Amyloidosis—Where Are We Now and Where Are We Heading?

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● *Context.*—Amyloidoses are disorders of diverse etiology in which deposits of abnormally folded proteins share distinctive staining properties and fibrillar ultrastructural appearance. Amyloidosis ultimately leads to destruction of tissues and progressive disease. With recent advances in the treatment of systemic amyloidoses the importance of an early diagnosis of amyloid, and a correct diagnosis of its type, has been realized.

Objective.—To summarize current recommendations for the diagnosis of amyloidosis.

Data Sources.—Presentation given at the 4th Annual Renal Pathology Society Satellite meeting in Istanbul based on discussions and recommendations formulated during an interactive diagnostic session held at the XIth International Symposium on Amyloidosis in Woods Hole, Massachusetts.

Conclusions.—Congo red stain is currently the gold standard for amyloid detection and the goal is to detect amyloid early. Diagnosis of the amyloid type must be based on the identification of amyloid protein within the deposits and not solely by reliance on clinical or DNA studies. However, the latter are recommended for confirmation of the amyloid type based on evaluation of the protein in deposits. Immunohistochemistry must be performed and interpreted with caution and inconclusive results must be evaluated further using the more sophisticated methods available in referral centers. An adequate amount and quality of tissue must be available for amyloid diagnosis and typing with emphasis on the use of fresh tissue and greater use of abdominal fat biopsy. The development of new technologies underscores the need for regular review of recommendations and standards for the clinical diagnosis of amyloidosis.

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A myloidoses are disorders of diverse etiology in which deposits of abnormally folded proteins share distinctive staining properties and fibrillar ultrastructural appearance.[1–3] Amyloidosis ultimately leads to destruction of tissues and progressive disease. Although they have been known since the time of Virchow in the 19th century, until relatively recently the amyloidoses were considered a medical curiosity of only academic interest rather than clinically relevant diseases. However, recent advances in the treatment of systemic amyloidoses have changed this outlook and, hence, the importance of an early diagnosis of amyloid, and a correct diagnosis of its type, has been realized.[2,4–9] Currently, more than 25 different proteins (and many more variants) are known to be involved in amyloidoses and additional protein types are continually being added to this list.[1–3] To accommodate this, a modern nomenclature has been developed, which is based on the type of protein involved. In this nomenclature, there is a prefix ''A,'' for amyloid, followed by an abbreviation derived from the name of the protein; thus, AL designates amyloid derived from immunoglobulin light chain, ATTR designates amyloid derived from transthyretin, AFib indicates amyloid derived from fibrinogen, and Aβ indicates amyloid derived from β protein, which is associated with Alzheimer disease[1] (Table).

Although this ''molecular-based'' classification of amyloidoses is very precise and allows for future expansion to include additional amyloid types, a simpler subdivision into systemic versus localized amyloidosis would be more relevant to clinical practice. However, although certain amyloid deposits are exclusively localized (several cerebral amyloidoses and amyloid associated with endocrine tumors), others (most notably AL) can be either systemic or localized. Therefore, amyloid typing must be followed by careful amyloid staging before distinction between localized and systemic amyloid can be made. Moreover, because at present it is not clear whether an apparently localized AL can progress to a systemic process, an appropriate follow-up may be indicated. Although, at present, management of localized amyloid deposits is mainly conservative, treatment of systemic amyloidoses involves radical approaches ranging from aggressive chemotherapy to liver transplantation; new pharmacologic therapies targeting systemic fibrillogenesis are also in trials.[2,4–17] The central concept of amyloidosis management is elimination of the supply of amyloidogenic protein. Thus, the treatment of AL, amyloidosis derived from immunoglobulin light chain and the most frequent type of systemic amyloidosis, targets the underlying neoplastic process with aggressive

Amyloid Protein	Precursor	Syndrome
AL/AH	Immunoglobulin; light/heavy chain	Multiple myeloma/plasma cell dyscrasia–associated, also known as primary
AA	Serum AA protein	Sporadic: secondary; familial: periodic fevers[b] (familial Mediterranean fever, other)
ALect 2	Leukocyte chemotactic factor 2	Renal—nephrotic syndrome and/or liver—chronic hepatitis[c]
$A\beta_2M$	β_2-microglobulin	Dialysis associated
ATTR	Transthyretin	Sporadic[d]; hereditary[e,f]
AFib	Fibrinogen A α-chain	Hereditary[e,g]
AApoAI	Apolipoprotein AI	Hereditary[e,h]
AApoAII	Apolipoprotein AII	Hereditary[e,i]
ALys	Lysozyme	Hereditary[e,j]
AGel	Gelsolin	Hereditary[e,k]
ACys	Cystatin C	Hereditary[e,l]

