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Immunohistochemical Identification of Amyloid, Using an Anti-human Serum Amyloid P Component (SAP) Antibody, is Possible in Ruminants but not in Dogs and Cats

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Summary

Amyloidosis represents a heterogeneous group of diseases that have in common the deposition of fibrils composed of proteins of beta-pleated sheet structure, a structure which can be specifically identified by histochemistry using the Congo red or similar stains. Amyloid consists primarily of the amyloid fibrils but also of the amyloid P component (AP). This component, which is identical with the serum counterpart (SAP), is found in all types of human amyloid, and immunohistochemical identification of AP has been proposed as an adjunct to the universal, type-independent diagnosis of human amyloidosis. In the present study of animal amyloidosis, we compared the amyloid-specific Congo red stain with an immunohistochemical protocol using an anti-human SAP antibody for the identification of amyloid in formalin fixed tissue samples. The species and types of amyloidoses investigated were: (i) seven cows, one yak (Bos grunniens), and one sheep affected with amyloidosis of presumed AA type, (ii) one dog with a pancreatic endocrine tumour producing amyloid of presumed AIAPP type, (iii) two cats with presumed AIAPP-amyloidosis of the islets of Langerhans, one cat with presumed AA-amyloidosis, and one cat with an amyloid-producing odontogenic tumour. Intense immunostaining co-localized with amyloid, identified by its congophilia and green birefringence, using a protocol without any antigen retrieval in each of the seven cows, the yak and the sheep. The method seemed more sensitive in the ruminants than the Congo red stain, but was unable to detect amyloid in the dog and the cats regardless of the application of various antigen retrieval protocols. However, specific identification of amyloid still rests on the Congo red method or similar histochemical techniques.

Introduction

Amyloidosis represents a heterogeneous group of diseases affecting humans and animals. These diseases have in common the deposition of fibrils composed of proteins of beta-pleated sheet structure (Zschiesche and Jakob, 1989). Amyloidosis can be either primary or elicited by some other disease process and thus be secondary. Disease is caused by the accumulated amyloid or by the eliciting disease in combination with the accumulated amyloid.

The specific diagnosis of amyloidosis is made, by using one of several histochemical staining techniques. One of these is the often used Congo red staining that gives amyloid a reddish colour and apple green birefringence (Puchtler et al., 1962) based on the selective binding of the dye to the beta-pleated sheets (Glenner, 1980). Although the Congo red method is preferred for the unambiguous diagnosis of amyloidosis, the method holds some problems. Examples of these are that prolonged formalin fixation prevents staining (Elghetany and Saleem, 1988), feline amyloid is reported to exhibit poor staining with Congo red – a problem which might be relieved by increasing the thickness of the tissue sections (Boyce et al., 1984), and animal amyloid generally gives staining of variable intensity (Nordstoga, 1975).

Classification of amyloid and thus amyloidosis is based on the identity of the fibril precursor protein. More than 20 types of amyloid have been identified in man, and in human pathology, immunohistochemistry (IHC) is the standard procedure used for classification (Picken, 2001). In addition to the amyloid fibril, amyloid consists of various other components (Kisilevsky, 2000). Thus amyloid P component (AP) is found in all types of human amyloid (Pepys et al., 1994), and it is quantitatively the most important of the non-amyloid fibril constituents, comprising up to 15% of the amyloid (Francis, 1996). The AP is identical with a normal plasma protein known as serum amyloid P component (SAP) (Pepys et al., 1994). The ligand for SAP in amyloid is dermatan sulphate and heparan sulphate, which themselves are integral parts of amyloid (Magnus et al., 1994). In man, the identification of AP in amyloid-suspected deposits could thus be a useful, universal, immunohistochemical diagnostic tool independent of the amyloid type, and Francis (1996) did, indeed, suggest the use of this procedure for the diagnosis of all types of human amyloidosis.
In domestic animals, SAP has been identified in swine, cattle, goat and sheep (Pepys et al., 1978), and SAP in cattle has been further characterized (Andersen et al., 1992). However, SAP has been identified in all vertebrate species in which it has been sought, and in some invertebrates too (Gewurz et al., 1995), so probably SAP is a normal plasma protein well conserved throughout the animal kingdom. The purpose of the present study was to assess the co-location of amyloid, identified by its specific tinctorial properties with the Congo red dye, with staining obtained by anti-human SAP IHC in selected cases of amyloidosis in cattle, yak (Bos grunniens), sheep, dog and cat. Evaluation of the intensity indicating sensitivity of the two tests was also attempted, and thus the study should indicate if anti-human SAP IHC is usable for the identification of animal amyloidoses.

