The Journal of Molecular Diagnostics, Vol. ■, No. ■, ■ 2018

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the Journal of Molecular Diagnostics
jmd.amjpathol.org

Noninvasive Molecular Monitoring in Multiple Myeloma Patients Using Cell-Free Tumor DNA

A Pilot Study

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Accepted for publication July 9, 2018.

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Novel treatments for multiple myeloma (MM) have increased rates of complete response, raising interest in more accurate methods to evaluate residual disease. Cell-free tumor DNA (cfDNA) analysis may represent a minimally invasive approach complementary to multiparameter flow cytometry (MFC) and molecular methods on bone marrow aspirates. A sequencing approach using the Ion Torrent Personal Genome Machine was applied to identify clonal *IGH* gene rearrangements in tumor plasma cells (PCs) and in serial plasma samples of 25 patients with MM receiving second-line therapy. The same clonal *IGH* rearrangement identified in tumor PCs was detected in paired plasma samples, and levels of *IGH* cfDNA correlated with outcome and mirrored tumor dynamics evaluated using conventional laboratory parameters. In addition, *IGH* cfDNA levels reflected the number of PCs enumerated by MFC immunophenotyping even in the complete response context. Patients determined by MFC to be free of minimal residual disease were characterized by low frequencies of tumor clonotypes in cfDNA and longer survival. This pilot study supports the clinical applicability of the noninvasive monitoring of tumor levels in plasma samples of patients with MM by *IGH* sequencing. *(J Mol Diagn 2018, ■: 1—12; https://doi.org/10.1016/j.jmoldx.2018.07.006)*

Q7 The treatment of multiple myeloma (MM) has changed during the past decade with the introduction of several classes of new effective drugs that have greatly improved response rates,[1,2] highlighting the need for more accurate methods of residual disease assessment. Several studies using multiparameter flow cytometry (MFC) or allele-specific oligonucleotide quantitative PCR have found that the presence of residual tumor cells after therapy is associated with shorter progression-free survival (PFS).[3] More recently, next-generation sequencing (NGS) techniques on bone marrow (BM) aspirates have been used.[4] Minimum residual disease (MRD) negativity by NGS of the *IGH* gene rearrangements is significantly associated with longer time to progression and better overall survival in patients treated with front-line novel therapies. Similar results were observed using MFC, although authors report the presence of a subset of patients who tested positive by MFC and negative by sequencing that probably reflects the patchy pattern of BM infiltration typically observed in MM.[4] This, together with extramedullary disease, represents a potential pitfall common to all techniques that use BM aspirates that may be nonrepresentative of the disease infiltration. The use of alternative methods for disease assessment, such as the monitoring of circulating cell-free tumor DNA (cfDNA), could improve the estimation of the risk of progression.[5] Ongoing studies are examining tumor cfDNA as a sensitive measure of small amounts of residual cells in lymphoma and myeloma.[6–8] The sensitivity of cfDNA analysis is still unknown, but the development of peripheral blood (PB)—based disease monitoring approaches should be

Supported by Associazione Italiana contro le Leucemie-linfomi e mieloma, Associazione Italiana Ricerca Cancro, and Fondazione Adiuvare. Q3 Q4 Q5

G.B. and S.G. contributed equally to this work.

C.C. and P.C. contributed equally to this work as senior authors.

Disclosures: None declared.

https://doi.org/10.1016/j.jmoldx.2018.07.006

a goal because it would allow for serial sampling without repeated BM biopsies (BMBs) in MM. In the present study, we describe a deep-sequencing method that allows identifying and quantifying residual tumor burden in patients with MM from plasma samples. The method was applied to a cohort of myeloma samples collected prospectively within a clinical trial.

Materials and Methods

Patients, Treatment Plan, and Sample Collection

We conducted a prospective randomized phase 3 trial in MM at first relapse comparing the activity of bortezomib, cyclophosphamide, and dexamethasone versus lenalidomide, cyclophosphamide, and dexamethasone as second-line therapy. Within this study, 25 patients who achieved complete response during therapy (International Myeloma Working Group guidelines)[9] and had longitudinal biological samples collected for disease monitoring were analyzed. Our institutional review board approved this study (INT 57/10), and patients provided informed consent.

BM and PB samples were obtained during routine clinical evaluations at study entry; after 3, 6, and 9 cycles of therapy; and at follow-up time points. Plasma was obtained processing PB samples collected in K2-EDTA tubes (BD Vacutainer, Becton Dickinson, Franklin Lakes, NJ) within 3 hours, with a first centrifugation at 1500 × *g* for 10 minutes and a second high-speed centrifugation at 16,000 × *g* for 10 minutes at 4°C. Plasma samples were stored at −80°C until extraction.[10] PB of 10 healthy donors was also collected. Namalwa (human Burkitt lymphoma; ACC 24) and JVM-2 (human chronic B-cell leukemia; ACC 12) cell lines were from DSMZ (Braunschweig, Germany).

Flow Cytometry and Immunomagnetic Separation

BM nucleated cells were isolated from a median of 8 mL (range, 7 to 14 mL) of BM aspirates after red blood cells lysis with a hypotonic solution (NH_4Cl, 1.5 mol/L; $KHCo_3$, 100 nmol/L; and Na_4EDTA, 10 nmol/L; pH 7.2 to 7.4). Plasma cells (PCs) were stained according to the European Myeloma Network guidelines[11] using eight-color monoclonal antibody combinations (Table 1) on a MACSQuant Analyzer (Miltenyi Biotec, Gladbach, Germany). Data were analyzed using MACSQuantify software version 2.6 (Miltenyi Biotec) and FlowJo software version 10.2 (FlowJo LLC, Ashland, OR). The target for collection was >500,000 cellular events in each tube. An immunomagnetic bead−based strategy was used to isolate BM $CD138^+$ PCs on the AutoMACS ProSeparator (Miltenyi Biotec).

Table 1 List of Monoclonal Antibodies Used for Flow Cytometric Analysis

Antigen	Fluorochrome	Clone
CD19*	PE	LT19
CD20†	VioBlue	LT20
CD27*	FITC	M-T271
CD28*	PE Vio770	15E8
CD38†	BV500	HIT2
CD45*	VioBlue	5B1
CD45*	APC Vio770	5B1
CD56*	PE Vio770	REA196
CD56†	PE Cy7	MEM-188
CD81*	PerCP Vio700	REA513
CD117*	PE	A3C6E2
CD117*	APC Vio770	A3C6E2
CD138*	APC	44F9

*Miltenyi Biotec, Gladbach, Germany.
†Biolegend.
APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phosphatidylethanolamine; PerCP, peridinin chlorophyll protein complex.

Genomic and Cell-Free DNA Extraction

Genomic DNA (gDNA) was extracted using Nucleospin Tissue kit (Macherey-Nagel GmbH & Co., Düren, Germany). cfDNA was extracted from 1 to 3 mL of plasma and eluted in 50 µL of Buffer AVE using a QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Extracted cfDNA was quantified on a high-sensitivity benchtop fluorometer (Qubit 2.0, Thermo Fisher Scientific, Waltham, MA). The size distribution of the fragments was tested by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, Böblingen, Germany). cfDNA was stored at −20°C until amplification and library preparation.

