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The dark side of current analytic methods for Bence Jones Proteinuria

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Abstract. – OBJECTIVE: Bence Jones proteinuria (BJP) refers to monoclonal free immunoglobulin light chains detected in urine, deriving from the clonal expansion of plasma cells in the bone marrow in patients with plasma cell dyscrasias, associated with monoclonal gammopathies of uncertain origin. This review summarizes routinely diagnostic procedures to assess BJP highlighting critical steps of pre-analytical, analytical, and post-analytical phases.

QUALITATIVE AND QUANTITATIVE METHODS: The best option for BJP detection is the first morning void urine sample and immunofixation electrophoresis detection technique (IFE) the recommended method, with the employment of specific polyvalent antisera. Other qualitative tests for a quick evaluation of BJP are currently available. Densitometric analysis performed on the 24-hour urine is the recommended method to quantify BJP. To overcome the 24-hour collection, it is possible to use morning urine sample and correlate the assessed value of BJP to creatininuria. In addition to the traditional ones, we here reviewed screening methods currently used to avoid false negatives and reduce the time around test (TAT), together with immunochemical quantification methods for increased sensitivity, after checking BJP by IFE. Mass spectrometry emerges as a new challenge in the determination of BJP.

CONCLUSIONS: The employment of different based-assays methods may be useful for diagnostic purposes to improve the accuracy of BJP monitoring in monoclonal gammopathies.

Key Words:
Immunofixation, Electrophoresis, Free light chains, Bence Jones protein, Monoclonal component.

Abbreviations

BJP: Bence Jonce protein; CZE: capillary zone electrophoresis; FLCs: free light chains; HR-UPE: High-resolution urine protein electrophoresis; Ig: immunoglobulins; IFE: immunofixation electrophoresis; IMWG: International Myeloma Working Group; MG: monoclonal gammopathy; MGUS: monoclonal gammopathies of undetermined significance; MM: Multiple Myeloma; MRD: minimal residual disease; MS: mass spectrometry; SMM: Smoldering Multiple Myeloma; TAT: Turn-around-time.

Introduction

Bence Jones proteinuria (BJP) is a sensitive and specific marker of monoclonality in lymphoproliferative diseases, characterized by B cell clone proliferation[1]. BJP was described for the

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first time in 1845, in a patient admitted to St. George's Hospital in London under the care of Drs. Watson and MacIntyre for a vague continuous pain referred to chest, back, and pelvis. They found that the addition of nitric acid in patient's urine was able to precipitate an "unknown" substance: it was the first tumor marker discovered[2]. Since then, BJP has been referred as the excretion of monoclonal free light chains (uFLCs) of human immunoglobulins (Ig) in urine.

Monoclonal FLCs play an effective role in diagnostics for screening, diagnosis and monitoring of disease and response to therapies for multiple myeloma and other plasma cell dyscrasias[3]. Different reports[4-9] suggest that polyclonal FLCs, produced by activated B-lymphocytes, could play a pathogenetic role in autoimmune, inflammatory, and cardiovascular diseases, so as in viral infection.

FLC consists of free κ (monomers with a molecular weight of 22 kDa) and λ (dimers of 44 kDa); other fragments existing as lower or higher -molecular weight polymers have also been described[10]. Megalin and cubulin receptors on surface of epithelial cells and of proximal convoluted tubules may bound and reabsorb FLCs. In this manner a valuable re-cycle of proteins and amino acid is possible[11].

Healthy subjects produce 0.5-1 g of FLCs everyday[12]. The efficiency of renal function takes in account the reabsorption of 10-30 g/day[13]. The catabolism of FLCs occurs by glomerular filtration and proximal renal tubular reabsorption. A monoclonal gammopathy (MG) produces a wide increase in FLCs level, which overwhelms renal re-absorption capacity resulting in leakage of BJ protein in urine. When the production of serum FLCs exceeds the capacity of tubular reabsorption[10,12], FLCs deposition, in turn, could lead to renal impairment and BJP: BJ protein leaks out in the urine due to FLCs overflow and reaches the distal tubule forming casts that could be resulting in obstruction (Figure 1).

