




Review

Is Circulating DNA and Tumor Cells in Myeloma the Way Forward?

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Abstract: Multiple myeloma (MM) is the second deadliest hematological cancer. Despite the enormous innovation on MM treatment in the last decades, still 48% of patients die within 5 years after diagnosis. MM diagnosis and therapeutic strategy mainly rely on direct bone marrow (BM) assessment. Given the MM heterogeneity, BM biopsies do not accurately reflect the whole disease status, hampering accurate disease prognosis. Moreover, biopsies are painful and invasive procedures, highlighting the need for non-invasive and more accurate source of biomarkers. Liquid biopsies are promising sources of biomarkers that may overcome these limitations. Peripheral blood carries circulating myeloma components that are being extensively explored since the last few years as an alternative to BM aspirates. These include circulating tumor cells (CTC), cell-free DNA (cfDNA), and extracellular vesicles containing miRNA and proteins. The current review summarizes scientific evidence establishing BM as a gold standard for the diagnosis, prognosis, and evaluation of minimal residual disease. We discuss the last advances regarding cfDNA and CTC biomarkers from peripheral blood in patients with MM as well as the statistical validations. This paper addresses the technological hurdles associated with liquid biopsies and examines the missing steps for their inclusion into the clinical practice.

Keywords: multiple myeloma; liquid biopsies; cell-free DNA; circulating tumor plasma cell; biomarker validation; statistical assessment; diagnosis; prognosis; minimal residual disease



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1. Introduction

Multiple myeloma (MM) is a malignant plasma cell disorder that accounted for 14% of all hematologic cancers in 2020 [1]. According to the Globocan 2020 report, the 5-year prevalence of myeloma in Europe is 18.44 per 100,000 habitants [2]. It usually evolves from a premalignant stage termed “monoclonal gammopathy of undetermined significance” (MGUS). According to the International Myeloma Working Group (IMWG) [3], MGUS is asymptomatic and characterized by less than 10% of plasma cell infiltration in the bone marrow (BM) and serum monoclonal protein (M-protein) below 3 g/dL. In some patients, a more advanced premalignant stage, referred to as “smoldering multiple myeloma” (SMM), is clinically recognized [4]. SMM patients are also asymptomatic but are characterized by BM plasma cell infiltration (BMPC) between 10 to 59% and/or serum M-protein above 3 g/dL and/or urinary M-protein above 500 mg per 24 h. Patients are diagnosed with active MM when they present a myeloma-defining event (end organ damage or biomarker of malignancy) and BMPC above 10%. Recently, BMPC above 60% without any myeloma-defining event has been included as a sufficient criterion for MM diagnosis [5]. The criteria defining a patient with MGUS, SMM, or MM are related to his/her risk to progress to the

symptomatic disease phase and expected overall survival (OS). MGUS and SMM patients have a risk of progression to MM of 1% [6] and 10% per year in the first 5 years and then lower risk [7], respectively. Current guidelines do not recommend treating these patients, as benefits would not be sufficient given the possible toxic side effects of treatment protocols. The decision to treat MM patients implies that the benefits in terms of decreased tumor load, increased quality of life, and increased life expectancy overcome the toxic side effects of drugs and the cost of treatment.

Despite continuous therapeutic progresses in the last decade, some MM patients still die rapidly, whereas others respond well to treatment. This is mostly related to the genetic heterogeneity of the disease [8] and to the immune profile of the BM microenvironment [9,10], making prognosis and therapeutic strategy difficult to accurately determine. The most common current strategy for genetic assessment and immune profile of MM relies on one-site invasive bone marrow biopsy that cannot reflect the whole status of the disease. This means that the decision to treat a patient, the choice of the therapeutic strategy, and the follow-up largely rely on the direct evaluation of clonal plasma cells from one site in the BM, or, rarely, from other organ biopsy, in case of extramedullary MM. Biopsies are painful and invasive procedures which increase the psychological burden associated with the disease.

Less invasive and more reliable alternative strategies are needed for MM tumor load quantification, genome assessment, and therapeutic monitoring.

Liquid biopsies are being considered as a relevant alternative source of information for tumor status. They provide an accessible and minimally invasive way for genomic tumor assessment and are an important source of biomarkers. Liquid biopsies include body fluids and blood containing tumor-derived sources of information, such as cell-free DNA (cfDNA), circulating tumor cells (CTC), cell-free RNA, and extracellular vesicles. Lately, efforts are being made to use liquid biopsies as adjuvant or even substitutes for BM biopsies. This review will first present the clinical foundations behind the direct evaluation of myeloma plasma cells using BM biopsies. In the second and third parts, we will review two types of liquid biopsies as promising ways for indirect assessment of myeloma status: cfDNA and circulating tumor plasma cell (CTPC) for diagnosis, prognosis, and therapeutic monitoring. Finally, the statistical methods to determine the clinical relevance of BM and liquid biopsies in MM will be discussed.

2. Direct vs. Indirect Assessment of Myeloma Plasma Cells

2.1. Myeloma Plasma Cells Direct Assessment through Bone Marrow Biopsies

The term “plasma cell” was introduced by W. Waldeyer in 1875 [11]. Marschalko published the first light microscopic description of plasma cells in 1895, including blocked chromatin, eccentric position of the nucleus, a perinuclear pale area, and a spherical or irregular cytoplasm [12]. Wright suggested that the tumor cells of myeloma consisted of plasma cells or immediate descendants of these cells [13]. Later, Santiago Ramon y Cajal [14] was the first to accurately describe the morphology and development of plasma cells.

The value of BM aspiration in the diagnosis of MM was presented for the first time in 1938 [15], after the introduction of the aspiration technique in the sternum [16,17]. The percentage of BMPC above 20% was stated as a diagnosis parameter only in 1973 [18] and then adjusted to the current cut-off value of 10% based on studies, such as those by Salmon and Durie [19] and Merlini et al. [20]. Upon this historical development, BM biopsy and aspirate have been established as the gold standards for myeloma diagnosis and prognosis [21,22] (Table 1). Of note, discrepancies between the percentage of plasma cells in BM aspiration and BM biopsy have been reported [23] and this information is incorporated in the current guidelines.

Table 1. Summary of BM and liquid biopsies evidence as diagnosis biomarkers.

