Clinical Chemistry 65:9
1084–1089 (2019)

Clinical Case Study

A Rapidly Deteriorating Patient with Gross Increase in Serum Free Light Chains

Angela W.S. Fung,[1*†] Mindy C. Kohlhagen,[2] Mari L. DeMarco,[1†] and John R. Mills[2]

CASE DESCRIPTION

A 71-year-old man presented with increasing confusion after dialysis. He was admitted for progressive decline in functional status over a 1-month period including delirium, a fall, and difficulty with pain, speaking, and walking. Four years ago, he was diagnosed with stage IIIB multiple myeloma with IgAκ M-protein and corresponding κ free light chain (FLC).[3] He was treated with combination chemotherapy of cyclophosphamide, bortezomib, and dexamethasone, and he achieved partial remission. Medical history was significant for myeloma-related end-stage renal failure, hypertension, osteonecrosis of the jaw secondary to bisphosphonate, and recent onset of squamous cell carcinoma.

At presentation, physical examination was unremarkable. The patient was alert and oriented, with a Glasgow coma scale of 15. Laboratory findings included hemoglobin concentration of 10.4 g/dL [reference interval (RI), 13.5–17.0 g/dL] and mean red cell volume of 114 fL (RI, 82–98 fL). Additional test results included a plasma total protein concentration of 6.5 g/dL (RI, 6.0–8.0 g/dL), albumin concentration of 4.2 g/dL (RI, 3.5–4.8 g/dL), total calcium concentration of 12.4 mg/dL (RI, 8.7–10.3 mg/dL; 3.11 mmol/L; RI, 2.18–2.58 mmol/L), ionized calcium concentration of 6.12 mg/dL (RI, 4.68–5.16 mg/dL; 1.53 mmol/L; RI, 1.17–1.29 mmol/L), phosphorus concentration of 7.3 mg/dL (RI, 2.5–5.0 mg/dL; 2.36 mmol/L; RI, 0.80–1.60 mmol/L), alkaline phosphatase concentration of 75 U/L (RI, 30–105 U/L), creatinine concentration of 10.2 mg/dL (RI, 0.68–1.13 mg/dL; 903 μmol/L; RI, 60–100 μmol/L), and blood urea nitrogen concentration of 69 mg/dL (RI, 7–22 mg/dL; 24.6 mmol/L; RI, 2.5–8.0 mmol/L).

QUESTIONS TO CONSIDER

1. What are causes of discordance between total protein and serum FLC quantification?
2. What are limitations of serum FLC assays?
3. What strategies can be used to clarify suspected inaccurate FLC results?

Serum protein electrophoresis (SPE) (Fig. 1, A and C) showed hypogammaglobulinemia, with a β region of 1.0 g/dL. Immunofixation electrophoresis identified the presence of IgAκ M-protein in the β region. Serum κ and λ FLCs concentrations were 4490.00 mg/dL (RI, 0.33–1.94 mg/dL) and 1.12 mg/dL (RI, 0.57–2.63 mg/dL), respectively, with a κ/λ FLC ratio of 4009.00 (RI, 0.26–1.65).

Three weeks later, a second SPE (Fig. 1, B and C) showed hypogammaglobulinemia but with an increased β region of 1.4 g/dL. In the β region, immunofixation electrophoresis identified an IgAκ M-protein along with a κ FLC band. The plasma total protein and albumin concentrations were 5.7 g/dL and 3.4 g/dL, respectively. Serum κ and λ FLC concentrations were 7830.00 mg/dL and 0.99 mg/dL, respectively, with a κ/λ FLC ratio of 7909.00. No urine specimens were submitted. Of note, the κ FLC and total protein concentrations were highly discordant, with the κ FLC concentration (7830.00 mg/dL or 7.83 g/dL) exceeding the total protein concentration (5.7 g/dL).