Amplification, Library Preparation, and Sequencing of *IGH* Gene Rearrangements

Amplification was performed by multiplex PCR using 500 ng of gDNA or a median of 20.3 ng of cfDNA (range, 8.6 to 45.9 ng) according to the BIOMED-2 concerted action with a consensus reverse *IGHJ* primer and in the forward direction a mix of framework region 1 (FR1) or a framework region 3 (FR3) *IGHV* family−specific primers[12,13] (Table 2). The gDNA PCR reactions were performed in 50 µL with the following components: 5 µL of dNTPs 2 mmol/L, 5 µL of buffer 10×, 5 µL of $MgCl_2$ (FR1: 20 mmol/L; FR3: 15 mmol/L), 1 µL of each primer 10 µmol/L (seven to FR1 or FR3 and one to *IGHJ*), 0.2 µL of Taq Gold 5 U/µL, and 1 to 26.8 µL of gDNA. The cfDNA PCR reactions were performed in 85 µL with the following components: 8.5 µL of dNTPs 2 mmol/L, 8.5 µL of buffer 10×, 8.5 µL of $MgCl_2$ (FR1: 20 mmol/L; FR3: 15 mmol/L), 1 µL of each primer 10 µmol/L (seven to FR1 or FR3 and one to *IGHJ*), 0.2 µL of Taq Gold 5 U/µL, and 45 µL of cfDNA. PCR conditions were 96°C for 10 minutes followed by 35 cycles of 96°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C (FR1: 10 minutes; FR3: 5 minutes) with cooling to 4°C.

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Table 2 Primers Used for *IGH* Gene Rearrangements' Amplification

Primer	Sequence
VH1D	5′-CCTCAGTGAAGGTCTCCTGCAAGG-3′
VH2D	5′-TCCTGCGCTGGTGAAAGCCACACA-3′
VH3D	5′-GGTCCCTGAGACTCTCCTGTGCA-3′
VH4aD	5′-TCGGAGACCCTGTCCCTCACCTGCA-3′
VH4bD	5′-CGCTGTCTCTGGTTACTCCATCAG-3′
VH5D	5′-GAAAAAGCCCGGGGAGTCTCTGAA-3′
VH6D	5′-CCTGTGCCATCTCCGGGGACAGTG-3′
VH1-FR3	5′-TGGAGCTGAGCAGCCTGAGATCTGA-3′
VH2-FR3	5′-CAATGACCAACATGGACCCTGTGGA-3′
VH3-FR3	5′-TCTGCAAATGAACAGCCTGAGAGCC-3′
VH4-FR3	5′-GAGCTCTGTGACCGCCGCGGACACG-3′
VH5-FR3	5′-CAGCACCGCCTACCTGCAGTGGAGC-3′
VH6-FR3	5′-GTTCTCCCTGCAGCTGAACTCTGTG-3′
VH7-FR3	5′-CAGCACGGCATATCTGCAGATCAG-3′
JHD	5′-ACCTGAGGAGACGGTGACCAGGGT-3′

Forward and reverse primers were pooled together in a multiplex PCR reaction. The expected amplicon size is in the range of 310 to 360 bp for framework region 1 (FR1) primer set and 100 to 170 bp for framework region 3 (FR3) primer set.

PCR products were controlled for quantity and quality on an Agilent 2100 Bioanalyzer using the high-sensitivity DNA kit (Agilent Technologies). Adapter ligation and barcoding were performed using Ion Plus Fragment Library kit and Ion Xpress Barcode Adapters 1-16/17-32 kits (Thermo Fisher Scientific) following the manufacturer's instructions. Barcoded libraries were purified using AMPure beads (Beckman Coulter, Brea, CA) according to the library size as reported in the manufacturer's instructions (FR1: 1×; FR3: 1.5×). Equimolar concentrations of each library were pooled and sequenced with a calibration standard panel (Thermo Fisher Scientific) on an Ion Personal Genome Machine (PGM) system with Ion PGM Hi-Q View Chef kit and Ion PGM Hi-Q View Sequencing kit using Ion 318 Chips v2 BC (Thermo Fisher Scientific). Reproducibility of the high-throughput sequencing method using the PGM was tested by running gDNA samples in duplicate with a different barcode in the same run and with the same barcode on a separate run. As for cfDNA, instead, given the limited availability, libraries were sequenced in duplicate in two runs using the same barcode. Namalwa cell line was used as internal positive control in each run.

Bioinformatics Workflow

Using the Torrent Suite software version 5.0.2 with default parameters (Thermo Fisher Scientific), raw reads were trimmed for low-quality 3′ ends and barcodes demultiplexed, thus obtaining the raw sequencing data (FASTQ format) produced by the Ion Torrent PGM. Quality assessment and reads filtering were performed using open-source tools (FastX-toolkit, Cutadapt) in a custom bash (Unix Shell) script (Supplemental Script S1). Specifically, raw sequencing reads were analyzed and filtered for base quality (median Phred score, >30; minimum quality score, 20) and sequence length (FR1: >255 bp, FR3: >70 bp) to get rid of incomplete reads or unspecific amplification products. Sequences were retained only if both forward and reverse primers could be identified, and then primer sequences were trimmed from the reads.

Filtered reads were converted to FASTA format (Supplemental Script S1) and aligned against IMGT germline database (IMGT, Montpellier, France) using the IMGT/HighV-QUEST online tool (*http://imgt.org/HighV-QUEST/login.action*, freely available on registration, last accessed October 11, 2017) with default parameters.[14]

Aligned reads were parsed using the open source VDJtools software[15] version 1.1.1. In detail, mapped reads [Q8] were aggregated into clonotypes based on their complementary-determining region 3 (CDR3) nucleotide sequence and the same IGH variable, diversity, and joining [*IGH V(D)J*] gene segment use. Frequency-based correction (Supplemental Script S1) was performed with default parameters (maximum number of mismatches allowed between clonotypes being compared, 2; child-to-parent clonotype size ratio threshold under which child clonotype is considered erroneous, 0.05) to eliminate erroneous clonotypes. Corrected samples were stored as a clonotype abundance tables for the subsequent analyses.

Statistical Analysis

Repertoire overlap was analyzed with VDJtools. Fisher's exact test, *U*-test, Kaplan-Meier analysis, log-rank test, and one-way analysis of variance with posthoc Tukey honestly significant difference test were performed in R version 3.3.2 (R Development Core Team, *http://www.r-project.org*, last accessed January 31, 2018)[16] and GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA). Correlation analyses were conducted using the Pearson's correlation coefficient (r). Venny (*http://bioinfogp.cnb.csic.es/tools/venny*, last accessed October 24, 2017) was used to generate Venn diagrams. [Q9] $P < 0.05$ was considered statistically significant.

Results

Patient Characteristics

Clinical features of the 25 patients at their first relapse are summarized in Table 3. Previous treatments included [T3] mainly bortezomib [nine patients (36%)] and high-dose melphalan [16 patients (64%)]. Median follow-up for the cohort is 17 months (range, 2 to 82 months). At accrual time point, patients had variable levels of $CD138^+$ PCs in the BM (median, 8.8%; range, 0.05% to 80%) but no circulating $CD138^+$ PCs in the PB.