Type of Biopsy	Cohort (Sample)	Method	Findings	Reference
PB	NDMM = 389	Kaplan–Meier curves	Shorter OS for patients with BMPC $\geq 10\%$ vs. $<10\%$. $p = 0.01$	Lee et al., 2017 [23]
BM	SMM = 651	Kaplan–Meier curves Log-rank test	Shorter TTP for BMPC $\geq 60\%$ vs. BMPC $< 60\%$, $p < 0.001$ 95% of patients with BM $\geq 60\%$ progressed to active myeloma within 2 years after diagnosis Median TTP = 7 months (95% CI, 1.0 to 12.9)	Rajkumar et al., 2011 [4]
	SMM = 96	ROC Multivariable Cox regression model	BMPC cut-off $\geq 60\%$ in patients progressing at 18 months (specificity = 95.5%) BMPC $\geq 60\%$ associated with high-risk progression ($p < 0.001$; HR = 13.7)	Kastritis et al., 2013 [24]
cfDNA	MM = 53 (64) Other cancers = 56	QIAamp Circulating Nucleic Acid kit (Qiagen)	MM = 20.1 ng/mL plasma Other cancers = 10.3 ng/mL $p < 0.001$	Kis et al., 2017 [25]
	MM = 37 HD = 21	QIAamp Circulating Nucleic Acid kit (Qiagen)	MM = 23 ng/mL (5–195) HD = 15 ng/mL (6–32) $p = 0.0085$	Mithraprabhu et al., 2017 [26]
	EM-MM = 8 (22) MM = 10 (23)	QIAamp Circulating Nucleic Acid kit (Qiagen)	MM = 16.4 ng/mL (4.3–214.7) EMM = 43.6 ng/mL (3.5–313.5) $p = 0.009$	Long et al., 2020 [27]
	MM = 77 SMM = 25	QIAamp Circulating Nucleic Acid kit (Qiagen)	MM = 25.2 ng/mL (0.2–467.14) SMM = 12 ng/mL (4.6–39.5) $p = 0.0001$ cfDNA concentration related to BMPC, $R^2 = 0.13$, $p = 0.001$	Deshpande et al., 2021 [28]
CTPC	Paired BM and PB samples MM = 72 SMM = 25 MGUS = 150 HD = 71	NGF	MM = 1.9 CTPC/ μ L (0.007–339.9) SMM = 0.16 CTPC/ μ L (0.005–12.9) MGUS = 0.008 CTPC/ μ L (<0.001 –9.8) $p < 0.05$ Correlation between BMPC and the absolute number of CTPC ($\rho = 0.78$; $p < 0.001$). Cut-off discriminating MGUS and MM—CTPC/ μ L ≥ 0.058 ($p < 0.001$).	Sanoja-Flores et al., 2018 [29]

The quantification of BMPC of newly diagnosed patients (NDMM) will first guide the decision of the hematologist to treat or not. Current guidelines recommend not treating MGUS and SMM patients. However, recent clinical trials have demonstrated the benefits of treating high-risk SMM patients with impact on OS [30], suggesting that BMPC criteria for diagnosis may need to be re-evaluated.

Second, BM aspirates are used for the immunophenotypic assessment of plasma cells, immune profiling, and the cytogenetic characterization of the clonal cellular content (Table 2). In NDMM patients, the cytogenetic evaluation is included in the clinical work-up to identify the presence of high-risk chromosomal alteration, including del(7p), t(4;14), and t(14;16) [31]. A meta-analysis of 3060 MM patients revealed that the risk of progression or death is almost twice higher in patients with high-risk vs. standard-risk (i.e., none of the high-risk alterations) cytogenetic alterations (CA; HR = 1.83 for progression and HR = 2.03 for death). In 2015, these high-risk CA have been included in the classification of NDMM patients under the Revised International Staging Score (R-ISS) [32]. Recently, a R2-ISS, including 1q copy number alterations, has been proposed [33]. Clinicians currently use R-ISS classification to better choose treatment options [34]. Although the new scoring system improved the accuracy of prognosis [32,33], a significant proportion of patient will relapse earlier or later than expected, implying that they will receive suboptimal treatment.

Table 2. Summary of BM and liquid biopsies evidence as prognosis biomarkers.

Type of Biopsy	Cohort (Sample)	Method	Findings	Reference
PB	MM = 10,750	Cox regression model Survival tree model	ISS model composed by: - Serum β 2-microglobulin (HR = 1.81) - Serum albumin levels (HR = 1.28)	Greipp et al., 2005 [35]
BM	MM = 3060	K-adaptive partitioning Univariable and multivariable Cox regression model	R-ISS model composed by: - ISS level (II vs. I: HR = 2.39/III vs. I: HR = 4.68) - Serum LDH level (HR = 2.55) - BMPC cytogenetic alteration risk (HR = 2.03)	Palumbo et al., 2015 [32]
	MM = 7077	Multivariable Cox regression model	R2-ISS model composed by: - R-ISS - BMPC 1q copy number alterations (HR = 1.45)	D'Agostino et al., 2020 [33]
cfDNA	Paired BM and blood samples MM = 53 Training = 23 MM Validation = 17 MM	LBSeq WGS	51 total mutations 49 (96.1%) BM-cfDNA mutations 2 (3.9%) BM-only mutations 5 (9.8%) cfDNA-only mutations >98% specificity	Kis et al., 2017 [25]
	Paired BM and blood samples MM = 18	Serum samples = ddPCR (NRAS, KRAS, BRAF) BM samples = WES + PCR	35 total mutations 34 (97.1%) BM-cfDNA mutations 1 (2.9%) BM-only mutation 0 (0%) cfDNA-only mutation Correlation of mutations levels between BM and cfDNA ($r = 0.507, n = 34, p < 0.002$) Covariation between M protein level and ctDNA	Rustad et al., 2017 [36]
	Paired BM and blood samples MM = 48 [NDMM = 15 RRMM = 33]	OnTarget Mutation detection (OMD)	128 total mutations 38 (29.7%) BM-cfDNA mutations 59 (46.1%) BM-only mutations 31 (24.2%) cfDNA -only mutations cfDNA-only mutations RRMM > NDMM (27.2% vs. 6.6%, $p = 0.25$ Chi-squared test)	Mithraprabhu et al., 2017 [26]
	MM = 22 (CR) Sequential cfDNA samples	NGS for IgH gene rearrangement MFC	Patients with $\geq 4.7%$ ($n = 12$) of IGH cfDNA had inferior PFS than patients with $< 4.7%$ ($n = 10$) (HR = 3.507, $p = 0.04988$, log-rank test)	Biancon et al., 2018 [37]
	Paired BM and cfDNA samples, MM = 9 Paired BM, cfDNA and CTPC samples, MM = 4	ULP-WGS WES	17% of BM-only mutations 12% of cfDNA-only mutations Tumor fraction in cfDNA correlate with disease stage ($p < 0.001$) and RISS ($p = 0.032$) 99% of BMPC mutations found in cfDNA or CTPC 94% of cfDNA or CTPC mutations found in BMPC	Manier et al., 2018 [38]
	Paired BM and blood samples EM = 8 (22) MM = 10 (23)	NGS and ddPCR	16 total mutations 12 (66.7%) EM-cfDNA mutations 5 (31.2%) EM-BM mutations ctDNA better represents EM mutations than BM biopsies (ROC = 0.873 vs. 0.621)	Long et al., 2020 [27]
	cfDNA MM = 77 ctDNA MM = 17	Ultra-Low Pass (ULP)-WGS	cfDNA > 25.2 ng/mL is related to shorter PFS (HR = 6.4) e OS (HR = 4.4) High ctDNA level correlates with high-risk GEP70 ($p = 0.0027$, Spearman $r = 0.69$)	Deshpande et al., 2021 [28]

Table 2. Cont.

