Chapter 4

Liquid Biopsy in Multiple Myeloma

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Additional information is available at the end of the chapter

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Abstract

Liquid biopsies represent an innovative methodology for cancer diagnostics and disease monitoring. The analysis of circulating cell-free nucleic acids (CFNA) and circulating tumour cells (CTC) are rapidly being adopted for quantitative and qualitative characterisation of the tumour genome and as a mode of non-invasive therapeutic monitoring. Circulating cell-free DNA (cfDNA) and CTC are representative of the underlying mutational profile of a cancer whereas the evaluation of extracellular RNA (exRNA) can be utilised as a prognostic biomarker thus providing critical biological information both at the time of diagnosis and during disease evolution. In this chapter, we will review the emerging utility of CFNA and CTC as biomarkers of prognosis and for both mutational characterisation and monitoring disease progression, and how these have the potential to provide additional information as an adjunct to bone marrow biopsies and conventional disease markers in multiple myeloma (MM). Emerging data suggest that liquid biopsies might offer a potentially simple, non-invasive, repeatable analysis that can aid in diagnosis, prognostication and therapeutic decision making in MM, with particular applicability in subsets of patients where conventional markers of disease burden may be less informative.

Keywords: cell-free nucleic acids, multiple myeloma, liquid biopsy, circulating tumour cells, non-invasive, diagnostics, disease burden

1. Introduction

1.1. Multiple myeloma

Multiple Myeloma (MM) is a multi-focal genetically heterogeneous clonal plasma cell (PC) malignancy that at diagnosis is present at multiple intra-medullary sites within the bone marrow (BM, **Figure 1**). It is the second most common haematological malignancy after lymphomas [1]. MM is preceded by a usually unrecognised asymptomatic clonal PC disorder

exclusively confined to the BM called monoclonal gammopathy of undetermined significance (MGUS) [2, 3]. During disease progression the PCs evolve the capacity to grow independently of the BM milieu and thus proliferate outside of the BM, manifesting as extramedullary (EM) MM and/or plasma cell leukaemia (PCL), with published observations showing that PCL represents a more genetically abnormal sub-clone that evolves from the original intra-medullary PC population [4]. The incidence of EM soft-tissue plasmacytomas in newly diagnosed (ND) patients ranges from 7 to 18% with an incidence of almost 20% at the time of relapse [5, 6]. The diagnosis and monitoring of MM relies on sequential BM biopsies and the quantitation of biomarkers of disease burden in the blood and/or urine - clonal immunoglobulin (paraprotein, PP) and/or isotype restricted free-light chains (Serum Free light chains, SFLC or Bence Jones Proteinuria) and in 15–20% of patients the MM cells secrete only SFLC, so called light chain MM [7]. Notably, the routine biomarkers of tumour burden are not informative in subsets of patients with oligo-secretory (OS)-MM or non-secretory (NS)-MM, which are subsets of MM that have low or no measureable levels of PP (<10 g/L) and/or SFLC (<100 mg/L), respectively, and constitute about 10% of MM patients at diagnosis (Australian National Myeloma and Related Diseases Registry, [8]). Moreover, patients with secretory MM at the time of initial diagnosis can transform to NS-MM or OS-MM at the time of relapse and patients with advanced disease are significantly more likely to have OS-MM disease compared to ND patients [8]. These low or immeasurable levels of PP prevent a patient's disease from

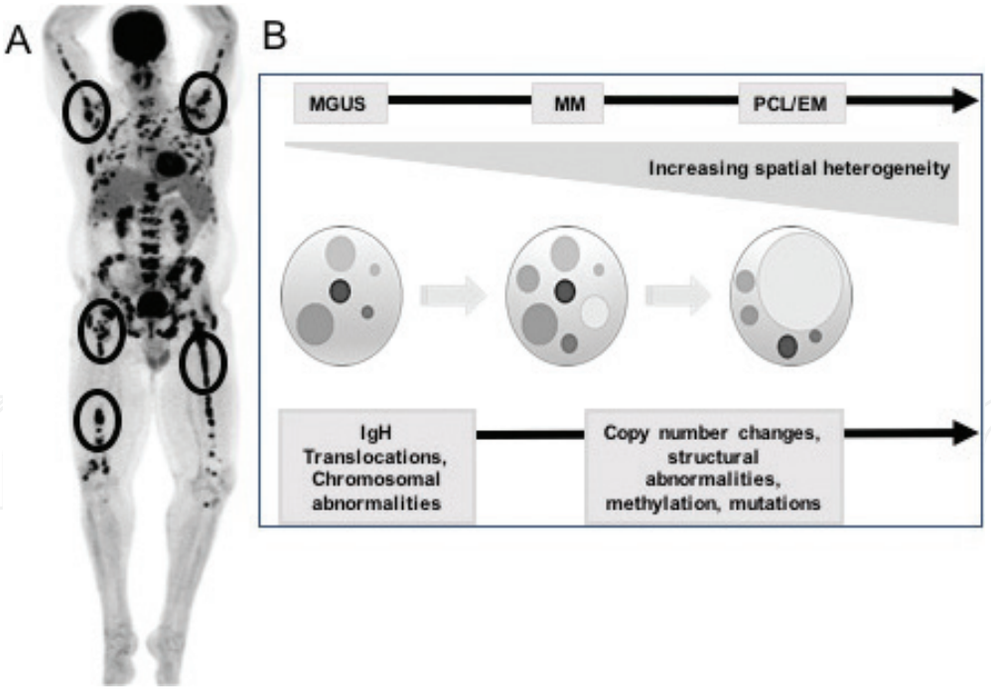

Figure 1. Multiple myeloma (MM) is a multi-focal malignancy manifesting at several sites black within the BM at diagnosis and in EM tissues in more advanced stages. (A) A PET image of a patient with MM is shown here, demonstrating disease foci at multiple sites (shown in the circles) with the potential that the tumour genome at these individual sites may demonstrate clonal heterogeneity. (B) Representation of the potential changes in clonal and sub-clonal fractions (represented in the different sized circles) are shown with the increasing spatial heterogeneity thought to be present during disease progression. Primary translocations of the IgH gene locus and chromosomal aneuploidies occur during MGUS and chromosomal abnormalities along with primary and secondary driver mutations emerge during MM progression.

