

Diversity of Cardiac Amyloidosis - A Comparative Histological, Histochemical, and Electron Microscopic Study of Systemic AA, AL and Isolated Atrial Myocardocyte Associated (Atrial Natriuretic Factor-AANF) Amyloidosis

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Abstract

Objective: This study focuses on various types of amyloidosis, characterized by amyloid A (AA), amyloid light chain λ or κ (AL λ or AL κ), amyloid transthyretin wild type (ATTRwt), amyloid β 2microglobulin (A β 2M) and localized (isolated) myocardocyte associated amyloid atrial natriuretic factor (AANF) protein deposits and discuss the possible involvement of the heart.

The aim our study was to describe a simple histochemical method for identification of systemic (AA, AL λ or AL κ , TTRwt, A β 2M) and localized myocardocyte associated AANF deposits for general departments of pathology.

Patients and Methods: Autopsy and biopsy material of patients with systemic or isolated amyloidosis were studied in one institute by light, polarization, and electron microscopy.

Classic histological, histochemical, immunohistochemical and electron microscopical methods were used.

Results: All mentioned types of amyloid deposits were eosinophilic, congophilic and birefringent by light or polarization microscopy, respectively. The amyloid filaments or fibrils of various deposits revealed no morphologic difference electron microscopically. However, there was a difference between systemic AA, AL λ or AL κ , ATTRwt, A β 2M, and isolated AANF amyloidosis in relation to the blood vessels, in the pattern of deposition, in cellular response of phagocytes, in the histochemical and ultrastructural characteristics of amyloid deposits.

Conclusion: The histological, immunohistochemical, histochemical and ultrastructural characteristics of systemic and isolated amyloidosis are different.

With simple histochemical methods the most important types of amyloid deposits (systemic AA, AL λ or AL κ , ATTRwt, A β 2M and isolated AANF) may be differentiated.

The histochemical methods are recommended for general departments of pathology, especially for those laboratories, where genomic DNA sequence analysis or amino acid sequence identification techniques are not available.

Keywords: Systemic AA, AL λ or AL κ ; ATTRwt; A β 2M; Isolated AANF Amyloidosis; Light and Electron Microscopy

Abbreviations

RA: Rheumatoid Arthritis; SLE: Systemic Lupus Erythematosus; SSc: Systemic Sclerosis; PsA: Psoriatic Arthritis; ACR: American College of Rheumatology; HE: Hematoxylin-Eosin Stain; PAS: Periodic Acid Schiff Reaction; AA: Amyloid A; AL λ : Amyloid Light Chain- λ ; AL κ : Amyloid Light Chain- κ ; ATTRwt: Amyloid Transthyretin Wild Type; ATTRm: Amyloid Transthyretin Mutant; A β 2M: Amyloid β 2Microglobulin; ANP or ANF: Atrial Natriuretic Peptide or Atrial Natriuretic Factor; AANF: Amyloid Atrial Natriuretic Factor

Introduction

The cardiovascular system is the main target of amyloid deposition in all types of systemic AA, AL, ATTR wild type (senile), hemodialysis-associated A β 2M, and hereditary amyloidosis [1,2]. Systemic amyloidosis may be accompanied by involvement of the heart, with disparate prevalence and extent of amyloid protein deposition of high risk of mortality [3-8].

Diversity of amyloid protein deposition in the heart is enhanced by amyloid atrial natriuretic factor (AANF) production, in case of isolated atrial myocardocyte associated amyloidosis. Different amyloid protein deposits may exist simultaneously in the same patient [1,2].

Objective of the Study

The objective of this study was to define the histological, histochemical, immunohistochemical, and electron microscopic characteristics of amyloid A (AA), amyloid light chain λ or κ (AL λ or AL κ), amyloid transthyretin wild type (ATTRwt), amyloid β 2microglobulin (A β 2M) and amyloid atrial natriuretic factor (AANF) amyloidosis, and to discuss the possible involvement of the heart.

Aim of the Study

The aim our study was to describe a simple histochemical method for identification of systemic (AA, AL λ or AL κ , TTRwt, A β 2M) and localized myocardocyte associated AANF deposits for general departments of pathology.

Patients and Methods

Autopsy and biopsy material of patients with systemic or isolated amyloidosis was studied in one institute. The patients were treated or died at the National Institute of Rheumatology between 1969 and 1998 (except patients with AANF and ATTRwt amyloidosis). The ethnicity of the populations reviewed was Caucasian without exception. The basic disease and the number of patients with amyloidosis and cardiac involvement are presented in table 1.

Light microscopic methods

From each autopsy patient 50 - 100 tissue blocks of 12 organs were studied microscopically, including 1 - 20 tissue samples of the heart. The size of tissue blocks was on the average 10 x 15 x 5 mm in each case.

The tissue blocks were fixed in 8% formaldehyde solution, embedded in paraffin. Serial sections were cut in sizes of 10 x 15 mm and stained with Hematoxylin and Eosin (HE) [9]. AA, AL λ or AL κ , ATTRwt, A β 2M, and AANF amyloidosis was diagnosed histologically according to Romhányi [10] by modified (more sensitive) Congo red staining [11].

Histochemical methods

At least 1 to 5 tissue samples were studied also histochemically (the size of the sections were 10 x 15 mm).

The histochemical identification of amyloid deposits was based on disintegration of amyloid deposits by performate pretreatment {85% HCOOH (8 ml), 30% H₂O₂ (3 ml) and 96% H₂SO₄ (220 µl)} according to Romhányi (1979) [12] or by oxidation induced degradation of amyloid deposits (0.25% KMnO₄ and 0.15% H₂SO₄, 1/1) according to Wright, *et al.* (1977) [13]. Introduction of different time period for breakdown of oriented amyloid filaments and fibrils according to Bély and Apáthy after performate pretreatment (at 20°C for 1, 5, 10, 15, or 20 seconds) or after oxidation (at 20°C for 30 seconds and 1, 3, 5 or 10 minutes) allows a more precise distinction of various types of amyloid proteins [14,15]. Amyloid deposits after performate pretreatment or oxidation were stained by Congo red without alcoholic differentiation, covered with gum Arabic and viewed with an Olympus BX51 polarized light microscope [11].

Immunohistochemical methods

The histochemical results were confirmed (if specific antisera were available) by classical immunohistochemical techniques using the streptavidin-biotin-complex/horseradish peroxidase method [16].

Electron microscopy

Selected cases were investigated with a JEM 100CX electron microscope.

From each patient 15 - 20 biopsy samples in sizes of 1 x 1 x 1 cubic millimeter were double fixed in Karnovsky's solution (2% paraformaldehyde and 2.5% glutaraldehyde mixture) at pH 7.2 for 90 minutes at room temperature and post-fixed in 1% osmium tetroxide for 60 minutes. Between the two fixations and after post-fixation the tissue blocks were rinsed with 0.2M sodium cacodylate buffer. After stepwise dehydration in ethanol the samples were embedded in Spurr's resin, applying propylene oxide as an intermediary solution. Ten to fifteen semi-thin (1 µm) sections were cut and 2 - 5 samples with characteristic fields were selected for ultra-thin (0.5 µm) sections respectively. The ultra-thin sections were cut with an ultramicrotome (Reichert), with interference colors in the silver spectrum, and stained with uranyl acetate and lead citrate.

Results

All mentioned types of amyloid deposits were eosinophilic, congophilic and birefringent, and were of a specific apple green color under polarized light. The color of polarized light produced by biochemically heterogeneous amyloid deposits after Congo red staining was influenced by the thickness of tissue sections.

In some cases, the intensity of birefringence varied within the same slide, even side by side in the same amyloid deposits [1,2].

Histological characteristics of amyloid deposits by light and polarisation microscopy

The histologic appearance of AA, ALλ or ALκ, and ATTRwt deposition was basically the same. Amyloid proteins in all forms of systemic amyloidosis were deposited in the wall of blood vessels, with or without extravascular deposition of amyloid (Figure 1-3).

Cellular reaction around AA or ATTRwt protein deposits was absent or moderate in contrast to ALλ or ALκ deposits, which was characterized by a more pronounced reaction of histiocytes and macrophages.

Aβ2M amyloid protein appeared in form of intra-capillary plugs or extravascular small conglomerates in tissues. The eosinophilic, congophilic deposits were globular in shape, and more often phagocytosed and/or surrounded by histiocytes and macrophages. Under polarized light a superficial (peripheral) ring of birefringence was noticed (Figure 4 and 5).

In contrast to the systemic types of amyloidosis the localized (isolated) myocardocyte associated amyloid atrial natriuretic factor (AANF) protein deposition was always extravascular, without involvement of the blood vessels. Around the AANF protein deposits there was no cellular reaction (Figure 6-8).

Immunohistochemical characteristics of amyloid deposits by light microscopy

Only with specific antiserum analyzed types of AA, ALλ or ALκ and Aβ2M amyloidosis have been considered.

The AA protein deposits showed a positive reaction for anti-human amyloid A-component with immunohistochemical staining.

The ALλ or ALκ deposits were positive for anti-human λ-light chain or anti-human κ-light chain (Figure 3) with a positive reaction of intravascular fluids, and tissues soaked by sera.

The deposits of globular Aβ2M protein showed with anti-human beta-2-microglobulin a ring like positive staining at the periphery, analogous to the superficial ring-like birefringence (Figure 5).

Histochemical characteristics of amyloid deposits by light microscopy

The amyloid A (AA) protein deposits accompanying systemic AA amyloidosis were sensitive to performate pretreatment (for 1 second) and resistant to KMnO₄ oxidation (for 30 seconds - 1 minute), resistant/sensitive (for 3 - 5 minutes) and sensitive (for 10 minutes) (Table 1a and 1b).

