Dynamics of tumor-specific cfDNA in response to therapy in multiple myeloma patients

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Abstract
Objectives: Progress in multiple myeloma treatment allows patients to achieve deeper responses, for which the assessment of minimal residual disease (MRD) is critical. Typically, bone marrow samples are used for this purpose; however, this approach is site-limited. Liquid biopsy represents a minimally invasive and more comprehensive technique that is not site-limited, but equally challenging.

Methods: While majority of current data comes from short-term studies, we present a long-term study on blood-based MRD monitoring using tumor-specific cell-free DNA detection by ASO-qPCR. One hundred and twelve patients were enrolled into the study, but long-term sampling and analysis were feasible only in 45 patients.

Results: We found a significant correlation of quantity of tumor-specific cell-free DNA levels with clinically meaningful events [induction therapy (P = .004); ASCT (P = .012)]. Moreover, length of cfDNA fragments is associated with better treatment response of patients.

Conclusions: These results support the concept of tumor-specific cell-free DNA as a prognostic marker.

KEYWORDS
cell-free DNA, liquid biopsy, multiple myeloma, qPCR
1 | INTRODUCTION

Multiple myeloma (MM) ranks as the second most common hematological malignancy, right after non-Hodgkin lymphoma. Its incidence varies globally, with higher frequency in Europe and North America. Over the past decade, great progress has been achieved not only in understanding of MM biology, but also in its treatment. This progress is best represented by the introduction of new drugs and multi-drug combinations, which induce deep treatment responses, including high proportion of stringent complete responses (sCR). Achievement of deep responses is the reason for growing interest in minimal residual disease (MRD), which has become the most important prognostic factor; evaluation of MRD requires development of reliable techniques. Currently, next-generation sequencing (NGS) and next-generation flow (NGF) are most common since MRD is primarily assessed from bone marrow (BM) samples. However, collection of BM is invasive, painful and occasionally can lead to false-negative results due to single-site collection or complication of patient’s condition. A newer approach, so-called liquid biopsy, has been intensively studied lately. It provides non- or minimally invasive alternative to diagnosis, treatment monitoring and MRD monitoring from body fluids. One of the most promising target molecules in liquid biopsies is cell-free DNA (cfDNA), short double-stranded DNA molecules circulating in various body fluids. Low levels of these molecules can be found even in healthy individuals; however, levels of cfDNA were found to be considerably elevated in various pathological conditions. The use of cfDNA for MRD evaluation in various hematological malignancies was described several times as having promising results. Moreover, cheap, reliable, and sensitive method of detection of these molecules and MRD in general is one of the top priorities in the field. Unfortunately, most of the studies evaluating MRD using cfDNA lack not only sufficient number of patients, but also longer observation times to draw conclusions. For these reasons, we performed a long-term study of dynamics of cfDNA in newly diagnosed MM patients using allele-specific (ASO) qPCR.

2 | MATERIALS AND METHODS

2.1 | Samples and patients

Two institutions were involved in patient recruitment and sample collection—University Hospital in Brno and University Hospital in Ostrava, Czech Republic. Before enrollment into the study, all patients signed the informed consent form approved by the Ethics committee of each hospital in accordance with the current version of the Helsinki declaration. To avoid sampling bias and to achieve maximum heterogeneity in the study, patient recruitment was consecutive and not restricted by any clinical or biological parameters. From the total number of 112 patients who were enrolled in the study, complete analysis was feasible in 45 (40.2%) patients. Bone marrow (BM) samples were collected at diagnosis, and CD138+ cell fraction was sorted using magnetic-activated cell sorting (MACS) as previously described. At diagnosis and at 3-month intervals, samples of peripheral blood (PB) were collected until a patient reached complete response (CR). If CR was not reached, samples were collected for 24 months after diagnosis. Two more samples of PB were collected (CR + 3, CR + 6) if patients reached CR (see Appendix S1 for more information). Due to short half-life of cfDNA, all PB samples were processed within 15 minutes after collection to separate and deep freeze serum (−80°C), further used for cfDNA extraction by QIAamp Circulating Nucleic Acid Kit (Qiagen). Commonly used protocol was used as previously described. Samples were centrifuged at 1300g/15 minutes/20°C and frozen as 0.5 mL aliquots. Samples were thawed only once.

