Analytical validation of new ELISAs for the quantitation of polyclonal free light chains and comparison to existing assays for healthy and patient samples

Jennifer L.J. Heaney[1], John P. Campbell[2], Margaret Goodall[1], Tim Plant[1], Meena Shemar[2], Christopher Hand[3], Mark T. Drayson[1]

1. Clinical Immunology Service, Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, UK.
2. Department for Health, University of Bath, Bath, UK
3. Abingdon Health Ltd., York, UK.

Corresponding author:

Dr Jennifer Heaney
Clinical Immunology Service
Institute of Immunology and Immunotherapy
College of Medical and Dental Sciences
University of Birmingham
Vincent Drive
B15 2TT
j.l.j.heaney@bham.ac.uk

Abstract

Background: Polyclonal FLCs can be used as a biomarker of inflammation and immune activation in a range of diseases. This study evaluated the performance of new FLC ELISAs (Seralite FLC ELISA) for the quantitation of polyclonal κ and λ FLC, including comparisons to existing assays.

Methods: Technical performance was assessed for the ELISA and reference ranges were generated using healthy donor serum (N=91). Patients with a range of conditions associated with polyclonal FLC dysregulation (N=164) were measured across platforms.

Results: The ELISAs generated references ranges of: 8.72– 23.0 mg/L κ FLC, and 8.52–25.24 mg/L for λ FLC. ELISAs demonstrated linearity across the calibration range and intra-assay (≤ 8.7%) and inter-assay (≤ 12.3%) imprecision was low. The limit of detection was 0.63 mg/L for κ and 0.57 mg/L for λ FLC. Minimal cross-reactivity was observed for interference agents, alternate FLC and whole immunoglobulin (median change ≤ 3.6 mg/L). Assays showed good batch-to-batch consistency. For patient samples, methods generated different κ and λ FLC concentrations and differences were seen between methods for the number of patients classified as below, with and above references ranges for κ and λ FLC. There was no significant difference in the FLC sum between the different techniques.

Conclusions: The ELISAs displayed good analytical and technical performance. The quantification of individual κ and λ FLC appears inherently different between platforms. These differences are attenuated if using the FLC sum, which was similar between methods and provided agreement in relation to patients having normal or elevated FLCs.

Key words: free light chain; polyclonal; quantitation; assay; methods; ELISA

1. Introduction

Immunoglobulins are produced by plasma cells and typically comprise two identical heavy chains and two identical light chains, of either a κ or λ isotype, with the exception of IgG4 where half-molecules exchange results in asymmetrical antibodies (Aalberse et al., 2009). Surplus free light chains are produced during the process of immunoglobulin synthesis, with production approximately 40% in excess of heavy chains (Suki and Massry, 1998). Free light chains (FLCs) that do not form whole immunoglobulins are released into the circulation until they are metabolised by the kidneys, where up to 10–30 g of FLC can be absorbed per day by the proximal convoluted tubule (Waldmann et al., 1972). The short half-life of FLCs in blood (2–4 hours for kappa and 3–6 hours for lambda) compared with whole immunoglobulins (5–8 days for IgA and IgM and 20 days for IgG) enables real time monitoring of immune suppression and stimulation, or disease progression and responses to treatment in conditions involving FLC dysregulation (Brekke and Sandlie, 2003; Davids et al., 2010). In a healthy state, depending on the assay used, serum FLC (sFLC) reference ranges lie between 3.3–22.66 mg/L for kappa and 3.7–27.0 mg/L for lambda (Katzmann et al., 2002; te Velthuis et al., 2011; Campbell et al., 2017).

FLCs are a key haematological biomarker in the diagnosis and monitoring of plasma cell disorders, where monoclonal light chains are secreted due to clonal plasma cell proliferation, usually resulting in overproduction of one type of light chain. Serum FLC analysis is used for the screening, prognosis and monitoring of multiple myeloma and other plasma cell dyscrasias, as recommended by international guidelines (Dispenzieri et al., 2009; Rajkumar et al., 2014). However, serum FLCs have been studied as a marker in a range of other diseases and polyclonal FLCs appear to be an important biomarker in their own right. Elevations in FLCs have been observed in a wide range of diseases, including rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, heart failure, diabetes, renal disease, asthma, chronic obstructive pulmonary disease, inflammatory bowel disease and HIV infection (van der Heijden et al., 2006; Nakano et al., 2011; Brebner and Stockley, 2013). These studies suggest that FLCs are useful as a marker of severity and or risk for certain diseases, monitoring disease activity and potentially predicting disease progression. In non-

clinical populations, FLCs also appear to have utility as a biomarker. The sum of kappa and lambda FLCs (FLC sum) negatively predicted survival in a large longitudinal data set of individuals aged ≥ 50 years without plasma cell malignancies. Individuals with the highest FLC sum levels had an increased risk of all-cause mortality: this risk of death remained after controlling for age, sex and renal insufficiency (Dispenzieri et al., 2012). This evidence from various diseases and the general population suggest polyclonal FLCs act as a biomarker of overall immune activation, general inflammation and infection.

Methods for the measurement of sFLCs include latex-enhanced nephelometric or turbidimetric laboratory assays, with Freelite® being the first available and most widely used laboratory assay (Bradwell et al., 2001). Alternative sFLC tests are now widely available, including Seralite®, a rapid lateral flow device (LFD) for the dual quantitation of κ and λ FLCs (Campbell et al., 2017). A new ELISA test for serum FLC has recently become available, Seralite®– FLC ELISA, which requires validation. An ELISA method has the benefits of not requiring any specialised equipment and therefore could help enhance accessibility of FLC testing. Various studies have compared different sFLC assays in relation to validating monoclonal FLC measurement, with many highlighting the differences in how they quantitate FLC (Jacobs et al., 2016; Te Velthuis et al., 2016; Campbell et al., 2017; Heaney et al., 2017a). However, there is a lack of data comparing different methods in relation to polyclonal dysregulation, despite being another important and growing application of sFLC testing. The aim of the present study was to I) review the technical and analytical performance of the FLC ELISA for polyclonal FLCs and II) compare polyclonal FLC quantitation across a range of patient samples between different platforms: ELISA, lateral flow, turbidimetry.

2. Materials and Methods

2.1. Serum free light chain quantitation methods

Concentrations of κ or λ sFLCs were measured using three different techniques*:* I) Freelite® (The Binding Site, UK) using a Roche Hitachi Cobas C501. Freelite was the first sFLC assay which became available in 2001 and uses sheep polyclonal antibodies specific for epitopes that are exposed on light chains when not bound to heavy chain. This test has undergone extensive

validation and is incorporated into the international guidelines for multiple myeloma (Durie et al., 2006; Dispenzieri et al., 2009) II) Seralite®-FLC (Abingdon Health Ltd, UK) lateral flow device (Seralite LFD). The Seralite LFD is a rapid test that quantitates serum κ and λ FLC levels simultaneously in 10 minutes using specific mouse anti-human FLC monoclonal antibodies (mAbs) (Campbell et al., 2013). This test has undergone clinical validation for diagnosis and monitoring of myeloma (Campbell et al., 2017; Heaney et al., 2017a; Heaney et al., 2017b) and development and test principle has been described in detail elsewhere (Campbell et al., 2017). III) Seralite®-FLC ELISA (Abingdon Health Ltd, UK). This test consists of two separate κ and λ FLC ELISAs and is described in detail below.

