Opinion Paper

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Is accuracy of serum free light chain measurement achievable?

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Abstract: The serum free light chain (FLC) assay has proven to be an important complementary test in the management of patients with monoclonal gammopathies. The serum FLC assay has value for patients with plasma cell disorders in the context of screening and diagnosis, prognostic stratification, and quantitative monitoring. Nonetheless, serum FLC measurements have analytical limitations which give rise to differences in FLC reporting depending on which FLC assay and analytical platform is used. As the FLC measurements are incorporated in the International Myeloma Working Group guidelines for the evaluation and management of plasma cell dyscrasias, this may directly affect clinical decisions. As new certified methods for serum FLC assays emerge, the need to harmonise patient FLC results becomes increasingly important. In this opinion paper we provide an overview of the current lack of accuracy and harmonisation in serum FLC measurements. The clinical consequence of non-harmonized FLC measurements is that an individual patient may or may not meet certain diagnostic, prognostic, or response criteria, depending on which FLC assay and platform is used. We further discuss whether standardisation of serum FLC measurements is feasible and provide an overview of the steps needed to be taken towards harmonisation of FLC measurements.

Keywords: free light chains; harmonisation; monoclonal gammopathy; M-protein; plasma cell dyscrasia; standardisation.

Introduction

The monoclonal gammopathies encompass a broad spectrum of clinical disorders ranging from benign monoclonal gammopathy of undetermined significance (MGUS) to life-threatening diseases such as multiple myeloma (MM) and light chain amyloidosis. Detection and quantification of monoclonal protein (M-protein) are integral parts of the diagnosis and monitoring of these diseases and have been traditionally performed by protein electrophoresis (PE) and immunofixation (IFE) [1, 2]. In the early 2000s serum free light chain (FLC) measurement became commercially available, and since its appearance has proven to be an increasingly important complementary test in the management of patients with monoclonal gammopathies [3–5]. Nonetheless, FLC measurements have analytical challenges and limitations that are mainly caused by the FLC heterogeneity [6]. The monoclonal FLC of each individual patient is unique, which hampers exact definition of the measurand. With the reports of new FLC assays across different analytical platforms [7–11], there is an increased need to harmonise FLC measurement results. In this opinion paper we discuss whether standardisation of FLC measurements is feasible and provide an overview of the steps that need to be taken towards harmonisation of FLC measurements.

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Lack of accuracy and harmonisation in current serum FLC measurements

The first commercially available serum FLC assays (Freelite, The Binding Site Group Ltd.) used polyclonal anti-human FLC antisera in automated immunonephelometric (INA) and immunoturbidimetric (ITA) assays [3, 12].

However, quantification of serum FLC by these methods is subject to analytical issues such as inconsistency in linear responses, interference, antigen-excess, imprecision and bias due to reagent lot-to-lot variation [9, 13–21]. The FLC result is further affected by the choice of analyser on which the Freelite assay is performed. Pair wise comparisons showed that different assay platforms result in significant differences in Freelite FLC values for patient samples [6, 19], and in different reference ranges for healthy controls [22]. Therefore it is advisable to perform FLC analysis of diagnostic and follow-up samples under the same analytical conditions, using more than one dilution, and on the same instrument.

The heterogeneity in FLC measurement has become more evident by the introduction in 2011 of a second commercially available immunonephelometric N Latex FLC assay (Siemens Healthcare) which is based on monoclonal antibody reagents [11]. Studies comparing both assays have demonstrated acceptable concordance in their capacity to differentiate patients with monoclonal gammopathies from healthy controls. However, various studies have confirmed differences in absolute values between Freelite and the N Latex FLC assay in patient samples [15, 23–28]. In Figure 1 we show a schematic

Figure 1: Schematic compilation of the method comparison of Freelite versus the N Latex FLC kappa:lambda ratio. FLC ratios are presented in Log-scale. Within the FLC ratio reference ranges for healthy controls (grey shaded areas) the regression line in blue almost overlaps the line of identity ($y=x$) and the best agreement is observed. In patients with highly abnormal FLC ratios (indicated by red dotted lines at FLC kappa:lambda ratios of 0.01 and 100), a larger measurement uncertainty is observed and the regression line deviates from the line of identity, hence leading to significant differences in FLC ratios between both methods.

