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Diagnosis of Amyloidosis: From History to Current Tools

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Abstract

The term amyloid encompasses a large variety of misfolded proteins with varying amino acids unified by the antiparallel beta-pleated sheet configuration and characteristic Congo red staining. The etiology of these proteins is equally varied, ranging from neoplastic plasma cell disorder, hereditary causes to inflammatory disorders. The protean clinical manifestation makes a high index of clinical suspicion the first crucial step in the diagnosis. A battery of investigations needs to be carried out for a complete diagnosis of amyloidosis and its underlying etiology. Biopsy with Congo red staining constitutes the most important modality for confirmation of amyloid. For further testing, varying modalities with increasing complexity, such as immunohistochemistry, electron microscopy, and mass spectrometry, need to be employed. We discuss, in the course of the current chapter, this fascinating protein from a clinical diagnosis perspective. The requisite investigations are also discussed in detail.

Keywords: amyloid, plasma cell, beta-pleated sheet, misfolded proteins, Congo red, mass spectrometry

1. Introduction

Amyloid is characterized by homogenous amorphous eosinophilic extracellular deposits in several tissues on routine histopathological examination. This amorphous proteinaceous substance is indeed very heterogeneous; however, all these types have some properties in common, which thereby define it as amyloid [1]. Understanding this core concept then makes it easy to understand the diagnostic approach for amyloid. Before proceeding with the diagnostic armamentarium available for amyloid, we briefly discuss various types of amyloid and their nomenclature.

2. Classification and nomenclature

Amyloid is characterized by some common properties as follows. These are fibrillary misfolded proteins that resist degradation by proteasome and macrophages leading to deposition. They can either be a normal protein with an inherent tendency to form amyloid when produced in excess (Example: ATTR) or result of genetic mutation giving rise to amyloidogenic protein. (Example: AApoAI) X-ray diffraction shows antiparallel β -pleated sheet configuration noted on X-ray diffraction and Congo red stained section shows diagnostic apple-green birefringence

under polarized light [2]. These properties bring them under one roof of amyloidosis. However, due to marked heterogeneity in chemical nature and therapeutic implications, amyloid is classified in several ways, as follows.

2.1 Systemic versus localized

Clinically, amyloid can be classified as systemic/generalized or localized based on whether it affects many organs/systems or affects only one site. Localized amyloid deposits are usually seen in the skin, larynx, and bladder. It is characterized by an indolent course requiring minimal intervention. However, recurrences can be a potential concern. At least 19 amyloid types are known to cause exclusive localized deposits. Systemic amyloidosis causes more generalized deposits, affecting the entire organ-like cardiac deposition in ATTR form or multisystem affection in AL amyloidosis. The systemic form is associated with 14 different forms of amyloidogenic proteins [3, 4]. Some forms like AL/AH/ATTR can cause localized deposits and more generalized systemic disease.

2.2 Primary versus secondary

Another popular way of classifying systemic amyloidosis is primary versus secondary amyloidosis. Primary is driven by clonal plasma cell proliferation, leading to excessive misfolded monoclonal light chain production, leading to fibril formation and amyloid deposition [5]. Whereas secondary amyloidosis is secondary to inflammatory processes like autoimmunity, infection likes tuberculosis.

Hereditary another distinct category is hereditary or familial amyloidosis where genetic mutation leads to the production of misfolded fibrillary protein (FAP).

We can also classify amyloidosis based on its biochemical nature. Thirty-six proteins have been identified as amyloidogenic in humans, and the list continues to grow with more types getting identified with sophisticated tests like mass spectrometry.

| | Systemic/ localized/ both | Amyloid type | Precursor protein | Organ predominantly affected |
|------------|------------------------------|--------------|-----------------------------------|---|
| Hereditary | Systemic | ATTR | Transthyretin, variants | PNS, ANS, heart, eye, leptomeninges |
| | Systemic | A β 2M | β 2 microglobulin, variants | ANS |
| | Systemic | AApoAI | Apolipoprotein A I, variants | Heart, liver, kidney, PNS, testis, larynx (C terminal variants), skin (C terminal variants) |
| | Systemic | AApoAII | Apolipoprotein A II, variants | Kidney |
| | Systemic | AApoCII | Apolipoprotein C II, variants | Kidney |
| | Systemic | AApoCIII | Apolipoprotein C III, variants | Kidney |
| | Systemic | AGel | Gelsolin, variants | Kidney, PNS, cornea |
| | Systemic | ALys | Lysozyme, variants | Kidney |
| | Systemic | AFib | Fibrinogen a, variants | Kidney |
| | Systemic | ACys | Cystatin C, variants | CNS, PNS, skin |
| | Systemic | ABri | ABriPP, variants | CNS |

