Amyloid Typing in Cardiac Amyloidosis Using Western Blotting

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ABSTRACT **Background:** Cardiac amyloidosis (CA) is characterized by the extracellular deposition of misfolded protein in the heart. Precise identification of the amyloid type is often challenging, but critical, since the treatment and prognosis depend on the disease form and the type of deposited amyloid. Coexistence of clinical conditions such as old age, monoclonal gammopathy, chronic inflammation, or peripheral neuropathy in a patient with cardiomyopathy creates a differential diagnosis between the major types of CA: light chain amyloidosis (AL), transthyretin amyloidosis (ATTR) and amyloidosis A (AA).

Objectives: To demonstrate the utility of the Western blotting (WB)-based amyloid typing method in patients diagnosed with cardiac amyloidosis where the type of amyloid was not obvious based on the clinical context.

Methods: Congo red positive endomyocardial biopsy specimens were studied in patients where the type of amyloid was uncertain. Amyloid proteins were extracted and identified by WB. Mass spectrometry (MS) of the electrophoretically resolved protein-in-gel bands was used for confirmation of WB data.

Results: WB analysis allowed differentiation between AL, AA, and ATTR in cardiac biopsies based on specific immunoreactivity of the electrophoretically separated proteins and their characteristic molecular weight. The obtained results were confirmed by MS.

Conclusions: WB-based amyloid typing method is cheaper and more readily available than the complex and expensive gold standard techniques such as MS analysis or immunoelectron microscopy. Notably, it is more sensitive and specific than the commonly used immunohistochemical techniques and may provide an accessible diagnostic service to patients with amyloidosis in Israel.

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KEY WORDS: amyloid typing, cardiac amyloidosis, mass spectrometry, transthyretin, Western blotting

Amyloidosis refers to a clinically heterogeneous group of disorders characterized by the extracellular deposition of amyloid proteins in various tissues of the body. Although the deposited amyloid proteins found in different clinical forms of amyloidosis share the same beta-pleated sheet configuration and common histochemical features (such as Congo red staining), they vary with respect to their primary structure. Approximately 42 different chemical types of amyloid proteins, originating from different precursor proteins, have been described so far [1]. In light chain amyloidosis (AL), the monoclonal immunoglobulin light chains are the precursor proteins of light chain deposits found in patients with plasma cell proliferative disorders. Serum amyloid A (SAA) accumulates in familial Mediterranean fever, chronic inflammations and infections, and certain malignancies (amyloidosis A/AA). Transthyretin (TTR) is deposited in senile wild-type transthyretin amyloidosis (ATTRwt) or when mutant, in familial TTRv amyloidosis. AL, AA, and ATTR account for approximately 98% of all types of amyloidosis and nearly 100% of cardiac amyloidosis.

Precise determination of amyloid type is critical in clinical practice since both treatment and prognosis are different in different amyloid diseases. However, in many cases the type of amyloidosis is not clear. Patients with cardiac amyloidosis may have a coexistence of plasma cell dyscrasia with a systemic inflammatory condition potentially causing AA, or neuropathy suggestive of ATTR amyloidosis. Elderly individuals with cardiomyopathy often have imaging studies compatible with ATTR but they concomitantly may have monoclonal gammopathy of unknown significance (MGUS), necessitating a definitive diagnosis of the type of amyloid to determine an appropriate therapy [2-4]. Thus, once a diagnosis of amyloidosis is confirmed by Con-

go red staining, the type of amyloid should be established by a precise analytical method. Unfortunately, the routinely used immunohistochemistry protocols are often non-effective, because of cross-reactivity due to tissue contamination by serum proteins, especially by immunoglobulins [5-8]. More precise technologies include immunoelectron microscopy (IEM) [9] and mass spectral (MS) analysis of laser micro-dissected amyloid fibrils [10]. However, these gold standard methods require advanced instrumentation and expertise, preventing their implementation in most medical centers.

We have developed a practical technique for amyloid typing based on Western blot (WB) analysis of the biopsy tissue extracted amyloid proteins by using commercial antibodies against different amyloid precursor proteins [11-18]. In this report, we describe the practical utility of WB-based amyloid typing in a series of patients diagnosed with cardiac amyloidosis where the type of amyloid was not obvious based on clinical data.

