid ferrocyanide (HAF), and oil red O. Kidneys were examined and graded by C.H., a specialist veterinary pathologist, and J.C.H.

Neonatal and puppy kidneys

Kidney lengths and widths were measured in neonates. The inner cortex was the part adjacent to the corticomedullary junction where there were mature but not immature glomeruli. The outer cortex extended from the inner cortex to the capsule. The numbers of immature and mature glomeruli in the inner and outer cortex were counted over 10 fields at high power magnification and averaged. 'Mature' glomeruli had distinct capsules and vascularized tufts [21], even if the visceral epithelial cells were attenuated and separate [22]. 'Immature' glomeruli had progressed beyond the S-shaped nephron but were not yet mature. 'Fetal glomeruli' were identical to those described in humans [14,23], that is, they had small tufts with only a few capillary loops and chain-like podocytes. The numbers of fetal glomeruli were expressed as a percentage of the total glomeruli in 10 fields in the outer cortex when examined at high power magnification. The average diameters of Bowman's capsules and mature glomerular tufts were measured in 10 fields at high power magnification using a calibrated Olympus graticule eyepiece.

Adult kidneys

The following histopathologic variables and cumulative glomerular, tubular, interstitial, and vascular scores were determined.

Glomerular indices included: the average number of glomeruli and average percent obsolescent/atrophic glomeruli

per field; the assessment of glomerular hypercellularity; mesangial thickening; glomerular sclerosis; large glomeruli; dilatation of Bowman's capsular space; periglomerular fibrosis; glomerular atrophy; and mineralization of the glomerular tuft and of Bowman's capsule. The average number of glomeruli per field represented the average count in five fields at low power magnification and the average percent obsolescent/atrophic glomeruli per field was based on the counts in five fields, and the percent of total glomeruli. Obsolescent/atrophic glomeruli had a retracted tuft within an apparently too large capsule, and have been described especially within bands of interstitial fibrosis in many types of renal diseases. Hypercellular glomeruli had clusters of >2 nuclei/glomerular capillary loop, and the mesangium was considered to be thickened when its width was greater than that of a normal capillary wall. Glomerular sclerosis was graded from segmental through to global. Glomerular size was semi-quantitatively assessed at low power magnification by comparison with the calibrated length of the arrow indicator (125 μm) in an Olympus model BH-2 micoscrope. The diameter of a normal glomerulus was ~190 μm, while atrophy was defined as a glomerular diameter $<25\%$ of this. Dilatation of Bowman's capsular space was enlarged compared with that of the normal dog, where Bowman's capsule is closely apposed to the glomerular tuft. Mineralization was demonstrated in HAF-stained sections.

Glomerular variables were graded: 0, no change; 1–3, mild, moderate, or severe focal change, where $<25\%$ of glomeruli were affected; 4–6, mild, moderate, or severe multifocal change where 25–75% of glomeruli were affected; and 7–9, mild, moderate, or severe diffuse change where $>75\%$ of glomeruli were affected. The glomerular score represented the sum of these individual variables.

Tubular indices were: cortical, corticomedullary, and medullary tubular dilatation; thickened tubular basement membrane (TBM); tubular inflammation; hyperplastic (pseudostratified) or atrophic tubular epithelium; tubular luminal protein; and mineralized TBM or epithelium.

Interstitial indices included: cortical, corticomedullary and medullary fibrosis; interstitial inflammation and mineralized interstitium. Tubular atrophy was not measured directly, but was reflected by the scores for cortical, corticomedullary, and medullary tubular dilatation.

Vascular indices examined were fibroelastic thickening of vessel walls and arteriolar hyalinosis/sclerosis.

Tubular, interstitial, and vascular variables were graded: 0, no change; 1–3, mild, moderate, or severe focal change; 4–6, mild, moderate, or severe multifocal change; and 7–9, mild, moderate, or severe diffuse change. Tubular, interstitial, and vascular scores were the sum of individual indices.

Individual variables and glomerular, tubular, interstitial, and vascular scores were compared to determine any differences between kidneys from affected dogs with normal renal function, affected dogs with renal impairment, and normal dogs. Then, glomerular, tubular, interstitial and vascular scores, and individual indices were examined to determine which parameter contributed most to the increase in UPC and serum creatinine.

Stereological assessment of glomerular size

Kidneys from three affected dogs with normal renal function and three normal dogs were examined using stereological techniques to compare glomerular tuft and corpuscle volume. One hundred sequential 3–4 μm sections were stained with periodic acid Schiff. The volumes of renal corpuscles and glomerular tufts were determined using the Cavalieri method [24,25]. For each dog, 30 complete glomeruli were randomly sampled from the inner, middle, and outer cortex using the disector principle. Each glomerulus was projected onto a grid and the number of points covered by the glomerular profile noted. This method enabled the volume of glomerulus (V_g) to be determined from the sum of points $\times$ a(p) $\times T$, where a(p) equalled the area per point and T was the section thickness (2 μm). Renal corpuscle and glomerular tuft volumes were then compared between the two groups.