| | Systemic/ localized/ both | Amyloid type | Precursor protein | Organ predominantly affected |
|----------|--------------------------------------|---------------------|--|--|
| | Localized | ADanb | ADanPP, variants | CNS |
| | Localized | A β | A β protein precursor, variant | CNS |
| | Localized | APrP | Prion protein, variant | CJD, GSS syndrome, fatal insomnia |
| | Systemic | | Prion protein variant | PNS |
| Acquired | Both | AL | Immunoglobulin light chain | All organs, usually except CNS |
| | Both | AH | Immunoglobulin heavy chain | All organs except CNS |
| | Systemic | AA | (Apo) Serum amyloid A | All organs except CNS |
| | Systemic | ATTR | Transthyretin, wild type | Heart mainly in males, lung, ligaments, tenosynovium |
| | Systemic | Ab2M | β 2-microglobulin, wild type | Musculoskeletal system |
| | Systemic | AApoAIV | Apolipoprotein A IV, wild type | Kidney medulla and systemic |
| | Systemic | ALECT2 | Leukocyte chemotactic factor-2 | Kidney primarily, Liver |
| | Localized | A β | A β protein precursor, wild type | CNS |
| | Localized | AaSyn | α -Synuclein | CNS |
| | Localized | ATau | Tau | CNS |
| | Localized | APrP | Prion protein, wild type | CJD, fatal insomnia |
| | Localized | ACal | (Pro)calcitonin | C-cell thyroid tumours |
| | Systemic | ACal | (Pro)calcitonin | Kidney |
| | Localized | AIAPP | Islet amyloid polypeptide | Islets of Langerhans, insulinomas |
| | Localized | AANF | Atrial natriuretic factor | Cardiac atria |
| | Localized | APro | Prolactin | Pituitary prolactinomas, aging pituitary |
| | Localized | AIns | Insulin | Iatrogenic, local injection |
| | Localized | ASPCd | Lung surfactant protein | Lung |
| | Localized | ACor | Corneodesmosin | Cornified epithelia hair follicles |
| | Localized | AMed | Lactadherin | Senile aortic, media |
| | Localized | AKer | Kerato-epithelin | Cornea, hereditary |
| | Localized | ALac | Lactoferrin | Cornea |
| | Localized | AOAAP | Odontogenic ameloblast- associated protein | Odontogenic tumours |
| | Localized | ASem1 | Semenogelin 1 | Vesicula seminalis |
| | Localized | AEnf | Enfurvitide | Iatrogenic |
| | Localized | ACatKe | Cathepsin K | Tumour associated |
| | Localized | AEFEMP1e | EGF-containing fibulin- like extracellular matrix protein 1 (EFEMP1) | Portal veins Aging associated |

Table 1.
 This table summarizes the common amyloid proteins identified in humans with brief comments related to each of them [6].

2.3 Nomenclature

International society of amyloidosis (ISA) recommended in 2018 that the name of any amyloidosis start with A standing for amyloid followed by suffix suggestive of underlying pathogenic protein. Example- AH stands for the heavy chain of immunoglobulin giving rise to amyloid [6, 7]. However AH, AL are amyloid proteins, and AH amyloidosis is the disease.

For amyloidosis caused by various inherited mutations leading to fibrillary protein production, hereditary is the favored term compared to familial. In hereditary subset nomenclature recommendation by ISA says single letter coding for amino acid substitution can be used with mention of amino acid position where substitution is happening. Example: ATTRV30M or ALysI56T. To indicate the mutation is driving the amyloidosis, vATTR (variant) is recommended by ISA over (mutant) ATTRm (**Table 1**).

3. History of diagnostic tools for amyloid

3.1 Early advances: till the development of Congo red as a diagnostic tool

Like many other diseases, the first description of amyloid came from autopsy rooms. It evolved from classical gross description to gross staining with iodine and sulfuric acid, suggesting starch-like amyloid properties [8].

Botanist Matthias Schleiden followed by Rudolf Virchow used this term amyloid, and confusion about its starch-like nature continued [9, 10].

Initially, metachromatic stains were utilized to detect amyloid. However, later chemist Paul Böttiger discovered a textile dye named Congo red which can bind to amyloid and Puchtler described the method for histological preparation [11]. Since then Congo red staining and apple-green birefringence became the gold standard for establishing the amyloid diagnosis. This early history of diagnosis of amyloidosis is dealt with in better detail in another chapter of this book. Hence, we intend to focus on the history of evolving tools for amyloid typing.

3.2 Modern history of amyloid diagnosis-evolution of tools for amyloid typing

1959, Alan S Cohen and Calkin decoded the fibrillary nature of amyloid under the electron microscope [12].

Amyloid extraction followed by X-ray diffraction demonstrated cross beta-pleated sheet structure of amyloid by Eanes and Glenner [13].

More important application of amyloid extraction opened the door for understanding biochemical nature and hence the pathogenesis of different types of amyloid. Glenner and colleagues decoded that AL amyloid was indeed due to the light chain of immunoglobulin getting misfolded and producing amyloid. AL is the most common type of amyloidosis affecting humankind.

Identifying these different types of amyloid proteins is what is referred to as amyloid typing in today's diagnostic practices. As emphasized earlier this is the crucial part of work up as treatment may vary from bone marrow transplant for AL amyloidosis to liver transplant for others.

Amyloid typing tools exploit two principles; one is the immune platform where antibody against the antigen of interest (amyloid here) is allowed to react. The immune complex formed is detected by either fluorescence in immunofluorescence (IF), chromogen in immunohistochemistry (IHC), or coupled this immune reaction with electron microscopy localizing exact deposition of the complex in immunoelectron microscopy [14].

Among the above investigations, IHC and IF have the biggest advantage of being widely available today. However, over time, researchers found several limitations of these typing techniques like the IHC panel is usually limited to target AL, AA, and ATTR amyloid. These tests do not detect mutated and truncated forms and have cross-reactivity with non-amyloid proteins [15]. Immunofluorescence as a technique for amyloid typing needs frozen tissue and antibodies used lack specificity giving inconclusive results [16].