2.2 | Identification of tumor-specific IgH rearrangement and patient-specific primers and probes design

The CD138+ cell fraction was sorted from BM samples collected at diagnosis, and genomic DNA (gDNA) was extracted. It was further used to identify a patient-specific rearrangement of variability (V), diversity (D), and joining (J) regions of immunoglobulin heavy chain (IgH), using PCR with a set of consensual primers. Combinations of the family of forward primers derived from the FR1 or FR2 region and reverse primer derived from the JH region of IgH were tested to find functional pair and to produce an amplicon (Table S6). This approach was already implemented in patients with acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphomas, and MM. After identification of functional primer sets, amplified PCR products were sequenced. Obtained sequences were used for analysis of specific VDJ rearrangements using IMGT/V-QUEST web tool. Patient-specific ASO primers and probes were designed using PrimerQuest tool (IDT). To ensure specific detection, probe design was based on the previously published probe sequences.

2.3 | qPCR of the IgH gene

Previously described strategy for MRD quantification using ASO-qPCR in MM was adopted in this study. Standard curve was prepared for each patient using plasmid DNA with incorporated patient-specific sequence. Patient-specific VDJ rearrangement was amplified, cloned into the vector and transformed to competent cells. First, colony PCR and agarose gel electrophoresis were used to check for bands of expected size. Subsequently, Sanger sequencing of colonies with correct band size was carried out and sequences of colonies were compared with those acquired from patient genomic DNA. Plasmids with confirmed insertion of target sequence were mixed with DNA obtained from mononuclear cells from pool of at least 5 healthy donors for correction of unspecific background and serially diluted to plot standard curve. qPCR was performed and
evaluated according to guidelines for interpretation of qPCR data.\textsuperscript{24}

qPCR reagents were mixed with extracted cfDNA, patient-specific ASO primers and probes, and reactions were cycled under following conditions: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minutes Based on the determined cfDNA levels, we classified samples as positive, negative, or positive non-quantifiable (PNQ). The PNQ status was used when at least one of two replications was positive outside of quantitative range of standard curve.\textsuperscript{24}

2.4 | Multiparametric flow cytometry (MFC) assessment of MRD status

BM samples were analyzed by 8-color panel protocol (CD38, CD45, cyκ, cyλ, CD138, CD19, CD56, CD27, or CD81, regarding original PC phenotype). The number of analyzed events was up to $10^{-7}$, if achieving sensitivity of $10^{-6}$ was possible.\textsuperscript{25} Required BM volume was lysed before staining of the surface markers, followed by fixation and permeabilization of cells and staining of the intracellular antigens. The samples were acquired by BD FACSCanto™ II (BD Biosciences) and analyzed by Infinicyt software (Cytognos SL).

2.5 | Fragment length analysis of total cfDNA

Fragment length of total cfDNA was analyzed using High sensitivity DNA analysis kit on 2100 Bioanalyzer (both Agilent Technologies) based on manufacturer’s instructions.

2.6 | Statistical analysis

Data were evaluated by absolute and relative frequencies of categorical variables and median (minimum–maximum) of quantitative variables. McNemar’s test was used to compare cfDNA data at diagnosis and at different time points during treatment and Mann-Whitney U test was used to determine correlation of cfDNA and M-protein. Time to reaching CR was assessed using Kaplan-Meier methodology. P-values less than .05 were considered statistically significant. Statistical analysis was performed in the SPSS software (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, version 25.0. Armonk, NY: IBM Corp.) and software R version 3.3.0 (www.r-proj.org).