Materials and Methods

Animals, organs and tissues

The material in this retrospective study consisted of 14 cases registered with a diagnosis of amyloidosis or lesions of amyloid nature (Table 1). No records of the types of amyloid were available. The 14 cases consisted of seven cows, one yak (Bos grunniens), one sheep, one dog and four cats. From these 14 cases, the following tissues were selected for histochemical re-evaluation [haematoxylin and eosin (HE), Masson trichrome and Congo red] and IHC: seven bovine kidneys, one bovine liver, kidney and thyroid gland tissue from one yak, one ovine kidney, one canine pancreatic tumour, two feline pancreatic glands, one feline kidney and one feline mandibular tumour. Recorded, relevant data including macroscopic and histological diagnoses are listed in Table 1.

Also included in the study were tissue samples of the kidneys from three cows with recorded diseases of non-amyloid nature (chronic proliferative glomerulonephritis, journal no. 33857; chronic interstitial nephritis, journal no. 33928; acute suppurative E. coli-induced pyelonephritis, journal no. 33948) and from one cow without lesions (journal no. 33999). These tissues served as controls and were stained with Congo red and according to the immunohistochemical protocol.

All tissue samples were from the archives of the Department of Veterinary Pathobiology, Laboratory of Pathology, The Royal Veterinary and Agricultural University, Copenhagen, Denmark. The samples had been fixed in 10% neutral buffered formalin, embedded in paraffin wax, and kept as paraffin wax blocks. The exact fixation time was not recorded, but probably was one to several days. All cases had been journalized during the period from 1995 to 2003.

Table 1. Cases registered with a diagnosis of amyloidosis or lesions of amyloid nature

<table>
<thead>
<tr>
<th>Species</th>
<th>Case no. (journal no.)</th>
<th>Filed tissue specimens</th>
<th>Recorded, relevant data including macroscopic and histological diagnoses</th>
<th>Tissues selected for histochemical re-evaluation and immunohistochemistrya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>1 (27234)</td>
<td>Kidney</td>
<td>Kidney submitted from slaughterhouse: renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>2 (28042)</td>
<td>Kidney</td>
<td>Kidney submitted from slaughterhouse: pigmentation of renal tubular epithelium, renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>3 (28147-3)</td>
<td>Kidney</td>
<td>Kidney submitted from slaughterhouse: renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>4 (32828)</td>
<td>Kidney</td>
<td>Kidney submitted from slaughterhouse: small calculi in renal calices, renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>5 (33243)</td>
<td>Kidney, lymph node</td>
<td>Kidney submitted from slaughterhouse: renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>6 (33647)</td>
<td>Kidney</td>
<td>Kidney submitted from slaughterhouse: renal amyloidosis</td>
<td>Kidney, liver</td>
</tr>
<tr>
<td></td>
<td>7 (28827)</td>
<td>Kidney, liver, spleen, pancreas, lungs, skin, heart</td>
<td>Necropsy: Holstein Friesian, 4 years old, haematoma, necrosis and fibrosis of several leg muscles, ulceration of the sole, multiple decubitus ulcers, choriocapitite infestation, chronic non-suppurative interstitial nephritis, hepatic amyloidosis</td>
<td>Kidney, liver</td>
</tr>
<tr>
<td>Yak</td>
<td>8 (27036)</td>
<td>Kidney, lungs, thyroid gland, liver, colon, uterus, oesophagus</td>
<td>Organs submitted: old animal, hydrothorax, thrombosis of both jugular veins, cough, multiple abscesses in liver, pyometra, oesophageal ulcers, abdominal fat necrosis, renal amyloidosis, amyloid in thyroid gland and in hepatic portal arteries</td>
<td>Kidney, thyroid gland</td>
</tr>
<tr>
<td>Sheep</td>
<td>9 (23315)</td>
<td>Kidney, liver, spleen</td>
<td>Necropsy: 2 years old, sternal decubitus ulcer, sternal subcutaneous abscesses, solitary lung abscess, Cysticercus tenuicollis on surface of liver and omentum, chronic focal peritonitis, amyloid in kidney, in liver and in spleen</td>
<td>Kidney</td>
</tr>
<tr>
<td>Dog</td>
<td>10 (28225)</td>
<td>Pancreatic tumour, liver, adrenal gland</td>
<td>Necropsy: Border Collie, 9 years old, low blood glucose and convulsions, beta-cell carcinoma of the pancreas, possibly amyloid in pancreatic tumour</td>
<td>Pancreatic tumour</td>
</tr>
<tr>
<td>Cat</td>
<td>11 (25720)</td>
<td>Pancreas, liver, kidney blood vessel, adrenal gland</td>
<td>Formalin fixed organs submitted. Main Coon, 16 years old, lymphocytic cholangiohepatitis, cystic kidney, chronic glomerulonephritis, amyloid in pancreatic islets of Langerhans</td>
<td>Pancreas</td>
</tr>
<tr>
<td></td>
<td>12 (26696)</td>
<td>Pancreas, kidney</td>
<td>Pancreas and kidney submitted: 11 years old, diabetes mellitus, glomerulosclerosis, amyloid in pancreatic islets of Langerhans</td>
<td>Pancreas</td>
</tr>
<tr>
<td></td>
<td>13 (33787)</td>
<td>Kidney</td>
<td>Necropsy: Persian cat, 8 years old, isostenuria, chronic interstitial nephritis, chronic membranoproliferative glomerulonephritis, renal amyloidosis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td>14 (33470)</td>
<td>Mandibular tumour</td>
<td>Tumour submitted: domestic shorthair, 4 years old, amyloid-containing odontogenic tumour in rostral mandibula</td>
<td>Mandibular tumour</td>
</tr>
</tbody>
</table>