Results

Affected neonatal and puppy Bull terriers (Table 1 and Figure 1)

Kidneys and kidney sections from six affected and five normal neonates, and five affected and four normal puppies were examined. The normal neonatal dog kidneys demonstrated a well-demarcated cortex and medulla and an active subcapsular nephrogenic zone with metanephric blastema cells, S-shaped nephrons, and maturing glomeruli. Fetal glomeruli were present. Some cortical and medullary tubular dilatation was present in kidneys from two normal animals, and there were small amounts of faintly staining primitive mesenchyme focally surrounding tubules throughout the kidneys. In affected neonatal kidneys, the nephrogenic zone was less dense than in normal neonates, with fewer and less prominent metanephric blastema cells and fewer immature glomeruli, but relatively normal numbers of mature glomeruli in the inner cortex. Fetal glomeruli were again noted. Overall there were significantly fewer glomeruli per unit area in affected neonatal kidneys compared with normal kidneys ($P<0.05$, Table 1), but the kidneys were of normal size, indicating that affected kidneys had significantly fewer total glomeruli than normal. There was no metaplastic tubular epithelium or evidence of dysontogenic derivatives. Cystic structures were present in three of the six affected neonates, and appeared to represent the cortical and medullary tubular dilatation seen in normal neonatal kidneys, but also dilatation of veins and peritubular capillaries.

In all the normal and affected puppy kidneys, the nephrogenic zone had disappeared, and the cortex and medulla were distinct. With the small numbers of animals it was not possible to demonstrate any difference in the number of glomeruli in affected and normal puppy kidneys. However there were significantly more fetal glomeruli per unit area in affected puppies than normal puppies ($P<0.05$, Table 1). The number of fetal glomeruli was increased in affected puppies compared with neonates because glomeruli continue to appear for at least the first 3 weeks of life in dogs. Again because of the small numbers of puppy kidneys available, it was not possible to demonstrate the separate population of glomeruli with increased Bowman's capsule diameter and tuft size that were present in adult affected kidneys. Primitive mesenchyme had disappeared from all but the most