3 | RESULTS

3.1 | Correlation of cfDNA with clinically meaningful events

To investigate whether the treatment response mirrors the dynamics of tumor-specific cfDNA, we compared patients’ cfDNA levels obtained at the time of diagnosis (entry) and following cfDNA levels at the time of clinically meaningful events (follow-up: Table 1). We found that positive or PNQ entry-level classification of tumor-specific cfDNA changed to negative in response to initiation of induction therapy ($P = .004$) in almost 33% of all patients. The impact of autologous stem cell transplantation (ASCT) was even greater. Out of 21 patients who received ASCT, 10 patients (47.6%) with previous positive or PNQ classification had negative cfDNA levels in the follow-up sample. We also compared the entry levels of cfDNA to levels determined in subsequent 3-month intervals to see a possible trend in changes (Table 2). This comparison showed that number of patients classified as negative is significantly higher in all but one time point of analysis indicating a positive effect of therapy.

| TABLE 1 | Comparison of matching samples and their cfDNA statuses determined at clinically meaningful events (follow-up status) to the cfDNA statuses at diagnosis (entry status) |
|--------|---------------------------------|-----------------|-----------------|
| Follow-up status | Entry status (Dg.) | (of total N) |
| Negative | PNQ/positive | P<sup>a</sup> |
| Induction therapy—3 mo (N = 43) | Negative | 7 (16.3%) | 14 (32.6%) | .004 |
| PNQ/positive | 2 (4.7%) | 20 (46.5%) |
| Induction therapy—6 mo (N = 20) | Negative | 4 (20.0%) | 6 (30.0%) | .125 |
| PNQ/positive | 1 (5.0%) | 9 (45.0%) |
| CR<sup>b</sup> (N = 18) | Negative | 5 (27.8%) | 5 (27.8%) | .063 |
| PNQ/positive | 0 (0.0%) | 8 (44.4%) |
| ASCT<sup>b</sup> (N = 21) | Negative | 3 (14.3%) | 10 (47.6%) | .012 |
| PNQ/positive | 1 (4.8%) | 7 (33.3%) |
| PD<sup>b</sup> (N = 18) | Negative | 3 (16.7%) | 6 (33.3%) | .125 |
| PNQ/positive | 1 (5.6%) | 8 (44.4%) |

Note: Values at intersections designate the number (% of total number) of patients with the same/changed status. Abbreviations: ASCT, autologous stem cell transplantation; CR, complete response; N, number of patients; PD, progressive disease; PNQ, positive non-quantifiable. Bold indicates statistical significant value. \textsuperscript{a}P-value of exact McNemar test. \textsuperscript{b}Assessed in time of first CR/ASCT/PD.

3.2 | Correlation of monoclonal protein and tumor-specific cfDNA levels

The standard for diagnosis and response evaluation in MM is level of monoclonal protein. Therefore, we compared M-protein levels to levels of tumor-specific cfDNA in respective samples. Unfortunately, no general correlation was found (Table S7). However, in 2/3 of patients,
this correlation was moderate; it was high or even statistically significant in six cases. In another six cases, the correlation could not be determined due to zero levels of one or both variables (Table S8).

### 3.3 Total cfDNA fragment length frequencies

To determine the source of the cfDNA fragments and whether the average fragment length changes during therapy, we measured total cfDNA size distribution (ranging from 180 to 10 000 bp). Our results show that total cfDNA fragment length changes during therapy especially in a group of patients who reached CR, where frequency of longer fragments increased significantly (Figures 1 and 2). The change in frequency of fragments of respective length at study entry and at time of CR is statistically significant in all but the shortest

![Graph showing frequency of fragments of respective length at study entry (baseline) and follow-up](image)

