C4d, a new diagnostic modality for cutaneous amyloidosis: a comparison of C4d expression in cutaneous amyloidosis and cutaneous lichen planus

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Background: Amyloidosis is a rare disease characterized by the extracellular deposition of a misfolded protein in multiple organs. Cutaneous amyloidosis (CA) is diagnosed by detecting amyloid deposition in the skin. Lichen amyloidosis (LA) and macular amyloidosis (MA) without visceral involvement are two of its more prevalent types.

Methods: This case-control study was conducted to evaluate C4d staining in amyloidosis to determine whether it could be used as a diagnostic tool for amyloidosis. Moreover, the results of C4d expression in amyloidosis with colloid bodies in lichen planus (LP) were compared. Therefore, 41 cases of CA and 43 cases of LP were selected. All samples were stained with C4d immunostain.

Results: 12 of 41 cases of CA had apple green birefringence; however, all of them were positive for C4d, the same as the LP group. The CA group had 100% C4d and 29% Congo red sensitivities ($P < 0.05$). C4d had 100% sensitivity for colloid bodies in LP. Therefore, the C4d stain could serve as a new IHC marker for highlighting the colloid bodies.

Conclusion: C4d immunohistochemical (IHC) staining could be a very valuable ancillary tool for diagnosing amyloidosis, although it did not differentiate amyloid deposition from colloid bodies of LP.

Keywords: lichen planus, cutaneous amyloidosis, C4d, Congo red, amyloid, colloid body

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INTRODUCTION

Amyloidosis is a rare disease that consists of a heterogeneous group of disorders. It is characterized by the extracellular deposition of misfolded protein, nonbranching linear fibrils (with an average 10 nm diameter) in a β-pleated sheet arrangement with typical

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apple-green birefringence on Congo red staining under polarized light, systematically throughout the body or in specific organs [1,2].

The pathophysiology of amyloid is very diverse. However, only these 6 types, including AA, ATTR, Aλ, Aκ, Aβ2M, and AK, are associated with pathology in the skin [3]. Amyloidosis is classified based on the type of precursor protein and its distribution (systemic or localized). To classify amyloid fibrils, the designated form of amyloid is followed by the abbreviated form of the fibril protein's name. AK is found in local cutaneous amyloidosis. CA deposits are hypothesized to be derived from degenerated keratin peptides of apoptotic keratinocytes transformed into amyloid fibrils by dermal macrophages and fibroblasts [4,5]. A hypothesis is that long-term scratching and rubbing causes epidermal trauma, which leads to keratinocyte degradation and amyloid formation [6].

CA can be primary or secondary. The presence of amyloid deposition in tissue confirms its diagnosis. It is characterized by small globular deposits of amyloid in the papillary dermis which has a pink appearance in routine H&E stain, metachromic purple in crystal violet stain, red in Congo red, and bright apple green birefringence under polarized light (PL). False positive results could be seen in sun-damaged skin. Moreover, insufficient thickness of sections (less than 8 microns) might result in false negative results [7].

Congo red has been the gold standard for detecting amyloid in tissue samples. Considering the mentioned limitations of this method, a more specific marker is required. Due to its role in complement activation and scaffolding for amyloid deposition, C4d IHC staining can be a useful ancillary tool for the definite diagnosis of amyloidosis.

METHODS

This case-control study was approved by the Medical Ethics Committee of Shahid Beheshti University of Medical Sciences (code: IR.SBMU.SRC.REC.1401.001). The study included 90 samples of skin punch and excisional biopsies with clinical and pathological diagnoses of cutaneous amyloidosis between 2011 and 2020 and cutaneous lichen planus between 2017- 2020. The paraffin embed blocks and slides were obtained from the pathology archives of Loghman Hakim Hospital and Shohada-e-Tajrish Hospital, affiliated with Skin Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran. 90 cases were selected for this study (45 cases for each group). H&E slides were reviewed to evaluate the quality of slides for the IHC study. Six cases were excluded due to their low-quality samples. C4d stain was conducted on all samples. Congo red slides were also available in the archive.