The amyloid light chain λ or κ (ALλ or ALκ) immunoglobulin deposits which accompanied systemic ALλ or ALκ amyloidosis were resistant to performate pretreatment (for 1 - 5 seconds), resistant/sensitive for 10 seconds and sensitive for (15 seconds) and resistant to KMnO₄ oxidation (for 1 - 3 minutes), resistant/sensitive (for 5 minutes) and sensitive (for 10 minutes) (Table 1a and 1b).

The amyloid beta-2-microglobulin (Aβ2M) globules of hemodialysis associated systemic amyloidosis were resistant to performate pretreatment (for 1 - 15 seconds), resistant/sensitive (for 20 seconds), and resistant to KMnO₄ oxidation (for 30 seconds), resistant/sensitive (for 1 minute) and sensitive (for 3 minutes or more) (Table 1a and 1b).

The amyloid transthyretin wild type (ATTRwt) deposits which accompanied systemic senile amyloidosis were resistant to performate pretreatment (for 1 - 20 seconds) and resistant to KMnO₄ oxidation (for 1 - 10 minutes or more) (Table 1a and 1b).

The extravascular localized myocardocyte associated amyloid atrial natriuretic factor (AANF) protein deposits were sensitive to performate pretreatment (for 1 second) and resistant to KMnO₄ oxidation (for 1 - 3 minutes) (Table 1a and 1b).

Table 1a summarizes the histochemical characteristics of systemic AA, ALλ or ALκ, ATTRwt, Aβ2M and of localized myocardocyte associated AANF protein deposits after performate pretreatment.

I. Performate pre-treatment					
Systemic amyloid protein deposits with cardiac involvement (basic diseases in parenthesis)					
Type of amyloid protein/Time	1 sec	5 sec	10 sec	15 sec	20 sec
(RA): AA n = 33 of 138 - cAAa n=29 of 33 [17] (SLE): AA n = 2 of 52 - cAAa n=2 of 2 (PsA): AA n = 2 of 12 - cAAa n=1 of 2 [18] (SSc): AA n = 1 of 12 - cAAa n=1 of 1 [19]	S	S	S	S	S

(SSc): AL λ n=1 of 12 - cAAa n = 1 of 1 [19] (multiple myeloma): AL λ n = 1 - cAAa n=1 of 1 (multiple myeloma): AL λ n = 1 - cAAa n=ND	R (1-5sec)	R	R/S*	S*	S*
(RA): AL κ n=1 of 161 - cAAa n = 0 of 1 [20] (multiple myeloma): AL κ n = 2 - cAAa n=2 of 2	R (1-5sec)	R	R/S*	S*	S*
A β 2M (β 2- microglobulin) n = 1**	R (1-15 sec)	R	R	R	R/S
ATTR wild type (Senile) n = 1 - cAAa n=1 of 1	R (1-25sec)	R	R	R	R
ATTR mutant variants n = 0	ND	ND	ND	ND	ND
Organ limited amyloid protein deposits (localized to the heart)					
Type of amyloid protein/Time	1 sec	5 sec	10 sec	15 sec	20 sec
AANF n = 2 - cAAa n = 2 of 2***	S	S	S	S	S

Table 1a: Histochemical characteristics of systemic and localized amyloidosis (according to the time of degradation by performate).

Remarks to table 1a

The “numbers” mentioned in the tables corresponds to the number of autopsy patients. The patients with AAa were treated or died at the National Institute, except autopsy patients with ATTRwt and biopsies of AANF amyloidosis; these cases were received for consultation from other institutes.

The low prevalence of systemic primary AL amyloidosis has been deceptive in our biopsies/autopsy population. Patients with lymphoproliferative disorders were transferred to an institution specialized in hematology.

Histochemically both AA and AANF deposits are sensitive to performate pretreatment (for 1 - 20 seconds). In each case of AA amyloidosis, the blood vessels are involved, while AANF amyloidosis is always an extravascular phenomenon

In advanced stages AL λ or AL κ deposits may be resistant to performate pretreatment (for 1 - 20 seconds) like A β 2M deposits, but histologically AL λ or AL κ and A β 2M deposits appear different.

Abbreviations: R: Resistant; R/S: Resistant/Sensitive; S: Sensitive.

“*”: Advanced stage of systemic AL λ or AL κ amyloidosis (at death) may be resistant to performate pretreatment (for 1 - 20 seconds).

“**”: Surgical specimens (20-25 tissue samples of synovial membranes and femoral heads) of one operated patient with hemodialysis-associated A β 2M amyloidosis: n = 1; tissue samples of the heart were not analyzed.

“***”: Amyloid deposits were present in the heart only (biopsy of gingiva, rectum, and abdominal fat were negative), and the blood vessels were spared. The size of the tissue samples or the size of the unstained sections of the heart with AANF amyloidosis was 3 x 6 mm.

Table 1b summarizes the histochemical characteristics of systemic AA, AL λ or AL κ , ATTRwt, A β 2M, and of myocardocyte associated AANF protein deposits by degradation of KMnO₄.

II. KMnO ₄ oxidation					
Systemic amyloid protein deposits with cardiac involvement (basic diseases in parenthesis)					
Type of amyloid protein/Time	30 seconds	1 min-ute	3 min-utes	5 minutes	10 min-utes
(RA): AA n = 33 of 138 - cAAa n = 29 of 33 [17] (SLE): AA n = 2 of 52 - cAAa n = 2 of 2 (PsA): AA n = 2 of 12 - cAAa n = 1 of 2 [18] (SSc): AA n = 1 of 12 - cAAa n = 1 of 1 [19]	R (30 sec - 1 min)	R*	R/S	R/S	S
(SSc): ALλ n = 1 of 12 - cAAa n = 1 of 1 [19] (multiple myeloma): ALλ n = 1 - cAAa n = 1 of 1 (multiple myeloma): ALλ n = 1 - cAAa n = ND	R (1 - 3 min)	R	R**	R/S**	S
(RA): ALκ n = 1 of 161 - cAAa n = 0 of 1 [20] (multiple myeloma): ALκ n = 2 - cAAa n = 2 of 2	R (1 - 5 sec)	R	R/S**	S**	S
Aβ2M (β2- microglobulin) n = 1***	R (30 sec)	R/S	S	S	S
ATTR wild type (Senile) n = 1 - cAAa n = 1 of 1	R (1 - 10 min)	R	R	R	R
ATTR mutant variants n = 0	ND	ND	ND	ND	ND
Organ limited amyloid protein deposits (localized to the heart)					
Type of amyloid protein/Time	30 seconds	1 min-ute	3 min-utes	5 minutes	10 minutes
AANF n = 2 - cAAa n = 2 of 2	R (1 - 3 min)	R	R	ND	ND

Table 1b: Histochemical characteristics of systemic and localized amyloidosis (according to the time of degradation by KMnO₄).

Remarks to table 1b

In advanced stages of systemic AA amyloidosis and in early stage of systemic ALλ or ALκ amyloidosis the resistance (sensitivity) may be practically the same.

In advanced stages of amyloidosis, the cardiovascular ALλ or ALκ amyloid deposits may be resistant to KMnO₄ oxidation (for 3 minutes) like AANF deposits, but deposition of AANF is an extravascular phenomenon without cellular reaction by macrophages.

The cardiovascular ATTRwt protein deposits are more resistant to KMnO₄ oxidation (for 1 - 10 minutes), than ALλ or ALκ deposits.

Abbreviations: R: Resistant, R/S: Resistant/Sensitive, S: Sensitive.

“*”: Minimal amyloid A deposits may be R/S to KMnO₄ oxidation (for 1 minute) in early stage of systemic AA amyloidosis.

“**”: In an advanced stage of amyloidosis, the massive ALλ or ALκ deposits may be resistant to KMnO₄ oxidation (for 3 - 5 minutes), like ATTRwt deposits. Systemic ALλ deposits seem to be more resistant to KMnO₄ oxidation (for 3 - 5 minutes) compared to ALκ deposits

“***”: Surgical specimens (synovial membranes and both femoral heads) of one operated patient with hemodialysis-associated Aβ2M amyloidosis: n = 1; tissue samples of the heart were not analyzed.

ND: Tissue samples were not available (ATTRm) or were too small for serial sections (AANF).

Summarized

The blood vessels were involved in all types of cardiovascular AA, ALλ or ALκ, ATTRwt and Aβ2M amyloidosis.

The resistance of AA (for less than 1 second), ALλ or ALκ (for 1 - 5 seconds), Aβ2M (for 1 - 15 seconds) and ATTRwt (for 1 - 20 seconds) to performate pretreatment increased gradually; the cardiovascular AA, ALλ or ALκ, ATTRwt protein depositions showed an enhanced stability, and the time of degradation increased.

An increased resistance to KMnO_4 oxidation was detected in case of AA amyloid deposits (for 30 seconds - 1 minute), AL λ or AL κ (for 1 - 3 minutes), and ATTRwt (for 1 - 10 minutes or more); the A β 2M deposits were less stable (only for 30 seconds).

AANF amyloidosis was an extravascular phenomenon, the walls of arteries and veins were always spared, and the AANF protein deposits were sensitive to performate pretreatment (for 1 second) and resistant to KMnO_4 oxidation (for 1 - 3 minutes).

Histological and histochemical characteristics of systemic (AA, AL λ or AL κ , A β 2M) and isolated AANF amyloid deposits are demonstrated by light and polarization microscopy in figure 5-8.

Original magnifications of microphotographs correspond to the 24 x 36 mm transparency slide, i.e. the correct height: width ratio is 2:3.

