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A new challenge for urinary free light chains: assessment of the upper reference limit in healthy subjects

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Abstract. – OBJECTIVE: Free light chains (FLCs) can be measured in both urine (uFLC) and serum (sFLC) in immunochemistry. We aim to compare FLC levels in serum and urine assessed among healthy volunteers and measured upper reference limits (URLs) of urinary FLC to creatinine ratio (uFLC/uCr) in mg/g to compare with the manufacturer's recommended URLs.

PATIENTS AND METHODS: Eligibility criteria: normal serum and urine FLC measure and negative serum/urinary immunofixation. Immunoturbidimetry was used to assess both κ and λ FLCs. The URLs were calculated with the 97.5th percentile of uFLC concentrations according to the Clinical and Laboratory Standards Institute recommendations.

RESULTS: 126 healthy subjects (median age 46 years, 62% females) met the inclusion criteria. Median concentrations of κ and λ sFLCs were similar both for males and females without significant differences. κ and λ uFLCs were significantly higher in males than in females ($p < 0.001$ and $p = 0.004$, respectively). Slower clearance for λ FLC compared to κ FLC was observed with an increased κ/λ uFLC ratio in both males and females. URLs for male and female subjects: κ uFLC mg/g uCr = 34.35 *vs.* 23.18, and λ uFLC mg/g uCr = 3.59 *vs.* 1.96, respectively compared well with manufacturer's URLs.

CONCLUSIONS: FLC catabolism is gender-dependent and occurs less rapidly in λ FLC than in κ FLC. The determination of the URL of uFLC, as uFLC/uCr, in healthy subjects in morning urine, proved to be consistent with the manufacturer's recommendations, but with a significant difference according to gender.

Key Words:
Bence Jones Protein, Free light chains, Urinary free light chains.

Abbreviations

BJP, Bence Jones protein; MG, monoclonal gammopathy; uFLC, urinary free light chains; uCr, urinary creatinine; IMWG, International Myeloma Working Group; uCr, urinary creatinine; sFLC, serum free light chains; eGFR, estimated glomerular filtration rate; uTP, urinary total proteins; uIFE, urinary immunofixation; CLSI, Clinical and Laboratory Standards Institute; CI, confidence interval; κ sFLC, serum free light chains kappa; λ sFLC, serum free light chains lambda; sFLC-ratio, serum κ/λ free light chains ratio; sCr, serum creatinine; κ uFLC, urinary free light chains kappa; λ uFLC, urinary free light chains lambda; MC, monoclonal component; MM, multiple myeloma.

Introduction

During immunoglobulin (Ig) synthesis, up to 500 mg per day of unbound serum-free light chains (sFLC) are produced in excess and released into circulation. Due to their low molecular weight, monomeric kappa (κ) FLC molecular weight ~22-25 kDa, and dimeric lambda (λ) FLC molecular weight ~44-50 kDa, are filtered

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at the glomerulus and then almost completely reabsorbed and metabolized in the proximal tubule. Therefore, only minimal amounts of polyclonal urinary FLC (uFLCs) are detectable in urine[1-4].

In contrast, when plasma cell dyscrasias occur, a plasma cell clone produces, in large amounts, only one class of Ig and/or one type of FLC, and, if its concentration exceeds the reabsorption capacity of the tubule (10-30 g/L per day), it passes into the urine as Bence Jones protein (BJP), resulting as a marker for plasma cell dyscrasias. According to the recommendations of the International Myeloma Working Group (IMWG), both serum and urine monoclonal FLC are useful markers in patient management and in the follow-up to assess treatment response: in serum, the paraprotein concentration and sFLC are κ/λ ratios to screening for monoclonal gammopathies, while in urine, BJP concentration[5-8].

