Biochemical markers in early diagnosis and management of systemic amyloidoses

Abstract: Systemic amyloid diseases are characterized by widespread protein deposition as amyloid fibrils. Precise diagnostic framing is the prerequisite for a correct management of patients. This complex process is achieved through a series of steps, which include detection of the tissue amyloid deposits, identification of the amyloid type, demonstration of the amyloidogenic precursor, and evaluation of organ dysfunction/damage. Laboratory medicine plays a central role in the diagnosis and management of systemic amyloidoses, through the quantification of the amyloidogenic precursor and evaluation of end-organ damage using biomarkers.

Keywords: amyloidosis; biomarkers; early diagnosis; monoclonal gammopathies.

DOI 10.1515/cclm-2014-0235
Received March 3, 2014; accepted April 22, 2014; previously published online May 28, 2014

Introduction

The amyloidoses are a group of diseases defined by tissue deposition of proteins as insoluble amyloid fibrils [1]. These are long, unbranched assemblies of misfolded protein monomers, which lose their native conformation and adopt a prevalently β-pleated secondary structure. In the systemic forms, this process leads to multiorgan dysfunction and is associated with widespread fibril deposition. Amyloidoses are classified on the basis of which protein is the core constituent of fibrils; an increasing number of different proteins is known to cause amyloid diseases in humans, and a dozen of these are etiological agents of systemic forms [2, 3] (Table 1). Despite the highly similar biochemical and biophysical features of fibrils originated from different proteins, the various types of amyloidoses differ profoundly in terms of pathogenesis, clinical course, prognosis and treatment. For several of these forms, important therapeutic progresses have been made in recent years, and effective treatments can now change the clinical course and improve survival. However, patient management approaches differ substantially according to the amyloidosis type, and precise diagnostic framing is a crucial prerequisite for correct treatment.

Light chain (AL) amyloidosis is the most common acquired systemic form (incidence approximately 10 patients per million/year) [4, 5]. In this disease, amyloid deposition is widespread and virtually every organ, except the central nervous system, can be targeted. AL is a complication of 15% of multiple myeloma cases, but in the majority of patients it is caused by a small, usually indolent, plasma cell clone [6, 7], which synthesizes an unstable, misfolding-prone light chain. The clinical presentation of AL amyloidosis is protean; however, the presence and severity of heart involvement (affected in more than 70% of patients) are the major prognostic determinants [5, 7, 8].

Hereditary amyloidoses, which include forms caused by deposition of transthyretin (ATTR amyloidosis), apolipoproteins A-I and A-II (ApoAI and ApoAII), variant β2-microglobulin, lysozyme, fibrinogen, gelsolin and cystatin C, are due to the production of unstable genetic variants of these proteins. In these forms, the family history is suggestive in approximately 50% of the cases.
Transthyretin (TTR) amyloidosis, the most frequent of the hereditary amyloidoses worldwide, can also develop sporadically, for deposition of wild type TTR in relation to aging [senile systemic amyloidosis (SSA)]. Hereditary ATTR often targets the heart, and in SSA cardiac involvement is constantly present [9], leading to echocardiographic profiles that can be difficult to differentiate from AL cardiomyopathy. Reactive (AA) amyloidosis, mainly targeting the kidney, liver, spleen and autonomic nervous system, is caused by deposition of the acute phase protein serum amyloid A (SAA), which occurs in presence of persistently elevated levels of this molecule [10]. This amyloidosis is, thus, a complication of conditions that determine a chronic systemic inflammation. Hereditary AApoAI most frequently manifests with asymptomatic cholestatic hepatopathy, renal failure, and testicular involvement with hypogonadism [11]. Certain mutations can cause progressive cardiomyopathy, leading to heart failure [12]. Another, recently identified, form of amyloidosis, ALect2 (derived from leukocyte chemotactic factor 2), has emerged as the third most common form of renal amyloidosis, and is distinct from common types of kidney amyloid deposition, since it causes renal failure in the absence of significant proteinuria [13–15]. An increasing number of rarer hereditary and acquired forms can also occur [2,16], recently recognized thanks to the availability of novel techniques for molecular analysis of amyloid deposits.

In clinical practice, the diagnosis of amyloidosis is a multistep process that involves three stages: assessment of the presence of amyloid deposits in tissues, identification of the type of amyloidosis, and evaluation of organ involvement/dysfunction (Figure 1). All these steps have recently witnessed the introduction of novel tools, in addition to those traditionally employed in clinical chemistry and pathology, which are providing new resources to physicians for accurate and sensitive diagnosis.