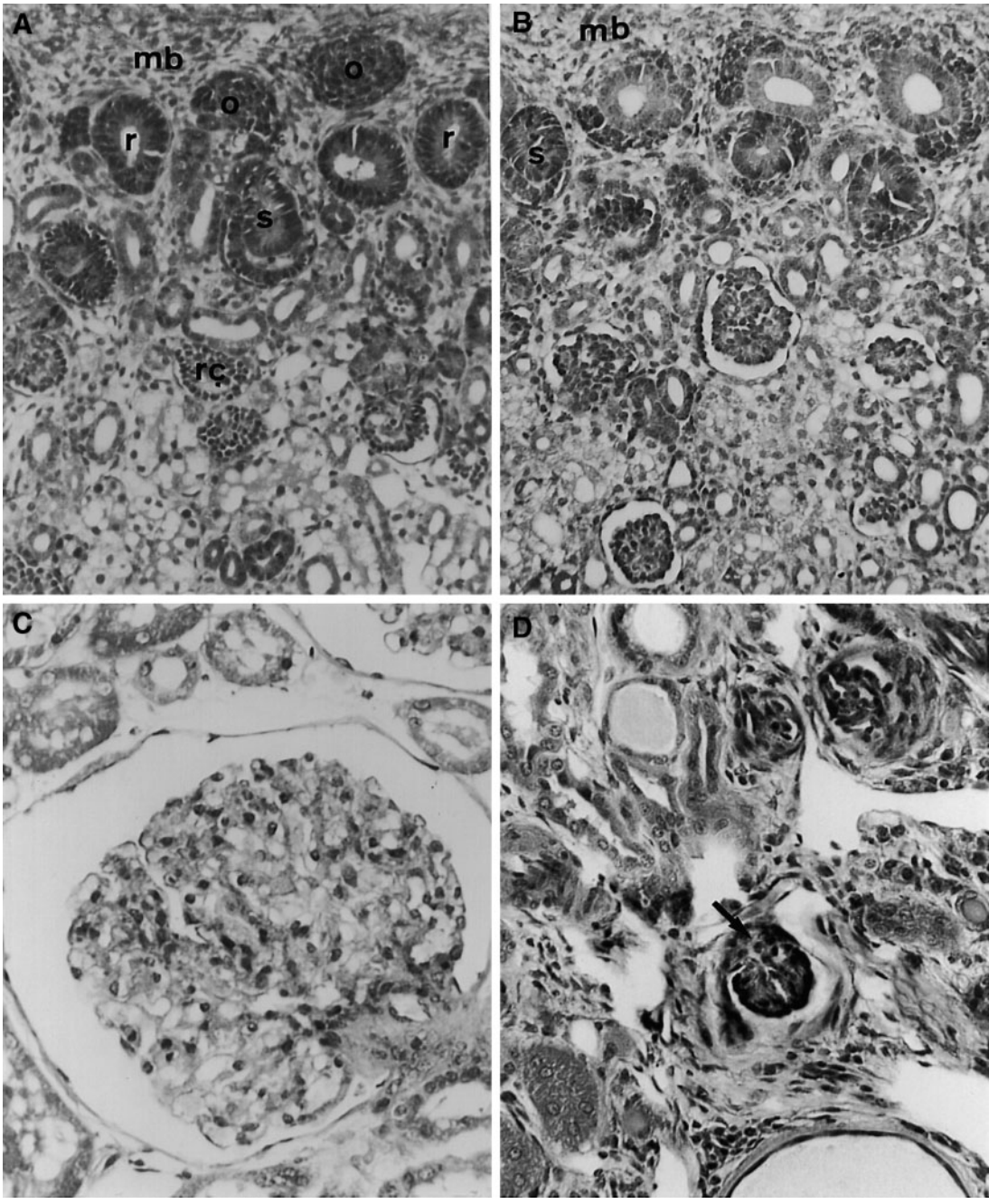

Fig. 1. (Above and opposite) (**A**) Kidney from normal dog neonate (1 day of age) showing active subcapsular cortex, with demarcated nephrogenic zone, metanephric blastema (mb), oval mesenchymal bodies (o), renal vesicles (r), S-shaped nephrons (s), and immature renal corpuscles (rc) (×200, Masson's trichrome). (**B**) Kidney from Bull terrier neonate (1 day of age) showing subcapsular cortex, with fewer developing nephrons and slighlty looser metanephric blastema (mb) (×200, Masson's trichrome). (**C**) Kidney from adult affected Bull terrier with normal renal function showing hypercellular glomerulus with large tuft, mild dilatation of Bowman's capsular space, and very mild thickening of Bowman's capsule (×200, Masson's trichrome). (**D**) Kidney from adult affected Bull terrier with normal renal function showing persisting fetal glomerulus (arrowed) (×200, Masson's trichrome). (**E**) Kidney from adult affected Bull terrier with normal renal function showing obsolecent/atrophic glomerulus with a paucity of capillary loops and chain-like arrangement of visceral epithelial cells resembling a fetal glomerulus, and with dilated Bowman's capsular space and a periglomerular cap of fibrosis (×200, Masson's trichrome). (**F**) Kidney from adult affected Bull terrier with normal renal function showing four obsolescent/atrophic glomeruli with capsular dilatation, and an apparently empty cyst within a band of fibrosis (×200, Masson's trichrome). (**G**) Kidney from adult affected Bull terrier with normal renal function showing glomerulus with capsular dilatation, and sclerotic glomerulus with periglomerular fibrosis (×200, Masson's trichrome). (**H**) Kidney from adult affected Bull terrier with impaired renal function showing a larger glomerular corpuscle and tuft (arrowed), glomerular atrophy, capsular dilatation, periglomerular fibrosis, cystic dilatation of Bowman's space and tubules, and interstitial fibrosis without inflammatory cell infiltrate (Masson's, ×100).

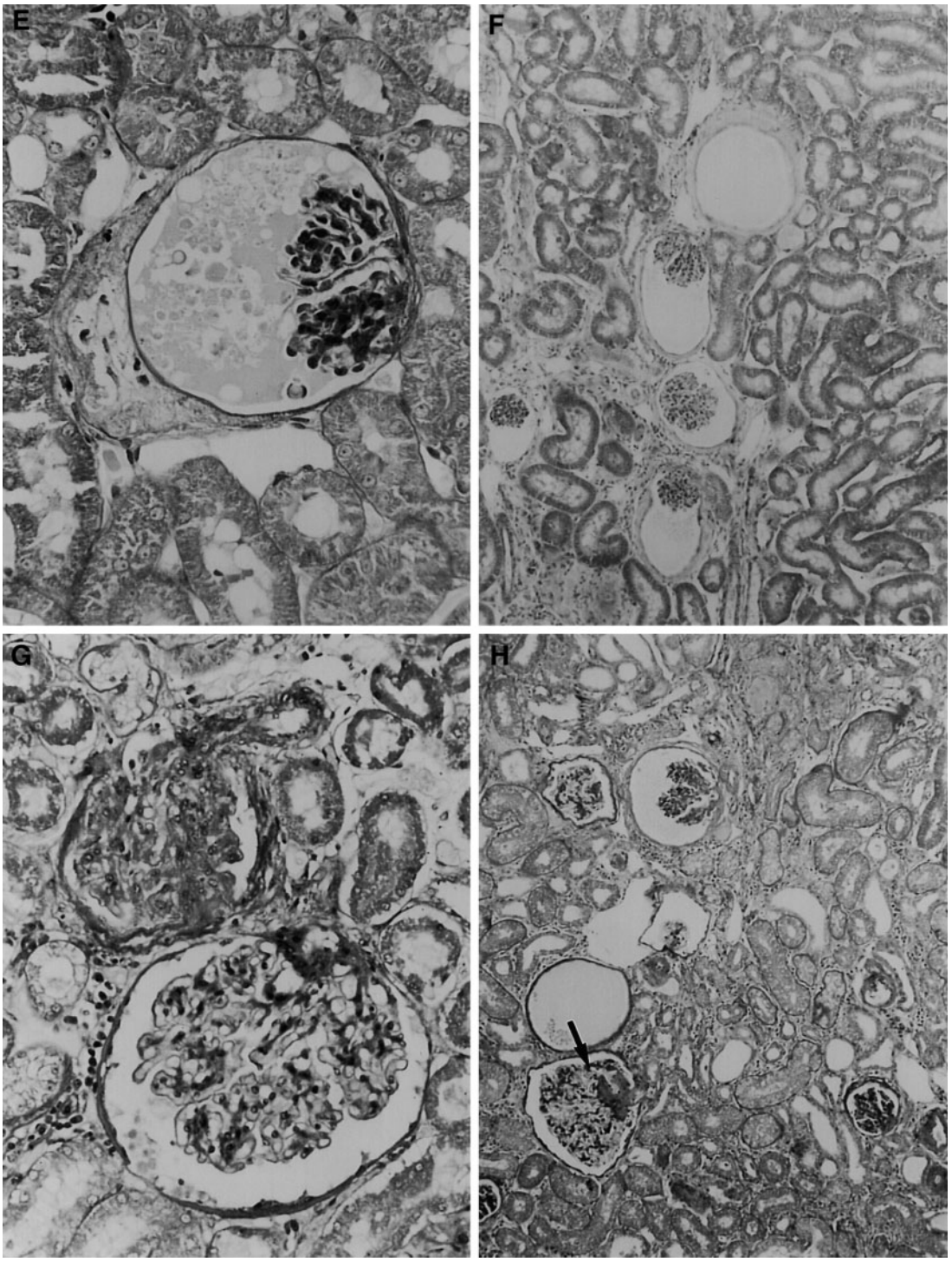

Fig. 1. Contd.