To overcome these shortcomings of immune methods for amyloid typing breakthrough technology of proteomics mass spectrometry was used. Vrana et al. used laser micro-dissection to extract the amyloid from Congo red positive bit of histopathology section and did a proteomic study with mass spectrometry to classify several types of amyloid by identifying the very nature of pathogenic protein giving rise to misfolded fibrils [17]. Modification of this technique is also developed for fat pad aspirate specimens. Details of these amyloid typing technologies will be discussed in the amyloid typing techniques section of this chapter.

Decoding the amyloid fibril to its true protein nature was possible with these typing tools. However, another aspect was to understand some types of amyloidosis that can be inherited and the treatment concerns they created.

Satoru Tawara and colleagues identified a point mutation in the gene coding for TTR mutant TTR-related FAP in 1983 [18]. To date, several hereditary types of amyloidosis have been identified, which are driven by various genetic events. Recent additions to this list have been fibrinogen A- α chain, lysozyme, apolipoprotein AII, and leucocyte chemotactic factor 2 [19].

This brings us to the end of a brief review of how the history of amyloid detection evolves from gross staining properties confusing it for starch to sophisticated tools understanding its true proteinaceous nature with typing of amyloid proteins and deciphering genetic events leading to a subset of these amyloid deposits. Knowing this background helps us to understand how we use these tools developed over decades and apply them today for the diagnostic workup of this rather unusual and mystifying disease.

4. Diagnostic approach to amyloidosis

4.1 Diagnosis starts by suspecting in clinic

Clinically it is relevant to first classify patients as having localized versus systemic amyloidosis. Localized amyloid is often only a cosmetic concern presenting as a nodule or a plaque most commonly in the skin. Localized cutaneous amyloid can be AD (keratin) depositing at the site of trauma or An Insulin at the site of injection, and many more. Rarely polyclonal plasma cells can give rise to a localized deposit of amyloidogenic light chain in skin, larynx, urinary bladder, and others. The localized form usually does not require extensive therapy, however, it can recur [3].

One of the challenges in establishing systemic amyloidosis diagnosis is suspecting it in the correct scenario and ordering the correct test. Coming across such patients should alert the treating physician to order broad screening tests for amyloid.

Patient may complain of nonspecific fatigue or weight loss, to symptoms and signs suggestive of the target organ involved. Classical presentations of systemic amyloidosis are nephrotic range proteinuria in renal amyloidosis, cardiac failure, sensorimotor and autonomic neuropathy without apparent cause [4].

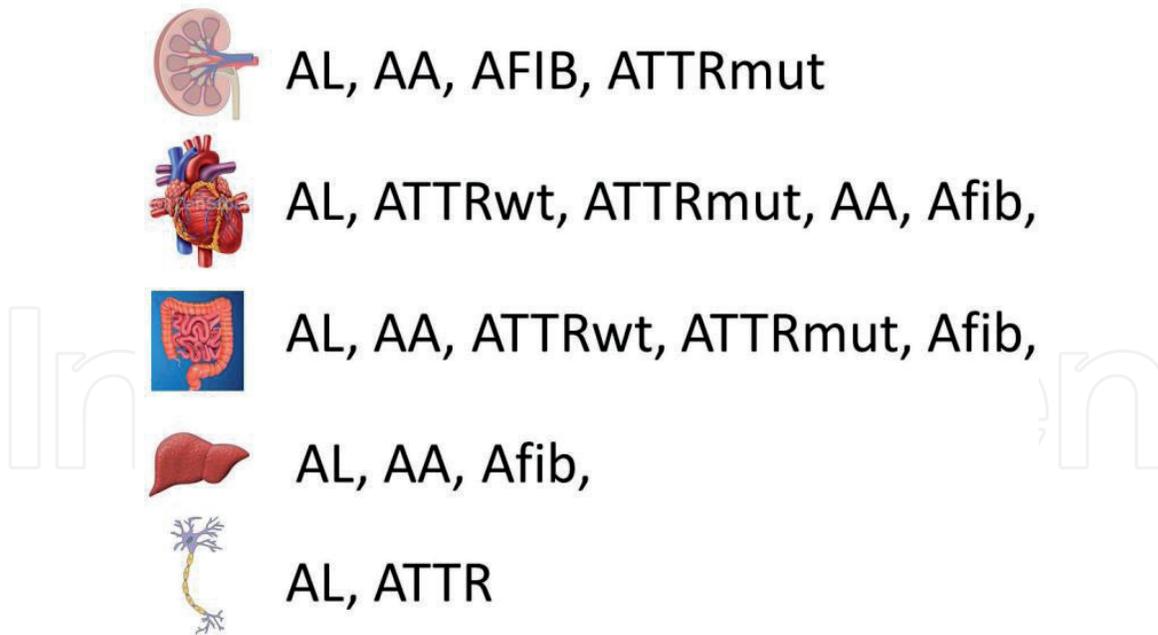


Figure 1.
Summary of the organ preference by various amyloidosis.

Primary amyloidosis (AL) is the most common type of systemic amyloidosis. The ratio of primary to non-primary is more skewed in America (20:1) than Europe (2:1), attributed to the common occurrence of hereditary familial Mediterranean fever in Europe. The kidney is the most common organ involved by all systemic amyloidosis except in ATTR. Other organs involved by AL type are the heart, liver kidney, and nervous system. Hepatomegaly with normal echotexture, easy bruising around eyes, and macroglossia are other classical pointers to amyloidosis, especially the commonest form, i.e. AL amyloidosis [3].

Several pointers in history help us to predict the type of amyloidosis. History of chronic inflammatory disease raises the possibility of secondary AA amyloid. Positive family history tilts the balance towards the genetic nature of illness initiating molecular testing for the same. The predominant organ involved also gives us the clue about the type of amyloidosis under investigation (**Figure 1**).

