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RNA based deep sequencing of the B-cell receptor for detection and monitoring of B-cell neoplasms

Dissertation

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Next-generation sequencing of peripheral B-lineage cells pinpoints the circulating clonotypic cell pool in multiple myeloma

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LYMPHOID NEOPLASIA

Next-generation sequencing of peripheral B-lineage cells pinpoints the circulating clonotypic cell pool in multiple myeloma

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Key Points

- Clonotypic B cells, long suspected to represent circulating stem-like cells, are consistently absent in the blood of myeloma patients.
- Malignant plasma cells frequently circulate in the peripheral blood, show evidence for clonal evolution, and may spread the disease.

The identity of the proliferative compartment of myeloma progenitor cells remains a matter of debate. Polymerase chain reaction-based studies suggested pre-switch "clonotypic" B cells sharing the immunoglobulin (Ig) rearrangement of the malignant plasma cell (M-PC), to circulate in the blood and possess stem cell-like properties. Here, we disprove this hypothesis. We screened peripheral blood IgM, IgG, and IgA repertoires of myeloma patients for the clonotypic rearrangement by next-generation sequencing. None of 12 cases showed pre-switch clonotypic transcripts. In the post-switch IgG/IgA repertoires, however, the clonotypic rearrangement was detected at high frequency in 6 of 8 patients with active disease, whereas it was undetectable after treatment, correlating with flow cytometric presence or absence of circulating M-PCs. Minor subclones with alternative post-switch isotypes suggested ongoing switch events and clonal evolution at the M-PC level. Our findings consistently show an absence of pre-switch clonotypic B cells, while M-PCs circulate in the peripheral blood and may contribute to spreading of the disease. (*Blood*. 2014;123(23):3618-3621)

Introduction

Improvement of long-term outcomes in multiple myeloma critically relies on a better understanding of the tumor-initiating cell in this disease. As in other malignancies, the cancer stem cell concept incriminates drug-resistant myeloma stem cells with tumor-initiating, self-renewing properties to feed the malignant plasma cell (M-PC) compartment in disease relapse and progression.[1,2] However, it remains a matter of controversy if the clonogenic population resides within the pool of terminally differentiated post-switch M-PCs or within a less differentiated (surface) immunoglobulin (Ig)-positive pre-switch B-cell compartment. The latter hypothesis has been fueled by the description of so-called clonotypic pre-switch (IgM^+) B cells postulated to express the same patient-individual variable region Ig rearrangement as the M-PC.[3-7] This finding even provided the rationale for therapeutic targeting of this postulated $CD20^+$ population with the monoclonal antibody rituximab.[8,9] However, inconsistent results of xenotransplantation experiments,[10-15] data on intraclonal evolution at the PC level,[16-22] the analysis of class switch junctions,[23] and the lack of benefit from CD20-directed targeted therapy[8,9] challenged the concept of such feeder cells in myeloma. Moreover, a recent study entirely failed to detect clonotypic rearrangements in highly purified B-cell populations devoid of contaminating M-PCs,[24] and our own data also pointed in this direction, because we could not phenotypically detect clonotypic B cells in the majority of patients with patient-individual Ig ligands as tracers.[25]

Here we used next-generation sequencing to definitively confirm or disprove the existence of this highly controversial cell population.

Materials and methods

Patients and samples

Blood and bone marrow samples of 12 myeloma patients (Table 1) visiting the Freiburg and Hamburg University Medical Centers were obtained after written informed consent as approved by the institutional review boards. This study was conducted in accordance with the Declaration of Helsinki.

Detection and Sanger sequencing of clonotypic Ig rearrangements

M-PC heavy-chain Ig rearrangements were determined as previously described from bone marrow and are shown in supplemental Table 1.[25] For detection of circulating clonotypic cells, peripheral blood mononuclear cells were used. Polymerase chain reaction (PCR) approaches for the qualitative detection of clonotypic rearrangements of all isotypes, of IgM isotype only, and for isotype subclass determination by Sanger sequencing are shown in supplemental Figure 1A-B,D and supplemental Table 2, available on the *Blood* Web site.

NGS of Ig repertoires

Ig transcripts were amplified for next-generation sequencing (NGS) from peripheral blood as described in the supplemental Detailed Methods section.

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B.T. and M.K. contributed equally to this study.

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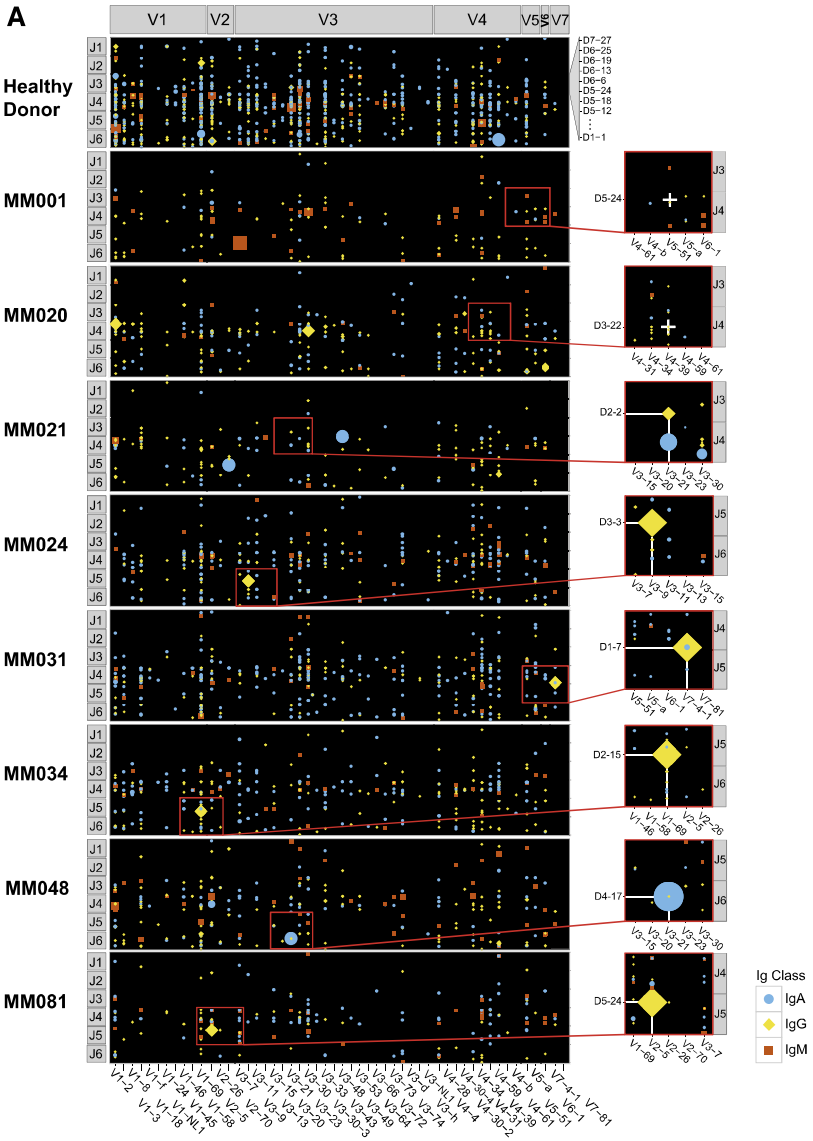

Figure 1. Targeted NGS of heavy-chain Ig repertoires from peripheral B-lineage cells of myeloma patients. (A) Clonotypic rearrangements in the peripheral blood of the myeloma patient cohort. Panel A shows patients MM001 and MM020, who were negative for the clonotypic rearrangement by a qualitative PCR approach. In patients MM021, MM024, MM031, MM034, MM048, and MM081, the clonotypic rearrangement was qualitatively detectable by PCR. For NGS, Ig transcripts were amplified with isotype-specific primers and multiplex sequenced on a MiSeq Illumina sequencer. Data were plotted using ggplot2 for R statistical software assigning a position to each potential V-D-J rearrangement. In patient MM020, a small clone was plotted at the expected site of the clonotypic rearrangement. However, this rearrangement was different from the clonotypic rearrangement (HCDR3 sequence and somatic hypermutation pattern). (B) Absence of clonotypic rearrangements in posttreatment follow-up samples of patients MM024 and MM031. Only IgG repertoires are shown. allo-HSCT, allogeneic hematopoietic stem cell transplant; auto-HSCT, melphalane high-dose chemotherapy followed by autologous stem cell transplant.