being monitored using traditional blood and urine tests and as such OS-MM and NS-MM patients are typically ineligible to participate in clinical trials of newer anti-MM therapeutics. Conventionally, therefore, treatment response in these patients is determined via sequential BM biopsies, where available, and whole-body PET/CT or MRI represent the only accepted non-invasive modality for following response in NS-MM. Therefore, despite well-established diagnostic and monitoring modalities, there remains a critical need to address specific subsets of patients where these conventional markers are inadequate.

The treatment of MM has witnessed significant progress with the introduction of proteasome inhibitors (PI) and immunomodulatory agents (IMID), however, the disease remains incurable. MM cells acquire resistance to systemic therapies through the accumulation of mutations that are often not present during the initial stages of the disease [9] with this genetic evolution providing the more resistant clones with a growth and survival advantage (**Figure 1**, [10]). Mutational characterisation of MM utilising whole genome sequencing (WGS) or whole exome sequencing (WES) of single-site BM biopsies predominantly of ND patients have indicated that the MM mutational spectrum is both complex and heterogeneous with recurring mutations in KRAS, NRAS, TP53, BRAF, IRF4, MAX, ATM, ATR, CCND1, CYLD, DIS3, BRAF, FGFR3, RB1, HIST1H1E, EGR1, TRAG3, FAM46C and LTB [11–14]. The MM mutational landscape is dominated by RAS-MAPK pathway mutations (45–50%) and while these mutations can be targeted therapeutically, the biological implication of the presence of these mutations remains controversial. Chng WJ and co-authors observed that the presence of KRAS mutations, but not NRAS mutations, was associated with inferior overall survival (OS) and a more aggressive disease phenotype [15]. However, it has been recently demonstrated that NRAS mutations may confer reduced sensitivity to bortezomib but not dexamethasone, while the presence of KRAS mutations does not appear to correlate with drug sensitivity [16]. Conversely, more recent studies have concluded that harbouring RAS mutations has no significant impact on disease outcome [13, 14]. In contrast, available data suggests that the presence of mutations involving the DNA damage response genes (TP53, ATM and ATR) is associated with a negative impact on survival [14]. Recently, mutational analysis of PCs from 33 MGUS patients indicated that while the number of somatic mutations was significantly lower when compared to MM the spectrum of mutations mirrored that of MM with KRAS, NRAS, DIS3, HIST1H1E, EGR1 and LTB mutations being observed [17]. Importantly, mutations in the DNA damage response genes were not found, indicating that these are secondary drivers accounting for disease progression and drug resistance. Notably, one patient harboured both KRAS and NRAS mutations highlighting that diversification and heterogeneity in this pathway may exist before the development of symptomatic MM [17]. To date the mutational characterisation of MM has utilised single-site BM biopsies, however, it is now increasingly recognised that such an approach may fail to capture the spatial and temporal genetic heterogeneity of this multi-focal disease, self-evidently with EM MM, but also, based on emerging data, in patients with 'typical MM' manifesting intra-clonal heterogeneity [11, 18–20]. Moreover, both spatial and temporal genetic heterogeneity are now recognised as adding to the genetic complexity of the disease as it evolves [21]. This is exemplified by recent observations from comparisons of BM and targeted biopsies of EM disease that sub-clones absent from the BM may be present at EM sites and critically, that these disease foci may respond differentially to treatment (**Figure 2**, [9, 22]). Therefore, despite the pivotal BM-based studies that currently inform our understanding of

