*original paper*
*Haematologica 1995; 80:409-415*

# DIAGNOSTIC APPROACH TO AND FOLLOW-UP OF DIFFICULT CASES OF AL AMYLOIDOSIS

***Vittorio Perfetti*°, Pietro Garini*, Maurizio Colli Vignarelli*, Maria Gabriella Marinone*, Irene Zorzoli*, Giampaolo Merlini****

**Research Laboratories of Biotechnology; Clinical Immunology°, I.RC.C.S. Policlinico S. Matteo, Department of Internal Medicine, Section of Internal Medicine and Medical Oncology, University of Pavia, Italy*

**ABSTRACT**

***Background.*** **Routine electrophoretic analysis fails to detect a monoclonal component (MC) in a considerable portion of AL amyloidosis patients. We investigated whether the combination of immunofixation (IF) on agarose gel electrophoresis and bone marrow plasma cell (BMPC) light chain κ/λ ratio analysis could contribute to diagnosis in these cases. The possible use of the BMPC κ/λ ratio in monitoring the clone was also investigated.**

***Methods.*** **We performed BMPC κ/λ ratio analysis and IF of serum and urine in 16 selected patients with no detectable MC at routine analysis, despite clinical features suggestive of primary amyloidosis. An anti-idiotypic monoclonal antibody specific for the amyloidogenic immunoglobulin and the BMPC κ/λ ratio were used to monitor the clone in a patient who underwent autologous peripheral blood stem cell transplantation.**

***Results.*** **Abnormal κ/λ ratios were found in 14 (sensitivity 87.5%), and a MC in 12 (sensitivity 75%). Combination of the two analyses confirmed diagnosis in all cases. In one patient changes in the size of the clone, monitored on serial bone marrow aspirates by an anti-idiotypic antibody, paralleled variations of the κ/λ ratio.**

***Conclusions.*** **This study demonstrates that the combined use of IF and the BMPC κ/λ ratio is extremely powerful in AL amyloidosis. In addition, the BMPC κ/λ ratio should be considered for monitoring the amyloidogenic clone when serum or urine MC is not quantifiable.**

*Key words: AL amyloidosis, plasma cell κ/λ ratio, immunofixation*



Diagnosis of AL amyloidosis can be very difficult.[1] Monoclonal components (MC) in the serum or urine, frequently consisting of free light chains (LC) only, can be present in minimal amounts and masked by polyclonal immunoglobulin or massive non-selective proteinuria, respectively. Therefore success strongly depends on the sensitivity of the electrophoretic techniques employed. Furthermore, a distinction should be made between primary and multiple myeloma-associated amyloidosis, since the latter is usually characterized by higher levels of MC which do not generally represent a diagnostic problem. Earlier studies employing immunoelectrophoresis found a MC in about 50%,[2] 63%,[3] 71%,[4] 85%,[5,6] of patients with primary amyloidosis. After the introduction of immunofixation (IF), a MC was found in 90% of patients with AL amyloidosis at the Mayo Clinic.[7]

Calculation of the bone marrow plasma cell (BMPC) LC κ/λ ratio can also be helpful in the diagnosis of AL amyloidosis. Demonstration of a clonal excess of PC[4,8-11] confirmed clinical diagnosis of primary amyloidosis in up to 85% of apparently MC-negative patients at immunoelectrophoresis.[10]

We exploited the diagnostic capabilities of a

*Correspondence: Giampaolo Merlini, M.D., Istituto di Clinica Medica II, University Hospital/I.R.C.C.S. Policlinico S. Matteo, p.le Golgi 2, 27100 Pavia, Italy, Tel. international +39.382.502947, Fax international +39.382.526223.*
*Acknowledgments: this work was supported by Associazione Italiana Ricerca Cancro (A.I.R.C.), Italian Ministry of Health (project n. 261RFM92/02), C.N.R. target projects A.C.R.O. and Biotechnology (grants n. 94.01322.PF39, 94.01195.PF39, 93.0134.PF70), Fondazione Ferrata-Storti, and I.R.C.C.S. Policlinico S. Matteo.*
*Received June 16, 1995; accepted July 29, 1995*

sensitive electrophoretic procedure, IF on high resolution agarose gel electrophoresis, and of the BMPC κ/λ ratio in 16 selected patients with biopsy-proven amyloidosis; the clinical histories and symptoms of this group suggested primary amyloidosis, but no MC could be found in the serum or urine at the Centers collaborating in our national protocol for the study and treatment of amyloidosis. Furthermore, we investigated how and to what extent the BMPC κ/λ ratio values are affected by changes in the clone size and, therefore, whether this technique could be useful in the follow-up.