Based on history and clinical examination when suspicion for amyloid is a very high battery of lab tests are advised. **Table 2** gives a comprehensive list of lab tests to be ordered in a case of amyloid to establish the diagnosis with amyloid typing [20].

4.2 Imaging for amyloid diagnosis/non-invasive amyloid diagnosis

Imaging tools are indispensable when cardiac amyloidosis is suspected. In patients with left ventricular wall thickness > 12 mm and red flag signs like unexplained right-sided heart failure, proteinuria, ECG showing reduced QRS voltage and conduction defect, and disproportionately increased N-terminal pro B-type natriuretic peptide (NT-proBNP), one should suspect cardiac amyloidosis. More than 97% of cases of cardiac amyloidosis are primary AL type or transthyretin type (both wild and mutant) [21]. Recent position statement of the European Society of Cardiology Working Group on Myocardial and Pericardial Diseases proposed non-invasive diagnostic criteria for cardiac amyloidosis though it is applicable for ATTR type only. Other types of cardiac amyloidosis still require demonstration on Congo red positive birefringent deposits to establish their diagnosis, which is discussed later.

For establishing diagnosis and typing of amyloid

- Target organ or surrogate site (abdominal fat pad) biopsy
- H & E and Congo red stain
- IHC/Immunoelectron microscopy/ proteomic study for amyloid typing
- Serum protein electrophoresis (SPEP), Immunofixation electrophoresis (IFE), Serum free light chain assay (sFLC) for evidence of monoclonal plasma cell proliferation
- Bone marrow aspirate and biopsy with flowcytometry for clonal plasma cells
- Mutation studies for various hereditary amyloidosis
- Work up for rheumatoid arthritis, tuberculosis and other inflammatory conditions associated with AA amyloidosis

For confirming systemic involvement, assess distribution and complications

- Renal function test
 - 24 hours urine protein/albumin
 - Cardiac magnetic resonance/Echocardiography for cardiac involvement
 - Pro NT-B-type natriuretic peptide
 - ECG
 - USG/ CT scan for liver span
 - Alkaline phosphatase
 - X ray or CT scan of lung
 - GIT biopsy with evidence of amyloid
 - SAP scintigraphy/technetium scintigraphy
 - Coagulation screen
-

Table 2.

List of investigations in case of amyloidosis (adapted with permission from Juneja et al. [20]).

Echocardiography and cardiac MRI (CMR) are important imaging tools to establish cardiac amyloidosis diagnosis. Classical findings on Echocardiography have left ventricle thickness > 12 mm with two or more of the below

1. Grade 2 or worse diastolic dysfunction
2. Reduced tissue Doppler S', E', and A' wave velocities (<5 cm/s)
3. Decreased LV global longitudinal strain (absolute value < -15%). Echocardiography findings have been recently given a scoring system to predict cardiac amyloidosis [22].

CMR shows diffuse sub-endocardial or transmural late gadolinium enhancement with abnormal gadolinium kinetics and/or extracellular volume $\geq 0.40\%$. These radiological findings need to be supported by either tissue evidence, i.e. endomyocardial or surrogate site biopsy showing Congo red positive deposits with amyloid typing for invasive diagnosis of cardiac amyloidosis.

However, for a second most common type of cardiac amyloidosis i.e. ATTR amyloidosis these Echocardiography and CMR findings can be supported with non-invasive radiotracer uptake base investigation like ^{99m}Tc -pyrophosphate (PYP) scintigraphy showing grade 2 or 3 myocardial radiotracer uptake and all three tests for monoclonal protein detection being negative [23]. The idea behind this approach is that these 3 tests namely serum protein electrophoresis, urine electrophoresis, immunofixation and serum-free light chain assay combined have >99%

sensitivity to pick up monoclonal protein [24]. These tests shall be discussed in more detail under amyloid typing as a test to demonstrate monoclonal protein detection. Negative results help us to exclude AL amyloidosis. High-grade tracer uptake with CMR/Echocardiography findings suggest the possibility of ATTR amyloidosis and pursue genetic testing to differentiate wild and mutant forms.

However, this again needs to be reemphasized that non-ATTR cardiac amyloidosis, any positivity for monoclonal protein, grade 1 scintigraphy results, and suspicious CMR/Echocardiography findings warrants invasive/tissue-based diagnosis of amyloid with amyloid typing which will be discussed in detail below and is applicable to different types of systemic amyloidosis.

4.3 Establish the tissue diagnosis of amyloidosis

4.3.1 Site of biopsy

When a clinical profile is highly suspicious of systemic amyloidosis, the first step in establishing the diagnosis is obtaining the tissue evidence of amyloid deposits.

Based on the complaints and lab findings, the organ involved can be targeted like liver or kidney. There is always a risk of bleeding, limited accessibility with these vital organs. However, for screening purposes either an abdominal fat pad or bone marrow can be targeted as it is involved in 80% of cases of systemic amyloidosis but may give a false negative result in ~15% cases [25].

4.3.2 Establishing the amyloid diagnosis on biopsy

Electron microscopy reveals the non-branching fibrillary nature of amyloid with a mean diameter of 10 nm (range 8 nm–12 nm). The fibrillary structure is not unique to amyloid as fibrils may be seen in other deposition diseases, such as immunotactoid glomerulonephritis and glomerular sclerosis. Electron microscopy shows differences in the characters of fibrils. However, we need a more widely available technique to confirm the deposits as amyloid [4].