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Table 3 Baseline Characteristics of the Study Cohort

Characteristic	Finding (n = 25)
Age in years, median (range)	65 (41–75)
Sex, n (%)	
Male	16 (64)
Female	9 (36)
MM subtype, n (%)	
IgG	13 (52)
IgA	8 (32)
Light chain	4 (16)
Laboratory findings	
BM infiltration on trephine biopsy, median (range), %	40 (10–95)
BM infiltration by flow cytometry, median (range), %	8.8 (0.05–80)
BM infiltration by NGS, median (range), %	50.1 (1.4–86.7)
Serum M protein, median (range), g/dL	2.60 (0.09–9.79)
Serum FLC ratio, median (range)	33 (0.01–344)
Serum LDH, median (range), U/L	294 (228–433)
Cytogenetic abnormalities, n (%)	
t4; 14	1 (4)
t14; 16	2 (8)
17p-	2 (8)
1q+	4 (16)
>1	6 (24)
None	5 (20)
NA	5 (20)
MM staging, n (%)	
ISS I	10 (40)
ISS II	6 (24)
ISS III	9 (36)
Time in months from diagnosis, median (range)	51 (22–117)
Follow-up, median (range), months	17 (2–82)

BM, bone marrow; FLC, free light chain; ISS, International Staging System; LDH, lactate dehydrogenase; MM, multiple myeloma; NA, not applicable; NGS, next-generation sequencing.

Identification and Characterization of Rearranged Clonal IGH by Ion PGM Sequencing in MM PCs

To identify the dominant tumor clonotype, rearranged *IGH V(D)J* loci in genomic tumor cell DNA (tcDNA) derived from isolated BM CD138$^+$ PCs were amplified by multiplex PCR using validated degenerate PCR primers complementary to *IGH* FR1 and the consensus *IGHJ* primer.[12,13,17] These oligonucleotides do not cause significant PCR amplification bias in multiplex PCR settings, allowing the correct representation of all the VH gene families.[18,19] The amplification generated products of expected sizes that were sequenced on Ion PGM. Sequencing yielded a means ± SD of 101,455 (79,377) reads that were subjected to quality assessment and filtering (Supplemental Figure S1). A means ± SD of 47,755 ± 32,787 sequences (47% ± 32% of total reads) passed quality filters and were used for subsequent downstream analyses. To assess clonality in the samples, the percentage of reads having the same *V(D)J* use and a unique CDR3 nucleotide sequence was determined. The most represented clone had a median frequency of 50.1% (range, 1.4% to 86.7%). Setting a frequency threshold >5% to define a clone as a tumor clonotype,[4,20] the sequencing method successfully identified a myeloma clonotype in 22 of 25 patients with MM (88%), indicating a specificity similar to the one reported in previously described NGS assays for clonal *IGH* identification (91%).[4,21]

In addition, consistent with previously published data,[22] in all 22 patients the *IGH V(D)J* gene segment use of the tumor clonotype was nonrandom: *IGHV3*, *IGHV4*, *IGHV1*, and *IGHV2* were the VH families expressed at the highest frequencies (Supplemental Figure S2A), and a preferential expression of VH3-23 [4 of 22 patients (18%)], VH1-69, VH2-5, VH3-30, VH3-48, and VH3-7 (each at 9%) was observed (Supplemental Figure S2B). *IGHD6-19* and *IGHD6-13* predominated (Supplemental Figure S2C), consistent with what seen in other B-cell malignant tumors.[23] Among the *IGHJ* subgroups (Supplemental Figure S2D), *IGHJ4* was statistically overrepresented (45% observed versus 16.7% expected, assuming a random choice for JH; $P < 0.001$, Fisher's exact test), whereas *IGHJ2* and *IGHJ3* were significantly underrepresented (5%; $P < 0.001$).

Validation Studies

Validation studies included i) comparison with traditional sequencing, ii) amplification with a different set of primers, and iii) evaluation of sensitivity. To validate the consistency of the NGS data, traditional Sanger sequencing of PCR products obtained using the same consensus sense primers complementary to FR1 and an antisense primer derived from the JH region at the 3′ end was performed.[17] Sequences were visualized with Chromas Lite version 2.1.1 (Technelysium, South Brisbane, Australia), and a single consensus sequence was generated, which was analyzed using IMGT/V-QUEST online tool (*http://imgt.org/IMGT_vquest/share/textes*, last accessed November 3, 2017).[24] In all 22 patients, the *V(D)J* identity and the CDR3 sequence of the tumor clonotype identified by NGS corresponded to that inferred with traditional Sanger sequencing approach. Moreover, in the remaining three patients for whom the NGS approach identified *IGH* sequences at frequencies under the validated threshold,[20] Sanger sequencing highlighted a polyclonal *IGH* repertoire (Table 4). [T4]

Consistent results were obtained when amplicons were generated using the FR3 primer set,[13] sequenced on the Ion PGM following manufacturer's instruction and analyzed with our custom bioinformatics workflow using a tailored length filtering. The absence of a tumor clonotype was

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Table 4 Genomic Tumor Cell DNA (Bone Marrow CD138$^+$ Plasma Cells) Sequencing Statistics

Sample	Reads, *n*	Reads after filtering, *n*	Most represented *IGH* clone, %	CDR3 sequence	Sanger sequencing
MM_1	106,941	61,651	57.5	5′-TGTGCACACAGCGAGGTCGGTGGTTATTACTAC-CCTAAACCCCTTGACTTCTGG-3′	Confirmed clonal
MM_2	122,011	68,574	80.1	5′-TGTGTACGTAGGGGGCAGCCTTCAATAGTCGCCG-ATGGAGGCATCTACTTTGACCACTGG-3′	Confirmed clonal
MM_3	112,528	63,862	76.4	5′-TGTGCGAGAGTTTCGGGGGGTATAGCAGTGGCTG-CGATCGACCGACCCATATTATACTACTACGGTATG-GACGTCTGG-3′	Confirmed clonal
MM_4	113,414	64,861	1.4	No *IGH* tumor clonotype (<5%)	Confirmed polyclonal
MM_5	50,607	16,530	69.4	5′-TGTGCGAGAGGTGTCGTGAGTGGTAGCTGCTACT-TTCGAAATTGGTTCGACCCCTGG-3′	Confirmed clonal
MM_6	81,978	35,170	8	5′-TGTGTGAAGTCTCTCGGGGCTTCTTGGCACTACG-CTATGGACGTCTGG-3′	Confirmed clonal
MM_7	68,208	22,751	63.1	5′-TGTGCGAGAGATAAGATAGGAGCAGCAGCTGGTA-GTTGGTTCGACCCCTGG-3′	Confirmed clonal
MM_8	44,827	14,832	42.7	5′-TGTGCGAGAGATTTAGGGGACGCTATGGACGTCTGG-3′	Confirmed clonal
MM_9	22,078	14,968	47.6	5′-TGTGCGAGAGTCACACGAGGGTACTACTTTGACT-ACTGG-3′	Confirmed clonal
MM_10	166,011	48,873	26.6	5′-TGTGCGAGAGGCCTGGGTAACGGAGCAGCTGCCC-AGGAAACTCACCTCGTCTGGTTCGACCCCTGG-3′	Confirmed clonal
MM_11	161,260	70,487	81.4	5′-TGTGCGAAAGATCATAACGAGTGGGAGCTGAGAC-GATCCGGGGACTGG-3′	Confirmed clonal
MM_12	101,841	32,718	85.8	5′-TGTGCGATGGACCGAACTGCAACGGAGGGGCTCG-ACCCCTGG-3′	Confirmed clonal
MM_13	113,960	54,211	86.7	5′-TGTGCGAGACATTCTGGAACAGTGGCTGGTATCT-TTGACAACTGG-3′	Confirmed clonal
MM_14	89,243	40,015	55.3	5′-TGTACCACCTGGGCGACCGCAGTGTCTGGGCGACTG-3′	Confirmed clonal
MM_15	190,832	76,913	50.1	5′-TGTGCAAAAGACGGGGGGTATAGCAGTGGCTGGG-CCCAAGAGGGCTTGGACTACTGG-3′	Confirmed clonal
MM_16	48,521	20,387	2.7	No *IGH* tumor clonotype (<5%)	Confirmed polyclonal
MM_17	37,374	17,433	21.0	5′-TGTGCGAAACTGCAGGGGCATTACTATGATAGTA-GTGGTTATCCGAACTGG-3′	Confirmed clonal
MM_18	49,426	21,320	51.4	5′-TGTGCGAGTTCTATTGTAGTAGTACCACCGGGC-GTCTGG-3′	Confirmed clonal
MM_19	36,952	14,994	9.1	5′-TGTGCGAGGGATCGTGATGGCAGTGGCTGGTCCT-TTGATTACTGG-3′	Confirmed clonal
MM_20	117,138	67,269	13.0	5′-TGTGCGAGAGCGGCGTCGGCAGCAGCTGGTACG-GAGGGGTTGTTCGACCCCTGG-3′	Confirmed clonal
MM_21	174,692	80,542	62.3	5′-TGTGCGACTATAGCAGTGGCTGGTCCCTACTGGT-ACTTCGATCTCTGG-3′	Confirmed clonal
MM_22	133,781	80,193	9.8	5′-TGTGCGGCGGGCCTGGAACCCCGCTACTGG-3′	Confirmed clonal
MM_23	159,121	71,957	74.9	5′-TGTGCGACCGCCCCAATACCGGACTCGGGAAGAC-TTGACAACTGG-3′	Confirmed clonal
MM_24	66,296	23,777	4.1	No *IGH* tumor clonotype (<5%)	Confirmed polyclonal
MM_25	167,322	69,586	17.8	5′-TGTGCGAGAACGACCAGCAGCTCCTTTGGCGACTGG-3′	Confirmed clonal