## *Materials and Methods*

### *Patients*

Sixteen patients with biopsy-proven amyloidosis were studied. Primary amyloidosis was suspected on the basis of clinical histories and presentation, despite the fact that repeated routine analyses performed in other Centers participating in the national study on amyloidosis failed to detect MC in the serum or urine. Hereditary amyloidosis was excluded on the basis of family history. Table 1 reports the characteristics of the 16 patients.

Table 1. Characteristics of the 16 patients with AL amyloidosis.

| *Patients* | *Sex* | *Age (yr.)* | *Biopsy* | *Clinical manifestation** |
|---|---|---|---|---|
| 1 | M | 62 | heart | HF |
| 2 | M | 58 | heart | HF |
| 3 | M | 54 | rectum | anorexia, diarrhea |
| 4 | M | 48 | heart | HF |
| 5 | F | 47 | kidney | nephrotic syndrome |
| 6 | F | 55 | heart | HF |
| 7 | F | 34 | rectum | HF |
| 8 | M | 65 | kidney | nephrotic syndrome |
| 9 | M | 73 | kidney | nephrotic syndrome |
| 10 | F | 55 | kidney | nephrotic syndrome |
| 11 | M | 57 | heart | HF |
| 12 | M | 59 | heart | HF |
| 13 | M | 63 | heart | HF |
| 14 | M | 65 | heart | GI bleeding, HF |
| 15 | M | 52 | kidney | nephrotic syndrome |
| 16 | F | 53 | liver | dyspepsia |

**predominant clinical manifestation at diagnosis. HF: heart failure; GI: gastrointestinal.*

### *High resolution agarose gel electrophoresis and immunofixation*

Agarose gel electrophoresis was performed as described.[12] IF on agarose gel was performed according to Alper and Johnson.[13] Undiluted rabbit antisera specific for human heavy and LC classes (Dako) were used for IF. The sensitivity of IF for MC as performed in our laboratory is 10 μg/mL. This result was obtained by serial dilutions of a pool of purified MC. Using these techniques in our laboratory a MC was detected in 103 out of 109 primary amyloidosis patients (sensitivity 94.5%). Patients with only free LC in the serum and/or urine constituted 34% of the total. λ LC were 2.32 times more frequent than κ. This series also included 15 of the 16 patients investigated in the present study: patient #3 had associated myeloma and was therefore excluded.

### *Determination of the immunoglobulin LC κ/λ ratio in bone marrow plasma cells*

All patients gave informed oral consent to BM aspiration. Analysis of the BMPC κ/λ ratio was performed by double-staining immunofluorescence. Ficoll-Paque (Pharmacia)-separated BM mononuclear cells were washed four times with RPMI 1640 medium (Sigma) at 37°C in order to reduce the background staining caused by serum immunoglobulins.[14] High cellular density cytocentrifuge slides were prepared, air dried, fixed in acetone for 2 minutes at 4°C and exposed to fluorochrome-conjugated antisera to human κ (FITC) and λ (TRITC) LC (1:50 final dilution) (Dako) for 30 min at room temperature. After two washings (10 min each) with PBS, PC were identified by their intense cytoplasmic staining using a Zeiss epifluorescent photomicroscope. Five hundred PC were counted for each patient. Morphology was evaluated on parallel slides stained with May-Grünwald Giemsa.