A key point to be considered, in the diagnosis of amyloid diseases, is time. Since amyloid deposition continues in the absence of specific treatments, organ damage is progressive; timely and correct diagnosis and prompt treatment are of vital importance for improving the quality of life and its expectancy. This is especially true for AL amyloidosis, the most rapidly evolving of the systemic forms, which often targets the heart, with more pronounced signs of myocardial sufferance than observed in other types. In AL, in fact, the development of effective therapeutic regimens has markedly improved survival during the last decade [17,18], but the 25%–30% early death rate (within the first year) has not changed, with patients dying within a few weeks of cardiac failure due to late diagnosis [7].

In order to achieve a timely diagnosis, it is crucial to alert physicians regarding this disease and make a more widely use of sensitive, early biomarkers. In fact, most of the clinical manifestations are non-specific (with the exception of pathognomonic manifestations, such as submandibular swelling, macroglossia or periorbital purpura in AL) [7], and diagnosis may be missed or delayed if not suspected through early, sensitive biomarkers of end-organ damage.

### Table 1 Most common human systemic amyloidoses (adapted from Sipe et al., Amyloid 2012;19:167–70).

<table>
<thead>
<tr>
<th>Fibril protein</th>
<th>Precursor protein</th>
<th>Acquired (A) or hereditary (H)</th>
<th>Target organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Immunoglobulin light chain</td>
<td>A</td>
<td>All organs except CNS</td>
</tr>
<tr>
<td>AH</td>
<td>Immunoglobulin heavy chain</td>
<td>A</td>
<td>All organs except CNS</td>
</tr>
<tr>
<td>Aβ2M</td>
<td>β2-microglobulin, wild type</td>
<td>A</td>
<td>Musculoskeletal system</td>
</tr>
<tr>
<td>ATTR</td>
<td>Transthyretin, wild type</td>
<td>A</td>
<td>Heart mainly in males, tenosynovium</td>
</tr>
<tr>
<td>AA</td>
<td>(Apo) serum Amyloid A</td>
<td>A</td>
<td>PNS, ANS, heart, eye, leptomeninges</td>
</tr>
<tr>
<td>AAPoAI</td>
<td>Apolipoprotein A-I, variants</td>
<td>H</td>
<td>Heart, liver, kidney, PNS, testis, larynx (C-terminal variants), skin (C-terminal variants)</td>
</tr>
<tr>
<td>AAPoAI</td>
<td>Apolipoprotein A-II, variants</td>
<td>H</td>
<td>Kidney</td>
</tr>
<tr>
<td>AAPoAIV</td>
<td>Apolipoprotein A-IV, wild type</td>
<td>A</td>
<td>Kidney medulla and systemic</td>
</tr>
<tr>
<td>AGel</td>
<td>Gelsolin, variants</td>
<td>H</td>
<td>PNS, cornea, cutis laxa</td>
</tr>
<tr>
<td>ALys</td>
<td>Lysozyme, variants</td>
<td>H</td>
<td>GI tract, liver, kidney</td>
</tr>
<tr>
<td>ALect2</td>
<td>Leukocyte chemotactic factor 2</td>
<td>A</td>
<td>Kidney, primarily</td>
</tr>
<tr>
<td>AFib</td>
<td>Fibrinogen α, variants</td>
<td>H</td>
<td>Kidney, primarily</td>
</tr>
<tr>
<td>ACys</td>
<td>Cystatin C, variants</td>
<td>H</td>
<td>CNS</td>
</tr>
</tbody>
</table>

ANS, autonomic nervous system; CNS, central nervous system; GI, gastrointestinal; PNS, peripheral nervous system.
Use of biomarkers for early diagnosis

The availability of markers of organ dysfunction/damage, as well as of the modern assays to quantify the amyloidogenic monoclonal precursor, has had a profound impact on the management of AL amyloidosis. In particular, the correct use of markers of cardiac and renal damage is the most efficient tool to identify patients developing systemic amyloidosis at early stages [19, 20]. Clinically overt symptoms, in fact, reflect advanced damage, whereas identification of organ involvement in a pre-symptomatic stage should be the goal, especially in forms in which available effective treatments can change the natural history of these progressive diseases. This is particularly important in individuals that can be classified as ‘at risk’ of developing amyloidosis, such as patients with MGUS, particularly in those with abnormal κ/λ ratio (at risk for AL) [7, 20], or with chronic inflammatory conditions (at risk for AA), or carriers of amyloidogenic mutations. In relation to this point, correct use of biomarkers and education of physicians to recognize early red flags (i.e., unexplained high NT-proBNP or/and albuminuria, Figure 1) have become the keystone for identifying pre-symptomatic patients with initial organ damage.