*Haematoxylin–eosin, Masson trichrome and Congo red.
Histochemical staining

Tissues were cut into 3- to 5-μm sections, which were mounted on adhesive slides and stained, according to the standard procedures with HE and the Masson trichrome technique. In addition, the slides were stained with Congo red as described elsewhere (Francis, 1996).

Immunohistochemical staining for AP

Staining for AP was performed by means of a two-layer indirect immunoperoxidase technique using a commercially available, polyclonal rabbit anti-human SAP antibody. Initially, all selected tissue specimens were stained according to a protocol that did not include any antigen retrieval. Thus, all tissues were cut into 3- to 5-μm sections, mounted on SuperFrost Plus slides (Gerhard Menzel, Brunschweig, Germany), dried at 60°C for 1 h, dewaxed in xylene, and rehydrated. Blocking of endogenous peroxidase activity was performed by incubating the sections with 3% (v/v) H2O2 for 5 min. The sections were subsequently incubated for 30 min with the primary, polyclonal rabbit anti-human SAP antibody, immunoglobulin fraction (A 0302; DakoCytomation Norden A/S, Glostrup, Denmark), diluted 1:200 with Tris-buffered saline (TBS: 0.05 M Tris–HCl; 0.15 M NaCl; pH 7.6) containing 1% (w/v) bovine serum albumin. Detection of bound antibody was performed with the peroxidase conjugated EnVision™ + system (K 3468; DakoCytomation Norden A/S) according to the instructions given by the producer. Finally, the sections were counterstained for 2 min with Mayers haematxolin and mounted with aqueous mounting medium (S 3025; DakoCytomation Norden A/S). The sections were washed with TBS for 5 min between each of the described steps.