CDR3, complementary-determing region 3; MM, multiple myeloma.

confirmed in the same three patients excluding, overall, a primer amplification bias under the conditions here reported.

To assess the sensitivity of the sequencing approach, samples generated by serial 10-fold dilutions of DNA extracted from the two cell lines Namalwa and JVM-2 bearing a known clonal IGH rearrangement, into healthy controls' polyclonal DNA were studied. The clonal sequences at dilutions as low as 10^{-5}, corresponding to a sensitivity of at least 0.001% [two Namalwa-specific sequence reads for a total of 60,624 filtered reads (0.003299%) and three JVM-2–specific sequencing reads for a total of 71,387 filtered reads (0.0042%)] were identified and quantified (Supplemental Table S1).

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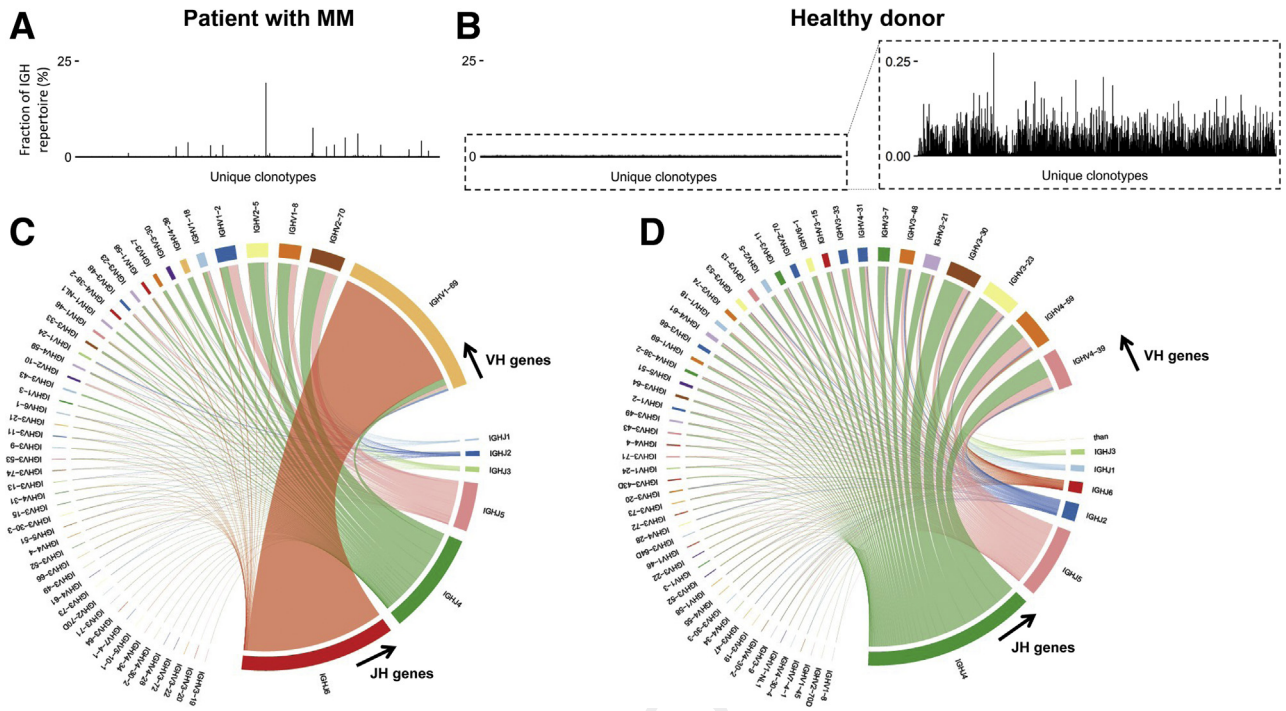

Figure 1 Sequencing of the *IGH* rearrangements in cell-free DNA of a representative patient with multiple myeloma (MM) and a healthy donor. Histogram of plasma clonotype frequencies detected in a patient with MM (**A**) and a healthy donor (**B**) with a zoom-in on lower frequencies (dotted box); unique clonotype frequencies are plotted on the *y* axis for both the patient with MM and the healthy donor (as a percentage of the total reads analyzed). V-J junction circos plot for a patient with MM (**C**) and a healthy donor (**D**). Arcs correspond to different VH and JH segments, scaled to their frequency in sample. Ribbons represent VH-JH pairings, and their broadness corresponds to the number of *IGH* sequences that exhibit this gene combination.

cfDNA Extraction and Amplification: Experimental Setup

cfDNA was extracted with QIAamp Circulating Nucleic Acid kit (Qiagen) starting from different volumes of plasma (0.5, 1, 2, 3 mL). A linear correlation between plasma volume and cfDNA concentration was not observed, probably because of a saturation effect of the silica membrane. The median cfDNA quantity extracted from healthy donors' PB samples was indeed 4.6 ng from 0.5 mL of plasma, 7.5 ng from 1 mL, 13.0 ng from 1.5 mL, and 20.4 ng from 3 mL.