The widely available biomarkers natriuretic peptide type-B (BNP), its N-terminal fragment (NT-proBNP) and troponins are routinely employed to evaluate, in particular, cardiac AL amyloidosis [5, 7, 19, 21–26]. In AL amyloidosis, NT-proBNP has 100% sensitivity in detecting cardiac involvement, anticipating echocardiographic abnormalities and preceding the onset of symptoms by several months [19, 27]. The sensitivity of NT-proBNP for detection of AL heart involvement at a very early stage has led to recommend checking this biomarker as a routine strategy to detect cardiac amyloidosis during follow-up of patients with MGUS and abnormal κ/λ ratio [7, 20]. Since renal function affects cardiac biomarkers, estimated glomerular filtration rate (eGFR) must be considered in interpreting their clinical meaning. Decreasing eGFR requires higher cut-offs of both NT-proBNP and BNP for detecting heart involvement and predicting survival [23], and BNP should be used instead of NT-proBNP in patients with eGFR <30.
Nevertheless, natriuretic peptides remain powerful and useful markers also in patients with renal failure [23]. Moreover, B-type natriuretic peptides should be used with great caution to evaluate amyloid heart dysfunction in patients with atrial fibrillation, since this condition is also associated with increased plasma concentration of these analytes [28–30]. High NT-proBNP is also observed in ATTR patients with cardiomyopathy [31–33], whereas troponin is elevated only in a minority of individuals in advanced stages [9, 31], and does not correlate with IVS-thickness or basal strain [31]. The role of biomarkers in early diagnosis of ATTR, however, has been scarcely explored; indeed use of NT-proBNP to predict cardiac abnormalities and monitor the onset of cardiac amyloidosis in carriers of amyloidogenic TTR mutations, or in ATTR patients with only neurologic symptoms, has recently been proposed [34]. An analysis of the Italian case series has shown the usefulness of NT-proBNP in follow-up of cardiac ATTR patients, with a powerful prognostic role [35]. It must be noted that the increase in NT-proBNP is usually lower in ATTR and SSA patients compared to AL ones, despite comparable or higher amyloid load (estimated by the cardiac walls thickness at echocardiography), suggesting that the cardiac toxicity exerted by immunoglobulin light chains is more pronounced that that caused by transthyretin [31, 36]. Indeed, heterogeneity in serum cardiac biomarkers concentration is observable among cardiac ATTR caused by different TTR variants [33, 37]. In particular, non-Val30Met variants as Val122Ile, and wild type TTR in SSA [33, 38], are associated with a more marked increase in B-type natriuretic peptides than in the most common variant Val30Met, suggesting more pronounced cardiomyocyte damage. In SSA, BNP increases in a time-dependent manner and correlates with diastolic dysfunction and cTnT; this evidence suggests that BNP could be important in the prognostic assessment of SSA patients [38].

Renal involvement in amyloidosis typically presents with signs of glomerular damage and is best evaluated by albuminuria and eGFR. Repeated measurements of microalbinurina and eGFR, in individuals at risk (see above), could help identify those who are developing renal amyloid deposits in early phases [7, 8, 10, 39].

This is of paramount importance, since recent evidence gathered on a large patient population of 461 patients shows that early diagnosis, when proteinuria is <5 g/24 h and eGFR >60 mL/min, allows intervention when renal damage is still reversible. Patients who obtain good quality response to treatment are protected from loss of renal function [40]. In AL amyloidosis, approximately 70% of patients present with proteinuria and two thirds are in the nephrotic range, whereas elevated creatinine is noted in one half of the patients [39, 41]. Serum cystatin C has also been evaluated as an early indicator of impaired GFR in patients with amyloidosis, in particular AA type [42, 43]. The available evidence suggests that cystatin C can identify a reduced GFR more accurately than serum creatinine-eGFR [42], and that it could be used as an early marker for detecting renal failure related to AA amyloidosis in specific categories at risk [43]. Renal insufficiency and tubular dysfunction due to amyloid deposits develop at later stages, with the exception of forms such as AApoAI and ALept2, in which tubulo-interstitial damage predominates from early phases [16, 44].

Liver can be involved in several amyloidoses, including AL [45–47], AA [10], AApoAI [11], and ALys [48, 49]. Increase in alkaline phosphatase and hepatomegaly document involvement of this organ, whereas transaminases are usually normal or slightly increased. Alkaline phosphatase values ≥1.5 times upper limit of the institutional normal value is a uniformly accepted criteria of hepatic involvement [45].

Moreover, the novel proteomic-based analyses of affected tissues have identified sets of tissue-resident (including extracellular matrix components, species involved in protein folding, and proteins implicated in energetic metabolism) and amyloid-associated proteins, whose representation changes in the presence of amyloid deposits [50–52]. If validated, these proteins, as single analytes or in combination, could represent novel, sensitive markers of amyloid deposition and indicators of amyloid-associated damage.