As controls for the normal κ/λ ratio, 14 marrows from BM donors and 5 from subjects without lymphoproliferative diseases or MC were examined following the same procedures. The

κ/λ ratios were not distributed normally, and the median was 1.65 (range 1.10-2.60). In addition, the κ/λ ratios were also calculated on PC from four reactive (AA) amyloidosis, two familial (AF) amyloidosis and one nonamyloidotic fibrillary glomerulopathy patient;[15] normal values were obtained in each case (see ***Results*** section). Other authors have reported slightly different normal κ/λ ratio values: Gertz *et al.*[10] found a median of 2.0 (range 1.0-4.0), Thielemans *et al.*[8] a mean of 1.3, whereas Gallo *et al.*[6] presented only a range of 1.6-2.2. The reasons for these discrepancies are unknown; however, it should be pointed out that previous studies did not use the double-staining technique on the same slide, rather reactions for κ and λ light chains were run on parallel slides with comparable cellular density. When very few PC are present, such as in normal BM, the double-staining technique may be more reliable. Furthermore, the relatively limited number of controls analyzed in our study as well as in those of other authors may contribute to these variations.

Positive controls for an abnormal κ/λ ratio included: PC from 14 multiple myeloma (8 IgGκ, 4 IgGλ, 1 IgAλ, 1 Bence Jones κ), 15 AL amyloidosis with detectable MC (5 IgGλ, 4 IgGκ, 2 IgAλ, 3 Bence Jones λ, 1 Bence Jones κ), 2 Waldenström macroglobulinemia (IgMk) and one case of benign monoclonal gammapathy (IgGκ). Of this series, only two patients with AL amyloidosis (both IgGκ) had a κ/λ ratio in the normal range (1.14 and 1.20), while λ amyloidosis patients had a median κ/λ ratio of 0.085 (range 0.01-0.35). The sensitivity of the κ/λ ratio for AL amyloidosis in the control group was 86.7%.

*Monitoring the amyloidogenic clone with the bone marrow plasma cell κ/λ ratio and with an anti-idiotypic monoclonal antibody*

Patient SCH was a 52-year-old male with cardiac AL amyloidosis (biopsy proven). He underwent 4 BM aspirates in 16 months: three before conditioning chemotherapy (melphalan 180 mg/m$^2$) for autologous peripheral blood stem cell transplantation, and the fourth four months later. An anti-idiotypic (Id) MoAb (12D11) was obtained and characterized as previously reported.[16,17] In order to monitor changes in the amyloidogenic clone the following parameters were considered: 1) BMPC percentages (%) on May-Grünwald Giemsa-stained slides; 2) BMPC κ/λ ratios; 3) Id+ PC% on total λ+ PC. This last value was calculated by double-staining immunofluorescence with anti-Id MoAb, revealed by TRITC-labelled rabbit anti-mouse Ig antiserum, and FITC-conjugated rabbit anti-human λ LC polyclonal antibodies (both from Dako). Densitometric quantification of the patient's MC was not possible because of overlapping with transferrin on serum electrophoresis.

## *Results*

*Immunofixation on high resolution agarose gel electrophoresis and bone marrow plasma cell κ/λ ratio in the diagnosis of AL amyloidosis*

Table 2 reports the results of the analyses (IF and BMPC κ/λ ratio) performed on 16 patients with amyloidosis whose clinical history and presentation were strongly suggestive of AL, but for whom routine immunoelectrophoretic techniques had failed to detect a MC.

Twelve of the 16 AL amyloidosis patients were found to have a MC at IF on agarose gel electrophoresis (sensitivity 75%): ten λ and two κ LC. Bence Jones proteinuria was present in all; seven also had free LC in the serum. Only one patient (#9) also showed a complete MC (IgAλ) in the serum.

κ/λ ratio analysis revealed a clonal expansion of PC in 14 of 16 patients and also showed abnormal results in three MC-negative κ (Table 2, patients #14-16; Fig. 1A), one MC-negative λ amyloidosis (Table 2, patient #11; Figure 1B), even in the presence of normal numbers of BMPC. Two patients (Table 2, patients #1 and 2) presented normal results despite urinary free monoclonal λ LC (Figure 2). The sensitivity of the BMPC κ/λ ratio was 87.5%, similar to that obtained by others (85%).[10]

Combination of the two analyses confirmed clinical diagnosis in all 16 cases, whereas these procedures were negative in the 6 control non-AL amyloidosis patients (Table 2, patients #17-

Table 2. Results of the analyses performed for the diagnosis of amyloidosis.