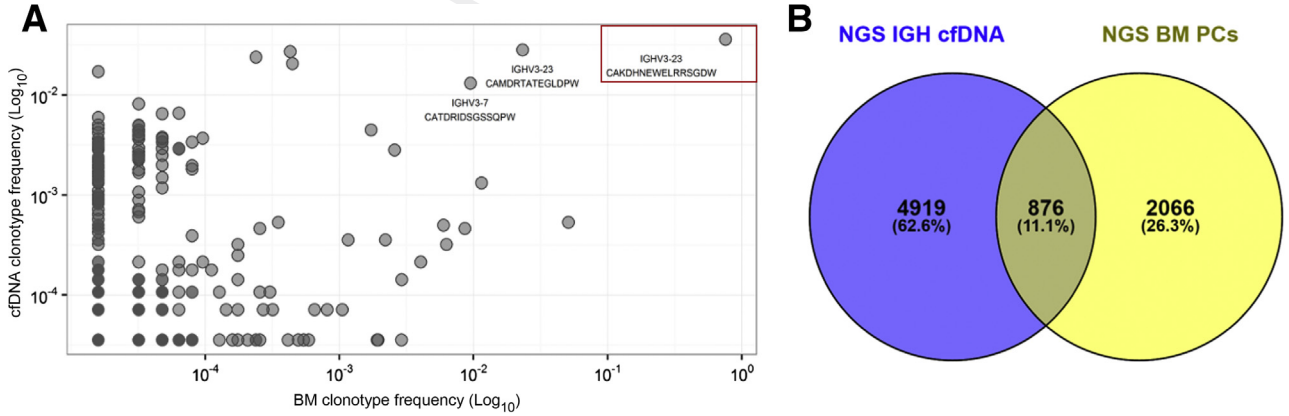

Figure 2 Overlap analysis of the clonotypes detected by next-generation sequencing (NGS) in tumor cell DNA and cell-free DNA (cfDNA). **A:** Scatterplot of the clonotype frequencies (log_{10} scale) observed in a representative patient with multiple myeloma (MM) [bone marrow (BM) clonotypes on the *x* axis and cfDNA on the *y* axis]. Most clonotypes are detected at low frequencies, whereas the tumor-associated clonotype (red box) can be identified as the one at the highest frequency in both compartments (plasma and BM tumor sample). Next to the top 3 most frequent shared clonotypes are the VH gene (*IGHV*x-x) and the related complementary-determining region 3 amino acid sequence. **B:** Venn diagram showing the number (x% calculated on the total clonotypes) of unique and shared clonotypes between the plasma (blue circle) and tumor (yellow circle) compartments. The Venn diagram was generated using Venny. PCs, plasma cells.

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To verify whether the plasma samples stored within the clinical trial for patients with MM at first relapse were suitable for cfDNA extraction and amplification, the quality and quantity of cfDNA extracted from plasma into K2-EDTA tubes (BD Vacutainer) or in Cell-Free DNA BCT tubes (Streck, Inc., Omaha, NE) that contained a cell-stabilizing agent that prevents cell lysis were compared.

PB from 10 healthy donors was collected in both tubes, processed as described above within 3, 6, 12, 24, 48, and 72 hours and 1 week, and stored at −80°C for at least 1 week. Of note, when plasma samples are processed within 3 hours (compatible with what was done for the samples stored within the MM clinical trial) both cfDNA BCT tubes and K2-EDTA tubes had similar performances, with only slightly higher concentrations of cfDNA from plasma samples collected in cfDNA BCT tubes (mean, 8.2 versus 7.5 ng with K2-EDTA tubes). It could clearly be confirmed that cfDNA stability is guaranteed only with the cfDNA BCT tube when samples are processed after 3 hours (median, 7.1 ng after 24 hours, 6.8 ng after 48 hours, 4.8 ng after 72 hours, 3.6 ng after 1 week), making them the best option for multicenter clinical studies in which samples are often centralized.

It was also studied whether plasma storage conditions might affect cfDNA recovery. No difference was observed when processing fresh or frozen samples (median cfDNA quantity from 1 mL of plasma: 7.8 ng after immediate processing, 7.5 ng after short-term storage of <6 months, 7.4 ng after >6 months).

Therefore, considering the minimum quantity of PCR products required for library preparation [10 to 100 ng to obtain 40 to 60 pM of final library (Ion Plus Fragment library kit and Ion PGM Hi-Q View Chef Kit protocols)], in the present study, the available 1 to 3 mL of plasma collected in K2-EDTA tubes was considered sufficient for extraction and downstream amplification.

Sequencing of the *IGH* Gene Rearrangements in Plasma Samples: Performance and Concordance of tcDNA and cfDNA Results

Matched genomic tcDNA and plasma cfDNA samples obtained at study entry were available for all 22 patients with a defined myeloma clonotype. *IGH* cfDNA sequencing yielded a means ± SD of 21,0056 (161,520) reads, with a means ± SD of 77,188 (55,058) sequences passing quality filters (37% of total reads versus 47% on tcDNA) and available for subsequent analyses. In MM plasma samples, the percentage of predominant clones ranged from 1.2% to 18% (Figure 1A). In comparison, in control plasma samples from healthy donors ($n = 10$), several different clones were present at low percentages, and no clonally related sequences were observed (mean, 0.006%; range, 0.002% to 1%) (Figure 1B).

To compare the recombinant repertoire in plasma of patients with MM and donors, Circos plots (VDJtools) showing the relative prominence of each VH-JH recombination within the repertoire of each subject were generated (Figure 1, C and D). These plots revealed that V genes are paired with many other J genes in control samples, whereas MM samples show few but broad connections, indicating the predominance of one VH-JH combination (one clonotype) in patients among other clones present at lower frequencies.

The tumor clonotype identified in cellular DNA in plasma samples was then searched. In all 22 cases (100%), the rearranged tumor-associated *IGH* sequence was present in plasma (Figure 2A) at percentages ranging from 1.2% to 18% of total filtered circulating *IGH* DNA reads (median frequency, 4.7%). As reported above for tcDNA, results were confirmed using an FR3 primer set. In addition, clonotypes (means ± SD, 10% ± 7%) were detected at high frequencies that are shared between paired plasma and tumor samples, whereas other clonotypes were found to be exclusive of each compartment (Figure 2B). Therefore, plasma cfDNA can accurately mirror the profiles of the most abundant clonotypes; furthermore, plasma cfDNA may reflect myeloma burden in BM but also in extramedullary sites.

Prognostic Implication of Clonotypic IGH cfDNA Levels in Patients with MM

Because there are different ways to determine the percentage of PCs in the BM (counting cells on trephine BMBs or on aspirate smears) and these methods often give discrepant results,[25] it was speculated that the analysis of clonal *IGH* in cfDNA may be used to help estimating tumor levels, overcoming BM sampling and analysis limitations. PCs on BMB were present in all patients before therapy at a median frequency of 40% of BM leukocytes (range, 10% to 95%): these frequencies did not correlate with clonotypic cfDNA frequencies ($r = 0.098$, $P = 0.6724$). Similarly, no significant correlation was found between M-protein levels evaluated by serum electrophoresis (SPEP) and clonotypic cfDNA frequencies ($r = -0.018$, $P = 0.9504$). *IGH* cfDNA levels and serum-free light chain (sFLC) ratios show a moderate positive but not significant correlation ($r = 0.4567$, $P = 0.2552$) (Supplemental Figure S3A). Likewise, no correlation was found between BMB PCs and SPEP levels ($r = 0.289$, $P = 0.295$), BMB PCs levels and sFLC ratios ($r = 0.148$, $P = 0.7793$), and SPEP levels and sFLC ratios ($r = -0.032$, $P = 0.8465$), supporting the idea that MM is a complex disease and none of these laboratory parameters alone accurately describes tumor levels in a single patient. Of interest, disease levels at baseline evaluated by NGS on plasma samples compare favorably to BM PCs infiltration by MFC ($r = 0.713$, $P = 0.0002$) and to NGS performed using tcDNA ($r = 0.45$, $P = 0.0354$) (Supplemental Figure S3B).