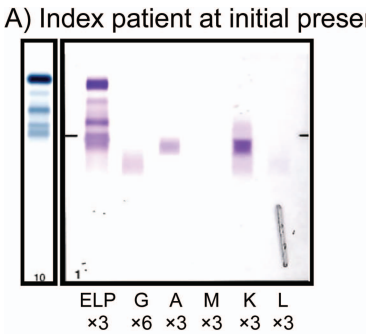
DISCUSSION

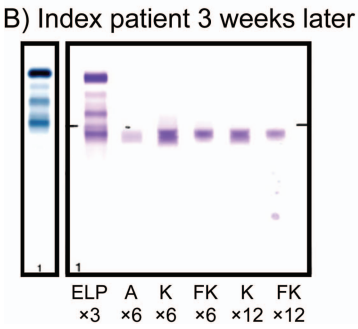
The discordance between total protein and serum FLC quantification may be explained by method-specific limitations and/or interferences. SPE coupled to total protein quantification has traditionally been used to estimate M-protein concentration. This method has limited analytical sensitivity and may either underestimate M-protein concentrations owing to dye saturation or overestimate M-protein concentrations owing to inclusion of background polyclonal immunoglobulin and/or comigrating proteins *(1)*.

Commercial serum FLC assays measure both polyclonal and monoclonal κ and λ FLCs. The calculated κ/λ

[1] Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Providence Health Care and University of British Columbia, Vancouver, BC, Canada; [2] Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, MN, USA.
* Address correspondence to this author at: Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Providence Health Care, 1081 Burrard St., Vancouver, BC, Canada V6Z 1Y6. Fax +604-806-9681; e-mail afung7@providencehealth.bc.ca.
† Member of the Society for Young Clinical Laboratorians (SYCL) (http://www.aacc.org/community/sycl).
Received November 4, 2018; accepted January 30, 2019.
DOI: 10.1373/clinchem.2018.299008

[3] Nonstandard abbreviations: FLC, free light chain; MASS-FIX, camelid-derived nanobodies enrichment-coupled MALDI-TOF mass spectrometry; RI, reference interval; SPE, serum protein electrophoresis.





C) SPE Quantification

	Initial presentation	3 weeks later
Total protein (6.0–8.0 g/dL)	6.5	5.7
Albumin (3.5–4.8 g/dL)	4.2	3.4
α 1 (0.1–0.2 g/dL)	0.2	0.2
α 2 (0.5–1.1 g/dL)	0.9	0.7
β (0.6–1.1 g/dL)	1.0	1.4
γ (0.5–1.5 g/dL)	0.2	0.2

Fig. 1. SPE (left) and immunofixation electrophoresis (right) from the index patient at initial presentation (A) and 3 weeks later (B).
For SPE, serum proteins were separated by gel electrophoresis, stained with amido black, and quantified by densitometry (C). For immunofixation electrophoresis, immunoglobulins were visualized with antisera against human immunoglobulins G, A, M, κ, λ and free κ and λ light chains. In the protein electrophoresis (ELP) lane, the proteins were stained with acid violet.

FLC ratio serves as an indirect measure of clonality. These assays are calibrated with either polyclonal or monoclonal antibodies that bind epitopes within the constant regions of light chains that are obscured in intact immunoglobulins but accessible when free or unbound from heavy chains. Quantification of monoclonal FLC is complicated by several factors: (*a*) the sequences of polyclonal and monoclonal FLCs are diverse; (*b*) FLC concentrations can vary by at least 6 orders of magnitude; and (*c*) FLC may exist in multiple quaternary structures including monomers, dimers, and higher-order polymeric aggregates. Ideally, anti-FLC antisera should be specific and differentiate unbound (free) light chains from intact immunoglobulins, maintain diverse reactivity to recognize various polyclonal and monoclonal subgroups of κ and λ FLCs, and have minimal reactivity to polymeric FLCs.

Immunonephelometric and immunoturbidimetric serum FLC assays are based on the principle of light scattering. Underestimation of serum FLCs may be a result of antigen excess or nonreactivity between anti-FLC antisera and antigen owing to, for instance, variations in the amino acid sequence or tertiary structure of the target epitope *(2–4)*. Overestimation may be caused by nonspecific interferences such as lipemia, cross-reactivity with intact light chains, and reactivity with aggregated FLCs *(2)*. Heterophile antibody interference and nonlinear dilution may result in either under- or overestimation depending on reaction parameters. Indicators of interference may include an unexpected change in the uninvolved FLC during disease monitoring, discordant FLC results between different dilutions, or a discrepancy when compared to other laboratory tests and clinical history *(2)*.