| *Pts.* | *PC%* | *Clinical diagnosis* | *Monoclonal component* | *κ/λ ratio* |
|---|---|---|---|---|
| 1 | 3 | AL | sλLC+BJλ | 1.20 |
| 2 | 4 | AL | BJλ | 1.36 |
| 3 | 32 | AL+MM | sλLC+BJλ | 0.01 |
| 4 | 12 | AL | sλLC+BJλ | 0.08 |
| 5 | 3 | AL | BJλ | 0.06 |
| 6 | 9 | AL | sλLC+BJλ | 0.14 |
| 7 | 15 | AL | sλLC+BJλ | 0.12 |
| 8 | 6 | AL | BJλ | 0.80 |
| 9 | 7 | AL | sIgAλ+sλLC+BJλ | 0.13 |
| 10 | 6 | AL | sλLC+BJλ | 0.07 |
| 11 | 4 | AL | neg | 0.09 |
| 12 | 13 | AL | BJκ | 49 |
| 13 | 7 | AL | BJκ | 22.81 |
| 14 | 10 | AL | neg | 16.24 |
| 15 | 6 | AL | neg | 9.87 |
| 16 | 4 | AL | neg | 61.5 |
| Controls | | | | |
| 17 | 2 | AA | neg | 1.40 |
| 18 | 5 | AA | neg | 1.51 |
| 19 | 8 | AA | neg | 1.99 |
| 20 | 4 | AA | neg | 1.40 |
| 21 | 8 | AF | neg | 1.15 |
| 22 | 6 | AF | neg | 1.72 |
| 23 | 5 | FG | neg | 1.45 |

*Normal values for κ/λ ratio are ≥ 1.1; ≤ 2.6. sλLC= serum λ light chains; BJ: Bence Jones proteins; FG = non amyloidotic fibrillary glomerulopathy.*

22), in the case of nonamyloidotic fibrillary glomerulopathy (Table 2, patient #23), and in the 19 controls (see ***Materials and Methods***). Eleven patients were found to have λ, and 5 κ amyloidosis. Pathology results obtained by κ/λ ratio analysis revealed that the sensitivity of IF was higher for λ (10/11) than for κ LC (2/5).

*Bone marrow plasma cell κ/λ ratio in the follow-up of AL amyloidosis*

Table 3 reports the analyses performed in order to assess whether the κ/λ ratio could be used to follow changes in clone size during disease course and therapy. This analysis demonstrated that the parameters paralleled.

Overlapping of the patient's MC with transferrin on serum electrophoresis prevented its densitometric quantification.

Table 3. Changes in bone marrow plasma cell percentage (% PC), idiotype-positive plasma cells (% Id+ PC) and the κ/λ ratio in patient SCH.

| *Parameters* | *Time 0* | *4 months* | *12 months* | *16 months** |
|---|---|---|---|---|
| %PC | 5 | 7 | 10 | 2 |
| %Id+PC | 2.5 | 4 | 6.4 | 0.9 |
| κ/λ ratio | 0.34 | 0.23 | 0.18 | 0.65 |

**4 months after high-dose chemotherapy.*

## *Discussion*

The percentage of AL patients with a MC varies according to the electrophoretic methodology employed. In negative cases, a diagnosis of AL amyloidosis can still be obtained by calculating the BMPC κ/λ ratio.[10]

In this study we exploited the diagnostic capability of an IF technique which has been routinely used in our laboratory since 1986 (sensitivity in AL amyloidosis control cases: 94.5%, see ***Materials and Methods***), as well as that of the BMPC κ/λ ratio (sensitivity in AL amyloidosis control cases: 86.7%, see ***Materials and Methods***). The objects of the study were 16 selected patients who had been referred to us by ten Centers collaborating in our national amyloidosis protocol because, despite a clinical suspicion of AL amyloidosis, no MC had been found in serum or urine at repeated routine analyses performed in their laboratories.