**TABLE 2** cfDNA status (positive/negative/PNQ) at diagnosis (entry status) compared with results in 3-mo intervals

| Time of assessment | Entry status (Dg.) | Negative | PNQ/positive | P<sup>a</sup> |
|--------------------|--------------------|----------|--------------|--------------|
| 3 mo (N = 43)      |                    | 7 (16.3%)| 14 (32.6%)   | .004         |
|                    | PNQ/positive       | 2 (4.7%) | 20 (46.5%)   |
| 6 mo (N = 40)      |                    | 7 (17.5%)| 11 (27.5%)   | .022         |
|                    | PNQ/positive       | 2 (5.0%) | 20 (50.0%)   |
| 9 mo (N = 38)      |                    | 5 (13.2%)| 14 (36.8%)   | .004         |
|                    | PNQ/positive       | 2 (5.3%) | 17 (44.7%)   |
| 12 mo (N = 34)     |                    | 5 (14.7%)| 12 (35.3%)   | .013         |
|                    | PNQ/positive       | 2 (5.9%) | 15 (44.1%)   |
| 15 mo (N = 33)     |                    | 6 (18.2%)| 17 (51.5%)   | <.001        |
|                    | PNQ/positive       | 1 (3.0%) | 9 (27.3%)    |
| 18 mo (N = 21)     |                    | 5 (23.8%)| 10 (47.6%)   | .002         |
|                    | PNQ/positive       | 0 (0.0%) | 6 (28.6%)    |
| 21 mo (N = 21)     |                    | 6 (28.6%)| 8 (38.1%)    | .109         |
|                    | PNQ/positive       | 2 (9.5%) | 5 (23.8%)    |
| 24 mo (N = 16)     |                    | 4 (25.0%)| 8 (50.0%)    | .008         |
|                    | PNQ/positive       | 0 (0.0%) | 4 (25.0%)    |

Note: Values at intersections designate the number (% of total number) of patients with the same/changed status. Bold indicates statistical significant value. <sup>a</sup>P-value of exact McNemar test.

![Graph showing frequency of fragments of respective length at study entry (baseline) and at time of reaching CR (only patients who reached CR analyzed)](image)
fragment range, which remained stable (180-220 bp, \( P = \text{n.s.} \), 350-400 bp, \( P < .001 \); 500-1000 bp, \( P < .003 \); 700-10000 bp, \( P < .021 \)) (Figure 2).

3.4 | Evaluation of time to complete remission

In a group of patients who reached CR, we investigated the connection between entry level of tumor-specific cfDNA and time to CR (Figure 3A). No statistically significant difference was found among the three groups, but interestingly, profile of group of PNQ patients was similar to the group of negative patients rather than to those who were positive. Therefore, we decided to estimate approximate quantities of PNQ samples and set a cut-off value of normalized quantities to 50 copies with respect to estimation error. Estimated quantities and cut-off value were used to identify potentially false-positive results caused by PCR error at high cycle number and split PNQ samples to groups of positive and negative patients. With this approach, we obtained a Kaplan-Meier estimate at 12 months, where all patients were classified as either positive or negative at entry (Figure 3B.). Estimates were supplemented by 95% confidence interval derived using Greenwood formula, and log-rank test was used to estimate statistical significance of the difference between curves (Colour figure can be viewed at wileyonlinelibrary.com)

4 | DISCUSSION

Analysis of tumor-specific cfDNA is a promising, emerging form of liquid biopsy in hematological malignancies.\(^2\) So far, several studies investigated cfDNA in MM patients,\(^2\) but only limited long-term data are available. Here, we performed a long-term comprehensive study of cfDNA dynamics, which extends previous findings about applicability of cfDNA analysis for minimally invasive testing of MM patients.\(^3\)\(^,\)\(^0\)\(^,\)\(^1\) Our results show that levels of tumor-specific cfDNA drop in response to therapy. Both, the induction therapy and ASCT significantly affected levels of tumor-specific cfDNA in follow-up samples. In both cases, positive/PNQ levels of cfDNA decreased to undetectable (negative) levels in 32.6% (\( P = .004 \)) and 47.6% (\( P = .012 \)) of concerned patients, respectively. These results confirm the high value of ASCT in treatment of MM patients demonstrated by various studies,\(^3\)\(^,\)\(^2\) but also indicate the ability of cfDNA to mirror patients’ response. The fact that majority of positive/PNQ patients in all assessed categories, except ASCT, remained positive (Table 1) is probably caused by various time to response of individual patients or possible non-responders to therapy. Response rates and survival
of patients may vary significantly, and fast response does not necessarily mean longer survival as recently reported. A study on more than 1000 individuals indicated that more gradual response (time to plateau >120 days) of MM patients is connected to longer survival compared with rapid responders.\textsuperscript{33} Since we observed a significant increase of negative cfDNA samples in time (Table 2), slower response to therapy, rather than resistance to therapy, is probably the reason why cfDNA levels remained stable in majority of patients at the time of assessment. On the other hand, a change from negative to positive cfDNA levels observed in some cases might indicate a possible non-responder to therapy.\textsuperscript{31,34}