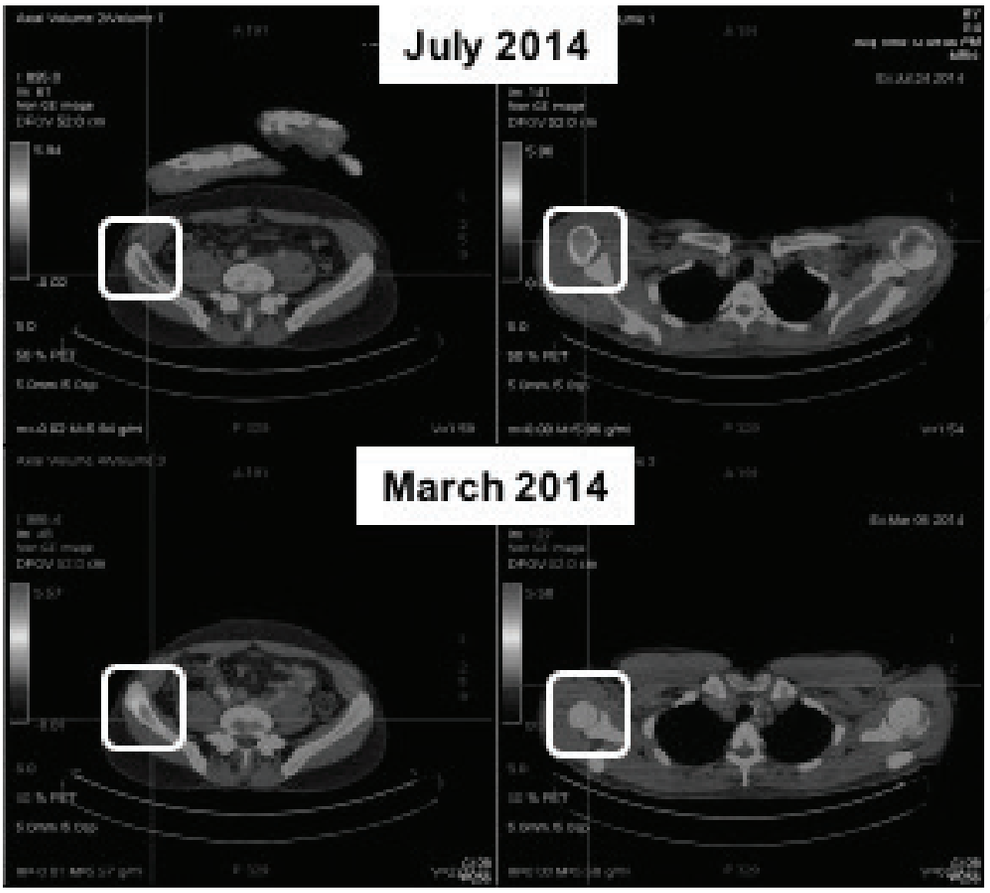

Figure 2. Different sites in a MM patient may respond differentially to treatment. PET image of a MM patient receiving thalidomide therapy taken 2 months apart showing regression of disease at one site (pelvis), while tumour growth is observed at another site (humerus).

the mutational spectrum in MM, the evident shortcomings of these approaches necessitates the adoption of newer and more novel strategies to enable the more comprehensive genomic characterisation of the disease.

1.2. Liquid biopsy

The utilisation of liquid biopsies continues to generate significant attention as it represents a platform that has the potential to provide rapidly evaluable and non-invasive, genomic characterisation of a patient's cancer. In simple terms, liquid biopsy refers to analyses that utilise blood or bodily fluids that contain circulating tumour cells (CTC) and/or fragments of nucleic acids or proteins that are derived from primary and/or secondary tumour sites. This material can then be interrogated to provide comprehensive information about the tumour genome and other biological characteristics of the disease. Moreover, liquid biopsies can be used to monitor the effects of therapy and may provide early evidence of recurrence or relapse of disease enabling early and informed changes in disease treatment. In this section, the two most widely studied types of liquid biopsy sources, CTC and cell-free nucleic acids (CFNA), will be discussed.

1.2.1. Circulating tumour cells (CTC)

CTC are intact tumour cells that disengage from tumour sites thus entering the circulation and are a recognised feature of metastatic cancers [23–25]. Historically, CTC were considered to be present only in a sub-set of ND, untreated or end-stage MM patients. Now with the advent of highly sensitive next-generation (NG) flow cytometry techniques the detection of CTC in pre-malignant MGUS and at different stages in MM has become feasible [26–28] and CTC can be detected in the peripheral blood of a substantial proportion of MM patients evaluated in this fashion [29, 30]. Importantly, the numbers of CTC in the peripheral blood in MM patients with active disease is known to be significantly higher than in patients with inactive disease, with higher numbers of CTC being a risk factor for progression to symptomatic MM for patients with either MGUS or smouldering MM (SMM) [29–33]. The number of CTC in ND and RR patients is also known to be an independent prognostic factor for survival [34–37]. Finally, the absolute numbers of CTC was found in one study to be a better predictor of disease activity than SFLC ratios [30].

1.2.2. Circulating cell-free nucleic acids (CFNA)

CFNA, refers to cfDNA and extracellular RNA (exRNA - includes mRNA and miRNA), and are being widely assessed in an ongoing fashion for their potential utility as blood biomarkers for cancer diagnostics. CFNA are derived predominantly through necrosis, spontaneous or chemotherapy induced apoptosis and active cellular release [38, 39]. Given that CFNA is also present at higher levels in certain physiological conditions and clinical scenarios (reviewed in [40]), it is critical to determine if the nucleic acids released are tumour-specific in cancer patients. In this section the different types of CFNA and their potential clinical utility will be discussed.

1.2.3. Cell-free DNA

The presence of CFNA in body fluids was first described by Mandel and Metais in 1948 [41]. One of the earliest observations in relation to cfDNA was that patients with malignant disease had higher levels of cfDNA in their serum than normal individuals, and that patients with persistently high levels of cfDNA demonstrated a lack of treatment response [42–44]. Mutated RAS fragments and microsatellite alterations in cfDNA were later observed in cancer patients and critically these alterations were not detected in paired tissue biopsies, highlighting the concept, very early-on, that isolated tissue biopsies may not represent the optimal material for defining the tumour genome [45, 46]. The development and utilisation of NGS technologies, WGS and/or WES of cfDNA containing tumour-derived DNA has identified mutations, tumour-derived chromosomal aberrations and gene rearrangements associated with acquired resistance to therapy without the need to perform sequential tumour biopsies [47–51]. Furthermore, it is evident that secondary mutations are more readily detectable in the plasma than via re-biopsy of primary tumours validating the utility of plasma-based analysis for the characterisation of potentially targetable oncogenes and the identification of resistance-associated mutations that are acquired during disease progression, thus informing therapeutic changes [48, 52, 53]. Available evidence would also suggest that cfDNA may be more representative of the entire tumour genome than the information derived from single BM or nodal/tumour biopsies, as

emerging evidence supports the notion that a range of haematologic malignancies including MM are likely to harbour significant sub-clonal and spatial genetic heterogeneity. In MM, levels of cfDNA are significantly higher than in both normal volunteers and non-MM cancers [54, 55] and the potential utility of cfDNA in MM will be discussed below.

