






## Tissue biopsy for the diagnosis of amyloidosis: experience from some centres

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### ABSTRACT

A reliable diagnosis of amyloidosis is usually based on a tissue biopsy. With increasing options for specific treatments of the different amyloid diseases, an exact and valid diagnosis including determination of the biochemical fibril nature is imperative. Biopsy sites as well as amyloid typing principles vary and this paper describes methods employed at some laboratories specialised in amyloidosis in Europe, Japan and USA.

**Abbreviations:** AEFEMP1: human fibulin-like extracellular matrix protein-1 amyloid protein; apoAIV: apolipoprotein A-IV; apoE: apolipoprotein E; ATTR: amyloid transthyretin; ATTRwt: wild-type amyloid transthyretin; DAB: 3,3'-diaminobenzidine; ELISA: enzyme-linked immunosorbent assay; AFib: fibrinogen A  $\alpha$  chain amyloid; FSB: (*E,E*)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy) styrylbenzene; IF: immunofluorescence; IHC: immunohistochemistry or immunohistochemical; LC-MS/MS: liquid chromatography with tandem mass spectrometry; LCOs: luminescent conjugated oligothiophenes; MALT: mucosa-associated lymphoid tissue; TTR: transthyretin; TTRwt: wild-type transthyretin

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## Introduction

Diagnosis of amyloidosis is usually based on demonstration of amyloid deposits in a tissue biopsy. A definitive diagnosis has become increasingly important since a number of impactful treatment options have developed. The clinical and biologic diversity of amyloidosis conditions emphasises the need for refined and exact analysis of the deposited material. Evolution of such analyses has occurred independently at the relatively few different specialised centres around the world. In this way, a number of distinct but partially complementary methods have emerged. The purpose of this clinical practice summary of experts is to describe some important steps in biopsy procedures and diagnostic tools in the diagnostic work-up of amyloid and amyloidosis and provide examples of alternative methods. In addition, we wish to point out some challenges and pitfalls. The specific signs, symptoms and conditions warranting suspicion

of amyloidosis that underlie the decision to obtain a biopsy fall beyond the scope of this article.

This paper is an inventory of methods used at laboratories specialised in the diagnosis of amyloidosis. Thus, it should not be regarded as a consensus article.

## Indication for a tissue biopsy

Local and systemic amyloidoses have a variety of symptoms reflecting the amyloidosis type, anatomical distribution and extent of the disease, i.e. the disease stage. It is noteworthy that amyloid and amyloidosis is not a single disease but a group of disorders with substantial variability in manifestation, anatomic distribution, progression and prognosis. Likewise, clinical presentation is highly variable, often vague, and difficult to interpret. Given the relative rarity of these diseases, amyloidosis is often not considered during clinical work-up and unfortunately often diagnosed at advanced stages of disease. Early diagnosis and treatment intervention

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are of paramount importance. It is our conviction that as soon as there is suspicion of amyloidosis, a tissue biopsy is indicated. Therefore, we strongly recommend a liberal attitude towards obtaining biopsies in suspected amyloidosis. The patient with cardiomyopathy who has grade 2 or 3 myocardial radiotracer uptake on bone scintigraphy is an exception, provided a monoclonal gammopathy is absent. It is important that the search for monoclonal protein should be done by serum and urine immunofixation in addition to serum free light chain quantification [1].

## Biopsy sites and methods

### Biopsy sites

Systemic amyloidosis is characterised by deposits in many different tissues even among subjects who initially have clinically overt symptoms in only one organ/tissue site, e.g. kidney or carpal tunnel ligament. Although clinical presentation and dynamics of disease progression vary with the different types of amyloidosis, there is significant overlap in organ involvement; for example, both ATTR and AL amyloid may deposit in the heart, nerves and carpal tunnels. The widespread deposition of amyloid provides the opportunity to biopsy tissue sites which are easily accessed and with little risk of biopsy-related complications which may occur when sampling the symptomatic organ. When screening for systemic amyloidosis the following sites are most commonly used in lieu of targeting affected organ:

- Subcutis (adipose tissue over abdomen; depending on method, epidermis and dermis may be included)
- Gastrointestinal tract (usually rectum, but stomach and duodenum are also used)
- Lip (accessory salivary glands)
- Bone marrow

Simply including Congo red staining in the evaluation of major organs biopsies performed for indications other than amyloidosis would increase the frequency of amyloid detection. A biopsy can also be directed towards a symptomatic organ (e.g. heart, liver, kidney) but in those cases usually as part of a thorough histopathological examination of a wider differential diagnosis and is often avoided due to potential risks. Our own expertise provides ample evidence that standard Congo red staining practices often lead to a diagnosis of amyloid/amyloidosis when it was not suspected clinically. Particularly, kidneys are subject to some deposits in most types of systemic amyloidosis, although not necessarily with clear clinical symptoms.

### Biopsy methods

It should be emphasised that clinical information must accompany a biopsy. The biopsy method depends on the chosen tissue site.

### Subcutis

For this site, there are several options

1. Fine needle aspiration biopsy. This is the original method by which a thin needle (22 gauge) is attached to a handle. No local anaesthesia is necessary. Obtained material is directly spread on microscopic slides, air-dried and stained for amyloid.
  - Advantages: Quick, usually painless and no special arrangement necessary.
  - Drawbacks: Training necessary, not useful for some amyloid types (ATTR, type A) since little connective tissue is included.
2. Needle biopsy with wider diameter (e.g., 16 gauge) attached to a syringe to create negative pressure. By this method more material is obtained that can be used for different purposes. Microscopic slides can be prepared by pressure methods (see below).
  - Advantages: More material is obtained, also containing some connective tissue and is therefore better for ATTR amyloid.
  - Drawbacks: More arrangements necessary, including local anesthesia. Greater risk for hemorrhage. To detect tiny ATTRwt amyloid deposits in adipose tissue, more subcutaneous tissue is often needed by means of surgical skin biopsy or punch biopsy in patients with suspected ATTRwt cardiomyopathy.
3. Punch biopsy. A wide punch (6–8 mm) should be used to obtain sufficient amount of adipose tissue. With some training, good amount of tissue can be obtained.
  - Advantages: All skin layers are obtained and material for pressure slides, for embedding and for other methods such as western blot or proteomics is gained.
  - Drawbacks: More arrangements necessary, including local anesthesia and one or two sutures to close the skin.
4. Surgical biopsy. Usually performed by a surgeon.
  - Advantages: More material is obtained.
  - Drawbacks: More arrangements necessary, including local anesthesia and sutures to close the skin.

### Salivary gland

Accessory salivary glands embedded in lamina propria of the inside of the cheek, are often involved in systemic amyloidosis. Amyloid is deposited in association with basement membranes surrounding each gland. An incisional biopsy from the mucosal side is commonly used, particularly for the diagnosis of ATTR amyloidosis. These biopsies are fixed in standard formaldehyde solution.

### Other sites for biopsy

Biopsies from the gastrointestinal tract or heart are usually put directly into buffered 4% formaldehyde solution for paraffin embedding unless some specific studies are planned. In case immune-electron microscopy is planned, samples are

best fixed in glutaraldehyde-containing solutions, such as Karnovsky's. For renal biopsies, sections from formalin-fixed, paraffin-embedded material, as well as frozen sections (for immunofluorescence) are used.