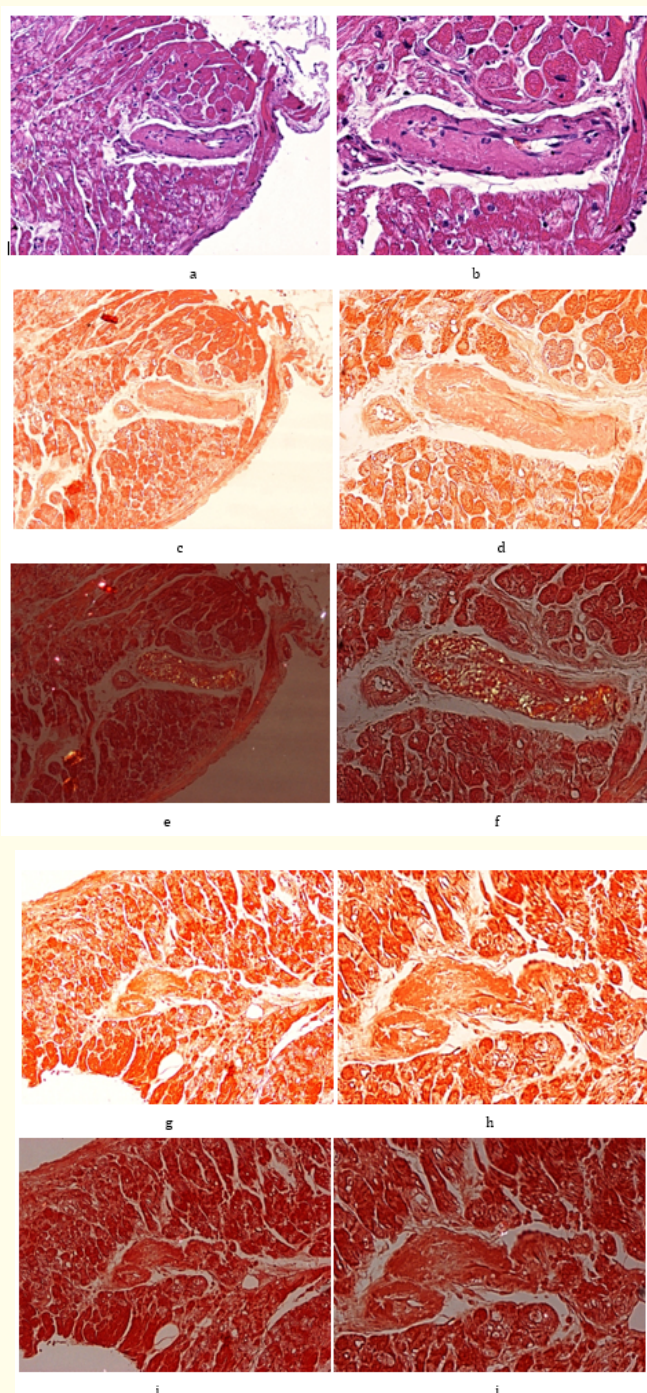


Figure 1: Systemic secondary AA amyloidosis. AA deposits are within the walls of small arteries; in all types of systemic amyloidosis the precursor peptides reach the target organ via the bloodstream, and the vessel walls are always involved. (a) HE, x100, (b) same as (a) x200, (c) Congo red staining, without alcoholic differentiation, covered with gum arabic, x100, (d) same as (c) x200, (e) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x100, (f) same as (e) x200. AA deposits are birefringent and show a specific apple green color under polarized light. (g) Performate pretreatment for 1 second and Congo red staining, without alcoholic differentiation, covered with gum Arabic, x100 (h) same as (g) x200. After performate pretreatment the staining intensity (congophilia) is increased, compared to the Congo red staining intensity without pretreatment. (i) Performate pretreatment for 1 second and Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x100 (j) same as (i) x200. The AA deposits are very sensitive to performate pretreatment for 1 second; the birefringence of AA deposits disappears immediately. The sensitivity is not influenced by the amount of deposited amyloid protein and is independent of the stage of AA amyloidosis.

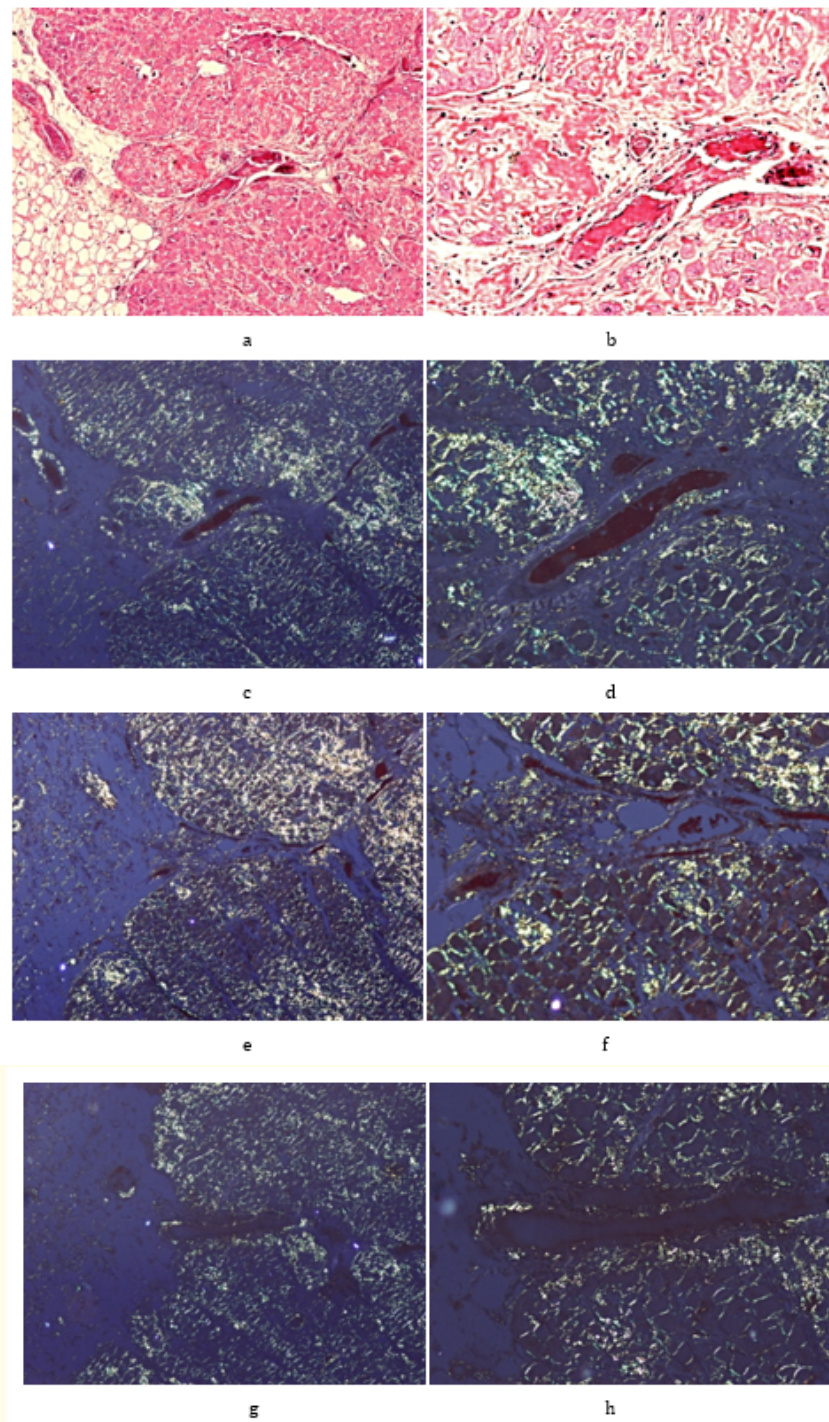


Figure 2a-2h: Heart, subepicardial region, systemic AL κ amyloidosis. Immunoglobulin light chain κ amyloid deposits in the wall of arterioles and on the interstitial reticulin and collagen fibers; the blood vessels are involved always in systemic amyloidosis. (a) HE, x 40, (b) same as (a) x100 (c) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x 40, (d) same as (c) x100. AL κ deposits are birefringent and show a specific apple green color under polarized light. (e) Performate pretreatment for 1 second, and Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x40 (f) same as (e) x100. The AL κ deposits are resistant to performate pretreatment for 1 - 5 second and retain the specific apple green color under polarized light. (g) KMnO₄ oxidation for 1 minute and Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x40 (h) same as (g) x100. The AL κ deposits are resistant to KMnO₄ oxidation for 1 minute. In advanced stage of amyloidosis, the massive AL λ or AL κ deposits may be resistant to KMnO₄ oxidation even for 3 - 5 minutes, like ATTRwt deposits.