1.2.4. Extracellular RNA (mRNA and miRNA)

The presence of extracellular RNA (exRNA) in plasma/serum was also described some decades ago [41, 56, 57] with significantly higher levels in cancer patients compared to healthy individuals [58]. Additionally, tumour burden was shown to correlated with the level of circulating mRNA [59]. The exRNA released into the circulation is surprisingly stable owing to its protection from RNAse-mediated degradation through packaging into exosomes, which are shed into the blood stream [60–64]. Biomarker studies measuring cancer-specific circulating mRNA have identified higher level of mRNA and correlation with disease status in a number of malignancies including nasopharyngeal carcinoma [65], prostate [66, 67], gastrointestinal [68–72], breast [73–76], hepatocellular carcinoma [77], lung [78, 79], cervical [80], lymphoma [81] and thyroid [82]. These findings highlight the potential utility of exRNA in cancer diagnostics, and while promising, it will be critical to assess exRNA in larger and annotated sample sets to establish specific circulating mRNAs as reliable non-invasive biomarkers.

Similarly, extracellular miRNA are found in virtually all fluid compartments of the body including blood where they can circulate bound to proteins, high-density lipoproteins or apoptotic bodies, or within exosomes, thus providing stability against RNases (reviewed in [83]). The levels of circulating miRNA have been described in a number of cancers with one of the earliest reports being in diffuse large B-cell lymphoma (DLBCL) demonstrating a correlation between the level of specific miRNAs and prognosis [84]. Subsequently, several publications of circulating miRNA in malignancy have demonstrated increased miRNA in cancer patients when compared to healthy individuals and a decrease in levels following tumour debulking surgery (reviewed in [85–87]). Circulating miRNA expression profiles are also known to have signatures correlative to disease stage, diagnosis and relapse [88–91]. The origin of circulating RNA subtypes is, however, not as well understood as for cfDNA, as these can potentially be sourced from tumour cells, the supporting micro-environment or immune cells [92]. While mRNA has not been assessed in MM, there are a number of studies of miRNA and the prognostic utility of these will be discussed in the next section.

2. Biological and clinical implications of liquid biopsy analysis in MM

Liquid biopsy is gaining momentum in MM due to the inherent nature of the disease and the obvious limitations associated with BM biopsy. A summary of the potential utility of CTC and CFNA evaluation are presented in this section (**Figure 3**).

2.1. Determining the tumour genome composition

Given the clonal genomic heterogeneity that exists within MM, particularly with disease progression, it is theoretically necessary to perform repeated BM biopsies to track tumour

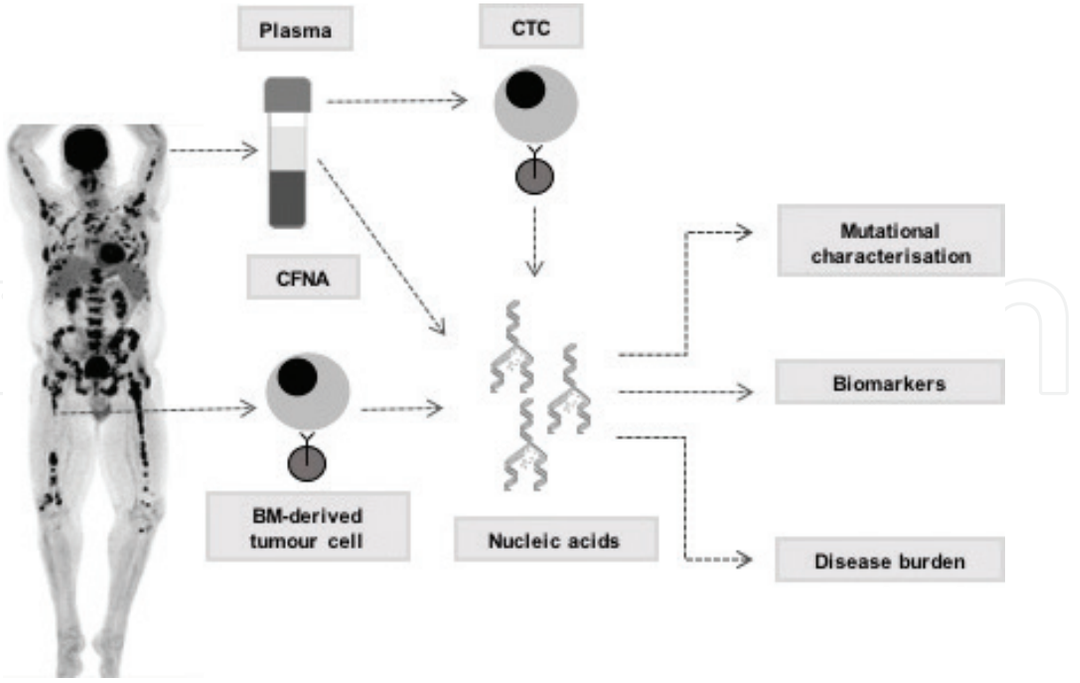

Figure 3. Currently in MM, BM-derived tumour cells are utilised for mutational characterisation, biomarker identification and to define disease burden. Peripheral blood can be utilised to obtain both CTC and CFNA. DNA and RNA can be derived from both sources and as both CTC and CFNA are derived from multiple tumour sites they theoretically will provide a more comprehensive profile of the disease in comparison to a single-site BM biopsy.