### Treatment of and shipping biopsies

When biopsies are performed at an amyloid centre where analyses will be performed, local routines will determine handling of the material. Since biopsies are most often performed outside of amyloid centres, biopsies are sent to external laboratories specialised in amyloidosis diagnosis. If, however, there are no local recommendations, biopsy material should be formalin-fixed. Some laboratories prefer to obtain material, particularly subcutaneous fat tissue, fresh in physiological sodium chloride solution. Since biopsies are obtained at sterile conditions, adipose tissue sent in this solution does not deteriorate even when kept at room temperature for a few days. Some laboratories have a fixed maximal time of 7 days. However, if an unfixed fat sample is to be analysed by proteomics, it must be stored frozen and shipped on dry ice. For mass spectrometry some centres collect samples in tubes containing RPMI 1640 with phenol red, heparin and 0.03% sodium azide. When biopsies are sent between laboratories, this is most commonly done with formalin-fixed and paraffin-embedded material.

### Making microscopic slides for amyloid diagnosis

#### Pressure preparations

For fresh fat tissue, pressure preparations are the easiest and quickest way to obtain microscopic slides for amyloid diagnosis. Fat tissue is washed with distilled water, small (a few mm<sup>3</sup>) pieces are taken from the biopsy and put on a microscopic slide (preferably positively charged) and cut with scissors into small fragments. Excess water is blotted off with filter paper and thereafter a second, clean slide is put on top and pressed firmly while fat and liquid material is removed from the edges. When no more liquid material is appearing, the slides are separated and both air-dried on a hot plate for 5 min. After cooling, the slides are defatted in acetone  $\times 2$  and can be stained in Congo red or any other stain for amyloid.

#### Sectioning

This can be performed directly as frozen sections or after embedding in paraffin. For routine, the latter is most common. Thicker sections, 4–10  $\mu\text{m}$ , are helpful in the detection of amyloid deposits by Congo red stain but are not obligatory. Small deposits are more likely to be seen in thicker sections, while they may be missed in thinner sections ('sampling error') and birefringence is also easier to recognise. Thus, in order to minimise sampling error, it is recommended that two (or more) sections be stained with Congo red, preferably cut from different levels within the block.

For laser microdissection followed by liquid chromatography with tandem mass spectrometry (LC-MS/MS), formalin-fixed and paraffin-embedded biopsies are used. Sections are placed on dedicated coated slides.

### Staining

#### Congo red

Congo red has been used for amyloid diagnostics for almost 100 years and is still the universal staining in surgical pathology. Combined with polarisation microscopy this is an excellent method to detect even minor amyloid deposits but only under strict conditions and by experienced examiners. Congo red is as such by no means specific for amyloid but can bind to many tissue components if used improperly. There are a number of recipes for Congo red solutions and staining, the most used is the one by Puchtler et al. [2], but with modifications. A simple modification for increased specificity is dilution of the dye solution 1:10 followed by prolonged staining. Commercial reagents are also available but optimisation is required. Whatever the method, to be specific it should not stain other components such as collagen, elastin or eosinophilic granules. If this happens, the section is over-stained and there is a risk of incorrect diagnosis of amyloidosis. Furthermore, Congo red should be used in combination with polarisation microscopy and there are also requirements for this equipment. Particularly, a strong light source, strain free optics and complete darkness obtained by crossed polars are needed. Using polarisation microscopy, at any given time, only a portion of the amyloid deposit typically shows diagnostic (orange–green–yellow) birefringence and only by further rotation of the stage will other parts of the deposit become visible while, in turn, the formerly visible areas will be obscured by the 'polarization shadow.' Birefringence may be difficult to observe in some types of amyloid, e.g. ATTRwt and human fibulin-like extracellular matrix protein-1 amyloid protein (AEFEMP1).

Pre-treatment of the specimen with potassium permanganate should not be used for amyloid typing.

Congo red can also be used with fluorescence microscopy, and this property is used by some laboratories for screening, or to select areas to be microdissected for mass spectrometry. At Congo red-fluorescence (and in thioflavin stains), the entire area is illuminated but there is a loss in specificity.