Type of Biopsy	Cohort (Sample)	Method	Findings	Reference
CTPC	MGUS = 325	PC immunofluorescence	MGUS with CTPC ($n = 63$, 19%) were twice as likely to experience progression to plasma cell disorder (HR = 2.1, $p < 0.03$)	Kumar et al., 2005 [39]
	RRMM = 42	6-color MFC	Shorter TTP (=51 days) and OS (=308 days) when CTPC have aberrant phenotype compared to other patients (TTP = 258 days, OS = 856 days; TTP = 581 days, OS = 1006 days; $p < 0.001$ and $p = 0.007$ for TTP and OS, respectively).	Peceliunas et al., 2012 [40]
	SMM = 91	PC Immunofluorescence	High CTPC increase risk of progression within 2 year (14/91, 15% of patients; risk of progression: 71% versus 24%, respectively, $p \leq 0.001$). High CTPC levels reduces OS (49 months versus 148 months; HR = 5.9, $p < 0.001$)	Bianchi et al., 2013 [41]
	NDMM = 157	6-color MFC (detection limite of 20/150,000 events)	CTPC $\geq 400/150,000$ ($n = 37$, 24%) associated with adverse cytogenetics, shorter TTNT and OS (14 months and 32 months vs. 26 months and not reached, respectively, $p < 0.001$).	Gonsalves et al., 2014 [42]
	ACST MM = 840	6-color MFC	Shorter PFS and OS in patients with CTPC (15.1 months vs. 29.6 months and 41.0 months vs. not reached, respectively, $p < 0.001$). CTPC is a predictive factor of mortality (HR = 2.5, $p = 0.001$) and sCR post-transplant (HR = 0.4, $p < 0.001$).	Chakraborty et al., 2016 [43]
	Paired BM and PB samples MM = 29 (8)	FACS WES	100% of clonal mutations in patient BM were detected in CTPC and that 99% of clonal mutations in CTPC were present in BM MM.	Mishima et al., 2017 [44]
	MM = 41 (104 PB; 29 BM) Clinical trial- EudraCT no. 2010-019173-16	ASO-PCR (Detection limit $\leq 10^{-6}$)	CTPC reduced by 97% after therapy induction and by 86% after ASCT.	Huhn et al., 2017 [45]
	SMM = 100	6-color MFC (Detection limit 20/150,000 events)	Patients with ≥ 150 CTPC ($n = 9$) with higher risk of progression to MM within 2 years (97% specificity and 78% sensitivity). TTP shorter for SMM ≥ 150 CTPC (9 months vs. not reached, $p < 0.001$).	Gonsalves et al., 2017 [46]
	NDMM = 247	6-color MFC (detection limit 10/150,000 events)	Less sCR for patients with CTPC (12% ($n = 48$) vs. 32% ($n = 117$) $p = 0.018$. Higher risk of mortality for patients with CTPC (HR = 5.7, $p < 0.001$) vs. patients without	Chakraborty et al., 2017 [47]
	Paired BM and PB samples MM = 72 (Solitary plasmacytoma = 17) SMM = 25 MGUS = 150	NGF (Detection limit $\geq 10^6$ /tube)	R-ISS III patients have higher CTPC counts vs. R-ISS I and II ($p = 0.001$ and $p = 0.004$, respectively). Increased PFS and OS in patients with < 0.1 CTPC/ μL (94% vs. 40%, $p = 0.014$; 100% vs. 67%, $p = 0.03$, respectively). Cut-off ≥ 0.058 CTPC/ μL discriminates MGUS vs. MM.	Sanoja-Flores et al., 2018 [29]
ASCT MM = 227	7-color NGF	Patients with CTPC ($n = 27$, 18.8%) have poorer PFS ($p = 0.031$) and higher risk of progression or death (43%, $p = 0.04$) when combined with high-risk cytogenetics and ISS.	Cowan et al., 2018 [48]	
Paired BM and PB samples MM = 53 (NDMM = 37; RRMM = 16)	NGF WES	First-time sequencing of triple-matched samples. CTPC detected in the PB of all patients (3.5 CTPC/ μL , range: 0.115–1248). Detected 537/658 mutations (82%) in CTPC present in BM tumor cells. Detected 48/52 altered genes (92%) in CTPC also present in BM or EM tumor cells.	Garcés et al., 2020 [49]	

Although 75% to 90% of MM patients currently reach a complete response (CR), the majority will eventually relapse. IMWG recommends quantification of minimal residual disease (MRD) to improve the assessment of response depth. For patients reaching a

complete response, MRD is a more sensitive measure of disease burden than a traditional definition of response. A negative MRD result on BM aspirates is associated with increased survival, regardless of disease staging and eligibility for transplant [50] (Table 3).

Table 3. Summary of BM and liquid biopsies evidence as drug response and MRD biomarkers.

Type of Biopsy	Cohort (Sample)	Method	Findings	Reference
BM	Start of maintenance therapy, MM = 224 After maintenance therapy, MM = 183	Kaplan–Meier curves Log-rank test Cox regression model	PFS and OS significantly extended in MRD negative vs. MRD positive at start and after of maintenance therapy PFS: HR = 0.22/OS: HR = 0.24 OS: HR = 0.18/OS: HR = 0.26 PFS longer in patients with MRD < 10 ⁻⁶ vs. MRD between 10 ⁻⁶ and 10 ⁻⁵ (HR = 1.94)	Perrot et al., 2018 [51]
	MM = (31) BM	Spearman’s test for paired data Log-rank test	Good correlation between NGF and NGS (rho = 0.62, p = 0.001). MRD negative patients by NGF presented extended PFS (p = 0.01) vs. patients with MRD positive.	Flores-Montero et al., 2017 [52]
cfDNA	Early relapse, MM = 28 Late relapse, MM = 25	QIAamp Circulating Nucleic Acid kit (Qiagen)	cfDNA level higher in late relapse than in early relapse (p = 0.016)	Kis et al., 2017 [25]
	MM = 7	ddPCR	in 3/7 patients ctDNA level coincided with serological changes of relapse in 4/7 patients, ctDNA level anticipated serological changes associated with relapse	Mithraprabhu et al., 2017 [26]
	Paired cfDNA and CTPC samples MM = 27	NGS for IgH gene rearrangement	Association between cfDNA/CTPC levels and response status (p < 0.001). Better clearance of cfDNA than M protein in responder patients.	Oberle et al., 2017 [53]
	MM = 22 MRD negative = 6	NGS for IgH gene rearrangement (5 × 10 ⁵ reads) MFC	n = 6 patients with negative MRD (<5 cells/10 ⁵) by MFC Correlation with IGH cfDNA (<10 ⁻⁵ , r = 0.5831, p = 0.0044, Pearson’s correlation test). Longer PFS for patients with lower level of IGH cfDNA (p < 0.001)	Biancon et al., 2018 [37]
	Paired BM and blood samples MM = 37 MRD negative = 11	NGS for IgH gene rearrangement	Negative predictive value (Specificity) = 36% (10/28) Positive predictive value (Sensitivity) = 89% (8/9) No quantitative correlation between ctDNA and BM mutations	Mazzotti et al., 2018 [54]
	Paired BM and blood samples MM = 12 MRD negative = 6	ASO-qPCR for IgH gene rearrangement MFC (10 ⁻⁶)	Negative predictive value (Specificity) = 83.3% (5/6) Positive predictive value (Sensitivity) = 66.7% (4/6) More patients with low level ctDNA have reached CR vs. patients with high level of ctDNA. ctDNA better reflect MRD status than M-protein level	Vrabel et al., 2020 [55]
	MM = 41 (29 BM;104 PB) EudraCT no. 2010-019173-16	ASO-PCR detection limit: ≤10 ⁻⁶	MRD negative = 27 MRD positive = 14 Correlation between BMPC and CTPC in MRD positive (tau = 0.604; p = 0.003).	Huhn et al., 2017 [45]
CTPC	NDMM = 458 PETHEMA/GEM2012MENOS65)	NGF detection limit ≤2.9 × 10 ⁻⁶	MRD negative = 205 MRD positive = 61 MRD negative: 82% reduction in the risk of progression or death (HR = 0.18; p < 0.001), 88% reduction in the risk of death (HR = 0.12; p < 0.001).	Paiva et al., 2020 [56]