In this case, the gross increase of κ FLC concentration on 2 different collections [4490.00 and 7830.00 mg/dL (4.49 and 7.83 g/dL)] was suspected to be a false increase because it was either approximate to or greater than the total protein concentration. The general agreement between total protein quantification (6.5 and 5.7 g/dL), albumin quantification (4.2 and 3.4 g/dL, respectively), and the results of SPE excludes the presence of a large M-protein. Previous serum FLC results for this patient during routine monitoring for the past 4 years had been concordant.

The κ FLC concentration from the most recent specimen measured at 7830.00 mg/dL, which exceeded total protein concentration, was derived from a high sample dilution at 1:160 000 with the Binding Site reagent on a Siemens BNII nephelometer. To investigate the discor-

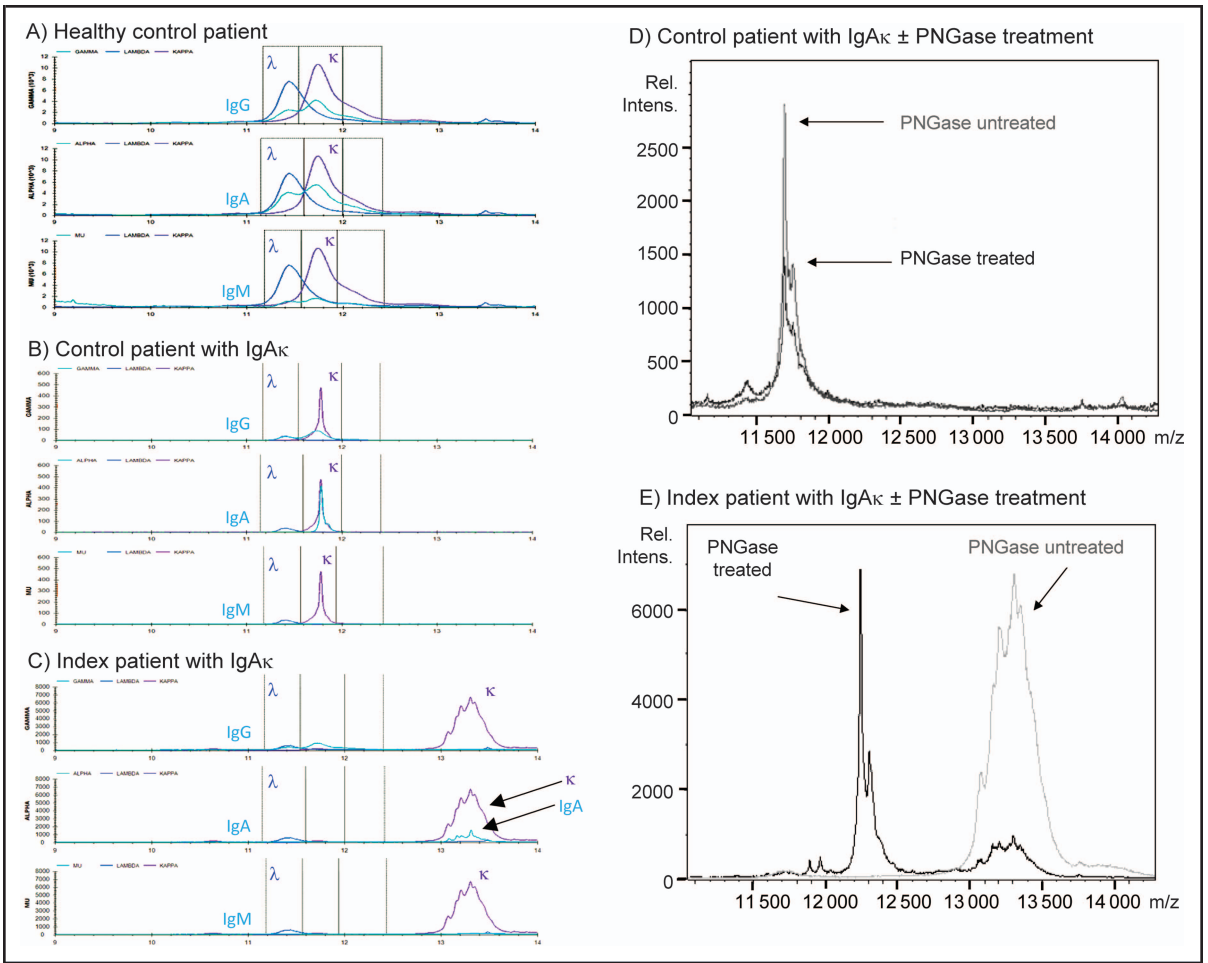


Fig. 2. MASS-FIX analysis of serum immunoglobulins from control and index patients.

(A–C), Serum immunoglobulin heavy chains G, A, M, or κ and λ light chains were enriched with nanobodies and spotted with α-cyano-4-hydroxycinnamic acid matrix for MALDI-TOF MS analysis in positive ion mode. For visualization, mass spectrum from each heavy chain is overlaid with the light chains. (D–E), MASS-FIX results with and without PNGase treatment. Four hundred microliters of serum was denatured at 50 °C in water bath for 10 min and incubated with 12.5 U of PNGase F for 2.5 h at 37 °C [lyophilized PNGase F from Sigma-Aldrich reconstituted in 20 mmol/L Tris-HCl (pH 7.5), 50 mmol/L NaCl, 5 mmol/L EDTA, and 50% glycerol]. The reaction was quenched at 75 °C for 10 min followed by MASS-FIX analysis.

dance, κ and λ FLCs were remeasured at multiple dilutions. There were no significant differences for λ FLC at different dilutions. The κ FLC concentrations varied as follows: 15.30 mg/dL at 1:100, >410.00 mg/dL at 1:2000, >1640.00 mg/dL at 1:8000, >8200.00 mg/dL at 1:40 000, and 7830.00 mg/dL at 1:160 000.