For Congo red staining, 8-10 µm thick sections were required. Modified Highman's Congo Red stain was used for the detection of amyloid on formalin-fixed, paraffin-embedded tissue sections. The amyloid showed an orange color in light microscopy and apple-green birefringence under polarized light. Medullary thyroid carcinoma was regarded as a positive control. In 29 cases of CA Congo, red staining was very faint, with no apple-green birefringence. In many situations, the diagnosis of amyloid required Congo red fluorescence and/or Thioflavin T, which was not available for us. These cases were first classified as negative Congo red birefringence (false negative); however, due to histomorphology features and significant clinical suspicion, they were counted as CA cases. Since the studied skin samples were small, we were unable to repeat the Congo red stain due to exhausted paraffin blocks.

All of the slides in both groups underwent immunohistochemistry C4d staining. A positive control for C4d was considered to be a kidney core needle biopsy. For C4d IHC staining, 4µm thick sections of formalin-fixed paraffin-embedded blocks were prepared on charged slides, and stained with C4d IHC stain (MAD-000672QD-3/V C4d, rabbit polyclonal; master diagnostic, Sevilla, Spain), and were considered to be positive if a brown amorphous staining pattern was present at the same site of the Congo red stained area. CA was diagnosed based on histological examination of H&E slides, Congo red slide with light microscopy, Congo red birefringence, and association with clinical findings Lichen planus was diagnosed using H&E slides and histologic criteria such as hyperkeratosis, irregular sawtooth acanthosis, wedge-shaped hypergranulosis, basal cell hydropic degeneration, lichenoid (band-like) inflammation, and apoptotic ("cytoid," "civatte," "colloid") bodies.

The data were analyzed using SPSS software version 26 (IBM Corp., Armonk, NY, USA). A comparison

of Congo red stain with C4d immunoreactivity was performed. $P < 0.05$ was considered statistically significant. Quantitative variables were expressed as mean ± SD, and qualitative variables were expressed as frequency and percentage. Test evaluation was performed with sensitivity.

RESULTS

Ninety paraffin-embedded blocks and related H&E and Congo red slides were selected for evaluation. Six cases were excluded from the study due to poor IHC quality. Eighty-four cases remained (41 cases in CA and 43 cases in the LP group). Table 1 summarizes the descriptions of CA and LP based on sex, age, and biopsies site. The age range of the CA group was 23-71 years, with a mean age of 43 ± 12 years, while the LP group had a mean age of 45.7 ± 20.7 years.

Cutaneous amyloid deposits were highlighted as orange depositions on Congo red-stained sections using light microscopy. Congo red staining of 29 cases in the CA group was faint, yielding negative results under polarized light microscopy. Amyloid diagnosis in these cases required Congo red fluorescence and/or Thioflavin T, which were not available for us. However, due to the histopathological findings and high clinical suspicion for CA, they were indicative of CA and regarded as false negative results.

C4d immunostaining was performed on all slides of both groups. All 41 cases of CA were positive for C4d in amyloid deposition (Figure 1). Brown staining of globular deposits was considered a positive result. No false negative staining was identified. In all 43 cases of lichen planus, C4d staining was positive in colloid bodies (Figure 2).

A comparison of Congo red stain with C4d immunoreactivity was performed. The CA group had 100% C4d and 29% Congo red sensitivities ($P < 0.05$). C4d sensitivity was 100% in both the CA and LP groups (Table 2).

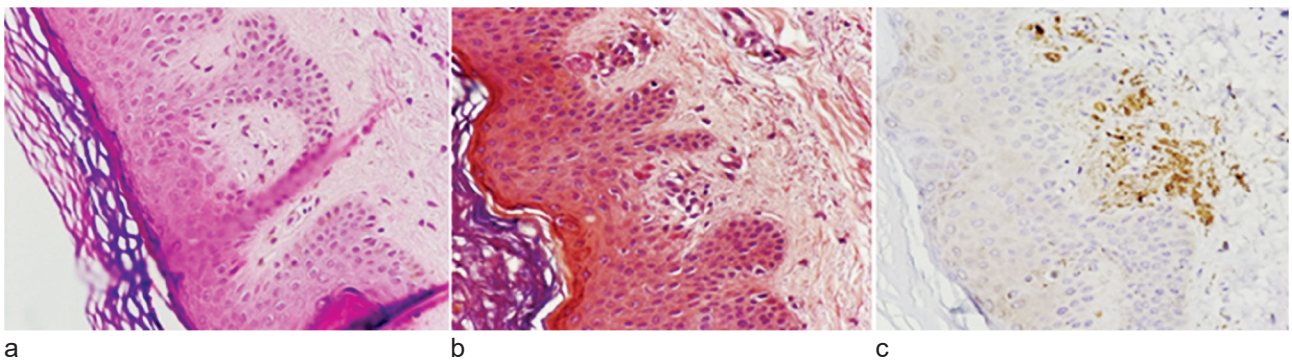
Table 1. Description of CA and LP by sex, age, and biopsies site