MRD quantification can be made through next-generation flow (NGF) or next-generation sequencing (NGS). These two methods are used in BM aspirates with

different degrees of expertise and, thus far, there are no clear advantages of one over the other. The quantification of MRD by multiparametric flow cytometry has been less sensitive than sequencing but, by using the optimal technical approach, as indicated by the EuroFlow Consortium, the sensitivity of immunophenotyping is now equivalent to sequencing [52]. Furthermore, flow cytometry is less labor-intensive, less time-consuming, and more readily available than sequencing.

The IMWG defines the MRD result as negative if no (0) myeloma cell is detected in 10^5 plasma cells using NGF or if no (0) IGH/IGK rearrangement is detected in 10^5 non-altered sequence by NGS on pre-sorted plasma cells [57]. Although the IMWG recommends an MRD sensitivity threshold of at least one in 10^5 , improved OS and PFS have been reported when a threshold of 10^{-6} is considered [51].

Thus far, BM samples have been used as a hallmark for diagnosis, prognosis, and response evaluation in MM. However, there is still potential for improvement by exploring new and more accessible biomarkers of disease.

2.2. Myeloma Indirect Assessment through Circulating Tumoral DNA

The first demonstration of cell-free DNA (cfDNA), also called circulating DNA (cirDNA), in healthy human blood was carried out in 1948 by Mandel and Metais [58]. It was only in 1966 that high levels of cfDNA were associated with a disease condition, i.e., systemic lupus erythematosus [59], and, in 1977, with the presence of tumor [60]. Analysis of circulating DNA sequence from cancer patients revealed that it partially originates from the tumor [61], which is now designated as circulating tumoral DNA (ctDNA).

The presence of cfDNA in MM patients was first described in 2006 where 65% of a cohort of 31 myeloma and lymphoma patients presented fragmented circulating nucleosomal DNA versus none in the control population ($n = 10$) [62]. Eleven years later, Kis et al. showed that MM patients have the highest cfDNA levels amongst cancers patients (median value in MM = 20.1 ng/mL plasma, $n = 53$ vs. 10.3 ng/mL in other cancers, $n = 56$ [25]—Table 1), opening the way for cfDNA as a non-invasive alternative to BM biopsies.

Since then, several studies showed a significant relation between the concentration of cfDNA in plasma and MM diagnosis (Table 1). Mithraprabhu et al. reported a significant increase in cfDNA in plasma from 37 MM patients compared to 21 healthy donors (MM = 23 ng/mL vs. HD = 15 ng/mL plasma, $p = 0.0085$ [26]). More specifically, Deshpande et al. found that the level of cfDNA is significantly higher in active MM than in SMM patients (MM = 25.2 ng/mL, $n = 77$ vs. SMM = 12 ng/mL plasma, $n = 25$, $p = 0.0001$ [28]). Importantly, they showed that increased cfDNA plasma content, although not linearly correlated, was significantly related to higher tumor burden in the BM of 77 MM patients ($R^2 = 0.13$, $p = 0.001$). Although they require further validation on larger cohorts at various stages of the disease, these studies suggest that cfDNA plasma concentration is related to disease stage and BMPC, making it a serious candidate for non-invasive MM diagnosis.

Since 2017, many efforts have been made to investigate cfDNA as a potential way to assess MM genetics (Table 2). The comparison of BM and cfDNA by NGS reveals that most mutations are detected in both types of biopsies (27 to 97.1% of total detected mutations are common between BM and cfDNA, Table 2) and that mutation levels are correlated between BM and cfDNA ($r = 0.507$, $n = 34$, $p < 0.002$ [36]). Interestingly, a significant proportion of mutation is only detected in cfDNA and not in the BM (0 to 40% of total mutations—Table 2). This ratio increases in relapsed and refractory (RRMM) patients (27.2% in RRMM vs. 6.6% in NDMM, $p = 0.25$ Chi-squared test [63]), supporting higher spatial and genetic heterogeneity in advanced diseases. It is noteworthy that the study of extra-medullary myeloma (EMM) revealed that mutations from EMM biopsies have more similarities with cfDNA than BM (66.7% vs. 31.2%; ROC = 0.873 vs. 0.621, respectively [27]), indicating that cfDNA may be more representative of EMM mutations than BM biopsies.

These studies suggests that cfDNA may permit the identification of additional mutant clones, distant from the BM biopsy site, which could be used for patient prognostication.

This potential is supported by studies showing a significant association of cfDNA levels with R-ISS [38], cytogenetic risks, and survival [28]. Deshpande et al. reported that patients with cfDNA concentration above 25.2 ng/mL plasma had shorter OS and progression-free survival (PFS, $n = 77$, HR = 4.4 and 6.4, respectively [28]).

However, results appear quite heterogeneous among the wide range of sequencing approaches (whole genome sequencing (WGS), ultra-low pass WGS, on-target mutation detection (OMD), ddPCR, and LBSeq) (see Table 2). The use of different blood processing protocols has been reported to produce important discrepancies in maternal plasma DNA concentrations [64]. Furthermore, some mutations detected in the BM are not found in cfDNA (2.9 to 46.1% of total mutations). The integration of cfDNA in the prognostic evaluation of NDMM will require protocol standardization, improvement of sensitivity, and an eventual association with additional parameters.