Only IF was capable of demonstrating a MC in 12 of these 16 patients (sensitivity: 75%), whereas high resolution agarose gel electrophoresis was negative. Free LC are more difficult to detect than complete MC because the former are usually present at much lower concentrations and are frequently detected only in the urine; eleven of the twelve IF-positive patients showed just free LC (91.7%), five of whom demonstrated Bence Jones proteins exclusively. The frequency of AL patients with a MC consisting of free LC in 109 consecutive, non selected patients was much lower (34%, see ***Materials***

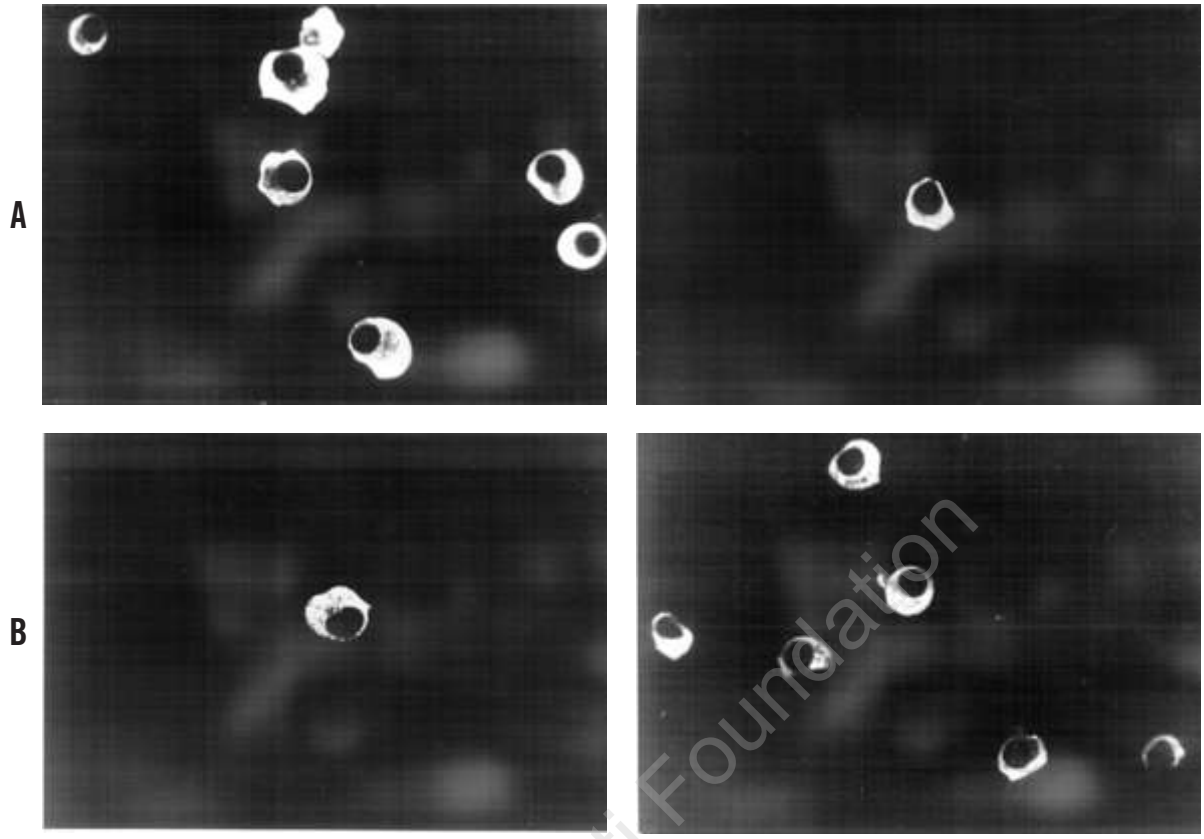


Figure 1. Bone marrow plasma cell κ/λ ratio by immunofluorescence analysis.
Left column: κ; right column: λ. A: Patient #15, B: Patient #11. Both were negative at IF on agarose gel electrophoresis.