genomic evolution within any particular patient. Such an approach, however, remains an unattractive proposition as BM biopsies are invasive, not without complications, subject to sampling error and cannot capture the increasing spatial heterogeneity present with disease evolution. Therefore, the capacity to perform sequential mutational characterisation using a more 'holistic' and non-invasive approach would be a highly desirable alternative option. Both CTC and CFNA have been explored for this purpose in MM, however, the field is relatively unestablished with only a small number of publications addressing this concept. One of the earliest reports by Zandecki et al. evaluated CTC and matched BM-MM cells and demonstrated that chromosomal abnormalities were consistent between these two compartments [93]. The recent development of both NG flow cytometry and contemporary sequencing technologies has enabled the analysis of CTC at the single-cell level [27] but to date only small numbers of patients have been evaluated using this approach. Analysis of 9 patients with single cell WGS revealed that all BM-defined mutations were similarly present in CTC [27]. Further analysis was done on CTC in two patients with low disease burden (one with treated MM and one with MGUS). The CTC of the MM patient harboured readily detectable somatic mutations in BRAF, TP53 and IRF4, with reappearance of the mutations when the patient relapsed, indicating that the treatment had not eradicated these particular sub-clones. CTC analysis of the patient with MGUS revealed the presence of a NRAS mutation, also present in the BM. The concordance with BM genetic composition was also confirmed by Mishima et al. utilising NGS when analysing 8 paired CTC and BM aspirates [28]. Clonal, defined as a > 0.90 mutant allele fraction (MAF), mutations were present in >99% of CTC and BM, while subclonal shared mutations (defined as >0.05 MAF) were concordant in >80%, with 16% of subclones (<0.05 MAF) discordant between the two compartments consistent with spatial

heterogeneity [28]. Similarly, copy number alternations (CNA) were assessed and found to be 92% concordant. Likewise, RNA-seq at the single cell level also accurately predicted the recognised non-random chromosomal translocations that manifest in MM responsible for overexpression of key MM-associated oncogenes [27]. Overall, for both somatic mutations and CNA, CTC appear to harbour more variances indicating that they are likely sourced from multiple tumour sites and the manifestations of spatial heterogeneity may thus be more accurately captured by this type of analysis.

The evaluation of cfDNA for mutational characterisation and monitoring of disease burden in MM has also recently been described [54, 55, 94, 95]. Importantly, and for the first time, spatial and clonal heterogeneity on a large scale in MM was confirmed by our study evaluating paired BM-derived CD138 selected MM cells and cfDNA utilising a highly sensitive targeted sequencing platform consisting of a panel of 96 cancer-associated mutations including mutations of KRAS, NRAS, BRAF and TP53. This demonstrated that 21% of MM patients had mutations detectable only in the plasma and that the prevalence of spatial heterogeneity increased with disease evolution [55]. Numerous signalling pathways are known to be mutated in MM [13, 14], with previous BM WES studies demonstrating that activating mutations of the RAS-MAPK pathway were present in approximately 50% of patients. In contrast, our study demonstrated RAS-MAPK activating mutations in 69% of cases and the co-existence of multiple mutated sub-clones in a significant proportion, with >3 activating mutations in 23% of patients (range, 3–17 mutations per patient) representing a hitherto unrecognised mutational convergence on the RAS-MAPK pathway. This had remained largely undiscovered in single-site BM WES studies owing presumably to the relative insensitivity of the methodologies used and the presence of clonal heterogeneity at sites distant to the BM biopsy site. While it is likely, with high-sensitivity approaches, that there will be a MAF threshold for minor BM sub-clones that enables them to be reproducibly detected in the plasma, this will not be relevant with less sensitive strategies like WES and targeted amplicon sequencing that cannot detect smaller sub-clonal mutations, thus explaining, and consistent with, the 96% concordance between BM and PL demonstrated using NGS technologies [54]. Interestingly, mutations in PIK3CA, which have only rarely been described in MM, were found in a recent study to be present, but only in the plasma, indicating that these could be a feature of EM disease [54]. However, confirmatory studies with larger cohorts of patients and more comprehensive and targeted panels of MM-associated mutations are required to validate these observations. Theoretically, therefore, plasma cfDNA analysis of MM patients, particularly in the case of EM-MM, would not only provide information on the underlying biology of this disease, but could also furnish information on response to therapy through quantitative sequential tracking of plasma-only mutations. Moreover, RNA-seq of plasma cfRNA derived from MM patients is also theoretically possible to provide information on single nucleotide polymorphisms (SNP), although this has not yet been described in the published literature. So, while in the short-term it is unlikely that plasma cfDNA evaluation will replace BM biopsy, as the latter remains necessary in MM for diagnostic purposes, we believe cfDNA analysis as an adjunct to BM biopsy for comprehensive mutational characterisation is likely to become a reality in the near future.

To date, the analysis of CTC or cfDNA with paired BM has largely been performed only at single time-points and has not been comprehensively utilised for longitudinal assessment using sequential specimens that would be critical to identify the evolution of the

clonal architecture of the disease during therapy. The prognostic significance of mutational profiling in MM also remains largely unknown, with only a few genes thought to have prognostic significance. Furthermore, it should be recognised that the prognostic impact of certain mutations has been based on studies profiling material sourced from BM aspirates and therefore may not have been representative of the entire tumour genome. This shortfall could be potentially overcome using CTC and/or ctDNA as an adjunct to BM biopsy for mutational analysis and consequently, for prognostic profiling.