The study of cfDNA for treatment monitoring is less documented but is producing promising results (Table 3). Plasma level of cfDNA is related to sFLC levels [38,65] and other serological biomarkers [26] during treatment. Kis et al. reported higher cfDNA levels in late relapse patients ($n = 25$) than in early relapse patients ($n = 28$, $p = 0.016$) [25], and Mithraprabhu et al. observed that reduced ctDNA level 5 days after treatment was correlated with increased PFS ($n = 24$, $p = 0.017$) [66,67]. Interestingly, Oberle et al. reported that cfDNA levels are more dynamic and better represent patient response than M-protein [53], which is the current gold standard for response monitoring. The rapid clearance of cfDNA, previously observed with fetal DNA in maternal plasma [68], may be an asset for close patient monitoring.

In terms of the depth of response measurement, the evaluation of MRD represents a real challenge as only bone marrow aspirates provide enough material to accurately quantify such a low tumor load. Among the few studies comparing MRD using BM to MRD using cfDNA, Mazzotti et al. reported a cfDNA specificity of 36% and a sensitivity of 89% ($n = 37$ MM patients) using NGS, with no correlation between ctDNA and BM mutation levels [54]. More encouraging, Vrabel et al. observed a cfDNA specificity of 83.3% and a sensitivity of 66.7% ($n = 12$ MM) using allele-specific oligonucleotide (ASO)-qPCR, with a correlation between ctDNA level and the number of patients reaching CR [55]. Of note, a study on 22 MM patients in CR described a strong correlation between MRD results from BM and IGH cfDNA ($r = 0.5831$, $p = 0.0044$, Pearson's correlation test), and a longer PFS in patients with low levels of IGH ctDNA ($p < 0.001$) [37]. Evidence supporting MRD assessment using cfDNA is still scarce and requires improvement in the sensitivity of detection methods. Hopefully, additional validations should come soon as the EUCADD clinical trial is currently evaluating the relevance of using cfDNA for the assessment of MRD on 100 MM patients [69].

2.3. Myeloma Indirect Assessment through Circulating Tumor Plasma Cells

Circulating tumor cells (CTC) were firstly observed under the microscope by Ashworth in 1869. Ashworth reported the presence of cells in the blood stream with similar aspect to the primary tumor of a metastatic cancer patient. He concluded that tumor cells must have passed through the circulatory system [70]. Later, the hypothesis of "the seed and soil" was proposed by Paget, suggesting that secondary tumors arise from CTC (the seed). The recipient tissue may have a permissive microenvironment (the soil), supporting the growth and the successful development a secondary tumor [71].

In MM, continuous trafficking of tumor plasma cells to multiple areas of the BM is thought to be the process behind the dissemination of the disease [72]. Several methods have been used for the detection and analysis of circulating tumor plasma cell (CTPC) in the peripheral blood (PB) of MM patients [73]. The most sensitive used technologies include conventional flow cytometry [74–76], NGF, PCR-based (p.e. ASO-PCR), quantitative PCR (qPCR), and NGS (p.e. whole exome sequencing (WES)) [38].

Several studies successfully demonstrated the myeloma clone representativity in the PB of the tumor burden in the BM [29,77,78] (Table 1). Using NGF in paired BM and PB

samples, Sanoja-Flores et al. were able to detect CTPC in 100% of MM and SMM patients (97/97 and 25/25, respectively) and in 59% of MGUS (30/50 patients, $p < 0.0001$) [29]. They report that CTPC concentration progressively increases from MGUS (median 0.008 CTPC/ μL) to SMM (median 0.16 CTPC/ μL) and to MM (median 1.9 CTPC/ μL). They proposed a cut-off of 0.058 CTPC/ μL to discriminate MGUS from MM patients with high accuracy (80% sensitivity and 80% specificity, $p < 0.001$) [29]. A non-linear correlation was observed between the percentage of tumoral PC (over total BMPC) and the absolute number of CTPC in the PB (Spearman's rho (ρ) = 0.78; $p < 0.001$). Interestingly, the correlation remained significant, even in patients with localized disease (solitary plasmacytoma and macrofocal MM, $\rho = 0.54$, $p = 0.02$), MGUS ($\rho = 0.64$, $p < 0.0001$), SMM ($\rho = 0.51$, $p = 0.02$), and MM ($\rho = 0.55$, $p < 0.0001$) [29]. Overall, these results support the potential use of CTPC to establish a diagnostic distinction between MGUS and MM without the need for a BM aspiration or biopsy.

The potential value of CTPC as a MM prognostic marker has been further explored. The number and the type of genetic alterations in CTPC have predictive value for the time to progression (TTP) and OS, and are correlated with R-ISS in MGUS, SMM, and MM patients [29,39,46,49] (Table 2). For instance, the number of CTPC detected by NGF using 244 paired BM and PB samples proved to efficiently discriminate between MGUS with high (≥ 0.058 CTPC/ μL) vs. low (< 0.058 CTPC/ μL) risk of progression to MM (median TTP of 31 months vs. not reached, respectively) [29]. Kumar et al. showed that MGUS patients with detectable CTPC were twice as likely (HR = 2.1) to experience progression to another plasma cell disorder [39]. Similarly, 14 MGUS patients with high numbers of CTPC ($> 5 \times 10^6/\text{L}$ of PB) were significantly more likely to progress to active disease within 2 years compared to 77 patients without high CTPC (71% versus 24% of progression, respectively, $p < 0.001$). SMM patients with a high level of CTPC have shorter OS compared to patients with low CTPC (49 months vs. 148 months, respectively, $p < 0.001$, HR = 5.9) [41]. A cut-off of ≥ 150 CTPC per 150,000 events has been proposed by Gonsalves et al. to discriminate SMM patients with a high risk of progression within 2 years ($n = 9$, 97% specificity and 78% sensitivity [46]).

In a study with 157 NDMM, the presence of ≥ 400 CTPC per 150,000 events was associated in 37 patients (24%) with higher ISS stage ($p = 0.002$), creatinine ($p = 0.045$), CTPC ($p < 0.001$), BMPC% ($p < 0.001$), and high-risk cytogenetics by FISH ($p = 0.016$), when compared to patients with < 400 CTPC/150,000 events. The median time to next treatment (TTNT) and OS in patients with ≥ 400 CTPC was 14 months and 32 months compared with 26 months and not reached for the rest ($p < 0.001$ [42]). Altogether, these studies suggest a relation between the concentration of CTPC with MM patient prognosis.

This potential was corroborated by a large study on 247 NDMM patients with paired evaluation of CTPC at diagnosis and before autologous stem cell transplantation (ASCT). Chakraborty et al. set the detection limit at 10 CTPC per 150,000 events assessed by multiparametric flow cytometry (MFC) [47]. The patients were grouped into three categories based on CTPC detection. Patients without detectable CTPC at both time points (CTPC-/-; $n = 117$) showed a rate of stringent complete response (sCR) of 32%: 82 patients (CTPC+/-) had a sCR rate of 30% and 48 patients (CTPC+/+, $n = 45$; CTPC-/+; $n = 3$) showed a 12% rate of sCR ($p = 0.018$). The multivariate analysis of OS revealed a higher risk of mortality for CTPC+/+ or -/+ (HR = 5.7, $p < 0.001$) and CTPC+/- (HR = 2.7, $p = 0.009$) patients when compared to the CTPC-/- group. These results emphasize the predictive factor of response to treatment of CTPC before ASCT [47].