Something as simple as routine histopathological evaluation of biopsy tissue with special stains comes to our rescue.

Amyloid is identified as an amorphous pink, homogenous, eosinophilic extracellular deposit on routine hematoxylin and eosin stain (**Figure 2a**). Though it has a classical appearance there are microscopic mimics. Fibrosis, Collagen, light chain and heavy chain deposits, and fibrin can potentially mimic amyloid; hence we need special stains to confirm amyloid [1].

As mentioned in detail in history, several stains enhance amyloid in different ways. Metachromatic stain to thioflavins showing fluorescence highlights amyloid. One stain that stood the test of time and is still most relevant to amyloid diagnosis is Congo red. X-ray diffraction revealed cross beta-pleated sheet structure of amyloid which potentially explains the mechanism of Congo red positivity and birefringence by amyloid.

The non-ionic hydrogen bond between amyloid and Congo red dye imparts it deep pink to red color. On polarization Congo red stained amyloid demonstrate apple-green birefringence, which is unique and diagnostic of amyloid unlike its mimics on histopathology (**Figure 2b and c**). Since then this text tile dye meant to stain cellulose fiber of fabric became an indispensable tool for amyloid diagnosis [11].

Congo red positivity with birefringence is now the part of the definition of amyloid. A routine light microscope can be easily converted to a polarizing microscope by adding a polarizer-analyzer pair in the light path [26]. However, one should remember that stain works better at alkaline PH on a thick cut section of 6 to 10 microns.

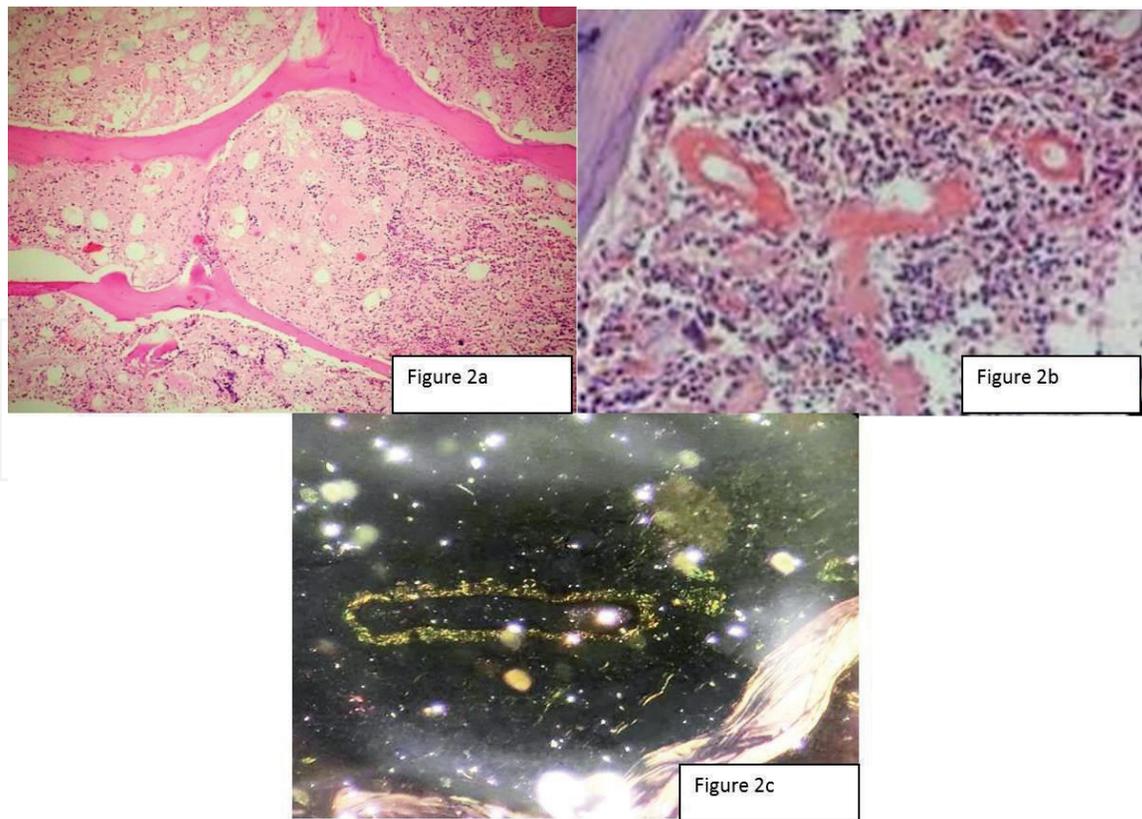


Figure 2.
a. H & E staining showing amorphous, extracellular, eosinophilic material (100X). b. Congo red stain showing deep pink to red-colored perivascular amyloid (400X). c. Amyloid deposits showing apple-green birefringence under polarized light (400X).

To conclude in the cases with high clinical suspicion one should order target organ or surrogate site biopsy and send for routine histopathological examination and special stain with Congo red. Documenting Congo red positive deposits with apple-green birefringence on polarization establishes the diagnosis of amyloidosis. It is also one of the diagnostic criteria for the most common systemic amyloidosis, i.e. primary AL amyloidosis by the International Myeloma Working group [27].