compilation of these currently published data on FLC kappa:lambda ratio comparisons. From this figure it can be appreciated that within the FLC ratio reference ranges for healthy controls (grey shaded areas) the regression line almost overlaps the line of identity ($y=x$) and the best agreement is observed, hence making comparisons within this reference range acceptable with only some discordance at both ends of the reference ranges. In patients with highly abnormal FLC ratios, bias is observed, as shown by the deviations from the line of identity, hence leading to significant differences in FLC ratios. Pair-wise analysis of individual samples can result in up to 10-fold differences in values between both methods.

Because the bias is not constant across the measuring range for the two FLC assays, it is not possible to apply a slope correction to harmonise their values. Therefore, methods from different vendors cannot be used interchangeably in individual patients, especially in monitoring response to therapy [11, 23, 24, 26]. In the case of a change of FLC method, it is imperative that the old and new methods are run concurrently for a period in which a new FLC baseline can be established for each individual patient monitored.

Clinical consequences of non-harmonised serum FLC measurements

The consequence of non-harmonised serum FLC measurements is that an individual patient may or may not meet certain diagnostic, prognostic, or response criteria, depending on the FLC assay and platform used.

In the International Myeloma Working Group (IMWG) guidelines for diagnosis of monoclonal gammopathies, the finding of an abnormal FLC ratio is incorporated in conjunction with serum and urine PE and IFE when screening for an M-protein [12, 29, 30]. In the updated IMWG criteria for the diagnosis of multiple myeloma, a fundamental change in terms of serum FLC interpretation was introduced. This change was based on several reports demonstrating that in patients with smouldering MM, a serum involved to uninvolved FLC ratio of at least 100 is a predictor of imminent progression to MM [31–34]. Therefore, in the new criteria a serum FLC kappa:lambda ratio ≤ 0.01 or ≥ 100 has become one of the myeloma defining events [35]. The IMWG guidelines report that the data on which these new cut-off points are determined are based on the serum Freelite assay. This change in the diagnostic criteria will directly affect therapy choice and have

serious clinical consequences. As can be seen in Figure 1, concordance between the currently available Freelite and N Latex FLC assays is acceptable in terms of abnormal versus normal FLC ratio with some discordance at both ends of the reference ranges (indicated with grey shaded blocks). A completely different situation is observed at both cut-off points of serum FLC kappa:lambda ratio 0.01 and 100 (indicated by red dotted lines). Concordance at these FLC ratio cut-off points is extremely poor. Most patients with a Freelite FLC kappa:lambda ratio of 100 or 0.01 would have an N Latex FLC kappa:lambda ratio <100 and >0.01 , respectively. The clinical consequence is that these patients may or may not meet the criteria of multiple myeloma requiring therapy depending on whether the FLC assay is performed at a diagnostic laboratory using Freelite or N Latex FLC reagents. It is also important to note that the FLC ratio in these patients is strongly affected by the value of the uninvolved FLC, which is often very low due to the immune suppressed state of the patient. FLC assay accuracy is especially problematic at these low FLC concentrations and variation of reported FLC concentrations in this low range may be very large [26, 36]. Other FLC cut-off points focus on absolute FLC concentrations and help to determine which treatment is indicated. For example, the International Kidney and Monoclonal Gammopathy Research Group recommends that serum FLC measurements are incorporated into the screening algorithm for monoclonal disease in acute kidney injury. A concentration of monoclonal serum FLC ≥ 500 mg/L in patients with acute kidney injury is indicative of tubular interstitial pathology, particularly cast nephropathy and requires initiation of disease-specific treatment to reduce FLC concentrations [37]. Concordance between Freelite and N Latex is low at a serum FLC ≥ 500 mg/L cut-off [23]. It is of note that this cut-off was based on data generated using Freelite FLC assays. Hutchison et al. indeed demonstrated that the serum FLC ≥ 500 mg/L cut-off cannot be applied when using N Latex FLC reagents [38]. Absolute FLC concentrations are also used to determine whether an individual patient is eligible for clinical trials. For patients with oligosecretory MM (serum M-protein <10 g/L; Bence Jones protein <200 mg/24 h) the IMWG introduced a criterion of measurable disease (involved-serum FLC ≥ 100 mg/L) [39], which allows an important proportion of these patients to be eligible for clinical trials.