2.2. ELISA composition and procedure

The anti-human FLC mAbs utilised in the ELISAs as capture agents, BUCIS 04 (anti-κ) and BUCIS 09 (anti-λ), are the same as those used in Seralite LFD that have undergone previous validation testing. The preparation and purification of these mAbs are described in detail elsewhere (Campbell et al., 2013). The mAbs were selected based upon reactivity with a wide range of κ or λ antigens without cross-reactivity with purified whole immunoglobulins. The specificity of these mAbs for FLC has been demonstrated previously (Campbell et al., 2013; Campbell et al., 2017; Heaney et al., 2017a). Anti-human light chain (bound and free) mAbs specific for κ (BUCIS 14) or λ (BUCIS 19) are used as secondary detection antibodies in the ELISAs. These mAbs recognise epitopes distinct from those targeted by the capture mAbs and have been validated for specificity for FLC in patient samples.

The FLCs κ and λ quantitative tests are separate enzyme-linked immunosorbent sandwich assays developed for use with serum specimens. All reagents and samples are brought to room temperature prior to use and all steps take place under room temperature conditions, without a plate shaker. Microplate wells are pre-coated with anti-human immunoglobulin FLC κ or λ capture mAbs. Samples, calibrators and quality controls are added to the plates (100 µL) and incubated for 1h. The plate is then washed 3 times: filling each well with wash buffer (300 µL). Anti-κ (BUCIS 14) and anti-λ (BUCIS 19) detection antibodies conjugated to horseradish peroxidase are

added to the appropriate plate (100 µL) and incubated for 30 min. The plates are then washed as before, 3 times with wash buffer. Substrate solution (tetramethylbenzidine, TMB) is then added to each well (100 µL) and incubated for 30 min under dark conditions, protected from light. The reaction between the substrate and HRP-antibodies (bound to FLCs in the sample) is stopped by adding 1M sulphuric acid to each well (50 µL) and the plate is read at 450nm wavelength. The absorbance is directly proportional to the concentration of FLCs contained in the sample. A 8-point standard curve is generated by plotting the absorbance against known FLC concentrations (mg/L) of the calibrators, with four parameter logistical curve fitting recommended. The calibrators for the standard curve provide a measurement range from approximately 0.01–1mg/L. It is up to the user to select sample dilution to provide a measurement range appropriate for their specimens. We diluted samples initially at 1 in 200 to provide a measurement range of 2mg/L up to 180 mg/L based on the calibrators provided. The concentrations of unknown samples can be determined using their absorbance values and the standard curve. For comparison, at the recommended initial dilution, Seralite has a measuring range of 2.5–200 mg/L and limits of detection of 1.4 mg/L for κ and 1.7 m/L for λ FLC; Freelite measuring range depends on the instrumentation used but is between 2.9–6 mg/L to 56.2–190 mg/L for κ FLC and 4.5–5.6 mg/L to 74.8–165 mg/L for λ FLC with a limit of quantitation of 0.45 mg/L.

Calibrator material was developed using a pool of normal serum samples. In the absence of International Reference material, the calibrator fluid was initially tested on Seralite to obtain a starting concentration. A calibration curve was generated on the ELISA using the fluid. A set of normal serum samples were measured in triplicate and the mean Kappa and Lambda concentrations were compared to Seralite. Based on the means an adjustment factor was applied to the calibrator fluid to give a new assigned value. Curves were then made using the new assigned value and the performance was checked using the same normal serum samples. For subsequent lots of calibrator fluid, assignment is based in on the existing approved calibrator fluid. Normal serum is pooled and run as a calibration curve on 3 κ FLC and 3 λ FLC ELISA plates; this curve is run alongside the existing calibrator fluid. For each plate the new curve standard concentrations are obtained from the curves with the existing calibrator. A concentration is applied

to STD 1 by doubling the mean concentration of standard 2, which should be equal to four times standard 3, eight times standard 4 an so on. A curve is produced with the newly assigned calibrator alongside the existing and approved calibrator. A set of samples (normal, and high to cover the assay range) are measured against both curves and the concentration on both curves are compared. The percentage difference between FLC values obtained from the new and current curves must be <10% for the assignment of the new calibrator to be approved.

2.3. ELISA assay dynamics

Linearity was assessed using serum samples containing either elevated κ or λ FLC and serially diluting in assay buffer. Samples were diluted in percentage decrements (from 90%–1.25%) and tested on the ELISAs; each dilution was tested in duplicate. The mean obtained values were plotted against the expected linear results to assess non-linearity.

Imprecision was assessed using 4 samples for κ and 4 samples for λ that contained lower end of normal, upper end of normal, elevated or high levels of FLC. Samples contained the following FLC levels: κ= 8.8/26.3/58.7/112.4 mg/L and λ = 9.4/33.6/77.1/114.3 mg/L. Each sample was tested in duplicate, on 3 plates, across 3 days (9 assays in total) using a single batch of plates.

A 'blank' concentration was obtained by measuring 20 replicates of assay buffer alongside a normal serum sample that had been serially diluted in percentage decrements (50%-5%) and tested on the ELISAs. The lowest κ and λ concentration of the normal sample detectable above the mean blank value was selected as the limit of detection.

Common interference agents of were added to serum samples containing normal κ and λ FLC levels. Purified agents (Sigma Aldrich, UK) were spiked individually into the serum samples at the following final concentrations: bilirubin (0.2 g/L), cholesterol (2 g/L), haemoglobin (2 g/L) and triglyceride (10 g/L). Purified immunoglobulins (University of Birmingham Monoclonal Antibody Production Service) were also added separately to the serum samples at the following final concentrations IgG-κ (0.73 g/L), IgG-λ (0.32 g/L), IgA-κ (1.48 g/L), IgA-λ (0.15 g/L), IgM-κ (0.45 g/L), IgM-λ (0.38 g/L), κ FLC (0.53 g/L) or λ FLC (0.73 g/L). Median change from the expected concentration of the normal samples after adding each interference agent was determined. The

concentrations of agents were selected based on thresholds typically used in the literature e.g. bilirubin 200 mg/L, triglyceride 5-10.0 g/L.

Variability between batches of ELISA kits was evaluated by tested 67 serum samples with a range of FLC levels using three consecutive lots of kits for both κ and λ. Data was analysed using Passing and Bablok regression and the difference for each sample was calculated between each batch to report a mean difference between batches in mg/L.

2.4. Reference ranges

91 serum samples that had been obtained from random donors from the NHS Blood and Transplant service (NHSBT Birmingham, UK) were used to generate reference ranges for the ELISAs for κ and λ FLC. These samples had previously undergone screening for renal function and monoclonal gammopathies.

2.5. Patient samples

A range of patient samples were retrospectively analysed using the ELISAs and LFD, to compare sFLC quantitation and diagnostic sensitivity between the laboratory platform and the portable lateral flow device. Serum samples from 164 patients without monoclonal gammopathies were evaluated. Patents were received by the Clinical Immunology Service (University of Birmingham, UK) and pertained to either diagnosis, follow-up or routine patient monitoring. Samples were stored at -20°C and had been through 2 cycles of freeze-thaw at the time of analysis in the present study. These patients had conditions associated with polyclonal FLC dysregulation: rheumatoid arthritis (n = 18); systemic lupus erythematosus (n = 16); Sjögren's syndrome (n =23); recurrent infections (n = 9); nephrotic syndrome (n =18); vasculitis (n = 11); B-cell non-Hodgkin lymphomas (n = 12), chronic lymphocytic leukaemia (n =13), acute kidney injury (n = 46). For all patients, κ and λ FLCs were measured. For polyclonal dysregulation patients, typically both κ and λ FLCs are elevated and the FLC sum, shown to be of importance in studies of polyclonal FLC (Dispenzieri et al., 2012), is reported throughout. The κ:λ ratio is typically not used for diagnostic purposes in relation to polyclonal dysregulation but is reported to enable comparison in future studies/cohorts with plasma cell dyscrasias.