#### Other stains

Although Congo red is used in most situations for the recognition of amyloid at microscopy, as mentioned it is not a stain without problems. The affinity for the dye varies strongly between, and sometimes within the different forms of amyloid. When the affinity is low, the resulting birefringence is also weak and can be difficult to discern. Therefore, some laboratories use additional stains, particularly for screening purposes. The old metachromatic stains, such as

cresyl violet, are rarely used by specialised laboratories. Alternatives are the Congo-red derived molecule (*E,E*)-1-fluoro-2,5-bis(3-hydroxycarbonyl-4-hydroxy) styrylbenzene (FSB), giving amyloid a strong fluorescence, or alcian blue, binding to the always present glycosaminoglycans. These two stains are used by some specialised laboratories. Finally, there are additional fluorescent ligands such as luminescent conjugated oligothiophenes (LCOs) with very promising properties but these are mainly yet used for research.

Thioflavin T (or S) stain is used extensively in the research setting but is relatively uncommon in clinical practice. Similar to Congo red-fluorescence, thioflavin stains are more sensitive for amyloid detection than Congo red polarisation. Again, this is in part due to visualisation of the entire area containing amyloid, at the same time, without the ‘polarization shadow.’

## Reporting

### Negative findings

When reporting the results of a biopsy submitted for amyloid diagnosis it is of great importance that the receiver understands that a negative finding (i.e. no amyloid in the biopsy) does not necessarily mean that the patient does not suffer from amyloidosis, unless the specimen originates from a specific target organ such as the myocardium. However, even myocardial biopsies carry the risk of a sampling error and a sufficient number of biopsies is mandatory to reduce the risk of a false negative result. Biopsies from subcutis or from gastrointestinal tract may be negative in some patients with systemic amyloidosis, partially depending on the amyloid type. With regard to colorectal biopsies amyloid often is present only in submucosal layers and superficial biopsies (only mucosa) carry a substantial risk of a sampling error, particularly in ATTR amyloidosis, reaching almost 60%. It is therefore wise to point out this possibility in the report.

### Positive findings

When amyloid is present in a biopsy, distribution and amount can be of importance to know although there is no strict correspondence of amyloid amount between different organs. For example, unusual large masses of amyloid in subcutis may point towards insulin type amyloid. In addition, some forms of AL amyloidosis may present as local tumour masses, some of which are even associated with plasmacytic B cell neoplasia, including extranodalmucosa-associated lymphoid tissue(MALT) lymphomas. It should also be underlined in the report that the nature of the amyloid and putative associated underlying diseases have to be determined.

### Determining the amyloid type

Several complementary methods can determine the biochemical nature of amyloid fibrils.

## Immunohistochemistry (IHC), including immunofluorescence (IF)

It must be stressed that antibody-based amyloid-type determination differs markedly from immunohistochemistry in other areas of general surgical pathology: experience, rigorous typing criteria and adequate quality assurance are mandatory.

Antibody-based methods can be applied to frozen or paraffin sections and analysed by light or fluorescence microscopy. Immunofluorescence on frozen tissue is the gold-standard, IHC technique for the evaluation of immune deposits in the kidney and amyloid typing using this method is a common first line approach in renal pathology. For evaluation of other types of biopsy, IHC is usually used. The most commonly utilised way to determine amyloid type and demonstrate binding of antibodies is visualisation by a dye-reaction, usually with 3,3'-diaminobenzidine (DAB). There are different antibodies commercially available against the most common amyloid fibril proteins including immunoglobulin light chains (kappa and lambda), transthyretin, protein AA and some others. Unfortunately, there are often problems with these commercial antibodies, which have not specifically been developed for amyloid diagnostics. Thus, such antibodies may have been raised against native, full-length protein in a normal, folded state while the fibrillary protein, in addition to having an abnormal configuration, very often consists of a fragment of the parent molecule. Therefore, many laboratories, specialised in amyloid diagnostics, have developed their own, specific monoclonal and polyclonal antibodies and perform controlled utility of them for determination of amyloid fibril type. Such antibodies can be raised against synthetic peptides corresponding to domains of a protein included in the deposited fibrils or against the purified amyloid protein itself. Even then, interpretation of results of staining requires extensive experience, which can only be obtained by reviewing a large number of specimens for diagnostics. This means in practice that type-determination of amyloid has to be concentrated in a few specialist laboratories and not performed in all pathology laboratories. Formic acid pre-treatment may enhance immunostaining of amyloid deposits.