The amplification strategy and primer sequences are shown in supplemental Figure 1C and supplemental Table 2. Amplicons were multiplex-sequenced on a MiSeq Illumina sequencer. Data were plotted using ggplot2 for R statistical programming by display on a matrix providing a specific position for each potential V-D-J rearrangement.

Multiparametric flow cytometry

Peripheral blood cells were stained with fluorescently labeled antibodies (CD45-ECD, CD138-PC5, CD38-FITC, CD19-PC7) to discriminate B-lineage cell populations.

Results and discussion

PCR-based detection of clonotypic peripheral blood cells

Peripheral blood clonotypic rearrangements were qualitatively detectable with HCDR3-specific primers in 6 of 8 patients with active disease, suggesting the presence of circulating clonotypic B-lineage cells (data not shown). None of the cases in remission after treatment was PCR-positive. All HCDR3 PCR-positive cases were also positive in an established semi-nested PCR approach using IgM-specific primers. Surprisingly, however, Sanger sequencing of PCR products revealed post-switch IgG/IgA transcripts despite the use of IgM-specific primers, indicating that the PCR may yield false-positive results in the presence of a dominant clonotypic rearrangement of alternative isotype (data not shown).

Targeted NGS of peripheral blood IgM, IgG, and IgA repertoires

This observation prompted us to comprehensively screen Ig repertoires of circulating cells expressing pre- and post-switch isotypes for the presence of the clonotypic rearrangement by NGS. Sequencing data were displayed on a matrix providing a specific position for each potential V-D-J rearrangement (Figure 1). We found highly skewed peripheral Ig repertoires in myeloma patients compared with the polyclonal repertoires of a healthy donor (Figure 1A), reflecting the

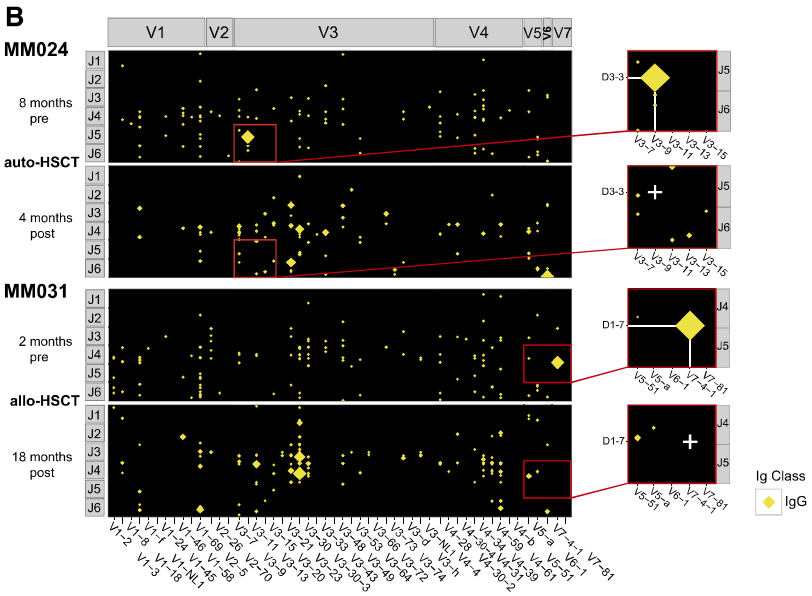

Figure 1. (Continued)

suppression of healthy polyclonal B-lineage cells by the M-PC clone in the bone marrow. None of the patients showed evidence for IgM^+ clonotypic rearrangements, suggesting absence of pre-switch clonotypic B cells in the peripheral blood. The clonotypic rearrangement could, however, be detected within the post-switch repertoire of the M-PC clone (IgG or IgA), indicating that M-PC circulate in the peripheral blood of these patients. In the majority of cases, the clonotypic rearrangement was the most abundant rearrangement detectable within the repertoire with a median of 48% of reads of the respective isotype (supplemental Table 3). PCR-negative control cases MM001 and MM020 (Figure 1A) as well as blood samples of patients MM024 and MM031 in complete remission after treatment (Figure 1B) were negative for the clonotypic rearrangement. In patients MM031 and MM048, we found, apart from the dominant clonotypic rearrangement of the M-PC, an identical rearrangement of alternative post-switch isotype (nonclinical rearrangement). Sequencing of the exact isotype subclass revealed a dominant IgG3 clone with a small IgA1 nonclinical isotype in patient MM031 and a dominant IgA1 clone with a small IgG1 nonclinical isotype in patient MM048. Considering the architecture of the Ig gene locus, this suggested that the MM031 IgG3 clone emerged from the preexisting IgA1 clone, while the small MM048 IgG1 clone had to be considered as precursor of the dominant IgA1 clone. This finding suggested ongoing sequential switch events in the progeny of post-switch myeloma cells and therefore clonal evolution at the PC level.

Flow cytometry-based detection of circulating PCs

To confirm our assumption that the clonotypic post-switch rearrangements may derive from circulating M-PCs, we analyzed peripheral blood cells by multiparametric flow cytometry. We found evidence of circulating M-PCs with the classical immunophenotype in all but one patient with a clonotypic rearrangement in ~1% of leukocytes (supplemental Figure 2A). In the control cases MM001 and MM020 and posttreatment samples of patients MM024 and MM031 (supplemental Figure 2B), no circulating M-PCs could be detected. Patient

Table 1. Clinical features and PCR detection of clonotypic rearrangement in the study cohort of 12 myeloma patients

Patient code	Treatment	Remission status at date(s) of sample acquisition	PCR-positivity for clonotypic rearrangement in peripheral blood
Patients in remission after treatment			
MM001	auto-HSCT, Rd	PR	—
MM020	auto-SCT	PR	—
MM023	Vel/Dex	PR	—
MM036	Vel/Dex	PR	—
Patients with active disease			
MM032	—	First diagnosis	—
MM050	Rd, Vel/Dex	Relapse	—
MM021	auto-HSCT, Rd, Vel/Dex	Relapse	+
MM024	auto-HSCT, Vel/Dex	Relapse (+ FU sample in CR)	+ (CR sample: −)
MM031	allo-HSCT	Relapse (+ FU samples in CR)	+ (CR sample: −)
MM034	—	First diagnosis	+
MM048	—	First diagnosis	+
MM081	—	First diagnosis	+

allo-HSCT, allogeneic hematopoietic stem cell transplant; auto-HSCT, melphalane high-dose chemotherapy followed by autologous stem cell transplant; CR, complete remission; FU, follow-up; MM, multiple myeloma; PR, partial remission; Rd, Revlimid (Lenalidomide) + Dexamethasone; Vel/Dex, Velcade (Bortezomib) + Dexamethasone.

MM021, who had a low burden of clonotypic rearrangements (only 2% of all IgG reads), was the only NGS-positive case with flow cytometric negativity for circulating M-PCs, most likely due to the different sensitivity levels of these assays.

Discussion of data in the context of previously published work

The hypothesis that pre-switch clonotypic B cells act as tumor-initiating and -propagating cells in myeloma has caused considerable debate for over decades. The results presented here should terminate this controversy. Using highly sensitive and highly specific state-of-the-art technology, we disprove the existence of such cells in the blood. This is in accordance with a recently growing body of evidence by other studies.[8-23]

Two methodological aspects most likely account for the discrepancy between prior reports[3-7] and our study. First, PCR with HCDR3- and isotype-specific primers may lack specificity in the presence of abundant clonotypic transcripts of alternative isotype. Without confirmation by direct sequencing of such transcripts, this finding may have falsely suggested the existence of IgM-positive clonotypic B cells, although the transcripts derived from IgG- or IgA-positive M-PCs. Second, many of the previous studies were primarily based on B-cell subpopulations isolated by fluorescence-activated cell sorting. Because M-PCs can circulate in the peripheral blood, such B-cell populations may easily have been contaminated by circulating M-PCs, which may then have provided the clonotypic sequences falsely attributed to B cells. Consequently, when highly purified B-cell populations devoid of contaminating M-PCs were investigated, no clonotypic transcripts were found.[24]

Taken together, our data show that pre-switch clonotypic B cells are inexistent in the blood of myeloma patients, while circulating M-PCs frequently occur in active disease and may contribute to spreading the disease.

