

Letter to the Editor

Laura Caponi*, Maria Franzini, Elona Koni, Silvia Masotti, Mario Petrini and Aldo Paolicchi

Discrepancy between FLC assays: only a problem of quantification?

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To the Editor,

Immunoglobulin light chains not associated with heavy chains free light chains (FLC) are found in serum. A growing clinical importance has been assigned to the quantification of the κ and λ FLC in serum in the management of plasma cell dyscrasias [1, 2].

The FLC may be detected as an isolated band on immunofixation, but this technique is unsuitable for quantification if the band is in trace amounts, nor can the FLC be quantified by protein electrophoresis unless the band significantly alters the electrophoretic profile of plasma proteins. At present, automated immunoassays are the only available techniques allowing quantitative determination of serum FLC [3].

Unfortunately, the two reagents available for FLC assay, Freelite® (The Binding Site Ltd., UK) and N Latex FLC (Siemens Healthcare Diagnostics GmbH, Germany), sometimes provide divergent results, especially for the λ FLC assay [4]. It has been proposed that the different results, unpredictably affecting individual serum samples, are due to the different reactivity of reagents against FLC oligomers that are known to be present to a variable extent in serum, especially when λ FLCs are involved [5]. Only indirect evidence has been provided for this hypothesis to date.

The clinical validity of the results of the two assays is considered equivalent but the quantitative discrepancy between the methods makes values for the two methods not interchangeable in the follow up of monoclonal

gammopathy [4, 6–8]. Improving the quantitative performance of the FLC assay might improve the clinical management of plasma cell disorders.

To establish to what extent the discrepancy between Freelite® and N Latex FLC is due to the different reactivity against oligomers, we decided to perform FLC analysis on plasma protein separated in fractions of different molecular weight obtained from the sample of a patient with multiple myeloma and elevated plasma λ FLC (Freelite 1360 mg/dL; N Latex 724 mg/dL).

The plasma sample was subjected to molecular size exclusion chromatography using a Superdex-75 10/300 column (volume: 24 mL; GE Healthcare, Little Chalfonts, UK). Fractions (0.5 mL) containing proteins in the range from 100 to 5 kDa, thus including FLC monomers and oligomers, but not intact immunoglobulins, were then collected and individually analyzed by a BNII nephelometer (Siemens) with the two FLC reagents.

Strikingly different elution profiles of FLC were seen (Figure 1A). The elution profile obtained by N Latex FLC revealed only one peak at 12.5 kDa, while Freelite® revealed a minute peak (<1% of the total reactivity) at 12.5 kDa, and almost all reactivity was concentrated in a peak at a molecular weight of approximately 50 kDa, apparently corresponding to FLC dimers. Both reagents failed to reveal a peak corresponding to the molecular weight expected for FLC monomers (25 kDa).

To check if the peaks corresponded to different forms of FLC, the same fractions were analyzed by nephelometry by an anti- λ light chains antiserum not restricted for FLC, provided by Siemens (N Antiserum to Human Ig/L-Chain, Type λ). The profile obtained was similar to the one obtained with the Freelite® (Figure 1A). To exclude a possible cross-reactivity, the same fractions were also analyzed by N Latex β_2 -microglobulin (Siemens) reagent, which showed that β_2 -microglobulin elutes later than the peak identified by N Latex FLC reagent (Figure 1A).

When a sample from the same patient, drawn 2 months later, and showing similarly discordant values of λ FLC (Freelite® 2400 mg/dL; N Latex 315 mg/dL), was treated with dithiothreitol (DTT) prior to chromatography both N Latex and Freelite® revealed only a wide peak at

*Corresponding author: Laura Caponi, Università degli Studi di Pisa, Laboratory of Clinical Pathology, University Hospital, Via Roma 67, 56126 Pisa, Toscana, Italy, E-mail: laura.caponi@med.unipi.it

Maria Franzini, Elona Koni and Aldo Paolicchi: Clinical Pathology Laboratory, University Hospital, Pisa, Italy