abnormal puppy kidney. In this puppy, normal glomeruli were sparse and there were frequent, empty dilated Bowman's capsular spaces in the cortex.

Affected adult Bull terriers with normal renal function (Tables 2–4 and Figures 1 and 2)

The light microscopic appearances of the kidneys from 11 adult affected Bull terriers with normal kidney function, 36 Bull terriers with impaired function, and 10 normal dogs were compared (Tables 2–4). Kidneys from adult affected Bull terriers with normal kidney function were grossly normal in size and shape, but there were prominent light microscopic changes affecting mainly the glomeruli and interstitium. There were fewer glomeruli per low power field than in normal dogs, and there were two populations of glomeruli, with about half having larger capsular

Table 1. Macroscopic and light microscopic characteristics of affected Bull terrier and normal neonatal kidneys

	Affected neonates ($n=6$)	Normal neonates ($n=5$)	Affected puppies ($n=5$)	Normal puppies ($n=4$)
Kidney length (cm)	2.2 (1.8–2.5)	1.7 (1.6–1.9) NS	N/A	N/A
Kidney width (cm)	1.1 (0.9–1.3)	1.0 (0.9–1.0) NS	N/A	N/A
Total no. glomeruli in unit area of outer and inner cortex combined	6.7 (5.0–7.2)	9.4 (8.6–12.0) ($P<0.05$)	2.4 (2.4–5.9)	4.6 (4.3–4.8) NS
Total no. glomeruli in unit area of outer cortex	4.9 (3.3–5.0)	6.7 (6.1–9.8) ($P<0.05$)	1.4 (0.9–3.4)	2.9 (2.8–4.9) NS
Average no. mature glomeruli in unit area of outer cortex	N/A	N/A	1.1 (0.7–3.1)	2.6 (2.1–4.8) NS
Averege no. immature glomeruli in unit area of outer cortex	4.9 (3.3–5.0)	6.7 (6.1–9.8) ($P<0.05$)	0.3 (0.1–1.0)	0.3 (0–0.9) NS
Average Bowman's capsular diameter in outer cortex (μm)	N/A	N/A	100 (68–115)	73 (72–83) NS
Average glomerular tuft diameter in outer cortex (μm)	N/A	N/A	75 (64–88)	71 (65–72) NS
Per cent fetal glomeruli	0 (0–2.1)	1.5 (1–4.5) NS*	21 (6–22)	0 (0–3.3) ($P<0.05$)**
Average no. mature glomeruli in unit area of inner cortex	2.1 (1.6–2.3)	2.5 (2–2.7) NS	1.5 (1.2–2.5)	1.9 (1.5–2.3) NS
Average Bowman's capsular diameter (μm)	115 (100–123)	103 (83–125) NS	120 (94–150)	106 (87–122) NS
Average glomerular tuft diameter (μm)	101 (91–110)	95 (76–113) NS	100 (87–121)	90 (76–108) NS

Medians and ranges are given. Measurements were compared with Wilcoxon's rank sum test for unpaired samples. N/A, not applicable. There were no mature glomeruli in the outer cortex of neonates, and consequently Bowman's capsule and glomerular tuft diameter were not measured. There were significantly fewer total glomeruli in affected compared with normal neonatal kidneys, and there were proportionally more fetal glomeruli in affected compared with normal puppy kidneys. (New glomeruli appear after birth in dogs.)

spaces and glomerular tufts than the others, and when compared with normal dogs, stereologically ($P<0.0001$, $P<0.0001$, Figure 2). In addition the percent obsolescent/atrophic glomeruli was increased compared with normal dogs, especially in the subcapsular cortex, in clusters within radially fibrotic bands and sometimes at the corticomedullary junction. Otherwise there was mild diffuse glomerular hypercellularity, mesangial thickening, and mild focal segmental and global sclerosis especially at the corticomedullary junction. Bowman's capsule was usually thickened. The other prominent feature was cortical, corticomedullary and medullary interstitial fibrosis. This was often seen as radial bands in the outer medulla sometimes extending into the cortex. Fibrosis was present in all kidneys, but was associated with inflammatory cell infiltrates in fewer than half, suggesting that the fibrosis was not secondary to inflammation. There was no primitive mesenchyme and foam cells were not seen. The TBM was thickened, but there was minimal tubular atrophy or dilatation. Arterial sclerosis and medial hypertophy of the arterioles at the corticomedullary junction were present in nearly all the affected adult kidneys even when renal function was normal. The extent of these changes varied in individual Bull terriers, and there were only occasional and mild changes in the normal dogs.