It was next determined whether cfDNA analysis might facilitate the early identification of clinically relevant risk

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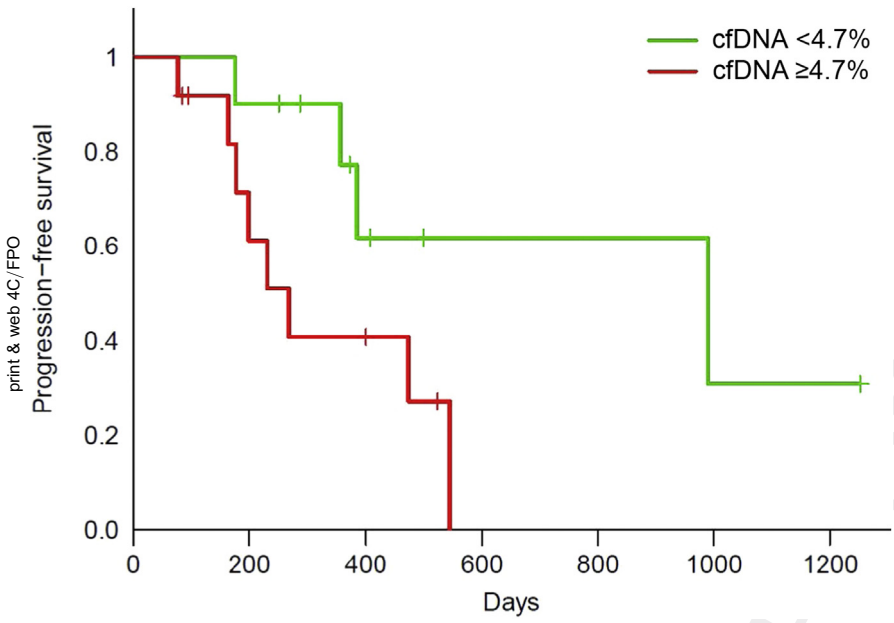

Figure 3 Correlation of *IGH* cell-free DNA (cfDNA) levels at baseline with progression-free survival. Data are expressed as estimated median values. $n = 10$ (patients with frequencies <4.7%); $n = 12$ (patients with frequencies ≥4.7%). Hazard ratio = 3.507 by Cox proportional hazards regression model, $P = 0.04988$ by log-rank test.

groups among the cohort of patients with MM enrolled in the study. Levels of the tumor-associated clonotype in cfDNA distinguish between groups of patients with different prognoses, with the median percentage of the tumor-associated clonotype in cfDNA as the cutoff value (4.7% of total reads). Patients with levels ≥4.7% ($n = 12$) of the tumor-associated *IGH* sequence before therapy had significantly inferior PFS [estimated median values, 268 versus 990 days; hazard ratio (HR) = 3.507, $P = 0.04988$, log-rank test] (Figure 3) than patients with levels <4.7% ($n = 10$). When considering baseline features, such as BMB PCs, SPEP levels, and sFLC ratios, no significant association was found with survival. Similarly, none of the variables known to affect clinical outcome in patients with MM, such as age, International Staging System status, or the Revised International Staging System status,[26] which includes serum lactate dehydrogenase and high-risk cytogenetic abnormalities (t4; 14, t14; 16, 17p- and 1q+),[27] significantly correlated with PFS or overall survival (Supplemental Table S2). On the other hand, as expected given the high concordance of cfDNA NGS results and MFC (Supplemental Figure S3B), higher numbers of PCs enumerated by MFC (median percentage as cutoff value, 8.8%) are associated with poorer PFS (estimated median values, 268 versus 545 days; HR = 3.745, $P = 0.04655$, log-rank test) (Supplemental Figure S4A), whereas no significant association was found between NGS results on tcDNA and survival (Supplemental Figure S4B).

Disease Monitoring by Clonotypic *IGH* cfDNA Sequencing

It was next hypothesized that longitudinal analysis of clonal *IGH* cfDNA may help understand tumor dynamics over time. Seventy plasma samples of the selected 22 patients with MM were analyzed. *IGH* cfDNA sequencing revealed that the tumor-associated clonotype could be tracked over time in plasma samples. The levels of the clonotypic *IGH* sequences in plasma reflected tumor dynamics evaluated using the International Myeloma Working Group criteria (BM PCs, SPEP levels, and sFLC ratios) (Figure 4). In addition, the phenotypic aberrancies detected in PCs at study entry for each patient (based on CD38/CD56/CD19/CD45, CD38/CD27/CD45/CD28, CD20/CD81/CD38/CD117) were used as patient-specific probes for residual disease assessment after therapy on consecutive samples. Cell-free DNA levels reflected the number of PCs enumerated by MFC immunophenotyping (Figure 4).

In the context of complete response time points ($n = 22$), MFC was used for MRD monitoring. At least 1×10^6 cellular events were collected and residual cells detected at a median frequency of 0.00065 (range, 0.00000668 to 0.027) tumor cells of the total events analyzed (range, 10,14,888 to 19,46,108). MRD negativity was defined when <50 aberrant-phenotype PCs were detected[28]; thus, six patients achieved MRD negativity during the timeframe of our analysis. When analyzing cfDNA *IGH* frequencies in these 22 patients at complete response time points (median, 0.0000395; range, 0.00000756 to 0.037), a high level of correlation was found between cfDNA NGS and MFC data ($r = 0.5831$, $P = 0.0044$, Pearson's correlation test) (Figure 5A). Accordingly, PFS was significantly prolonged ($P < 0.001$) for the six patients who achieved MRD negativity, displaying frequencies of the clonotypic cfDNA rearrangement $<10^{-5}$ (for clonotypic cfDNA frequencies $<10^{-5}$: $n = 6$, means ± SD PFS, 714 ± 327 days; for clonotypic cfDNA frequencies $\geq 10^{-5}$-$\leq 10^{-4}$: $n = 9$, 325 ± 75 days; for clonotypic cfDNA frequencies $>10^{-4}$,