The high sensitivity and wide measurement range of immunometric methods (immunonephelometry or immunoturbidimetry) allows the measurement of both the very high sFLC concentrations of patients with plasma cell dyscrasia and very low uFLCs concentrations of the healthy subjects (HS) that are several orders of magnitude lower. According to the manufacturer, these techniques are very sensitive (< 1 mg/L)[3] and allow the detection of uFLCs in the urine of HS using the first-morning void without the need for urine concentration. The manufacturer provides a reference limit for uFLCs in HS expressed as mg/L. However, we measured uFLCs as uFLC to urine creatine ratio (uFLC/uCr) in mg/g, as already reported by other authors[2,3,9]. Moreover, the quantification of other proteins in urine, such as albuminuria and total proteinuria, is usually expressed as a ratio with creatininuria, as Global Outcome guidelines (KDIGO) suggest[10].

The purpose of the current study is to measure and calculate reference limits of polyclonal uFLCs as uFLC/uCr in mg/g in the morning urine of HS and compare our results with the reference values recommended by the manufacturer according to good laboratory practice. Since we assumed that uFLCs should be practically absent in HS, we focused our analysis on the upper reference limit (URL) of uFLCs. Furthermore, this study aims to directly compare FLC concentrations in serum and urine in HS measured by the same immunometric methods.

Patients and Methods

Study Design

We conducted this prospective study at the Department of Laboratory Medicine of Health Local Unit of Modena, Italy. The study complied with the Declaration of Helsinki and was approved by the local Ethics Committee (Comitato Etico Area Vasta Emilia Nord, Italy, Prot. 32/2021/SPER/AUSLMO-UFLC2021) applying informed consent.

Voluntary HS were enrolled: paired samples of serum and urine were collected from each subject and, after centrifugation at 3,000 rpm for 10', were stored at -20°C and -80°C, respectively, and analyzed within two months. All sera were tested to detect κ and λ FLC and creatine; the estimated glomerular filtration rate (eGFR) was calculated according to the CKD-EPI equation[11]. All urine samples were tested for κ and λ FLC, urinary total proteins (uTP) and urinary creatinine (uCr) to calculate uFLC and uTP to uCr ratio.

Study eligibility criteria included sFLC κ/λ ratio between 0.26-1.65[12], negative urinary immunofixation (uIFE); serum creatinine (sCr) <0.95 mg/dL (84 μmol/L) and <1.17 mg/dL (104 μmol/L) for females and males respectively (according to manufacturer's indications), eGFR > 60 ml/min/1.73 m^2, uTP < 150 mg/g-uCr [10].

Analytical Methods

Urine and serum FLCs detection was performed by turbidimetric assay on Optilite (The Binding Site, Birmingham, UK). Freelite Kappa Free kit and Freelite Lambda Free kit for serum and Freelite Mx Kappa Free Kit and Freelite Mx Lambda Free kit (The Binding Site, Birmingham, UK) were used. The reagent consists of a sheep polyclonal monospecific antibody coated onto a polystyrene latex particle to enhance the reaction. Freelite reacts only with exposed free light chain epitopes hidden when the light chain is bound to the heavy chain. As a kinetic reaction, seven readings are taken at different times and coded by software that automatically obtains the sample concentration and detects excess antigen that could produce falsely low results, based on the reaction rate. The manufacturer reference ranges of serum-free k and λ were 3.3-19.4 mg/L and 5.71-26.3 mg/L, respectively, and for urinary-free and k λ reference ranges were < 32.9 mg/L and < 3.79 mg/L, respectively.

Urine immunofixation was performed by Easy-Fix G26 (Sebia-Interlab Lisses, FR), a fully auto-

mated gel electrophoresis system performing for immunofixation (IFE) either in serum and/or urine specimens. A bivalent antiserum (Sebia-Interlab, Lisses, FR) using two lanes for sample. The first lane was fixed in order to reveal the urine protein pattern, whereas the second lane was treated with a bivalent antiserum consisting of a mix of anti-total k and anti-total λ antiserum. The presence/absence of discrete bands in the second lane reveals the presence/absence of monoclonal components. In the case of positivity, BJP characterization was performed on three lanes: the first with antiserum anti-IgA-IgG-IgM, the second with antiserum anti-total k, and the third with antiserum anti-total λ (Sebia-Interlab, Lisses, FR).