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Authorship

Contribution: B.T., M.K., M.A., and D.I. performed experiments; M.B., B.T., M.K., M.T., and A.G. interpreted data; and M.B., B.T., and M.K. wrote the manuscript.

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II. Supplementary material

*Supplementary Table 1: Clonotypic Ig rearrangement of malignant PC clone.**

Patient Code	Isotype	V Gene (HC)	D Gene (HC)	J Gene (HC)	HCDR3 Sequence
MM001	IgG κ	5-51	5-24	4	CVRPRIRERGPIPLDFW
MM020	IgG κ	4-39	3-22	4	CAGRGSNFDSDSRDFIIFDSW
MM023	IgG κ	5-51	6-19	4	CACPSRYSSVWRIDYW
MM036	IgG κ	1-f	3-16	5	CTRSVPSTVHNNWFDPW
MM032	IgG λ	2-5	4-23	2	CVHRRMGQLQDWYFDLW
MM050	IgG κ	3-15	6-13	3	CATEISSGASVGSVKVLW
MM021	IgG λ	3-21	2-2	3	CARVQIPAALDSW
MM024	IgG κ	3-9	-	5	CVQAIRFVF
MM031	IgG λ	7-4	1-7	4	CAREYYYNYVRYFDSW
MM034	IgG λ	1-69	2-15	5	CARDTDILVVDVATGFDPW
MM048	IgA λ	3-21	4-17	6	CARGGYGDNPYYHYGLDVW
MM081	IgG κ	3-23	2-15	5	CAQSNVAAAPRGWFDPW

* Ig = immunoglobulin, PC = plasma cell, MM = multiple myeloma, HC = heavy chain, HCDR3 = heavy chain complementarity determining region 3.

*Supplementary Table 2: Primer sequences.**

Category	Denomination	Primer Sequence (5' to 3')
VH family-specific forward primer	VH1a	CAGGT**k**CAGCTGGTGCAG
	VH1b	CAGGTCCAGCTTGTGCAG
	VH1c	**s**AGGTCCAGCTGGTACAG
	VH1d	CA**r**ATGCAGCTGGTGCAG
	VH2a	CAGATCACCTTGAAGGAG
	VH2b	CAGGTCACCTTGA**r**GGAG
	VH3a	GA**r**GTGCAGCTGGTGGAG
	VH3b	CAGGTGCAGCTGGTGGAG
	VH3c	GAGGTGCAGCTGTTGGAG
	VH4a	CAGGTGCAGCTACAGCAG
	VH5a	GA**r**GTGCAGCTGGTGCAG
	VH6a	CAGGTACAGCTGCAGCAG
	VH7a	CAGGTsCAGCTGGTGCAA
VH patient-individual HCDR3 reverse primer	MM001 HCDR3 reverse	CTCTCCCGGATTCGTGGTC
	MM020 HCDR3 reverse	GGCAGGGGGTCAAATTTTGAC
	MM021 HCDR3 reverse	GATCCTCGAGACGGTGACCAGGGTTCC
	MM023 HCDR3 reverse	CAGACACTGCTATATCTAGAAGG
	MM024 HCDR3 reverse	CGAAGCGAATGGCTTGTACAC
	MM031 HCDR3 reverse	GAATATTACTACAACTACGTACGATAC
	MM032 HCDR3 reverse	GTCTTGGAGTTGCCCCATTC
	MM034 HCDR3 reverse	GCAACATCCACCACTAAAATATCC
	MM036 HCDR3 reverse	CTGTGGAAGGGACTGATCTTG
	MM048 HCDR3 reverse	GGATTGTCACCGTACCCTCC
	MM050 HCDR3 reverse	GATGCTCCAGACGATATTTCTG
	MM081 HCDR3 reverse	GCAGCTGCTACGTTAGATTGC
VH patient-individual HCDR3 forward primer	MM001 HCDR3 forward	GACCACGAATCCGGGAGAG
	MM020 HCDR3 forward	GTCAAAATTTGACCCCCTGCC
	MM021 HCDR3 forward	GGAACCCTGGTCACCGTCTCGAGGATC
	MM023 HCDR3 forward	CCTTCTAGATATAGCAGTGTCTG
	MM024 HCDR3 forward	GTGTACAAGCCATTCGCTTCG
	MM031 HCDR3 forward	GTATCGTACGTAGTTGTAGTAATATTC
	MM032 HCDR3 forward	GAATGGGGCAACTCCAAGAC
	MM034 HCDR3 forward	GGATATTTTAGTGGTGGATGTTGC
	MM036 HCDR3 forward	CAAGATCAGTCCCTTCCACAG
	MM048 HCDR3 forward	GGAGGGTACGGTGACAATCC
	MM050 HCDR3 forward	CAGAAATATCGTCTGGAGCATC
	MM081 HCDR3 forward	GCAATCTAACGTAGCAGCTGC
VH constant region reverse primer	IgM reverse	CCAGGACACCTGAATCTGCC
	IgG reverse	GGCTCACGTCCACCACCACGCA
	IgA reverse	GGGATTCGTGTAGTGCTTCACGTG
NGS primer forward	PlugOligo specific forward	ACGCAGAGTGGCCATTACGGC
	PlugOligo overhang I forward	ACACTCTTTCCCTACACGACGCTCTTCCGATCTACGCAGAGTGGCCATTAC
	PlugOligo overhang II forward	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC
reverse	VH constant region reverse primer	
	IgM overhang I reverse	TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGGAATTCTCACAGGAGACG
	IgG overhang I reverse	TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCAGGGGGAAGACSGATG
	IgA overhang I reverse	TGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCTCAGCGGGAAGACCTTG
	Barcorde primer	CAAGCAGAAGACGGCATACGAGAT(N)^{6}GTGACTGGAGTTCAGACGTGTG

* VH = heavy chain variable region, HCDR3 = heavy chain complementarity determining region 3.

*Supplementary Table 3: Detection of the clonotypic rearrangement in peripheral B-lineage cells by next-generation sequencing.**

Patient Code	Remission Status at Time Point of Sample Collection	Total Number of Reads	Isotype	Reads per Isotype	Clonotypic Rearrangement (% of total Reads)	Clonotypic Rearrangement (% of Reads from respective Isotype)
HD		40248				
			IgM	33.02 %		
			IgG	26.98 %		
			IgA	40.00 %		
MM001	remission after treatment	10664				
			IgM	87.66 %		
			IgG	7.36 %	0 %	0 %
			IgA	4.98 %		
MM020	remission after treatment	8173				
			IgM	5.43 %		
			IgG	87.94 %	0 %	0 %
			IgA	6.63 %		
MM021	active disease	3120				
			IgM	14.29 %		
			IgG	33.62 %	0.67 %	2 %
			IgA	52.08 %		
MM024	active disease	2324				
			IgM	37.05 %		
			IgG	37.74 %	23.49 %	62.26 %
			IgA	25.22 %		
MM024	remission after treatment	3972				
			IgM			
			IgG	82.25 %	0 %	0 %
			IgA			
MM031	active disease	6284				
			IgM	32.48 %		
			IgG	41.55 %	28.09 %	67.60 %
			IgA	25.97 %	0.05 %	0.18 %
MM031	remission after treatment	9090				
			IgM			
			IgG	32.18 %	0 %	0 %
			IgA			
MM034	active disease	7609				
			IgM	22.43 %		
			IgG	44.01 %	29.87 %	67.87 %
			IgA	33.55 %		
MM048	active disease	4677				
			IgM	44.92 %		
			IgG	26.88 %	0.06 %	0.24 %
			IgA	28.20 %	13.41 %	47.54 %
MM081	active disease	36133				
			IgM	24.74 %		
			IgG	51.42 %	50.34 %	97.88 %
			IgA	23.83 %		

* HD = healthy donor, MM = multiple myeloma.