Absence of staining (−) of amyloid with the initial protocol, i.e., no detectable staining of extracellular, congophilic and green birefringent material, gave in an attempt to provoke a positive response rise to four new immunohistochemical stainings with expanded protocols. These consisted of the initial version of the protocol supplemented with the four following antigen retrieval steps commonly used for unmasking antigens (McNicol and Richmond, 1998): (i) microwave boiling in a pH 6.0 buffer (0.01 M citrate), (ii) microwave boiling in a pH 10.0 buffer (S 3307; DakoCytomation Norden A/S), both at 750 W for 15 min followed by a 20-min cooling period where the slides were left in the buffer, (iii) Proteinase K (S 3307; DakoCytomation Norden A/S) digestion for 5 min, (iv) trypsin (S 3307; DakoCytomation Norden A/S) digestion for 30 min. Staining, whether weak (+), moderate (+++) or strong (+++) of the sections resulted in restaining of three serial sections with Congo red, anti-human SAP antibody, and a similarly diluted normal rabbit antibody, immunoglobulin fraction (X 0903; DakoCytomation Norden A/S), for the assessment of specificity of the reactions. Specificity was also assessed in serial sections of kidney tissue from the three cows with recorded diseases of non-amyloid nature and from the cow without lesions. All test rounds included staining of human renal amyloidotic tissue with the recommended protocol for the detection of human AP, using both the anti-human SAP and the normal antibodies (A 0302 data sheet, 10.10.02; DakoCytomation Norden A/S). This secured essentially that the antibodies worked well.

Results

Histochemical staining

A spectrum of lesions was present in the kidneys from the seven cows (case numbers 1–7, Table 1). However, all kidneys exhibited deposition of an eosinophilic, amorphous, homogeneous, proteinaceous substance that gave the amyloid-specific red colour and green birefringence with the Congo red stain. The location of the amyloid varied and was seen interstitially in the medulla (Fig. 1a,b) and in glomeruli. Other lesions included hyaline droplets in proximal tubular epithelial cells, dilated tubules, hyaline casts in tubular lumina, interstitial fibrosis and interstitial infiltration with mononuclear cells. The liver from one of the cows (no. 7) with renal amyloidosis had accumulated amyloid between the liver cell cords. The amyloid had a preference for the perilobular zone. The intensity of the Congo red stain (red colour) was generally low in the tissues from the cows, and in some of the tissues the staining was barely visible (Figs 1a and 2a). However, the red colour intensified slightly under polarized light and also gave rise to a just visible green birefringence (Fig. 1b).

In the kidney from the yak (no. 8) and the sheep (no. 9), amyloid deposition and other lesions were essentially identical with those described for the cows. In the thyroid gland from the yak, amyloid was deposited in the interstitial tissue outlining the follicles. The intensity of the Congo red staining (red colour) was moderate in the yak, intense in the sheep, and always with green birefringence.

In the pancreas from the dog (no. 10), an infiltratively growing tumour was seen, which in some areas showed an adenoid growth pattern. The tumour was criss-crossed by connective tissue septa, and amyloid was occasionally present here. The amyloid stained intensely and specific (green birefringence) with Congo red.

In pancreatic tissue from the two cats (nos 11 and 12), amyloid deposits were seen within the islets of Langerhans. In the kidney from cat no. 13, amyloid deposition and other renal lesions were essentially identical with those described for the cows. However, in contrast to the cows, extensive glomerulosclerosis was observed. The mandibular tumour from cat no. 14 showed expansive growth and consisted of odontogenic epithelial cells, some of which showed evidence of keratinization. The cells were located within a mesenchyme of fusiform to stellate cells. Within the epithelial component, amyloid was occasionally seen. The amyloid stained intensely and specifically with Congo red in all cats (Fig. 3).

Kidney tissue from all three cows with recorded diseases of non-amyloid nature and from the cow without lesions showed no evidence of amyloid deposits as judged by negative-Congo red staining.

Immunohistochemical staining

The renal tissue from the seven cows, the yak and the sheep showed the same pattern of staining. All stained, using the protocol without antigen retrieval, moderately (+ +) to strongly (++++) with the anti-human SAP antibody in the areas with amyloid, i.e., those areas displaying congophilia and green birefringence (Figs 1c and 2b). However, in some of the animals, a focal staining, weak (+) or moderate (+ +), of tubular epithelium (mainly cortical tubules; Fig. 2b), tubular
luminal content and blood-vessel luminal content (plasma) was also seen. However, these foci did not stain positive for amyloid with Congo red.