PATIENTS AND METHODS

BIOPSY TISSUES

Patients were managed in the cardiomyopathy clinic of the Heart Failure Institute, and all procedures were performed according to clinical indications. Congo red positive endomyocardial biopsy specimens of five patients with a questionable type of cardiac amyloidosis were examined by the described techniques.

EXTRACTION

Amyloid proteins were extracted from fresh-frozen biopsy specimens using an aqueous solution of 20% acetonitrile and 0.1% trifluoroacetic acid [11-13,18]. The extracted material was dried by lyophilization. Of note, recovery of amyloid from tissue samples was increased by repeating the extraction step for two more times.

ELECTROPHORESIS AND WESTERN BLOTTING

Extracted proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 4–20% Tris-glycine gels (Gradipore, Frenchs Forest, Australia) under reducing conditions. The electrophoretically separated proteins were transferred to nitrocellulose membranes and immunodetected using a panel of commercial antibodies against the precursors of amyloid proteins in the most common amyloid diseases (ATTR, AL, and AA): rabbit antibodies to human immunoglobulin κ and λ light chains, to prealbumin (TTR), and to SAA (DAKO, Carpinteria, CA). Since amyloid fibrils are composed of relatively small molecular weight (MW) proteins (15 kDa TTR monomers, light chain fragments of MW < 25 kDa, or 8 kDa AA proteins), the interpretation of the obtained results was restricted on analysis of proteins within the MW range from 5 to < 25 kDa. Under our experimental conditions, these proteins were electrophoretically separated from the irrelevant higher MW contaminants allowing straightforward typing of amyloid proteins based on their specific immunoreactivity and characteristic MW.

MASS SPECTROMETRY

MS, considered to be the gold standard for amyloid typing, was used for validation. Extracted proteins were separated by SDS-PAGE and stained with Coomassie blue. The revealed protein bands of interest were excised. The proteins in the gel slices were reduced with 3 mM DTT (60°C for 30 minutes), modified with 10 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, room temperature for 30 minutes) and digested in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin (Promega) at a 1:10 enzyme-to-substrate ratio, overnight at 37°C. The resulting peptides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The peptides were resolved by reverse-phase chromatography on 0.075 × 180-mm fused silica capillaries packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted with linear 30 minutes gradient of 5–28% acetonitrile with 0.1% formic acid followed by 15 minutes gradient of 28–95% and 25 minutes at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.15 µl/min. Mass spectrometry was performed by Q Exactive plus mass spectrometer (Thermo Fisher, USA) in a positive using repetitively full MS scan followed by high collision dissociation of the 10 most dominant ions (> 1 charges) selected from the first MS scan. The obtained data were analyzed using Proteome Discoverer 2.4 software (Thermo Fisher Scientific, USA) against the human proteome and the unreviewed light chains of the uniport database. Peptide- and protein-level false discovery rates (FDRs) were filtered to 1% using the target-decoy strategy. The data were quantified by label free analysis using the same software [19]. Amyloid type was determined by comparing the relative intensities of peptides sequences matching TTR, constant and variable domains of κ light chains (LCCκ and LCVκ, respectively) and λ light chains (LCCλ and LCVλ), and SAA.

RESULTS

The results of amyloid typing using cardiac biopsy tissue extracts of five patients with an equivocal type of amyloidosis are presented. In WB analysis, amyloid typing is based on specific immunoreactivity of tissue extracted and the electrophoretically separated proteins and their characteristic MW. MS was applied for confirmation of WB findings by measuring the abundance of the peptide sequences characterizing the major forms of systemic amyloidosis in the protein-in-gel bands.

PATIENT 1

Patient 1 was an 83-year-old male with suspected ATTRwt due to progressive cardiac hypertrophy on echocardiography and a Perugini grade 2 cardiac uptake on technetium diphosphono-propanodicarboxylic acid (Tc DPD) scintigraphy. However, the patient was previously diagnosed with monoclonal gammopathy; therefore, the question of AL vs. ATTR remained unresolved.