Affected adult Bull terriers with impared renal function (Tables 2–4 and Figure 1)

Kidneys from affected adult Bull terriers with impaired renal function were macroscopically small, pale, and had an irregular outline. In some cases, the capsular surface had a finely granular texture, and the cortex was thinned and contained multiple cysts <1 mm in diameter. All histopathological changes seen in affected dogs with normal renal function were also present in animals with renal impairment, but the abnormalities were more widespread and more pronounced. In many cases, early changes were partially obscured or obliterated by fibrosis, inflammation, or nephron dropout. There were fewer glomeruli per low power field in kidneys from dogs with impaired renal function, and both normal and large glomeruli appeared to 'drop out' at the same rate.

Most kidneys demonstrated obsolescent/atrophic glomeruli where there was dilatation of Bowman's capsular space and glomerular atrophy, in some cases so severe that the tufts were not evident within hugely dilated capsular spaces. In just over half the kidneys examined there were also sclerotic glomeruli where Bowman's space and capillary loops could not be identified. There was marked nephron loss and extensive cystic tubular dilatation probably due to obstruction from fibrous distortion as well as dilated Bowman's capsular spaces. Markedly dilated tubules and collecting ducts were prominent in the outer medulla where they were sacculated and distorted with either attenuated flattened epithelium or pseudostratified columnar, proliferative epithelium. Similar but less dilated tubules and ducts were seen in both the inner medulla and the cortex. Mild thickening of the TBM was present, and abnormal tubules and collecting ducts were filled with protein and surrounded by dense collagen. Interstitial fibrosis was prominent throughout

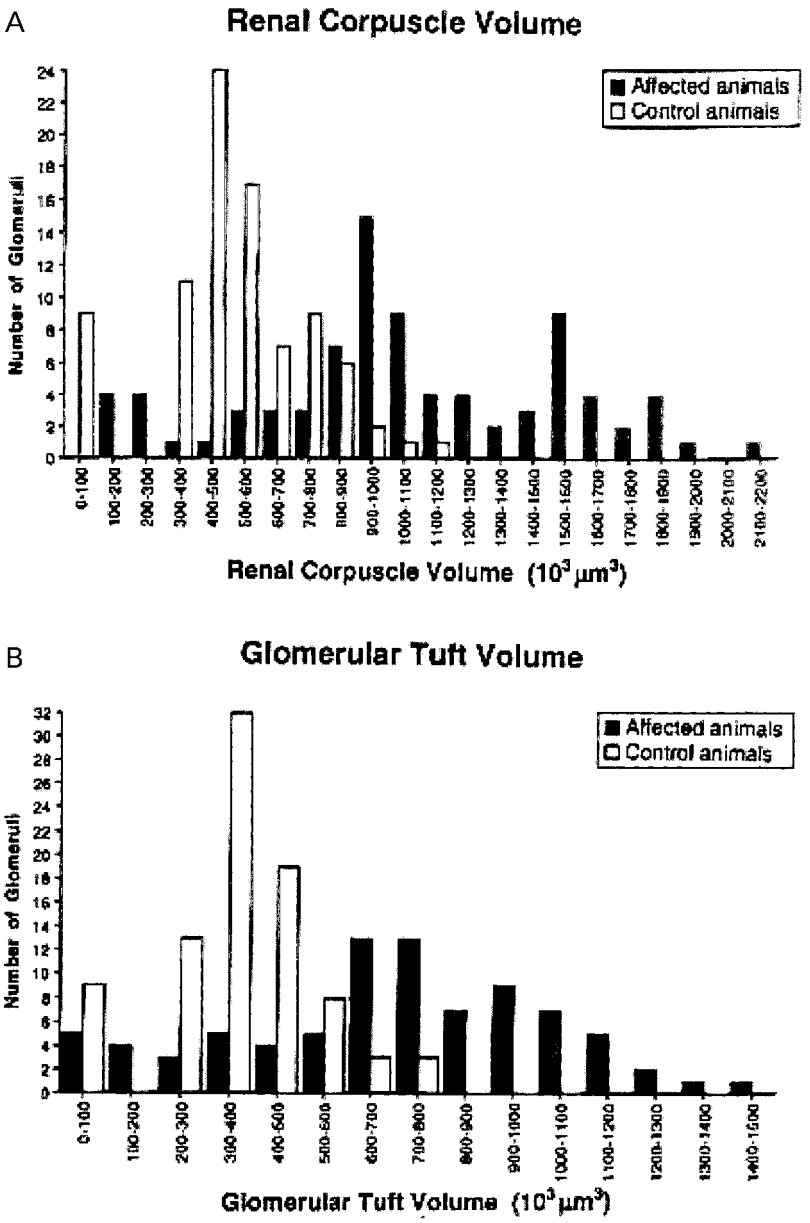

Fig. 2. Stereological comparison of (**A**) renal corpuscle volume and (**B**) glomerular tuft volume in three affected adult Bull terriers with normal renal function and three normal age- and size-matched dogs. This shows populations of normal and significantly larger corpuscles and tufts in affected dog kidneys ($P < 0.0001$ for both).

the kidney especially at the corticomedullary junction. Varying degrees of radial and segmental cortical fibrosis were present too, sometimes with horizontal fibrosis between adjacent bands. The fibrosis was accompanied by varying degrees of inflammation, and tubular cystic formation was most marked in the areas with the worst fibrosis. Foam cells were not present. Arterial sclerosis was present in nearly all the kidneys from affected adult Bull terrier kidneys, where there was renal impairment, with sclerosis of the arterioles mainly in the connective tissue around the arcuate arteries.