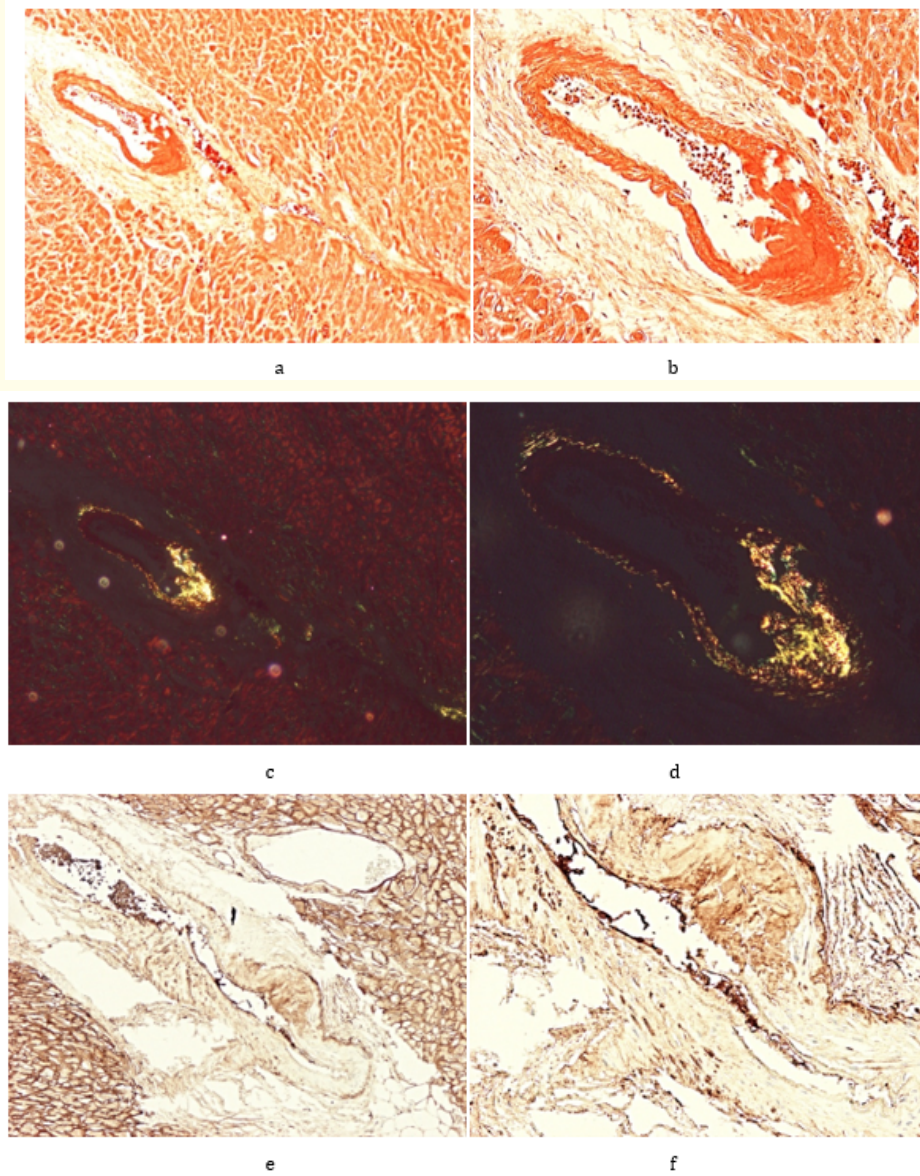
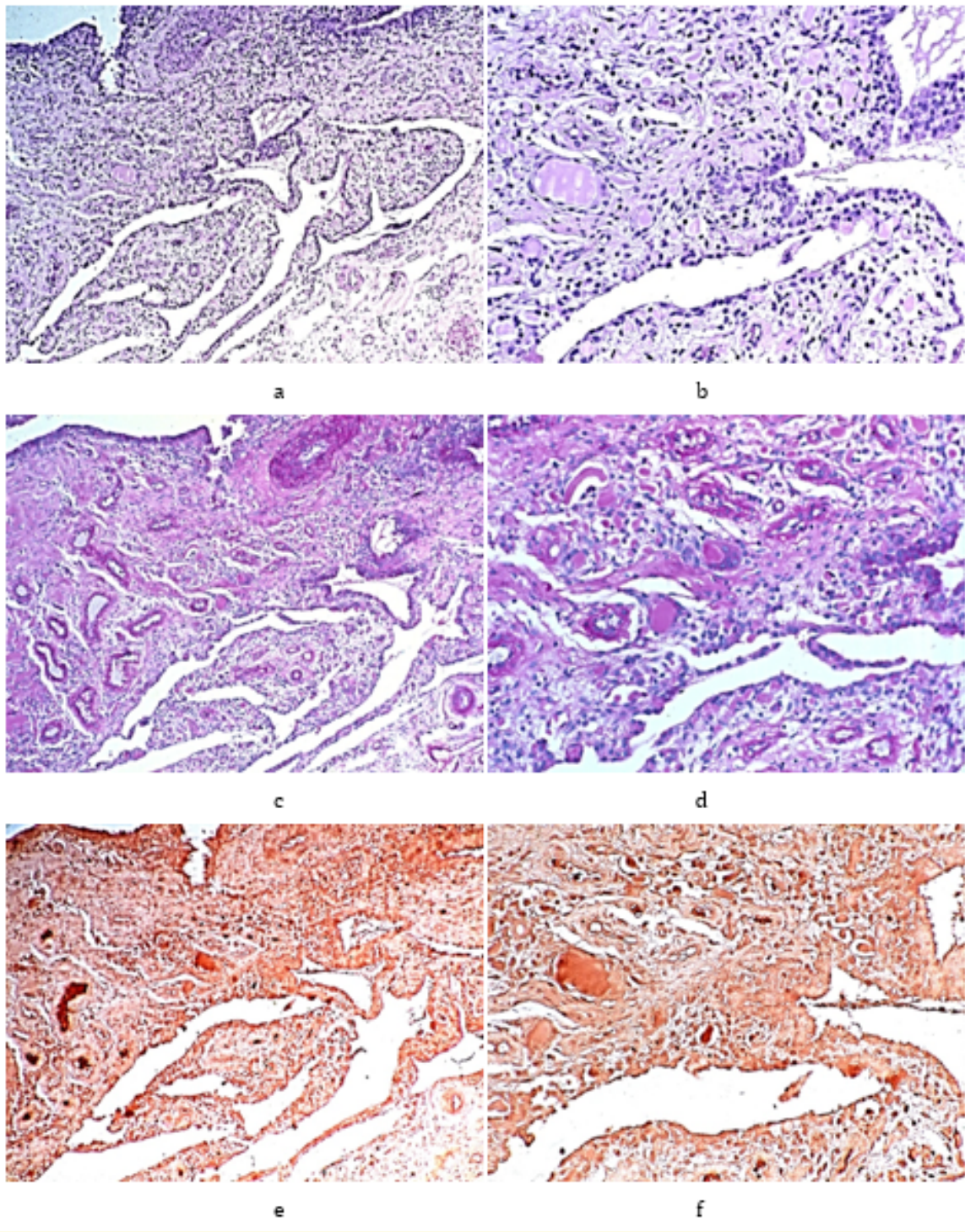


Figure 3a-3f: Heart, systemic AL κ amyloidosis. Immunoglobulin light chain κ are deposited in the wall of a small artery. (a) Congo red staining, without alcoholic differentiation, covered with gum arabic, x40, (b) same as (a) x100, (c) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, x40, (d) same as (c) x100. The birefringent AL κ deposits show of a specific apple green color under polarized light. (e) a-hu κ -light chain diluted [monoclonal antibody N1568; DAKO, Glostrup, Denmark], Streptavidin-biotin-complex/horseradish peroxidase reaction, x40, (f) same as (e) x100. Amyloid deposits of light chain κ in the wall of a small artery are positive for anti-human κ -light chain. The intravascular contents and tissues soaked by sera are also positive for anti-human κ -light chain. Immunohistochemical analysis of AL κ or AL λ reaction may be difficult (in contrast to the clear immunohistochemical staining for AA deposits), because of the extensive background staining (note the non-specific staining of interstitial reticulin and collagen fibers).