Supplementary Figure Legends

Supplementary Figure 1: Illustration of PCR amplification approaches for detection, sanger sequencing and next-generation sequencing (NGS) of Ig

A: Isotype-independent qualitative detection of clonotypic Ig rearrangements. The PCR was performed as schematically shown. PCR products were subjected to electrophoresis on agarose gels and qualitatively detected by ethidium bromide staining.

B: Semi-nested PCR approach for detection and sanger sequencing of clonotypic pre-switch IgM rearrangements. The PCR was performed as schematically shown with PCR product clean-up performed between the two PCR steps. Products were visualized by ethidium bromid staining on agarose gels, cloned into pJET1.2 vector and sequenced.

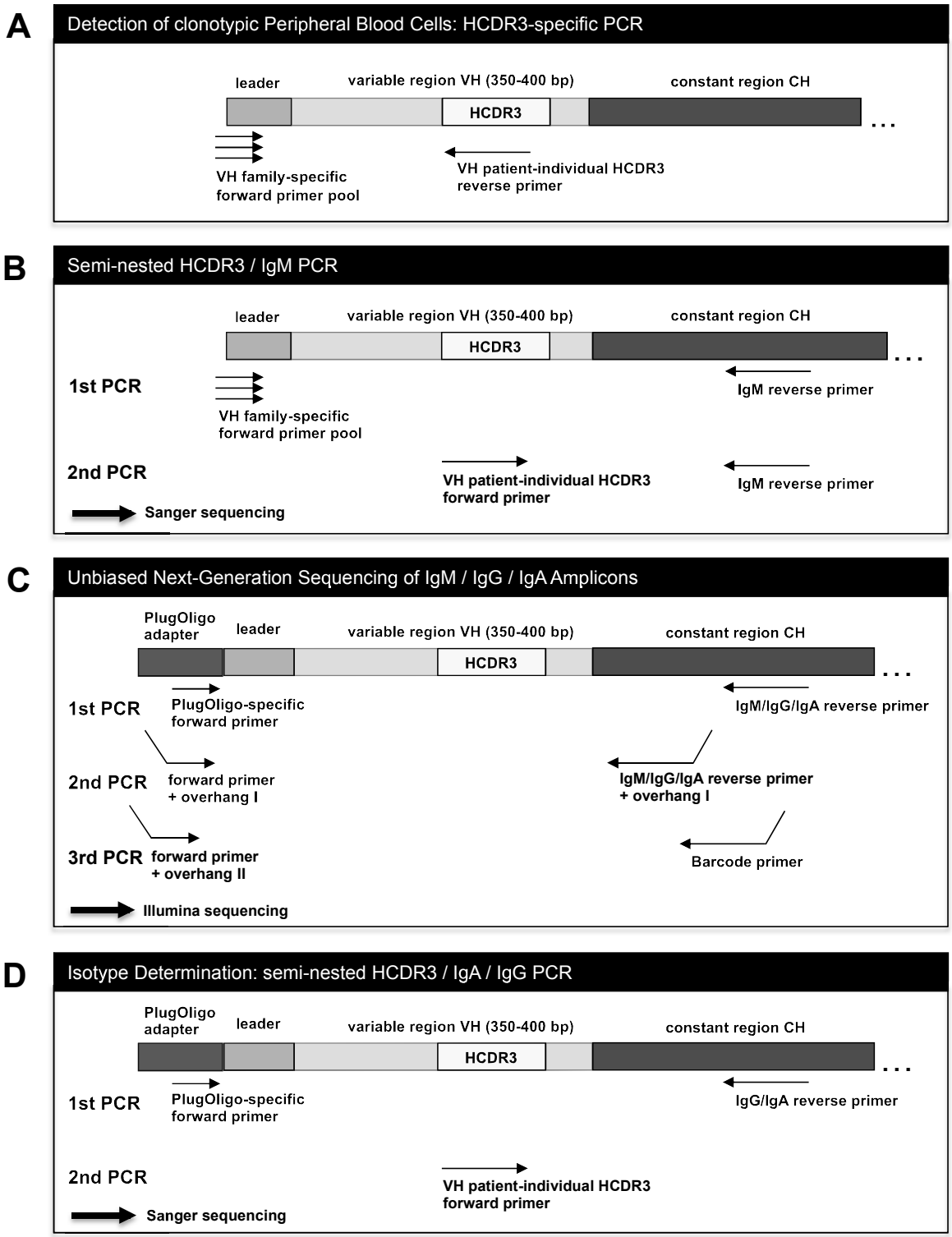
C: Amplification approach for NGS of heavy chain Ig repertoires. The Mint-2 kit was used to attach a PlugOligo sequence to each cDNA. The PCR was performed as shown with primers containing Illumina-specific overhangs. All products were quantified, pooled and subjected to next-generation sequencing.

D: Amplification approach for Sanger sequencing to determine the exact Ig isotype subclass of the clonotypic rearrangement. The PCR was performed as shown. The products were cloned into pJET1.2 vector (as in B) and sanger sequenced.

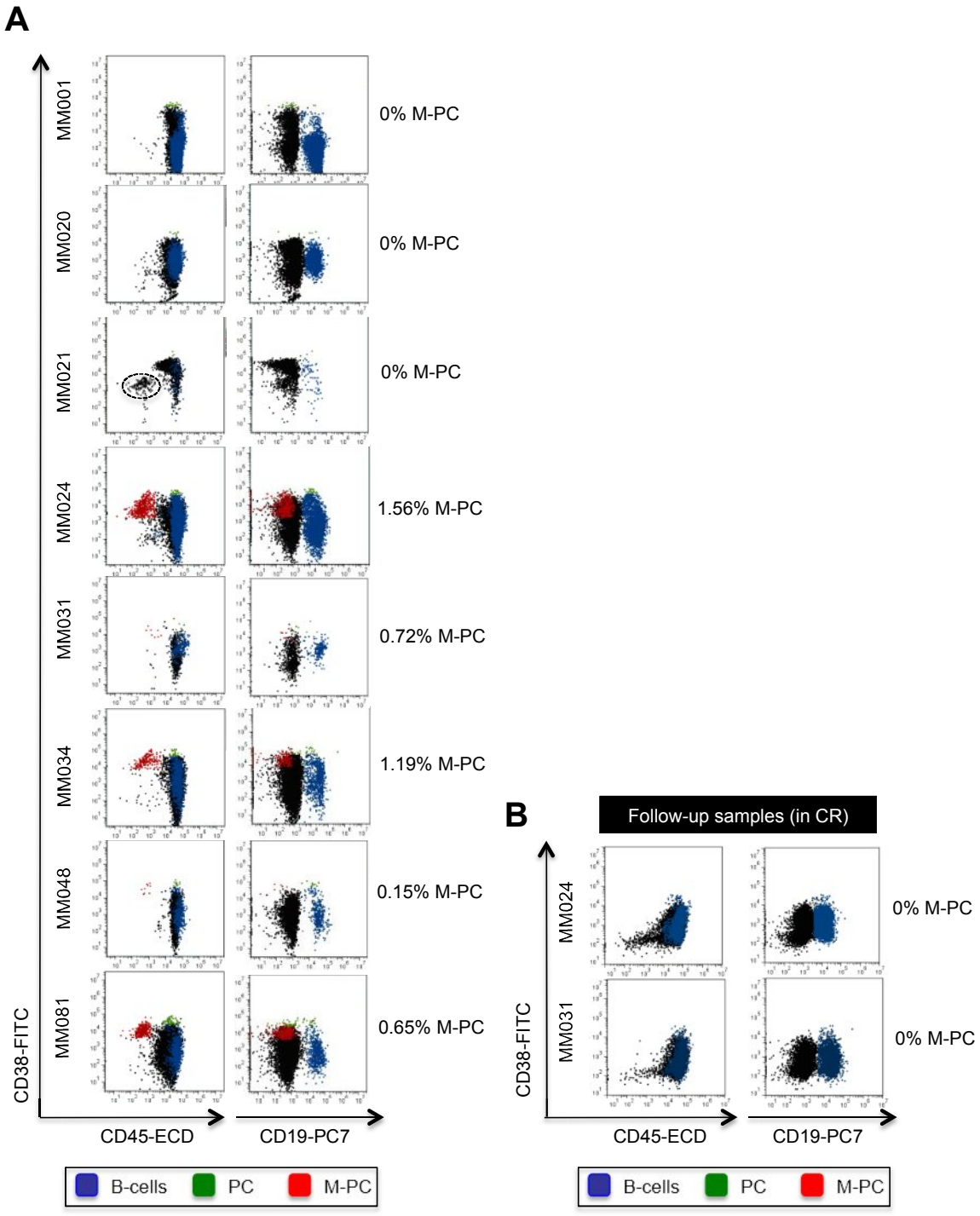
Supplementary Figure 2: Multiparametric flow cytometry for the detection of circulating malignant plasma cells (M-PC)