Abnormalities in the normal kidneys were occasional, mild and focal, and there were no vascular changes.

Analysis of histopathological and biochemical variables (Tables 2–4)

In adult dogs, all biochemical and histopathological variables, except the number of glomeruli per low power field and per cent fetal glomeruli, were described as ordinary scaled data. Data were tested for homogeneity of variance before parametric tests were used. Logarithmic transformations were applied to the UPC and serum creatinine, and glomeruli per low power field and per cent fetal glomeruli to reduce the skewedness and improve linearity.

UPC values were significantly different between affected Bull terriers with normal or impaired renal function, and normal dogs. Likewise the serum

Table 2. Clinical and laboratory features of affected adult dogs with or without renal impairment, and normal dogs

	Affected adult Bull terriers		Normal dogs ($n=10$)	P
	Non-end-stage ($n=11$)	End-stage ($n=36$)		
Gender	M 6, F 5	M 22, F 14	M 8, F 2	
Age (years)	4.5 (0.8)	3.6 (0.4)	2.2 (0.3)	0.052
UPC	0.49 (0.11)	3.73 (0.53)	0.15 (0.02)	<0.001
Serum creatinine (μmol/l)	76 (8)	1038 (82)	N/A	<0.001
Glomerular score (0–81)	32.3 (2.1)	42.3 (1.0)	4.2 (2.0)	<0.0001
Tubular score (0–81)	21.3 (3.8)	62.6 (1.1)	0.0 (0.0)	<0.0001
Interstitial score (0–45)	24.7 (1.9)	41.5 (0.5)	0.8 (0.7)	<0.0001
Vascular score (0–12)	6.4 (1.2)	11.1 (0.2)	0.0 (0.0)	<0.0001

Data are means and SE. Normal range for serum creatinine 44–32 μmol/l; and for UPC <0.3. P values refer to one-way ANOVA between three groups. All *post-hoc* test comparisons between groups using Fisher's PLSD method were highly significant ($P<0.0001$).

creatinine values were also significantly different between dogs with normal or impaired renal function ($P<0.001$, Table 2). (These values were not available for normal dogs.)

With the glomerular, tubular, interstitial, and vascular scores, a one-way ANOVA was performed between the three groups, and *post-hoc* comparisons were made using Fisher's PLSD. Glomerular, tubular, interstitial, and vascular scores were all greater in kidneys from affected dogs with impaired renal function than from affected dogs with normal renal function or from normal dogs ($P<0.0001$, Table 2).

Individual histopathological parameters were then compared between the three groups of dog kidneys (Table 3) using the Kruskal–Wallis test, and *post-hoc* comparisons were made using the Mann–Whitney test with α adjusted down to 0.0167. All P values were corrected for ties. This analysis showed that all histopathologic parameters were worse in affected dogs with renal impairment than in normal dogs ($P<0.0167$) except glomerular tuft mineralization and tubular inflammation, which were infrequent. This was true also when both groups of Bull terriers were compared with normal dogs. In addition, there was no difference in the amount of glomerular sclerosis, glomerular hypercellularity, mesangial thickening, glomerular tuft mineralization, tubular inflammation, or lumen mineralization between affected Bull terriers with impaired or normal renal function, while all other parameters were significantly different. There were fewer glomeruli per low power field in kidneys from affected dogs with normal function than in normals, and even fewer in kidneys from dogs with impaired function ($P<0.001$). Deteriorating renal function did not affect the per cent obsolescent/atrophic glomeruli, but the actual number of these glomeruli per field was less in kidneys with renal impairment than with normal renal function reflecting the lower number of glomeruli per field.

Both UPC and serum creatinine correlated inversely with the number of glomeruli present per unit area which is explained by worsening renal failure being accompanied by nephron loss.

The UPC correlated strongly with glomerular, tubular, interstitial, and vascular scores ($r=0.77$, 0.92, 0.88, and 0.81, respectively). The correlation was best for the tubular score, and this was the only variable that remained significant after performing a multiple regression analysis adjusting for all four scores. This correlation was confirmed by a stepwise regression. Ten individual histopathological indices correlated strongly with the UPC (Spearman's rho correlations $\geqslant 0.80$, Table 4). These were dilatation of Bowman's capsular space, thickening of Bowman's capsule, cortical, corticomedullary junction and medullary tubular dilatation, tubular protein, hyperplastic or atrophic tubular epithelium, cortical and corticomedullary fibrosis, and thickening of vessel walls. Five of these parameters were tubular, which was consistent with the previous finding that the tubular score correlated best with UPC after adjustment by multiple regression. Five of these scores reflected tubular atrophy, namely cortical, corticomedullary and medullary tubular dilatation, and cortical and corticomedullary fibrosis. It was not, however, possible to determine which of these variables correlated most strongly with UPC.