5. Subtyping of amyloid

5.1 Need of amyloid typing

While detection of amyloid is undoubtedly essential, equally important is to identify its subtype. Rather than being a separate disorder, amyloid deposition is often a manifestation of an underlying disease process. Amyloidosis causes symptoms based on organ involvement. However, some of the symptoms might be attributable to the underlying disease process independent of the amyloid deposition. Moreover, treatment of the underlying disorder is the only way to stop the progression of amyloid deposition. The therapy varies widely from liver transplant to bone marrow transplant depending on the type of amyloidosis. Sub-typing of the amyloid gives vital clues about the underlying disorder, highlighting the importance of identifying its subtype.

While apple-green birefringence in a Congo red section under the polarized microscope is sufficient for the detection of amyloid, its sub-typing is an uphill task.

Following issues need to be addressed after tissue diagnosis of amyloidosis is established.

- a. Sub-typing of the amyloid.
- b. Evaluate for the organs affected by amyloid deposits.
- c. Evaluate for amyloid-independent symptoms attributable to the underlying cause.
- d. Diagnose the underlying cause and formulate the best possible treatment option based on it.

Numerous techniques have evolved ranging from simple, widely available immunofluorescence to complicated scarcely available mass spectrometry to identify the subtype of amyloid. Each of these techniques has its own merits and will be discussed subsequently.

5.2 Tools of amyloid typing-immunofluorescence

Immunofluorescence (IF) is based on antigen–antibody reaction. The difference is that the antibody is tagged to a fluorescent substance necessitating a fluorescent microscope to visualize the reaction. The staining is done typically on frozen sections of unfixed tissue. Immunofluorescence can also be performed on formalin-fixed tissue (when fresh unfixed tissue is unavailable), albeit at a slightly lower sensitivity [28]. Since the fluorescence fades with time and exposure to light, it is necessary to view the slides immediately on staining and digitally archive them before it fades. Moreover, the repertoire of antibodies available for sub-typing by IF is limited.

Due to these numerous limitations, IF is not used for amyloid sub-typing. It is used to assess kidney and skin biopsies in which kappa or lambda staining may reveal restriction.

5.3 Immunohistochemistry

Immunohistochemistry (IHC) is a method that helps to detect and localize specific antigens in a tissue based on antigen–antibody reaction. In the current era, most of the IHC is being performed on formalin-fixed material for convenience and durability. The specific antibody against the antigen of interest is added to the tissue. After washing the unbound antibody, an enzyme (mostly horseradish peroxidase) tagged secondary antibody is added. This enzyme converts the chromogenic substance giving a colored reaction. When viewed under a microscope, the presence of the colored reaction product indicates the presence of the desired protein. Its location in relation to cell viz. extracellular, intra-nuclear, intra-cytoplasmic can also be determined [29].

The antibodies available to detect amyloid have increased steadily. Anti-amyloid P component antibody is used to detect the glycoprotein P that is associated with the amyloid deposits. It is independent of the type of amyloid and the anti-AP antibody is positive in virtually all the cases of amyloidosis [30]. Anti-kappa light chain, anti-lambda light chain, and anti-AA are the commonly used antibodies for amyloid sub-typing. Other antibodies include those directed against calcitonin, fibrinogen, lysozyme, transthyretin, β -2 microglobulin, and apolipoprotein A1.

The most significant advantage of IHC is the ease and convenience it offers in the diagnostic process. IHC for sub-typing is performed on the same tissue used for the detection of amyloid. No separate tissue processing needs to be done. Moreover, IHC is a widely available and routinely performed test. The slides can be easily photographed for digital archiving. The slides themselves can be preserved for a few years for review, if necessary. The diagnostic yield of immunohistochemistry is highly variable. While some centers have reported sensitivity and specificity

nearing 90% [30, 31], others have reported being as low as 75% [32]. With correct standardization of the staining procedure, IHC is a very powerful and convenient tool for amyloid sub-typing.

However, IHC staining does come with some potential pitfalls that one needs to be aware of. In a large study including 169 biopsies, Anja et al. found that 13 biopsies could not be stained either due to insufficient tissue or failure of all the antibodies to stain the tissues [31]. The antibodies are typically manufactured using native protein as the antigen. Hence, in some cases with significant alteration in the protein structure during amyloidogenesis, the antibody may fail to stain the amyloid deposit leading to lower sensitivity [33]. The other important pitfall encountered by Anja et al. was non-specific staining in 51 cases. In these, the tissue showed positive staining with more than one antibody from among the panel of antibodies. In such cases, the antibody that stains strongly and homogeneously was considered positive while others were negative. However, in almost 33 cases the staining was inconclusive and clinical and other patient details had to be considered for final sub-classification. Intense staining with more than one antibody or inhomogeneous staining with 2 or more antibodies (in absence of strong staining by anyone antibody) are some of the pitfalls of immunohistochemistry. This inconclusive false-positive staining is either due to non-specific binding to non-amyloid protein or the presence of normal proteins within the amyloid deposits containing the epitope targeted by the antibody (e.g. plasma proteins entrapped in amyloid deposits may give false-positive reaction with anti-kappa and anti-lambda antibody). After thorough standardization of the procedure, a dedicated center may be able to reduce (but not eliminate) such inconsistencies.

In summary, due to easy availability, IHC is an excellent tool for sub-typing of the amyloid. However, both false positive and false negative staining reactions make it necessary to employ other diagnostic tools. A dedicated center with rigorous validation protocols for IHC may be able to achieve high sensitivity and specificity.

5.4 Immunoelectronmicroscope (IEC)

In principle, IEC is similar to IHC relying on the binding of a specific antibody to the amyloid. However, IEC does offer certain advantages over IHC that make it a very sensitive and specific modality for confirmation of the presence of amyloid and its sub-typing.