A heart biopsy was Congo red positive [Figure 1A, 1B]. The results of the immunohistochemistry amyloid typing were inconclusive. There was no evidence for AA amyloidodosis [Figure 1E], but weak immunostaining was observed with the anti-κ [Figure 1C], anti-λ [Figure 1D], and anti-TTR [Figure 1F] antibodies. WB analysis was applied to study proteins in the MW area < 25 kDa typical of amyloid [Figure 2, left box] where a 15 kDa band reacted specifically with anti-TTR, but not with anti-κ or anti-λ antibodies. The immunoreactivity of this protein band, as well as its MW (characteristic of TTR monomer), supported the diagnosis of ATTR amyloidosis. No monoclonal light chain fragments immunoreactive with either anti-κ or anti-λ antibodies were found. MS analysis of the protein-in-gel 15 kDa band revealed the very abundant TTR sequences compared to those of κ and λ light chains [Figure 2, right box]. No detectable SAA sequences were found. The obtained results supported diagnosis of ATTR, in concordance with the WB findings.

PATIENT 2

Patient 2 was a 53-year-old female carrier of a TTR V32A mutation who had family history of ATTR amyloidosis. She presented with cardiomyopathy and paresthesia in her hands due to carpal tunnel syndrome but no evidence of small fiber neuropathy. Her cardiac status was inconclusive as she had left bundle branch block (LBBB) and deteriorating systolic function and positive cardiac uptake on technetium pyrophosphate (Tc PYP) bone scan but no

Figure 1. Patient 1. Endomyocardial biopsy showing Congo red staining under non-polarized **[A]** and polarized **[B]** light. Results of amyloid typing by immunohistochemistry **[C]** to **[F]** were uncertain. Weak immunostaining was observed with both anti-κ **[C]** and anti-λ **[D]** antibodies. No immunostaining was found with anti-SAA antibodies **[E]**, while staining with anti-TTR antibodies was weakly positive **[F]**.

AA = amyloid A, anti-TTR = anti-transthyretin

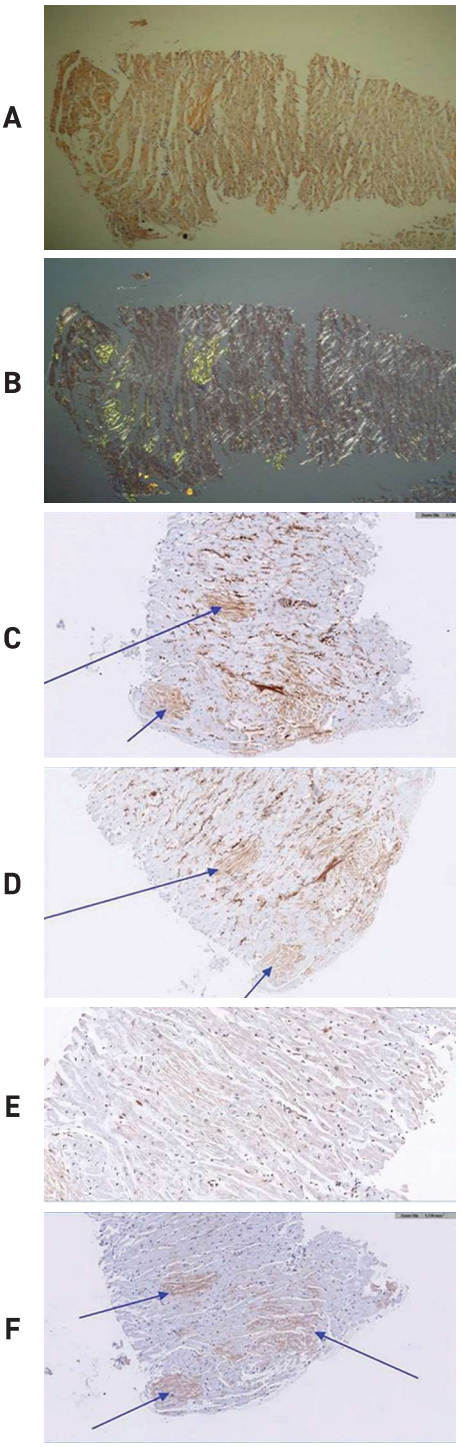
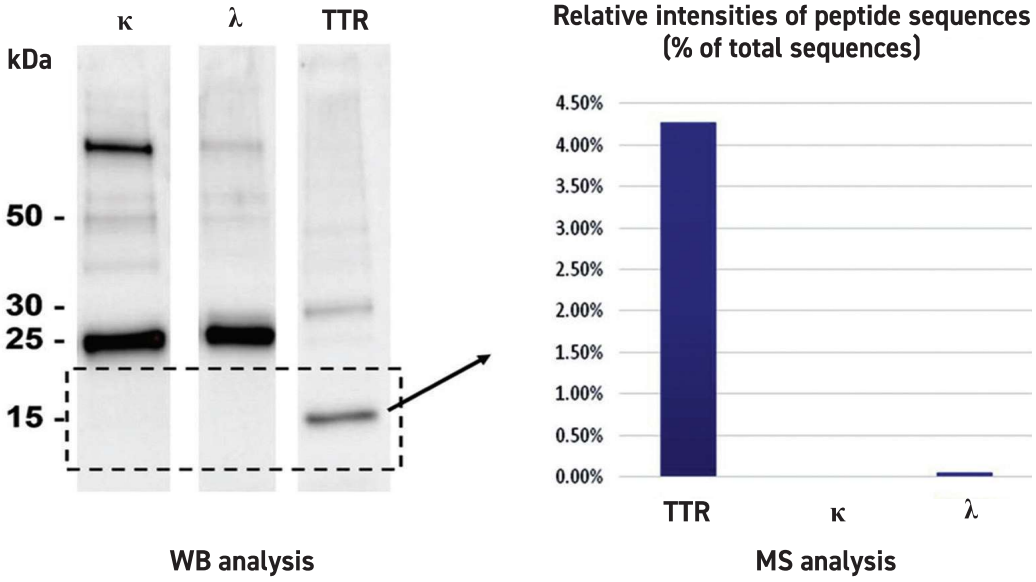


