LYMPHOID NEOPLASIA

A PRACTICAL APPROACH TO THE
DIAGNOSIS OF SYSTEMIC AMYLOIDOSES

Running title: IMMUNO-ELECTRON MICROSCOPY IN AMYLOIDOSIS

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KEY POINTS
-First wide prospective report of the role of immuno-electron microscopy (IEM) in the differential diagnosis of systemic amyloidosis
-IEM allows to characterize correctly the amyloid protein in virtually all cases and represents a viable alternative to mass spectrometry
ABSTRACT

Accurate diagnosis of systemic amyloidosis is necessary for both assessing the prognosis and to delineate the appropriate treatment. It is based on histological evidence of amyloid deposits and characterization of the amyloidogenic protein. We prospectively evaluated the diagnostic performance of immuno-electron microscopy (IEM) of abdominal fat aspirates from 745 consecutive patients with suspected systemic amyloidoses. All cases were extensively investigated with clinical and laboratory data, with a follow-up of at least 18 months. The 423 (56.8%) cases with confirmed systemic forms were used to estimate the diagnostic performance of IEM. Compared to Congo-red based light microscopy, IEM was equally sensitive (75-80%), whereas IEM revealed significantly more specific (100% vs. 80%; p<0.001). In AL amyloidosis, kappa cases were more difficult to diagnose (sensitivity 71%), while the analysis of abdominal aspirate was informative in only 40% of patients with transthyretin amyloidosis. We found a high prevalence (20%) of a monoclonal component in patients with non-AL amyloidosis highlighting the risk of misdiagnosis and the need for unequivocal amyloid typing. Notably, IEM identified correctly the specific form of amyloidosis in more than 99% of the cases. Immuno-electron microscopy on abdominal fat aspiration is an effective tool in the routine diagnostic of systemic amyloidoses.

Key words: systemic amyloidosis, immuno-electron microscopy, abdominal fat aspirate, AL amyloidosis.
INTRODUCTION

Amyloidosis is a heterogeneous group of diseases that share the deposition of amyloid fibrils in organs and tissues, with the same characteristic cross-β sheet secondary structure, independently of their protein primary structure. More than 30 unrelated autologous proteins can produce systemic amyloidoses, either localized or systemic. The various forms differ in pathogenesis and prognosis, but usually show overlapping clinical manifestations, making their differentiation on a clinical ground very difficult. Precise amyloid typing is crucial for patient adequate treatment because the various forms require different approaches, which can range from autologous stem cell transplantation in AL amyloidosis to liver transplantation in transthyretin (TTR) amyloidosis (ATTR).

The diagnosis and classification are based on histologic demonstration of amyloid deposits and characterization of the amyloid precursor. Abdominal subcutaneous fat aspiration with fine needle is fast, harmless and the most common diagnostic tool when a systemic form is suspected, being a convenient alternative to organ biopsy. The resulting tissue smear is examined by polarized light-microscopy after Congo red staining, in order to detect the presence of amyloid. The second step is to identify the amyloidogenic protein, in order to unequivocally establish the type of amyloidosis. Traditional histochemistry-based typing techniques have shown relevant limitations, with improved results in organ biopsies examined in a highly specialized laboratory in a recent series. Mass-spectrometry techniques have paved the way for novel automated proteomic assays that are now considered a diagnostic standard, but this complex and expensive technology is not yet available in most institutions.

Immuno-electron microscopy (IEM) is a technique that combines immunohistochemistry with electron microscopy. Using gold-labeled secondary antibodies, IEM can co-localize the protein within amyloid fibrils greatly reducing the background staining, which is the most common cause of reduced specificity of immunohistochemistry. As a reference center in Italy, we developed a program devoted to the immune-characterization of amyloid in abdominal fat, based on the routine use of electron and immuno-electron microscopy. In a small series, we previously had reported that IEM study of abdominal fat could reliably characterize amyloid deposits in suspected cardiac amyloidosis. The aim of the present study was to prospectively evaluate the diagnostic value of IEM in a consecutive series of abdominal fat aspirates from patients with suspected systemic amyloidoses.
METHODS