2.6. Statistical Analyses

To evaluate differences between the 3 methods, Friedman tests were used to evaluate if significant differences were evident for FLC parameters, with Dunn's multiple comparisons tests where appropriate. Spearmans rank correlation was used to look at the strength of associations between the different sFLC techniques. Assay lot-to-lot variability between the 3 batches was assessed using Passing and Bablok regression analysis. Analysis was conducted using the Microsoft Excel add-in Analyse-it software (version 4.60, Method Evaluation, www.analyse-it.com) for Passing Bablock and linearity analysis and IBM SPSS (Version 21) to tests differences between methods. SigmaPlot version 12.0 (SystatSoftware Inc., USA) and GraphPad Prism (GraphPad Software Inc., USA) were used to produce figures.

3. Results

3.1. Assay dynamics

Samples from random blood donors were tested to obtain references ranges for the ELISAs (Table 1). The ranges are based upon the 5–95th percent range: 8.72– 23.0 mg/L κ FLC, 8.52–25.24 mg/L λ FLC. Results from the blood donors were measured across the three tests (Figure 1). There was a significant difference between the tests for both κ FLC ($\chi^2 = 29.1$, $p < .001$), where the Seralite ELISA returned higher values compared with Seralite LFD and Freelite; there were no significant differences between Seralite LFD and Freelite. For λ FLC ($\chi^2 = 19.8$, $p < .001$), Freelite was significantly different to the two other methods. For the FLC sum ($\chi^2 = 36.4$, $p < .001$), all methods differed between each other. For the κ:λ ratio ($\chi^2 = 13.1$, $p < .001$), the LFD differed between Freelite and the ELISA.

Table 1. FLC values and ranges in healthy random donors obtained using the Seralite ELISA, Seralite LFD and Freelite

	κ FLC (mg/L)	λ FLC (mg/L)	FLC sum	κ:λ ratio
Seralite ELISA				
Median	14.7	13.8	28.4	1.0
Min	6.8	7.3	15.1	0.5
Max	37.1	33.6	68.4	1.90
5^{th}–95^{th} percentile	8.7–23.0	8.5–25.2	18.7–45.5	0.65–1.6
Seralite LFD				
Median	11.7	13.4	25.0	0.9
Min	4.3	5.0	12.0	0.3
Max	31.9	34.0	53.5	2.5
5^{th}–95^{th} percentile	6.8–20.0	7.6–23.3	16.3–41.6	0.4–1.8
Freelite				
Median	11.2	11.3	22.4	1.0
Min	4.5	6.6	13.3	0.4
Max	27.4	17.6	45.0	1.6
5^{th}–95^{th} percentile	6.2–18.2	8.1–15.9	15.1–34.4	0.7–1.4

Samples with FLC concentrations at the upper end of the calibration range were serially diluted in sample buffer. Each concentration was measured on the ELISA. κ FLC was linear between 6 and 135.8 mg/L and λ FLC between 3.7 and 142.4 mg/L. The maximum difference between expected linear and observed concentrations was 14.9% for κ FLC and 18.2% for λ FLC. The limit of detection was 0.63 mg/L for κ and 0.57 mg/L for λ FLC.

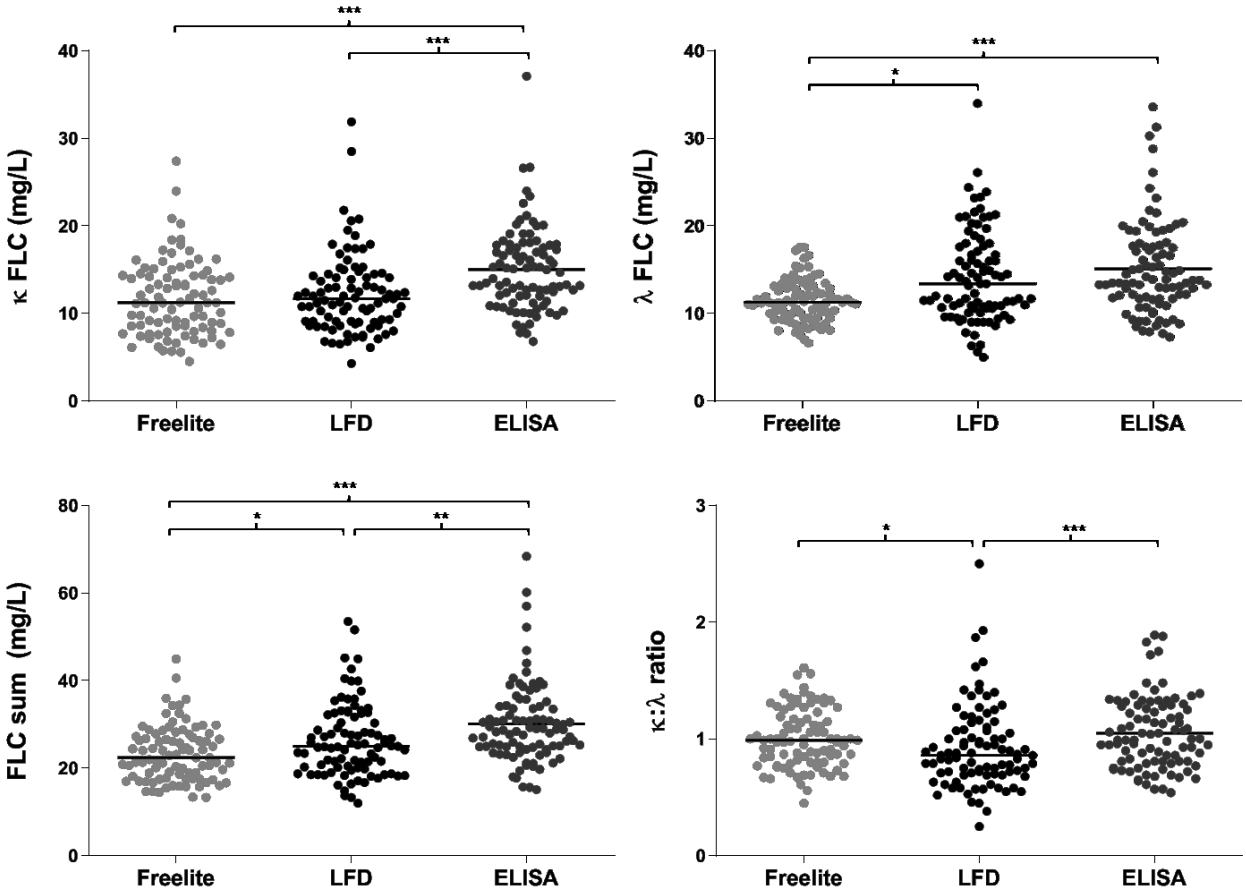

Figure 1. κ and λ serum FLC levels, the FLC sum and κ:λ ratio in random donors measured using different FLC methods

Data is shown for 91 serum samples obtained from apparently healthy random donors measured using Freelite, Seralite LFD test and Seralite ELISAs. Line indicates the median. *p > .05' **p <.01; ***p <.001

Three samples with normal, upper limit of normal, elevated and highly elevated samples for κ and λ FLC were assessed and corresponding precision data is presented in Table 2. Both within- and between-plate imprecision was low for the κ and λ ELISAs. Across the 4 samples, intra-assay CV was between 3.5–8.7% for κ FLC and 3.2–4.8% for λ FLC. Inter-assay precision was between 5–8% for κ FLC and 6.7–12.3% for λ FLC.