Renal Involvement in Human Systemic Amyloidoses[a]

[a] Updated with permission from S. Karger AG, Basel, Switzerland.[40]
[b] Genetic defect in proteins involved in the inflammatory response but not the amyloid precursor protein per se.[25]
[c] Apparently isolated renal involvement with nephrotic syndrome and liver involvement with chronic hepatitis, closely mimics AL.[3]
[d] Senile form derived from wild transthyretin with cardiomyopathy; renal involvement in medulla and vessels.[39]
[e] Genetic defect involving the amyloid protein precursor.[1,2,35]
[f] Polyneuropathy and cardiomyopathy, some mutants with severe renal involvement.[27–30]
[g] Severe nephropathy with glomerular involvement.[31,32]
[h] Hepatic, cardiomyopathy, nephropathy with involvement of renal medulla, some mutants with neuropathy.[12,33]
[i] Nephropathy.[11]
[j] Gastrointestinal, nephropathy.[34]
[k] Cutis laxa, cranial neuropathy, renal failure in homozygotes.[1,2]
[l] Clinically silent.[1,2]

chemotherapy and stem cell rescue.[4–6] In the second most common type of amyloidosis worldwide, AA, targeting the underlying inflammatory disease leads to a reduction of the circulating fibril precursor, serum AA protein.[18] In patients with familial AA amyloidoses, biologic drugs appear to be effective. A subset of patients with hereditary amyloidoses will benefit from liver transplantation.[10–13] A new class of antiamyloid agents, currently in clinical trials, also appear to be amyloid type specific for AA and ATTR.[14–17] In patients with dialysis associated amyloidosis, kidney transplantation is considered the best therapeutic option. Early treatment of amyloidosis is associated with much better outcomes and survival and may not only halt amyloidogenesis but even reverse established deposits.[18] Thus, currently, the challenge is to detect amyloid early and to type it correctly. This requires an increased awareness of the disease among both pathologists and clinicians. Although systemic amyloidoses involve multiple organs, kidneys are the most frequently affected organ.[18,19] This underscores the role of nephrologists and renal pathologists in the detection and typing of systemic amyloidoses. Thus, one of the major differential diagnoses of proteinuria is amyloidosis. Clinically, the presence of proteinuria, renal insufficiency, heart failure, orthostatic hypotension, peripheral neuropathy, or unexplained kidney, heart, or systemic disease are suspicious for amyloidosis.[2] Hepatomegaly, splenomegaly, hypothyroidism, and hypofunction and/or enlargement of the adrenal gland are also suspicious for amyloidosis. The symptoms associated with gastrointestinal amyloidosis can be quite diverse, ranging from bleeding, malabsorption, weight loss, and constipation to diarrhea. Although these symptoms are largely nonspecific, the fact that gastrointestinal biopsies are frequently performed provides a vast pool of material that is potentially diagnostic for amyloidosis. From the pathologist's perspective, the differential diagnosis of collagenous colitis and ischemia should also include amyloid.

The present review is based on a presentation given at the 4th Annual Renal Pathology Society Satellite meeting in Istanbul, Turkey, summarizing current recommendations for the diagnosis of amyloidosis. These recommendations were originally discussed and formulated during an interactive diagnostic session held at the XIth International Symposium on Amyloidosis. An extended abstract from this diagnostic interactive session was published in the proceedings of the meeting.[20]

In this review I address the following issues: (1) detection of amyloid, (2) the choice of tissue for diagnosis, (3) the role of genetics in the evaluation of patients with systemic amyloidosis, and (4) amyloid typing—immunohistochemistry versus proteomics (or both).

DETECTION OF AMYLOID

Diagnosis of amyloidosis is based on the detection of deposits in tissues.[1,2,20] Thus far, no biochemical markers in body fluids, diagnostic of amyloidosis, are available.