Considering abovementioned results, we hypothesized that even CR will be reflected in tumor-specific cfDNA levels, but this was not confirmed. Since there is a trend of increasing negative cfDNA in time and \( P \)-value is almost significant, this result might be affected by low number of patients who reached CR (\(N = 18\)) and nature of used method. ASO-qPCR is a powerful method for MRD evaluation with sensitivity comparable to other methods.\textsuperscript{35,36} The sensitivity of our assay reached \(10^{-4}-10^{-6}\) and corresponds to sensitivities described by other studies.\textsuperscript{30,37} However, applicability of ASO-qPCR is often limited by technical complexity and variety of technical problems.\textsuperscript{28} In our study, lower applicability mirrors in the number of analyzed vs. enrolled patients (45/112) with main reason for exclusion being the inability to identify patient-specific VDJ rearrangement (see Appendix S1 for details).

Despite strict rules for defining MRD positivity and thorough standardization,\textsuperscript{24} ASO-qPCR still harbors a risk of false-positive and false-negative results.\textsuperscript{39,40} Preanalytical factors were demonstrated to have a significant effect on cfDNA analysis regardless of used method.\textsuperscript{41} An important factor is yield of cfDNA. We observed high intra- and inter-patient variability (range 1.75-405.6 ng per mL of serum), which is consistent with previous studies among MM patients and other malignancies.\textsuperscript{28} A wide range of biological and physiological factors, including tumor burden, disease stage or even half-life of cfDNA can influence release and clearance of tumor-specific fraction of total cfDNA and play a role in this variability.\textsuperscript{42} As a result, scarce amounts of cfDNA are isolated in some cases in which target molecules may be under-represented causing false-negative results.\textsuperscript{6,43} This at least partially explains why some patients were found cfDNA negative at diagnosis and put emphasis on standardization of preanalytical factors which can be influenced. Regardless of these issues, our results show relation of tumor-specific cfDNA and therapy response. We also hypothesized that cfDNA will reflect decreasing tumor burden caused by applied therapy. Our data confirm this hypothesis, since number of samples with undetectable levels of tumor-specific cfDNA significantly increased in time (Table 2).

Comparison of M-protein and cfDNA levels did not show any significant correlation. These results are in agreement with recent findings.\textsuperscript{27} It is not surprising since half-life of these two variables is entirely different. While cfDNA half-life ranges from minutes to a few hours,\textsuperscript{44} M-protein levels remain stable for longer, since its half-life ranges from 7 to 25 days depending on the type of immunoglobulin.\textsuperscript{45} Therefore, occasional correlation depends on the dynamics of the disease and frequency of sample collection.