The above mentioned problems of discordances of FLC values outside the normal reference ranges are increasingly relevant in the risk assessment and monitoring of monoclonal gammopathies. As recently reviewed by Graziani et al. each monoclonal gammopathy has risk cut-off points outside the normal FLC ranges, that are independent risk

factors predictive of progression of disease and survival [5]. For example, based on reports that demonstrate the utility of the FLC ratio to predict survival in MM patients [40–42], it was proposed to incorporate the FLC ratio into the International Staging System with cut-off points of <0.03 or >32 [40]. From Figure 1 it can be appreciated that concordance between Freelite and N Latex results is not optimal at these cut-off points. In the IMWG guidelines on risk stratification in MM, no cut-off points are applied but only the statement that the FLC ratio may be useful for prognostication in MM patients [43].

It is evident that non-harmonised serum FLC measurements cause poor between-laboratory agreement, which is demonstrated with CVs ranging from 25% to 70% in external quality assessment (EQA) programs [21, 44]. This has implications for multicenter clinical trials that are not centrally monitored, where results from different laboratories supporting these studies may not be strictly comparable.

Meanwhile, other FLC immunoassays across different analytical platforms have been developed [7–10]. Recently, van Duijn et al. have introduced mass spectrometry (MS) as a novel technological platform to measure FLC [45]. This liquid chromatography-MS method quantifies FLC independent of immunoassay, as it relies on the measurement of proteolytic immunoglobulin peptides, rather than an affinity interaction with a detection antibody. Mills et al. made use of the unique molecular mass of an M-protein and established a MS based method to detect monoclonal light chains with high sensitivity [46]. For clinical practice it is important to realise that most current guidelines are based on studies that were performed using Freelite reagents. Present data indicate that the various FLC assays cannot be used interchangeably, especially in monitoring response to therapy, which warrants clinical validation of these novel FLC assays. Assay validation in one clinical group of patients does not necessarily imply validity in all groups of patients. Chronic kidney disease (CKD) is one particular disease in which the Freelite and N Latex FLC performance is significantly different. In CKD patients with no monoclonal gammopathy it is observed that compared to healthy controls, the Freelite FLC ratio is significantly higher than the N Latex FLC ratio [15]. This further stresses the importance of clinical validation studies across various patient groups, such as in renal failure, AL amyloidosis and myeloma.

Accuracy

Accuracy refers to measurement results that reflect the measured substance's actual amount. The Vocabulary of International Metrology defines 'measurement accuracy'

as the closeness of agreement between a measured quantity value and a true quantity value of a measurand [47]. The transfer of accuracy is dependent on having available a reference measurement system consisting of higher order reference materials (RM) and reference measurement procedures (RMP) through to the manufacturer's selected and standing measurement procedures (MP) to the routine MP used by clinical laboratories [48]. Traceability of measurement results to higher order RM and RMP is achieved by an unbroken sequence of calibrations for which bias should be eliminated [49]. The use of the same RM by different manufacturers can enable different assays to give equivalent measurement results within clinically specified acceptable limits. This is provided that the measurand has been defined, remains the same throughout the traceability chain and different immunoassays have the same selectivity for the measurand [49]. In addition, commutability of RM and intermediate calibrators is required to minimise any systematic bias between measurement procedures calibrated with them. Currently there is no internationally accepted RM or RMP for serum FLC. Calibration traceability by diagnostic manufacturers is variable as shown for three commercial FLC assays in Table 1. A calibrator consisting of a pool of different monoclonal FLCs prepared for example by purification of a pool of urinary FLC (Bence Jones protein, BJP) gives increased antigenicity of an RM but is unlikely to be able to be reproduced [51]. In contrast, use of a pool of BJPs to

assess polyclonal FLC and clonally different monoclonal FLC may be flawed due to differences in antigenic expression by monoclonal FLC whereas use of a serum pool of polyclonal FLC from subjects without B-cell disorders would give a diverse range of FLC. The serum matrix better reflects clinical patient samples although it is important that there is minimal interference from other serum proteins including intact immunoglobulin light chains that are bound to heavy chains and may possibly contaminate FLC material [51]. Nakano et al. recommended therefore use of urine rather than serum as the matrix for RM; however, serum FLC may differ chemically from urine FLC due to post translational protein differences [51].