Congo red stain continues to be the gold standard for detection of amyloid deposits.[1,2,20] In bright field, deposits of amyloid stained with Congo red typically have a salmon-pink color. However, small deposits, in particular in thinner sections, may not be apparent in bright light. Importantly, the bright field appearance in itself is not diagnostic. Congo red–stained slides must be examined under polarized light and only the presence of apple-green birefringent deposits is considered diagnostic of amyloid.[19,20] Caution is advised regarding ''overinterpreting'' collagen as amyloid. In addition to the experience of the observer, good fixation, a proper staining protocol (alkaline Congo red), and appropriate optics are required for the examination of Congo red–stained slides. Thus, a strong light source and a rotating table are recommended. Moreover, reading slides in a darkened room, after pupil acclimation, facilitates the detection of smaller deposits. Thicker sections (5–10 μm) may be helpful but are not essential if the previously listed conditions are met.[19,20] Other stains, or techniques, such as fluorescence, thioflavin S and T, methyl violet, and sulphonated Alcian blue are less specific and at times also less sensitive (most notably
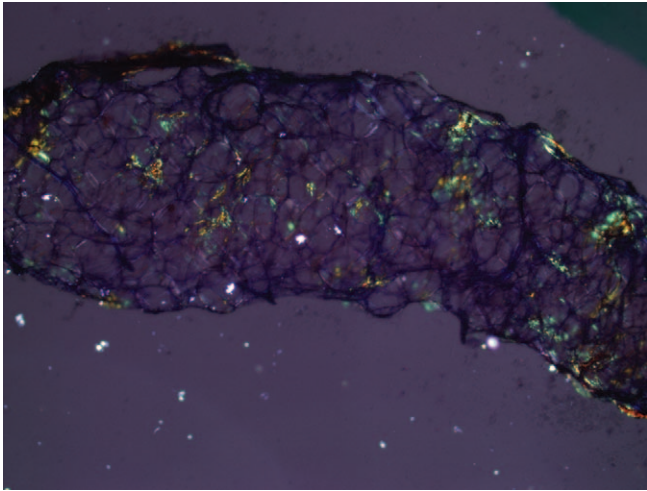
Diagnosis of Amyloidosis—Picken

Figure 1. *Abdominal fat pad aspirate with deposits birefringent under polarized light (Congo red viewed under polarized light, original magnification ×200).*

sulphonated Alcian blue or methyl violet) for the detection of amyloid, and confirmation with Congo red stain is required. For example, sulphonated Alcian blue identifies glycosaminoglycans (which form scaffolding for the amyloid fibrils) rather than amyloid fibril protein per se. Immunofluorescence may increase sensitivity (especially in the case of minute deposits) but its specificity is lower.[20] Among the stains commonly used in renal pathology, periodic acid–Schiff and silver stain may be helpful in raising a suspicion of amyloid: Deposits of amyloid are weakly periodic acid–Schiff positive and negative in a silver stain. However, Congo red stain should be examined not only to confirm a suspicion of amyloid deposits but also to rule out the possible presence of early deposits, which are otherwise inconspicuous in hematoxylin-eosin or these special stains. Because deposits of amyloid are frequently very focal and irregularly distributed in tissue sections, multiple sections may need to be examined.[2,20]

In keeping with the definition of amyloidosis, a generic diagnosis of amyloid requires either Congo red positivity or electron microscopic demonstration of the fibrillar nature of deposits. Knowledge of the ultrastructural appearance of amyloid is important in the differential diagnosis of various organized deposits (addressed by Herrera and Turbat-Herrera elsewhere in this issue).

THE CHOICE OF TISSUE SPECIMEN: FAT AS AN UNDERUSED SOURCE OF TISSUE FOR AMYLOID DETECTION AND BEYOND

For the detection of amyloid, biopsy of a clinically affected organ is the most sensitive method and may also detect concomitant pathologies.[2] However, such a biopsy is invasive and carries the risk of complications, in particular bleeding. Thus, if amyloidosis is clinically suspected, a less invasive procedure is desirable. In the early 1970s, Westermark and Stenkvist (recently reviewed in reference 21) demonstrated that amyloid can be detected in subcutaneous fat. During the decades, subcutaneous fat pad biopsy, obtained via fine-needle aspiration, being safe, cheap, and rapid, has been introduced as a screening test for the detection of amyloidosis (Figure 1). van Gameren et al[22] have shown that by examination of 3 Congo red–