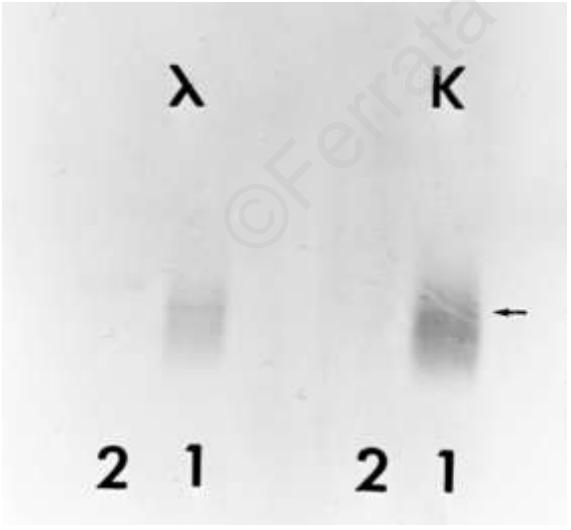


Figure 2. Immunofixation on agarose gel electrophoresis demonstrating free monoclonal light chains in a patient (#2) with a normal BMPC κ/λ ratio; a faint λ light chain is present in row 2 (concentrated urine). Row 1: serum. The arrow indicates the point of sample deposition. No monoclonal heavy chains were detected with antisera to the various classes (data not shown).

***and Methods***). These results explain the difficulties encountered by general hospital laboratories in detecting a MC. This series of patients represents those *difficult* primary amyloidosis cases reported in the literature.[1,5]

It is important to stress that IF sensitivity was higher for λ (10/11) than for κ LC (2/5). This is probably due to the lower background staining of polyclonal λ LC at IF since their concentration is about half of that of κ.

Most patients with AL amyloidosis had nephrotic syndrome or proteinuria higher than 0.3 g/L; for this reason concentration of urine samples was not performed except in three cases (patients #2, 7, 13). It has been reported that concentration of urine with massive proteinuria may mask minute amounts of monoclonal free LC.[8]

Calculation of the κ/λ ratio in BMPC was extremely sensitive, with only two cases showing a normal result, despite serum and/or uri-

nary λ LC. The κ/λ ratio proved better than IF in this series (sensitivity: 87.5 vs. 75%).

Combined use of the two methods allowed diagnosis in all 16 *difficult* primary amyloidosis cases. In certain AL cases we suggest first performing IF on high resolution agarose gel electrophoresis; if this is negative, then determination of the κ/λ ratio should be performed. These tests are in fact complementary. IF detects increased amounts of MC and its sensitivity is influenced by the following factors: a) the amount of secreted MC; b) its rate of catabolism, kidney excretion and tissue deposition; c) LC class (higher sensitivity for λ LC); d) the amount of polyclonal Ig. The κ/λ ratio depends on the clone size. That the BMPC κ/λ ratio is not influenced by the same factors as IF and therefore that the two tests are independent is clearly demonstrated by the fact that the sensitivities of the κ/λ ratio were almost identical in the group of 16 selected *difficult electrophoresis* patients and in the 15 unselected patients used as positive controls (87.5 and 86.7%, respectively). If the clone is a high LC producer, a few PC will suffice to support the disease. These few monoclonal PC may be difficult or impossible to detect within a polyclonal PC population. If, on the other hand, the clone is a low secretor, the circulating MC may be very low or undetectable, even if the clone is well expanded in the bone marrow and therefore easily recognizable by monospecific antisera.

In patient SCH, using an anti-Id MoAb we obtained a formal demonstration that abnormal κ/λ ratio values were due to the amyloidogenic clone, and we found a very good correlation between changes in this parameter over time and variations in the number of Id+ PC (see Table 3). At time 0, amyloidogenic PC constituted 50% of total PC. This accounted for the altered κ/λ ratio observed (0.34). Id+ PC were only 2.5% of total BM cells. It must be emphasized that these numbers of clonal cells are below the detection limits of Southern blot analysis,[18] as well as those of most diagnostic PCR methods based on the amplification of rearranged DNA heavy chain variable regions,[19] which are usually around 5-10%. Changes in the PC%, Id+ PC% and the κ/λ ratio over 16 months followed the same trend. Slight increases in the Id+ PC% were accompanied by detectable changes in the κ/λ ratio during the period without treatment. High-dose chemotherapy for autologous peripheral blood stem cell transplantation – an approach previously described by Majolino *et al.*[20] – resulted in a seven fold decrease in the number of amyloidogenic PC; the κ/λ ratio improved markedly but did not reach normal values. Id+ PC still constituted almost half of the BMPC population and only 0.9% of total BM cells. This result demonstrates that the BMPC κ/λ ratio can detect the persistence of Id+ PC.