Urinary total proteins were performed using a pyrogallol red/molybdenum method on the AU 800 chemistry platform (Beckmann Coulter, Brea, CA, USA). Both serum and urine creatinine were assayed by the kinetic Jaffé compensated method, traceable by the isotope dilution mass spectrometry (IDMS) reference method[13] on the Beckman Coulter AU 800 analyzer.

Statistical Analysis

URLs of uFLC/uCr were determined following the EP28-A3c guideline published by the Clinical and Laboratory Standard Institute (CSLI)[14]. According to CLSI, the best method for the establishment of a reference interval is to collect samples from a sufficient number of qualified reference individuals to yield a minimum of 120 samples for analysis by non-parametric data, for each partition (e.g., sex, age range). For cohorts including less than 120 specimens, it is recommended to use the robust method for small samples based on percentile calculation for non-parametric distributions. We calculated the 97.5th percentile of uFLCs/uCr in mg/g, for a healthy population URL.

To evaluate the normal distribution, we applied the Shapiro-Wilk test, and outliers were evaluated with the Turkey test. A confidence interval (CI) of 90% was calculated by bootstrap (10,000 iterations; random number seed: 978) as recommended for the robust method[15]. Variables were expressed as median and interquartile ranges (continuous) or percentages (categorical). Comparison among groups was assessed with non-parametric Mann-Whitney U test. Correlations of uFLCs with age and gender were performed with Spearman's rank correlation coefficient (r). p-value < 0.05 was considered statistically significant. The MedCalc Statistical Software, version 14.8.1, and Microsoft Excel 2019 version 2110 were used to perform the analyses and to create graphs.

Results

One hundred and thirty-seven healthy subjects were approached. All of them were proven negative at uIFE, but 11 subjects were excluded according to values outside of the specified inclusion criteria: FLC ratio > 1,65 (n = 2), eGFR < 60 ml/min/1.73 m^2 (n = 3); and uTP > 150 mg/guCr (n = 6). A total of 126 HS met the inclusion criteria and were enrolled. Demographic characteristics, serum, and urine analyses are outlined in Table I.

There was no relationship found between uFLC/uCr according to age for both males and females for k uFLC (r < 0.01; p = 0.95) and λ uFLC (r = 0.04; p = 0.65) (Figure 1). We did not find any significant differences according to gender among parameters analyzed in serum except for creatinine, whereas all urinary parameters, except uTP/uCr, showed differences significantly associated with gender. Despite the normalization of uFLC to uCr ratio, differences persisted according to gender for both k uFLC/uCr and λ uFLC/uCr (males *vs.* females): 10.97 (IQR 6.70 - 17.22) *vs.* 4.87 (IQR 3.23 - 11.48); p < 0.001 and 1.04 (IQR 0.49 - 1.57) *vs.* 0.55 (IQR 0.43 - 0.98); p = 0.004, respectively (Figure 2).

Therefore uFLC/uCr URL were calculated according to gender. For males, URL for k uFLC/uCr was 34.35 (90% CI 27.48 - 39.99) and for λ uFLC/uCr was 3.59 (90% CI 2.51 - 4.48); for females the URL for k uFLC/uCr was 23.18 (90% CI 17.92 - 27.63) and for λ uFLC/uCr was 1.96 (90% CI 1.60 - 2.33) (Table II).

It is interesting to note that the URL of k FLC specified by the manufacturer is also lower in serum than in urine for k FLC: 19.4 *vs.* 32.9 mg/L, respectively; whilst, in contrast, for λ FLC, the value is higher in serum than in urine: 26.3 *vs.* 3.79 mg/L.