stained slides per patient, amyloid can be detected with 100% specificity and 80% to 90% sensitivity. However, many pathologists have encountered less than optimal results due to inadequate samples and technically poor smears; misinterpretation of Congo red stains because of the autofluorescence of collagen under polarization microscopy is also not uncommon. For a description and instruction video of the fat aspiration procedure please refer to www.amyloid.nl (ie, www.amyloid.nl/investigations. htm; accessed May 7, 2009). Recently, a semiquantitative grading system for the amount of amyloid in fat tissue has been proposed by Hazenberg et al.[23] The authors propose that the amount of amyloid in the examined fat tissue be graded using a Congo red score from 0 (negative) to 4+.[23] In addition to the Congo red score, Hazenberg and colleagues[23] have also developed the quantitative assessment of amyloid in fat in AA amyloidosis using an ELISA method. Both Congo red score and quantitative assessment were validated clinically and shown to correlate with disease severity, progression, and response to treatment, at least in patients with AL and AA amyloidosis.

In recent years, fat has reemerged as an underused source of tissue not only for amyloid diagnosis but also for amyloid typing and staging. With an adequately diagnostic fat specimen, it is possible to perform not only a Congo red stain but also immunofluorescence, electron microscopy, and, if necessary, a Western blot or other molecular studies for amyloid typing. Dr G. Gallo's personal experience in the typing of amyloid deposits in fat aspirates, with a 94% success rate, is very encouraging.[24] Subsequent molecular analysis of extracted amyloid deposits confirmed the accuracy of the immunofluorescence method.[24]

Not infrequently, following the initial detection of amyloid in biopsy material, the amount of tissue available for further characterization of deposits is insufficient and a second biopsy must be performed. In such cases, an abdominal fat biopsy should be considered. Moreover, because frozen tissue is preferred for amyloid typing (please see later) a second biopsy provides the opportunity to secure fresh, rather than formalin fixed, material. In this respect, a surgical abdominal fat pad biopsy is preferred rather than an aspirate. As well as being a simple procedure, surgical biopsy secures more abundant tissue for additional studies.

Renal amyloidosis is usually associated with systemic amyloidosis, whereas amyloid elsewhere may be localized or part of a systemic process. Thus, the diagnosis of extrarenal amyloid requires subsequent staging to address the issue of whether we are dealing with a systemic or a localized form of amyloidosis. Again, an abdominal fat biopsy is the specimen of choice for amyloid staging purposes. Interestingly, amyloid deposits in other parts of the genitourinary system are most commonly localized and mimic tumors clinically.

THE ROLE OF GENETIC TESTING IN THE EVALUATION OF PATIENTS WITH SYSTEMIC AMYLOIDOSIS

The role of genetics in systemic amyloidoses has recently been reevaluated. Genetics may be associated with amyloidosis in several ways: either as a mutation in nonamyloid protein or as a mutation involving amyloid protein itself; a potential role for genetics in "sporadic amyloidoses" is also suspected.[18,19,25–28] The first of these mechanisms occurs in patients with familial AA, which is as-

sociated with various periodic fevers, of which familial Mediterranean fever is the best known.[25] These patients have an inborn error of inflammatory response in the innate immune system and mutations in genes for nonamyloid fibril proteins play a permissive role in the development of amyloid. The pathology of sporadic versus familial AA is similar and, therefore, diagnosis of familial AA has to be based on clinical grounds. However, the distinction between sporadic versus familial AA has implications for treatment and prognosis and should also involve genetic counseling. See also INFEVERS, the registry for familial Mediterranean fever and hereditary inflammatory disorder mutations (http://fmf.igh.cnrs.fr/ISSAID/infevers/; accessed May 7, 2009).[25]

Systemic amyloidoses that develop as a consequence of a mutation involving the amyloid protein itself are referred to as hereditary amyloidoses and several proteins have been shown to be amyloidogenic following mutation.[1,2,18,19,26–36] Many proteins have been shown to have multiple amyloidogenic mutations and phenotypes may vary depending on the mutation.[26–34] Interestingly, the kidney may be involved by all types of hereditary amyloidoses and the distribution and concentration of deposits may vary, including glomerular and/or extraglomerular deposits, some being limited to extraglomerular vasculature or to deep medulla.[27–34] In certain hereditary amyloidoses, the degree of renal involvement is also greater in homozygotes than in heterozygotes (Table).

