Chapter 7
Amyloidosis: Pathogenesis, Types, and Diagnosis

Shereef Elmoamly and Laura Obici

Abbreviations

DPD	^{99m}Tc-labeled 3,3-diphosphono-1,2-propanodicarboxylic acid
FLC	Free light chains
FMF	Familial Mediterranean fever
HSPG	Heparan sulphate proteoglycan
IEM	Immunoelectron microscopy
IHC	Immunohistochemistry
ISA	International Society of Amyloidosis
LCM-MS	Laser capture microdissection and mass spectrometry
NAC	National Amyloidosis Centre
NGS	Next-generation sequencing
PYP	^{99m}Tc-labeled pyrophosphate
SAP	Serum amyloid P

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Introduction

Amyloidoses are heterogeneous pathological conditions characterized by the extracellular deposition of insoluble protein fibrils that cause progressive organ damage. Amyloidosis may be a systemic disorder, resulting in a spectrum of clinical presentations, or a localized disease associated with single tissue or organ involvement. The term amyloid was first adopted by Rudolph Virchow in 1854 to describe the deposition of a starch-like material with hyaline appearance under light microscopy [1]. The fibrillar nature of amyloid was revealed only in the second half of the twentieth century and the first amyloid protein, namely a fragment of a monoclonal light chain, was isolated from natural amyloid deposits in 1970 [2].

Amyloid has a pathognomonic microscopic appearance, showing apple-green birefringence under polarized light after Congo red staining. Amyloidosis may be hereditary or acquired. The latter also includes an iatrogenic form such as β2-microglobulin amyloidosis occurring in patients on chronic haemodialysis.

Pathogenesis

Amyloid fibrils are derived from globular precursor proteins that undergo misfolding and aggregation into a highly ordered structure. Under electron microscopy amyloid fibrils are rigid, non-branching and around 8–12 nm in diameter (Fig. 7.1) [3]. X-ray diffraction demonstrates a crossed beta-sheet structure.

In vitro studies indicate that the fibrillogenesis process involves protein misfolding, generation of partially unfolded intermediates, aggregation into oligomeric species and/or protofibrils, and ultimately formation of mature, crossed beta-sheet fibril structures. Fibril formation is accelerated in the presence of amyloid seeds, according to nucleation kinetics [4].

Several mechanisms, often acting in combination, are known to play a role in promoting *in vivo* amyloidogenesis. These include a persistent increase in the concentration of the circulating precursor, mutations that perturb the stability of the native structure, an intrinsic misfolding propensity of the protein in its wild-type form and/or proteolytic remodelling leading to the generation of amyloidogenic fragments [5, 6]. In hereditary amyloidosis, fibrillogenesis is promoted by a genetic variant, mostly consisting of a point mutation leading to single amino acid substitution or a premature stop codon. Amyloidogenic mutations result in protein structural changes that destabilize the native conformation. Acquired forms of amyloidosis associated with increased or abnormal production of the precursor include, for example, AA amyloidosis secondary to chronic inflammation and AL amyloidosis resulting from underlying plasma cell dyscrasia.

Moreover, amyloidogenesis is favoured when the proteostasis system, that targets misfolded and aggregated proteins to degradation in the cellular and extracellular compartments, is overwhelmed and/or reduced in its capacity by ageing [7].