Both the serum and urine λ FLCs concentrations are strongly reduced during the filtration from serum to urine, whereas the concentrations of κ FLCs are less reduced. As a result, κ/λ FLC ratios in serum were not significantly different [1.16 (IQR 0.99-1.40) in males *vs.* 1.13 (IQR 0.99-1.32) in females] whereas there was a significant difference according to gender in urine [11.34 (IQR 8.68-13.46) in males *vs.* 9.58 (IQR 7.42-15.57) in females; p = 0.036].

Table I. Baseline, serum, and urine characteristics of the study population, with comparisons according to gender.

Characteristics	Total median (IQR)	Males median (IQR)	Females median (IQR)	*p*-value
Gender, number (%)	126	48 (38)	78 (62)	-
Age, years (range)	46 (18-69)	45 (18-69)	46.5 (22-64)	Ns
Serum analyses:				
κ sflc, mg/L	13.59 (11.57-16.24)	14.41 (11.88-16.84)	12.88 (11.42-15.82)	Ns
Λ sflc, mg/L	11.83 (9.89-14.23)	11.66 (10.36-14.98)	11.86 (9.82-13.82)	Ns
κ/λ sflc ratio	1.15 (0.99-1.33)	1.16 (0.99-1.40)	1.13 (0.99-1.32)	Ns
Scr, mg/dl	0.80 (0.70-0.90)	0.93 (0.85-1.02)	0.72 (0.66-0.79)	< 0.001
Scr, µmol/L	70.74 (61.89-79.58)	82.23 (75.16-90.19)	63.66 (58.36-69.85)	< 0.001
Egfr, ml/min/1.73 m^2	96.55 (88.39-107.21)	92.90 (84.48-108.14)	97.50 (92.18-106.73)	Ns
Urine analyses:				
Urine IFE	Negative	Negative	Negative	-
uCr, mg/dL	112.00 (79.92-160.08)	139.5 (102.83-204.58)	102.00 (73.00-132.33)	< 0.001
uCr, µmol/L	9,903.04 (7,066.52-14,154.27)	12,334.59 (9,092.22-18,088.96)	9,018.84 (6,454.66-11,700.62)	< 0.001
uTP, mg/dL	5.50 (3.70-7.23)	6.60 (5.04-9.85)	4.75 (3.50-6.20)	< 0.001
uTP, g/L	0.06 (0.04-0.07)	0.07 (0.05-0.09)	0.05 (0.04-0.06)	< 0.001
uTP/uCr, mg/g	48.23 (40.14-58.95)	45.94 (39.66-56.11)	48.57 (40.16-61.33)	Ns
κ uFLC, mg/L	8.19 (3.48-19.35)	18.63 (7.29-25.97)	5.81 (2.62-12.49)	< 0.001
λ uFLC, mg/L	0.79 (0.35-1.64)	1.61 (0.50-2.39)	0.48 (0.32-1.13)	< 0.001
κ uFLC/uCr, m/g	8.02 (4.14-14.63)	10.97 (6.70-17.22)	4.87 (3.23-11.48)	< 0.001
λ uFLC/uCr, mg/g	0.61 (0.44-1.20)	1.04 (0.49-1.57)	0.55 (0.43-0.98)	0.004
κ/λ uFLC ratio	10.62 (7.85-13.21)	11.37 (8.68-13.46)	9.58 (7.42-12.57)	0.036

IQR, interquartile; κ sFLC, serum kappa free light chains; λ sFLC, serum lambda free light chains; κ/λ sFLC ratio, serum κ/λ free light chains ratio; sCr, serum creatinine; eGFR, estimated glomerular filtration rate; IFE, immunofixation; uTP, urinary total proteins; uCr, urinary creatinine; κ κ uFLC, urinary kappa free light chains; λ uFLC, urinary lambda free light chains; ns, not significant.