Figure 2. Patient 1. Amyloid typing using WB (left box) and MS (right box). In this and in the following blots [Figures 3 and 4] fresh-frozen cardiac biopsy tissue was extracted and run by SDS-PAGE under reducing conditions. The dashed-line area of MW < 25 kDa (characteristic of amyloid proteins) was used for interpretation of the obtained results. WB analysis: Presence of 15 kDa band reactive only with anti-TTR (but not with anti-κ and anti-λ antibodies supports the diagnosis of cardiac ATTR amyloidosis. MS analysis of the 15 kDa protein band: abundant TTR sequences (> 4% of total sequences) were identified. Only trace amounts of constant and variable domain sequences of κ and λ LC were detected.

ATTR = amyloidosis transthyretin, MS = mass spectrometry, MW = molecular weight, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, TTR = transthyretin, WB = Western blot



left ventricular hypertrophy and no delayed enhancement with magnetic resonance imaging (MRI) [Figure 2].

A heart biopsy showed Congo red positive perivascular staining and weakly positive immunohistochemistry staining for TTR. Using WB typing, the extracted proteins clearly showed the 15 and 30 kDa anti-TTR reactive bands [Figure 3, left box]. No immunoreactivity was observed with anti-κ and anti-λ in the MW region < 25 kDa. MS analysis showed that the intensity of TTR sequences identified in the 15 kDa proteins-in-gel band [Figure 3, right box] was high compared to that of light chains. No SAA sequences were found. These findings supported the diagnosis of ATTR and confirmed the WB amyloid typing results.

PATIENT 3

Patient 3 was an 84-year-old male with an indolent course of dyspnea. Scintigraphy with Tc DPD showed a Perugini grade 3 cardiac uptake, suggestive of ATTRwt. However, the nephelometric FLC assay showed markedly elevated κ FLC with an abnormal κ/λ ratio, suggesting a diagnosis of plasma cell dyscrasia. Thus, the type of amyloidosis in this patient (i.e., AL or ATTR) remained undetermined.