Detection of amyloid deposits in tissues

The diagnosis of amyloidosis is biopsy based. Although the clinical picture, biochemical parameters and imaging techniques can lead to suspect the presence of a systemic amyloid disease, it is only through demonstration of amyloid fibrils in tissues that the diagnosis can be established. Under optical microscopy, this task exploits the peculiar tinctorial properties of fibrils [1, 2]. All amyloids, independently from the protein by which they are originated, specifically bind to dyes as Congo red and thioflavins. The appearance of ‘apple-green’ birefringence, upon observation under polarized light of Congo red-stained specimens, is pathognomonic and is considered the diagnostic gold standard [53]. Electron microscopy allows the identification of characteristic 10 nm-wide non-branching fibrils [1].
In the presence of a positive biopsy at any site, organ involvement is defined by clinical, laboratory or imaging findings consistent with amyloid-related organ dysfunction. However, organ biopsy carries a significant risk of hemorrhagic complications. In systemic AL amyloidosis patients, the hemorrhagic risk may be increased by the frequent presence of coagulation abnormalities, such as acquired factor X deficiency [54], and infiltration of vessel walls by amyloid [55]. However, the outcome of a large series of renal biopsies in patients with systemic amyloidosis did not show increased bleeding risk [56]. When systemic amyloidosis is suspected, elective examination of subcutaneous abdominal fat is the recommended screening procedure [53, 57–60]. The diagnostic sensitivity of fat pad examination is high (70%–80% overall) [59] and acquisition of this tissue by fine needle biopsy is substantially innocuous, rapid and inexpensive. If clinical suspicion stands despite a negative fat pad biopsy, examination of minor labial salivary glands [61, 62] can be considered the preferred second step (being positive in >50% of patients with negative fat pad) [61]. This procedure has also high sensitivity for detecting amyloid deposits in subjects with molecular diagnosis of variant TTR [62].

A novel class of amyloid ligands, luminescent-conjugated thiophene polymers (LCP), has recently been described [63, 64]. These conformation-sensitive compounds interact with amyloid fibrils in tissue sections, and were shown to be highly sensitive for visualization of amyloid deposits. LCP were also claimed to be useful for detection of early species during the pathway to amyloid formation, possibly opening a way to early diagnosis [65]. Moreover, the fluorescent emission spectrum by amyloid-bound LCP is characteristic for each amyloidosis type, providing a ‘spectroscopic signature’ of the deposited protein. This peculiar property proved useful for classifying amyloid subtypes in patients’ biopsies [64], making LCP interesting as potential future tools for amyloid subtyping in clinical practice.

**Amyloidosis typing**

Amyloidosis typing consists in identifying which protein is the main constituent of the amyloid fibrils. As mentioned above, precise assessment of the amyloid type has fundamental clinical implications for patients and families, which include choice of treatment, assessment of prognosis, genetic counseling, and follow-up. Most importantly, the amyloidoses differ substantially in terms of treatment options, which range from chemotherapy in the case of AL, to transplantation of the organ (liver) producing the amyloidogenic protein in ATTR. Despite the different etiology, however, the clinical presentation is not diriment: amyloid organ dysfunction is non-specific, and the same organ can be affected by different types of amyloidosis, with similar manifestations, but different clinical implications. The field of tissue amyloid typing has recently witnessed novel developments, the most important of which is the use of proteomics in clinical setting [51, 52, 66–69]. Proteomics is now complementing the traditional histological techniques.

Antibody-based identification of the protein constituent of the fibrils has been the mainstay until recently. This can be performed under optical microscopy, by immunohistochemistry or immunofluorescence [3, 70], or through immunoelectron microscopy (IEM) [71]. IEM, coupled with immunogold labeling, allows visualizing the co-localization between the antibody signal and the amyloid fibrils, and is routinely used for amyloid diagnosis on various tissues, including abdominal fat obtained by fine needle aspiration [71].

Immunohistochemistry-based methods, however, present specific issues, related principally to the altered conformation and extensive post-translational processing of the deposited proteins, which may not be recognized by the available antibodies (raised against the native proteins), and to the presence of serum contamination in fixed biopsies, which may lead to high background staining and false identifications. These issues can significantly impair the specificity and sensitivity of the analysis, as pointed out by several reports [72–74]. Moreover, antibody-based techniques can only be informative if the panel of antibodies includes those directed against the specific amyloid protein present in the sample, and are blind towards novel or unexpected amyloid types. Due to the specific challenges associated with amyloid immunotyping, samples should be referred to experienced laboratories, specialized in such testing. Indeed, a recent analysis, performed by a reference center using commercial and custom antibodies, has documented that, under optimal conditions, immunohistochemistry has elevated sensitivity and specificity in this task [70].

The mentioned drawbacks of the antibody-based techniques have also prompted the search for novel typing methods. In particular, proteomic amyloid typing has already moved into clinical practice and has been implemented as a clinical test [75].