While serological FLC detection is increasingly used by clinicians as a marker of inflammation and systemic and/or organ specific autoimmunity[5,6], BJP detection is essentially limited in diagnosis of plasma cell dyscrasias and in the monitoring of treatment efficacy and response. BJP quantification is important in defining the diagnosis of Smoldering Multiple Myeloma (SMM) and in the evaluation of therapy response in Multiple Myeloma (MM). In Waldenstrom macroglobulinaemia, consensus is lacking about the usefulness of BJP determination, for both diagnosis and monitoring of disease[8,14-17].

Qualitative and quantitative strategies for determination of BJP as recommended by international guidelines will be here discussed and compared with emerging methods that, although unconventional, display both technical and management advantages.

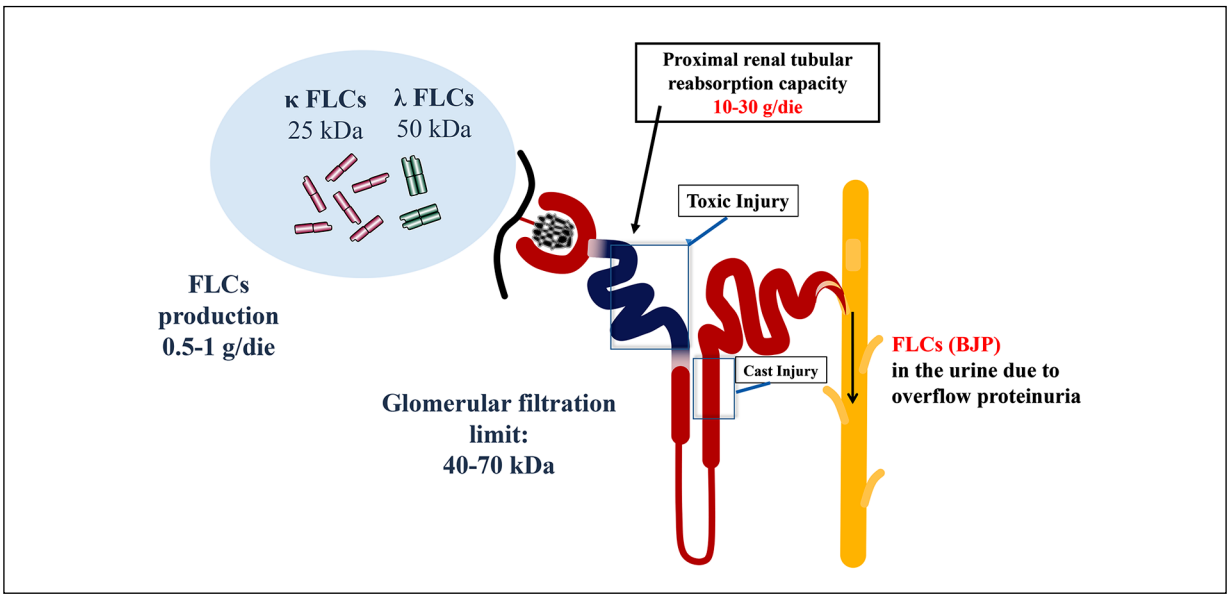

Figure 1. Catabolism of FLCs by glomerular filtration and proximal renal tubular reabsorption.

Qualitative Analysis

BJP is detected by IFE and may be quantified by densitometry of urinary electrophoresis (UPE)[10]. The best option for detection, as suggested by updated recommended guidelines, is the first morning void and BJP concentration should be referred to urinary creatinine[10,18,19] similarly as stated by "Kidney Disease Guidelines: Improving Global Outcomes for proteinuria and albuminuria"[20].

The qualitative BJP-IFE, performed with heavy (γ, α, μ, ε or δ) and light (κ or λ) chains, still represents the "gold standard" for identifying monoclonal FLCs even when heavy chains and other proteins are present. Several manual steps characterize BJP analysis, making it expensive and time-consuming, despite the availability of semi-automated and fully automated instruments[21]. Moreover, it requires dedicated staff with advanced training and expertise in interpretive commenting in a protein laboratory performing all areas of bench work[19,22].

Hight resolution urinary electrophoresis (HR-UPE), capillary zonal electrophoresis (CZE) is not sensitive enough, failing the recommended limit of 10 mg/L. These procedures are time-consuming and expensive requiring purification systems to increase their sensitivity, despite which the risk of producing false negatives remains still very high[10,18,19,23].