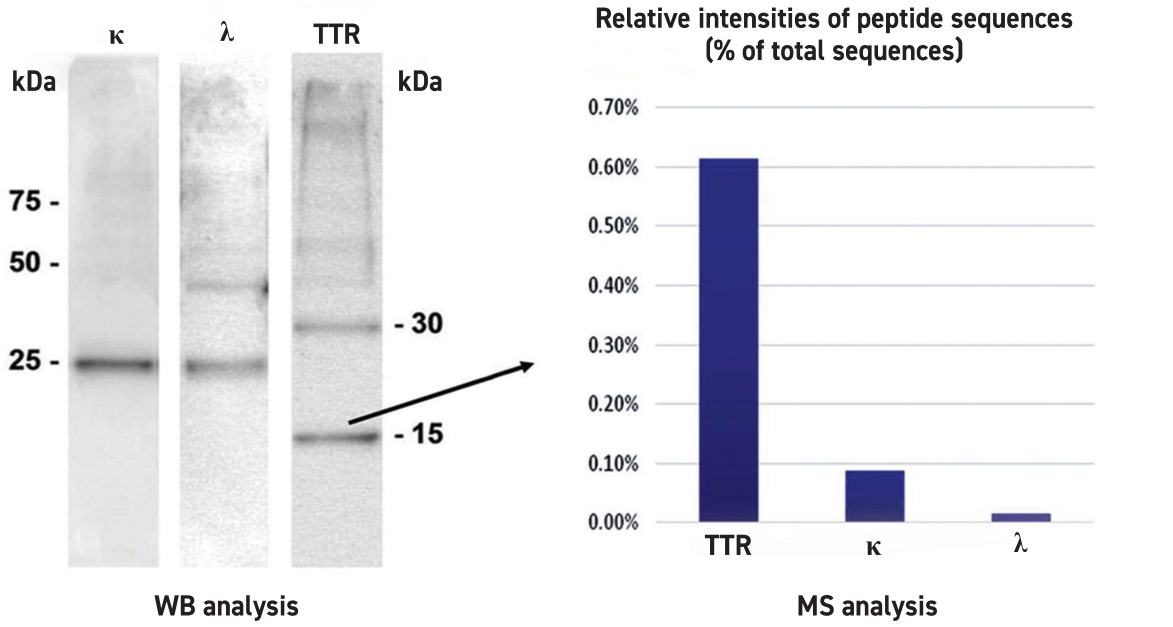
The patient underwent endomyocardial biopsy which showed Congo red positive staining. The results of amyloid typing by immunohistochemistry were inconclusive. WB typing showed that the 15 and 30 kDa bands reacted specifically with anti-TTR antibodies [Figure 4A, left box]. No immunoreactive monoclonal light chain fragments were detected using anti-κ and anti-λ antibodies. The WB data were compatible with a diagnosis of ATTR amyloidosis. MS analysis of the 15 kDa proteins-in-gel band revealed amino acid sequences characteristic of TTR. The intensity of identified TTR sequences was high compared to that of light chains [Figure 4A, right box]. No SAA sequences were detected. The obtained results confirmed the diagnosis of ATTR and were in concordance with the WB findings.

PATIENT 4

Patient 4 was an 80-year-old male who was treated for multiple myeloma with cardiac amyloidosis. Despite hematological remission, he showed progressive cardiac hypertrophy and conduction disease. Because of a +2

Figure 3. Patient 2. Amyloid typing using WB (left box) and MS (right box). WB analysis showed that the 15 and 30 kDa bands (TTR monomer and dimer, respectively) reacted only with anti-TTR antibodies, thus pointing to the diagnosis of cardiac ATTR amyloidosis. MS analysis of the 15 kDa protein band: the presence of abundant TTR amino acid sequences (comparing to those of κ and λ light chains) confirms the diagnosis of ATTR.

ATTR = amyloidosis transthyretin, MS = mass spectrometry, MW = molecular weight, TTR = transthyretin, WB = Western blot



cardiac uptake on Tc DPD bone scan, cardiac biopsy was performed to define the type of cardiac amyloid. Biopsy examination showed Congo red positive staining, but no specific immunostaining was observed by immunohistochemistry. WB analysis of the biopsy tissue extract demonstrated a15 kDa band reactive specifically with anti-λ, but not with anti-κ antibodies [Figure 4B]. No immunoreactivity with anti-TTR was observed. These findings indicated the presence of the tissue deposited monoclonal immunoglobulin light chain fragments typically observed in AL-λ amyloidosis. This diagnosis of AL amyloidosis was confirmed by Mass Spectroscopy Service at the Amyloidosis Center of Pavia University Italy (courtesy of Prof. Giampaolo Merlini).