Serum creatinine measurements were available for five affected dogs with normal renal function and 25 affected dogs with renal impairment. The measurements were not log transformed in this data set. Serum creatinine correlated with glomerular, tubular, interstitial, and vascular scores ($r=0.64$, 0.65, 0.62, and 0.64, respectively). Stepwise regression indicated that both glomerular and tubular scores were independent predictors of serum creatinine. The interstitial and vascular scores were not significant after adjusting for glomerular and tubular scores. Thus, there was no obvious predictor of serum creatinine as there was for UPC. Multiple regression including only glomerular and tubular scores showed a stronger correlation with tubular scores than for glomerular scores ($P<0.02$, $P=0.03$, respectively). However, both glomerular and tubular scores correlated with each other. When the effect of individual histopathological variables on the serum creatinine was evaluated, Spearman's rho

Table 3. A comparison of glomerular, tubular, interstitial, and vascular variables in adult affected dogs with or without renal impairment, and normal dogs

	Affected adult Bull terriers		Normal dogs ($n=10$)	P
	Normal renal function ($n=11$)	Impaired renal function ($n=36$)		
Glomerular indices				
Glomeruli/low power field[a]	9.0	4.22	12.9	<0.001
Per cent obsolescent/atrophic glomeruli	1.66	1.85	0.58	0.032
Glomerular hypercellularity	7	8	0	<0.0001
Mesangial thickening	7	6	0*	<0.005
Sclerosis	2	2	0*	<0.005
Large glomeruli	7	2	0.5	<0.0001
Dilated Bowman's space	2	7	0*	<0.0001
Thickened Bowman's capsule	7	8	0*	<0.0001
Glomerular atrophy	1	5	0*	<0.0001
Mineralized glomerular tuft	0	0	0	0.11
Mineralized Bowman's capsule	0	5	0	<0.0001
Tubular indices				
Cortical tubular dilatation	0	9	0	<0.0001
Corticomedullary junction tubular dilatation	0	8	0	<0.0001
Medullary tubular dilatation	0	9	0	<0.0001
Tubular protein	0	9	0	<0.0001
Thickened TBM	7	8	0*	<0.0001
Tubular inflammation	0	0	0	0.16
Hyperplastic or atrophic tubular epithelium	0	8	0	<0.0001
Mineralized TBM	0	8	0	<0.0001
Mineralization in lumen	0	7	0	<0.0005
Interstitial indices				
Medullary fibrosis	7	9	0*	<0.0001
Corticomedullary fibrosis	7	9	0*	<0.0001
Cortical fibrosis	7	9	0*	<0.0001
Interstitial inflammation	0	8	0	<0.0001
Mineralized interstitium	0	7	0	<0.0001
Vascular indices				
Thickened small/medium vessel walls	7	9	0*	<0.0001
Arteriolar hyalinosis/sclerosis	2	3	0*	<0.0001

[a]Includes obsolescent/ atrophic glomeruli. TBM, tubular basement membrane. Values are medians except for glomeruli/low power field and % fetal glomeruli where means are given. P values refer to the Kruskal–Wallis test (non-parametric ANOVA) for all three groups. *Post-hoc* comparisons were performed using the Mann–Whitney test with the level of alpha adjusted downwards to 0.0167. *$P<0.0167$ for normal *vs* BT groups. All P values were corrected for ties.

correlation was <0.80 for all of them, and the best correlation was with medullary fibrosis ($r=0.72$, $P=0.0002$).

Stereological assessment of glomerular size

Both corpuscle and tuft volumes in kidneys from affected dogs with normal renal function varied across the observed range, and were approximately bimodal (Figure 2). Overall, corpuscle and tuft volumes in affected kidneys were larger than in kidneys from normal dogs ($P<0.0001$ for both by unpaired t-test).

Discussion

The Bull terrier model of autosomal dominant Alport syndrome demonstrated many of the histopathological abnormalities described previously in X-linked and autosomal recessive disease. However, it suggested in addition that there are fewer nephrons in autosomal dominant Alport syndrome, and that hyperfiltration even before birth may contribute to glomerular hypertrophy, and eventually glomerular scarring, and the interstitial fibrosis that correlates with renal impairment.

The demonstration of fewer glomeruli in affected neonatal kidneys and the suggestion of fewer glomeruli in puppy kidneys was confirmed in affected adult Bull terriers with normal renal function. The reduced nephron number in neonates is consistent with a direct effect of the genetic mutation and protein abnormality rather than the glomerular 'dropout' associated with renal impairment. The mutation has already affected the ultrastructural appearance of Bull terrier GBM by the time of birth [20].

Affected Bull terrier puppy kidneys had increased fetal glomeruli despite the overall reduction in glomerular numbers compared with normal animals. Fetal glomeruli comprise tufts of single layers of capillaries with rounded chain-like podocytes that normally occur only in the very young [26], and may represent developmental arrest at the stage where glomerular epithelial cells separate to form vascular

Table 4. UPC and serum creatinine *vs* individual glomerular, tubular, interstitial, and vascular variables in affected adult dogs with normal and impaired renal function

