The Interpretation of Congophilia in Tissue Biopsies: Caution Required

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Amyloidoses are diseases caused by tissue deposits that consist of an abnormally folded protein. Currently at least 36 protein types, and many more variants, have been identified in human amyloidoses. Despite the chemical diversity of the amyloid fibril-forming proteins, all amyloid deposits share an affinity for Congo red dye and have the same ultrastructural properties. Recent decades have seen remarkable progress in the treatment of patients with several types of systemic amyloidosis, as evidenced by a durable treatment response and enhanced long-term survival when the disease is diagnosed early, before significant organ injury occurs. Hence, there is an urgent need for the early diagnosis of amyloidoses by detection of amyloid deposits in tissue.[1]

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Besides deposits of amyloid, various other organized deposits have been shown to occur in human tissues (in particular in the kidney), forming crystals, tactoids/tubules, or fibrils that are different from those of amyloid. These other fibrillar deposits, diagnostic of fibrillary glomerulonephritis (FGN), are morphologically similar to amyloid in that they are rigid and nonbranching, but differ from amyloid in that they are thicker. The pathogenesis, prognosis, and treatment of these various deposition diseases are different, so their correct differentiation from amyloidosis is important.[2,3] Uniquely in the case of amyloid, Congo red, a fabric dye, has been used for the detection of amyloid deposits since the early 20th century. In contrast, fibrillary deposits in FGN (as well as all other organized deposits) have been shown to be noncongophilic.

However, in this issue of *AJKD*, Alexander at al[4] suggest that, based on their experience with 18 cases of FGN, certain rare cases may mimic the appearance of amyloid by Congo red stain. Specifically, they encountered rare cases of FGN showing congophilia and anomalous colors under polarization. Similarly suspicious images were obtained by Congo red combined with fluorescence microscopy.[4] This finding therefore appears to undermine the specificity of the Congo red stain for amyloid. One could argue that the images presented in these cases of "congophilic FGN" fail to show convincing congophilia and birefringence, with both being variable but generally weak. However, in view of the variability of congophilia in otherwise typical deposits of amyloid already reported in the literature, findings in the 18 cases under discussion must be carefully considered.

Although Congo red staining has long been accepted as the gold standard for amyloid identification, the interpretation of this stain is not always easy because the staining procedure requires expertise and the use of polarized light microscopy. It is well known that polarization can be difficult to perform and interpret and that it requires good-quality optics. Upon binding of the Congo red dye, amyloid fibrils exhibit green birefringence when viewed by polarization microscopy. However, the classic "apple green" color is typically seen only under ideal optical conditions, whereas other anomalous colors may appear/disappear owing to strain birefringence (yellow) and/or during uncrossing of the polarizer and analyzer (orange). Thus, the dye's diagnostic "apple green birefringence" may be difficult to visualize, resulting in lower sensitivity of detection. Hence, the definition of amyloid staining was expanded in the most recent update on amyloid nomenclature issued by the International Society of Amyloidosis to read: "an amyloid fibril must exhibit affinity for Congo red and with green, yellow or orange birefringence when the Congo red-stained deposits are viewed with polarized light."[1 (p 210)]

Moreover, it has also been reported that not all amyloid deposits display an equal intensity of polarization. Thus, some deposits have been shown to exhibit "sparkly" green birefringence (amyloid derived from leukocyte chemotactic factor 2 [ALect2], seminal vesicle amyloid), whereas others are only weakly birefringent.[5,6] In a survey by Picken,[7] variability of Congo red stain intensity was reported by 75% of renal pathologists.

Among Swedish patients with amyloid derived from the transthyretin (TTR) Val30Met mutation, Suhr et al[8] noted marked variation between different individuals in the intensity of Congo red staining of deposits and their birefringence pattern when examined under polarized light. Upon further investigation, it was shown that this difference in affinity for the Congo red stain was a consequence of 2 distinct types of amyloid fibrils. One of these (type A) consisted of carboxy-terminal TTR-related amyloidosis (ATTR) fragments and full-length TTR, whereas the other (type B) consisted exclusively of full-length TTR. The latter type was characterized by a strong affinity for Congo red staining and a glittering appearance when visualized under polarized light, whereas ATTR deposits from patients with type A fibrils only displayed a weak affinity for Congo red and an absence of glittering birefringence.

Differences in the level of congophilia were also reported in a rare case with 2 co-existing types of amyloid in myocardium.[9] Further studies using immunohistochemistry (IHC) and laser microdissection-tandem mass spectrometry showed that areas with intense congophilia corresponded to deposits of amyloid derived from the λ light chain (AL-λ), whereas pale deposits were derived from TTR.

In the author's own experience, among the cases referred for a second opinion, many show weak congophilia.[10] This illustrates the difficulties encountered in the evaluation of Congo red–stained slides as a consequence of basic differences in congophilia, as well as a lack of experience by many pathologists. Such cases require careful evaluation using good-quality optics to demonstrate diagnostic polarization. Evaluation of the Congo red–stained slides must be done using maximum microscopic illumination, preferably in the dark, with the observer's pupils dark adapted; a commercial polarizer set, with a rotatable table, is strongly recommended.