A screening uIFE test with a mixture of anti-κ and λ light chain[24], or a mixture of albumin, α1-microglobulin, anti κ and λ antisera[25] is performed to detect BJP prior to setting up traditional urine BJP-IFE with pentavalent anti-IgG, -IgA, -IgM and anti-κ and -λ chains antiserum. This procedure displays the advantage that free light κ and λ chains may be tested in a single lane, identifying negative samples, which can be rapidly ruled out. Non-negative samples are further investigated using traditional uIFE that provides the advantages of rapidity, cost-effectiveness, and reduced TAT.

The effect to screen BJP on the workflow seems to be a TAT reduction due to the method. The TAT obtained from the laboratories information system is based on the elapsed time between the initial recording of request and the final validation of the electrophoresis report[26-28].

A novel approach for testing monoclonal proteins is provided by mass spectrometry (MS), that for its sensitivity and specificity could potentially transform currently employed procedures for characterization and assessment of monoclonal proteins[26,27].

Quantitative Analysis

The urinary levels of FLCs do not reflect serum concentration, due to reabsorption process and/or renal impairment that may result in an inaccurate valuation of tumor burden[28].

BJP level in urine is not associated with myeloma burden but rather with renal function and protein deposition rate in different tissues[29]; its rapid detection together with an earlier start of treatment may ameliorate the prognosis.

The consensus documents and the most representative guidelines of the International Myeloma Working Group (IMWG)[3,30,31] recommend the measurement of BJP to monitor and to evaluate response to therapy, in 24-hour urine collection. Different authors described major issues related to this procedure, starting with the difficulty of collection in elderly and frail patients. Furthermore, urine samples would require refrigeration from collection until arrival in laboratory and then the addition of an antibacterial agent, but these pre-analytic steps are always disregarded[32,33].

The recent updated kidney disease guidelines "Improving Global Outcomes (KDIGO) on chronic kidney disease and acute kidney disease" recommend urine sample from the first morning void for determination of albuminuria and total proteinuria, expressing the relative concentration of urinary creatinine[20]. In analogy, it seems reasonable to use early morning urine sample for BJP quantification correlating the resulting concentration to urinary creatinine.

Conventional Methods

The IMWG guidelines refer to the quantification of BJP by densitometric technique, as described by the American College of Pathologist (CAP)[34]. The CAP guidelines suggest the 24-hour urine collection of specimen[34], although the issues previously discussed.

BJP quantification can be achieved through the densitometric scanning of electrophoresis peaks, determining the ratio between BJP peak percentage to total protein amount: in this way, BJP is expressed in mg/L as a percent of total protein detected in urine. Methods for measuring total proteins in urine are insensitive and not accurate for detecting BJP. A range of critical issues is currently described referring to precipitating reagents for urinary proteins and dye-binding reagents, due to differences in reaction of tubular versus glomerular proteins and in calibrator composition, resulting in the underestimation BJP concentration[19]. Dipsticks currently used to detect

proteinuria are soaked with a buffered dye identifying albumin and not BJP[10].

Electrophoretic methods are performed on agarose gel and differ in volume of sample loading and protein sensitivity depending on staining. The background represents a limitation for all staining methods. Urinary specimen must be concentrated before analysis to ensure appropriate sensitivity using Coomassie Blue. Colloidal Gold is the most sensitive dye, but it displays demanding drawbacks as incubation time and lack of automation step[12]. Crystal violet represents a good compromise between sensitivity and specificity, and it is employed on automated protein gel electrophoresis systems[21].

The electrophoretic technique currently used to highlight the monoclonal peak of BJP is the high-resolution urine protein electrophoresis (HR-UPE). Although this method is more sensitive than traditional electrophoretic ones, it is often not enough to achieve the recommended limits of 10 mg/L[10]. Urinary samples may require to be concentrated (recommended 10- up to 100-fold); and the devices currently employed to this purpose should have a cut-off range of 5-10 kDa.

The limits occurring in HR-UPE produce a heterogeneous range of urinary protein patterns depending on the presence and relative concentration of albumin, glomerular and tubular proteins, BJP, serum paraprotein, polyclonal FLCs, sometimes myoglobin and hemoglobin. Furthermore, light chain ladders, that are not BJPs but can be confused with BJP, could appear in elderly populations suffering from tubular proteinuria due to chronic inflammatory diseases. Sometimes BJPs could co-migrate with these ladders, and the experienced laboratory staff must carefully control to guarantee absence of concomitant BJP[12].