Patients
This was a prospective study enrolling 745 consecutive patients (432M/313F; median age 63 years, range: 24 to 89), referred to the Amyloidosis Research and Treatment Center, Foundation I.R.C.C.S. Policlinico S. Matteo, Pavia, for suspected systemic amyloidosis from May 2003 to December 2010. Patients were referred for suspected cardiac (332), renal (360), peripheral or autonomous nerve (130), liver (50), soft-tissue (152), gastrointestinal (26) and/or other organ involvement (56) in systemic amyloidosis. Two hundred and seventy-two (36.5%) patients had two or more suspected organ involvement. Baseline demographics, clinical and laboratory data, including possible organ involvement, were recorded. Abdominal fat aspirate was performed in all patients and samples were submitted for light and immuno-electron microscopy. Biopsies from other organs beside abdominal fat tissue were available for IEM in 323 cases. Analysis of the genes encoding transthyretin, Apo-A1 and Apo-A2 lipoprotein, fibrinogen and lysozyme was performed by automated sequencing of all gene coding regions. All patients were followed for at least 18 months; 22 (2.9%) were lost to follow-up and were excluded from the study. At the end of follow-up, all patient data were reviewed by the study clinicians (G.M. and G.P.) and the diagnosis and type of amyloidosis were confirmed or rejected on the basis of clinical and laboratory findings. Written informed consent was obtained for all patients, in accordance with the Declaration of Helsinki, for the use of biological samples and clinical data for research purposes, according to the institutional review board guidelines.

Light microscopy examination (LM)
Abdominal fat smears were stained with alkaline Congo red saturated alcoholic solution as previously described, with the Westermark’s modifications. Amyloid was demonstrated by the typical apple-green birefringence under polarized light microscopy.

Immuno-electron microscopy (IEM)
Specimens were fixed by immersion in a modified Karnovsky’s solution (0.5% glutaraldehyde, 2% paraformaldehyde in 0.2 M cacodylate buffer, pH 7.3) for at least 4 hours, and post-fixed in 1% osmium tetroxide in the same buffer. Samples were then dehydrated through a graded series of ethyl alcohols and embedded in epoxy resin. 600-800 Å thick ultrathin sections were cut, mounted on nickel grids and stained with 5% uranyl acetate and lead citrate (Reynold’s solution). A
minimum of 5 sections for each patient were observed with a Philips CM12 electron microscope. Selected sections were then processed for post-embedding immunogold as previously reported. Briefly, sections were etched with 3% H2O2 for 10 min at room temperature. Anti-κ light chain and anti-transthyretin immunostain required enzymatic predigestion (0.05% trypsin in TRIS buffer with 0.05% CaCl2, 37°C, 15 min) to unmask antigenic epitopes. The section were then rinsed in 0.05 M TRIS/HCl buffer, pH 7.3, incubated with either 1:20 normal goat serum or 1% egg albumin for 15 min at room temperature, and subsequently overnight at 4°C with the primary antibodies (Dako, Glostrup, DE), rinsed with the same buffer and then incubated for 60 min at room temperature with the secondary antibody (anti-rabbit or anti-mouse IgG) or with protein-A, both of which were conjugated to 15 nm colloidal gold particles (British Biocell International, UK). The panel of primary antibodies covers the commonest forms of amyloidosis: AL (kappa and lambda), AA, and ATTR; in selected cases, immunogold labeling was performed also with antibodies directed against apolipoprotein AI. Fibrinogen and lysozyme were investigated when clinically indicated. Sections were stained with 5% uranyl acetate and lead citrate (Reynold’s solution) and scanned with a Phillips CM12 electron microscope (Figure 1). Specificity of immunoreactions was verified using either normal goat serum or egg albumin instead of primary antibody. Each reaction was also checked by immunostaining known positive samples with primary antibodies.