A: Detection of circulating M-PC in the peripheral blood of the patient cohort. Panel A shows patients MM001 and MM020, who were negative for the clonotypic rearrangement by NGS. In patients MM021, MM024, MM031, MM034, MM048 and MM081 the clonotypic rearrangement was detectable in peripheral blood by NGS. Fluorescently labeled antibodies CD45-ECD, CD138-PC5, CD38-FITC, CD19-PC7 were used for staining. CD45-or(+)/CD138+/CD38++/CD19- cells were classified as M-PC (red), CD45+or(+)/CD19+/CD138+/CD38++ as physiological PCs (green), CD45+/CD19+/CD138-/CD38+/- cells as B-cells (blue). Selected plots are shown. In patient MM021 myeloid blasts were detectable in peripheral blood (dotted circle) as this patient had concomitant myelodysplastic syndrome.

B: Panel B shows follow-up samples of patients MM024 and MM031 after treatment.



Suppl. Figure 1



Suppl. Figure 2

Detailed Methods section

Next-generation sequencing (NGS) of Ig repertoires

Single-stranded cDNA was generated from myeloma patients' peripheral blood mononuclear cells using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia). At the 5' end of the cDNA a common PlugOligo-3M adapter was incorporated which allowed for subsequent unbiased forward priming. In this way, Ig was amplified without V-gene specific primers which may otherwise introduce a bias for certain Ig genes. As reverse primers, isotype-specific IgG, IgA or IgM heavy chain constant region primers were used. In a second step, a semi-nested PCR was performed using a different set of constant region primers annealing directly adjacent to the variable region (after framework region 4). These primers contained NGS-compatible sequences for later hybridization of amplicons to the Illumina flow cell and for sequencing-primer annealing (Supplementary Figure 1D). In a third step, a patient and sample-specific barcode was incorporated. All primer sequences are shown in Supplementary Table 2. PCRs were carried out with the Phusion® High-Fidelity DNA Polymerase (NEB, Frankfurt, Germany) in combination with GC-buffer and addition of 3% DMSO taking advantage of the enzyme's low error rates. Amplicons of correct size were excised from agarose gels and purified (HiYield $^{(R)}$ PCR Clean-up/Gel Extraction Kit by SLG, Gauting, Germany). All amplicons were multiplex-sequenced with a 500-cycle single indexed (8 nucleotides) run on a MiSeq Illumina sequencer. No merging of forward and reverse reads was applied since the reverse reads already provided the necessary information for V-/D-/J-gene usage analysis by IMGT/HighV-QUEST as evidenced by high alignment scores for gene identification. Data were plotted according to the V-/J-/D-gene usage of each clone using ggplot2 package for R statistical programming language. Each J gene is subcategorized by the 27 IMGT listed D-genes, schematically shown in Fig 1A for J3 of the Healthy Donor. Since individual rearrangements may differ even if identical V-D-J genes are used, HCDR3 sequences were manually compared between overlapping clones to confirm identity. Symbol sizes of IgM, IgG and IgA transcripts reflect the abundance of the clone normalized to overall reads allowing for size comparisons between all clones in individual patients.

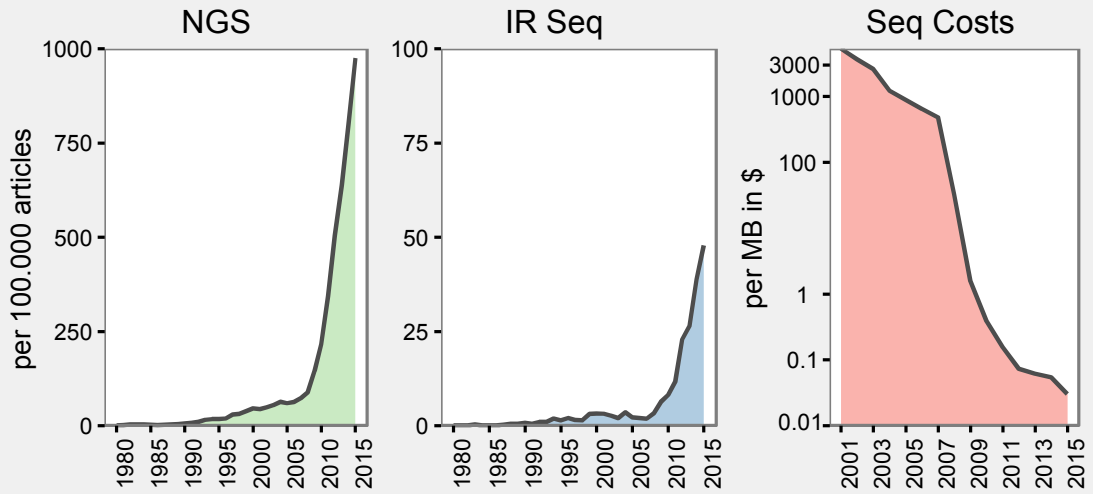
III. Summary in a broader context

1. Introduction

The emergence of next generation sequencing (NGS), also known as deep sequencing, has revolutionized the way genomic information can be gathered. After having started with single sequence based Sanger sequencing in the ‘80s, we are now capable of sequencing billions of bases in parallel in just a fraction of the time, and with constantly decreasing costs.

As one of a multitude of different applications, next generation sequencing of B-cell and T cell receptors – commonly referred to by the term immune repertoire sequencing (IR Seq) – quickly became a substantial tool for the characterization and observation of the immune system (cf. Fig. A).

Fig. A



Number of articles mentioning NGS or immune repertoire sequencing (IR Seq) per 100.000 PubMed listed articles. Usage of these terms in the literature has grown exponentially in the last ten years underlining the revolutionary character of these techniques. At the same time sequencing costs (Seq Costs) per mega base (MB) decreased massively after introduction of NGS techniques and broader adaptation in 2007. *Source: PubMed and NHGRI (see Appendix).*

Rearrangements of V, (D) and J genes form the basis for the most specific antigen recognition sites of B and T cell receptors; these are termed complementarity determining region 3 (CDR3) (Murphy et al. 2012). A process called somatic hypermutation further increases the variability of the B-cell receptor. Overall, these processes generate a vast amount of diverse B and T cell receptors (Shlomchik and Weisel 2012). BCRs and TCRs are composed of two heavy and two light chains forming paired dimers with one binding site each. The CDR3 of the heavy chain (HCDR3) has a higher variability compared to the light chain due to the introduction of a D gene. Therefore, the HCDR3 mainly contributes to the specificity and avidity of

the BCR/TCR and perfectly serves as a highly specific clonal marker in immune repertoire profiling (Xu and Davis 2000).

Next generation sequencing of these HCDR3 regions has already changed our understanding of the immune system's diversity and dynamics to an unprecedented level (Weinstein et al. 2009), (Oliveira et al. 2015), (Laserson et al. 2014).

Besides providing astonishing insights into the pathophysiology of several diseases (Kirsch et al. 2015), (Parameswaran et al. 2013), (Srivastava et al. 2012), IR Seq has also proven to be a valuable tool for diagnostics and even prognostics in malignancies of the B and T-cell compartments (Logan et al. 2011, Kirsch et al. 2015). All of these examples highlight IR Seq's great potential for advancing our knowledge about the immune system's role in health and disease and for future clinical practice.