The amyloid in the liver of cow no. 7 showed a moderate to strong reaction (+ +, ++++) with the anti-SAP antibody, again using the protocol without antigen retrieval. However, the hepatocytes and blood-vessel luminal content (plasma) showed a weak (+) reaction with the antibody as well, but without evidence of amyloid. The amyloid in the interstitial tissue of the thyroid gland from the yak stained moderately (+ +) with the antibody (no antigen retrieval), and staining of other structures was not observed.
Identification of AP in Animal Amyloid

Using the SAP antibody, focal staining of tubular epithelium (mainly cortical tubules) tubular luminal content and blood-vessel luminal content (plasma) was seen in the renal tissue from the cow without lesions (+) and in the cows with diseases of non-amyloid nature (+ +). These areas were without amyloid by the Congo red stain.

Substituting the anti-SAP antibody with the normal antibody in the tissues from the ruminants revealed absence of staining (−) or weak (+) staining of amyloid and other structures. However, amyloid always stained with anti-SAP antibody with an intensity that was at least two grades higher than the staining generated with normal antibodies (+ + versus − or ++ + versus +).

The amyloid in the pancreatic tumour from the dog (no. 10) and amyloid within the pancreatic islets from the two cats (nos 11 and 12) revealed absence of staining (−) with the anti-SAP antibody, whether antigen retrieval was used or not. Amyloid within the kidney from the cat no. 13 and within the mandibular tumour from the cat no. 14 showed weak (+) staining with low pH microwave boiling and high pH microwave boiling protocols respectively. However, the intensity of the staining did not differ from that with the normal antibodies.

Amyloid in human renal tissue always stained strongly (++ +) with the anti-SAP antibody, as opposed to no (−) or weak (+) reaction with the normal antibodies.

Discussion

Immunohistochemical detection of amyloid in tissues from cow, sheep and yak was possible using an anti-human SAP antibody with a protocol without antigen retrieval. Specificity of the staining was assessed by evaluating the concord of the immunohistochemical and the Congo red stains, by substituting the anti-SAP antibody with normal antibodies and by performing the immunohistochemical stain on bovine renal tissue without lesions and on bovine renal tissues with lesions of non-amyloid nature. The immunohistochemical protocol performed well on amyloid identified by the Congo red stain (congophilia and green birefringence) giving the deposits an intensity of the staining did not differ from that with the normal antibodies.

Amyloid in human renal tissue always stained strongly (++ +) with the anti-SAP antibody, as opposed to no (−) or weak (+) reaction with the normal antibodies.

Typing of the amyloid, and thus classification of the amyloidosis, was not performed in any of the presented cases, but generalized, reactive AA-amyloidosis is considered to be the most commonly observed type in domestic animals (Maxie, 1993). Several reports on generalized, reactive AA-amyloidosis in cattle exist, and the incidence of this type of amyloidosis, which has the kidneys as the primary target organ, is ranging from 0.4% to 2.7% (Zschiesche and Jakob, 1989). Thus, based on the relatively high frequency of AA-amyloidosis in cattle, the cases of bovine amyloidosis described in the present study are probably all caused by AA. It is possible to identify AA histochemically as the protein is sensitive to KMnO 4 treatment which prevents the binding of Congo red to the amyloid (Wright et al., 1977). The method, as described by Francis (1996), was applied on some of the tissues from the cows, but sensitivity to KMnO 4 treatment was not observed (results not shown). However, the method is associated with technical problems that can lead to either false-negative (pseudoresistance) or false-positive results (Van de Kaa et al., 1986).

To our knowledge, no reports on amyloidosis in yak exist. However, the recorded macroscopic findings of abscesses in the liver and pyometra, and the finding of multiple organs affected with amyloidosis, points to AA-amyloidosis.

In sheep, three types of amyloidosis have been described: (i) generalized AA-amyloidosis has been reported by several authors, most recently by Ménsoa et al. (2003), (ii) local amyloidosis in C-cell carcinoma of the thyroid gland (Zschiesche and Jakob, 1989), (iii) local AP-amyloidosis found in nervous tissue in sheep affected with scrapie (Zschiesche and Jakob, 1989). Based on the recorded histological findings of amyloid in kidney, liver and spleen of the sheep of the present study, the amyloid here probably was of the AA type.