PATIENT 5

Patient 5 was an 84-year-old male with a history of coronary artery disease and an apparently silent Crohn's disease. He was suspected of having cardiac amyloidosis because of compatible cardiac MRI findings and monoclonal gammopathy but had no evidence of plasma cell dyscrasia on bone marrow biopsy. His Tc DPD bone scan was negative. Endomyocardial biopsy was performed to confirm the diagnosis of amyloid and determine its type.

Tissue biopsy was Congo red positive. Unexpectedly, immunohistochemistry showed a positive immunoreactivity with anti-SAA antibodies and weak immunoreactivity with both anti-κ and anti-λ antibodies. No immunoreactivity for TTR was observed. WB typing: the tissue-extracted and electrophoresed proteins showed immunoreactivity to anti-AA, but not to anti-κ and anti-λ antibodies, thus indicating AA amyloidosis [Figure 4C]. The diagnosis of AA amyloidosis was established, leading to re-evaluation of patient Crohn's disease by intestinal MRI, which identified active inflammation, prompting a change in medical management.

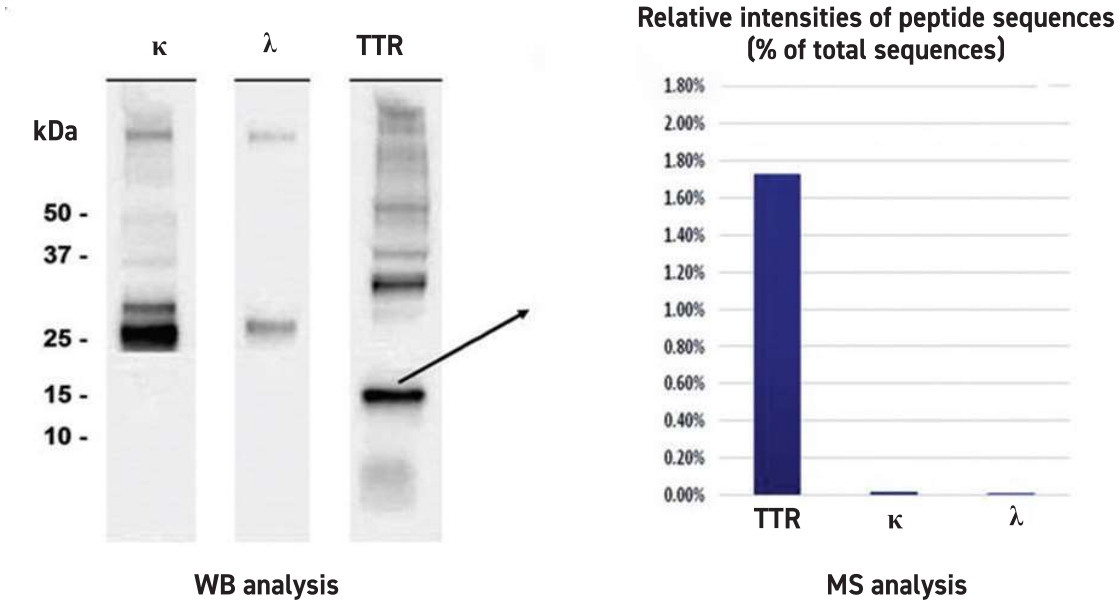
DISCUSSION

In this study, we demonstrated the diagnostic utility of our WB-based amyloid typing method in the equivocal cases of cardiac amyloidosis. We have previously applied this method successfully in different non-cardiac tissues and validated it against the standard techniques [11,13,15-17]. In this report, the WB-based approach allowed differentiation between AL, AA, and ATTR in car-