In the following work, I will focus on the development and application of this technology for the B-cell compartment. Malignant B-cells in B-cell neoplasms share a clonal CDR3. Through deep sequencing of this unique B-cell marker, we can detect and monitor B-cell neoplasms (Mailankody et al. 2015). Furthermore, our RNA-based deep sequencing approach allows us to identify the corresponding immunoglobulin class of a specific B-cell. With this information, we are also able take a closer look at the dynamics of immunoglobulin class switching events in B-cell neoplasms like multiple myeloma. In our publication, we successfully applied this technique to the controversial hypothesis that clonotypic pre-switch (IgM positive) B-cells form a progenitor and potentially regenerate the cell pool in multiple myeloma (Thiele et al. 2014).

We further advanced our technique to gather even deeper insights into the B-cell repertoire by including information about the immunoglobulin subclass. This could enable an even deeper look at the B-cell compartment.

2. Materials and Methods

2.1 Patient material

Blood and bone marrow samples were collected from patients after they had signed a written consent form approved by the ethics committee and in accordance with the Declaration of Helsinki.

2.2 mRNA isolation and cDNA synthesis

Peripheral blood mononuclear cells (PBMCs) were separated from whole blood and bone marrow samples by FiColl density centrifugation. The cells were either stored at -80°C or used immediately for mRNA isolation with the RNeasy Mini Kit plus (Qiagen)

to reduce RNA loss by enzymatic degradation. The mRNA was then reverse transcribed to single stranded cDNA using the Mint-2 kit (Evrogen), with the specific use of the provided CDS-1 and PlugOligo-3M Adapters, according to the kit's protocol.

In this way the Mint-2 kit for cDNA synthesis allowed us to take advantage of one universal forward primer due to a 5' end ligated consensus DNA sequence, thereby eliminating the potential bias introduced by multiplexed V gene family specific priming (Bashford-Rogers et al. 2014).

2.3 Library preparation for next generation sequencing

Due to unspecific forward priming, the reverse primers were very specifically designed to only amplify immunoglobulin sequences. The priming site was chosen to be in close proximity to the B-cell clone-specific identifier, the HCDR3, to maximize deep sequencing coverage of that region.

Three reverse primers specific for the immunoglobulin IgA, IgG and IgM classes, with priming sites in the constant region of the heavy chain (CH1), were developed to cover every heavy chain allele listed in the IMGT database (Giudicelli et al. 2006).

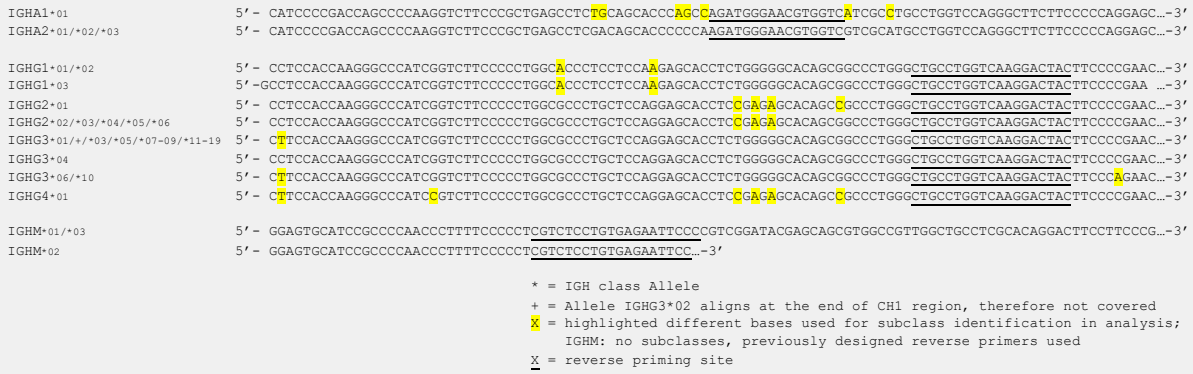
Specificity for immunoglobulin sequence amplification was further increased by a three-step PCR approach: i) specific amplification of the target sequences with a touch down PCR, ii) specific semi-nested addition of illumina adapters (Overhang I), and iii) unspecific addition of a patient- and sample-specific barcode (Overhang II) (see Suppl. Fig. 1). To reduce PCR errors, PCRs were carried out with the proofreading enzyme Phusion High-Fidelity DNA Polymerase (NEB). 3% DMSO and GC-Buffer were added to allow for better amplification of GC-rich regions. Cycling counts were kept at a minimum level to avoid potential PCR bias introduced by exponential amplification. PCR products were gel purified, specific bands were excised and gel extraction was performed using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Finally, the samples were pooled equally according to Qubit fluorometric quantitation (ThermoFisher) and loaded onto a MiSeq system (Illumina) using a MiSeq Reagent Kit v2 (Illumina).

2.4 Immunoglobulin subclass primer design

Reverse priming sites were chosen to i) cover a sufficient amount of subclass sequence differences for analysis, ii) cover almost all subclass alleles, and iii) still be close enough to the CDR3 upstream of the CH1 for sequencing accuracy. Immunoglobulin subclass allele sequence data was taken from the IMGT Immunoglobulin Reference Database (Giudicelli et al. 2006). Sample preparation from

one healthy donor and subsequent processing was performed according to the aforementioned protocol. For subclass identification, sequences were matched by aligning differing base sequences (see Fig. B) and then pulled from the final sequencing pool in the following sequential order: IgA1>IgA2; IgG1>IgG3>IgG2>IgG4. It was thereby possible to subsequently separate IgG3, IgG2 and IgG4 based on differing bases highlighted in Fig. B, using the Biostrings package for R statistical language (Pagès et al. 2016).

Fig. B



Immunoglobulin heavy CH1 region subclass allele alignment. *Source: IMGT*

2.5 Data processing, analysis and plotting

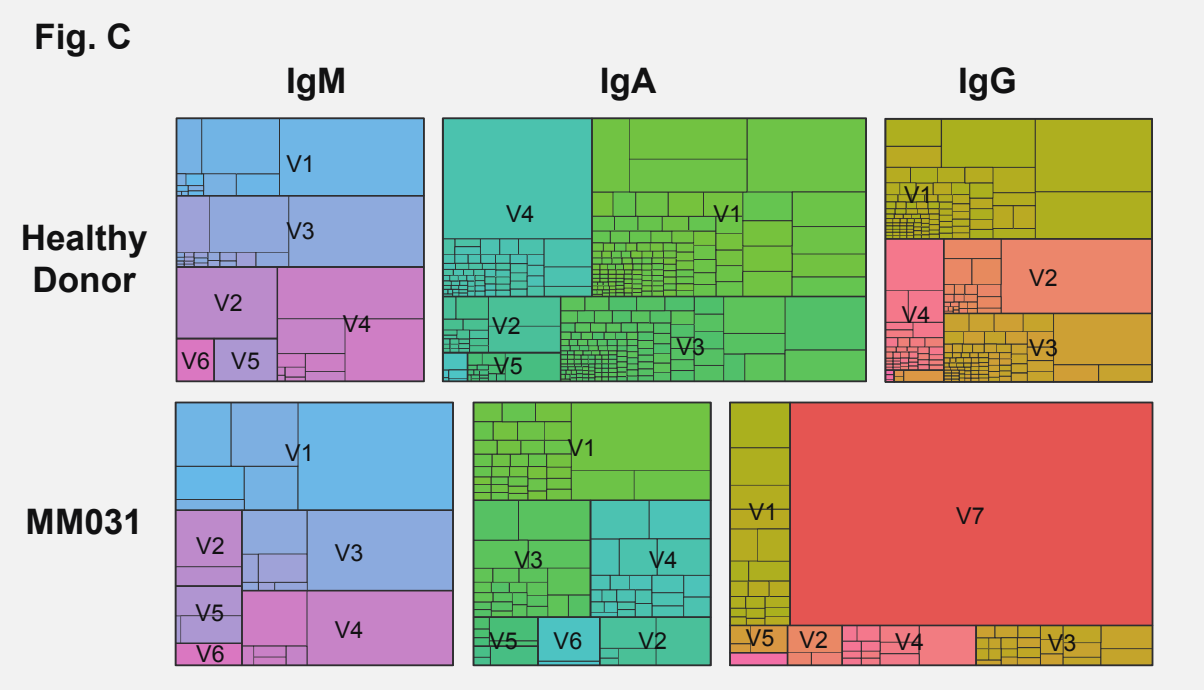
Sequencing data demultiplexing and illumina adapter trimming was performed by the MiSeq system. No forward and reverse read alignment was performed. Instead, only reverse reads were used for further analysis. In that way, we were able to increase the amount of harvested immunoglobulin sequences and obtain more accurate reads for the HCDR3 region due to the proximity of the reverse priming site. In addition, permissive parameters for quality trimming (phred score <5) also contributed to higher immunoglobulin sequence output to be used for further analysis (MacManes 2014).