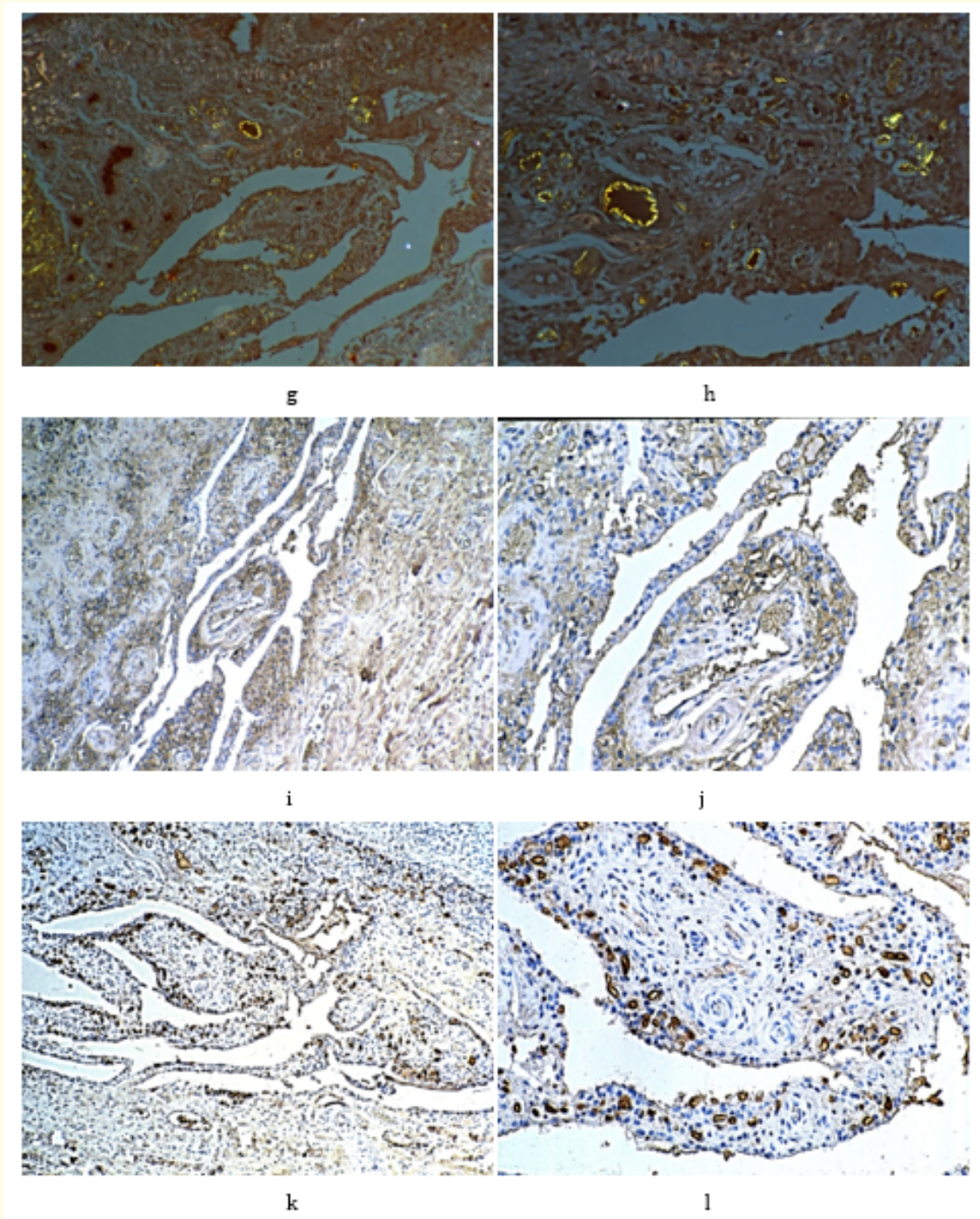


Figure 4a-4l: Synovial membrane, hemodialysis-associated $\beta 2$ microglobulin ($A\beta 2M$) amyloidosis. (a) HE, x50 (b) same as (a) x125. $A\beta 2M$ protein in form of intra-capillary globules and plugs or in extravascular localization as extracellular deposits or incorporated by macrophages. (c) PAS, x50 (d) same as (c) x125. $A\beta 2M$ deposits are positive for Periodic Acid Schiff reaction, like AA, AL λ or AL κ , and ATTRwt amyloid deposits. (e) Performate pretreatment for 15 sec, and Congo red staining, without alcoholic differentiation, covered with gum arabic, x50, (f) same as (e) x125. The eosinophilic, congophilic deposits are globular in shape, and are accompanied by a pronounced cellular reaction of histiocytes and macrophages. (g) Performate pretreatment for 15 sec, and Congo red staining, viewed under polarized light, x50 (h) same as (g) x125. Under polarized light the $A\beta 2M$ deposits show a characteristic peripheral ring of birefringence. (i) a-hu beta-2-microglobulin 1:400 [polyclonal antibody A0072; DAKO, Glostrup, Denmark], Streptavidin-biotin-complex /horseradish peroxidase reaction, x50 (j) same as (i) x125. Systemic $A\beta 2M$ deposits are positive for anti-human beta-2-microglobulin. (k) a-hu amyloid P Component 1:300 [polyclonal antibody A0302; DAKO, Glostrup, Denmark], Streptavidin-biotin-complex/horseradish peroxidase reaction, x50 (l) same as (k) x125. $A\beta 2M$ deposits are positive for anti-human amyloid P Component, like AA or others.

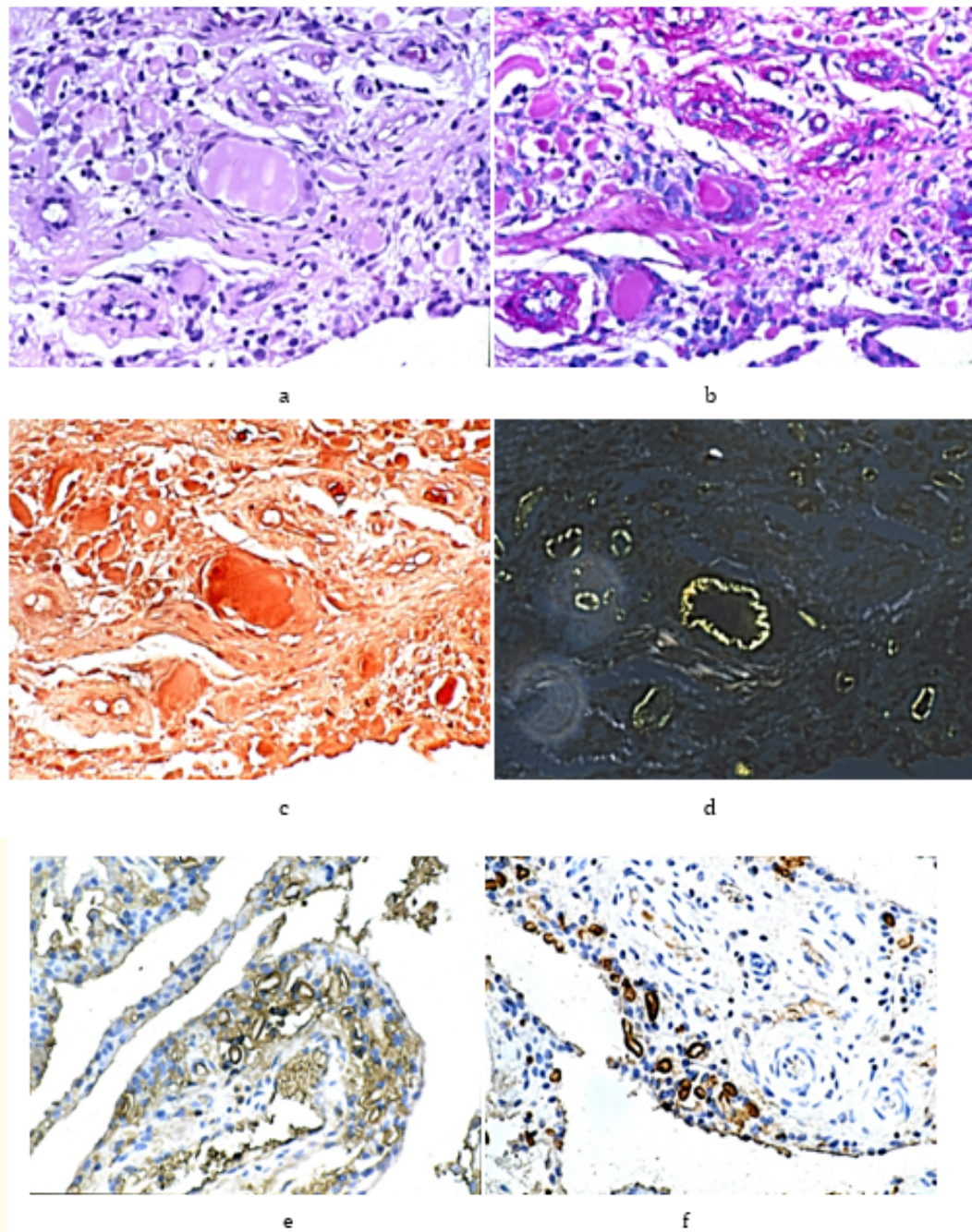


Figure 5a-5f: Synovial membrane, systemic $\beta 2$ microglobulin ($A\beta 2M$) amyloidosis. (a) HE, x200. $A\beta 2M$ protein masses in form of intra-capillary globules or plugs. The extravascular deposits are in extracellular localization or incorporated by macrophages. (b) PAS, same as (a) x200. (c) Performate pretreatment for 15 sec and Congo red staining, same as (a) x200. The eosinophilic, congophilic deposits are globular in shape, and are more often phagocytosed or surrounded by histiocytes and macrophages, than AA, AL λ or AL κ amyloid deposits. (d) Performate pretreatment for 15 sec and Congo red staining, viewed under polarized light, same as (a) x200. Under polarized light the $A\beta 2M$ protein deposits show a superficial (peripheral) ring of birefringence. (e) a-hu beta-2-microglobulin 1:400 [polyclonal antibody A0072; DAKO, Glostrup, Denmark], Streptavidin-biotin-complex/horseradish peroxidase reaction, x200. The deposits of globular $A\beta 2M$ protein show with anti-human beta-2-microglobulin a ring like positive staining at the periphery, analogous to the superficial ring-like birefringence. (f) a-hu amyloid P Component 1:300 [polyclonal antibody A0302; DAKO, Glostrup, Denmark], Streptavidin-biotin-complex/horseradish peroxidase reaction, x200. The deposits of globular $A\beta 2M$ protein show with anti-human amyloid P Component a ring like positive staining at the periphery, analogous to the superficial ring-like birefringence.