The recorded data on the canine pancreatic tumour, supported by the results of the histological re-evaluation, points to the diagnosis of a pancreatic endocrine tumour. Beta-cell adenoma and carcinoma are the most frequently occurring tumours and tubular epithelium is probably the result of proteinuria and resorption of SAP by the epithelium. A similar argument has previously been offered to explain the finding of AA in renal tubular epithelium from human patients with AA-amyloidosis (Van de Kaa et al., 1986), i.e. polyclonal antibodies raised against AA cross-reacting with SAA. It is stated that normal tissue AP is found in man (spleen, liver and kidney), but not in any other species (Pepys et al., 1997). This is in contrast to the above cited work of Niewold et al. (1991), in which AP is described to be associated with normal basal membranes of glomeruli and tubular epithelium in cattle, a finding which was explained by the presence of heparan sulphate in basal membranes and the binding of AP to this substance (Niewold et al., 1991; Magnus et al., 1994).

Detection of amyloid with the anti-human SAP antibody in the dog and the cats was unsuccessful, despite the use of various antigen retrieval methods. However, SAP seems to exist in all animal species (Gewurz et al., 1995). Thus, failure of staining of dog and cat amyloid could indicate that SAP does not accumulate here. However, I-123 labelled human SAP accumulates in amyloid-affected livers of Oriental cats (Pirisalu et al., 1994); the type of amyloid was not determined, but was probably AA. A more plausible explanation of the lack of staining is that the anti-human SAP antibody does not react with SAP in the two species.

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The recorded data on the canine pancreatic tumour, supported by the results of the histological re-evaluation, points to the diagnosis of a pancreatic endocrine tumour. Beta-cell adenoma and carcinoma are the most frequently occurring tumours
arising from pancreatic islets (Capen, 2002). Amyloid is found in approximately 25% of canine pancreatic endocrine tumours (O’Brien et al., 1987), and the amyloid probably is derived from islet amyloid polypeptide (IAPP) (O’Brien et al., 1990).

The recorded data and the histological re-evaluation of the two feline cases of amyloidosis of the islets of Langerhans, correspond well with the diagnosis of AIAPP-amyloidosis (O’Brien et al., 1993). Familial, systemic AA-amyloidosis occurs in Siamese, Oriental and Abyssinian cats (Niewold et al., 1999). Sporadic, systemic amyloidosis has also been described in other breeds (Blunden and Smith, 1992; Beatty et al., 2002), and the cat in our study was probably affected with renal AA-amyloidosis. The histological re-evaluation of the feline mandibular tumour clearly points to the diagnosis of an amyloid-producing odontogenic tumour (Head et al., 2002). The type of amyloid in these tumours is of epithelial origin (Breuer et al., 1994).

The use of the immunohistochemical protocol applying antibodies against human SAP and without antigen retrieval, seems to be a promising method for identification of amyloid in cows and possibly in all ruminants. Antigen retrieval was not assessed as the method without retrieval worked well. However, antigen retrieval might have improved the method further, but also will make the immunohistochemical protocol more laborious. In comparison with the immunohistochemical identification of the fibril precursor proteins, the general identification of amyloid with anti-SAP IHC will reduce the need for a vast panel of species and fibril precursor protein-specific antibodies. Moreover, the anti-SAP immunohistochemical protocol seems more sensitive than the Congo red method, as the latter method gave weak, barely visible (but specific) staining of amyloid in the cows of our study. This makes it laborious to scan the Congo red dyed sections in order to select areas for evaluating the presence of green birefringence. Optimization of the Congo red stain was not attempted in our study, but weak staining was not a general problem at our laboratory as amyloid in some animals stained intensely. In short, these findings are in agreement with the recorded superior sensitivity of IHC for the identification of amyloid fibrils (Linde et al., 1984), and that Congo red staining seems to hold some inborn technical problems that results in low sensitivity (Francis, 1996). Although immunohistochemical detection of AP is more sensitive than the Congo red stain, the latter specifically identifies the beta-pleated sheet structure of the deposits, and thus amyloid and amyloidosis, and IHC should always be co-evaluated with histochemistry (Pepys, 1992).

Acknowledgements

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References


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