After processing, the sequencing datasets were uploaded to the IMGT/ HighV-Quest platform, which i) aligns the data to IMGT germline genes to identify V, D and J genes and the hypermutation status with a certain accuracy, ii) identifies the HCDR3 region of each sequence by the IMGT unique numbering system, and iii) clusters immunoglobulin sequences by HCDR3 regions into clonotypes (Li et al. 2013). Reads of individual clonotypes were set in relation to total reads per sample, allowing for comparable dot sizes between different samples. Clonotypes with reads fewer than four were excluded from plotting due to an increasing influence of artificial BCR rearrangements created by PCR errors. The output data was plotted with the ggplot2 (Wickham 2009) and treemap package (Tennekes 2016) for R statistical language (Team and others 2013).

3. Results

3.1 Successful B-cell receptor library preparation and mapping of a B-cell receptor repertoire

We were able to successfully establish a library preparation and deep sequencing platform based on the methods outlined above. Overall, we investigated samples from one healthy donor and eight multiple myeloma patients through deep sequencing. All samples yielded an acceptable amount of sequencing depth, as measured by the number of HCDR3 regions, identified by IMGT/HighV-Quest analysis (average: 13248 reads, min: 2324, max: 40248) (see Suppl. Tab. 3). Interestingly, we observed a striking difference in diversity when comparing the BCR repertoire of a healthy individual to our multiple myeloma study cohort patients. The eight myeloma patients had an average of 348 different BCR clones, compared to 1,537 for the healthy donor. Fig. C illustrates these differences and also demonstrates the diversity of the different immunoglobulin class compartments.



Peripheral BCR repertoire of a healthy donor and myeloma patient (cf. Fig.1A). Compared to the healthy donor, the repertoire diversity in multiple myeloma is clearly reduced. The BCR of the MP-C comprises most of the V gene family 7 which is usually not very abundant. This treemap is hierarchically plotted according to immunoglobulin class, V gene and CDR3. Relative field sizes correspond to sequencing count of a certain CDR3. Colors indicate immunoglobulin class and V gene families.

3.2 Detection of circulating malignant plasma cells and analysis of their clonotypic composition in multiple myeloma

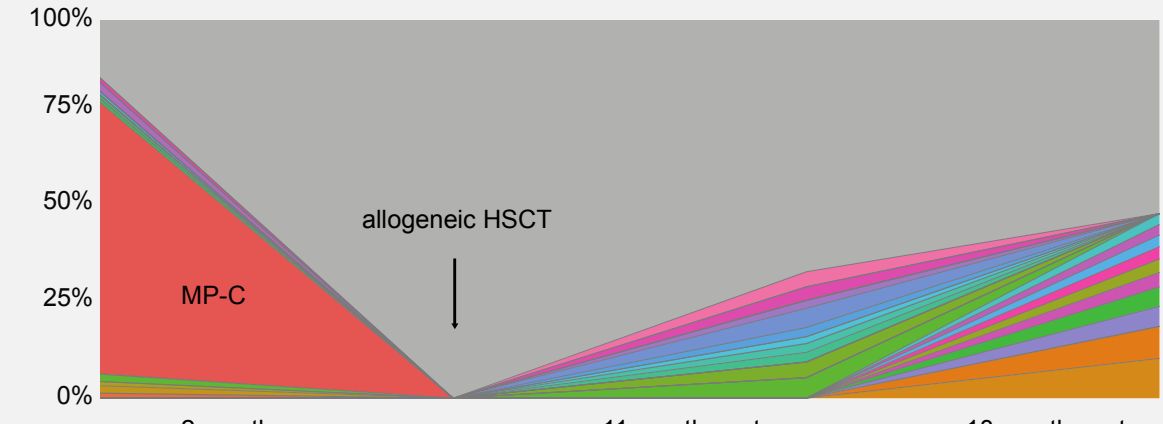
In 6 out of 8 myeloma patients, we were able to detect the malignant plasma cell (M-PC) in the peripheral blood. In 5 of the 8 patients, malignant cells were identified only by the dominant abundance of one HCDR3 sequence. Although multiple myeloma is

a disease that primarily manifests in the bone marrow, our data shows that M-PCs also circulate in the peripheral blood of these patients. One of the main questions addressed by our publication was the controversial hypothesis of so-called clonotypic pre-switch B-cells (Matsui et al. 2008). Having not undergone class switching, they have been thought to form a progenitor and potentially a regeneration cell pool in multiple myeloma, thereby being responsible for relapse of the disease (Reiman et al. 2001). Applying our BCR deep sequencing technique, we did not find any evidence for clonotypic pre-switch B-cells in our myeloma patient cohort. Instead, we detected a small population of malignant plasma cells that seemed to have undergone further class switching, possibly indicating ongoing evolution in the M-PC pool (see Fig. 1A). These findings underline the great potential of deep sequencing for the assessment of heterogeneity in B-cell neoplasms.

3.3 Monitoring circulating B-cells in myeloma patients during treatment course

Two patients in our myeloma patient cohort underwent allogeneic hematopoietic stem cell transplantation (HSCT) and remained in complete remission at follow-up examinations, as determined by FACS analysis of the bone marrow. Deep sequencing analysis for malignant cells in the peripheral blood revealed no evidence of ongoing disease, in concordance with the bone marrow analysis. Performing such analysis of the peripheral B-cell repertoire at several time points might provide valuable insights into the dynamics of the engraftment process following HSCT. The findings from patient MM031 are illustrated in Fig. D.

Fig. D



Clonal composition of the peripheral IgG BCR repertoire for myeloma patient MM031 during allogeneic HSCT treatment course. Colored areas represent the ten most abundant CDR3s at each of the three sampling times. Less abundant CDR3s are summed up in the grey area. Prior transplantation the CDR3 of the MP-C comprises 70% of the peripheral BCR repertoire. The MP-C is not detectable at post transplantation sampling times, reflecting a complete remission status. Also the dynamic of a reconstituting IgG compartment is observable with a complete turnover of the ten most abundant clones between the two post treatment sampling points.

3.4 Expanding the technique to determine immunoglobulin subclasses

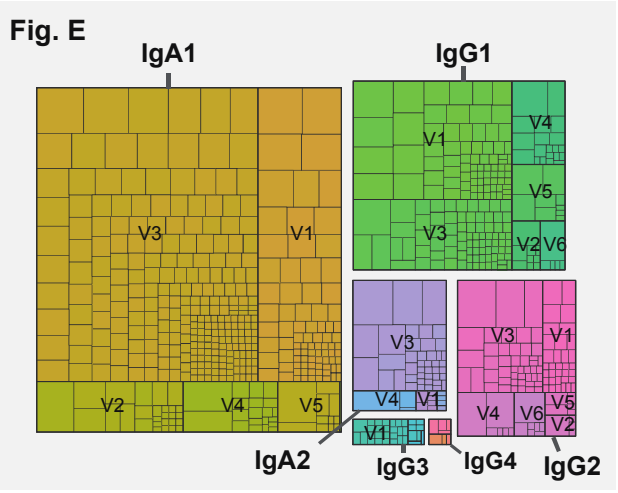
We modified our method to also include information about the immunoglobulin subclasses, in order to gather more information about the potential effector function of a certain BCR. In a proof of principle experiment, we were able to show that the subclass distribution measured using our deep sequencing platform was comparable to the results of (Schanz et al. 2014), who used a technically different deep sequencing approach (Schanz et al. 2014). Though comparing transcription and expression levels, our subclass distribution was also in the range of routine reference values for serum immunoglobulins measured by nephelometry (Schauer, Stemberg, Rieger, Borte, Schubert, Riedel, Herz, Renz, Wick, and Herzog 2003; Schauer, Stemberg, Rieger, Borte, Schubert, Riedel, Herz, Renz, Wick, Carr-Smith, et al. 2003) (cf. Tab. A).