Urinary proteins separated by electrophoresis display some drawbacks, they can have different affinities for the dyes used for staining electrophoretic strips, and lack of linearity of the densitometric response. Presence of multiple bands of BJP in the urine or co-migration of BJP with other proteins, may render it difficult to define the BJP peak by densitometry[34].

The dye does not work with same sensitivity along different electrophoretic sessions, showing false lower concentrations upon detection. Elevated levels of BJP can show areas of prozone that require additional electrophoresis with the diluted specimen.

The inaccuracy of the colorimetric methods used to measure total proteinuria is due to different affinities for different proteins, showing better linearity with albumin concentration with low sensitive and specific to microproteins and BJP. Moreover, major limitations of immunological methods arise from structural differences between monoclonal BJP and polyclonal light chains, usually used as antigen and calibrants respectively. This makes it hard to distinguish between monoclonal and polyclonal FLCs, resulting in a major limitation at low BJP concentrations[12,35].

The assessment of BJP is a clinical index value and therefore the challenge for the laboratory must be to reduce the imprecision of different steps of method, not being able to change its inaccuracy. It is widely suggested that patient's follow-up should be performed in the same laboratory to minimize analytical variability[35].

BJP quantification is limited by metabolic and analytical items, as urinary excretion of FLCs is influenced by degree of protein polymerization, by renal function, and by protein deposition rate in different tissues; for these reasons urinary levels of BJP cannot be related to the tumor cell mass[10,34].

Unfortunately, an accurate measurement of BJP cannot be easily achieved with currently employed laboratory techniques. The MS-based methods for measurement of monoclonal proteins seem to be promising but with limitations due to the relatively small sample sizes analyzed so far[28,36]. The occurrence of fully automated systems on the worldwide market of electrophoresis techniques still requires an educational module suitable for continuing professional development staff expertise, with relatively long analytical times and devoted human resources.

Alternative Methods

An alternative method to agarose gel electrophoresis for quantitative determination of BJP is the zonal capillary electrophoresis (CZE). This procedure shows some limitations due to the difficulty of analyzing samples with low protein concentrations and the presence of many non-protein substances interfering with quality and resolution of electrophoretic separation.

These problems have not been completely solved because the pre-treatment of the urine sample, aimed mostly at the removal of salts alone (usually partial), showed to be very long, complex, and expensive[23]. The CZE can be only used after sample pretreatment and its sensitivity, ideally better than densitometric method's one, is

around 7-12 mg/L for samples, with total urinary protein concentration ≥100 mg/L[23,37].

Immunochemical direct assay represents another approach to detect uFLCs that is able to recognize a hidden antigen not exposed in intact immunoglobulin molecule. The Freelite assay by the Binding Site Group (Birmingham, UK) has been the first immunochemical assay, launched on the market in 2001, initially validated only on serum samples. Serum FLCs quantification performed on an automated nephelometer or turbidimeter[33,34] (Figure 2) has changed diagnostic monoclonal gammopathy criteria, disease monitoring and assessment of stringent or complete response according to the International Myeloma Working Group response criteria[13,38].

Recent international guideline updates for diagnosis of symptomatic MM include at least 60% clonal bone marrow plasma cells or extra medullary plasmacytoma, i.e., the SLiM criteria. SLiM is an acronym for 60% or more clonal plasma cells (S), light chains (Li), and more than one focal lesion on Magnetic Resonance Imaging (M) in addition to the existing CRAB requirement (calcium elevation, renal dysfunction, anemia, and bone disease)[3,39,40]. Serum FLC (sFLC) levels outside the reference range were included (sFLC involved/uninvolved ratio >100; normal reference intervals: free κ 3.3-19.4 mg/L; free λ 5.7-26.3 mg/L; and κ/λ ratio <0.26 or >1.65) to identify patients who might benefit from an early treatment.

The IMWG guidelines do not recommend the determination of uFLC by immunochemical direct methods[13,41], although this procedure is currently validated for the determination of FLC in urine. Noteworthy, the IMWG guidelines indicating the quantification of uFLCs for the assessment of response to therapy predate the validation of the urinary FLC immunochemical determination method in 2018[30,42,43]. The major problem with this assay is the prozone effect that occurs with antigen excess and a falsely negative result may be produced[44]. Furthermore, the most recent automated analyzers – turbidimeters or nephelometers – can detect prozone effect with the availability of software that can recognize and manage with serial dilutions the excess of antigen[45,46] and provide some advantages. The quantitative immunochemical evaluation of urinary FLCs could show a high sensitivity (<1 mg/L)[47,48], a wide measuring range, due to lower and upper linearity limits. The assessment of FLCs in unconcentrated urine samples allows to avoid the interference of complete Igs or other proteins on the measure[38].