### Pro and cons for use of immunohistochemistry in determination of amyloid fibril protein

#### Pro

- When good antibodies are available it is a comparably cheap method
- No expensive equipment necessary
- Immunoreactivity of amyloid and tissue components can be evaluated directly
- Best method in case of double amyloid (not extremely rare!)

#### Cons

- Many commercial antibodies are not reliable in amyloid diagnostics

- Some good antibodies are not commercially available (difficulties to use in-house antibodies in accredited laboratory)
- Great experience necessary
- Only types of amyloid that are looked for will be found

### Mass spectrometry

Mass spectrometry (MS) has emerged as a method of choice for amyloid type determination, particularly after laser dissection of FFPE samples, pioneered by researchers at the Mayo Clinic. After dissection, material is dissolved and usually proteolytically cleaved by trypsin and obtained peptides identified. In addition, also non-fixed fat aspirates can be analysed, without microdissection. It is an extremely sensitive method which identifies not only major fibril proteins but also a wealth of tissue components. A number of such components have been identified as typically associated with amyloid and are now known as 'amyloid signature component'. These include apolipoprotein A-IV (apoAIV), apolipoprotein E (apoE) and serum amyloid P-component (SAP).

### Pro and cons for use of MS in determination of amyloid fibril protein

#### Pro

- Sensitive – always many proteins detected
- Exact protein information
- Associated protein can be detected
- With an extended database also variants or mutations can be identified
- Possibility to detect also rare or novel forms

#### Cons

- Expensive equipment demanding expertise handling
- Great experience in evaluation of results necessary
- Some proteins less easy to identify, especially highly variant ones
- Sometimes difficult to know which among proteins are from amyloid
- Has to be centralised (not necessarily a disadvantage).

### Additional methods

#### Immune electron microscopy

Some laboratories have successfully developed immune electron microscopy for diagnostic purposes. A big advantage is that fine needle subcutaneous fat aspirate can be used, thus with minimal problems for patients. As for immunohistochemistry, specific antibodies are necessary.

### Western blot analysis

Western blot analyses of extracts from subcutaneous fat tissue is used at some centres. Non-fixed tissue is preferred. Presently, this is the only method to distinguish between type A and type B ATTR amyloid fibrils.

### Enzyme-linked immunosorbent analysis

ELISA methods are used in one centre to quantify amyloid proteins in guanidine extracts from non-fixed subcutaneous fat tissue.

One method does not necessarily exclude another. Currently, in many laboratories amyloid typing by mass spectrometry is recommended for the typing of amyloid deposits when routine immunofluorescence or immunohistochemistry/immune electron microscopy is negative or equivocal, for the detection of unusual types, and for the confirmation of an unusual or unexpected amyloid type.

### Conclusions

This paper addresses biopsy methods for diagnosis of amyloidosis. Results have always to be evaluated together with other findings, e.g. including presence of monoclonal plasma cells, findings at MR, DNA analysis, and many others. ATTR amyloidosis with cardiomyopathy is a specific example where bone scintigraphy can be successfully used in diagnostic work.

The contemporary approach to amyloid protein typing represents a consideration of available technologies, experience, practicality and cost effectiveness, rigorous typing criteria and adequate testing quality assurance. Misdiagnosis of the amyloid protein type must be avoided. This has led to a high degree of specialisation of the tissue-based diagnostic work which tends to be increasingly concentrated to few centres.

In addition, molecular biological techniques can be performed on tissue samples, when it comes to classify amyloid as a hereditary variant (e.g. hereditary ATTR or fibrinogen A  $\alpha$  chain (AFib) amyloidosis). This, however, necessitates written informed consent and genetic counselling of the patient prior to tissue based diagnostics.

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