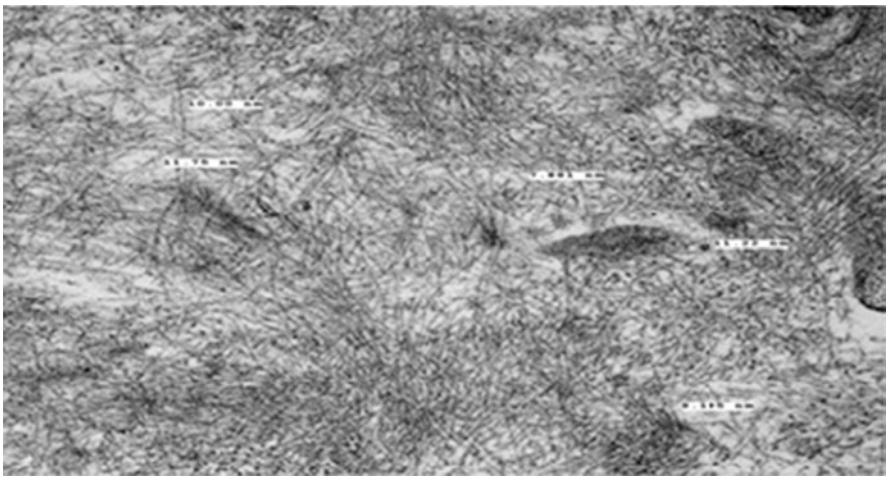

Fig. 7.1 Electron microscopy appearance of amyloid (in renal amyloidosis). Photo courtesy of Dr. B Vydianath, University Hospitals Birmingham NHS Foundation Trust

Amyloid deposits not only consist of one key precursor protein among those indicated in Table 7.1 but also contain additional constituents including heparan sulphate proteoglycan (HSPG), serum amyloid P component (SAP), apolipoprotein E, and vitronectin. These components, that are invariably present, serve as universal amyloid signatures [8]. The pathological roles of these additional molecules in amyloidogenesis are not fully clarified although there is evidence that glycosaminoglycans may act as scaffold for amyloid aggregation and SAP inhibits fibril degradation [9].

Whereas systemic forms of amyloidosis are caused by circulating precursor proteins, in localized amyloidosis, such as AL amyloidosis involving the lungs, the skin, or the genitourinary tract the precursor immunoglobulin light chain is synthesized and processed at affected local sites [10, 11].

Amyloid Protein Nomenclature and Classification

According to the International Society of Amyloidosis (ISA) nomenclature, all amyloid fibril proteins are named protein A together with the specific protein name as a suffix, for example, AL (L for immunoglobulin light chain), ATTR (TTR for transthyretin), or AFib (Fib for fibrinogen alpha chain). The protein name may be further specified, for example, ATTRwt or ATTRv (wt for wild type and v for variant). Hereditary amyloidosis protein variants are named according to the mature protein substitution or deletion, with the amino acid involved and the change position listed, for example, ATTRV30M (methionine replacing valine). The main

Table 7.1 Amyloid fibril proteins and their precursors in human [8]

Fibril protein	Precursor protein	Systemic and/or localized	Acquired or hereditary	Target organs
AL	Immunoglobulin light chain	S, L	A, H	All organs, usually except CNS
AH	Immunoglobulin heavy chain	S, L	A	All organs except CNS
AA	(Apo) serum amyloid A	S	A	All organs except CNS
ATTR	Transthyretin, wild type	S	A	Heart mainly in males, lung, ligaments, tenosynovium
	Transthyretin, variants	S	H	PNS, ANS, heart, eye, leptomeninges
Ab2M	b2-microglobulin, wild type	S	A	Musculoskeletal system
	b2-microglobulin, variants	S	H	ANS
AApoAI	Apolipoprotein A I, variants	S	H	Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants)
AApoAII	Apolipoprotein A II, variants	S	H	Kidney
AApoAIV	Apolipoprotein A IV, wild type	S	A	Kidney medulla and systemic
AApoCII	Apolipoprotein C II, variants	S	H	Kidney
AApoCIII	Apolipoprotein C III, variants	S	H	Kidney
AGel	Gelsolin, variants	S	H	Kidney, PNS, cornea
ALys	Lysozyme, variants	S	H	Kidney
ALECT2	Leukocyte chemotactic factor-2	S	A	Kidney, primarily
AFib	Fibrinogen a, variants	S	H	Kidney, primarily
ACys	Cystatin C, variants	S	H	CNS, PNS, skin
ABri	ABriPP, variants	S	H	CNS
ADan	ADanPP, variants	L	H	CNS
Ab	Ab protein precursor, wild type	L	A	CNS
	Ab protein precursor, variant	L	H	CNS
AaSyn	a-Synuclein	L	A	CNS
ATau	Tau	L	A	CNS
APrP	Prion protein, wild type	L	A	CJD, fatal insomnia
	Prion protein variants	L	H	CJD, GSS syndrome, fatal insomnia
	Prion protein variant	S	H	PNS