The measurement of urinary FLCs does not properly provide an indication of BJP concentration because polyclonal FLCs are not distinguished from monoclonal ones. First, it is necessary to check the presence of BJP by uIFE and only subsequently, for the purpose of disease monitoring and therapy, go on with the measure-

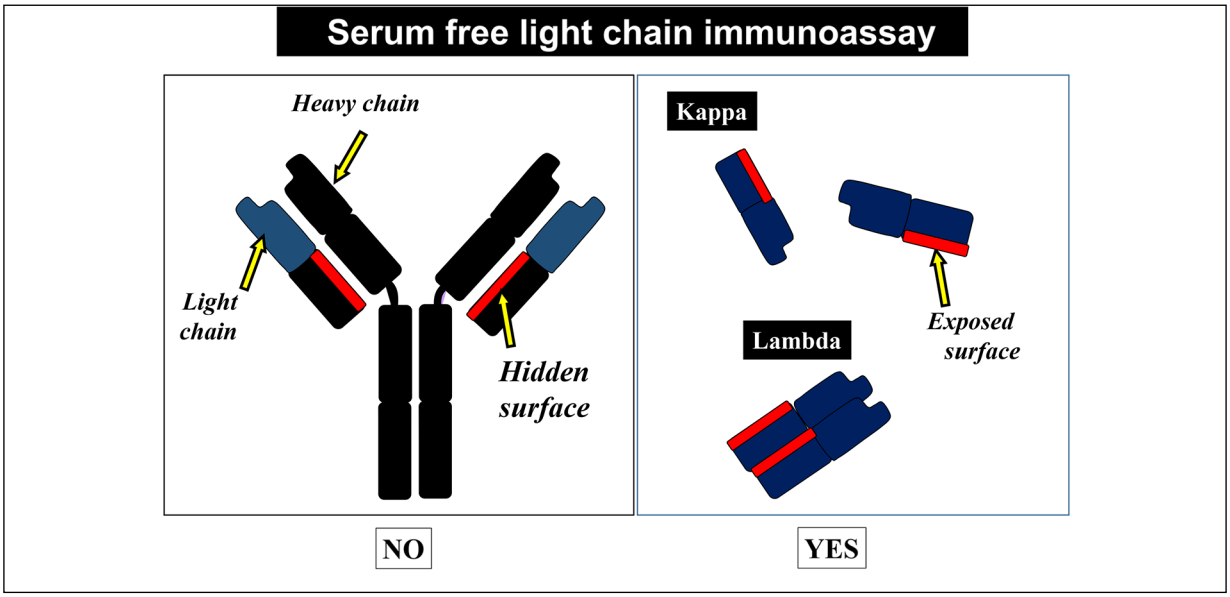

Figure 2. Serum FLCs quantification as performed by the Freelite immunochemical assay (Binding Site Group, Birmingham, UK).

ment of uFLCs, particularly of the involved chain that can be useful. The conventional quantitative method to measure BJP displays an inaccurate, imprecise, and empirical performance discouraging its use in daily laboratory practice. In contrast, the measurement of uFLCs is automated, standardized, and reproducible. By performing this measurement in association with a preliminary uIFE to confirm the presence of BJP, the immunochemical method could be an aid in monitoring response to therapy in patients with preserved renal function[49]. Serum FLC measurements are directly related to light chain production, whereas urinary FLC measurements take in account renal reabsorption. The different catabolism of FLC κ and λ, in subjects with nephropathy, as well as a possible κ and λ biclonality, could cause a false alteration or normalization of the urinary FLC κ/λ ratio[38,50]. Therefore, to prevent this bias, it is advisable to measure only the free chain involved in patients, taking care to ensure that the patient's renal function is preserved.

Despite all limitations due to the automated method, respect to the quantification of BJP obtained with densitometric and colorimetric techniques, detection of urinary FLCs seems to be a reliable alternative to HR-UPE and CZE for monitoring BJP concentrations in multiple myeloma patients[48,49].