In routine practice, it is very helpful to combine fluorescence microscopy with Congo Red stain to screen for amyloid deposits.[10] Thus, using a red fluorescence filter (tetramethylrhodamine [TRITC]), deposits of amyloid appear bright red and are therefore much easier to see than under polarization alone (see the figure in reference[10]). Although this technique is not entirely specific and polarization is still required for confirmation, it facilitates the detection of small amyloid deposits.

Taking into account the foregoing information, the following question must be asked: can we diagnose (or rule out) amyloid with confidence, and if so, how? In this issue of *AJKD*, Alexander et al have provided answers to these questions by conducting an extended workup of their cases to include not only electron microscopy (EM) and thioflavin stain, both of which are routinely used in nephropathology practice, but also IHC for a newly discovered marker for FGN and laser microdissection-tandem mass spectrometry (Table 1).

When using EM, amyloid fibrils and the fibrils present in FGN, despite being morphologically similar, differ in width: amyloid fibrils are typically 10 (range, 8-12) nm wide, while the fibrils present in FGN are said to measure about twice as much. In their cases of "congophilic FGN," Alexander et al reported that the mean fibril width was 14 (range, 11-18) nm and hence showed some overlap with amyloid fibrils. This highlights the fact that in individual cases in clinical practice, diagnosis of amyloidosis based on fibril width alone may be difficult in the absence of unequivocal/convincing Congo red positivity. Thus, caution is required when interpreting some fibrillary deposits as amyloid in specimens in which Congo red stain is negative for amyloid. In particular, in the kidney, amyloid mimickers and "look-alikes" are not uncommonly seen. For these reasons, the pursuit of additional testing, as practiced by Alexander et al in this report, is appropriate and not surprising.

The fluorochrome dye thioflavin-T (and its derivatives) has been known as a potent histologic marker of amyloid, with specificity similar to that of Congo red stain.[11] Interestingly, in the current study by Alexander et al, thioflavin staining was negative in 5 of 6 cases tested, with only 1 case considered to be weakly positive in an apparently nonblinded evaluation. It should be noted that thioflavin stains are not entirely specific for amyloid in tissue sections because they may stain other structures. Thus, results obtained with thioflavin must be correlated with Congo red stain itself or with EM. Other histochemical stains (eg, crystal violet and sulfated alcian blue) are not only less sensitive, but also less specific and are not recommended.

Alexander et al, in their quest to solve the current diagnostic challenge, set out to look for molecular signatures of amyloid and FGN by proteomic methods and IHC.[12-14] DNAJB9 (DnaJ homolog subfamily B member 9), one such marker, has been shown to be invariably present in deposits of FGN by proteomics methods. This was subsequently confirmed by IHC and immune EM.[13,14] In the current study, DNAJB9 was also detected using laser microdissection-tandem mass spectrometry in tissues in all 18 cases of congophilic FGN, while proteins considered to be amyloid signatures (serum amyloid P component, apolipoprotein E, and apolipoprotein AIV) were essentially absent. IHC, performed on selected cases, was also positive for DNAJB9.

What does all this tell us? Although Congo red stain remains at the forefront of testing in the diagnosis of amyloidosis, its interpretation may be challenging in certain instances involving genuine cases of amyloidosis that show weak congophilia, as well as rare cases of FGN that may be also weakly congophilic. However, in cases with less than optimal results ensuing from the initial workup, further studies must be performed to reach a clinically acceptable level of confidence in the diagnosis. New staining and imaging techniques are being tested,[15]

Table 1. Differential Diagnosis Between Congophilic and Noncongophilic FGN Versus Amyloidosis

	Congophilic FGN (n = 18)	Noncongophilic FGN (n = 56)	Amyloidosis (n = 145)
Congo red	Suspicious	Negative	Positive
Thioflavin	5/6 negative 1/6 weakly positive	Negative[a]	Positive[a]
Monotypic IF	6%	9%	92.7% in AL
EM	11-18 nm; mean, 14 nm	10-26 nm; mean, 16 nm	8-12 nm
IHC DNAJB9	4/4 positive	Positive[a]	Negative
LMD-MS/MS "amyloid signature"	Absent	Absent	Present

Abbreviations: AL, immunoglobulin light chain amyloidosis; DNAJB9, DnaJ homolog subfamily B member 9; EM, electron microscopy; FGN, fibrillary glomerulonephritis; IF, immunofluorescence; IHC, immunohistochemistry; LMD-MS/MS, laser microdissection-tandem mass spectrometry.
[a]Historical data, based on published literature.
Based on data from Alexander et al.[4]

and it is hoped that these new optically active conformation-sensitive ligands will be introduced into clinical practice in the near future. Until such time, we must make the most of the methods that are currently available and, in the case of congophilia, some caution in its interpretation is required.

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Support: None.

Financial Disclosure: The author declares that she has no relevant financial interests.

Peer Review: Received April 24, 2018, in response to an invitation from the journal. Direct editorial input from an Associate Editor and a Deputy Editor. Accepted in revised form May 17, 2018.

 doi: 10.1053/j.ajkd.2018.05.009

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