Data analysis
Descriptive statistical analysis of patient demographic, clinical and laboratory data was performed. Sensitivity, specificity and predictive values of light and electron microscopy were assessed against the gold standard of expert clinical evaluation on patient chart and follow-up. The diagnostic performance of the tests was compared with respect to amyloid types and involved organs with the Pearson’s chi-squared test. Agreement analyses according of IEM to final diagnosis were performed using kappa statistics and exact logistic regression was used to identify predictors of disagreement. Statistical tests were performed with PASW software 18.0 for Windows® (Chicago, IL, USA) and STATA 13.1 (Stata Corp, College Station, Texas, USA). A p value of < 0.05 was required for statistical significance.
RESULTS

Overall diagnostic performance of light and electron microscopy
After extensive clinical and laboratory analysis, 458 out of 745 patients (61.5%) were diagnosed with amyloidosis. In the remaining other 287, the absence of any evidence of amyloidosis and a clinical follow-up of at least 18 months allowed to rule out the diagnosis. Taking into account only systemic forms, 423 (56.8%) positive cases were included in the study. The distribution and characteristics of the different forms of amyloidosis are summarized in Table 1. The diagnostic performance, in terms of sensitivity, specificity and predictive values, is reported in Table 2. Both techniques showed good sensitivity (79% and 76.1%, respectively) but IEM was significantly more specific than LM (100% vs 79.7%, p<0.001). Forty-five patients with systemic amyloidosis and positive abdominal fat on LM were negative in IEM: 33 AL amyloidosis (23 λ, 10 κ), 7 ATTR, 4 AA and one fibrinogen amyloidosis. The diagnosis in these cases was based on the characterization of amyloid deposits in 31 biopsies from other organs beside fat tissue (20 renal, 3 liver, 3 salivary glands, 2 bone marrow, and lymph node, endomyocardial and rectal biopsies one each) and/or DNA sequencing (8 cases). Of the 89 patients with negative LM in whom a diagnosis of amyloidosis was finally achieved, 33 had a positive fat aspirate at IEM. These comprised 28 AL (21 λ, 7 κ) and 5 AA cases. In the other 56 cases, systemic amyloidosis was diagnosed by biopsy of one or more affected organs (33 renal, 9 endomyocardial, 4 liver, 4 nerve, 4 gastrointestinal, 3 lymph nodes, 2 bone marrow and 1 salivary gland and pulmonary one each biopsy) and/or DNA sequencing (12 cases).

Performance of IEM in amyloid typing
IEM correctly identified the specific form of amyloidosis in more than 99% of the cases. Agreement of IEM results with final diagnosis was substantial (κ=0.79; p<0.001). Gender was a significant cause of disagreement, having males higher probability of false negative results (p<0.001, at logistic regression); age at diagnosis was not significant in this regard.

Only in two out of 423 positive cases (0.5%) the amyloid component was misclassified by IEM. One was a 73 year-old male patient without history of chronic inflammation, suspected heart and kidney involvement (including proteinuria and renal failure) and kappa light chain M-protein by immunofixation. IEM showed kappa immunoreactive deposits associated with weak positivity for SAA; proteomic analysis of abdominal fat identified AA amyloidosis. No specific inflammatory disease could be diagnosed. The second case was a 76 year-old male with isolated cardiac involvement and kappa light chain M-protein by immunofixation. IEM was positive for kappa light
chains; however, the Ile68Leu DNA mutation in TTR gene was found. ATTR amyloidosis was finally confirmed by proteomic analysis of abdominal fat.

The presence of monoclonal components in serum or urine was a potential confounding factor in the diagnosis of patients with non-AL systemic amyloidoses. Of 103 patients with non-AL systemic amyloidoses, 22 had a monoclonal component in serum and/or urine (21.4%). The final diagnosis was AA (16) and mutated ATTR (6) amyloidosis. The predominantly involved organs in these patients were heart (13), kidney (15), and peripheral/autonomous nervous systems (9).

The presence of macroglossia was a positive predictor for AL amyloidosis diagnosis. Thus, among 39 patients with macroglossia and positive LM, 38 (27 kappa / 11 lambda) were diagnosed with AL amyloidosis. Only one patient with chronic arthritides and AA amyloidosis had macroglossia.