Finally, a new method that is still under validation for its application in daily laboratory diagnostics is Mass Spectrometry. It is based on the enrichment of Igs, followed by a reduction step to separate light from heavy chains, and then a micro flow liquid chromatography coupled to mass spectrometry instrument. In this way, MS allows monitoring monoclonal light chain; subsequently, the top-down MS isotyping provides both quantification and identification, displaying a better sensitivity and specificity for detection the minimal residual disease if compared with conventional methods[27]. Thanks to the increased accuracy and analytic specificity, the IMWG Mass spectrometry Committee recommends MS for the detection of monoclonal proteins; it allows to distinguish residual monoclonal component from monoclonal antibodies employed in the drug treatment[51].

Conclusions

Addressing the limitations and performance of a diagnostic technique is important for better interpretation of laboratory data. Automated tests in diagnostic routines reduce TAT, facilitating results that are more accurate. uIFE screening in qualitative BJP detection shows good results, encouraging these methods as a first-line diagnostic testing strategy. The employment of different based-assays methods may be useful for diagnostic purposes to improve the accuracy of BJP monitoring in monoclonal gammopathies. Nephelometric/turbidimetric assays may be applied as a quantitative method only after the identification of positive samples by laboratory screening with uIFE. A workflow for the qualitative determination of BJP and the quantitative determination of uFLC is proposed in Figure 3, especially recommended in laboratories with high throughput, where the large number of samples cannot be to the detriment of the accuracy and precision of the results. Screening approach with immunofixation evaluates positive samples for BJP, subsequently confirming the results with uIFE assay. In regard to the quantitative uFLC, the measurement with immunochemistry platforms allows to improve

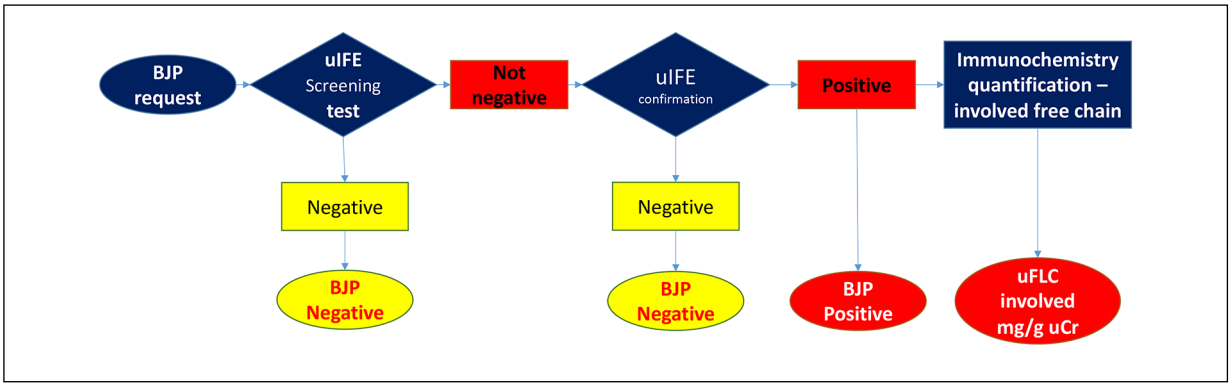

Figure 3. A workflow proposal for the qualitative determination of BJP and the quantitative determination of uFLC.

data repeatability and organizational skills by automation. Additionally, assaying only the involved urinary free light chain ensures a higher accuracy and precision.

Finally, in the future, a third-level test can be added through the widespread use of mass spectrometry methods to perform a standardized assay for monitoring therapy, assessing response to treatment, and detecting minimal residual disease (MRD).

The precision medicine era is focused on identifying those approaches, more effective for patients based on genetic, environmental and lifestyle factors, considering residual myeloma cells as clinically relevant for disease progression and relapse. In MM, MRD refers to myeloma cells that are hidden in the bone marrow after a clinical response has been measured and the patient is in remission. The future goal of urinary FLCs must be a more sensitive methodological approach for MRD identification. New procedures and methods could open new scenarios in therapy monitoring consistent with the presence of residual disease and perhaps predictive of relapse by improving survival of a disease that still appears incurable.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Authors' Contribution

P.N. and G.C. analyzed literature and prepared the original draft of the manuscript; C.N., V.B., D.D., K.P., M.S. collected and analyzed data from literature; M.M. critically reviewed and submitted the final version; F.G. and U.B.: coordinated and supervised the group.

Informed Consent and Ethical Approval

Not necessary for this type of study.

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