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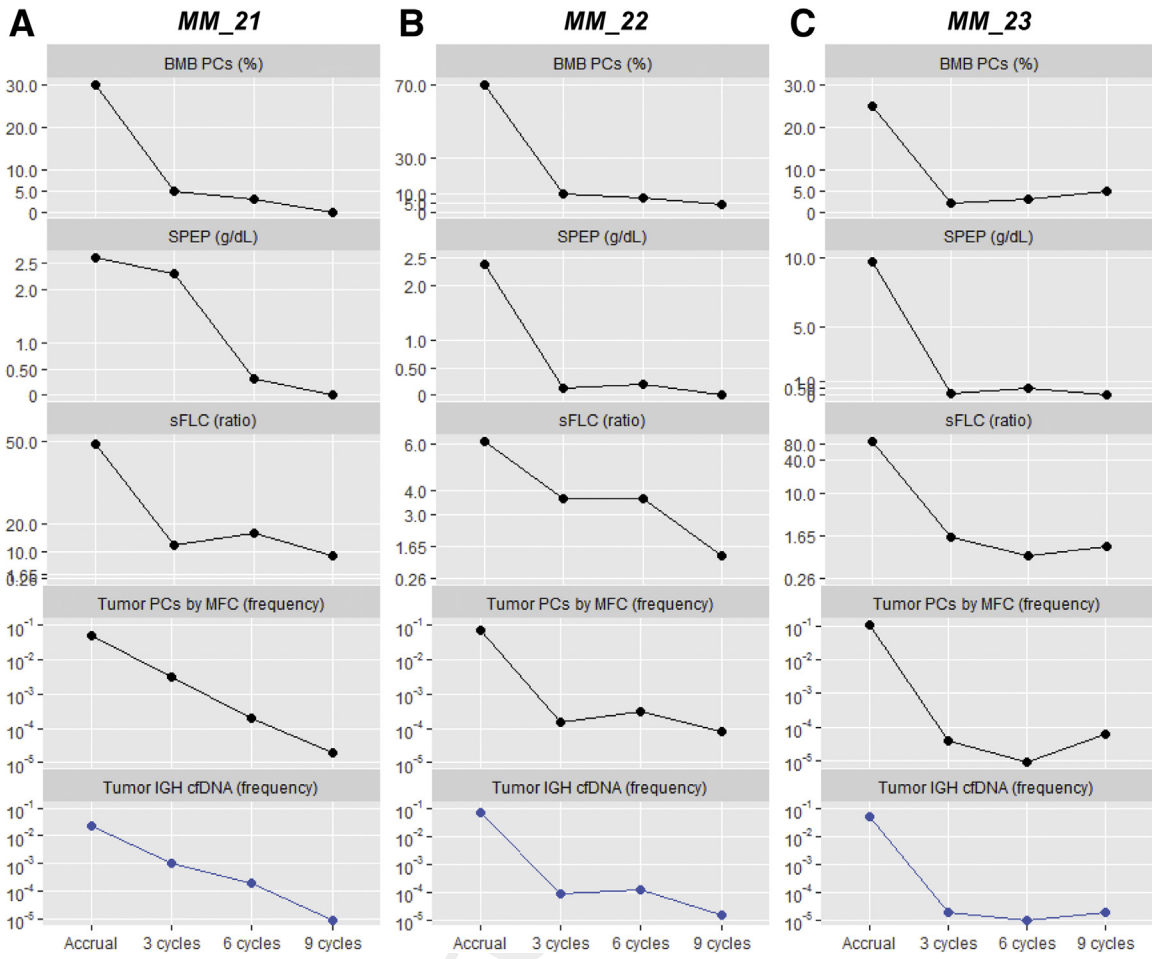

Figure 4 Quantification of *IGH* cell-free DNA (cfDNA) levels in relation to multiple myeloma (MM) clinical indexes. Following a top-down disposition, the *y* axes of the line plots are respectively related to percentage of bone marrow plasma cells on trephine biopsies (BMB PCs) (linear scale), M-protein concentrations evaluated by serum electrophoresis (SPEP) (linear scale), serum-free light chain (sFLC ratio) (linear scale for **A** and **B**, $\log_2$ scale for **C**; reference range, 0.26 to 1.65); bone marrow PCs enumerated by multiparameter flow cytometry (MFC) (tumor PCs by MFC, frequency, $\log_{10}$ scale); and plasma *IGH* cfDNA levels (tumor IGH cfDNA, frequency, log10 scale). Time points are labeled on the *x* axis. **A:** Case MM_21: plasma *IGH* cfDNA levels decrease during treatment, paralleling the decrease of BMB PCs and SPEP levels, which become undetectable after nine cycles of treatment. This finding is consistent with the MFC trend and contrasts with the sFLC ratio, which is stable during treatment. **B:** Case MM_22: Plasma *IGH* cfDNA levels decrease rapidly after the initial cycles of therapy, stabilize from cycles 3 to 6, and then decrease again after the sixth cycle. These dynamic changes are reflected by SPEP, sFLC, and MFC. BMB PCs levels instead steadily decrease during treatment. **C:** Case MM_23: Levels of *IGH* cfDNA decrease after three cycles of therapy and become stable and present a slight increase after nine cycles, mirroring specifically the trend of sFLC and MFC and also of BMB PCs. In this case, the SPEP levels contrast as they become undetectable at the end of therapy.

$n = 7$, 143 ± 59 days) (Figure 5B). In these cases, to increase theoretical sensitivity, NGS of cfDNA samples was performed with a lower level of plexing (maximum of eight samples on an Ion 318 Chip v2 BC) to obtain at least 5×10^5 reads per sample.

Discussion

cfDNA is emerging as a noninvasive disease biomarker in solid tumors and lymphomas.[29,30] However, its clinical utility is still under investigation, and few data are available in the context of MM. We provide the first report describing the clinical significance of detecting and monitoring cfDNA in patients with relapsed or refractory MM using an *IGH* deep-sequencing method. We extend previous findings[8] by demonstrating that this approach allows i) the identification of the tumor-associate *IGH* clonotype in BM tumor PCs and consequently in plasma samples, ii) the assessment of tumor levels at study entry and during therapy from plasma samples, and iii) the analysis of residual disease using cfDNA with results comparable to standard MFC.

The clonally rearranged *IGH* genes of mature B-cell malignant tumors offer specific somatic DNA sequences that can serve as molecular markers for tumor cells. Standardized primers developed by us[12,31] and by the BIOMED-2 concerted action[13] were used to amplify all *IGH* sequences in patients' tumor samples. Libraries were sequenced on an Ion PGM bench-top system and data analyzed with a set of web-based specific bioinformatic