Several methods for amyloid proteomic typing have been proposed, which allow analysis of both paraffin-embedded and fresh tissue [51, 68, 76]. All these approaches are centered on the concept of protein identification by
mass spectrometry (MS), which is based on the acquisi-
tion of the mass of protein/peptide fragments, followed 
by alignment (through database search engines and algo-
rithms) of the experimental data with protein sequences 
deposited in databases. A probability score provides the 
confidence of correct identification for each of the protein 
species found in the sample.

The most widely employed proteomic approaches 
for amyloid typing are based on the so-called ‘shotgun’ 
methodology, in which all proteins from a clinical sample 
are digested, separated by liquid chromatography and 
directly analyzed in the mass spectrometer, followed by 
database search [51, 68, 76]. However, major differences 
among the methods exist, principally involving the stra-
egies used to fractionate the tissue proteins prior to MS, 
and the data analysis criteria that allow classifying one 
of the identified proteins as the amyloid constituent. 
A widely employed approach is based on selection of 
amyloid-positive areas by laser capture dissection/micro-
dissection (LCD/LCM), retrieval/extraction of proteins, 
and shotgun liquid chromatography-tandem mass spec-
trometry (LC-MS/MS analysis) [50, 67, 68, 75–77]. In the 
final step of the process, a pathologist calls the amyloid 
subtype from the list of identified proteins (which include 
both fibrillar and fibril-associated proteins), by consider-
ing the most abundant amyloidogenic species detected 
across all microdissections. LCD/LCM-based proteomics 
has led to the identification of novel amyloidosis types. 
In fact, identification is not bound to the a priori choice 
of the correct specific reagents, such as antibodies, but to 
the existence in databases of the amino acid sequences of 
proteins present in the analyzed sample. Thus, proteins in 
samples can be identified without prior hypotheses about 
their nature, as long as they are present in databases. 
Novel forms classified with this approach include heredi-
tary β₂-microglobulin [78] and apolipoprotein A-IV [79]. 
Microdissection highly reduces the generic background of 
tissue proteins; however, this also lengthens the analysis, 
may be cumbersome when the deposits are scanty, and 
does not allow evaluating molecular changes in the target 
tissue.

In the differential proteomics approaches [51, 76, 
80], such as in the MudPIT-based method developed by 
investigators of the Pavia Amyloid Research and Treat-
ment Center in collaboration with colleagues at CNR in 
Milan [51, 66, 76], identification of the deposited protein 
is instead achieved on the whole tissue, without prior 
selection of the amyloid-positive areas. Typing is obtained 
through comparison of the diseased sample protein profile 
with a corresponding reference profile from control tissue 
(derived from non-affected individuals). The assump-
tion behind this approach is that the deposited proteins 
should be more abundant in patients than in controls. 
Sample preparation is less cumbersome in this phase, but

![Workflow of MudPIT (Multidimensional Protein Identification Technology)-based diagnostic proteomics procedure for tissue amyloid typing](image)

Figure 2: Workflow of MudPIT (Multidimensional Protein Identification Technology)-based diagnostic proteomics procedure for tissue amyloid typing [51, 66].

The approach is based on acquisition of the complete proteome profile of the diseased sample. Identification of the amyloidosis type is achieved through semiquantitative differential analysis with the average proteome profile of the corresponding control tissue. α-Value provides a quantitative score of the predominance of a specific amyloidogenic protein in the sample, thus allowing diagnosis. FFPE, formalin-fixed paraffin-embedded; 2DC-MS/MS, two-dimensional liquid chromatography–tandem mass spectrometry.
chromatographic separation is generally longer. Given the higher complexity of the proteome maps, this type of analysis requires the introduction of a mathematical algorithm, to assess and rank the over-represented amyloidogenic proteins in patients (Figure 2).

Although both approaches are already employed in specialized proteomic laboratories, the methods required for tissue processing and/or protein fractionation are lengthy. Shortening the sample preparation steps, while achieving efficient enrichment of the target proteins, and reducing the length of the chromatographic separations, would be the technical premises to fit these methods into the time constraints of a clinical chemistry laboratory.

A few caveats in the use of proteomics for amyloid typing are to be underlined. From a practical point of view, the requirement of specialized equipment and personnel translates in the fact that proteomic typing is not as widely and easily available as antibody-based typing methods, and samples are currently to be sent to the few centers worldwide where this technique is established. Moreover, inter-laboratory standardization of the analytical procedures would be necessary to guarantee standardization of typing. In fact, instead of being dependent on good histology or immunohistochemistry, proteomics is dependent on factors including enzymatic protein fragmentation, chromatographic separation, mass accuracy and resolution of the mass spectrometer, protein sequence database and searching algorithms, and criterion for interpreting the protein lists. All these factors vary between laboratories and can make it difficult to standardize patients’ results. An additional specific issue is related to the chance that the primary sequence of the amyloidogenic proteins differs from that of the normal counterpart deposited in common databases. This problem is especially relevant in the case of immunoglobulin light chains and genetic protein variants, and may prevent identifying and assigning significant peptides belonging to the amyloidogenic species [76]. The use of composite protein databases, supplemented with known human immunoglobulin variant domains and known amyloidogenic mutations, can partially overcome this issue [75].