Table 2. Imprecision analyses of Seralite ELISAs.

CV %	**Intra-assay**	**Inter-assay**
κ FLC		
Lower end of normal (8.8 mg/L)	3.5	5.0
Upper end of normal (26.3 mg/L)	6.2	8.4
Above normal (58.7 mg/L)	4.1	7.3
Elevated (112.4 mg/L)	8.7	8
λ FLC		
Lower end of normal (9.4 mg/L)	3.3	7.8
Upper end of normal (33.6 mg/L)	3.2	6.7
Above normal (77.1 mg/L)	4.8	12.3
Elevated (114.3 mg/L)	4.4	9.8

Results from interference testing are reported in Table 3. No more than a median change of 1.2 mg/L for κ FLC and 1.3 mg/L for λ FLC was observed when adding common interference agents (bilirubin, cholesterol, haemoglobin, and triglyceride) to a normal sample. In addition, minimal cross-reactivity was observed with light chains bound to whole immunoglobulin and alternate FLC, with 3.6 ≤ mg/L median change for found for both κ and λ when adding IgG, IgM, IgA or alternate FLC to a normal sample.

Table 3. Seralite ELISA interference data showing median change from expected concentration in FLC level when common interference agents were added to serum samples containing normal FLC levels.

	Median change (mg/L)
κ FLC	
Haemoglobin (2.0 g/L)	-1.2
Bilirubin (0.2 g/L)	0.1
Cholesterol (2.0 g/L)	1.2
Triglyceride (10.0 g/L)	0.7
λ FLC (0.73 g/L)	2.7
IgG κ (0.84 g/L)	-0.2
IgA κ (1.48 g/L)	3.2
IgM κ (0.45 g/L)	2.3
λ FLC	
Haemoglobin (2.0 g/L)	-1.3
Bilirubin (0.2 g/L)	-0.3
Cholesterol (2.0 g/L)	0.6
Triglyceride (10.0 g/L)	0.1
K FLC (0.53 g/L)	2.5
IgG λ (0.32 g/L)	1.3
IgA λ (0.15 g/L)	3.6
IgM λ (0.38 g/L)	0.7

Samples with a range of FLC levels across the calibration curve were assessed using 3 separate lots for κ and λ. Passing and Bablok regression analysis returned the following slopes (95% CI) and intercepts (95% CI), respectively: 0.97 (0.90 to 1.07) and -0.04 (-0.65 to 0.42) for batch 1 vs 2; 0.90 (0.76 to 1.00) and -0.40 (-1.05 to 0.27) for batch 1 vs 3; 0.90 (0.78 to 1.02) and -0.25 (-1.05 to 0.31) for batch 2 vs 3 for κ FLC; 1.05 (0.97 to 1.13) and -0.02 (-0.51 to 0.33) for batch 1 vs 2; 0.92 (0.86 to 1.02) and 0.03 (-.37 to 0.22) for batch 1 vs 3; 0.92 (0.86 to 0.95) and -0.10 (-0.37 to 0.02) for batch 2 vs 3 for λ FLC. The mean differences between batches (mg/L) and CV (%) were: 4.4 mg/L and 11.1% (A vs B) , 3.3 mg/L and 15.9% (A vs C), 4.0 mg/L and 11.4% (B vs C) for κ FLC; 1.6 mg/L and 7.5% (A vs B), 1.8mg/L and 7.6% (A vs C) and 2.3 mg/L and 10.7% (B vs C) for λ FLC.

3.2. Patient samples

Serum from patients with a range of clinical disorders associated with dysregulated polyclonal FLC secretion and or clearance were compared between the ELISA and the other established FLC tests, Seralite LFD and Freelite. Differences in absolute FLC concentrations between the assays are shown in Figure 2. Significant differences were found between Freelite and both the ELISA and LFD for κ FLC ($p < .001$ for both comparisons): κ FLC were higher on Freelite compared to the other methods. Conversely, for λ FLC, Freelite values were significantly lower than both methods and the LFD was also significantly lower than the ELISA ($p < .001$ for all significant comparisons). These opposing higher and lower differences for κ and λ FLCs meant that when the FLC sum was calculated, there was no statistical difference in this parameter between the methods ($p = .17$). For the κ:λ ratio, Freelite was significantly higher compared to the LFD and ELISA and ($p < .001$); the LFD was also significantly higher compared to the ELISA ($p < .05$).

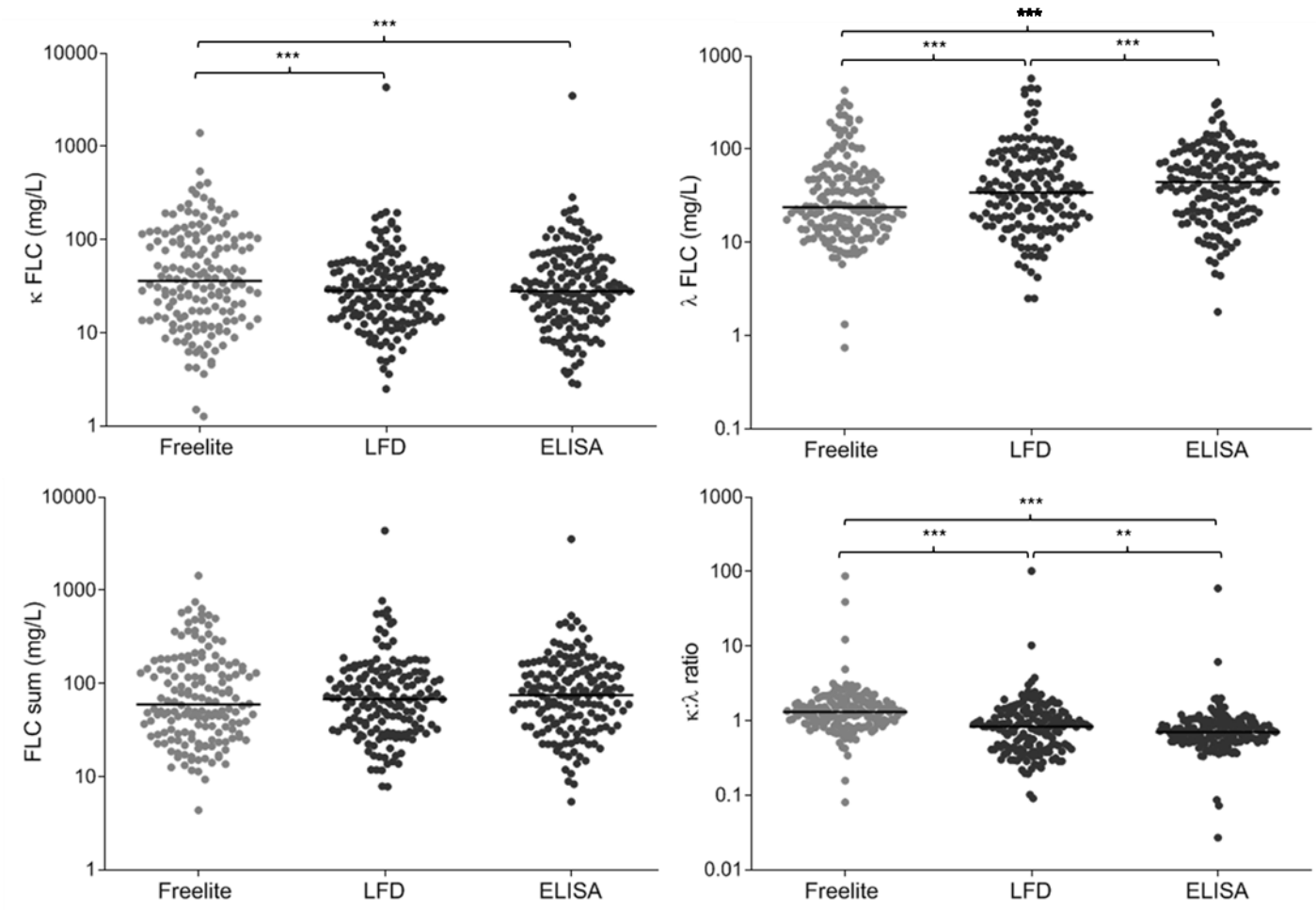

Figure 2. Patient samples κ FLC, λ FLC, FLC sum and κ:λ ratio according to different FLC quantitative methods