Silvia Masotti: Scuola Superiore S. Anna Pisa, Pisa, Italy

Mario Petrini: Haematology Unit, University Hospital, Pisa, Italy

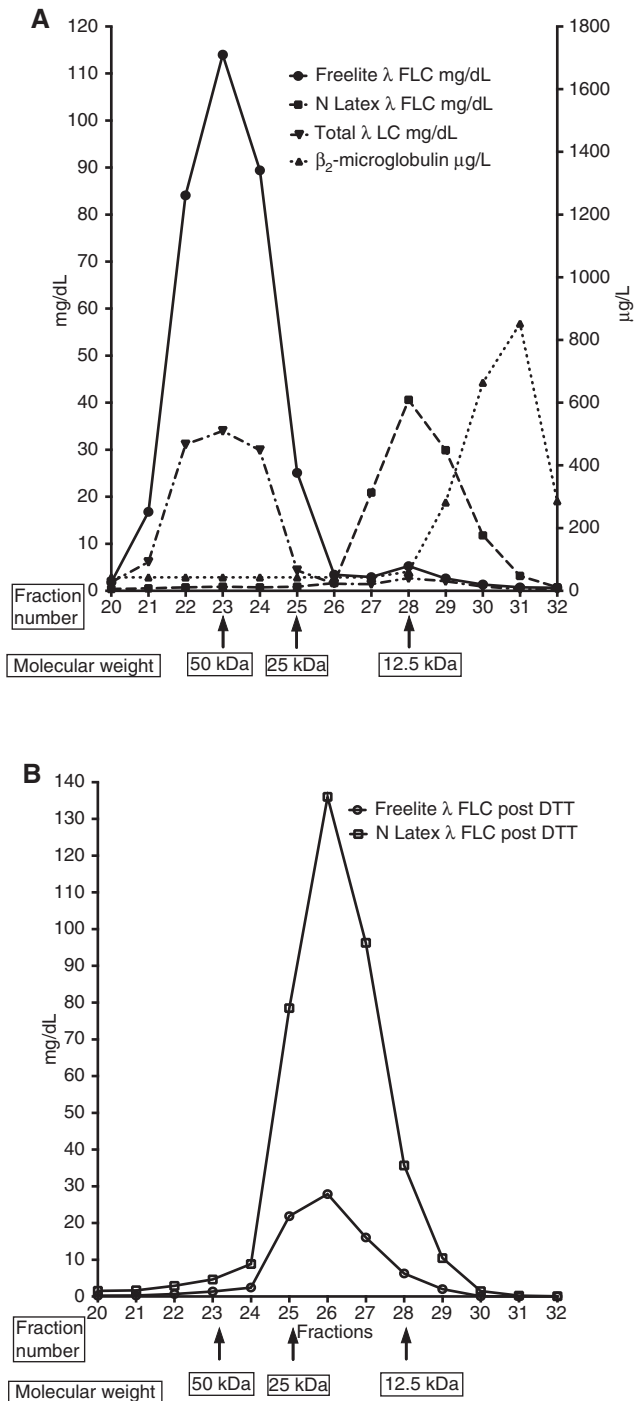


Figure 1: Gel-filtration chromatography elution profiles. The amount of λ FLC or whole λ light chains or β_2 -microglobulin measured in each fraction under non-reducing conditions are shown in (A); elution profiles after dithiothreitol (DTT) treatment of the samples and the amount of FLC measured in each fraction is plotted in (B).

25 kDa, while not only the peak at 50 kDa but also the one at 12.5 kDa had disappeared (Figure 1B).

Western blot analysis of the fractions collected was then performed by using an anti- λ light chains antiserum

as a primary antibody (Figure 2A–D). In completely non-reducing conditions (i.e. sample not treated with DTT, nor β -mercaptoethanol in the sample buffer), a high molecular weight band was seen, more abundant in fractions 23–24, corresponding to the 50 kDa peak detected by the Freelite[®] reagent, while another distinct band at a lower molecular weight appeared in later fractions, mostly in fraction 28, corresponding to the 12.5 kDa peak detected by N Latex (Figure 2A). The cross reactivity with β_2 -microglobulin was clearly also excluded by immunoblot (Figure 2D and E).

When the same fractions were loaded under reducing conditions, with β -mercaptoethanol included in the loading buffer, the same band of FLC was seen in all fractions, at a molecular weight of approximately 25 kDa, thus corresponding to the one expected for monomeric FLC. In these conditions, not only the band corresponding to FLC dimers, but also the FLC band at lower molecular weight (in Western blot appearing at 20 kDa, probably following the denaturing action of SDS) disappeared (Figure 2B).

These findings allowed us to exclude that the FLC immunoreactive protein detected by N Latex FLC at 12.5 kDa corresponds to an FLC fragment, while it is likely that intra-molecular protein disulfide bonds decrease the apparent molecular weight of the FLC monomer, which shows its expected value only upon reduction by DTT or β -mercaptoethanol.

Indeed, when we subjected it to non-reducing Western blot analysis the gel filtration fractions obtained from samples treated with DTT before chromatography, a major band of FLC was found in fraction 26, corresponding to an apparent molecular weight of 25 kDa and no immunoreactive FLC protein was found in the fractions corresponding to 12.5 kDa and 50 kDa (Figure 2C).