Variable	CA		LP	
	N	%	N	%
Sex				
Female	18	21.4	24	28.6
Male	23	27.4	19	22.6
Age				
0-20	0	0.0	6	7.1
21-40	17	20.2	14	16.7
41-60	20	23.8	11	13.1
61-80	4	4.8	12	14.3
Location				
Upper Ext	5	6	14	16.7
Lower Ext	14	16.7	11	13.1
Trunk	15	17.9	9	10.7
Other	7	8.2	9	10.7
Total	41	48.8	43	51.2

Abbreviations: CA: Cutaneous amyloidosis, LP: Lichen planus, Ext:Extermties

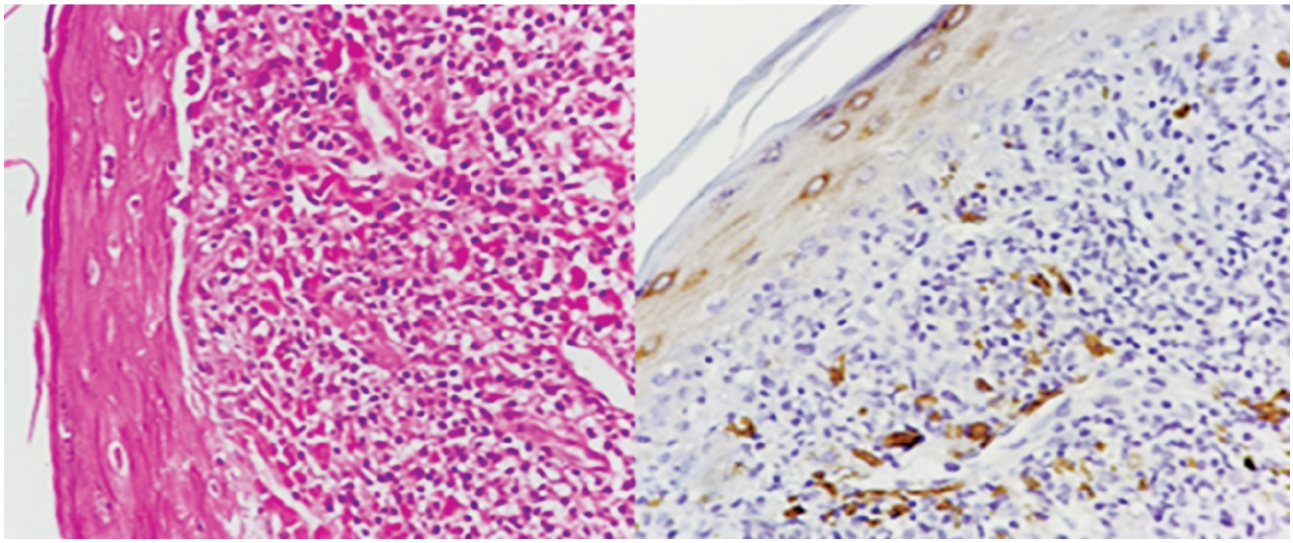
DISCUSSION

Amyloidosis is characterized by small globular amyloid deposits in the papillary dermis. CA might be primary or secondary. The presence of amyloid deposition in the tissue confirms the diagnosis. The most prevalent types are lichen amyloidosis and macular amyloidosis without visceral involvement [7]. Another form is nodular CA, in which amyloid deposits in the deep dermis and subcutis, sometimes in blood vessel walls, and is typically associated with AL-type amyloidosis [8]. CA is characterized by small globular deposits of amyloid in the papillary dermis which has a pink appearance in routine H&E stain, metachromic purple in crystal violet stain, red in Congo red with bright apple green birefringence



a b c

Figure 1. Scant amyloid deposition in CA; a) H&E (× 40); b) Congo red stain (× 40); c) C4d immunostain (× 40).



a b

Figure 2. Lichen planus: a) H&E (× 40), b) C4d immunostain (× 40).