Nonlinear dilution anomalies have previously been described *(3)*. Typically, up to a 4-fold difference between calculated concentrations have been described with up to 5.4% of patients having a >4-fold difference *(3)*. Antigen excess, matrix effects, and propagation of serial dilution errors may be responsible for dilutional anomalies *(3, 4)*. Cases of grossly exaggerated increases (>10-fold difference compared to SPE) have been reported for λ FLC *(5)* and κ FLC *(6)*, in which polymerization of FLCs was demonstrated to be the cause for overestimation. Usually, κ FLCs preferentially exist as monomers and noncovalent dimers, whereas λ FLCs preferentially exists as covalent dimers *(5)*. Multimeric light-chain complexes have been reported in patients with monoclonal gammopathy and may exist as homomeric polymers or in complex with other proteins such as α_1-antitrypsin *(5)*. Polymerization has been reported to be more prevalent with monoclonal κ than monoclonal λ FLCs *(7)*. Polymerized FLCs are postulated to accelerate the nephelometric reaction, which

results in an overestimation compared to the unpolymerized calibrators *(7)*. However, the exact mechanism of polymer formation or pathophysiological relevance remains elusive *(5)*.

To better understand the discordant results in this case, serum from our index patient was further investigated by camelid-derived nanobodies enrichment-coupled MALDI-TOF mass spectrometry (MASS-FIX) *(1)*. Serum from a healthy control showed smooth gaussian-like peaks for each subtype, characteristic of polyclonal immunoglobulins (Fig. 2A). Serum from a control patient with IgAκ M-protein demonstrated sharp peaks for IgA and κ light chains, characteristic of a monoclonal IgAκ (Fig. 2B). The κ and λ light chains' $[M+2H]^{2+}$ ions in the controls were within the expected mass region of 11 100–12 500 *m/z* (boxed area). Contrary to control sera, mass spectra from our index patient's serum (Fig. 2C) showed an atypical "polytypic-like" pattern in which broad, irregular-shaped peaks (arrows) corresponding to IgA and κ light chains were shifted to a higher *m/z* than expected, characteristic of N-glycosylation *(8, 9)*. Consistent with the heterogeneity of N-glycans, N-glycosylation of FLCs appeared to increase FLC heterogeneity in serum, as demonstrated by the smearing band in SPE and the presence of multiple spikes in the mass spectra.