Hereditary amyloidoses have traditionally been considered to be rare and believed to be associated with a positive family history. This misconception has been challenged recently. Although, in the United States, the frequency of diagnosis of hereditary amyloidoses has increased 5-fold during the last 3 decades (from 2% to 10%), they are still believed to be underdiagnosed (reviewed in reference 2). For comparison, in the United Kingdom 16% of amyloidoses were recently diagnosed as hereditary.[2,33] Although hereditary amyloidoses have an autosomal dominant mode of inheritance, owing to variable penetrance, the clinical presentation can be quite diverse and the onset of clinical disease quite late.[29,35] This, coupled with the pervasive lack of awareness of amyloidosis, accounts for the fact that a family history of amyloidosis is often missing.[35] Clinically, hereditary amyloidoses may mimic AL, which is also the most common type of systemic amyloidosis. The biggest challenge that has emerged during recent years is the detection and correct diagnosis of these hereditary amyloidoses and their differentiation from AL. In view of the differences in treatment, the need for such distinction cannot be overemphasized.

The question may arise as to how to detect these hereditary amyloidoses? Can we diagnose amyloid type based on molecular studies alone? The short and decisive answer to this question is no. The presence of a mutation does not always correlate with the amyloidosis type in some patients. Thus, patients can have AL and carry a genetic variant that is not the cause of their amyloidosis.[20,36–38] On the other hand, some 25% of patients with hereditary amyloidosis may have a coincidental monoclonal gammopathy.[20,33,37] Thus, currently, genetic testing should always be complementary to other diagnostic techniques that allow unequivocal identification of the amyloid protein. In cases in which DNA sequencing detects a mutant amyloid precursor protein, protein analysis must be the definitive evidence.[20]

However, DNA analysis is mandatory to confirm a diagnosis of hereditary amyloidosis based on identification of the protein type present in amyloid deposits (please see later). Precise identification of the mutation is important for treatment and prognosis. Only patients with hereditary amyloidoses associated with proteins produced by the liver (exclusively or predominantly) may benefit from liver transplantation as a form of a surgical gene therapy.[10–13] There are also differences between phenotypes, including prognosis, associated with different mutations of the same protein. Importantly, the full spectrum of hereditary amyloidoses is still being discovered and the absence of any currently known amyloidogenic mutation does not rule out hereditary amyloidosis associated with a new, hitherto-unknown mutation or the involvement of entirely new proteins.[3]

ATTR, amyloidosis derived from a variant of transthyretin, is the most common hereditary amyloidosis in the United States and worldwide.[27–29] Although ATTR is typically associated with polyneuropathies and cardiac involvement, some mutations show significant renal involvement as well.[27] Interestingly, even the wild-type transthyretin can undergo fibrillogenesis in older individuals, targeting predominantly the heart.[39] ATTR, whether hereditary or senile, continues to be underdiagnosed. AFib, amyloidosis derived from a mutant of fibrinogen, first emerged as the most common type of hereditary systemic amyloidosis in Europe, but since then its worldwide distribution is unraveling. AFib is derived from a mutant of the fibrinogen A α chain.[31,32] Other hereditary amyloidoses include AApo A-I, AApo A-II, AApo AIV, ALys, and AGel (Table; recently reviewed in reference 2).

AMYLOID TYPING: PITFALLS AND EMERGING PROSPECTS

What issues are involved in the differential diagnosis of amyloid type? How should one approach the ever-expanding diversity of amyloidoses?