Data is shown for patients with a range of conditions associated with polyclonal FLC dysregulation (n = 164). Lines indicate the median. κ FLC levels were higher on Freelite compared to the other methods (p < .001). λ FLC levels were lower on Freelite compared to other methods; the LFD was also significantly lower than the ELISA (p < .001 for all significant comparisons). There was no significant difference between the methods for FLC sum. Freelite returned a higher κ:λ ratio compared to the other methods (p < .001); the κ:λ ratio by LFD was also significantly higher compared to the ELISA (p <.05).

Figure 3 illustrates κ and λ FLC quantitation on the ELISA compared to Freelite and the LFD for individual patients. The majority of patients had FLC levels either within or above the reference ranges of the assays but as the reference lines indicate, concordance was not always seen for individual patients. As shown in Table 4, differences were seen between the methods in the proportion of patients below, within and above the reference ranges of each technique. There was a difference in the distribution of patients below, within and above the reference range for κ FLC ($X^2 = 28.8$, $p < .001$): a higher number of patients fell above the κ FLC reference range for Freelite compared to the LFD and ELISA, and also for the ELISA compared to the LFD. The distribution of patients in relation to reference ranges was also significantly different between the methods for λ FLC ($X^2 = 30.6$, $p < .001$). In contrast to κ FLC, a lower number of patients were above the reference range for Freelite compared to the LFD and ELISA. The LFD was also lower than the ELISA. There was no significant difference between the methods for the number of patients in the reference range categories for FLC sum. The same proportion of patients (70.1–71.3%) had an elevated for FLC sum across the methods. The κ:λ ratio showed a significant difference in the number of patients classed as normal/abnormal ($X^2 = 6.6$, $p < .05$): Freelite returned a higher number of patients with normal ratios compared to the LFD and ELISA. Differences between methods were also confirmed by Passing-Bablok regression (Table 5). Overall, systematic and proportional differences were seen between all methods across FLC parameters. However, no differences were observed in the case of FLC sum for ELISA vs LFD and for the κ:λ ratio for Freelite vs LFD.

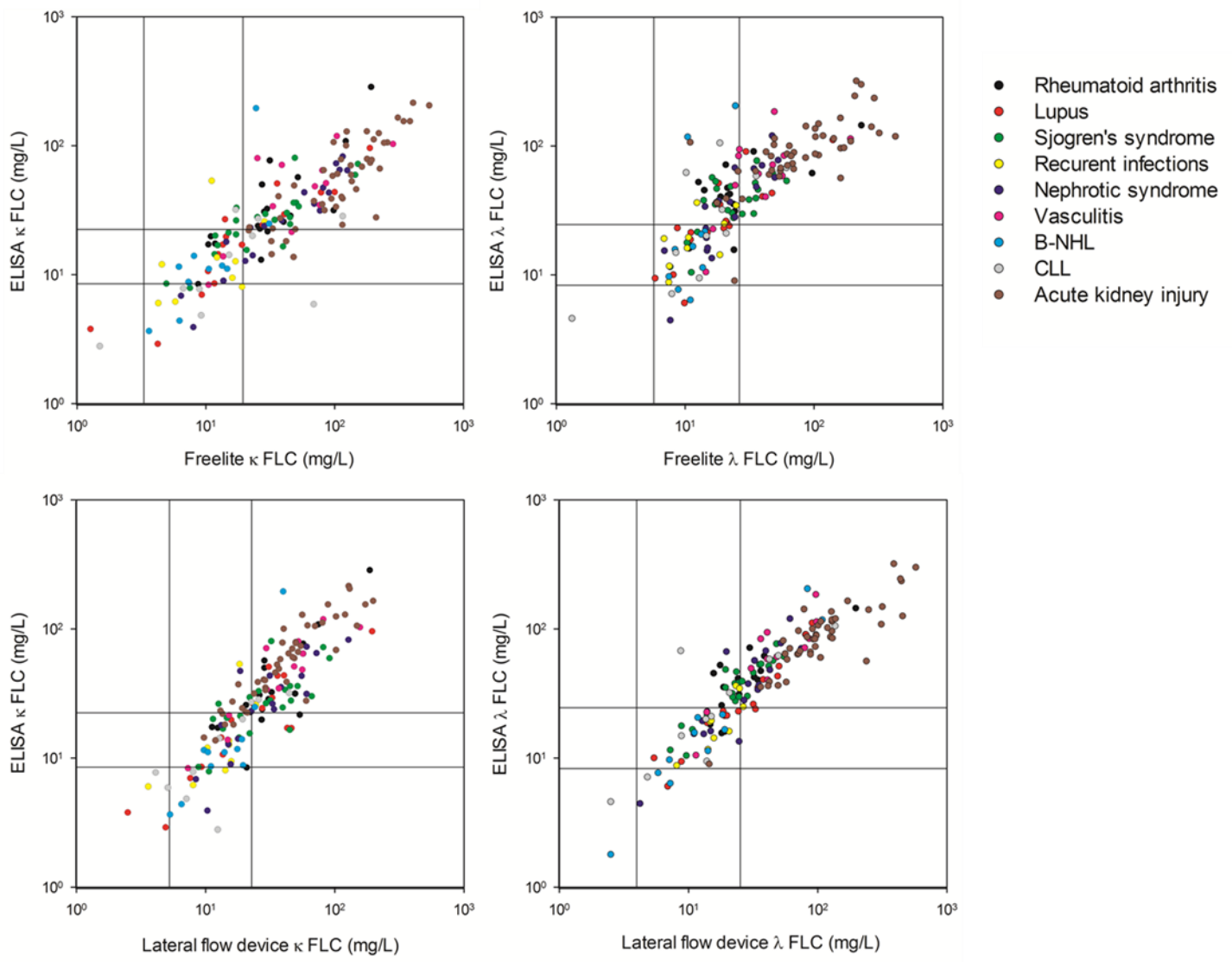

Figure 3. Distribution of K and λ FLC concentrations between methods in relation to reference ranges

Serum FLC concentrations are shown for the Seralite ELISA vs Freelite (top panels) and the Seralite ELISAs vs Seralite LFD (bottom panels) for a range of samples from patients with a range of conditions associated with polyclonal FLC dysregulation (n = 164). Lines indicate the normal reference ranges for each assay.