Table 7.1 (continued)

Fibril protein	Precursor protein	Systemic and/or localized	Acquired or hereditary	Target organs
ACal	(Pro)calcitonin	L	A	C-cell thyroid tumours
		S	A	Kidney
AIAPP	Islet amyloid polypeptide[c]	L	A	Islets of Langerhans, insulinomas
AANF	Atrial natriuretic factor	L	A	Cardiac atria
APro	Prolactin	L	A	Pituitary prolactinomas, ageing pituitary
AIns	Insulin	L	A	Iatrogenic, local injection
ASPC	Lung surfactant protein	L	A	Lung
ACor	Corneodesmosin	L	A	Cornified epithelia, hair follicles
AMed	Lactadherin	L	A	Senile aortic, media
AKer	Kerato-epithelin	L	A	Cornea, hereditary
ALac	Lactoferrin	L	A	Cornea
AOAAP	Odontogenic ameloblast-associated protein	L	A	Odontogenic tumours
ASem1	Semenogelin 1	L	A	Vesicula seminalis
AEnf	Enfurvitide	L	A	Iatrogenic
ACatK	Cathepsin K	L	A	Tumour associated
AEFEMP1	EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1)	L	A	Portal veins Ageing associated

clinical presentation may be added to the amyloidosis name, for example, ATTRv cardiomyopathy or ATTR polyneuropathy [8].

To date, at least 36 proteins have been identified that form amyloid deposits in humans. As mentioned before, amyloid deposits may be systemic, affecting various organs and tissues throughout the body, or localized, with deposits being formed in a single organ or tissue.

Exclusively localized amyloid deposits have been associated with 22 proteins, while 18 proteins (and many more variants) are classified to be associated with systemic amyloidosis. Interestingly, some protein types (most notably AL/AH, amyloidosis derived from immunoglobulin light or heavy chain, respectively, and amyloidosis derived from prion protein) can occur as either localized or systemic forms (Table 7.1). This list is periodically updated. In addition, novel potential amyloidogenic precursor proteins are currently under investigation [8].

Certain amyloid proteins have specific target organs resulting in typical clinical manifestations. In general, however, systemic amyloidoses are clinically heterogeneous, with considerable overlap in presenting features. The potential contribution of genetic and/or environmental factors to the phenotypic variability that characterizes these diseases has been suggested, particularly in hereditary transthyretin amyloidosis and in AL amyloidosis [7].

Fifteen inherited amyloid types are known that are caused by genetic variants of the precursor proteins, mostly represented by missense mutations. Genetic variants result in protein products more amyloidogenic than their wild-type counterparts [12]. Some polymorphisms are also associated with increased amyloid risk, such as certain alleles of the apolipoprotein E gene in acquired cerebral amyloid angiopathy [13] and some specific isoforms of serum amyloid A protein in systemic AA amyloidosis [14]. Moreover, hereditary autoinflammatory disorders such as familial Mediterranean fever (FMF) or cryopyrinopathies may result in chronic inflammation and development of AA amyloidosis [15].

Epidemiology

Amyloidosis is a rare disorder for which it is still difficult to obtain a reliable estimate of incidence and prevalence [16]. In 2013, the UK National Amyloidosis Centre (NAC) reported that the estimated incidence of systemic amyloidosis was exceeding 8.0 per million inhabitants per year with around one third diagnosed with AL amyloidosis [17]. In the developed world, AL amyloidosis has long been the most prevalent with significant increases in diagnosis in the last decade [18]. Moreover, wild-type ATTR amyloidosis, which typically affects male subjects over the age of 60, is more and more recognized nowadays, with a prevalence that increases according to age. On the contrary, AA amyloidosis is more frequent in developing countries where endemic infectious diseases (e.g. tuberculosis or leprosy) and/or autoinflammatory diseases are relatively frequent (e.g. familial Mediterranean fever in the Mediterranean countries) [19]. Hereditary amyloidoses are much rarer, constituting approximately 10% of all systemic forms. However, their prevalence is still likely underestimated due to lack of clinical suspicion and insufficient detection of genetic mutations [20]. ATTRv is the most common hereditary amyloidosis worldwide.