	UPC		Serum creatinine	
	rho	*P*	rho	*P*
Glomerular indices				
Glomeruli/low power field[a]	0.77	<0.0001	−0.40	0.03
Per cent obsolescent/atrophic glomeruli	0.17	0.34	0.01	0.94
Glomerular hypercellularity	0.51	0.003	0.27	0.14
Mesangial thickening	0.16	0.35	0.22	0.24
Sclerosis	0.64	0.0002	0.35	0.06
Large glomeruli	0.22	0.20	−0.49	<0.01
Dilated Bowman's space	0.86	<0.0001	0.31	0.09
Thickened Bowman's capsule	0.82	<0.0001	0.54	<0.01
Glomerular atrophy	0.70	<0.0001	0.29	0.12
Mineralized glomerular tuft	0.08	0.63	0.27	0.15
Mineralized Bowman's capsule	0.62	0.0004	0.53	<0.01
Tubular indices				
Cortical tubular dilatation	0.80	<0.0001	0.55	0.001
Corticomedullary junction tubular dilatation	0.80	<0.0001	0.64	0.0006
Medullary tubular dilatation	0.87	<0.0001	0.64	0.001
Tubular protein	0.85	<0.0001	0.29	0.12
Thickened TBM	0.75	<0.0001	0.57	0.003
Tubular inflammation	0.21	0.22	−0.02	0.90
Hyperplastic or atrophic tubular epithelium	0.81	<0.0001	0.45	0.03
Mineralized TBM	0.44	0.01	0.32	0.09
Mineralization in lumen	0.52	0.003	0.10	0.59
Interstitial indices				
Medullary fibrosis	0.79	<0.0001	0.72	0.0002
Corticomedullary fibrosis	0.84	<0.0001	0.54	0.004
Cortical fibrosis	0.88	<0.0001	0.57	0.002
Interstitial inflammation	0.77	<0.0001	0.39	0.04
Mineralized interstitium	0.73	<0.0001	0.38	0.05
Vascular indices				
Thickened small/medium vessel walls	0.82	<0.0001	0.50	<0.01
Arteriolar hyalinosis/sclerosis	0.72	<0.0001	0.45	<0.02

[a]Includes obsolescent/atrophic glomeruli. UPC, urinary protein:creatinine; rho, Spearman's rho correlation; *P* values adjusted for tied values.

loops [13,27]. Twenty per cent of biopsies from adults with X-linked Alport syndrome have up to five fetal glomeruli [14,26], but the numbers can be underestimated in end-stage kidneys because of glomerulosclerosis and fibrosis. Fetal glomeruli typically undergo obsolescence by sclerosis, but their resemblance to obsolescent/ atrophic glomeruli suggests that fetal glomeruli also undergo atrophy and capsular dilatation. Fetal glomeruli have not been demonstrated previously in autosomal or canine forms of Alport syndrome.

The reduced number of mature glomeruli in puppy kidneys and the increased proportion of fetal glomeruli suggest that the abnormality in Bull terrier hereditary nephritis results in developmental arrest, although the demarcated cortex and medulla and the loss of the nephrogenic zone indicate that the normal maturation sequence occurs in parallel. In X-linked Alport syndrome the affected GBM contains only the fetal α1(IV) and α2(IV) isoforms which are thought to inhibit glomerular maturation and result in persisting fetal glomeruli [28]. Subsequently Alport glomeruli appear to be resistant to physiological growth stimuli, as their capillary loops are smaller and thinner walled than normal [14]. These abnormalities may further contribute to functional glomerular hyperfiltration and secondary glomerulosclerosis. While all type IV collagen chains are present in Bull terrier GBM, amounts of individual isoforms have not been quantified, nor have the capillary loops been studied to determine whether they too are smaller and more thin walled than normal.

Nephrons with larger renal corpuscles were first noted in neonatal Bull terrier kidneys, and larger corpuscles and glomerular tufts were also present in puppy kidneys. A separate population, which accounted for about half of the glomeruli was confirmed stereologically in the kidneys from adult dogs with normal renal function. These were still present in the kidneys from dogs with end-stage disease and thus did not appear to undergo sclerosis at a faster rate than the smaller glomeruli. It is not clear how these two populations of glomeruli develop.

Radial fibrosis was first seen in kidneys from adult Bull terriers with normal renal function and became more prominent in dogs with renal impairment. Cystic changes were an early and prominent abnormality. In neonates, cysts appeared to represent dilatation

of veins and peritubular capillaries, but in puppies and older dogs they corresponded to dilatation of Bowman's capsule probably occurring at least partly because of obstruction from fibrotic bands [29]. Similar cystic lesions have been observed in human Alport kidneys, but neither radial fibrosis nor cystic changes have been described previously in canine forms of Alport syndrome [17,18]. Foam cells were not present, but have not been described before in dogs with proteinuria from any cause.

Interestingly arterial and arteriolar sclerosis were common in affected Bull terrier kidneys even when renal function was normal, although dogs rarely became hypertensive. The mineralized membranes probably reflected the untreated metabolic disturbance of renal failure.

In Bull terriers with hereditary nephritis, tubular abnormalities were the best predictor of increased UPC and thus impaired renal function, and five of the 10 individual histological variables that correlated best with an increased UPC were direct or indirect measures of tubular atrophy and interstitial fibrosis. In contrast, there was no obvious predictor of serum creatinine but, of all the variables, tubular scores and, in particular, medullary fibrosis correlated best. These results are consistent with previous studies of X-linked Alport syndrome, where the degree of renal impairment depended on the severity of tubulointerstitial change, and correlated inversely with the amount of cortical interstitial volume [29] and thus with what we measured as tubular dilatation and cortical and corticomedullary fibrosis.

The demonstration in Bull terrier kidneys of fewer nephrons in neonates, increased fetal glomeruli and a separate population of glomeruli with larger corpuscles and tufts reflect the effects of the underlying genetic mutation that are first manifest antenatally. The major determinant of renal impairment in adult affected Bull terriers is, however, progressive tubulointerstitial damage after birth.

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