In patients not on dialysis, who are diagnosed with systemic amyloidosis, therapeutic options center on the recognition of 1 of 3 main categories of systemic amyloidosis: AL, AA, and the ever-expanding group of hereditary amyloidoses (reviewed in reference 2). Immunohistochemistry is currently the standard for amyloid typing in routine clinical practice. AA can be reliably typed in frozen and/or paraffin sections. However, immunohistochemical typing of AL is still challenging and the difficulties frequently compounded by truncation of the light (or heavy) chain. Commercial antibodies are raised against the constant regions of the respective immunoglobulin light chains. Therefore, a subset of AL, in which amyloid fibrils are derived from a truncated light chain (ie, containing only variable regions), will be expected to be nonreactive with commercial antibodies.[2,19,40–45] At the same time, a limited antibody panel will also miss a number of hereditary amyloidoses as well. In such cases, the major differential diagnosis is between AL and hereditary types. Regrettably, in the past, some pathologists rushed to diagnose nonreactive cases as AL, seemingly by default.[43] Given the implications for patient treatment, such an approach is a sure way of discrediting not only immunohistochemistry but also pathologists as well. The second troublesome issue is the presence in the tissue of background stain, which in paraffin sections in particular can be significant due to the ''locking-in'' of serum proteins during fixation. However,
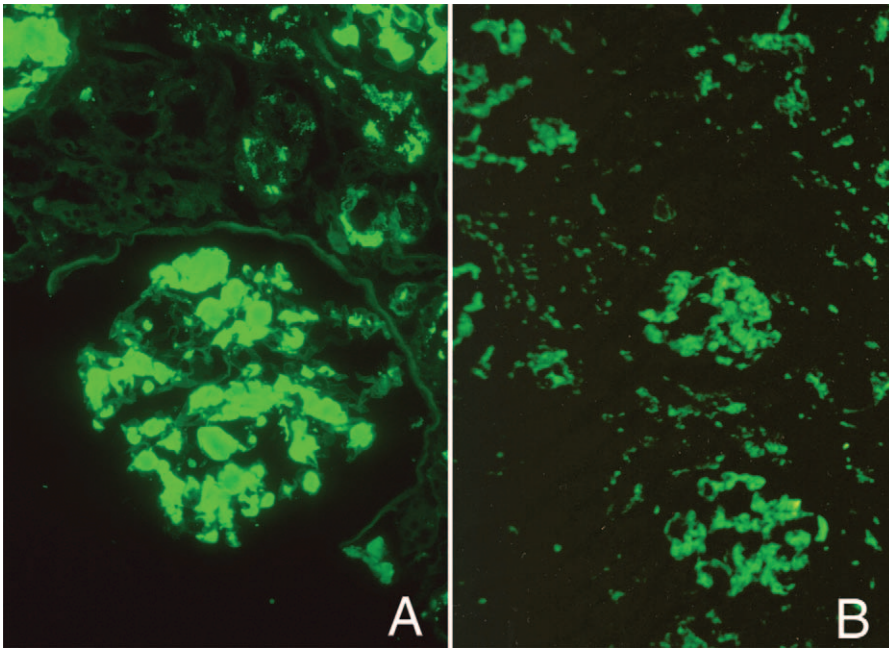
Figure 2. *Diagnostic results of amyloid typing for λ light chain. A, Strong, 3+ stain for λ light chain; stain for κ light chain was negative (not shown). B, Stain for amyloid P component. Amyloid P component is present in all types of amyloid deposits regardless of their chemical composition. Both stains, for λ light chain and amyloid P component, correspond to areas that are Congo red positive and exhibit birefringent under polarized light (frozen sections, immunofluorescence, original magnifications ×100 [A] and [B]). Reprinted with permission from S. Karger AG, Basel, Switzerland.[40]*

this issue can be alleviated by the use of frozen sections and immunofluorescence stains, which provide a cleaner background (Figure 2). Moreover, immunofluorescence stains on frozen sections have a higher sensitivity when compared with immunoperoxidase stains on paraffin sections (reviewed in reference 2). Therefore, in inconclusive cases, acquisition of an additional sample of fresh tissue should be considered. Abdominal fat surgical biopsy, being essentially a noninvasive procedure, is a clinically acceptable source of additional diagnostic material for amyloid typing, which has hitherto been largely used only for screening purposes.[2,20]