Diagnosis

Diagnosis of systemic amyloidosis relies on the critical combination of clinical findings, histopathological evidence, genetic results, and imaging studies. Family history may guide towards an inherited form, leading to further investigations.

Histologic demonstration of amyloid deposits and characterization of the amyloid precursor protein in tissue is the cornerstone for a definitive and accurate diagnosis (Fig. 7.2). First, a tissue sample should be examined by polarized light microscopy after Congo red staining in order to detect the presence of amyloid (Figs. 7.3 and 7.4). Biopsies can be initially obtained from abdominal fat, minor salivary glands, skin, or rectal mucosa. If negative, an affected organ (e.g. kidney, heart) should be investigated to definitively confirm or exclude the diagnostic suspicion.

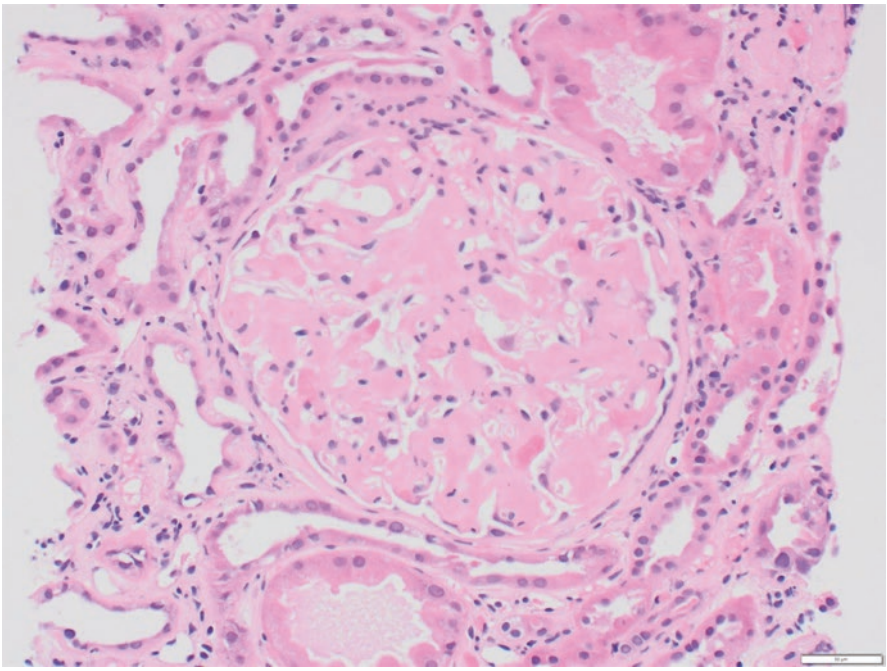

Fig. 7.2 Amyloid deposition in a renal glomerulus as seen on Haematoxylin and Eosin stain. Original magnification x 200, Photo courtesy of Dr. Y Hock, University Hospitals Birmingham NHS Foundation Trust

Subcutaneous fat pad aspiration or biopsy is suggested as a good initial sampling source as it's a safe, rapid, simple, and cheap procedure with no risk of serious bleeding. Congo red staining and examination using polarizing microscopy of subcutaneous fat pad aspiration or biopsy has an overall sensitivity of 57–85% and a specificity of 92–100% for light chain (AL) and secondary (AA) amyloidosis [21–23]. Single organ involvement may yield lower sensitivity of fat pad aspiration or biopsy [24]. Other potential sites of biopsy have variable degrees of sensitivity, rectal biopsy reported to be 84% sensitive in one large series. The sensitivity of kidney, liver, and carpal ligament biopsies were 90% or more in the same cohort (Fig. 7.4) [25].

When a positive biopsy is identified, the second step is to define the amyloidogenic protein in order to unequivocally establish the type of amyloidosis, as this guides its specific treatment. Amyloid typing can be performed by light microscopy immunohistochemistry (IHC), immunoelectron microscopy (IEM) characterization or proteomics, the latter being now considered the gold standard.