Regarding mutational assessment, WES results suggest that CTPC can provide the same genetic information as bone marrow MM cells and even reveal mutations with greater sensitivity in some cases [79] p.e. (KRAS, NRAS, or BRAF [44]). Recently, Garcés et al. sequenced exomes of triple-matched samples of MM patients (BMPC, CTPC, and extra medullary plasmacytoma). The results showed that 537 (82%) mutations were detectable in CTPC among the 658 mutations identified in BM tumor cells. CTPC carried mutations in 48 (92%) of the 52 genes that were altered in BM or EM tumor cells, including BRAF, KRAS,

NRAS, or TP53 [49]. The validation of these results and the availability of sequencing methodologies may accelerate the clinical use of CTPC to assess genetic prognosis markers in MM patients without the need to perform BM aspirations.

Besides the high rate of complete responses achieved with current treatments, the majority of patients relapsed, implying the presence of remnant neoplastic plasma cells and positive MRD [57] (Table 3). To better understand the sensitive borderline between positive and negative MRD using CTPC, two important studies are worth mentioning. Huhn et al. performed a longitudinal quantitative analysis of CTPC and BMPC using a highly sensitive ASO-PCR. Following 41 MM patients receiving ASCT, they reported a significant correlation between the number of BMPC and CTPC in MRD-positive patients ($\tau = 0.604$; $p = 0.003$). They also found a strong correlation between the number of CTPC and the depth of response to therapy ($\tau = 0.41$; $p = 1.07 \times 10^{-10}$) [45].

The work of Paiva et al. reports MRD assessment in 458 NDMM patients after ASCT. They confirm the applicability of the NGF technique to the IMWG flow of MRD-negative response criterion to evaluate treatment efficacy in Myeloma (PETHEMA/GEM2012MENOS65). Among the 205/458 (45%) patients with negative MRD, only 14 (7%) progressed until two years after the end of the clinical trial. Patients with negative MRD presented 82% reduced risk of progression or death ($HR = 0.18$, $p < 0.001$) and 88% had a reduced risk of death ($HR = 0.12$, $p < 0.001$) [56].

In accordance with these results, the potential of CTPC for MRD quantification is being evaluated by the clinical study NCT02627261 [80]. It aims to compare CTPC with two other methods for MRD detection on 100 NDMM patients who will receive ACST. Matched BM and PB samples are being collected before treatment, after induction, after intensification, and after consolidation. The quantification of MRD will be assessed by (i) BMPC% by flow cytometry; (ii) BMPC% by NGS; and (iii) the concentration of CTPC in blood (method not specified). This study will bring insight regarding the relevance of using CTPC for MRD assessment and confirm the relation between CTPC concentration with PFS and OS of MM patients, as previously described [43,45,48].

2.4. Statistical Validation of Biomarkers for Multiple Myeloma

The inclusion of myeloma cells parameters into current IMWG guidelines is based on solid statistical analysis. The acceptance of biomarkers, such as cfDNA and CTPC, as new standards will require thoughtful statistical validation. The following section reviews historical statistical methods analysis of BM as biomarker for diagnosis, prognosis, and MRD assessment, and discusses the statistical requirements for new biomarkers to be used as substitutes or adjuvants to BM biopsies.

2.4.1. Validation of Diagnostic Biomarkers

For a variable to be considered as a diagnostic biomarker, it is necessary that there are significant differences between diseased and non-diseased groups (or between disease stages) for that same variable. Such determination can be made using Wilcoxon or Friedman tests and the Mann–Whitney U or the Kruskal–Wallis tests. Both are used to assess the two-sided statistical significance of differences observed between ≥ 2 groups for paired and unpaired variables, respectively. This first step to assess differences between the interest groups was accurately performed by Lee et al. [23]. In their paper, a pre-analysis of the variables assessed the differences between patients with $BMPC < 10\%$ and patients with $BMPC \geq 10\%$ through the Mann–Whitney test. Then, and as performed by Wu et al. in 2018, it is important to assess the association between the variable under study and the time until progression from one disease stage to the other [81]. The difference of time to progression between groups may permit to consider the characteristic under study as a diagnostic biomarker.

The criteria of $\geq 10\%$ of BMPC for asymptomatic SMM or symptomatic MM comes as a pragmatic cut-off, as we have not been able to find a clear statistical validation of this value. As stated by Kyle et al. in 1980, “in practice it is usual to use an arbitrary figure

of less than 10% plasma cells in the BM aspirate and minimal infiltration in a trephine biopsy” [22]. They also suggest that the presence of 5% symptomatic patients with less than 10% of infiltration is probably related to sampling bias and that asymptomatic patients with $\geq 10\%$ infiltration are likely to enter active MM soon and should be treated.

The cut-off for BMPC above 60% in the absence of any symptoms was first introduced by Rajkumar, Larson, and Kyle in 2011 [4] after a reassessment of the 2007 original Mayo Clinic cohort of SMM [82]. The same group validated this new cut-off in 651 SMM patients [4]. Through the computation of Kaplan–Meier (KM) curves and respective log-rank test, they proved that the median time to progression from SMM to MM was significantly shorter ($p < 0.001$) for the patients with a level of BMPC of at least 60% when compared to patients with less than 60% of BMPC. Amongst patients with $\geq 60\%$ BMPC, 95% progressed to active myeloma within 2 years after diagnosis, with a median time to progression of 7 months. This cut-off was confirmed by Kastritis et al. from the Greek Myeloma Group on a cohort of 96 SMM patients [24]. Through a receiver-operating characteristic analysis (ROC), they proved that a BMPC $\geq 60\%$ could identify patients who progressed at 18 months, with a specificity of 95.5%. A multivariable Cox regression analysis demonstrated that BMPC $\geq 60\%$ was significantly associated with a high risk of progression (HR = 13.7, 95% CI [4.44–42.2]) (Table 1).

To determine if the BMPC could be replaced by CTPC or cfDNA as a diagnostic biomarker, the first step is to study its relation to the time until progression from the asymptomatic disease stage as SMM to active myeloma by means of KM curves and respective log-rank test. Then, univariable and multivariable Cox regression models may be employed to evaluate the hazards of progression alone and in combination with other factors.

The adoption of a new biomarker over the use of BM-related values is only possible if their discriminative power is equal or superior to those that are already previously established. It may be established through the ROC curves and the measurement of the associated area under the curve (AUC). The AUC assesses how much the variable is capable of distinguishing between groups (in this case, between patients and non-patients or between the various stages of the disease). AUC represents the performance of the ROC curve and summarizes the performance of the biomarker by evaluating the trade-offs between true positive rate (sensitivity) and false positive rate (1—specificity).