### Diagnostic performance by amyloid type

**Systemic AL amyloidosis**

Three-hundred twenty patients (76% of systemic amyloidoses) were diagnosed with AL amyloidosis, mainly of lambda isotype (68.9%). Serum and/or urine immunofixation were positive in 96.3% of the patients with AL amyloidosis, and a serum or urine monoclonal component was more frequently found by immunofixation in patients with lambda (99.1%) than in those with kappa AL amyloidosis (89.9%; \( p < 0.001 \)). The addition of serum free light chain (FLC) ratio together with serum and urine immunofixation allowed the identification of a monoclonal protein in 99.4% of AL patients.

As reported in Table 3, LM and IEM of abdominal fat aspirates diagnosed about 80% of the patients with AL amyloidoses, but the sensitivity in AL kappa cases (74% and 71%) was significantly lower in comparison to lambda (84% and 83%) for both techniques (\( p = 0.03 \) and \( p = 0.016 \), respectively). The diagnostic performance of the two techniques also differed according to the involved organ. Abdominal fat LM and IEM were more sensitive in patients with cardiac involvement compared with patients with non-cardiac amyloidosis (89.7% vs. 77.3%; \( p < 0.001 \)), and less sensitive in patients with any renal involvement compared to other patients (74.4% vs. 83.3%; \( p = 0.037 \)).

Predictors of disagreement at logistic regression were AL type and specific organ involvements, together with gender: AL kappa type was shown to be associated with a higher rate of disagreement in IEM than AL lambda (\( p < 0.001 \), sex adjusted model). Also, the rate of disagreement was lower in the presence of isolated heart involvement (\( p = 0.047 \), sex adjusted model). It was higher in the presence of kidney involvement (\( p = 0.019 \), sex adjusted model).
AA amyloidosis

AA amyloidosis was the second more common type in this series. Reactive amyloidosis occurred in rheumatoid arthritis (13 patients), inflammatory bowel disease (8 patients) other polyarthrites (14 patients with ankylosing spondylitis, polymyalgia rheumatica, psoriatic arthritis and others), familial periodic fever syndromes (4 patients), Castleman disease (2 patients), polyserositis (2 patients), Schnitzler syndrome (2 patients), cystic fibrosis (2 patients) and other inflammatory conditions (5 patients). In 17/69 patients (25%) it was not possible to identify any underlying cause. LM and IEM sensitivities (77%) were very similar to those observed in AL amyloidosis (Table 3); the sensitivity of IEM for AA amyloidoses was significantly higher when compared with that for ATTR (77% vs. 43%; p=0.002). A monoclonal protein was found by immunofixation in almost one-quarter of the AA patients. Five patients with negative fat aspirate by LM and IEM were confirmed to have AA amyloidosis by IEM on different organ biopsies (3 kidney, 1 endomyocardium and 1 minor salivary gland).

ATTR amyloidosis and other familial forms

Most ATTR amyloidosis cases in the present series were due to mutations in the TTR gene (25 patients), whereas only 5 senile wild-type ATTR forms were identified. The reason for this relatively low number of ATTR amyloidosis cases in our series is that in patients with positive family history of ATTR amyloidosis and consistent clinical presentation, the identification of the mutation is often considered sufficient to make the diagnosis. The most frequent mutations were Val30Met (7 patients), Ile68Leu (4 patients), Tyr78Phe (3 patients) and Val122Ile (3 patients). Abdominal fat LM sensitivity for these forms was slightly lower (particularly in senile forms) than for AL and AA amyloidosis. IEM sensitivity was significantly lower for ATTR than for the other types. Considering 33 other patients diagnosed with ATTR in our center during the study period by means of gene sequencing, but without IEM analysis, fat aspirate LM sensitivity for ATTR amyloidosis reached 71.4% (IC 95% 58.5-81.8). A monoclonal protein was found by immunofixation in 24% of patients with mutated ATTR. Three out of the 5 patients with senile ATTR forms were confirmed by IEM on endomyocardial biopsies.