To investigate the presence of N-glycosylation, serum from controls and index patients, both with IgAκ M-proteins, were subject to MASS-FIX analysis with and without PNGase F treatment. PNGase F is an amidase that hydrolyzes the innermost N-acetylglucosamine of high mannose, hybrid, and complex oligosaccharides from asparagine residues of N-linked glycoproteins. Upon PNGase F treatment, the control samples (Fig. 2D) showed no change in peak shape or *m/z* shifts, whereas the index samples (Fig. 2E) showed a change in peak shape from broad, irregular-shaped peaks to a narrow peak that is shifted to a lower *m/z*, consistent with the removal of N-glycans on PNGase F treatment *(9)*. Moreover, the post-PNGase F-treated κ FLC concentration (911.00 mg/dL or 0.91 g/dL) correlated better with SPE quantification of the β region (1.0 g/dL) for the index patient.

FOLLOW-UP

This case presents a rapidly deteriorating myeloma patient with a spurious high increase in serum κ FLC concentration likely owing to N-glycosylation of the involved FLC. N-glycosylation of light chains is estimated to occur in 17%–49% of amyloidosis cases and 4%–15% of other plasma cell disorders *(8, 9)*. The pathogenic effect of glycosylated light chains has been postulated to have increased amyloidogenic potential, and it may have prognostic implications *(10)*. Glycosylation has been previously hypothesized to facilitate aggregation of FLC *(10)*, which further supports the explanation of gross overestimation of κ FLC concentration in this case. A week later, the patient succumbed to the disease.

POINTS TO REMEMBER

- Serum FLC quantification has high clinical sensitivity for screening, prognostic stratification, and monitoring for relapse.
- Analytical limitations of serum FLC assays include underestimation due to antigen excess and low affinity for certain monoclonal FLCs and overestimation due to interferences, reactivity to polymeric FLC, cross-reactivity with intact immunoglobulins, and nonlinear dilution.
- Serum FLC results are best interpreted with clinical findings and compared to supplementary laboratory tests (e.g., SPE, urine protein electrophoresis, bone marrow biopsy).
- PNGase F treatment of serum can be used in combination with the MASS-FIX assay to investigate the presence of immunoglobulin light-chain glycosylation.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: None declared.
Research Funding: None declared.
Expert Testimony: None declared.
Patents: J.R. Mills, 2017222399, 10-2018-7027703, BR 11 2018 067435 4, 17708320.1, 3,015,296, 201780013491.5, 16/078,986, PCT/GB2017/050489, 201647037235, 15/301,633, PCT/US2015/02437.

References

1. Mills JR, Kohlhagen MC, Dasari S, Vanderboom PM, Kyle RA, Katzmann JA, et al. Comprehensive assessment of M-proteins using nanobody enrichment coupled to MALDI-TOF mass spectrometry. Clin Chem 2016;62:1334–44.
2. Tate J, Bazeley S, Sykes S, Mollee P. Quantitative serum free light chain assay–analytical issues. Clin Biochem Rev 2009;30:131–40.
3. Vercammen M, Meirlaen P, Broodtaerts L, Broek IV, Bossuyt X. Effect of sample dilu-