Discussion

One hundred and twenty-six HS (median age 46 years, 62% females) met the inclusion criteria for this study. Median concentrations of κ and λ FLCs assessed in serum were similar in males and females, without significant differences. In contrast, κ and λ FLCs in urine were significantly higher in males than in females: while in males, the κ FLC concentration in serum and urine was almost equal, in females, it was half as low. A slower clearance was observed for λ uFLCs compared to κ uFLCs; almost 10- and 20-fold lower concentrations of λ FLCs were found in the urine of males and females, respectively. The result was that the κ FLC concentration in the urine for both males and females was

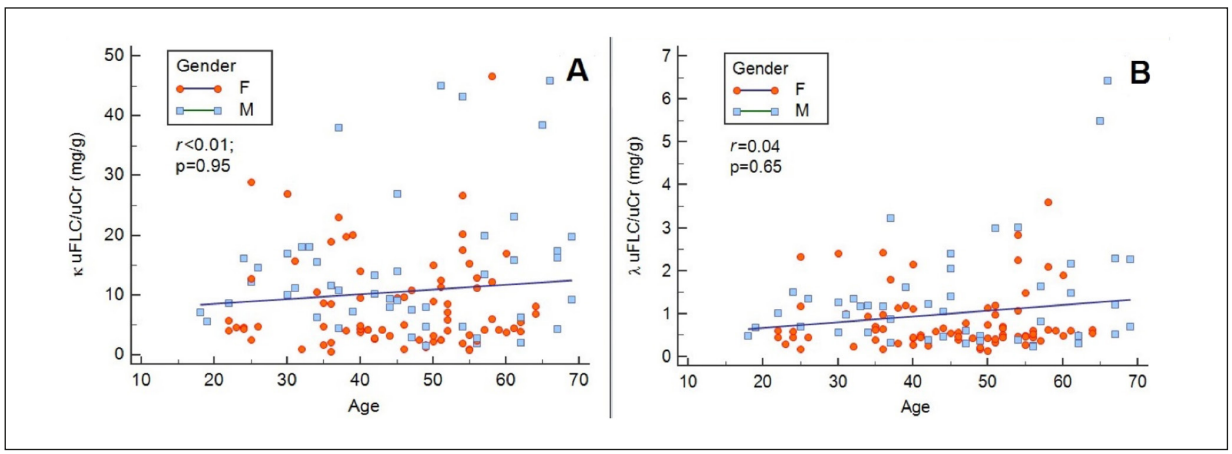

Figure 1. Correlation between urinary free light chains and age. **A**, κ uFLC, urinary kappa free light chains; **B**, λ uFLC, urinary lambda free light chains; uCr, urinary creatinine; r, Spearman's rank correlation coefficient.

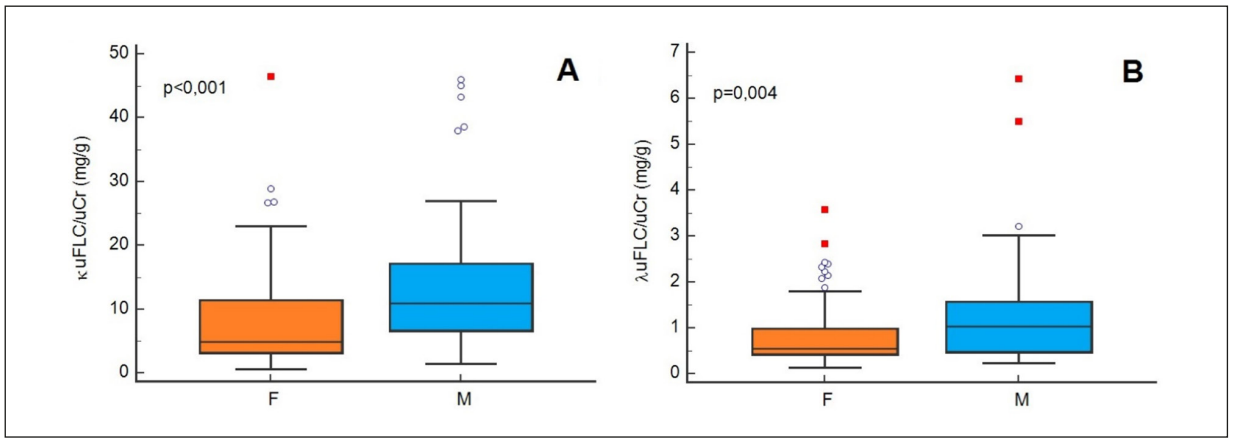

Figure 2. Comparison between urinary free light chains by gender. **A**, κ uFLC, urinary kappa free light chains; **B**, λ uFLC, urinary lambda free light chains; uCr, urinary creatinine, M, males, F, females.