In conclusion, the present findings suggest that the different estimation of FLC oligomers is not the only bias of FLC assays: gel filtration chromatography allowed us to distinguish in the same sample two different molecular forms of λ FLC with different molecular weights. These forms, apparently dimer and monomer of the same monoclonal protein, besides displaying different molecular weight, display different antigenic properties, resulting in the monomeric form being selectively recognized by the N-Latex assay and the dimeric form by the Freelite[®] assay.

Thiol reduction experiments and Western blot analysis allowed us to conclude that the formation of protein disulfides between FLC monomers may hide epitopes critical for the N Latex FLC, while the Freelite[®] assay may fail to recognize FLC monomers with oxidized protein thiols.

No conclusions can be drawn concerning the nature or the position of the protein disulfides involved, and

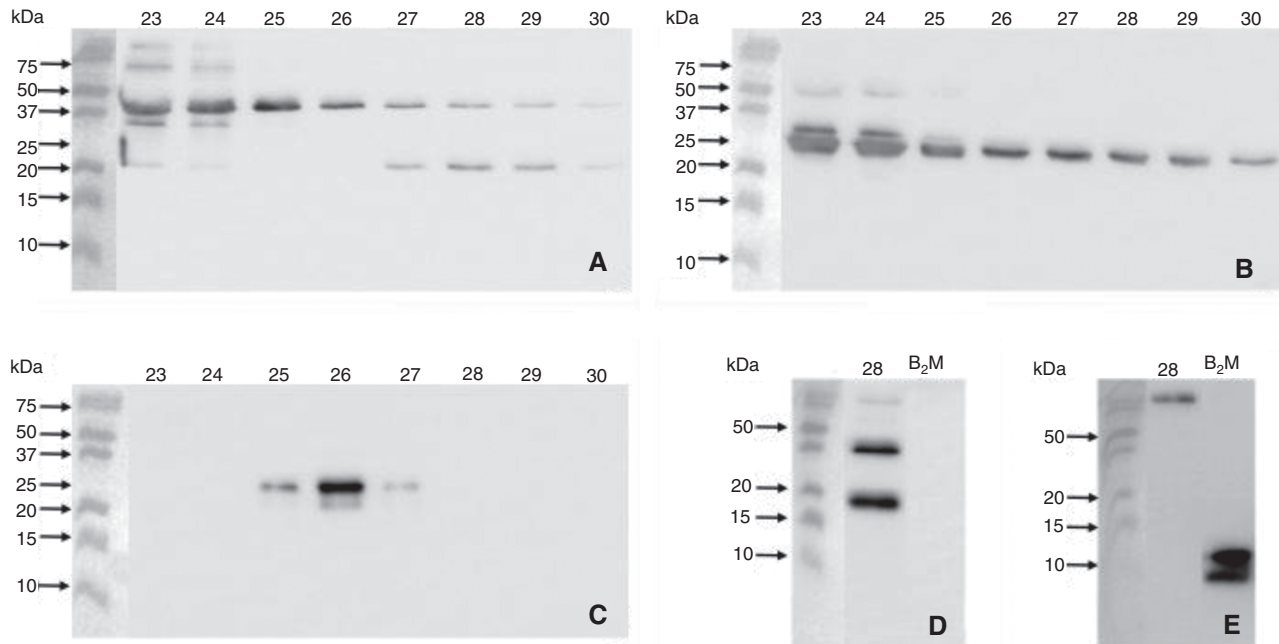


Figure 2: Western blot analysis of fractions obtained by gel-filtration chromatography. Fractions obtained by gel-filtration chromatography were loaded onto polyacrylamide gel in the absence (A) or presence (B) of β -mercaptoethanol. Fractions obtained by gel-filtration chromatography performed in the presence of dithiothreitol were loaded without β -mercaptoethanol (C). Nitrocellulose membranes (A–C) were incubated with the Siemens anti- λ antiserum not restricted for FLC as a primary antibody; Western blot analysis performed to exclude the possible cross reactivity of the N Latex λ FLC reactive with β_2 -microglobulin: in (D) the anti- λ light chains antiserum reveals FLC monomers and dimers but not purified β_2 -microglobulin; in (E) the anti- β_2 -microglobulin antiserum (anti- β_2 antiserum HRP labeled; The Binding Site) reveals only its specific antigen (B2M).

further study is needed to establish to which extent the redox chemistry of protein thiols influences FLC assay in the clinical setting, and if this is a general feature of FLC or affects only specific samples. Hopefully, such future studies will allow a better understanding of the relationship between structure and pathogenetic properties of the monoclonal FLC.

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