Defining the measurand for free light chain measurement

Monoclonal FLC are a heterogeneous group of protein molecules that have large charge and size variation due to the unique amino acids that constitute the variable region subgroups [52]. The isoelectric point (pI) of urinary BJP can range from acidic (≈ 4.5) to basic (8.5–9.5) pH with pI values being patient-specific (Figure 2). Amyloidogenic LCs show a wide range of charge heterogeneity, which is reflected in pI values from 5.0 to 8.6 [53]. The size of monoclonal FLC can range from low molecular weight fragments of 5–18 kDa

Table 1: Calibration traceability for three serum free light chain (FLC) assays.

Assay	Calibration materials	Value assignment measurement procedures
Freelite [3]	Purified (polyclonal FLC) material: – Monomeric FLC prepared by reduction/acetylation of serum polyclonal IgG and purified by affinity chromatography.	Amino acid analysis.
	Secondary, serum-matrixed (polyclonal FLC) material: – A pool of elevated serum FLC prepared from SLE sera.	Manufacturer's serum FLC standing MP and using purified FLC material as calibrator.
	Master and product calibrators (polyclonal kappa and lambda FLC): – SLE sera.	Manufacturer's serum FLC standing MP and using secondary FLC material as calibrator.
N-Latex [11]	Master calibrator (polyclonal kappa and lambda FLC): – Contains purified FLC in PBS and 1% human albumin.	Value-assigned against Freelite assays by manufacturer's serum FLC INA method.
	Product calibrators (polyclonal kappa and lambda FLC): – Contains purified FLC in PBS and 1% human albumin.	Value-assigned against master calibrator by manufacturer's serum FLC INA method.
Campbell et al. [7, 50]	Purified (monoclonal kappa and lambda FLC) materials: – Prepared from 9 urines with high Bence Jones protein concentration (5 kappa/4 lambda).	Absorbance at 280 nm at 1/100 dilution of freeze-dried urines redissolved in saline.
	Assay calibrators (monoclonal kappa and lambda FLC): – Diluted pools.	Equal concentrations of each kappa FLC and each lambda FLC were combined and diluted to 7 g/L.

INA, immunonephelometric assay; MP, measurement procedure; PBS, phosphate buffered saline; SLE, systemic lupus erythematosus.

Figure 2: Broad-range (pH 3–9.5) agarose gel isoelectric focusing (IEF) with acid fixation and Coomassie Brilliant Blue R staining of urines from patients with Bence Jones protein.

Arrows represent the multiple FLC forms detected (confirmed by immunofixation). The number of monoclonal FLC may vary and consist of major and minor monoclonal bands which can appear evenly spaced (like a ladder) or randomly spaced. Isoelectric points vary from highly anodic (pH 4.5–5) to highly cathodic (pH 8–9).

(found in amyloid fibrils) to monomers, dimers or higher levels of polymerisation [53, 54]. Kappa FLC exist as monomers (≈ 25 –30 kDa), non-covalent dimers (≈ 45 –50 kDa), or both, whereas lambda FLC are predominantly in the form of disulphide-linked dimers (≈ 45 kDa) although monomers (≈ 22 –25 kDa) are present as well as other higher molecular weight forms in some cases of AL amyloidosis [53].

In nonsecretory myeloma (NSMM) FLC are reported to be present in multimeric forms in addition to the usual kappa FLC monomers and lambda FLC dimers [55]. Abraham et al. have reported on monoclonal lambda FLC presenting as hexameric aggregates that cannot be cleared by renal excretion in a case of light chain myeloma (LCMM) [13]. Harding et al. noted in two MM patients that ~80%–90% of FLC was present as polymers of molecular weight between 25 and >100 kDa (monoclonal kappa FLC) and between 50 and >200 kDa (monoclonal lambda FLC) which may have prevented adequate removal of excess FLC by high cut-off haemodialysis [56]. Kaplan et al. have recently shown that monoclonal FLC from MM patients consistently display an increased degree of FLC-dimerization compared to either the monoclonal FLC in patients with MGUS or the polyclonal FLC in healthy controls [57].