Transmission electron microscopy relies on a beam of electrons instead of light to visualize the tissue. The high-energy electrons make it possible to visualize the tissue at a much higher magnification without losing the resolution. Consequently, the processing of the tissue for electron microscopy differs vastly from processing for routine light microscopy. Fixative for electron microscopy is either glutaraldehyde or Karnovsky fixative (glutaraldehyde+formaldehyde). Though fresh tissue is preferred, formalin-fixed tissue can be re-processed for electron microscopy. The selected areas from semi-thin sections are further subjected to immunoelectron microscopy. The antigen-antibody reaction is visualized by using protein A/G gold conjugates. These gold conjugates appear as sharp electron-dense deposits [34].

The biggest advantage of IEC is the direct visualization of the amyloid fibrils. Thus, any non-specific binding of the gold-labeled antibody to non-amyloid proteins can be detected improving the specificity to more than 95%. Electron microscopy not only provides confirmatory detection of amyloid but immunostaining can also help in its sub-typing. In a large study involving 423 cases of systemic amyloidosis, the sensitivity of IEM was comparable to light microscopy (75–80%). The specificity in detection and sub-typing was however virtually 100% [35].

The limited availability of electron microscopes precludes its routine use. Also, the expertise needed for tissue processing and interpretation makes it out of most

laboratories' reach. However, the use of IEM at a referral laboratory for the diagnosis of cases indeterminate on IHC is possible and may overcome the economic and technical constraints.

5.5 Mass spectrometry

Mass spectrometry is a powerful, modern tool for proteomics study. Proteomics, similar to genomics, is the study of all the proteins produced by an organism, an organ system, or an individual cell. Mass spectrometry uses a sophisticated multi-step process to isolate and identify the proteins by determining the molecular mass. Modern developments in mass spectrometry are towards devising ways to accurately quantify the various isolated proteins in the mixture [36].

The initial step is to isolate and concentrate the tissue of interest. In the context of amyloidosis, it involves careful separation of the congophilic amyloid deposits from the rest of the tissue. This is usually achieved through laser micro-dissection on a thick (6–8 micron) section of formalin-fixed tissue. The micro-dissected predominantly contains amyloid proteins along with other proteins. These proteins are extracted and then subjected to trypsin digestion to break them into fragments. The proteins need to be broken down to fragment peptides because intact proteins are too large to be analyzed efficiently by mass spectrometry. Once generated, these fragments are then separated from one another using high-performance liquid chromatography. These uncharged peptides cannot be subjected to mass spectrometry. The next step, probably the most significant, is imparting a charge to the peptides without dissociating or fragmenting them. Commonly done by electrospray ionization (ESI), the peptide can also be charged using matrix-assisted laser desorption/ionization (MALDI). These ionized peptides are then subjected to “flight” in an electromagnetic field. The movement of these peptides is dependent on their mass to charge ratio, the software analyses the data to determine the molecular mass. An expert interpretation is needed to make sense of the data and the software analysis to reach a definite conclusion about the various constituents of the protein mixture [37].

The detection and diagnosis of amyloidosis by MS relies on the detection of “amyloid signature” and detection of specific subtypes. Amyloid signature detects non-amyloid proteins that are universally associated with amyloid proteins irrespective of the amyloid subtype. Detection of these proteins gives proof of the presence of amyloid in the sample. These proteins are Serum Amyloid P component (SAP), apolipoprotein A4 (APOA4), and apolipoprotein E (APOE). Indeed, in a study by Vrana et al., 13 out of 20 Congo red negative subcutaneous fat aspirates showed the amyloid signature implying its higher sensitivity than conventional Congo red staining to detect amyloidosis [38]. Detection of a specific subtype depends on the detection of a specific protein. The sensitivity and specificity of MS for amyloid subtyping are about 90% and nearly 100% respectively [39]. In some cases, the amyloid signature protein may be detected but sub-typing may not be possible by MS.

Apart from high sensitivity and specificity, the most significant utility is the identification of unknown and novel proteins. Unlike antibody-based methods (like IHC or IEM), MS characterizes all the protein in the micro-dissected material and hence rare cases of novel amyloid subtypes can also be diagnosed by MS. However, similar to IEM, high cost, necessary technical expertise makes MS out of reach for most laboratories.

6. Diagnosis of underlying disorder

Management of amyloidosis targets the underlying disorder. No definitive treatment can reverse the amyloid protein that is already deposited, and thus treatment

aims to control the underlying disorder, preventing further amyloid deposition. Sub-typing of the amyloid is crucial for the diagnosis of the underlying disorder. Every effort to subtype the amyloid should be made. In case IHC is indeterminate, the sample is sent for immunoelectron microscopy or preferably mass spectroscopy to a referral lab. Once the amyloid is sub-typed, relevant testing can be performed to confirm the presence of the underlying disorder.

6.1 AL amyloid

AL amyloid is derived from the immunoglobulin light chain, either kappa or lambda. It is practically secondary to underlying plasma cell dyscrasia, rarely due to some other type of lymphoma. Plasma cell dyscrasias have been classified into different sub-types with specific diagnostic criteria [40]. Being a disorder that affects multiple systems, a battery of tests is needed for a detailed investigation of plasma cell dyscrasia. Amyloid deposition can be secondary to multiple myeloma or other monoclonal gammopathies like MGUS or asymptomatic myeloma. However, occasionally, it may occur in the absence of any detectable plasma cell dyscrasia in which case it is termed as Primary Amyloidosis. It must, however, be noted that even primary amyloidosis is due to an underlying clonal plasma cell proliferation that is so small that routine tests cannot detect it. In such cases, other investigations like urine electrophoresis and serum-free light chain assay may be helpful.