IHC involves the use of antibodies for a panel of amyloidogenic proteins. It is not expensive, and it is potentially widely available. However, due to its low specificity and sensitivity, it needs to be performed by a highly specialized pathologist usually

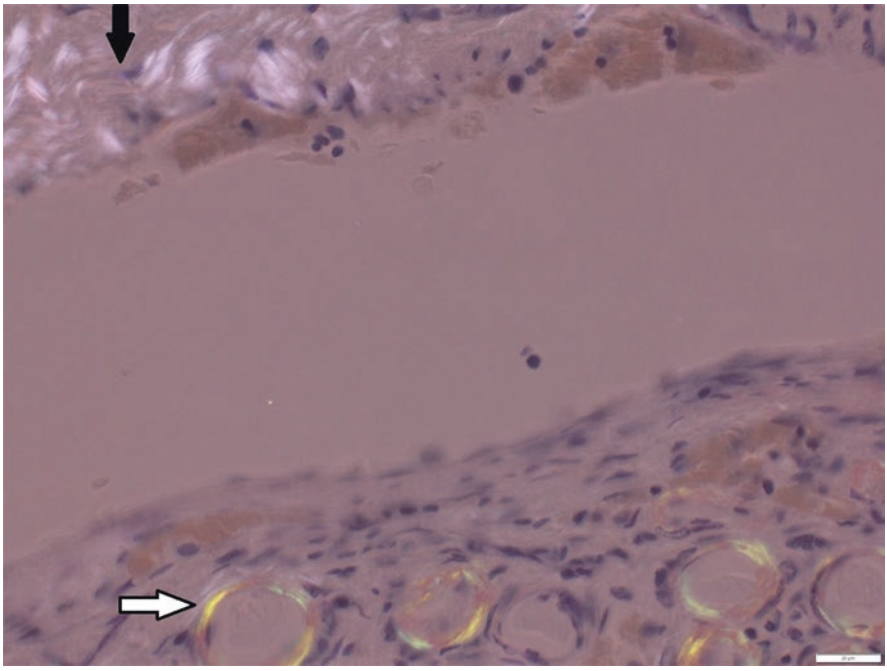

Fig. 7.3 Abundant amyloid deposition in renal blood vessels as seen by Congo red staining (with polarization). Apple green birefringence of amyloid in blood vessels can be seen in the lower part (white arrow) with non-birefringent collagen fibres in the upper part for comparison (black arrow). Original magnification x 200, Photo courtesy of Dr. Y Hock, University Hospitals Birmingham NHS Foundation Trust

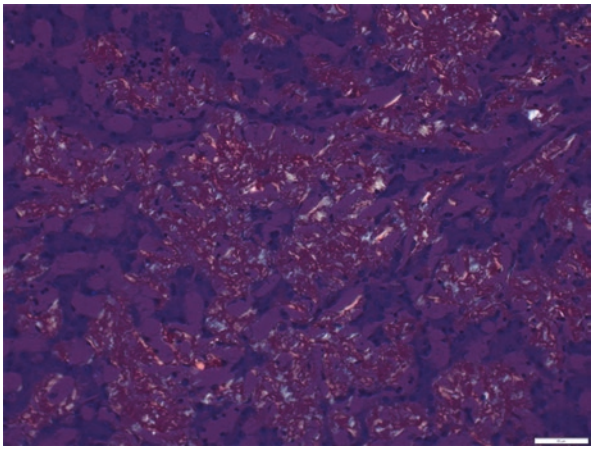

Fig. 7.4 Abundant amyloid deposition in liver as seen on Congo red stain (with polarization demonstrating apple green birefringence). Original magnification x 200, Photo courtesy of Dr. Y Hock, University Hospitals Birmingham NHS Foundation Trust

in expert centres. It is mandatory to use antibodies specifically developed for the recognition of amyloidogenic proteins to avoid false-positive or false-negative results.