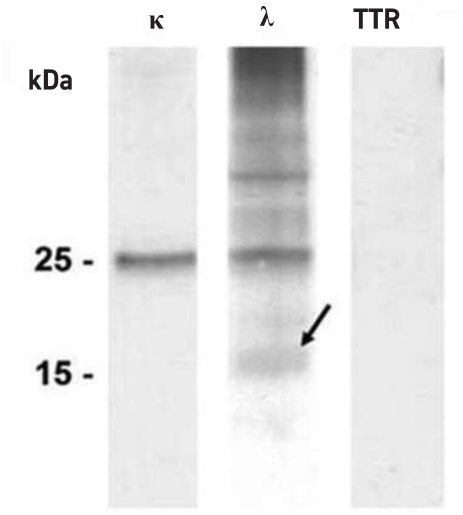
Figure 4. WB-based amyloid typing of patient 3 [A], patient 4 [B], and patient 5 [C]
[A] Specific immunoreactivity of the 15 kDa band with anti-TTR on WB points to the diagnosis of ATTR amyloidosis. MS analysis revealed highly abundant TTR peptides sequences, thus supporting the WB results.
[B] The 15 kDa band reacted specifically only with anti-λ indicating the diagnosis of AL.
[C] The 8 and 16 kDa bands reacted only with anti-AA, consistent with AA amyloidosis.

AA = amyloid A, ATTR = amyloidosis transthyretin, MS = mass spectrometry, MW = molecular weight, TTR = transthyretin, WB = Western blot

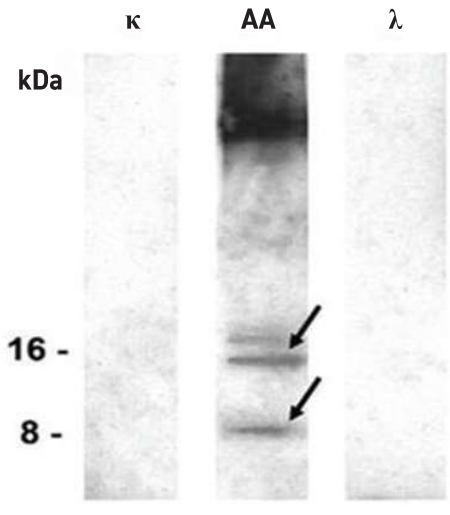
[A] Patient 3



[B] Patient 4



[C] Patient 5



diac biopsies by relying on the specific immunoreactivity of the extracted and electrophoretically resolved proteins and their characteristic MW. The obtained results were confirmed using MS technique.

The WB-based amyloid typing method is cheaper and more readily available compared to the complex and expensive gold standard typing methods. Notably, it is more sensitive and specific than the routine immunohistochemistry techniques. Furthermore, it allows a straightforward amyloid protein determination by MS analysis of the electrophoretically separated protein-in-gel band.

In contrast to the existing amyloid typing methods, our technique involves extraction of amyloid proteins from biopsy tissues prior to their subsequent analysis. Such an approach offers a useful platform for both the immunochemical and proteomic examination of the extracted tissue material. In this study, proteomic analysis was used to confirm our WB amyloid typing data. We found that electrophoretic separation of extracted proteins simplifies the preparation of sample for MS analysis by avoiding expensive and laborious laser micro-dissection of amyloid fibrils used in the proteomic gold standard amyloid typing method. The electrophoretically separated proteins-in-gel bands were then processed and analyzed in a collaborating MS facility in Israel. For accurate MS data interpretation, we determined relative intensities of the identified sequences matching different amyloid precursor proteins (κ and λ light chains, TTR, and SAA). For example, in the ATTR patient 3, MS analysis of the extracted heart tissue proteins showed that the intensity of identified TTR sequences was 100 times higher compared to that of κ or λ sequences, while no SAA sequences was detected. These data suited the WB findings. The 15 kDa band (MW characteristic of TTR monomer) reacted specifically only with the anti-TTR antibodies, while no immunostaining with anti-κ and anti-λ antibodies was observed in the MW region characteristic to light chain fragments (< 25 kDa) typically observed in AL.