2.4.2. Validation of Prognostic Biomarkers

Establishing a simple and uniform model to assess the risk of progression for NDMM patients, as well as the time until it occurs has always been one of the main goals. Several models of risk stratification were presented over the years (Table 2). Based on 10,750 patients from 15 institutions and groups from Asia, Europe, and North America, Dr. Greipp et al. developed the international staging system (ISS) [35], a model with significant improvements over the Durie–Salmon staging (DSS) system [19]. Although it is a very practical and simple model, it proved to be insufficient as it does not consider some known prognostic markers, such as cytogenetic abnormalities and LDH, which were included in 2015 in the R-ISS [32].

Interestingly, Palumbo et al. did not implement recursive partitioning based on binary-tree representations to stratify the variables of interest and group them into new groups, due to the difficulty of this methodology in dealing with continuous variables. Instead, they implemented a K-adaptive partitioning algorithm especially conceived for censored survival data (minimax-based partitioning rule by log-rank test) to obtain subgroups that combine the levels of each of the variables under study. This methodology was developed by Eo, Hong, and Cho in 2014, and implemented in the package kaps (K-Adaptive Partitioning for Survival data) of R statistical software [83]. The developed algorithm allows the discovery of an optimal set of cut-off points for each prognostic factor and their combination in K heterogeneous subgroups by evaluating all the possible splits. If not specified, the algorithm also selects the number of K subgroups based on permutation

tests. This methodology is of special interest in cases where the survival data component is relevant and needs to be considered.

Through this algorithm, three groups were defined and called R-ISS I, II, and III. Then, and through an univariable Cox analysis, it was proven that ISS stage III, high-risk CA, and elevated levels of LDH were associated with a poorer OS. The combination of these levels on R-ISS showed to improve the stratification and impact on OS, independent of age and therapy.

To this day, this is still the main model of risk stratification for newly MM-diagnosed patients. Despite the undoubted value of this model, patients within similar prognostic groups display heterogeneous outcomes, which may be a sign that the current prognostic factors used in patient stratification are suboptimal. In late 2020, D'Agostino and colleagues proposed a new risk stratification model, the R2-ISS, based on 7077 patients enrolled in clinical trials all over Europe [33]. Through the application of a multivariable Cox regression model, adjusted for age, sex, and therapy, the impact of risk features on OS as well on PFS was assessed and showed the impact of 1q copy number alteration (CNA) inclusion, since this variable presents an HR of 1.45 ($p < 0.001$) for OS and HR of 1.37 ($p < 0.001$) for PFS. Although very promising, this study has not yet been externally validated and the R-ISS is still the main model of risk stratification for newly MM diagnosed patients.

To assess the prognostic value of a biomarker, its relationship with the survival of the cohort under study is commonly determined. The comparison between risk groups is made through the analysis of Kaplan–Meier survival curves and the existence of differences between different survival curves is assessed through the log-rank test. This test evaluates the null hypothesis that there is no difference between several groups regarding the probability of the event occurrence at any time point. In this way, it is evaluated which of the groups under study presents a worse prognosis (generally associated with a greater probability of death), as performed by Kyle et al. [82]. Often, death before progression to symptomatic myeloma is treated as a competing risk, as implemented by Kastritis et al. [24].

To model the association between variables with survival, the most appropriate method involves the application of Cox regression models (also called Cox's proportional hazards model), since the log-rank test only compares survival functions between stratified groups. Factors that are significantly predictive of survival in a univariable Cox regression model are often tested together in a multivariable model. A good example of this application can be observed in the work developed by Kyle et al., Kastritis et al., and Rajkumar et al. [24,82,84], to mention a few.

The validation of cfDNA and CTPC as good prognostic biomarkers requires a significant association with patient survival and/or the presence of CA. The evaluation of CA using CTPC or cfDNA may be a new approach for the assessment of progression risk in MM, but the added value of including these new variables in detriment of CA must be significant and undoubtable. First, the presence of the CA already included in the R-ISS and/or validated MM-associated mutations must be validated in cfDNA and CTPC. Next, it is necessary to re-evaluate the existing relation between these biomarkers and OS, as well as their impact on PFS, by means of univariable Cox analysis. Once their predictive value for survival is established, methods based on classification algorithms that can consider the inherent censoring character of survival analysis, such as the one applied by Palumbo et al. [32] can be used to build a new risk score. The study of this new score may be based on the new markers under study in combination with markers whose predictive value is already established, such as LDH or ISS. Then, it is imperative to evaluate this new score as a predictor of OS and PFS, and the last step is to assess its predictive performance against the already established R-ISS through the comparison of the ROC curves and respective AUC.

2.4.3. Validation of Biomarkers for Depth of Response

The response to treatment is usually assessed by PFS. The mechanisms for assessing PFS are similar to those for assessing prognosis, with the difference that the endpoint

in this case is not exclusively the death of the patient but also disease progression. For diseases such as MM, where the average life expectancy of patients is around 6 years, the evaluation of the impact of biomarkers on OS often requires a large follow-up period, which is not always possible. For that reason, the use of the time from random assignment until the second disease progression or death, commonly referred as PFS2, allows the estimation of the impact of variables whose impact takes longer to manifest, but when using the OS as an end point is not feasible. This strategy proved to be of great value by Palumbo et al. [32], especially when dealing with the impact of new treatment regimens, as PFS2 can estimate the impact of both first- and second-line therapies on outcome with a shorter follow-up time.

However, the mechanisms that could allow a better assessment of patient prognosis after the first relapse, guiding towards a better selection of the second-line treatment, are still under study. Nowadays, and in the absence of a more fitted strategy, the R-ISS and ISS are often used to stratify patients with MM at relapse, although the value of considering such algorithms after the first relapse is unclear. In 2019, Hájek et al. [85] proposed a novel risk stratification algorithm (RSA) to allow a better assessment of patient prognosis at first relapse. Data were collected from 1418 patients treated in 20 Czech centers from 2007 to 2016. A complete explanation of the methodology implemented by Hájek [85] can be found in the paper from Bouwmeester et al. [86]. A multivariable Cox regression model was implemented, considering non-correlated factors as predictors and OS from initiation of second-line treatment the dependent variable. The final model was obtained after backward selection based on Akaike information criterion (AIC), and 16 factors were identified as independent predictors of OS: 3 corresponded to characteristics at diagnosis, 10 corresponded to characteristics at the initiation of second-line treatment, and 3 were related to patient experience during first-line treatment.

Patient-specific risk scores were calculated for each of the selected predictors, considering the HR value (for categorical variables) or the $\exp[\beta \times \text{patient value}]$ (for continuous variables). The total risk score for each patient was obtained by multiplying each predictor-specific risk score with each other. This value was then used as input for the K-adaptive partitioning for survival (KAPS), i.e., the same methodology as implemented by Palumbo et al. for the development of R-ISS [32]. Through KAPS, four groups were defined, based on survival expectations and total risk scores with significant differences. This new risk stratification was then validated on a different cohort of 998 patients and proved to be an improvement regarding the established models, since RSA outperformed ISS and R-ISS (C-indices of 0.72, 0.60 and 0.59, respectively). The C-index value of 0.72 for the RSA indicates that the predicted scores can effectively describe the observed sequence of events and may be used as an additional tool to assess the prognostic future after their first relapse.