almost 10-fold higher than λ FLC concentration, confirming a slower clearance for λ FLC. This could be due to significant differences in physicochemical properties between κ and λ FLC[16-18]. The physiological mechanism for which κ FLC is excreted more rapidly than λ FLC is probably due to quaternary structural differences exhibited by the two light-chain isotypes and to the molecular polymorphism of urine FLCs; k FLCs usually are monomeric (22-25 kDa), renal clearance is faster than for the dimeric λ FLCs (44-50 kDa)[17].

Additionally, the serum synthesis of κ FLCs is almost twice as high as that of λ FLCs (k:λ = 1.8:1)[5,19]. Differences between the excreted amounts of κ FLCs and λ FLCs can be explained by the dimeric structure of λ FLC molecules limiting their filtration through the glomerular membranes[20,21]. Nowrousian et al[22] demonstrated that the 24-hour clearance for κ FLC is almost 2.5-fold higher than λ FLC clearance. Consequently, the lower serum production and slower clearance of λ FLCs compared with κ FLCs may result in lower urine concentrations of λ FLCs compared with κ FLCs[22-24].

The advantage of monitoring FLC in serum and urine on the same analytical platform is that it allows a more accurate comparison between the results of the two biological fluids, eliminating bias due to different methods.

Calculated URLs for male and female subjects were: κ uFLC/uCr mg/g = 34.35 *vs*. 23.18, and λ uFLC/uCr mg/g = 3.59 *vs*. 1.96, respectively. Even considering the different measurement units (mg/g uCr *vs*. mg/L), the calculated URL agrees well with the one suggested by the manufacturer. Both cases show a 10-fold higher concentration of κ uFLC than of λ uFLC. Furthermore, both the URLs calculated in our study and those suggested by the manufacturer show that κ FLCs are higher in urine than in serum, while the opposite is observed for λ FLCs. However, the manufacturer's recommendations are not gender specific.

Interestingly, in our study, uFLCs concentration correlates with gender despite standardization with creatininuria. Some previous studies[21] have reported that sFLCs and uFLCs concentrations are independent of age and gender. On the other hand, a recent study[2] involving patients

Table II. Urine kappa and lambda free light chains upper reference limits in male and female healthy subjects, calculated according to CLSI EP28-A3c guidelines.

	Males range	Males 97.5th percentile upper limit (90% CI)	Females range	Females 97.5th percentile upper limit (90% CI)
κ uFLC/uCr mg/g	1.49-45.99	34.35 (27.48-39.99)	0.58-46.58	23.18 (17.92-27.63)
λ uFLC/uCr mg/g	0.24-6.43	3.59 (2.51-4.48)	0.13-3.59	1.96 (1.60-2.33)

CI, Confidence Interval; κ uFLC, urinary kappa free light chains; λ uFLC, urinary lambda free light chains; uCr, urinary creatinine.

with chronic kidney disease found a higher uFLC/uCr in male patients than in females. We also found no correlation between uFLCs and age, but it should be noted that our cohort does not represent the over 70s group (age = 18-69), the age at which the prevalence of gammopathies increases[25,26]. In addition to the lack of elderly subjects, this study is also limited by an insufficient number of individuals for each gender group; indeed, CLSI recommended the number of 120 subjects per group[14]. Therefore, further studies should include more subjects and may find age-related differences not found in our study for the relatively young age of enrolled subjects.