The other forms of familial amyloidosis included two apolipoprotein A1 mutations (Leu75Pro), one lysozyme mutation (Trp64Arg) and a case of renal involvement by fibrinogen amyloidosis (Glu526Val). Only this last case was positive by LM in fat aspirate, and all cases were negative by IEM.
Proposed approach to the diagnosis of systemic amyloidoses

According to our models to identify predictors of disagreement, the patients with higher risk of false negative are those with AL kappa type amyloidosis, renal involvement, particularly if isolated, and/or male gender. Accordingly, we may recommend a practical approach to the diagnosis of systemic amyloidosis as reported in Figure 2, which takes into account other biopsies, in addition to fat aspirate, such as surgical abdominal fat samples, labial salivary glands, or biopsies of involved organs. For instance, we have previously shown that the biopsy of minor salivary glands has a 58% diagnostic sensitivity in patients with negative abdominal fat aspirate, and the combined negative predictive value of the two biopsies is greater than 90%24. However, the biopsy of clinically affected organs can be required in selected cases to achieve the final characterization of amyloid deposits.

DISCUSSION

This is the largest study on diagnostic performance of abdominal fat and any other biopsy site in systemic amyloidosis. Abdominal fat analysis by IEM allowed reaching a definitive diagnosis in 79.4% of AL, in 76.8% of AA and in 43.3% of ATTR amyloidosis. The sensitivity of IEM on abdominal fat aspirate in our tertiary reference center was 76.1%, with 100% specificity and 99% correct classification of the amyloid type. Sensitivity was however higher in AL lambda amyloidosis, the most frequent protein type. In a recent report, 110 out of 117 cases (94%) were correctly classified using immunohistochemical stains of involved organ biopsies, with 96% sensitivity and 100% specificity in a subgroup of patients with a known amyloid type.11 In our study, IEM on abdominal fat aspirates yielded similar specificity (100%), a slightly lower sensitivity (80%), and typed correctly the amyloid deposits in 99.5% of cases. In their study, all the biopsies studied were “positive”. However, in the cited report11, calculation of sensitivity and specificity was done only in a subgroup of 51 patients in whom they were confident enough of the final diagnosis. The 94% figure is the proportion of patients classified in the whole group, including cases lacking a clinical final diagnosis.

Using abdominal fat aspirate samples for amyloid typing has several relevant advantages over organ biopsies: fat aspiration is usually performed on the same day of the clinical consultation, and time needed for completing the IEM typing is about 7-10 days, allowing for prompt therapy initiation, particularly important in AL amyloidosis. Abdominal fat is the elective biopsy site because it is easily accessible, minimally invasive, and can be repeated during follow-up, if necessary25,26. In cases where more tissue is needed due to the focal deposition of the amyloid fibrils, a surgical
abdominal fat biopsy allowing larger sample might be indicated. Although it has been reported that, once hematostatic disorder is appropriately ruled out, bleeding risk during kidney biopsy is not increased in patients with systemic amyloidosis\textsuperscript{27}, liver biopsy carries a significant risk of bleeding that may have fatal consequences. Liver biopsy should be avoided or undertaken via transjugular route. Furthermore, some biopsies can be performed only in specialized centers, such as endomyocardial biopsy. Moreover, abdominal fat aspirate is also suitable for the analysis of amyloid protein composition through proteomics, becoming an interesting source of information on the interaction between amyloidogenic and non-amyloidogenic proteins in tissue\textsuperscript{12,28}. In this series, proteomics was useful in 2 cases, one AA and one ATTR, misdiagnosed as AL due to non-specific precipitation of serum immunoglobulin on the amyloid deposits\textsuperscript{14,29}.