2.2. Biomarkers

The establishment of single-cell RNA-seq methodology in CTC from MM patients [27] presents an important opportunity for the identification of biomarkers that may define differing sub-sets and stages of the disease. Theoretically, this may represent a technically feasible and hence more reliable strategy than evaluation of exRNA as the RNA would be derived from tumour cells that should overcome some of the stability issues that may be associated with exRNA. Furthermore, CTC-derived RNA should address the issue of spatial heterogeneity and hence provide a more inclusive RNA-signature during disease evolution, as compared to present data based on single-site BM biopsies. To date, the potential of exRNA for differentiating the continuum of plasma cell dyscrasias ranging from MGUS to intra-medullary, EM MM and PCL has been explored in MM, but mainly through the analysis of miRNA and not mRNA (reviewed in [83]). Available evidence would suggest that MM cells contain differing populations of miRNA species at defined disease stages [96–100], therefore an approach identifying miRNA biomarkers non-invasively utilising exRNA would represent a readily accessible and novel approach. However, while circulating miRNA biomarker studies, including patients from different disease stages, have defined relative differences in both the expression of specific miRNAs and the absolute levels of circulating miRNAs as potential biomarkers [101–109] there is widespread discordance between the published studies with respect to the specific miRNAs identified [83]. For example, Yoshizawa et al. found significant downregulation of miR-92a in patients with symptomatic MM when compared to normal subjects, conversely, Besse et al. when comparing serum miRNA profiles between healthy, ND and EM patients, demonstrated that miR-130a expression was significantly decreased in the EM patients, hence a potential biomarker for EM disease, but no correlation with patient outcome was evident [101]. Jones et al., identified that a combination of miR-1246 and miR-1308 could distinguish MGUS from MM patients [106], whereas miR-25 was shown to correlate with conventional serum markers of MM at both diagnosis and at complete response (CR) post autologous stem-cell transplantation (ASCT) [103]. Some of the studies also identified a prognostic correlation with the actual levels of circulating miRNA. Rocci et al. demonstrated that miR-16 and 25 were significantly associated with OS in ND MM [102]. Qu et al. identified miR-483-5p as a potential predictor of MM survival [110]. Hao et al. showed that lower expression of miR-19a predicted poor OS [107], while Kubiczkova et al. concluded that a combination of miR-34a and let-7e can distinguish MM patients from normal individuals, and that patients with lower levels of miR-744 and let-7e had shorter OS [105]. In parallel with these studies, an evaluation of exosome-derived miRNA demonstrated that both let-7b and miR-18a were significantly associated with progression-free survival (PFS) and OS [111]. Unlike CTC, one of the significant shortcoming of assessing circulating miRNA levels is that expression levels might be

impacted upon by both the biological characteristics of the disease and the tumour burden, thereby presenting significant challenges when it comes to identifying correlations with prognosis. Moreover, the discrepancy between these studies highlights the lack of concordance between the analytical methodologies, study populations and treatment regimens employed. A recent publication that provided a systematic review and meta-analysis of 7 circulating miRNA studies in MM established that utilising a combination of miRNAs rather than single miRNAs would be more effective in the diagnosis and prognostic classification of MM [112].

2.3. Therapeutic monitoring

Longitudinal and dynamic monitoring of CTC and CFNA could provide an avenue for detecting loss of response in patients before the emergence of clinical relapse. This could be particularly useful in patients with minimal residual disease (MRD) or when conventional markers are inadequate. MM treatment has witnessed significant progress with the utilisation of novel therapies including PI and IMID with approximately 75% of patients achieving a near-complete response (CR) or CR with front-line therapy. In this context the attribution of MRD negativity has emerged as a paradigm that may be critical in informing treatment decisions. While MM invariably relapses, MRD-negative patients consistently demonstrate more prolonged PFS and may represent a group where therapeutic de-escalation or modification can be safely considered [113, 114]. Consequently, the necessity for highly sensitive assays to detect MRD is critical and this has led to the development and increasing adoption of NGS MRD approaches [115–119]. Currently, MRD status is determined via single site BM biopsy, which clearly has its limitations. Given that the numbers of CTC in the peripheral blood in MM patients with active disease is known to be significantly higher than in patients with inactive disease and the developments in the detection of CTC in MM, the possibility of assessing the overall numbers/presence of CTC in patients during therapy for MRD assessment is imminent [29–33]. Recently, CTCs were monitored to predict response to in MM patients treated within an open-label, randomised, multicenter phase III clinical trial MM5 of newly diagnosed MM patients with the authors concluding that CTC detection in conjunction with BM for MRD detection [120]. Likewise, cfDNA has been used for dynamic monitoring following surgery/radiotherapy for the detection of residual disease in a number of solid cancers, with persistently high or increasing levels of cfDNA detectable mutations post-surgery/radiotherapy associated with a lack of treatment response and a much greater risk of relapse [42], while patients with undetectable levels of somatic mutations in cfDNA have demonstrated fewer or no recurrences [121]. The monitoring of MRD using cfDNA in diffuse large-cell B Lymphoma has also demonstrated that the persistence of cfDNA mutations identifies patients at risk of recurrence before the emergence of clinical relapse [122]. However, any correlation between the presence or re-emergence of cfDNA detectable mutations and disease progression has not yet been assessed in MM.

Currently, only three studies in MM have utilised liquid biopsy in comparison with conventional markers of disease burden in sequential plasma samples, of which only one study has compared CTC and ctDNA for therapeutic monitoring. All three studies utilised different approaches. Oberle and colleagues assessed clonotypic V(D)J rearrangement in CTC and cfDNA in a cohort of 27 MM patients. Overall, an association between both the presence of cfDNA and CTC V(D)J detectable rearrangements with response was demonstrated [94].

Notably, the study detected V(D)J rearrangement in cfDNA in all patients assessed while rearrangements in CTC were only detectable in 70% of patients, indicating that cfDNA, derived from CTC, BM and EM tissue, may represent a better medium for disease evaluation than CTC alone. Long-term monitoring of recurrently occurring mutations in sequential serum samples from 11 patients over a period of 7 years has also been performed in MM [95]. While the majority of patients assessed demonstrated a correlation between PP and the quantifiable levels of specific mutations in sequential plasma samples, there were clearly some patients where analysis of cfDNA was able to identify impending relapse prior to the emergence of clinical relapse, showing the potentially higher sensitivity of cfDNA analysis for disease monitoring. However, a major limitation of sequentially monitoring an initially identified mutant clone is the possibility a sub-clonal mutations not detectable at diagnosis evolving during relapse or being present at an undetectable level initially and subsequently predominating due to selection pressure. Indeed, this appeared to be evident in one of the patients studied, where the initial detection of a BRAF V600E mutation was not maintained despite the continued serologically persisting disease [95]. The evolution of the tumour genome during therapy was also clearly demonstrated in our study, in which 7 patients were monitored sequentially [55]. One patient in particular demonstrated the potential complexities presented by genomic disease evolution. Initially the patient manifested both TP53 R273H and NRAS G13R mutated clones that were responsive to therapy with lenalidomide and dexamethasone (Rd). Subsequently a rapid increase in SFLC consistent with light-chain escape was coincident with the emergence of two new KRAS clones, KRAS G12A and KRAS G12 V. The clonal fraction of both KRAS clones reduced with a switch to Ixazomib-cyclophosphamide-dexamethasone (ICd) therapy coinciding with a serological response. However, the NRAS G13R clone that was responsive to Rd. progressed on ICd in contrast to the TP53 R273H clone that continued to respond,