The consequences of this variability of the measurand are that unless an FLC immunoassay can recognise all structural forms and conformations of the FLC with equimolar reactivity, the different forms will not produce accurate FLC measurement results and values may vary markedly for some monoclonal FLC among different FLC assays. Various studies have confirmed differences in absolute values between Freelite and N Latex immunoassays and concordance for monoclonal FLC [24, 26]. No data are available from the manufacturers of FLC assays comparing the reactivity of equal concentrations of different monoclonal FLC isolated from patient sera. Di Noto et al. compared polyclonal versus monoclonal FLC

quantification by Freelite and N-Latex assays [58]. They showed that while the assay correlation was good for polyclonal FLC (correlation coefficient ≥ 0.90) it was poor for monoclonal FLC in MM (0.56–0.82 for lambda and kappa FLC at >500 mg/L, respectively), thereby suggesting that it is the heterogeneity of the monoclonal FLC measurand which is contributing to differences in values and the lack of concordance between FLC assays. We recommend that manufacturers of serum FLC assays quantify the immunoreactivity of equal concentrations of polyclonal and monoclonal FLC obtained from various monoclonal gammopathies to determine if an assay's responses are equimolar.

Selectivity of assays for FLC measurement

Quantification by immunoassay

Anti-FLC antibodies target the constant domains of the light chains (C_L) in C κ allotypes and C λ isotypes which in general have little structural variation. FLC antibodies should have equivalent immunoreactivity for all variable region (V_L) subgroups, high specificity to differentiate FLC from bound immunoglobulin light chains, and high affinity to ensure assay reproducibility. Each monoclonal FLC differs in its structure and antigenic expression. Hence, diverse reactivity with heterogeneous FLC is required to avoid underestimating FLC concentration [51]. Examples of false negative results are where a monoclonal FLC fails to react because of a lack of antigen recognition (large monoclonal FLC diversity) or due to limited reactivity (conformation of FLC) by the anti-FLC antibody [18, 59].

Interferences in FLC assays may cause analytical false positive results. FLC antibodies must not react with epitopes hidden in intact immunoglobulins to avoid falsely elevated FLC from cross-reaction. As there is only a slight difference in antigenicity between free and bound light chains associated with heavy chains, intact immunoglobulins present in g/L in serum can potentially cross-react in FLC assays and give falsely elevated FLC concentration. Current data indicate that normal serum immunoglobulin levels cause overestimation of only ~10% (3–4 mg/L, representing a cross-reactivity of about 0.05%) at normal FLC concentrations [51]. High concentrations of intact M-protein, in contrast, interfere with FLC reactivity of the involved FLC [16].

Differences in absolute values can occur between different FLC immunoassays for some samples depending on the type of immunoassay used and the assay platform. INA and ITA assays which are based on antigen-antibody mediated complex-formation, may overestimate FLC possibly due to excess cross-linking in solution. The presence of polymeric forms of FLC may further accelerate this process and result in overestimation of FLC. It is speculated that polymerisation is more common among kappa FLC resulting in repeating antigenic epitopes compared with unpolymerised calibrator [9]. INA methods, but not ELISA, are apparently affected because there are fewer epitopes available for binding in an ELISA sandwich assay format. It is reported that Freelite kappa FLC concentrations consistently deviated from the concentrations measured with ELISA and serum protein electrophoresis and the effect became more apparent at high FLC concentrations [60, 61].