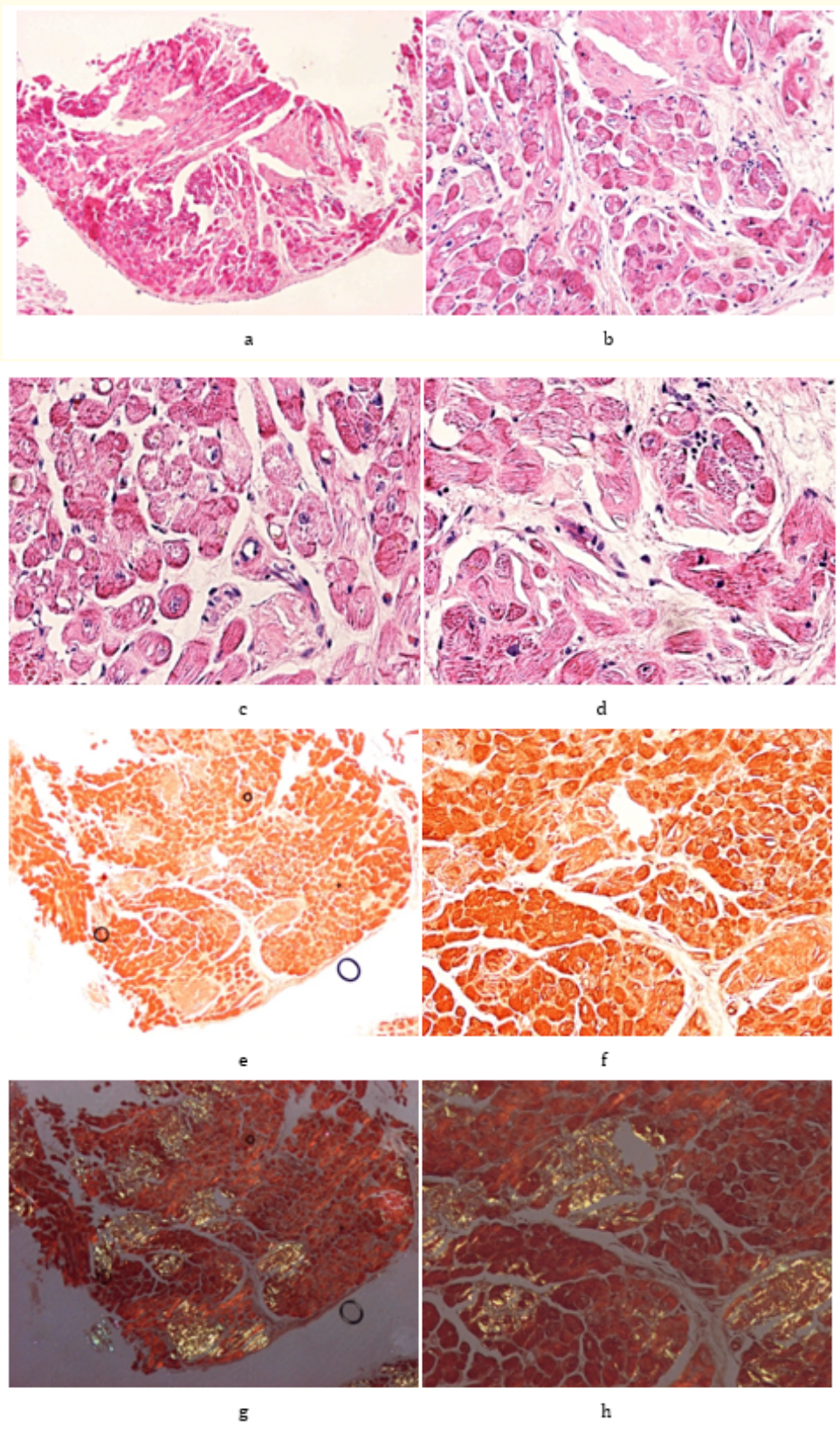


Figure 6a-6h: Isolated myocardocyte associated amyloid atrial natriuretic factor (AANF) amyloidosis. The AANF protein deposits involve only the heart and are always extravascular. Amyloid deposits are localized interstitially or within the myocardocytes, without involvement of the blood vessels. Around the AANF protein deposits there is no cellular reaction. (a) HE, x40, (b) same as (a) x100, (c) same as (b) x200, (d) same as (b) x200. (e) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, same as (a) x40, (f) same as (e) x100. (g) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, same as (e) x 40, (h) same as (f) x100.

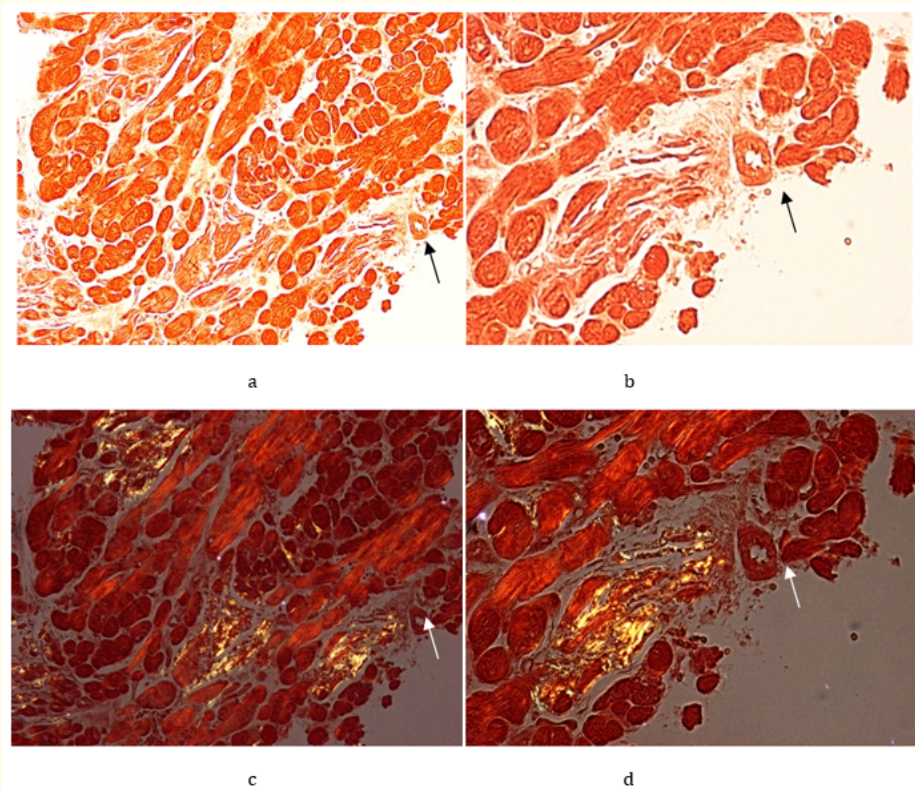


Figure 7a-7d: Isolated myocardocyte associated amyloid atrial natriuretic factor (AANF) amyloidosis. In AANF amyloidosis the blood vessels (arrow head) are not involved. (a) Congo red staining, without alcoholic differentiation, covered with gum arabic, x100, (b) same as (a) x200, (c) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, same as (a) x 100, (d) same as (b) x200.

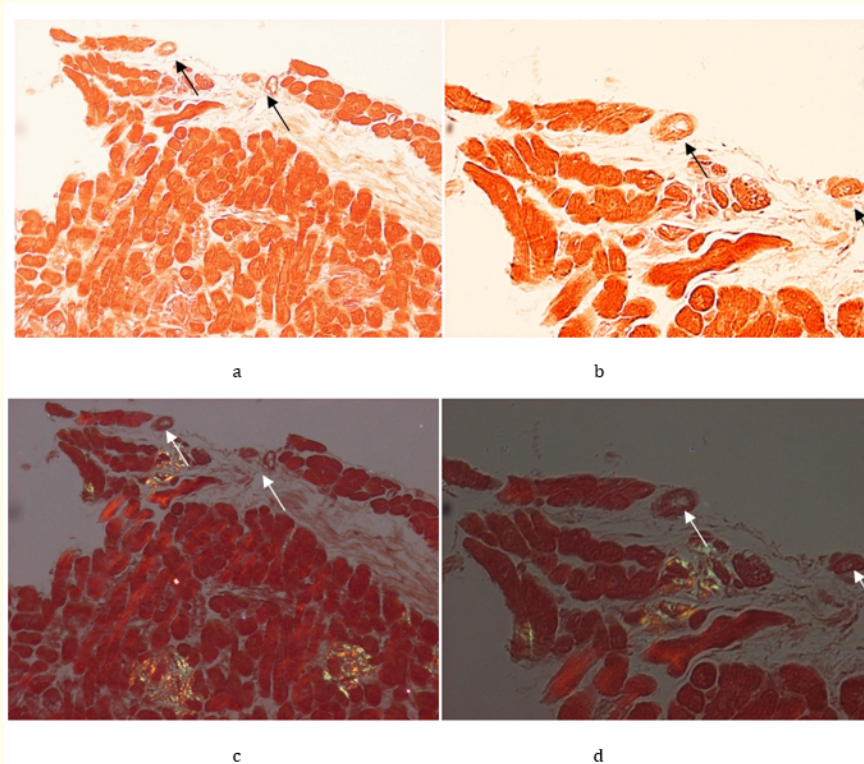


Figure 8a-8d: Isolated myocardocyte associated amyloid atrial natriuretic factor (AANF) amyloidosis. In AANF amyloidosis the blood vessels (arrow head) are not involved. (a) Congo red staining, without alcoholic differentiation, covered with gum arabic, x100, (b) same as (a) x200, (c) Congo red staining, without alcoholic differentiation, covered with gum arabic, viewed under polarized light, same as (a) x 100, (d) same as (b) x200.

Electron microscopic characteristics of amyloid deposits

Only selected cases of AA, AL λ or AL κ and A β 2M amyloidosis were examined electron microscopically.

Electron microscopically the filaments or fibrils of various amyloid deposits revealed no morphologic difference. Amyloid filaments (7 - 10 nanometer in diameter) and fibrils (10 - 40 nanometer in diameter) were present in all types of amyloidosis.

However, there was a difference:

- In relation to the blood vessels,
- In the pattern of deposition (parallel array or pool-like),
- In the arrangement of filaments or fibrils within the amyloid deposits (loose filamentous areas, single or multiple dense cores, closely packed dense clusters of filaments, crystalloid arrangement), and
- In cellular response of phagocytes.

Electron microscopic characteristics of systemic AA amyloid deposits

In systemic AA amyloidosis the amyloid A filaments and fibrils were always within vessel walls or around capillaries with or without deposits between the collagen and reticulin fibers (collagen IV). Amyloid A filaments and fibrils were arranged in parallel circular layers. The dense cores of amyloid A deposits were composed of closely packed parallel arranged fine filaments of indeterminate length (Figure 9 and 10). Cellular reaction of macrophages was absent or moderate.

Electron microscopic characteristics of systemic AL λ deposits

In systemic AL λ amyloidosis extracellular lake-like amyloid deposits dominated the picture; these were located within the vessel walls or around the capillaries and between the collagen bundles.

The extracellular lake-like amyloid deposits between the collagen bundles were less compact than the deposits located near the vessels (similarly to systemic AA amyloidosis) (Figure 11 and 12).

Prominent cellular reaction of macrophages and fibroblasts was present in some areas.