As previously stated, the correct typing of amyloid deposits is essential for patient management. Several techniques have been developed for typing, such as enzyme-linked immunosorbent assay (ELISA), Western blot, immunohistochemistry, IEM or mass spectrometry\textsuperscript{30}. Mass spectrometry is now considered a standard for amyloid typing, allowing the unequivocal identification of the amyloid protein and is independent of availability of specific antibodies. It can be done profitably centralized and even performed on paraffin-embedded fat samples. However, dedicated mass spectrometers are very expensive, require highly qualified personnel, and are available in very few institutions worldwide. Our IEM protocol utilizes commercial antibodies, and can be applied in referral hospital’s pathology laboratory. However, only certain Amyloidosis Units in the world apply IEM systematically. Therefore, specificity and PPV may not be as good in other centers with less experience with this technique. IEM allows the “in situ” correlation of antibody localization and fibril morphology, overcoming the limit of non-specific stain of light immunohistochemistry and of custom-made antibodies used in other studies\textsuperscript{11}.

The diagnostic performance in the general series was good; all positive patients by IEM were affected by systemic form of amyloidosis, with 100% positive predictive value. Diagnostic agreement was significantly lower in males. The negative predictive value was 74%. This relatively low negative predictive value can be the consequence of the sparse, focal distribution of less abundant amyloid deposits, which can be absent in small samples of fat aspirates of patients with systemic amyloidosis. For this reason, when systemic amyloidosis is strongly suspected on clinical grounds and abdominal fat aspirate is negative, the diagnostic hypothesis should not be ruled out. Thus, diagnostic performance of abdominal fat aspirate in male patients with AL kappa type amyloidosis and renal involvement is suboptimal, and in these patients surgical biopsy from abdominal subcutaneous fat may be more informative\textsuperscript{31} (Figure 2).
As far as amyloid typing is concerned, AL lambda cases were the most easily detected by IEM, in contrast to kappa cases, where IEM sensitivity was significantly lower. This previously unreported observation suggests that IEM negative results should be considered carefully when AL amyloidosis of kappa type is suspected. In our experience in 2 recent cases with kappa light chain monoclonal components, kappa light chains could be identified in abdominal fat aspirate by proteomics while in one of them IEM was negative and in the other the result was non conclusive. LM positivity correlated with the number of involved organs, a tendency not confirmed by IEM. Independently of light chain isotype, patients in whom the kidney was the prevalently involved organ were more likely to have negative abdominal fat aspirates than those affected by diffuse systemic or predominantly cardiac involvement. Of note, high sensitivity of IEM on fat tissue for cardiac amyloidosis has been described previously.

Interestingly, IEM analysis of abdominal fat aspirates was less sensitive (43%) in patients with ATTR amyloidosis. This may be due to the focal pattern of amyloid deposition in ATTR amyloidosis, which raises the possibility of false negative results in small samples of fat tissue. On the other hand, IEM has a less crucial role in the diagnosis of hereditary ATTR amyloidosis, since in patients with a known family history the diagnosis could be supported by DNA analysis. In patients with senile systemic amyloidosis (SSA), cardiac biopsy remains the gold standard, since the abdominal fat aspiration has shown low sensitivity. It has been reported, in a previous small series of 11 patients, that a surgical skin biopsy including the deep subcutaneous fat pad, can be useful for the histopathological diagnosis of SSA. Scintigraphy with 3,3-diphosphono-1,2-propanodicarboxylic acid (DPD) or pyrophosphate (PYP) can also provide useful diagnostic hints in an appropriate clinical context.

The prevalence of hereditary amyloidosis (ATTR) associated with a benign monoclonal gammopathy reached 24%. This phenomenon has been recognized with variable prevalence in other series, ranging from 3 out of 7 patients to “low grade” monoclonal gammopathies in 24% of familial amyloidosis patients, as in our series. This high prevalence may be due to a referral bias, since most patients refer to our hematology-oriented center. It is worth emphasizing that IEM was able to classify correctly more than 99% of these patients. Only two cases had non-specific reaction to kappa antibody, possibly due to entrapment of serum immunoglobulins within the amyloid fibrils, while proteomic unequivocally classified them as ATTR and AA amyloidosis. On the other hand, 99% of AL lambda and 90% of AL kappa amyloidosis patients had a detectable monoclonal component by serum or urine immunofixation; which increased to 99% when serum
FLC was combined with immunofixation. It means that AL kappa cases are more frequently oligosecretory, as it has already reported in multiple myeloma. This subgroup of patients would be more difficult to diagnosis by either immunofixation or IEM, with a more relevant diagnostic value of serum FLC ratio determination in these cases.