In 2016, Kumar et al. proposed guidelines for the assessment of MRD in MM [57]. The key question regarding MRD is the level of minimal residual disease that is undetected by conventional methods based on plasma-cell phenotype, genotype, or both. As stated by the authors, the optimal test to assess MRD should be suitable for most patients, present a high sensitivity and specificity, be easily accessible, and be obtained in most patients. To this day, none of the existent techniques can fulfill all the requirements, although NGS and NGF of the BM satisfy most of them. The possibility of assessing MRD using cfDNA and/or CTC opens novel and easier options.

In 2018, the prognostic value of MRD during maintenance therapy on patient's outcome was evaluated on BM samples by Perrot and colleagues by NGS [51] (Table 3). They defined a negative MRD assessment as the absence of tumor plasma cells within 10^6 BM cells in patients who achieved at least a very good partial response. In line with what is common practice in these cases, survival functions were graphically computed by the Kaplan–Meier method and the comparison between them was performed using the log-rank test. The prognostic value of MRD on OS and PFS was assessed by a multivariable Cox regression model adjusted for stratification factors and treatment groups. This study

showed that PFS and OS were significantly extended in MRD negative vs. MRD positive patients independently of the treatment group, the CA profile, or the ISS level (PFS: HR = 0.22; OS: HR = 0.24, $n = 224$ patients starting maintenance; PFS: HR = 0.18; OS: HR = 0.26, $n = 183$ patients with complete maintenance). The risk of progression in patients with an MRD level between 10^{-6} and 10^{-5} was almost twice as high in patients with MRD $< 10^{-6}$ at the start of maintenance therapy (HR = 1.94), and almost three times higher when MRD was evaluated after complete maintenance. This shows that MRD levels below 10^{-6} are predictive of improved PFS when compared to 10^{-5} .

NGF can also achieve a 10^{-6} sensitivity. In 2017, Flores-Montero et al. [52] proposed a novel next-generation flow approach for highly sensitive and standardized MRD detection on 31 BM patient samples to validate NGF vs. NGS. By applying the Spearman's test for paired data, a good correlation was found between the percentage of residual myeloma cells measured by NGF and NGS ($\rho = 0.62$, $p = 0.001$), although MRD assessment showed a higher applicability for the NGF approach: 31/31 versus 27/31 for NGF and NGS, respectively ($p < 0.001$).

As current MRD evaluation involves BM biopsies, alternative minimally invasive biomarkers, such as cfDNA, were evaluated. The study of Vrabel et al. [55] evaluated the dynamics of cfDNA in BM and PB samples at diagnosis and during follow-up. For those who were true MRD positives, MRD determination using MFC and cfDNA corresponded to each other and were MRD positive in most cases, with a sensitivity of 66.7% (true positive rate) and a specificity of 83.3% (true negative rate) (CI = [22.3%–95.7%] and [35.9%–99.6%], respectively). This combined approach validates the prognostic value of cfDNA for MM patients.

The adoption of CTPC over BM was also investigated. The study conducted by Huhn et al. [45] showed that, although CTPC may act as a substitute of BM assessments until PB is MRD negative, it cannot yet be considered as a mechanism for detection of MRD by itself. Data were analyzed according to risk strata based on ISS as well as cytogenetics at the time of diagnosis. The results and respective conclusions already outlined were achieved by the implementation of the R package NADA [87], conceived for the analysis of left-censored data, by which Kendall's tau correlation coefficient for censored data was computed.

3. Discussion and Conclusions

Clinical evidence indicating that cfDNA and CTPC are serious candidates to substitute BM biopsies for MM assessment are available. Nevertheless, the establishment of cfDNA and CTPC as new standards and their incorporation in diagnosis, prognosis, and response evaluation still require further techniques of harmonization, inter-laboratory validations, large-scale clinical studies, and strict statistical validation.

The use of cfDNA has been approved by the FDA in 2016 for the detection of mutations in non-small cell lung cancer [88]. Recent studies suggest that cfDNA-based applications could also be developed for MM patients, providing greater representativity of the mutational landscape than BM biopsies. However, as liquid biopsies are collected from a site distant from the tumor, greater efforts are necessary to prove the relation between the measured variable and the disease. Comorbidities or aging can alter cfDNA production from other cells than the tumor and cancer-associated mutations can occur without causing cancer in the patient. Levels of cfDNA and specific mutations need to be discriminated according to their association with OS and PFS. Due to the limited sensitivity of similar methods applied on PB, MRD investigation has been restricted to BM. This may change soon with the continuous increase in accuracy of the currently available NGF and NGS methods. The use of cfDNA for MRD quantification is still poorly documented although promising. Preliminary results suggest that sensitivity of detection will be a limiting factor and may require significant improvement before its translation to the clinic. A clinical trial currently assessing MRD detection using cfDNA on 100 MM patients may bring new insight to the topic [69].

The quantification of circulating plasma cells and CTPC have been included in the diagnosis of plasma cell leukemia (PCL) since the late 1900s. Interestingly, the IMWG recently highlighted the similar adverse prognostic impact of the presence of $\geq 5\%$ circulating plasma cells in patients with PCL or MM. This new cut-off may permit the early identification of PCL patients that were previously misdiagnosed as MM [89] and highlights the strong potential of CTPC quantification for prognosis in MM. The use of CTPC in MM, at least for MRD assessment, has been recently assessed on a large cohort of patients, giving very promising results [56]. This suggests that CTPC may be more readily integrated to the clinical setting than cfDNA.

So far, few studies have compared the predictive potential of cfDNA and CTPC, and no clear predictive superiority of one over the other has been reported. It is noteworthy that, when used in combination, these liquid biopsies were found to strongly increase the strength of prognosis over BM biopsies. For instance, Manier et al. reported that mutations detected in cfDNA and CTPC are highly concordant, and that the combination of both more effectively captures the mutational landscape of BMPC than individually (99% together vs. 83% for cfDNA alone) [38]. Another study revealed that CTPC and cfDNA are, together, positively associated with the level of response of patients [53]. Interestingly, cfDNA and CTC almost always carry mutations not detected in the BM samples, confirming their potential to cover clonal heterogeneity of MM and extra medullary MM, which are possibly better than BM biopsies. Unfortunately, studies combining two or more types of liquid biopsies are still scarce to permit a clear validation of their strength in association.

Regarding the statistical validation methods, the most appropriate statistical approach is based on several factors: the number and type of observations available for analysis, the questions we want to answer, the intended outcomes, and so on. Although there is no recipe for statistical analysis, the most important is that it fits the purpose, whether it is the validation of the biomarker as a diagnostic, prognostic, or depth of response tool.

Liquid biopsies present undeniable advantages over BM aspirates in terms of spatial and temporal tumoral representativity, as well as for patient well-being. Incorporation of cfDNA and CTPC into MM guidelines will require additional effort from the scientific community, which is surely about to happen.

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