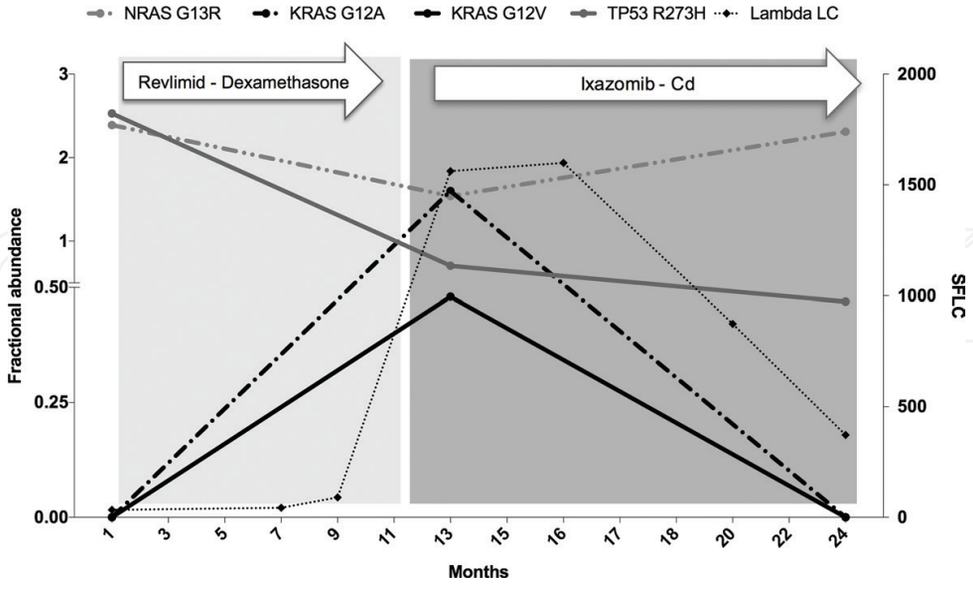

Figure 4. Line graph represents the clonal fraction of 4 mutant clones (left Y-axis) and lambda LC (right Y-axis) in sequential PL of relapsed patients collected at months 1, 13 and 24 during therapy. Patient relapsed on revlimid and dexamethasone with increase in levels of two mutant clones KRAS G12 V and KRAS G12A at month 13 coinciding with lambda LC, however, TP53 R273H and NRAS G13R were found to decrease. A switch to Ixazomib, cyclophosphamide and dexamethasone (cd) at month 13 decreased levels of KRAS G12A and KRAS G12 V with increasing levels of NRAS G13R suggesting differential response of mutant clones to treatment. Image reproduced from [55].

highlighting the differential responses of the 4 mutant clones to two different lines of therapy (**Figure 4**). These observations signify that comprehensive mutational analysis in sequential plasma is critical to define therapeutic response in a timely manner. Plasma ctDNA analysis may also represent a novel and informative strategy for disease monitoring in OS-MM and NS-MM patients as demonstrated in our recent publication [55]. Sequential plasma ctDNA analysis in a ND NS-MM patient over a period of 19 months showed that relapsed disease was associated with the reappearance of mutant KRAS G12 V and KRAS G12D clones that had been present at diagnosis in the BM and the emergence of two new clones, NRAS G13D and NRAS Q61K, with the former showing refractoriness to both Thalidomide – dexamethasone, cyclophosphamide, etoposide and cisplatin (T-DCEP) and re-treatment with bortezomib (velcade) – cyclophosphamide – dexamethasone (VCD) and persisting until the patient died shortly thereafter from progressive disease (**Figure 5**). Interestingly the BM biopsy at month 19 showed apparent reduction in disease burden coincident with reintroduction of VCD but droplet digital PCR (ddPCR) of plasma ctDNA showed an increasing clonal fraction of the NRAS G13D clone consistent with VCD-refractory disease distant to the site of BM biopsy. This first-time observation indicates that ctDNA represents a readily accessible non-invasive biomarker of disease burden that may be superior to BM biopsy for monitoring treatment response in NS-MM. Overall, all three studies demonstrated the potential for ctDNA to be used in monitoring MM patients. Clearly, there were limitations due to small sample sizes, lack of homogenous patient treatment and the critical need to be more comprehensive in mutational identification to address tumour genome evolution. Future studies should therefore incorporate sequential cfDNA assessment adopting NGS-based approaches,