In conclusion, IEM increased to 100% the specificity of abdominal fat aspirates analysis for the diagnosis of systemic amyloidosis, with a high level of agreement. IEM can correctly characterize amyloid deposits in 99.5% of cases, thus representing an efficient and more readily available alternative to mass spectrometry. The high prevalence of a monoclonal component in patients with non-AL amyloidosis emphasizes the risk of misdiagnosis and the need for an unequivocal amyloid typing.
ACKNOWLEDGMENTS
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AUTHORSHIP CONTRIBUTIONS
C.F.L., L.V., G.P., and G.M. conceived and designed the study; L.V., P.M., F.L., A.F., L.O., P.M., G.L.C. and M.P. provided study materials and patients; C.F.L., L.V., A.F., L.O., P.M. and G.L.C. collected and assembled the data; C.F.L., G.P., C.K. and G.M. analyzed and interpreted the data; C.F.L., P.M., G.P., M.P., C.K. and G.M. wrote the manuscript; and all authors gave final approval of the manuscript.

CONFLICT OF INTEREST
All the authors have not disclosures to declare.
REFERENCES


Table 1. Characteristics of the patients with systemic amyloidosis according to amyloid protein involved

<table>
<thead>
<tr>
<th></th>
<th>AL λ (%)</th>
<th>AL κ (%)</th>
<th>AA (%)</th>
<th>Mutated ATTR (%)</th>
<th>Senile ATTR (%)</th>
<th>Others ¥ (%)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n (%)</td>
<td>221 (52)</td>
<td>99 (24)</td>
<td>69 (16)</td>
<td>25 (6)</td>
<td>5 (1)</td>
<td>4 (1)</td>
<td></td>
</tr>
<tr>
<td>Age median (range)</td>
<td>63 (30-87)</td>
<td>63 (28-86)</td>
<td>63 (34-89)</td>
<td>61 (40-79)</td>
<td>75 (65-84)</td>
<td>67 (66-70)</td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>121/100*</td>
<td>63/36*</td>
<td>26/43*</td>
<td>21/4*</td>
<td>5/0</td>
<td>3/1</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>MC by serum and urine IFE + serum FLC (%)</td>
<td>99.5*</td>
<td>98.9*</td>
<td>23.2*</td>
<td>24*</td>
<td>0</td>
<td>0</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

| Organ involvement    |          |          |        |                  |                |              |       |
| Heart (%)            | 66.7*    | 56.6*    | 26.1*  | 80               | 100            | 50           | p<0.001 |
| Urinary proteins >500 mg/24h (%) | 55.4* | 55.6* | 84* | 0*              | 0              | 75           | p<0.001 |
| Kidney failure (%)   | 22.5*    | 41.4*    | 78.3*  | 0*               | 0              | 75           | p<0.001 |
| Liver (%)            | 11.7     | 22.2     | 7.2    | 0                | 0              | 50           |       |
| GI (%)               | 4.5      | 4        | 5.8    | 0                | 0              | 0            |       |
| PNS/ANS (%)          | 20.3*    | 22.2*    | 5.8*   | 76*              | 0              | 0            | p<0.05 |
| Soft tissues (%)     | 30.2*    | 24.2*    | 0*     | 0*               | 0              | 0            | p<0.05 |

MC, monoclonal component; IFE, immunofixation electrophoresis; FLC, free light chain; Kidney failure defined by estimated creatinine clearance < 60 ml/min; GI, gastrointestinal tract; PNS/ANS, peripheral nervous system/autonomic nervous system.

¥Two cases of mutated apolipoprotein Al, one with mutated lysozime and one with mutated fibrinogen.
Table 2. Diagnostic performance of light microscopy and immunoelectron microscopy of abdominal fat in systemic amyloidosis.