Electron microscopic characteristics of systemic A β 2M deposits

Electron microscopically the A β 2M protein deposits were present within macrophages or extracellularly, sometimes surrounded by remnants of capillary endothelium or bordered by membranes of dead apoptotic macrophages (Figure 13 and 14). The extracellular A β 2M protein deposits with remnants of endothelial cells may correspond to the intracapillary globules (spherules) or plugs observed by light microscopy.

The extracellular A β 2M amyloid deposits showed a loose filamentous-fibrillar arrangement. The deposits were inhomogeneous and one or several focal dense, core-like accumulations of filaments were seen, surrounded by a loose interdigitating or peripheral marginal zone. The dense cores were composed of fragmented filaments of different sizes (of indeterminate length) and variable thickness. The filaments were more compactly arranged in the dense areas than in the peripheral, marginal or interdigitating zones (Figure 13 and 14).

The loose filamentous zone at the periphery of amyloid deposits corresponded to the superficial (peripheral) ring of birefringence observed under polarized light, while the multifocal dense, core-like accumulations of fragmented filaments noticed in the central area of the deposits did not appear birefringent under polarized light.

Intracellular β 2-microglobulin amyloid deposits were present in macrophages, bordered by membrane (Figure 13 and 14).

Electron microscopic characteristics of systemic AA, AL κ and A β 2M amyloid deposits are demonstrated in figure 9-12. The original magnification (O) of electron microphotographs correspond to the 60 x 90 mm negatives.

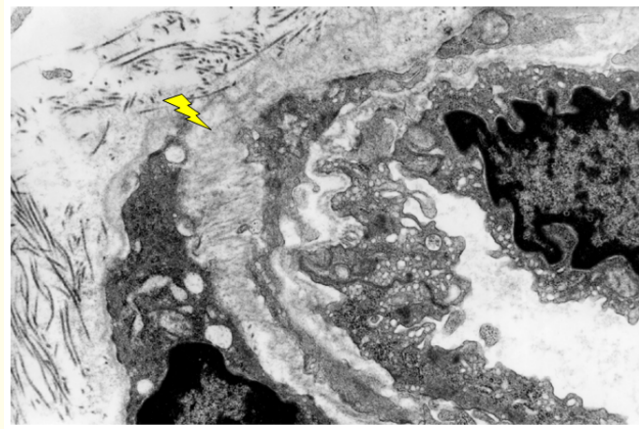


Figure 9: RA, systemic AA amyloidosis, part of a cross sectioned arteriole with collagen fibers and fibroblast. Condensed amyloid A filaments and fibrils are located within the vessel wall (arrow), and between collagen fibers. O: x8300.

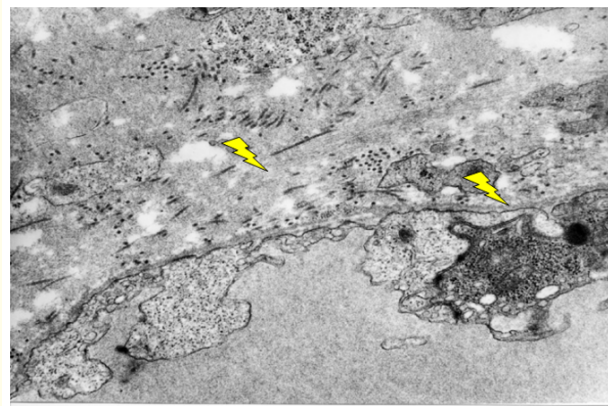


Figure 10: RA, systemic AA amyloidosis, dilated capillary lined by damaged endothelial cells. The amyloid A filaments and fibrils are arranged parallel with the subendothelial basal lamina and collagen fibers (arrows). (O) x13000.

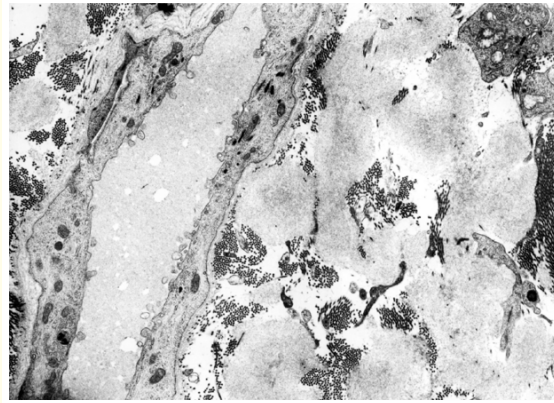


Figure 11: Multiple myeloma, systemic ALλ amyloidosis, dilated capillary. Perivascular pools of ALλ deposits between collagen fibrils near the basal lamina. (O): x3300.

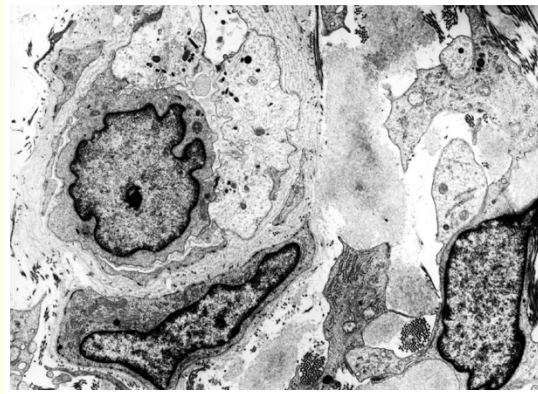


Figure 12: Multiple myeloma, systemic ALλ amyloidosis, cross section of a capillary. Endothelial cells are swollen and necrobiotic, basal lamina is multi-layered. Pools of amyloid ALλ deposits with loose filamentous structure are seen between fibroblast and cell processes. O: x3300.

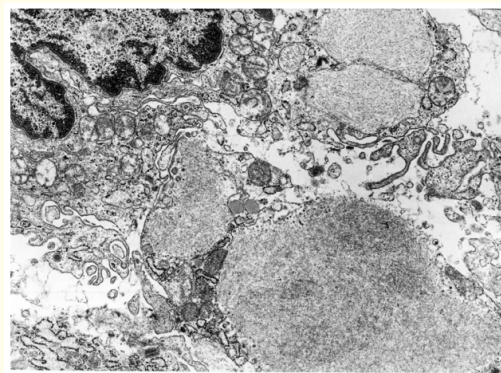


Figure 13: Hemodialysis-associated systemic Aβ2M amyloidosis, synovial membrane. Degenerated macrophages, intra- and extracellular Aβ2M amyloid deposits. The incorporated filamentous (2 - 3 nm thick) amyloid deposits are bordered by membrane of degenerated macrophages. O: x6600.

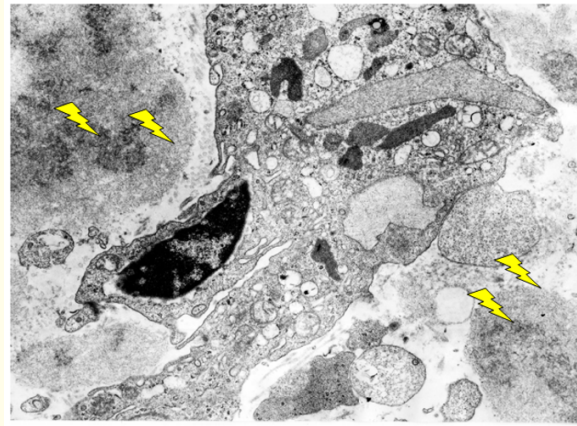


Figure 14: Hemodialysis-associated systemic $A\beta_{2M}$ amyloidosis, synovial membrane. Macrophages, intra- and extracellular filamentous $A\beta_{2M}$ amyloid deposits with loose marginal zone and dense central core (arrows). The extracellular filamentous (2 - 3 nm thick) $A\beta_{2M}$ amyloid deposits are bordered by remnants of damaged capillaries or dead apoptotic macrophages. O: x5000.

Discussion

It is difficult to estimate the true incidence of AA, AL, ATTRwt, $A\beta_{2M}$, etc. amyloidosis in a general population or involvement of the heart (prevalence of cardiac amyloidosis) since it depends on the cohort of patients, on the number of autopsies/biopsies, on the specificity and sensitivity of the demonstration techniques, etc.

Using a less sensitive staining method some positive cases may remain undetected. A more specific method potentially detects more cases and reveals earlier stages. Amyloidosis in most studies is diagnosed with various staining methods of diverse specificities and sensitivities, such as thioflavin T or S, toluidine blue, crystal violet, Sirius red, Congo red staining according to Romhányi [10], Bennhold, Puchtler or Bély, etc. [1,11]. The limited number of autopsies and/or of microscopic examinations of various organs may lead to underestimation of amyloidosis.

The rate of AL amyloidosis per million people per years was in Olmsted County, Minnesota 6.1 from 1950 to 1969, but rose to 10.5 from 1970 to 1989 [21]. According to Banypersad, *et al.* (2012) in the United Kingdom and United States AL amyloidosis has an annual incidence of 6 to 10 cases per million populations [22].

By extrapolation from multiple myeloma cases an incidence of 3.2 per million could be ascribed to AL amyloidosis, the incidence of AA amyloidosis was likely to be about 2.0 per million (based on rheumatoid arthritis death rates, an incidence of 2.0) and the incidence for ATTRwt (senile) amyloidosis may account 3 per million persons per years [23].

Wild-type TTR amyloid (ATTRwt) deposits were found at autopsy in 25% of the population older than 80 years [24].

Röcken, *et al.* (2002) analyzed 245 patients undergoing cardiac bypass surgery or mitral/aortic valve replacement and found 40 (16.3% of 245) cases with isolated atrial AANF amyloidosis. All patients were excluded with a known history of AL or ATTRwt amyloidosis, and all deposits were immunoreactive for atrial natriuretic peptide (ANP) [25].