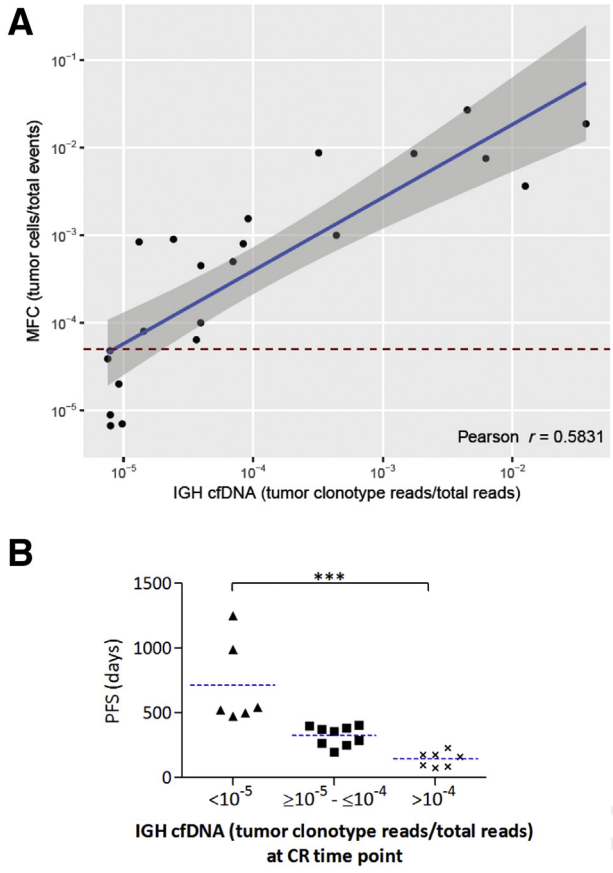

Figure 5 Comparison between *IGH* cell-free DNA (cfDNA) sequencing and multiparameter flow cytometry (MFC) data. **A:** Scatterplot showing correlation between *IGH* cfDNA sequencing (IGH cfDNA, $\log_{10}$ on the *x* axis) and eight-color flow cytometry (MFC, $\log_{10}$ on the *y* axis) in assessing residual disease in the selected 22 patients (black dots). The frequencies of *IGH* cfDNA (*x* axis) are expressed as the number of patient-specific tumor clonotype sequencing reads over the total sequencing filtered reads. The frequencies of bone marrow (BM) multiple myeloma (MM) plasma cells (PCs) detected by MFC are expressed as the number of cells bearing the aberrant immune-phenotype (detected at study entry) over the total collected events. **Blue line** represents the best-fit line obtained using the linear method; shaded area indicates the 95% CI of the best-fit line. Pearson's correlation test was used to quantify the strength of the association ($P = 0.0044$). **Red dashed line** set the sensitivity threshold for MFC (frequency $<5 \times 10^{-5}$). **B:** Group analysis shows that levels of disease detected by *IGH* cfDNA sequencing are significantly correlated with progression-free survival (PFS). Dashed blue lines represent the mean value of each category. The strength of the correlation (*P*) was calculated using one-way analysis of variance with the posthoc Tukey honestly significant difference test. $n = 6$ (patients with frequencies $<10^{-5}$); $n = 9$ (patients with frequencies $\geq 10^{-5}$-$\leq 10^{-4}$); $n = 7$ (patients with frequencies $>10^{-4}$). ***$P < 0.001$. CR, complete response.

tools openly available. *IGH V(D)J* gene segment use in our cohort of patients with MM resembles that seen by other studies[22,23] and supports the idea that the approach used can be applied for identification, characterization, and quantification of myeloma-associated *IGH* clonotype in tumor samples.

Clonotypic *IGH* sequences could be determined from baseline tumor cells in 88% of patients with MM at first relapse, and results were consistent with those obtained by PCR and Sanger sequencing, indicating a specificity similar to that reported using commercial sequencing approaches.[4] Consistently, in all patients with the characterized *IGH* gene rearrangement in tumor samples, the NGS approach was able to detect the same rearrangement in plasma samples. A predominant clonotype was detected among other clones likely representative of normal B-cells. In contrast, in healthy donors no clonally related sequences were observed, most likely reflecting the enormous diversity of *IGH V(D)J* combinations generated during B-cell development.

A significant advantage of cfDNA analysis in the care of patients with MM would be the ability to measure tumor burden by avoiding invasive BMBs. Quantification of BM PCs is crucial for diagnosis and prognostication, as reemphasized in the revised International Myeloma Working Group guidelines.[32] However, the degree of BM infiltration by conventional morphologic or immunohistochemical analysis may vary significantly not only among patients but also within the same patient. This has been attributed to the patchy pattern of PCs infiltration, a factor that could also explain the inconsistency of PC counts on BM aspirates as a prognostic factor.[33] In the present study, we demonstrate the clinical implication of the detection of cfDNA: patients with MM with a high cfDNA level of the tumor-associated *IGH* sequence before treatment have a shorter PFS than the others. This finding suggests that cfDNA levels might indeed reflect tumor burden and represent potentially relevant prognostic biomarkers at study entry. This strategy could complement current methods used to determine tumor levels that are limited by invasive biopsies and suboptimal results attributable to sampling variability.

Molecular disease monitoring using cfDNA has a clear clinical relevance even during therapy and might also complement the longitudinal assessment of BM PCs counts, serum monoclonal proteins, or sFLC concentration for response evaluation. In fact, our findings reveal the ability of cfDNA analysis to track tumor kinetics as already shown in lymphoma[5] and suggest that cfDNA may be further useful in cases where disease is predominantly confined to the tissues (eg, solitary plasmacytomas, extramedullary and nonsecretory myeloma).

The fact that cfDNA levels well mirror results obtained with MFC prompted us to evaluate whether such analysis may be relevant even in the MRD context. The current gold standard for MRD monitoring in MM involves immunophenotyping using flow cytometry on BM samples to detect the residual MM cells bearing an aberrant phenotype.[34,35] The use of a PB-based assay for MRD analysis has numerous advantages over any method relying on invasive BMBs.[36] In our study, results obtained analyzing MRD by MFC on BM samples using consensus antibody panels[37] showed complete concordance with cfDNA analysis in all cases. Of note, patients defined as MRD negative by MFC ($<5 \times 10^{-5}$ BM PCs) were also characterized by very low frequencies of the tumor-associated clonotype in plasma

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samples ($<10^{-5}$) and had a significantly longer PFS than patients with $>10^{-5}$ BM PCs.

Several studies have shown that NGS of the *IGH* represents an effective and highly promising tool for MRD detection in BM samples of patients with lymphoma and MM with sensitivity that may approach $\geq 10^{-6}$.[4,37] Sensitivity is a critical aspect in MRD detection and is limited by the number of input cells or DNA. However, the sensitivity that can be obtained using cfDNA is still unknown because the origin of cfDNA has yet to be fully understood. In this study, our sequencing approach on cfDNA allowed us to analyze at least 5×10^5 reads among which the clonal rearrangement was searched for. Increasing the amount of cfDNA will in the future serve to further improve the theoretical sensitivity. Another reasonable option would be to focus on shorter *IGH* fragments using the FR3 primer set. In cfDNA, because the most adundant DNA fragments are 160 bp long (length of DNA on one nucleosome), one could imagine that shorter amplificates (100 to 170 bp) would be more suitable and that such an approach would reach higher sensitivity than restricting the analysis to less abundant longer DNA molecules. On the other hand, as comparable results were obtained using FR3 primers in cfDNA of patients and healthy donors, this study was conducted analyzing FR1 amplified fragments that would potentially provide additional sequence information relevant to monitor clonal dynamics. A formal comparison between sensitivity of FR1- and FR3-based strategies on cfDNA is still lacking. In addition, it still remains to be determined whether enhanced sensitivity will provide supplementary clinical information in the context of current therapy protocols for MM.[38]

In summary, results of this pilot study support the clinical applicability and utility of quantifying tumor levels by our deep sequencing of *IGH* gene rearrangements present in plasma of patients with MM. This method can be implemented in any laboratory with NGS capability, can be applied to most patients with MM with a short turnaround time, and can be exploitable for the study of MRD.

Acknowledgments

We thank Anisa Bermema, Vittorio Montefusco, and Niccolò Bolli for their assistance in data acquisition and many useful discussions on this research.

C.C. and P. C. conceived and designed the study; G.B., S.G., and A.V. performed experiments and collected data; G.B., S.G., C.C., and P.C. analyzed and interpreted data; GB, S.G., C.C., and P.C. wrote and edited the manuscript.

Supplemental Data

Supplemental material for this article can be found at *https://doi.org/10.1016/j.jmoldx.2018.07.006*.

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