<table>
<thead>
<tr>
<th></th>
<th>Light microscopy % (CI 95%)</th>
<th>Immuno-electron microscopy % (CI 95%)</th>
<th>p significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>79 (74.7-82.7)</td>
<td>76.1 (71.7-80.1)</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>79.7 (74.4-84.2)</td>
<td>100 (98.4-100)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Negative predictive value</td>
<td>71.6 (66.2-76.4)</td>
<td>74 (69.2-78.2)</td>
<td></td>
</tr>
<tr>
<td>Positive predictive value</td>
<td>85.4 (81.4-88.7)</td>
<td>100 (98.4-100)</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>
Table 3. Sensitivity of light and immuno-electronic microscopy according to amyloidosis type.

<table>
<thead>
<tr>
<th>Amyloidosis type</th>
<th>n</th>
<th>Light microscopy % (CI 95%)</th>
<th>Immuno-electron microscopy % (CI 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>320</td>
<td>80.9 (76.1-85)</td>
<td>79.4 (74.4-83.6) @</td>
</tr>
<tr>
<td>λ</td>
<td>221</td>
<td>84.2 (78.5-88.6) #</td>
<td>83.3 (77.5-87.8) &amp;</td>
</tr>
<tr>
<td>λ</td>
<td>99</td>
<td>73.7 (63.8-81.8) #</td>
<td>70.7 (60.6-79.2) &amp;</td>
</tr>
<tr>
<td>AA</td>
<td>69</td>
<td>76.8 (64.8-85.8)</td>
<td>76.8 (64.8-85.8) §</td>
</tr>
<tr>
<td>ATTR</td>
<td>30</td>
<td>66.7 (47.1-82.1)</td>
<td>43.3 (26.6-62.3) @ §</td>
</tr>
<tr>
<td>Mutated ATTR</td>
<td>25</td>
<td>72 (50.4 – 87.1)</td>
<td>40 (21.8 – 61.1)</td>
</tr>
<tr>
<td>Senile ATTR</td>
<td>5</td>
<td>40 (7.2 – 83)</td>
<td>40 (7.2 – 83)</td>
</tr>
<tr>
<td>Others</td>
<td>4</td>
<td>25 (1.3-78.1)</td>
<td>0</td>
</tr>
</tbody>
</table>

Statistical significance between groups: #, p=0.03; @, p<0.001; &, p=0.016; §, p=0.002.
FIGURE LEGEND

Figure 1. Immuno-electron microscopy of abdominal fat aspirate from patients with different amyloid diseases: postembedding immunostaining with polyclonal anti-λ light chains (A), anti-k light chains (B), monoclonal anti-SAA (C) and polyclonal anti-transthyretin (D) antibodies. Secondary antibodies are conjugated with gold particles 15 nm in diameter. Uranyl acetate, lead citrate. Bar = 1 μm

Figure 2. Suggested diagnostic approach and fat tissue biopsy procedure in patients with suspected systemic amyloidoses, according to gender, organ involvement and involved light-chain isotype. Genetic testing for hereditary amyloidosis is indicated in patients with isolated cardiac (to exclude transthyretin and apolipoprotein A1 amyloidoses) or renal (for fibrinogen and lysozyme) involvement, as well as in those with peripheral neuropathy either isolated or associated with cardiac involvement (transthyretin).
Figure 1.
Clinical suspicion of systemic amyloidosis

Heart involvement*
Lambda LC
Female

Abdominal fat aspirate
Biopsy of salivary glands

Biopsy of involved organ

All other patients with suspected systemic amyloidosis*

Surgical biopsy of abdominal fat

Biopsy of involved organ

* Consider DNA genomic tests for hereditary amyloidosis if indicated
A practical approach to the diagnosis of systemic amyloidoses

Carlos Fernández de Larrea, Laura Verga, Patrizia Morbini, Catherine Klersy, Francesca Lavatelli, Andrea Foli, Laura Obici, Paolo Milani, Gian Luca Capello, Marco Paulli, Giovanni Palladini and Giampaolo Merlini