Table 2. Congo red and C4d results in CA and LP groups

	CA		LP	
	n	%	n	%
Congo Red				
Positive	12	29		
Negative	29	71		
C4d				
Positive	41	100	43	100
Negative	0	0	0	0
Total	41		43	

Abbreviations: CA: Cutaneous amyloidosis, LP: Lichen planus

under polarized light (PL). It has a pink stain in routine H&E slides, a meta-chromic purple stain in crystal violet stain, and a red stain in Congo red with bright green birefringence. C4d staining is a new modality for identifying amyloidosis [9].

Congo red stain is considered the gold standard for confirming amyloid deposition [10]. It is used by most histopathology laboratories to demonstrate amyloid in tissue samples. False positive results could be found in sun-damaged skin. Collagen and elastin fibers polarize the light, which might be misinterpreted for amyloid deposits. In the early stages of the disease, low amyloid deposition between collagen bundles is difficult to detect. Therefore, Congo red stain results might be negative [11]. Insufficient thickness of sections (less than 8 microns) might also result in false negative results [7].

The pro-inflammatory environment, as well as some amyloid fibril components, could activate the complement system [12]. The amyloid fibrils in the skin act as a scaffolding for the deposition of serum proteins, such as the complement system [13]. C4d is a stable product of the complement system after activation in the classic or lectin pathway and remains in the tissue for a longer time due to its adherence to endothelial cells, extracellular matrix, and vascular basement membrane. C4d is an available IHC marker that is being investigated as an ancillary method for cutaneous amyloidosis [14,15].

Lichen planus is a disease of unknown etiology that presents with pruritic, violaceous, subacute to chronic papulosquamous dermatitis mostly seen on the flexor surfaces of the arms and the legs. The mucosa and nails may also be involved [16]. LP can be resolved within 12 months. However, post-inflammatory pigmentation may persist longer than in cases of CA. The basal cell damage causes a form of multiple scattered colloid bodies with a mean diameter of 20 µm in the papillary dermis which is one of the diagnostic signs of lichen planus in the microscopic view, seen as eosinophilic round bodies. They represent tonofilament-rich bodies extruded into the dermis which occur as a result of the destruction of keratinocytes. This is one of the differential diagnoses of amyloid deposition in the skin [17].

In CA, depositions occur either at the dermo-epidermal junction or within the dermis, with no underlying systemic disease [18]. CA has three

main clinicopathological types, including lichen amyloidosis, macular amyloidosis, and nodular amyloidosis. The first two types, lichen amyloidosis and macular amyloidosis, are regarded as keratin-type amyloidosis. They have been related to keratinocytes' degeneration due to the epidermal trauma induced by long-term scratching and rubbing associated with chronic diseases and are positive for High Molecular Weight-Cytokeratin (HMWKs). The third type, nodular amyloidosis, is related to the deposition of light-chain immunoglobulins, associated with plasma cell infiltration [18,19].

In the present study, pink globular depositions were seen in H&E slides in the CA group. However, they were quite small in most cases. These cases had faint Congo red stain in light microscopy without green birefringence under polarized light, but even in cases with slight staining, the results were positive for C4d stain.

According to the findings of the present study, Congo red stain showed 29% birefringence positivity in the CA group. Using polarized light, Banu *et al.* found 70% positivity of Congo red stain [7]. According to Woo Jung Sung, all cases of CA were positive in Congo red stain, whereas only 35% positivity was detected in the fibrosis grou*p* [21]. Abdullah Alhumid *et al.* studied 19 cases of CA, and only one case showed apple-green birefringence under polarized light [22]. In the Sari Aslani *et al.* study, none of the patients in the MA group showed apple-green birefringence in Congo red stain [23]. Tissue sections (with less than 8 µm thickness) might result in false negative results. Since early diagnosis of amyloidosis is critical to achieving better treatment outcomes, it seems necessary to find specific markers with fewer chances of false results [21].