In the perspective of early diagnosis, the relative usefulness of proteomics and antibody-based methods is also still matter of debate. In particular, the performance of proteomic technologies in biopsies with minor amounts of deposits is still poorly defined [3]. The observation that some amyloid-associated proteins, such as apolipoprotein A-IV, serum amyloid P and apolipoprotein E, are invariably observed in amyloid deposits analyzed by shotgun proteomics, however, led to hypothesize their use as general proteomic amyloid biomarkers (amyloid tissue fingerprint), possibly useful for early diagnosis [50–52].

Measurement of the amyloidogenic precursor

According to the intrinsic features leading to fibril deposition, the amyloidogenic proteins can be divided into four main classes: 1) proteins that become amyloidogenic in relation to aging, in the absence of mutations or changes in concentration, as in the case of wild type TTR causing SSA; 2) proteins that form amyloid deposits only when their circulating concentration is persistently high, as in the case of SAA in reactive amyloidosis, or of β2-microglobulin in dialysis-related amyloidosis; 3) proteins in which the presence of amino acid substitutions is the primary determinant of amyloidogenicity, as in hereditary amyloidoses; and 4) proteins in which both the presence of destabilizing features in primary sequence and high concentration concur to amyloidogenicity, as in immunoglobulin light chains causing AL amyloidosis.

The search for the amyloidogenic precursor is an important step in the diagnostic process of amyloidoses. In some forms, such as AL and AA, the quantitative evaluation of the amyloidogenic precursor is also required for assessing the prognosis, during disease follow-up, and to define response to treatment.

In light chain amyloidosis, the diagnosis requires demonstration of the plasma cell clone producing monoclonal light chains. In contrast with what observed in multiple myeloma, the amyloidogenic λ clones predominate over κ ones (λ to κ clones ratio 4:1) [7, 8]. The clonal plasma cell population can be detected both by bone marrow analysis and by the identification of a monoclonal light chain in the patient’s serum and/or urine. Bone marrow analysis, with evaluation of plasma cell infiltrate and fluorescence in situ hybridization (FISH) [81–83], should be performed at baseline evaluation. However, the amyloidogenic clone in AL amyloidosis often is of small size (median bone marrow plasma cell infiltrate 7%–10%) [6–8], and detection of the low concentrations of circulating amyloidogenic precursor requires the combined use of several techniques to increase sensitivity [84, 85]. Moreover, in half of patients, the clone produces monoclonal free light chains (FLC) only [7, 8]. Current evidence recommends combining the use of serum and urine electrophoresis and immunofixation (IFE), together with free light chains quantification and evaluation of the κ/λ FLC ratio, in order to reach the best diagnostic sensitivity for
the amyloidogenic monoclonal protein [84–87]. In this dreadful disease, study of serum alone misses a fraction of patients that, although small, cannot be overlooked [88]. The concentration of FLC at diagnosis has also prognostic significance and has been included in a staging system along with NT-proBNP and cardiac troponins (cTn) [26, 89]. FLC quantification is now an indispensable tool also to evaluate response to treatment. In AL patients, changes in FLC concentration after chemotherapy were shown to predict survival and improvement or progressive worsening of organ dysfunction [22, 90–93], and new response criteria based on FLC measurement have recently been validated [21]. It is important to note that all these studies were performed using the Freelite immuno-nephelometric assay (The Binding Site), based on the use of polyclonal anti-FLC antibodies [94]. More recently, a novel assay for FLC quantification (N latex FLC, Siemens) based on monoclonals, has been developed and introduced on the market [95]. The performance of the two assays for FLC quantification in AL amyloidosis patients has recently been compared [96, 97]. The results showed that the assays have similar diagnostic sensitivity; however, they are not interchangeable and the dynamic range of the N Latex FLC assay is limited.

The polyclonal anti-FLC antibodies have also been employed to purify monoclonal FLC from serum. The primary sequence and post-translational modifications of circulating FLC can be described using mass spectrometry; although still experimental, proteomic analysis of these proteins is a promising tool to detect features related to their pathogenicity [98, 99].

In AA amyloidosis, the formation of amyloid deposits is subtended by a condition of chronic inflammation, and quantification of serum amyloid A protein allows direct evaluation of the fibril precursor. SAA is a sensitive marker for the acute-phase, with a high dynamic range [100, 101]. Repeated measurement and close monitoring of this protein is advised in AA amyloidosis patients; mortality, amyloid burden, and renal prognosis, in fact, correlate with the SAA concentration during follow-up [102, 103]. However, only a minority of patients with chronic inflammation will eventually develop AA amyloidosis; thus, evaluation of the known disease-modifying factors, such as SAA genotype, may allow selecting the subset of patients who are at higher risk for this complication. In particular, specific SAA1 alleles, in different populations (SAA1.1 in Caucasian and SAA1.3 in Japanese populations), significantly increase the risk of AA [10].