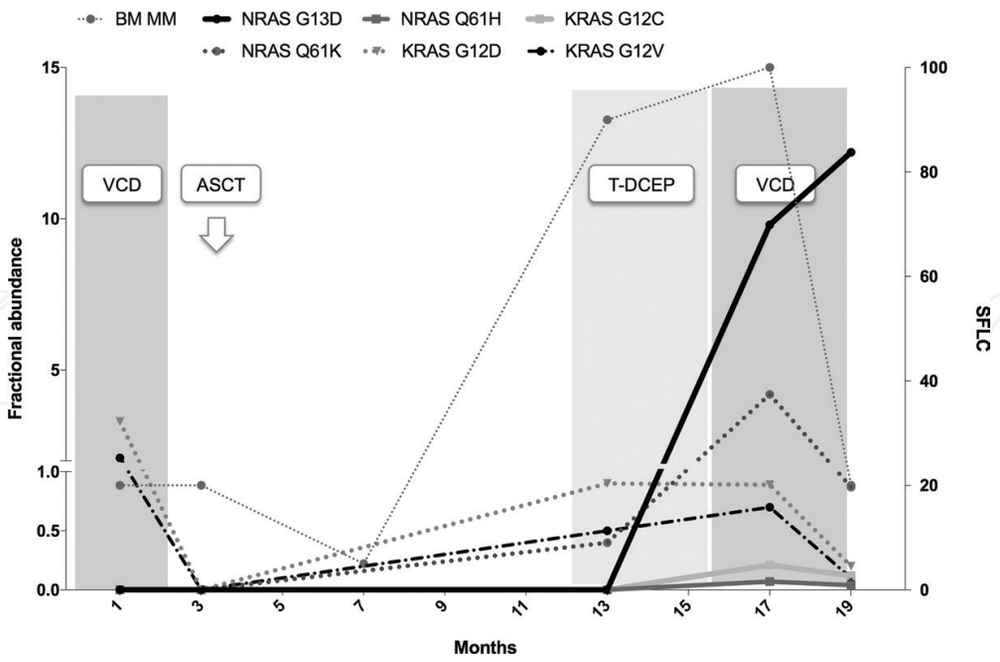

Figure 5. Line graph represents the clonal fraction of mutant clones by ddPCR in a non-secretory patient, patient #2. PL was collected at 1, 3, 13, 17 and 19 months post- diagnosis. The proportion of BM MM cells is shown with an increasing clonal fraction of 4 clones coinciding with BM relapse at month 13, only 9 months post-autologous stem cell transplantations (ASCT). At month 19 a BM response to VCD was evident but with an increasing abundance of the NRAS G13D clone. The patient succumbed to refractory progressive disease shortly afterwards. Image reproduced from [55].

at diagnosis and subsequent relapses, to quantify previously detected and emergent clones, in larger annotated sample sets, thus enabling the comprehensive tracking of mutations and assessment of disease burden with correlation to conventional serum biomarkers.

3. Methodologies and challenges

An increasing number of publications have proven the utility of liquid biopsy for cancer screening, diagnosis and disease monitoring. However, for this paradigm to be more widely incorporated into the clinical setting a standardised approach for both the pre-analytical processing and the analytical platforms utilised is necessary. Identification and isolation of MM CTC has been established through the utilisation of ultra-sensitive NGS multicolour flow cytometry techniques [26–28]. Lohr et al. devised a combination methodology that allowed for isolation of CTC using the standard markers (CD138+ CD38+ CD56 variable, CD45 low) and serial dilutions to single cells through fluorescence microscopy to improve the detection of CTC [27]. Currently, these are the only described methodologies for isolation of CTC and more studies are required to standardise the methodologies described.

CFNA analysis relies on the standardisation of collection, processing and optimal storage of cfDNA and exRNA, which can now be streamlined with commercially available specialised collection tubes that can stabilise blood at room temperature and preserve the white blood cells to avoid rupture and therefore contamination with cellular DNA in the plasma (Streck, PAXgene, Roche, NORGEN BIOTEK, etc.). Isolation of genomic DNA or RNA from both CTC and CFNA, to a large extent, has also been simplified through the use of commercially available specific isolation kits, although the performance of each of these kits in comparison to other conventional methods is controversial [123]. The choice of the NGS methodologies to address specific questions is however, more complicated and is confounded by the type of nucleic acid being analysed, assay sensitivity and specificity and the heterogeneous content of tumour-derived nucleic acids. A substantial technical challenge to overcome in optimising cfDNA analysis is the level of sensitivity of the various NGS methodologies, as cfDNA may contain low-frequency mutant alleles that might not be readily detectable in WES and targeted amplicon sequencing, which have a sensitivity between 1 and 5%. This is being addressed with the utilisation of unique molecular indices that provide further resolution [48, 124] and such approaches are currently offered by a number of commercial companies (Rubicon genomics, QIAGEN, Agilent).

Analysis of exRNA is more complicated as a technology-of-choice needs to be determined for large-scale identification of potential biomarkers. Additionally, the validation and diagnostic implication of circulating RNA sub-types currently relies on 'internal controls' which are often not suited for this type of analysis. This could be addressed using absolute quantification methods, a plausible approach utilising ddPCR, which provides a more robust approach by quantitating targets per unit in serum or plasma, as done in cfDNA studies.

For CFNA and CTC, unfortunately, to date, few of the described methodologies are as yet validated or standardised, complicating generalisability and inter-study comparisons. Overall, the field would benefit from establishment of certain guidelines for each aspect of sample

analysis to allow for comparison of studies of similar kind. These limitations not withstanding, the analysis of ctDNA has gained significant momentum is the past couple of years, with numerous commercial companies offering 'liquid biopsy' testing. Such analysis is now frequently integrated into clinical trials and plasma DNA EGFR mutation testing for non-small cell lung cancer has recently been approved by the FDA [125–127]. Further commercialisation of these 'liquid biopsies' as diagnostics is rapidly evolving, but currently is largely limited to informing treatment choices in late stage cancers.

4. Conclusions

Liquid biopsy analysis can provide a dynamic and comprehensive picture of the genomic landscape in MM. Specifically, serial analysis can provide a non-invasive approach to monitor tumour burden and genomic evolution that also incorporates characterisation of the spatial and temporal genomic heterogeneity, which predominates over time, in this multi-focal disease. Accumulating published evidence and imminent developments in the field indicate that this type of analysis will provide critical information for precision medicine and likely transform the management of more problematic sub-groups of MM including NS, OS patients and those with EM disease. In the future, instead of utilising extensive imaging and invasive and potentially misrepresentative BM biopsies, liquid biopsies could be used to inform real-time clinical decision thus further improving the outcome for MM patients.

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