Interpretation of amyloid typing must be done in the context of Congo red positivity, in which areas that are positive for amyloid by Congo red stain are correlated with immunohistochemistry. To address this issue, an elegant ''overlay technique'' has been developed, in which a Congo red stain and immunohistochemistry are performed on the same slide—at least in the case of AA amyloidosis.[22] Permanganate pretreatment of Congo red–stained slides for amyloid typing is completely obsolete and should not be used or reported.[20] The interpretation of immunohistochemistry performed in paraffin sections and immunofluorescence in frozen sections is not a simple matter and also depends on the experience and expertise of the operator. It is important to use an antibody panel and a built-in control for evaluation of these studies, as well as positive and negative controls (Figure 3). In addition to antigenic preservation and sensitivity of detection, stringency of diagnostic criteria and technical issues
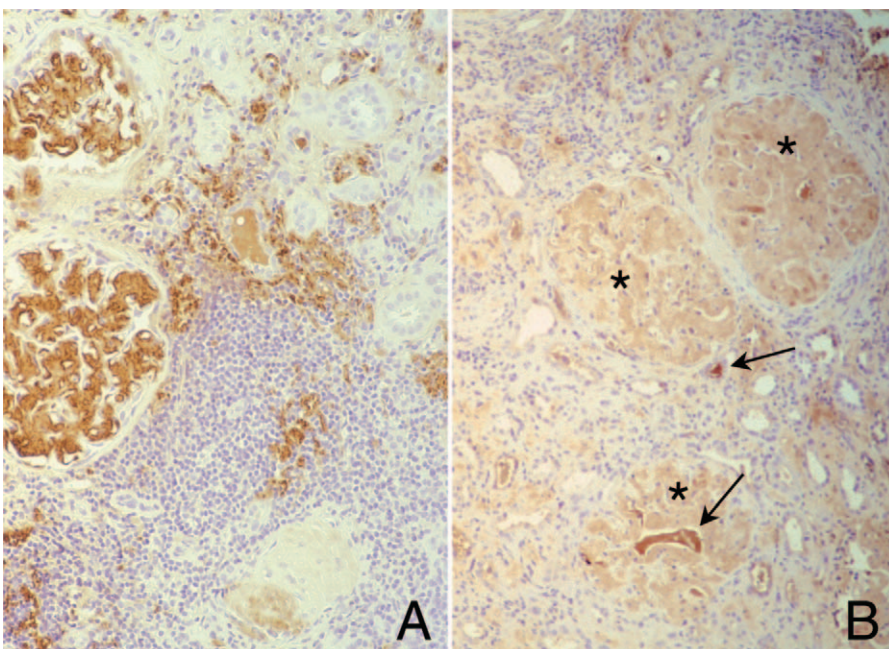

Figure 3. *Amyloid typing in paraffin sections. A, Strong, 3+ stain for λ light chain. B, Stain for κ light chain with only focal positivity, corresponding to serum proteins (arrows) and only a blush stain in areas corresponding to amyloid (asterisks) (immunoperoxidase, original magnifications ×150 [A and B]). Reprinted with permission from S. Karger AG, Basel, Switzerland.[40]*

also play a role (recently reviewed in reference 2). Good results for amyloid typing using amyloid-specific antibodies are reported with other antibody-based techniques, such as immunoelectronmicroscopy[46] and Western blotting.[21] Recently, antibodies raised against recombinant peptides corresponding to the variable region of immunoglobulin light chains were also tested for their potential utility in amyloid typing.[47]

To conclude, immunohistochemistry, in particular immunofluorescence on frozen sections, is a fast and valid methodology for amyloid typing but should be done with caution and with a full awareness of its limitations and pitfalls. In cases that are inconclusive or negative, evaluation by a reference laboratory, using more sophisticated methods, should be pursued.[2,20]

Direct typing of amyloid protein extracted from formalin-fixed, paraffin-embedded specimens has also been reported.[48–50] Using proteomics techniques, amyloid typing can be successful in small samples, including biopsies.[51–53] Again, even though such studies are feasible in paraffin-embedded biopsies, fresh tissue is preferable and fat may be an excellent source of such samples.[51] Although these technologies, which are currently available only in highly specialized laboratories, have not yet been validated in large numbers of samples at multiple centers, their development is a welcome advancement in the diagnosis of amyloidosis.[54–56] In current pathology practice the molecular characterization of amyloid proteins represents a valuable complement to immunohistochemistry.

Finally, processing and interpretation of all amyloid specimens is best relegated to renal pathologists who are more familiar than general pathologists with polarization microscopy, immunofluorescence microscopy, and electron microscopy.

The current recommendations regarding amyloid diagnosis can be summarized as follows:

1. Congo red stain is currently the gold standard for amyloid detection and the goal is to detect amyloid early.
2. Diagnosis of the amyloid type must be based on the identification of amyloid protein within the deposits and not solely by reliance on clinical or DNA studies. However, the latter are recommended for confirmation of the amyloid type based on evaluation of the protein in deposits.
3. Immunohistochemistry must be performed and interpreted with caution and inconclusive results must be evaluated further using the more sophisticated methods available in referral centers.
4. An adequate amount and quality of tissue must be available for amyloid diagnosis and typing with emphasis on the use of fresh tissue and greater use of abdominal fat biopsy.
5. The development of new technologies underscores the need for regular review of recommendations and standards for the clinical diagnosis of amyloidosis and the need to address the regulatory (accreditation, licensing, validation, analyte-specific reagent status) and reimbursement issues connected with these emerging technologies.

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