tion on serum free light chain concentration by immunonephelometric assay. Clin Chim Acta 2011;412:1798–804.
4. Bossuyt X, Delforge M, Reynders M, Dillaerts D, Sprangers B, Fostier K, et al. Antigen excess detection by automated assays for free light chains. Clin Chem Lab Med 2018; 56:235–8.
5. Abraham RS, Charlesworth MC, Owen BA, Benson LM, Katzmann JA, Reeder CB, et al. Trimolecular complexes of lambda light chain dimers in serum of a patient with multiple myeloma. Clin Chem 2002;48:1805–11.
6. de Kat Angelino CM, Raymakers R, Teunesen MA, Jacobs JF, Klasen IS. Overestimation of serum kappa free light chain concentration by immunonephelometry. Clin Chem 2010;56:1188–90.
7. Mead GP, Carr-Smith HD. Overestimation of serum kappa free light chain concentration by immunonephelometry. Clin Chem 2010;56:1503–4.
8. Milani P, Murray DL, Barnidge DR, Kohlhagen MC, Mills JR, Merlini G, et al. The utility of MASS-FIX to detect and monitor monoclonal proteins in the clinic. Am J Hematol 2017;92:772–9.
9. Kumar S, Murray D, Dasari S, Milani P, Barnidge D, Madden B, et al. Assay to rapidly screen for immunoglobulin light chain glycosylation: a potential path to earlier AL diagnosis for a subset of patients. Leukemia 2019;33:254–7.
10. Bellotti V, Mangione P, Merlini G. Review: immunoglobulin light chain amyloidosis–the archetype of structural and pathogenic variability. J Struct Biol 2000; 130:280–9.

Commentary

Christopher R. McCudden*

Serum free light chain assays have long been known to have a variety of analytical issues. These issues include excessive lot-to-lot variability, nonlinearity, hook effect, and overrecovery. Depending on the vendor, reagents are produced as either polyclonal antibodies by immunizing sheep or monoclonal antibodies generated by hybridomas. Polyclonal antibodies rely on a mixture of free light chains for immunization, whereas monoclonal antibodies are specifically targeted toward the constant portion (CL domains) of the light chain. In either case, consistent calibration and definition of a reference standard is defied by the variability in the serum free light chain target in patients *(1)*.

Free light chains can polymerize, thus forming large immunoreactive complexes. These multireactive polymers can cause overestimation of serum free light chain concentration through excess light scattering. Gel filtration studies have identified aggregates, which have been attributed as the cause of gross overestimation of light chain concentration, at times in vast excess of the total protein concentration.

Within this case study is an elegant experiment identifying a mechanism of antigen excess. Here, the recently developed matrix-assisted laser desorption/ionization time-of-flight mass spectrometry method was used to elucidate the underlying cause of serum free light chain overestimation. Using peptide:N-glycosidase F, the authors confirm that the formation of aggregates involves N-glycosylation. N-glycosylation sites are associated with increased risk of aggregation and amyloidosis. Indeed, mutations anywhere within the immunoglobulin light chain sequence that generate N-glycosylation sites (asparagine-X-serine/threonine) have been identified as structural risk factors that increase the likelihood of amyloidosis *(2)*. Thus, in addition to explaining the cause of antigen excess, it is conceivable that the findings reported in this case study could eventually be used as a prognostic factor, in which the degree of amyloidogenicity would confer higher risk analogous to genetic studies.

Department of Pathology and Laboratory Medicine, Division of Biochemistry, University of Ottawa, Ottawa, Canada.
* Address correspondence to the author at: 501 Smyth Rd. Ottawa ON, Canada, K1H 8L6. E-mail cmccudde@uottawa.ca.
Received April 10, 2019; accepted April 24, 2019.
DOI: 10.1373/clinchem.2019.302935

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met thefollowing 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

Authors' Disclosures or Potential Conflicts of Interest: *No authors declared any potential conflicts of interest.*

References

1. Tate J, Bazeley S, Sykes S, Mollee P. Quantitative serum free light chain assay–analytical issues. Clin Biochem Rev 2009;30:131–40.
2. Poshusta TL, Sikkink LA, Leung N, Clark RJ, Dispenzieri A, Ramirez-Alvarado M. Mutations in specific structural regions of immunoglobulin light chains are associated with free light chain levels in patients with AL amyloidosis. Plos One 2009;4:e5169.