We found that WB-based amyloid typing was especially useful in the analysis of fresh-frozen rather than formalin-fixed cardiac biopsy tissues. Although extraction of amyloid proteins from formalin-fixed paraffin-embedded tissues is possible using formic acid [14-18], the yield of the recovered amyloid proteins was low compared to that using fresh-frozen cardiac biopsies. Since just a minute amount of cardiac tissue is needed for amyloid typing by our technique, we suggest obtaining a tiny portion of a freshly collected biopsied material to be fresh-frozen for further analysis. This procedure does not require sophisticated equipment and can be performed in an ordinary clinical laboratory using easily available commercial antibodies to the common amyloid precursor proteins.

From a clinical perspective, our method is especially applicable to patients with cardiac TTR amyloidosis because specific therapy is currently available for this entity. Up to 10% of elderly patients with amyloid cardiomyopathy have a concomitant monoclonal gammopathy creating a differential diagnosis between ATTRwt and AL amyloidosis. Furthermore, AL may cause a false positive uptake on bone scintigraphy. Considering the availability, reliability and low-cost of the WB amyloid typing, our method may provide a solid basis for diagnostic service accessible to patients with amyloidosis in Israel [3-5,20].

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Capsule

CD19 CAR T-cell therapy in autoimmune disease: a case series

Muller and colleagues evaluated 15 patients with severe systemic lupus erythematosus (SLE) (8 patients), idiopathic inflammatory myositis (3 patients), or systemic sclerosis (4 patients) who received a single infusion of CD19 chimeric antigen receptor (CAR) T cells after preconditioning with fludarabine and cyclophosphamide. The median follow-up was 15 months (range 4–29). The mean duration of B-cell aplasia was 112 ± 47 days. All the patients with SLE had DORIS remission. All the patients with idiopathic inflammatory myositis had an American College of Rheumatology/European League Against Rheumatism (EULAR) major clinical response, and all the patients with systemic sclerosis had a decrease in the score on the EUSTAR activity index. Immunosuppressive therapy was completely stopped in all the patients. Grade 1 cytokine release syndrome occurred in 10 patients. One patient each had grade 2 cytokine release syndrome, grade 1 immune effector cell–associated neurotoxicity syndrome, and pneumonia that resulted in hospitalization. The authors concluded that CD19 CAR T-cell transfer appeared to be feasible, safe, and efficacious in three different autoimmune diseases, providing rationale for further controlled clinical trials.

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Capsule

Case fatality risk among individuals vaccinated with rVSVΔG-ZEBOV-GP in patients with confirmed Ebola virus disease in Congo

The rVSVΔG-ZEBOV-GP vaccine constitutes a valuable tool to control Ebola virus disease outbreaks. In this retrospective cohort study **Coulborn** and colleagues assessed the protective effect of the vaccine against death among patients with confirmed Ebola virus disease. The authors analyzed all 2279 patients with confirmed Ebola virus disease. Of these 2279 patients, 1300 were female (57%) and 979 were male (43%). Vaccination significantly lowered case fatality risk (vaccinated: 25% [106/423] vs. not vaccinated: 56% [570/1015]; $P < 0.0001$). In adjusted analyses, vaccination significantly lowered the risk of death compared with no vaccination, with protection increasing as time elapsed from vaccination to symptom onset (vaccinated ≤ 2 days before onset: 27% [27/99], adjusted relative risk 0.56 [95% confidence interval 0.36–0.82, $P < 0.0046$]; 3–9 days before onset: 20% [28/139], 0.44 [0.29–0.65, $P < 0.0001$]; ≥ 10 days before onset: 18% [12/68], 0.40 [0.21–0.69; $P < 0.0022$]; vaccination date unknown: 33% [39/117], 0.69 [0.48–0.96; $P < 0.0341$]; and vaccination status unknown: 52% [441/841], 0.80 [0.70–0.91, $P < 0.0001$]). Longer time from symptom onset to admission significantly increased the risk of death (49% [1117/2279], 1.03 [1.02–1.05; $P < 0.0001$]). Cycle threshold values for nucleoprotein were significantly higher indicating lower viraemia among patients who were vaccinated compared with those who were not vaccinated. The highest difference was observed among those vaccinated 21 days or longer before symptom onset (median 30.0 cycles [IQR 24.6–33.7]) compared with patients who were not vaccinated (21.4 cycles [18.4–25.9], $P < 0.0001$).

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