In conclusion, this study shows that the combination of IF on high resolution agarose gel electrophoresis and κ/λ ratio analysis of BMPC is an extremely powerful tool in the diagnosis of AL amyloidosis. Furthermore, κ/λ ratio analysis can reliably detect even small changes in clone size and may be useful in patient follow-up.



## *References*


1. Gertz MA, Kyle RA. Primary systemic amyloidosis - a diagnostic primer. Mayo Clin Proc 1989; 64:1505-19.
2. Cathcart ES, Ritchie RF, Cohen AS, Brandt K. Immunoglobulins and amyloidosis. An immunologic study of sixty-two patients with biopsy-proved disease. Am J Med 1972; 52:93-101.
3. Vital Durand D, Touraine JL, Levrat R, et al. Immunoelectrophoretic investigations in 55 patients with systemic amyloidosis. Blut 1984; 49:91-4.
4. Preud'homme JL, Ganeval D, Grünfeld JP, Striker L, Brouet JC. Immunoglobulin synthesis in primary and myeloma amyloidosis. Clin Exp Immunol 1988; 73:389-94.
5. Kyle RA, Greipp PR. Amyloidosis (AL). Clinical and laboratory features in 229 cases. Mayo Clin Proc 1983; 58:665-83.
6. Gallo GR, Feiner HD, Chuba JV, Beneck D, Marion P, Cohen DH. Characterization of tissue amyloid by immunofluorescence microscopy. Clin Immunol Immunopathol 1986; 39:479-90.
7. Kyle RA. Primary systemic amyloidosis. J Intern Med 1992; 232:523-4.
8. Thielemans C, Aelbrecht W, Verbeelen D, Somers G, De Waele M, Van Camp B. Intracellular immunoglobulin distribution of bone marrow plasma cells as a diagnostic aid for primary amyloidosis. J Clin Pathol 1982; 35:285-8.
9. Wolf BC, Kumar A, Vera JC, Neiman RS. Bone marrow morphology and immunology in systemic amyloidosis. Am J Clin Pathol 1986; 86:84-8.
10. Gertz MA, Greipp PR, Kyle RA. Classification of amyloidosis by the detection of clonal excess of plasma cells in the bone marrow. J Lab Clin Med 1991; 118:33-9.
11. Wu SS-H, Brady K, Anderson JJ, et al. The predictive value of bone marrow morphologic characteristics and immunostain-

ing in primary (AL) amyloidosis. Am J Clin Pathol 1991; 96:95-9.
12. Merlini G, Piro P, Pavesi F, Epis R, Aguzzi F. Detection and identification of monoclonal components: immunoelectrophoresis on agarose gel and immunofixation on cellulose acetate compared. Clin Chem 1981; 27:1862-5.
13. Alper CA, Johnson AM. Immunofixation electrophoresis: a technique for the study of protein polymorphism. Vox Sang 1968; 17:445-52.
14. King MA, Wells JV. Cell-bound immunoglobulin on peripheral blood mononuclear cells of patients with myeloma. Clin Exp Immunol 1981; 45:552-6.
15. Verani RR. Fibrillary glomerulopathy. Kidney 1993; 2:63-6.
16. Bellotti V, Stoppini M, Perfetti V, et al. Use of an anti-idiotypic monoclonal antibody in studying amyloidogenic light chains in cells, urine and fibrils: pathophysiology and clinical implications. Scand J Immunol 1992; 36:607-15.
17. Perfetti V, Bellotti V, Garini P, et al. AL amyloidosis: characterization of amyloidogenic cells by anti-idiotypic monoclonal antibodies. Lab Invest 1994; 71:853-61.
18. Beishuizen A, Verhoeven M-AJ, Mol EJ, Breit TM, Wolvers-Tettero ILM, van Dongen JJM. Detection of immunoglobulin heavy-chain gene rearrangements by Southern blot analysis: recommendations for optimal results. Leukemia 1993; 7:2045-53.
19. Deane M, Norton JD. Detection of immunoglobulin gene rearrangement in B cell neoplasias by polymerase chian reaction gene amplification. Leuk Lymphoma 1991; 5:9-22.
20. Majolino I, Marcenò R, Pecoraro G, et al. High-dose therapy and autologous transplantation in amyloidosis AL. Haematologica 1993; 78:68-71.