Table 4. Measurement of patient samples (n = 164) using different methods in relation to reference ranges (RR) of the different tests

Proportion of patients (%)	**Freelite**	**Seralite LFD**	**Seralite ELISA**
κ FLC			
Below RR	1.2	3	12.8
Within RR	30.5	39.6	26.2
Above RR	68.3	57.3	61.0
λ FLC			
Below RR	1.2	1.2	4.3
Within RR	54.9	40.2	26.2
Above RR	43.9	58.5	69.5
FLC sum			
Below RR	4.9	3	6.7
Within RR	23.8	25.6	26.2
Above RR	71.3	71.3	70.1
κ:λ ratio			
With RR	68.3	63.4	55.5
Abnormal ratio	31.7	36.6	44.5

Reference ranges for each of the assays were taken from previous publications for existing assays (Freelite and Seralite LFD) for κ and λ FLC levels and κ:λ ratio. Reference ranges for FLC sum have not been published previously and were based on the analysis of healthy donors across the platforms as part of the present study. κ FLC: 3.3–19.4 mg/L (Freelite); 5.3–22.7 mg/L (Seralite LFD) 8.7–23.0 mg/L (Seralite ELISA); λ FLC: 5.7–26.3 mg/L (Freelite); 4.0–25.1 mg/L (Seralite LFD); 8.5–25.2 mg/L (Seralite ELISA); κ:λ ratio: 0.26–1.65 (Freelite); 0.5–2.5 (Seralite LFD); 0.65–1.6 (Seralite ELISA); FLC sum: 15.1–34.4 mg/L (Freelite); 12.2–36.6 mg/L (Seralite LFD); 18.7–45.5 mg/L (Seralite ELISA)

Table 5. Passing-Bablok regression results for comparison of patient samples (n = 164) between methods

	Seralite ELISA vs Freelite	ELISA vs Seralite LFD	Freelite vs Seralite LFD
κ FLC			
Intercept	-12.51 (-18.24 to -7.43)	2.68 (0.58 to 4.63)	-15.65 (-22.94 to -11.04)
Slope	1.98 (1.79 to 2.22)	0.78 (0.70 to 0.88)	2.10 (1.84 to 2.47)
λ FLC			
Intercept	-1.92 (-4.50 to 0.36)	-5.27 (-7.55 to -2.79)	2.71 (0.50 to 4.64)
Slope	0.79 (0.69 to 0.86)	1.06 (0.97 to 1.15)	0.71 (0.65 to 0.79)
FLC sum			
Intercept	-13.67 (-22.58 to -7.59)	2.05 (-1.88 to 5.63)	-10.21 (-16.54 to -5.14)
Slope	1.26 (1.12 to 1.43)	1.03 (0.96 to 1.11)	1.24 (1.14 to 1.37)
κ:λ ratio			
Intercept	-0.63 (-1.15 to -0.24)	0.32 (0.26 to 0.37)	0.28 (-0.06 to 0.48)
Slope	2.73 (2.27 to 3.46)	0.44 (0.37 to 0.54)	1.26 (0.96 to 1.67)

4. Discussion

The present study evaluated the performance of ELISAs for the measurement of polyclonal FLCs and compared FLC quantitation of patient samples to existing FLC platforms. The ELISAs have a wide calibration range and low limit of detection, comparable to existing commercial assays, to enable the measurement of low and normal levels of FLCs alongside sera with elevated FLCs. For any method of polyclonal FLC measurement, it is important that quantitation captures lower concentrations as levels are typically a lot lower than in neoplastic plasma cell disorders. Sensitive measurement of samples with low levels of FLCs is also required if investigating immunosuppression. In relation to measurement at the bottom of the calibration curve, the ELISAs may have an advantage as the Freelite assay has been demonstrated to have gaps at the lower end (Bradwell, 2008) and the LFD test analytical range does not go below 2.5 mg/L (Campbell et al., 2017). The ELISAs demonstrated minimal cross-reactivity with common interference agents, including light chains forming part of intact immunoglobulins, and good linearity was shown. Further, the assays displayed a high level of reproducibility with good intra- and inter-assay precision and lot to lot consistency.

For the measurement of healthy donors, the ELISAs returned higher FLC concentrations compared to Freelite and the LFD with median values 2.5–3.5 mg/L above the other methods. The values obtained in this study showed slight variations with those published previously (Katzmann 2002; Campbell 2017). This is to be expected when measuring different cohorts of healthy individuals or similar donors at a different time point. As is the case with all assays, reference ranges are specific to the group of individuals at the specific time investigated and users should establish reference ranges for their own laboratory.

Clinical samples across a range of conditions associated with FLC dysregulation were investigated and results from the ELISA were compared to Freelite and Seralite LFD. Results confirmed that κ and λ FLC quantitation was consistently different between methods across the range of analyses performed. The classification of patients in relation to reference ranges of κ and λ FLC was also significantly different between methods. However, when computing the FLC sum, differences in κ and λ FLC cancelled each other out and resulted in no significant difference between platforms for

this parameter in terms of both concentration and classification of normal/elevated. In plasma cell disorders there is typically a significant imbalance between monoclonal and polyclonal FLC production and thus the ratio and FLC difference are key markers (Durie et al., 2006; Dispenzieri et al., 2009; Rajkumar et al., 2014). In contrast, in conditions relating to polyclonal light chains, both kappa and lambda FLCs typically increase and the FLC sum is more relevant for diagnosis, prognostication and monitoring. In studies relating to polyclonal FLCs and mortality prediction, FLC sum is the marker of interest (Eisele et al., 2010; Anandram et al., 2012; Dispenzieri et al., 2012).

The information presented as part of this study provides useful information for current and potential users of FLC assays to measure polyclonal FLCs. As perhaps expected, κ and λ FLC quantitation is inherently different when employing different test formats and technologies. This has been shown previously for samples containing monoclonal FLCs. Consequently, the same consistent test should be used in studies with multiple timepoints or tracking individuals over time and methods are not interchangeable. When FLC sum is the measure of interest, different methods are likely to return similar concentrations and provide broad agreement regarding the classification of FLC elevation.

ELISAs can offer several practical benefits for laboratories and researchers. They are a simple method that only requires a plate reader as opposed to any large or expensive equipment. Nephelometric and turbidimetric methods are generally only available in specialised clinical laboratories; ELISAs are more accessible to a wider range of researchers and potential FLC users. They are a flexible platform that can be run manually or on automated systems depending on sample numbers and resources. Others have utilised both polyclonal anti-FLC antibodies and monoclonal anti-FLC antibodies to make ELISA formats for quantitation of FLCs in serum (Nakano and Nagata, 2004; Davern et al., 2008; de Kat Angelino et al., 2010). Although the concept of using ELISAs for the measurement of FLCs is not novel, these previous ELISA methodologies have not been translated into commercially available assay kits. The present study provides initial data regarding the use of the Seralite ELISA in polyclonal clinical samples and provides comparison data with two different FLC methods – Freelite and Seralite LFD test.

It should be noted that while in practice users may choose to use the same system for measuring monoclonal and polyclonal samples, the intended use of Freelite and Seralite LFD is for myeloma and related disorders. Alternatively, Combylite™ (Blinding site) is a turbidimetric immunoassay that quantitates polyclonal sFLCs and provides the FLC sum, designed for testing inflammatory conditions associated with elevated FLC (Faint et al., 2014). Although Combylite has high concordance with Freelite, direct comparison with the ELISAs, which are intended for polyclonal use, would be useful as part of future studies. It should be noted that other assays are also available. N Latex (Siemens) is a nephelometric assay (te Velthuis et al., 2011) that has mainly been validated for use in a range of plasma cell dyscrasias and renal impairment. There is also a new assay based on ELISA technology (Sebia) that has initially been validated in myeloma and CKD samples (Jacobs et al., 2018). Future studies should establish the performance of the ELISAs in relation to these methods. It may be particularly interesting to see how Seralite ELISA compares to the new Sebia ELISA and if using the same platforms produces greater agreement between methods or if the different antibodies are main contributor to variability, regardless of assay system. In addition, to assess the utility of the Seralite ELISA in relation to monoclonal samples, separate in-depth evaluation would be required as part of additional studies. The growing range of sFLC technologies can promote use of FLC testing and users' choice of method will most likely incorporate the nature of the samples to be tested and resources available.