In all types of systemic amyloidosis the precursor peptides reach the target tissue via the bloodstream, and the vessel walls are always involved [1,2,14,17,26]. Cardiac amyloidosis may be associated with any type of systemic amyloidosis [17].

In contrast to systemic amyloidosis isolated amyloidosis is limited to an organ or tissue and is an extravascular phenomenon; the precursors are not connected to the systemic circulation, and the vessel walls remain always spared, the process is localized without involvement of the blood vessels [1,2,14,17,26].

According to Banyersad., *et al.* (2012) in primary AL amyloidosis the heart is affected in 40% to 50%, in ATTRwt amyloidosis almost all patients are over 70 years, and in AA amyloidosis the heart is rarely involved [22].

Boldrini., *et al.* (2013) analyzed 344 consecutive patients with AL amyloidosis; cardiac amyloidosis was present in 240 (69.77%) cases [27].

Numerous early publications discuss the prevalence of AA amyloidosis in RA autopsy patients with or without its role in mortality, but only a few of these studies mention cardiac involvement. In most of these early publications the prevalence of AA amyloidosis in patients with RA at autopsy was between 10 - 20% (excluding the publications analyzing only a few cases with extreme high prevalence of AA amyloidosis) [17]. In this previous study we discussed the relationship between systemic and cardiac amyloidosis. AA amyloidosis complicated RA in 33 (23.91%) of 138 patients and was histologically excluded in 105 (76.08%) cases. The heart was involved in 29 (21.81%) of 138 patients or (87.88%) of 33 patients [17].

At the National Institute of Rheumatology 11558 patients died between 1969 and 1998 (in 30 years); among them 234 with RA, 52 with SLE, 12 with SSc, 12 with PsA, and all of them were autopsied.

The incidence of AA amyloidosis was: in RA (n = 48) 20.51% of 234, in SLE (n = 2) 3.84% of 52, in PsA (n = 2) 16.66% of 12 [18] and in SSc (n = 1) 8.33 % of 12 [19]. Thus in our institute the incidence of AAa in autopsy patients with autoimmune disease was: (n = 53) 0.458% of 11558 pts in this time period.

The clinical symptoms and consequences depend on the type of amyloid, on the extent of systemic involvement, and on the treatment options [28]. Early diagnosis is important, because the amyloid filaments and fibers will be fragmented and aggregated during deposition and in advanced stage of amyloidosis the chance of resolution will be diminished [1].

Electron microscopically the closely packed and fragmented filaments may lead to a core-like, electron dense accumulation within amyloid deposits. The AA, AL λ or AL κ deposits in the wall of blood vessels were more compact, compared to the less compact extravascular amyloid deposits between the collagen bundles (Figure 11 and 12).

These changes are, in our opinion, related to the chronology of amyloid deposition (early and late), and to the stage-dependent maturation (fragmentation) of deposited amyloid filaments (fresh and old); i.e. the loose arrangement of amyloid filaments reflect an early stage, and the core-like electron dense accumulation of amyloid filaments reflect suggest an advanced stage of amyloid deposition.

This assumption is supported by the increased time of disintegration in the histochemical demonstration of old deposits by performate pretreatment or by KMnO₄ oxidation, in contrast to fresh ones [1].

In most clinical studies the diagnosis of amyloidosis is based on the evaluation of clinical symptoms, and only some cases are confirmed by biopsy, and even fewer by autopsy [29-38].

Clinical suspicion of AA amyloidosis is raised in case of unexplained weight loss, fatigue, anemia, impaired renal function, restrictive cardiomyopathy, hepatomegaly, or gastrointestinal complaints (malabsorption, malnutrition, diarrhea, constipation disturbed motility, bleeding) or reduced respiratory capacity; a biopsy is needed for confirmation [29].

AL or AH amyloidosis can affect primarily the heart (dyspnea, orthopnea, fatigue, leg edema, syncope), kidneys (albuminuria, leg edema), skin (thickening, easy bruising, periorbital ecchymosis) nerves (paresthesias), liver (hepatomegaly, elevated alkaline phosphatase level) and gastrointestinal tract (dysphagia, loss of appetite) accompanied by weight loss and fatigue. Characteristic hallmarks are also macroglossia and periorbital ecchymosis [30].

The senile, non-hereditary systemic ATTRwt amyloidosis is usually associated with cardiac disease, but amyloid TTR protein deposition is not limited to the heart, and also found in other organs [31]; association of carpal tunnel syndrome is characteristic [22,32].

The hereditary genetically determined mutant TTR variants deposition (ATTRm amyloidosis) preferentially affects the heart and nervous system; heart failure is often associated with progressive sensorimotor neuropathy (familial amyloidotic polyneuropathy) [33].

In A β 2M amyloidosis osteo-articular structures, cervical intervertebral discs, the large bones close to the joint spaces, synovial membranes, carpal tunnel tissue are preferred sites [34,35], but other organs and viscera may be involved as well [36-38]. The risk of cardiovascular involvement is increased in A β 2M amyloidosis [39].

The diagnostic panel of amyloidosis of the heart is wide including electrocardiography, echocardiography, cardiac magnetic resonance, nuclear imaging, laboratory testing, biomarkers, etc. [27,32,33,40,41]. Unfortunately the results are not specific for amyloid itself, and we cannot state more today than Alan S Cohen wrote 50 years ago: "There are no laboratory abnormalities specific to or unique for amyloid"... "There is no one finding in the blood, urine, electrocardiogram or x-ray that is specific for this disease, however", and in agreement with him, "the diagnosis should be based upon a biopsy using an "appropriate staining procedure" [42].

Genomic DNA sequence analysis is required for hereditary amyloidosis, and amino acid sequence identification for amyloid protein deposits [3]. Different amyloid protein deposits may exist simultaneously in the same patient [1,2]. Thus it is insufficient to identify a mutation in the gene of a candidate amyloid protein without confirming the variant changes in the amyloid fibril protein [3]. With other words, to detect mutations for example in the gene of transthyretin peptides does not necessarily mean that the actual amyloid peptides are transthyretin mutant variants (TTRm) and the amyloid protein deposits in the heart contain amyloid transthyretin mutant fibrils (ATTRm). Exact identification of amyloid deposits is possible by histochemical methods or identification of amino acid sequence is necessary.

Genomic DNA sequence analysis or amino acid sequence identification or other techniques, like electron microscopy, are not always available whereas relatively simple histological, immunohistochemical and histochemical methods are.

At least five serially cut tissue sections are necessary for exact identification of the main types of amyloid proteins.

The first section of the formaldehyde fixed and paraffin embedded tissue blocks will be stained with Hematoxylin and Eosin (HE) to demonstrate the eosinophilic deposits.

The second slide should be stained with Congo red, and viewed under polarized light [11]. Under polarized light the apple green color in a dark field is specific, and more sensitive to demonstrate minimal amyloid deposits, than light microscopic staining methods. A polarizing microscope with high brightness (at least 100 watt) is indispensable.

Identifying the relation of amyloid deposits to blood vessels is critical to differentiate the systemic and localized types of amyloidosis which the first and second tissue sections allow.

The third slide is used to performate pretreatment for 1 second.

Performate pretreatment for 1 second is a very sensitive and specific method to identify AA amyloidosis. The birefringence of AA deposits immediately disappears in slides stained with Congo red and viewed under polarized light, while other forms of amyloid deposits (AL λ or AL κ , A β 2M and ATTRwt) are resistant and retain the specific apple green color under polarized light.

Performate pretreatment, using different times for degradation, for example 3 or 5 seconds would be sufficient to identify the various types of systemic amyloidosis (See table 1a). AL λ or AL κ amyloidosis is R/S, ATTRwt and A β 2M deposits are resistant, but the hemodialysis associated deposits are morphologically different.

KMnO₄ oxidation may present a second independent confirmation of the diagnosis based on performate pretreatment.

A fourth slide may be used for KMnO₄ oxidation for 5 minutes for independent identification of amyloid deposits.

Systemic senile (ATTRwt) amyloidosis is resistant to 5 minutes KMnO₄ oxidation (under polarized light the specific apple green color still exists).

Systemic AL λ or AL κ amyloidosis is resistant/sensitive to 5 min KMnO₄ oxidation (under polarized light the specific apple green color decreases); while of β 2-microglobulin deposits will be disintegrated, A β 2M is sensitive to 5 min KMnO₄ oxidation (under polarized light the specific apple green color disappears).

KMnO₄ oxidation alone (without performate pretreatment) is not sufficient to differentiate between systemic AA and systemic AL λ or AL κ amyloidosis, because the resistance of AA and AL deposits may be practically the same.

The fifth slide may be used for another independent identification of amyloid deposits with immunohistochemical staining.

The AA protein deposits showed a positive reaction for anti-human amyloid A-component with immunohistochemical staining. The anti-human amyloid A immunohistochemical reaction is specific, but minimal deposits of amyloid A may be missed with it.

The immunohistochemical analysis of AL λ or AL κ , or A β 2M deposits may be difficult especially in case of minimal deposits because of the extensive background staining.

The histochemical identification of amyloid deposits is recommended in laboratories where genomic DNA sequence analysis or amino acid sequence identification techniques are not available [1,14,15,43].

Conclusion

There is a difference between systemic AA, AL λ or AL κ , ATTRwt, A β 2M and isolated AANF amyloidosis in relation to the blood vessels, in the pattern of deposition, in cellular response of phagocytes, in histochemical and in ultrastructural characteristics of amyloid deposits.

Based on relation to the blood vessels and histochemical differences of amyloid deposits the main types of systemic and isolated amyloidosis may be distinguished on serial histological sections. The histochemical identification of amyloid deposits is recommended for laboratories where genomic DNA sequence analysis or amino acid sequence identification techniques are not available.

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