In hereditary amyloidoses, the formation of deposits is sustained by a genetically determined variant of the amyloidogenic protein, in which amino acid substitutions make the protein prone to misfolding and aggregation. The most common and convenient approach for demonstrating the presence of variants is through genetic testing. More than 100 different TTR mutations, distributed across the whole length of the protein sequence (127 amino acids), are known to cause ATTR amyloidosis (http://amyloidosismutations.com/mut-attr.php). In this form, in parallel to DNA analysis, alternative strategies have been developed to assess the amyloidogenic variant in serum. Isoelectric focusing has been used as a rapid screening test [104]. Other methods combine serum TTR immunoprecipitation with mass spectrometry [76, 105]; this allows assessing mass shifts attributable to amino acid substitutions, and, through peptide mass fingerprinting or MS/MS sequencing, to map the variant. Although useful to confirm that the amyloidogenic TTR circulates in blood, these methods are not routinely employed in clinical practice. Quantification of circulating transthyretin is not employed in the diagnostic workout, but will become essential to monitor the efficacy of the novel, gene-silencing treatments against ATTR, based on the use of RNAi to reduce the production of this protein [106]. To this aim, distinct measurement of mutant and wild-type transthyretin can be achieved through the use of liquid chromatography–tandem mass spectrometry [106].

The degree of post-translational proteolytic processing of TTR in the amyloid fibrils has also been shown to correlate with the phenotype. TTR fragments in tissue amyloid deposits, detected by Western blot, correlate with late onset of the disease and myocardial involvement [107, 108].

A peculiar issue in demonstrating the presence of the amyloidogenic precursor is posed by SSA, caused by deposition of wild type transthyretin (wtTTR), usually in men aged >60 years, mainly in the heart [9]. Although proteomic analysis of TTR immunoprecipitated from serum revealed a number of potentially destabilizing post-translational modifications, such as S-sulphonation and S-thiolation [109], no specific features that differentiate amyloidogenic from non-amyloidogenic wtTTR have yet been reported. Moreover, the serum concentrations of total TTR in SSA patients did not differ from age, gender and ethnically matched controls or from a group of AL patients with significant clinical cardiac involvement [110]. Scintigraphy with 99mTc-3,3-diphosphono-1,2-propanodiacetyl acid (99mTc-DPD) and 99mTc-pyrophosphate can help in differentiating transthyretin-related familial and senile cardiac amyloidoses from light chain cardiac amyloidosis [111, 112]. However, the diagnosis of SSA requires endomyocardial biopsy, with demonstration that the deposited fibrils are constituted by TTR, in absence of mutated TTR by DNA sequencing.
In some instances, more than one possible amyloid-forming protein is detected in a patient [74, 113]. This is the case, e.g., of patients who carry an amyloidogenic TTR variant, who are concomitantly carriers of monoclonal gammopathy of undetermined significance (MGUS). In these instances, definitive typing of amyloid fibrils in a tissue biopsy is mandatory to avoid misdiagnosis. In hereditary amyloidosis, DNA sequencing demonstrates amyloidogenic mutations in the genes encoding for the protein found in the deposits.