FLCs have been shown to exert a range of biological functions (Nakano et al., 2011; Brebner and Stockley, 2013). These roles include interactions with immune cells such as neutrophils, mast cells and B cells; stimulation of inflammatory cytokine release; mediation in allergenic responses and assistance in antigen uptake (Cohen et al., 2001; Nakano et al., 2011; Braber et al., 2012; Hutchinson et al., 2012 ; Brebner and Stockley, 2013). Due to these activities, FLCs have been proposed to stimulate chronic inflammation via immune activation (Redegeld et al., 2012). This mechanism would account for the various relationships observed between FLCs and inflammatory and autoimmune diseases. FLCs also serve as a non-specific inflammatory marker for mortality in the general population, although it's not clear if an elevated FLC sum acts as a proxy for disease or a mediator of disease processes (Dispenzieri et al., 2012). Other studies have also highlighted the

use of FLCs as a biomarker in inflammation, ageing and health research (Drayson, 2012 ; Heaney et al., 2016a; Heaney et al., 2016b). The growing evidence of the utility of FLCs across a range of inflammatory and autoimmune conditions and its emergence as a biomarker in different fields of research may increase the need for and adoption of FLC assays. Indeed, it is possible to assess FLCs in a range of biological fluids, including saliva and CSF, which may increase the scope of research involving FLCs further in the future. The ability to detect low levels of polyclonal FLCs with the ELISAs could assist with quantification in alternative specimens but this would need to be validated in separate studies.

The present study was a retrospective analysis of stored clinical samples that had undergone 2 cycles of freeze-thaw. FLCs have been shown to be stable when analysed on the LFD (Campbell et al., 2017) and various studies have analysed FLCs after long-term storage (Dispenzieri et al., 2012; Turesson et al., 2014; Horber et al., 2019). However, users should determine the effects of applicable sample storage and processing on their chosen platform.

5. Conclusions

The Seralite ELISAs for the measurement of polyclonal κ and λ FLCs demonstrated good performance characteristics. When compared with Freelite and Seralite LFD tests, quantitation was found to be different for κ and λ FLC concentrations, providing further evidence that FLC techniques are not interchangeable for individual light chain measurements. Only the FLC sum returns comparable results and concordance regarding FLC elevation across the methods.
This study provides initial analytical validation of the Seralite ELISAs and provides information on how they compare to existing tests for the purpose of polyclonal FLC measurement. Platforms for FLC measurement are becoming more diverse and this growing range of FLC measurement techniques may help improve access to FLC testing and enhance choice to suit user needs and testing in different populations.

Declarations of interest

MS is an employee of Abingdon Health Ltd. MD has an advisory role with Abingdon Health Ltd and reports personal fees from Abingdon Health Ltd. JC, MG, and TP own shares in Abingdon Health Ltd. CH is Chairman of Scientific & Medical Advisory Board of Abingdon Health Ltd.

References

Aalberse, R.C., Stapel, S.O., Schuurman, J. and Rispens, T., 2009, Immunoglobulin G4: an odd antibody. Clin Exp Allergy 39, 469-77.

Anandram, S., Assi, L.K., Lovatt, T., Parkes, J., Taylor, J., Macwhannell, A., Jacob, A., Handa, S., Harding, S. and Basu, S., 2012, Elevated, combined serum free light chain levels and increased mortality: a 5-year follow-up, UK study. J Clin Pathol 65, 1036-42.

Braber, S., Thio, M., Blokhuis, B.R., Henricks, P.A., Koelink, P.J., Groot Kormelink, T., Bezemer, G.F., Kerstjens, H.A., Postma, D.S., Garssen, J., Kraneveld, A.D., Redegeld, F.A. and Folkerts, G., 2012, An association between neutrophils and immunoglobulin free light chains in the pathogenesis of chronic obstructive pulmonary disease. Am J Respir Crit Care Med 185, 817-24.

Bradwell, A.R. 2008. Serum Free Light Chain Analysis (Plus Hevylite). Binding Site Birmingham

Bradwell, A.R., Carr-Smith, H.D., Mead, G.P., Tang, L.X., Showell, P.J., Drayson, M.T. and Drew, R., 2001, Highly sensitive, automated immunoassay for immunoglobulin free light chains in serum and urine. Clinical chemistry 47, 673-80.

Brebner, J.A. and Stockley, R.A., 2013, Polyclonal free light chains: a biomarker of inflammatory disease or treatment target? F1000 Med Rep 5, 1.

Brekke, O.H. and Sandlie, I., 2003, Therapeutic antibodies for human diseases at the dawn of the twenty-first century. Nat Rev Drug Discov 2, 52-62.

Campbell, J.P., Cobbold, M., Wang, Y., Goodall, M., Bonney, S.L., Chamba, A., Birtwistle, J., Plant, T., Afzal, Z., Jefferis, R. and Drayson, M.T., 2013, Development of a highly-sensitive multi-plex assay using monoclonal antibodies for the simultaneous measurement of kappa and lambda immunoglobulin free light chains in serum and urine. J Immunol Methods 391, 1-13.

Campbell, J.P., Heaney, J.L., Shemar, M., Baldwin, D., Griffin, A.E., Oldridge, E., Goodall, M., Afzal, Z., Plant, T., Cobbold, M., Jefferis, R., Jacobs, J.F., Hand, C. and Drayson, M.T., 2017, Development of a rapid and quantitative lateral flow assay for the simultaneous measurement of serum kappa and lambda immunoglobulin free light chains (FLC): inception of a new near-patient FLC screening tool. Clin Chem Lab Med 55, 424-434.

Cohen, G., Rudnicki, M. and Horl, W.H., 2001, Uremic toxins modulate the spontaneous apoptotic cell death and essential functions of neutrophils. Kidney Int Suppl 78, S48-52.

Davern, S., Tang, L.X., Williams, T.K., Macy, S.D., Wall, J.S., Weiss, D.T. and Solomon, A., 2008, Immunodiagnostic capabilities of anti-free immunoglobulin light chain monoclonal antibodies. Am J Clin Pathol 130, 702-11.

Davids, M.S., Murali, M.R. and Kuter, D.J., 2010, Serum free light chain analysis. Am J Hematol 85, 787-90.

de Kat Angelino, C.M., Raymakers, R., Teunesen, M.A., Jacobs, J.F. and Klasen, I.S., 2010, Overestimation of serum kappa free light chain concentration by immunonephelometry. Clinical chemistry 56, 1188-90.

Dispenzieri, A., Katzmann, J.A., Kyle, R.A., Larson, D.R., Therneau, T.M., Colby, C.L., Clark, R.J., Mead, G.P., Kumar, S., Melton, L.J., 3rd and Rajkumar, S.V., 2012, Use of nonclonal serum immunoglobulin free light chains to predict overall survival in the general population. Mayo Clin Proc 87, 517-23.