6.2 AA amyloid

AA amyloidosis is generally due to an underlying inflammatory condition such as chronic autoimmune diseases, including rheumatoid arthritis and inflammatory bowel disease, and chronic infections, like tuberculosis. Occasionally, familial Mediterranean fever may be the cause of AA amyloidosis. The cause of AA amyloid deposition is often dependent on the geography-inflammation associated with AA commoner in developing countries and familial Mediterranean fever is a commoner cause in people of the Mediterranean region especially Turks, non-Ashkenazi Jews, Arabs, and Armenians. Thus, in a case with AA amyloid deposits, the ethnicity of the patient along with a careful clinical examination of the patient will give a clue to the possible diagnosis. Further investigations in acquired amyloidosis will be directed towards identifying the underlying cause. In almost one-fifth of cases, the underlying cause may remain undiagnosed despite extensive investigations [41].

6.3 Hereditary amyloidosis

Diagnosis of hereditary disorders leading to amyloidosis hinges on the integration of information obtained by amyloid sub-typing, the clinical details of the patient, and the genetic test to detect the specific gene mutation.

6.4 Cases when amyloid sub-typing not possible

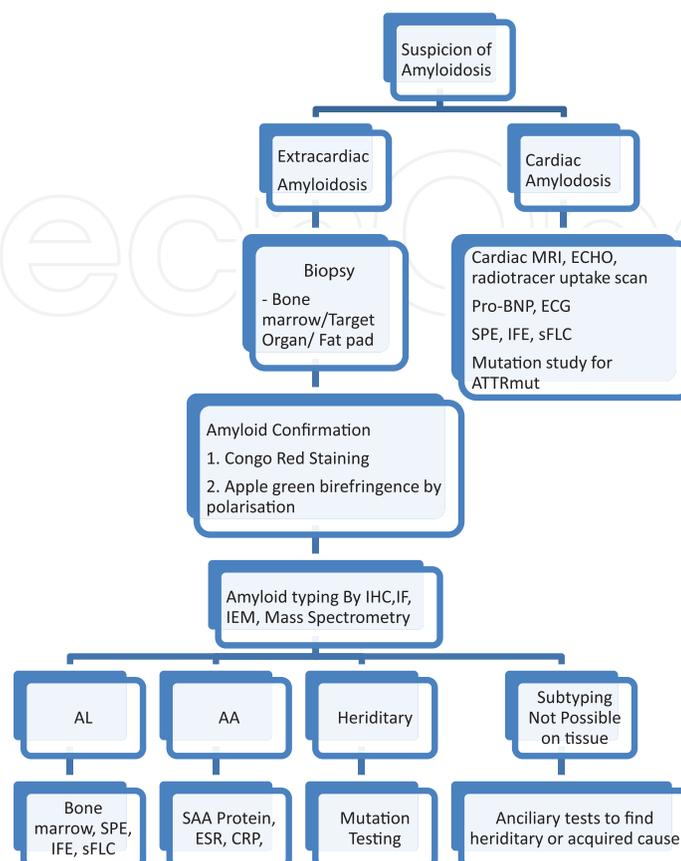
In occasional cases, despite best efforts, it may not be possible to subtype the amyloid on tissue biopsy. In such cases, identification of the underlying disease would be based on the clinical details and ancillary investigations. However, it must be noted that this approach may lead to misdiagnosis as more than one condition may co-exist. Most commonly, monoclonal gammopathy may co-exist with other causes of amyloidosis, especially the hereditary forms [42]. Some of the difficulties encountered in cases where amyloid typing is not possible on biopsy include:

1. Hereditary forms often present late in life and have variable penetrance making family history unreliable.
2. Monoclonal gammopathy is a common incidental finding in many elderly individuals. Its presence does not imply that the amyloid is AL type.
3. The pattern of organ involvement, though helpful, is often overlapping in many different types of amyloid.

Thus it is imperative that every effort must be made to subtype the amyloid on the biopsy material.

1. **Summary:** Amyloidosis is a disorder of deposition of misfolded protein in extracellular spaces. Heart, kidney, liver, blood vessels in various organs are principally affected organs. Chemically amyloid has vast heterogeneity, the common feature being misfolding of the abnormal protein to form beta-pleated sheets. The chemical heterogeneity stems from equally varying causes ranging from hereditary to acquired and malignant disorders.

The clinical manifestations usually pertain to the organ involved. Except for ATTR cardiac amyloidosis, tissue biopsy is crucial for confirmation and chemical sub-typing of amyloid. Characteristic apple-green birefringence under polarized light is a Congo red stained tissue is still the most widely used technique for amyloid detection. IHC, immune-electron microscopy, and mass spectrometry are increasingly complex but efficient tools for chemical sub-typing of the amyloid. Apart from that, a battery of investigations is required for confirmation of underlying disorder and to assess the organs involved by amyloid. Diagnosis of amyloidosis starts from suspicion in the clinic. Following algorithm summarizes the diagnostic approach for a suspected case of amyloidosis.



7. Conclusions

Science has evolved, and there has been significant improvement in our understanding of this complex disease called amyloidosis. This has led to better diagnostic tools, therapeutic options, and eventually better care for patients suffering from this group of illnesses. However, we still strive to develop techniques for early detection, preventive strategies, and curative options for this mysterious disease.

Conflict of interest

Nil.

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