Aforementioned limitations of ASO-qPCR are best reflected in the evaluation of time to CR. Surprisingly, no significant difference was found when comparing positive, negative and PNQ groups. Nevertheless, even though PNQ results are generally considered to be positive rather than negative, the Kaplan-Meier curve representing this cohort is more similar to negative group. Since empirically, the PNQ samples are more prone to be false-positive than other classifications, we performed estimation of cfDNA quantities of these samples and used a cut-off value to split them to remaining two groups. After division, two groups have significantly different times ing this cohort is more similar to negative group. Since empirically, we performed estimation of cfDNA quantities of these samples and used a cut-off value to split them to remaining two groups. After division, two groups have significantly different times

| Time of assessment (mo) | CR status | M-protein (g/L) | Flow MRD status | cfDNA | cfDNA quantity |
|------------------------|-----------|----------------|----------------|-------|---------------|
| 6                      | Positive  | 0              | Negative       | Negative | 0          |
| 9                      | Positive  | 0              | Negative       | Negative | 0          |
| 9                      | Positive  | 0              | Negative       | PNQ    | 76.9         |
| 12                     | Positive  | 0              | Negative       | Negative | 0          |
| 15                     | Positive  | 0              | Negative       | Negative | 0          |
| 21                     | Positive  | 0              | Negative       | Negative | 0          |
| 3                      | Positive  | 0              | Positive       | PNQ    | 56.2         |
| 6                      | Positive  | 0              | Positive       | PNQ    | 9.0          |
| 9                      | Positive  | 0              | Positive       | 2397.8 |
| 12                     | Positive  | 0              | Positive       | Negative | 0          |
| 15                     | Positive  | 0              | Positive       | 142.5  |
| 18                     | Positive  | 0              | Positive       | Negative | 0          |

Note: Matching data of cfDNA and MFC analyses were available for 12/18 (66.7%) patients with CR. Sensitivity = 66.7% (22.3%–95.7%), specificity = 83.3% (35.9%–99.6%).

TABLE 3 MDR status from flow cytometry compared to M-protein and cfDNA quantity
the MRD status may serve as a good example. MFC, commonly used for MRD evaluation, and our cfDNA data provide comparable, but not identical results contrasting M-protein levels (Table 3) (sensitivity = 66.7% [22.3%-95.7%], specificity = 83.3% [35.9%-99.6%]). Nevertheless, the results of cfDNA testing in recent publications are inconsistent, so more studies are needed.46

Lastly, our analysis of total cfDNA fragment length frequencies provided interesting results. As confirmed previously, spectrum of short fragments of approximately 180-220 bp is connected to apoptosis, while long fragments originate mainly from cells dying via necrosis.47 Our results suggest that in addition to apoptosis, a contemporary increase of necrosis of tumor cells is present in patients who reached CR. Since necrosis is generally a pathological process that occurs after serious physical or chemical damage, these results correspond to cell death of malignant cells after effective therapy. This also explains the highest effect in the group of patients with CR, where therapy is highly effective. Moreover, a programmed form of necrosis—necroptosis may be partially responsible for elevated fraction of longer cfDNA fragments. Proteasome inhibitors are widely used for treatment of MM.48 Majority of patients in our study have also had proteasome inhibitors included in their treatment regimens, with bortezomib being the most prevalent. Nevertheless, a recent study suggests that bortezomib may induce necroptosis instead of apoptosis in some cases.49 Surprisingly, apoptosis remained at the same level throughout the time. Reasons remain unknown; however, secondary necrosis might play its role. Apoptotic cells are physiologically rapidly phagocytosed. Nevertheless, if immune system is overwhelmed or some chemotherapeutic agents and types of radiation therapy are used,50 cells can undergo secondary necrosis as an outcome of apoptosis more often.50,51

In conclusion, our study confirms the prognostic value of cfDNA for MM patients. Our analysis shows that not only levels of cfDNA change in reaction to therapy, but also that overall distribution of cfDNA fragment lengths changes especially in cohort of patients with best response. Moreover, despite the low number of samples with MFC measurements, MRD status determined using cfDNA also corresponded to results acquired by MFC better than M-protein levels. Analysis, however, requires optimization or implementation of novel methods since ASO-PCR is too time-consuming and does not allow analysis of polyclonal MM patients. Likewise, as cfDNA was most relevant in CR patients, further studies on larger cohorts of patients with CR are needed.

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CONFLICT OF INTEREST
No conflict of interest to declare.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.