In the present study, to avoid missing amyloid deposition, all the slides for Congo red stain were prepared with 8-10 µm thickness. Although all of the slides were examined with a standard clinical microscope, 29 cases were negative for Congo red birefringence. As stated by Ashraf El-Meanawy *et al.*, the apple green birefringence was more readily visible with higher intensity by using a metallurgical microscope compared to the standard clinical microscope [20]. Congo red stain is not specific for amyloid and can also stain elastotic dermis collagen, hyaline deposits in colloid milium, lipid proteinosis [7], and sun-damaged skin [11]. Additionally, due to variability in the assessment of the slides between different pathologists, Congo red stain in different studies might yield various results [20]. Thus, Congo Red stain is insufficient on its own for diagnosing amyloidosis [24]. In cases with low amyloid deposition, the IHC method can overcome this issue [7].

As a byproduct of C4 degradation, C4d is a biomarker of complement activation in antibody-mediated classical pathways [25]. It has been used to predict the prognosis of kidney transplants, however, a few studies have been done on its role in amyloidosis, especially in the skin [24]. For accurate and early diagnosis of amyloidosis, it could be a useful modality [21].

The C4d IHC is easier to perform and interpret than Congo red staining. Sung *et al.* described the utility of C4d in the recognition of systemic amyloidosis [21], although there were only a few investigations on C4d in CA in the medical literature. In Vijaya *et al.* study, all of the 32 patients with primary cutaneous amyloidosis (PCA) showed apple-green birefringence under polarized light [26]. Abdullah Alhumid *et al.* studied 19 cases of CA, and only one case indicated apple-green birefringence under polarized light [22]. In the present study, 29 out of 41 cases (71%) of CA had negative Congo red birefringence results. Banu *et al.* reported that Congo red polarization was pale in 14 cases. Therefore, Congo red fluorescence and/or Thioflavin T staining were employed to confirm the diagnosis of amyloidosis in these cases. Immunofluorescent (IF) was more expensive and required IF microscope evaluation, which was not easily available. Thus, the C4d marker which was easier to apply and interpret was utilized [7].

Previously a few IHC markers were suggested to be helpful in the diagnosis of MA, including cytokeratin5 (CK5), CK5/6, CK18, and HMWK (34betaE12) [27]. Sari Aslani *et al.* found that none of the patients' samples showed apple-green birefringence under polarized light, with a positive rate of 50% using HMWK and 52.4% using CK5 (23). In Woo Jung Sung *et al.* study, C4d staining (94.4% positivity) was found to be a more effective modality for detecting amyloid deposits and distinguishing them from fibrosis (C4d negative staining) than Congo red stain [21].

In the present study, C4d utility in amyloid

deposits and colloid bodies in LP were compared. The findings showed 100% sensitivity in C4d satin compared to 29% sensitivity in Congo red stain ($P < 0.05$). Banu *et al.* also found 100% positivity for C4d. Thus, C4d as a practical method for studying amyloidosis, particularly in the early stages of the disease and/or in cases with low amyloid depositions is suggested. It is also recommended to use this method in cases with small-sized samples, such as skin punch biopsies, which might be missed in Congo red stain. To get better clinical outcomes, treatment should be initiated at an early stage.

The findings of the present study indicated that C4d stain could not differentiate between amyloid deposition and colloid body in LP (100% in both groups). Moreover, C4d was 100% positive in colloid bodies. Therefore, it could be proposed as a new IHC marker for highlighting colloid bodies.

CONCLUSION

Congo red stain might result in missing low amyloid depositions in the early stages. Therefore, it is recommended to use the C4d IHC marker as a useful practical tool for diagnostic purposes in CA. It could be also a new IHC marker for highlighting colloid bodies. The C4d stain showed no preference for differentiating between amyloid depositions and colloid bodies of LP.

Author's Contribution

RMR conceived the research idea, supervised this study and revised the manuscript.

FBZ and AR collected the histopathology data and prepared the first draft

MG collected data and prepared the first draft

ZR collected the statistical analysis and prepared the first draft

MRT collected data and prepared the first draft

SS conceived the research idea and revised the manuscript carefully

All authors discussed the results and contributed to the final manuscript.

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