We analyze FLCs among HS on a first-morning urine sample, despite the 24-hour urine being the currently recommended specimen for determining renal protein excretion[7,8,27]. However, several problems have been associated with 24-hour urine collection, including frequent incomplete collections, bacterial degradation, problems with storage and transport to the laboratory, and difficulty in sample collection, especially among elderly and frail patients. Additionally, fluid intake, amount of physical activity, diet, body, and environmental temperatures, amount of liquids lost through sweating, and other lifestyle-related variables may affect the reliability of results obtained from 24-hour urine collection[1,28-31]. The official guidelines for chronic kidney disease, Kidney Disease Improving Global Outcomes (KDIGO), recommend albuminuria and proteinuria measurement with a first-morning urine sample and that results should be normalization to creatininuria to reduce intra- and interindividual variability[10,32]. Therefore, we considered the measurement and evaluation of the URL of uFLC as the uFLC-to-uCr ratio in mg/g appropriate in this case.

Future applications of immunochemical measurements of FLCs in urine could also include the monoclonal involved uFLCs of which BJP is composed. This fully automated method allows easier standardization than densitometry[33], the currently recommended method for quantifying BJP[1,34,35]. However, it does not distinguish between monoclonal and polyclonal FLCs[36]. BJP-positive urine must be pre-detected by urinary immunofixation, the only currently available method to detect and characterize urinary monoclonal FLCs[1,35]. The immunochemical measurement of the only involved monoclonal uFLC could be clinically significant only in BJP-positive urines. In particular, the modification in percent of the involved uFLC concentration during treatment could be indicative[37], similar to the modification in percent of BJP recommended by the IMWG to assess treatment response in patients with multiple myeloma (MM)[7,8].

Automated tests in diagnostic routines reduce turn-around time and allow results that are more accurate. Nephelometric/turbidimetric assays may be applied as quantitative methods only after the identification of positive samples by laboratory screening with uIFE[37].

Serum and urine results obtained by the same immunochemical procedure would provide a consistent view of the FLCs produced and excreted by the patient. However, specific studies should be performed in selected patients to investigate the extent of variations in the involved FLC, measured by immunochemical methods, which can be useful to assess therapeutic treatment response in patients with MM with preserved renal function and to demonstrate the clinical effectiveness of this approach[38,39]. Finally, the calculation of the concentration of uFLCs in HS can serve as a basis for further future studies in patients with renal failure[9].

Conclusions

The potential benefits of FLC immunoassays for assessing monoclonal gammopathies, in terms of improved sensitivity, accuracy, cost savings, and the use of serum as a test medium, are considerable. Our study suggests that FLC catabolism is gender-dependent in HS and occurs less rapidly in λ FLC than in κ FLC. Determining of the URL of uFLC, expressed as uFLC/uCr, in HS in morning urine proved to be consistent with the manufacturer's recommendations but with a significant difference according to gender. The automated immunochemical assay can enable rapid and reliable assessment, suitable for a high-intensity laboratory, allowing for measurement of the concentrations of FLC involved, which helps monitor treated MM patients. However, further studies will be helpful to consolidate the results obtained in this study.

Conflict of Interest

The authors declare no conflict of interest.

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Authors' Contribution
Conceptualization and original draft preparation: P.N., U.B., Methodology: V.C., D.D., M.R.C., V.N. Analysis: G.R V.N. Data curation: T.T, G.C. Review and Editing: V.C., U.B., M.M. The first draft of the manuscript was written by P.N., U.B., M.M. All authors commented on previous versions of the manuscript. All authors have read and agreed to the published version of the manuscript.

Informed Consent
Informed consent was obtained from all individual participants included in this study.

Data Availability
The original data presented in the study are included in the manuscript. Further requests should be addressed to the corresponding author..

Ethics Approval
The study was approved by the local Ethics Committee (Comitato Etico Area Vasta Emilia Nord, Emilia-Romagna, Italy. Prot. 32/2021/SPER/AUSLMO-UFLC2021).

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