**Biomarkers in assessment of amyloid organ dysfunction**

Accurate assessment of the pattern and severity of organ involvement is the third key step in the diagnostic and prognostic definition of systemic amyloid diseases. While the clinical profile is very reproducible in some amyloidosis types (i.e., in AA, in which more of 90% of cases present with glomerular proteinuria), other types, such as AL, can have extremely protean manifestations, and organs can be involved in variable combinations [7, 8]. In this form, definition of the severity of organ (heart, in particular) damage is mandatory during the evaluation stage, in order to assess the risk of therapy, the patients’ prognosis, and response to treatment [5, 7, 21]. Biomarker-based staging and frequent response assessment is crucial to improve the survival of patients with AL amyloidosis. Since cardiac damage determines survival and treatment tolerability, the staging system based on NT-proBNP (>332 ng/L) and cardiac troponin (cTn) T/troponin-I (cTnT, >0.035 ng/mL; cTnI >0.1 ng/mL) [24], or more recently high-sensitivity cTnT (hs-cTnT) [22, 114] is the most robust method for risk stratification [5, 7]. High-sensitivity cTnT is the single most important prognostic factors in AL amyloidosis and the Mayo Clinic group has proposed an ‘hs-cTnT only’ staging system, with excellent discriminating ability [114]. Recently, the clinical role of novel cardiac biomarkers in AL amyloidosis has been the focus of targeted studies. Our group reported that midregional proadrenomedullin (MR-proADM) is a powerful prognostic marker, which may not only reflect cardiac dysfunction but also widespread systemic disease, and can be combined with cTn for detecting patients at risk of early death [115]. Soluble ST2 (sST2) was also reported to be a powerful prognostic factor for AL amyloidosis patients; moreover, being a decoy receptor that neutralizes the benefits of IL-33, ST2 has been hypothesized to play a role in the myocardial hypertrophy and reduction of contractility seen in patients with AL [116]. Low-risk patients tolerate chemotherapy better and are significantly more likely to respond to treatment, whereas high-risk subjects need gentle but rapidly acting regimens. Patients with early heart involvement and only moderately elevated cardiac biomarkers (NT-proBNP <5000 ng/L and troponin T <0.06 μg/L) can safely undergo autologous stem cell transplantation [5, 7, 117], further highlighting the critical importance of early detection of amyloid cardiomyopathy. In contrast, stage III patients with NT-proBNP >8500 ng/L are at high-risk of death within a few weeks, are extremely fragile and sensitive to treatment toxicity [118]. Clinical benefit depends on profound clonal response translating into organ, especially cardiac or renal, response. Hematologic and cardiac response to therapy should be assessed frequently; cardiac response can be reliably evaluated using NT-proBNP, whose clinically significant decreases and increases demonstrate strong correlations with survival [21]. An NT-proBNP decrease of >30% and >300 ng/L (if baseline is ≥650 ng/L) [21] guides treatment adequacy, mindful of increases in renal failure [23].

Other factors are being studied as possible novel prognostic indicators in AL amyloidosis. In this perspective, von Willebrand factor antigen (vWFag) has been assessed under the rationale that endothelial dysfunction may play a pathophysiological role [119]. Indeed, vWFag concentration is elevated in AL patients and associates with a high risk of early death and shorter survival, improving the prognostic ability of cardiac markers; of note, this analyte does not correlate with cardiac biomarkers and pattern of organ involvement.

The prognostic role of biological features of the amyloidogenic plasma cell clone has also been investigated. Presence of >1% bone marrow plasma cells at diagnosis, quantified using multiparameter flow cytometry immunophenotyping, is associated with poorer prognosis, whereas >5% normal plasma cells identifies a subgroup of AL patients with better survival [120]. A recent exploratory study [121] confirms that defining the plasma cell immunophenotype may help refine the current staging of AL amyloidosis and response to treatment. In particular, expressions of CD56, CD27 or both on plasma cells with an abnormal phenotype were independent markers of poorer prognosis. FISH can also provide additional prognostic information in this disease. Recently, the gain of chromosome 1q21 was proven to be an independent adverse prognostic factor [122].

Besides having a central role in clinical management of systemic amyloidosis, the organ dysfunction biomarkers have also provided a key contribution for understanding the biological bases of cardiac damage in patients,
in particular in AL amyloidosis. The observation that changes in serum FLC concentration are paralleled by concordant and proportional variations of NT-proBNP, reflecting improvement or worsening of heart dysfunction even in the absence of changes in cardiac amyloid load (estimated by echocardiography) [19, 92], lead to hypothesize a significant pathogenetic role of soluble light chain species. This evidence confirms experimental observations obtained in vitro [123–125] and indicates that cardiac damage in amyloidosis results from the sum of multiple factors; in particular, direct toxicity of soluble cardiotoxic FLC towards cardiac cells significantly contributes to the overall dysfunction, along with the anatomical/mechanical damage due to deposition of the stable amyloid fibrils.

Conclusions

Systemic amyloidoses are complex diseases that pose unique challenges to physicians and laboratory medicine. Their clinical severity and etiologic complexity require precise diagnostic definition through an integrated procedure. However, the goal is to achieve not only an accurate, precise diagnostic definition through an integrated procedure, but also a timely diagnosis, and to detect organ dysfunction at early stages. It is crucial, in these progressive diseases, to block organ damage before irreversible decline. The use of biomarkers of cardiac, renal and liver involvement in populations at risk, such as individuals with MGUS and abnormal FLC ratio, and in carriers of amyloidogenic mutations, is now entering the clinical practice with the aim of anticipating the diagnosis of these progressive diseases. The routine use of organ dysfunction biomarkers has had a deep influence on the clinical management of amyloidoses, changing the clinical course of many forms and providing important hints about the biological bases of damage.

Acknowledgments: This work was supported by Associazione Italiana per la Ricerca sul Cancro, special program ‘5 per mille’ (N° 9965), the Italian Ministry of Health (GR-2010-2317596), Fondazione Cariplo (2013-0964), Amyloid Foundation and Fondazione Mintas, Ghisleri College, Pavia.

Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

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