In a study conducted by the national amyloidosis centre in the UK, IHC was diagnostic in 76%, and showed 100% concordance with the results of laser capture microdissection and mass spectrometry (LCM-MS) performed on the same samples [26]. The rate of false positives and negatives may be unacceptably high without using a validated panel of antibodies and proper methods. Background staining may occur due to non-immunological binding or to the presence of normal proteins containing epitopes targeted by the antibody in the extracellular space, such as normal immunoglobulins, resulting in false-positive results [27].

IEM is a technique available in few referral centres that combines immunohistochemistry and electron microscopy. Using gold-labelled secondary antibodies, IEM can co-localize the protein within amyloid fibrils and greatly reduce background staining, increasing accuracy. In an Italian study, IEM was equally sensitive (75–80%) but significantly more specific (100% vs 80%; $P < 0.001$) in diagnosing the type of systemic amyloidosis compared to light microscopy [28].

In cases where IHC is equivocal and not decisive or additional information is considered useful for clinical or diagnostic purposes, samples should be analysed by mass spectrometry-based proteomics. This approach relies on laser microdissection of Congo red positive amyloid deposits followed by mass spectrometry of digested proteins (LMD-LC-MS/MS), a sophisticated and highly accurate approach to determine the amyloid fibril type based on accurate measurement of the molecular mass of the more abundant peptides. This technique requires accurate sample preparation, mass spectrometry analysis, and protein identification by bioinformatics tools [29]. It is highly effective in recognizing all amyloid types in a single assay, increasing the diagnostic accuracy from 76 to 94% compared to IHC [26, 30]. Limitations include costs, accessibility, the need for experience and longer turnaround time [29].

In parallel with histological investigation and tissue amyloid typing, the corresponding protein precursor should be identified and quantified in blood by means of biochemical and/or genetic tests. The presence of a monoclonal protein in patients with suspected or biopsy-proved AL amyloidosis should always be investigated by serum and urinary immunofixation coupled with measurement of monoclonal free light chains (FLC). Only the combination of these tests allows detecting and quantifying the culprit monoclonal protein in 100% of cases.

Molecular genetic testing is essential for the diagnosis of hereditary amyloidosis. This is usually performed by Sanger sequencing of selected genes but next-generation sequencing (NGS) is becoming an increasingly available tool to test a panel of potentially involved genes at the same time.

Diagnosis of ATTRv amyloidosis, the most common form of hereditary amyloidosis worldwide, still takes several months or years from symptom onset in people with no known family history from non-endemic regions. This is still mostly due to limited disease awareness and/or misdiagnosis with other more common diseases. However, once an index patient has been identified in a family,

genetic counselling and pre-symptomatic testing in at-risk relatives can be undertaken to identify possible mutation carriers, ensuring close monitoring and early diagnosis [31].

Bone tracer scintigraphy, particularly ^{99m}Tc-labelled 3,3-diphosphono-1,2-propanodicarboxylic acid (DPD) and ^{99m}Tc-labelled pyrophosphate (PYP), is now a well-acknowledged tool for the diagnosis of cardiac transthyretin amyloidosis and has substantially contributed to increasingly identify this disease in the past few years. The mechanisms underlying the specific tropism of these tracers for ATTR amyloid deposits are still elusive but a positive DPD or PYP scan can permit a non-invasive diagnosis of TTR amyloidosis according to validated diagnostic algorithms, avoiding the need for a positive biopsy. Briefly, a positive scan defined by a Perugini score higher than 1, in the absence of a monoclonal protein, is diagnostic for cardiac transthyretin amyloidosis [32]. As wild-type ATTR is not distinguished from hereditary ATTR based on this tool, genetic analysis is ultimately always needed to accurately differentiate acquired from hereditary TTR amyloidosis.

Scintigraphy using radioisotope-labelled serum amyloid P component (SAP) can demonstrate the presence of amyloid within some organs and provide an estimate of amyloid burden [33]. This procedure is however available only in a few centres. It is safe and can be repeated every 6–12 months to monitor the course of the disease, particularly in patients with secondary AA amyloidosis, therefore guiding treatment strategy. Sensitivity of SAP Scintigraphy is higher in AA and AL amyloid (90%) compared to 48% for hereditary transthyretin-related (ATTR) amyloidosis, with 93% specificity in all these conditions [34].

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