Dispenzieri, A., Kyle, R., Merlini, G., Miguel, J.S., Ludwig, H., Hajek, R., Palumbo, A., Jagannath, S., Blade, J., Lonial, S., Dimopoulos, M., Comenzo, R., Einsele, H., Barlogie, B., Anderson, K., Gertz, M., Harousseau, J.L., Attal, M., Tosi, P., Sonneveld, P., Boccadoro, M., Morgan, G., Richardson, P., Sezer, O., Mateos, M.V., Cavo, M., Joshua, D., Turesson, I., Chen, W., Shimizu, K., Powles, R., Rajkumar, S.V. and Durie, B.G., 2009, International Myeloma Working Group guidelines for serum-free light chain analysis in multiple myeloma and related disorders. Leukemia 23, 215-24.

Drayson, M.T., 2012 Using single protein biomarkers to predict health and disease in diverse patient populations: a new role for assessment of immunoglobulin free light chains. Mayo Clin Proc 87, 505-7.

Durie, B.G., Harousseau, J.L., Miguel, J.S., Blade, J., Barlogie, B., Anderson, K., Gertz, M., Dimopoulos, M., Westin, J., Sonneveld, P., Ludwig, H., Gahrton, G., Beksac, M., Crowley, J., Belch, A., Boccadaro, M., Cavo, M., Turesson, I., Joshua, D., Vesole, D., Kyle, R., Alexanian, R., Tricot, G., Attal, M., Merlini, G., Powles, R., Richardson, P., Shimizu, K., Tosi, P., Morgan, G. and Rajkumar, S.V., 2006, International uniform response criteria for multiple myeloma. Leukemia 20, 1467-73.

Eisele, L., Dürig, J., Huttmann, A., Dührsen, U., Führer, A., Kieruzel, S., Bröcker-Preuss, M., Bokhof, B., Erbel, R., Mann, K., Jöckel, K.-H. and Moebus, S., 2010, Polyclonal Free Light Chain Elevation and Mortality In the German Heinz Nixdorf Recall Study. Blood 116, 3903-3903.

Faint, J.M., Basu, S., Sutton, D., Showell, P.J., Kalra, P.A., Gunson, B.K., Jackson, C.E., Mushtaq, A., Assi, L.K., Carr-Smith, H.D., Cockwell, P. and Harding, S.J., 2014, Quantification of polyclonal free light chains in clinical samples using a single turbidimetric immunoassay. Clin Chem Lab Med 52, 1605-13.

Heaney, J.L., Campbell, J.P., Griffin, A.E., Birtwistle, J., Shemar, M., Child, J.A., Morgan, G., Jackson, G. and Drayson, M.T., 2017a, Diagnosis and monitoring for light chain only and oligosecretory myeloma using serum free light chain tests. British Journal of Heamatology 178 220-230.

Heaney, J.L., Campbell, J.P., Punit, Y., Griffin, A.E., Shemar, M., Pinney, J. and Drayson, M.T., 2017b, Multiple myeloma can be accurately diagnosed in acute kidney injury patients using a rapid serum free light chain test. BMC Nephrol 18, 247.

Heaney, J.L., Gleeson, M., Phillips, A.C., Taylor, I.M., Drayson, M.T., Goodall, M., He, C.S., Svendsen, I.S., Killer, S.C. and Campbell, J.P., 2016a, Salivary immunoglobulin free light chains: reference ranges and responses to exercise in young and older adults. Exerc Immunol Rev 22, 28-41.

Heaney, J.L., Phillips, A.C., Drayson, M.T. and Campbell, J.P., 2016b, Serum free light chains are reduced in endurance trained older adults: Evidence that exercise training may reduce basal inflammation in older adults. Exp Gerontol 77, 69-75.

Horber, S., Klein, R. and Peter, A., 2019, Effects of Long-Term Storage on Serum Free Light Chain Stability. Clin Lab 65, 181107.

Hutchinson, A.T., Jones, D.R. and Raison, R.L., 2012 The ability to interact with cell membranes suggests possible biological roles for free light chain. Immunol Lett 142, 75-77.

Jacobs, J.F., Tate, J.R. and Merlini, G., 2016, Is accuracy of serum free light chain measurement achievable? Clin Chem Lab Med 54, 1021-30.

Jacobs, J.F.M., de Kat Angelino, C.M., Brouwers, H., Croockewit, S.A., Joosten, I. and van der Molen, R.G., 2018, Evaluation of a new free light chain ELISA assay: bringing coherence with electrophoretic methods. Clin Chem Lab Med 56, 312-322.

Katzmann, J.A., Clark, R.J., Abraham, R.S., Bryant, S., Lymp, J.F., Bradwell, A.R. and Kyle, R.A., 2002, Serum reference intervals and diagnostic ranges for free kappa and free lambda immunoglobulin light chains: relative sensitivity for detection of monoclonal light chains. Clinical chemistry 48, 1437-44.

Nakano, T., Matsui, M., Inoue, I., Awata, T., Katayama, S. and Murakoshi, T., 2011, Free immunoglobulin light chain: its biology and implications in diseases. Clin Chim Acta 412, 843-9.

Nakano, T. and Nagata, A., 2004, ELISAs for free human immunoglobulin light chains in serum: improvement of assay specificity by using two specific antibodies in a sandwich detection method. J Immunol Methods 293, 183-9.

Rajkumar, S.V., Dimopoulos, M.A., Palumbo, A., Blade, J., Merlini, G., Mateos, M.-V., Kumar, S., Hillengass, J., Kastritis, E., Richardson, P., Landgren, O., Paiva, B., Dispenzieri, A., Weiss, B., LeLeu, X., Zweegman, S., Lonial, S., Rosinol, L., Zamagni, E., Jagannath, S., Sezer, O., Kristinsson, S.Y., Caers, J., Usmani, S.Z., Lahuerta, J.J., Johnsen, H.E., Beksac, M., Cavo, M., Goldschmidt, H., Terpos, E., Kyle, R.A., Anderson, K.C., Durie, B.G.M. and Miguel, J.F.S., 2014, International Myeloma Working Group updated criteria for the diagnosis of multiple myeloma. The Lancet Oncology 15, e538-e548.

Redegeld, F.A., Thio, M. and Groot Kormelink, T., 2012, Polyclonal immunoglobulin free light chain and chronic inflammation. Mayo Clin Proc 87, 1032-1033.

Suki, W.N. and Massry, S.G. 1998 Suki and Massry's Therapy of Renal Diseases and Related Disorders. In. Kluer Academic Publishers

Te Velthuis, H., Drayson, M. and Campbell, J.P., 2016, Measurement of free light chains with assays based on monoclonal antibodies. Clin Chem Lab Med 54, 1005-14.

te Velthuis, H., Knop, I., Stam, P., van den Broek, M., Bos, H.K., Hol, S., Teunissen, E., Fischedick, K.S., Althaus, H., Schmidt, B., Wagner, C. and Melsert, R., 2011, N Latex FLC - new monoclonal high-performance assays for the determination of free light chain kappa and lambda. Clin Chem Lab Med 49, 1323-32.

Turesson, I., Kovalchik, S.A., Pfeiffer, R.M., Kristinsson, S.Y., Goldin, L.R., Drayson, M.T. and Landgren, O., 2014, Monoclonal gammopathy of undetermined significance and risk of lymphoid and myeloid malignancies: 728 cases followed up to 30 years in Sweden. Blood 123, 338-45.

van der Heijden, M., Kraneveld, A. and Redegeld, F., 2006, Free immunoglobulin light chains as target in the treatment of chronic inflammatory diseases. Eur J Pharmacol 533, 319-26.

Waldmann, T.A., Strober, W. and Mogielnicki, R.P., 1972, The renal handling of low molecular weight proteins. II. Disorders of serum protein catabolism in patients with tubular proteinuria, the nephrotic syndrome, or uremia. J Clin Invest 51, 2162-74.