In NSMM it is reported there is a nephelometric overestimation of FLC concentration of between 1.5- and 3.5-fold for NSMM sera relative to other myeloma sera due to the presence of oligomers [55]. In LCMM the concentration of monoclonal FLC can be 10–20-fold higher than by protein electrophoresis and grossly overestimated if large aggregates of FLC are present [13]. Di Noto et al. determined the kappa and lambda FLC proportions of monomer to dimer in several monoclonal samples using non-reducing SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting and compared FLC concentrations obtained by staining SDS-PAGE gels with Coomassie Brilliant Blue versus values by Freelite and N Latex assays [58]. Their conclusions were that densitometric quantification of monoclonal FLC, i.e. total monomeric and dimeric FLC, showed a discrepancy with both FLC immunoassays and it was likely that FLC polymers do influence FLC measurement. Interestingly, the authors did a similar examination of molecular forms in normal and elevated

polyclonal FLC samples and showed a closer correlation between all FLC methods. In order to avoid the effect of FLC polymers causing overestimation of FLC by INA and ITA methods, it would be an option to pre-treat samples so as to break di-sulphide bonds and form monomeric FLC. However, this would then require clinical revalidation of assays which would be an onerous task.

Apart from differences in measurement results between manufacturers' FLC assays, different assay formats may also contribute to differences in values for the same monoclonal FLC and the same assay antibodies. Such differences are best illustrated in Quality Assurance Programs [21, 44]. For example, measurement of a monoclonal FLC kappa sample by the Freelite assay using Prospec (Siemens Healthcare, Marburg, Germany) and IMMAGE (Beckman Coulter, Brea, CA, USA) nephelometry can give quite different FLC values (Figure 3A and B). In the same line, Figure 3 demonstrates that FLC values on the same BNII analyser (Siemens Healthcare) vary depending on which FLC assay is used. Differences in sample dilutions, sample to antibody volume, and kinetics, i.e. rate vs. endpoint INA, may contribute to the variation in final FLC concentration.

Quantification by serum protein electrophoresis

Measurement of serum monoclonal FLC by densitometric quantification of the M-protein on serum PE can differ to values obtained by immunoassay [62]. Kappa and lambda FLC concentrations may be several-fold higher by immunoassay than by serum PE and occasionally are even greater than the patient's serum total protein concentration [9, 58, 60]. When measuring monoclonal FLC by densitometry from protein-stained electrophoresis gels, the M-protein may present as more than one band, or may overlay other protein bands, or because of the limited sensitivity of serum PE it may not present as a discrete band at all as occurs in NSMM. Thus FLC quantification in serum and urine may be underestimated by PE. It is generally accepted that M-protein quantification of a single discrete electrophoretic band is most accurate and reproducible when performed by the gold standard method serum PE. However, there are factors that may also affect the accuracy of serum PE quantification such as non-linearity of dye-binding due to dye saturation and differential dye binding in which the dye-binding characteristics of albumin for example is different to those of the immunoglobulins [63]. A comparison with FLC quantification using capillary electrophoresis would be interesting as this technique does not experience

Figure 3: Serum free light chain (FLC) results for an FLC kappa monoclonal patient sample assessed by the Royal College of Pathologists of Australasia Immunology Quality Assurance Program (modified from reference #44).

(A) Individual FLC kappa:lambda ratio results from 24 laboratories. (B) Mean FLC values and ranges are shown for eight platforms and two immunoassays (The Binding Site Freelite™ and Siemens Healthcare N Latex). The Binding Site reagent was used for the in-house Architect assay.

problems linked to dye-binding. IFE is a qualitative technique only and similar to the reactivity of FLC in INA and ITA the FLC antibody can give a variable reaction in the gel or not react at all.

Conclusions

From an accuracy perspective, immunoassay FLC methods cannot distinguish between FLC multimers and depending on the reactivity of monomeric and dimeric FLC, values may vary between assays and between platforms and do not indicate the actual amount of FLC. It is for these reasons that when monitoring disease response, FLC should be measured using the same assay and the

same platform. As noted by Murng et al. in their study of individual sample variability on serum FLC measurement, “It is impossible to verify the calibration in all samples on an individual basis. Each FLC is potentially a different substrate with unique properties in the assay” [21].

In summary:

- At the present time serum FLC assays are not accurate and measurement results are not equivalent between different methods.
- There is a need for improvement of serum FLC assays by manufacturers including information in kit inserts on the assay reactivity of monoclonal and polyclonal kappa and lambda FLC in specific disease groups.
- For these reasons it is important that serum FLC results are not used in isolation but interpreted in the

clinical context together with other relevant laboratory results.

- Whether accuracy of FLC measurement is achievable, it remains to be seen.

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