Tab. A
Relative distribution of immunoglobulin subclasses in peripheral blood (%)

Subclass	Deep sequencing our approach	Deep sequencing Schanz et al.	Nephelometry serum mean
IgA1	89,7	-	87,4
IgA2	8,9	-	12,6
IgG1	63,5	56,1	55,6
IgG2	21,2	39,5	33,4
IgG3	6,6	4,0	7,1
IgG4	0,3	0,5	3,8

Comparison of relative immunoglobulin subclass distribution in peripheral blood of healthy donors, determined with: our deep sequencing approach, a technically different RNA based deep sequencing technique of Merle et al. and routine serum assessment with immunonephelometry. Schanz et al. only sequenced IgG subclasses.

Fig. E



Treemap of a peripheral BCR repertoire of a healthy donor hierarchically distributed by immunoglobulin subclasses and V genes. Field sizes represent BCR abundance, coloration subclasses and V gene families like indicated.

4. Discussion

Although we were very successfully able to apply our RNA-based BCR deep sequencing technique to several questions, as outlined in the results section, the method also has certain shortcomings that should be noted.

Certainly one common problem with RNA-based techniques is the difficulty of obtaining consistently stable read counts between different samples (see Supp. Tab. 3). This is most probably attributable to the use of RNA, which is difficult to store and handle. In addition, sequencing-expressed BCR does not allow for conclusions about actual B-cell numbers due to great differences in B-cell activity (Klein, Küppers, and

Rajewsky 1997) (Pabst, Hazanov, and Mehr 2015). However, sequencing the RNA/cDNA does allow a smaller amount of sample input to be used for library preparation due to a focus on the active transcriptome instead of the entire genomic DNA (gDNA) (Georgiou et al. 2014). The frequency of rearranged nonproductive BCRs is also lower due to the degradation of resulting nonsense mRNA (Larimore et al. 2012).

In comparison, gDNA-based techniques have the advantage of i) providing more consistent sequencing results, ii) allowing the calculation of actual B-cell numbers in the originally drawn blood sample, and iii) not requiring preprocessing by reverse transcription, which adds another source of PCR bias (Woodsworth, Castellarin, and Holt 2013), (Pabst, Hazanov, and Mehr 2015). However, these techniques do not allow immunoglobulin class and subclass identification due to a large intron between the CH1 and the variable region. Therefore, a gDNA-based approach was not applicable to the question of pre-switch clonotypic B-cells addressed in our publication.

Cross-contamination between samples is another frequently experienced problem in RNA as well as gDNA-based approaches, and it can happen at every step of sequencing library preparation and even in the sequencing machine itself (Longo, Berninger, and Hartley 1990; Tan et al. 2014; Laurence, Hatzis, and Brash 2014), (Urban et al. 2000). Besides the use of very strict cleaning protocols, different locations for library preparation steps, and thorough washing protocols for the sequencing machine, these problems can also be addressed through the introduction of certain DNA tags that allow contamination to be identified (Seitz et al. 2015).

An additional common obstacle is PCR bias. It is introduced in the library preparation steps, especially in gDNA-based approaches, which use multiplexing PCRs with V and J gene family specific primers for library preparation (Glanville et al. 2009; Shugay et al. 2014). Carlson et al. successfully addressed this issue by adjusting primer ratios according to the sequencing results of a synthetic human V/J gene library (Carlson et al. 2013). In our RNA-based approach, we attempted to minimize this potential source of PCR bias by utilizing only one universal forward primer and three different immunoglobulin-class-specific reverse primers (see Materials and Methods section). However, a different bias is introduced by the blood sampling process itself. Deep sequencing of the B-cells from one blood draw only provides a small excerpt of the overall diversity of the circulating B-cell repertoire; therefore, the overall circulatory diversity can only be estimated (Benichou et al. 2012). In addition, sampling of circulating B-cells might miss immunological processes taking place in harder-to-sample tissues like the lymphatic system. In the end, the particular scientific

questions being asked determine whether an RNA- or gDNA-based approach should be chosen (Pabst, Hazanov, and Mehr 2015; Georgiou et al. 2014).

Despite the technical problems and shortcomings of BCR deep sequencing, there exists a broad range of future clinical applications, especially in the detection and monitoring of B-cell neoplasms. The technique can be applied to minimal residual disease (MRD) monitoring in a variety of B-cell derived malignancies. In multiple myeloma, deep sequencing MRD monitoring has a proven prognostic advantage compared to conventional methods (Martinez-Lopez et al. 2014) and was therefore recently recommended by the International Myeloma Working Group (Kumar et al. 2016). Furthermore, deep sequencing MRD has already demonstrated diagnostic benefits in diffuse large B-cell lymphoma and chronic lymphatic leukemia, both B-cell neoplasms that primarily occur in the lymphatic system but also in the blood, facilitating easy sampling (A. C. Logan et al. 2013; Thompson and Wierda 2016; Bartlett et al. 2013). These techniques allow, with unprecedented sensitivity, the collection of deeper insights into disease development, progression and therapy response. As such, MRD status assessed through deep sequencing might guide future treatment decisions more precisely, enabling, for example, the delivery of additional chemotherapy to patients who do not show a deep response. Nevertheless, the possibility of cross-contamination outlined above must be kept in mind, as it has far reaching consequences for possible treatment decisions.

The technique might also aid in the elucidation of B-cell engraftment in transplantation settings like those demonstrated in this work, responses to infections or vaccinations (Wilson and Andrews 2012), and may even enable the development of potent, fully humanized monoclonal antibodies for the treatment of diseases (Schieferdecker et al. 2016).

Extending our technique to also cover immunoglobulin subclass information might enable further elucidation of questions around hereditary subclass deficiencies, B-cell differentiation and class switching, subclass effector function, and more recently described IgG4 related diseases, for example (Kamisawa et al. 2015, 4).

Immune repertoire deep sequencing has already become a powerful research tool with concrete clinical applications. Although it remains a costly method, we can expect it to become an important part of future precision-guided medicine. Knowledge about the advantages as well as disadvantages of these techniques will help safely guide the process of translation into clinical practice.

5. Conclusion/ Summary

With our findings, we have established a functioning RNA-based BCR deep sequencing platform that allows:

- Detection of malignant clones in the peripheral blood of patients with multiple myeloma and potentially other B-cell neoplasms (e.g. CLL, DLBCL, AL-Amyloidosis, Waldenström's macroglobulinemia)
- A potentially higher sensitivity yield compared to routine FACS analysis
- Tracking of the diversity of a circulating B-cell repertoire and of the presence of malignant clones throughout treatment
- Identification of the immunoglobulin class and subclass of a specific BCR

Nevertheless, regardless of the promising clinical applications, one must also be aware of the technological limitations of this technique.

5. Zusammenfassung

Wir konnten erfolgreich eine RNA-basierte Tiefensequenzierungsplattform des B-Zell Rezeptors in unserer Arbeitsgruppe etablieren. Diese Plattform ermöglicht uns:

- maligne Klone von B-Zell Neoplasien wie dem multiplen Myelom zu detektieren
- potentiell höhere Sensitivitäten bei B-Zell Detektion zu erzielen, als dies mit herkömmlichen Methoden wie der FACS Analyse möglich wäre
- den Verlauf von B-Zell Neoplasien unter Therapie näher zu verfolgen mit potentiellen Implikationen für die Therapie
- die Immunglobulin Klasse wie auch Subklasse aller B-Zell Rezeptoren in einer Blutprobe zu bestimmen

Neben den beschriebenen vielseitigen potentiellen zukünftigen Anwendungen dieser Technologie, dürfen die aufgeführten Limitationen nicht vernachlässigt werden.

IV. Appendix

PubMed query expressions for Fig. A:

"Next-generation sequencing":

(exome OR whole OR deep OR high-throughput OR (next AND generation) OR (massively AND parallel)) AND sequencing

"Immune repertoire sequencing":

((Immune AND repertoire) OR Repertoire OR Immunome OR (bcr OR (b cell receptor) NOT (BCR-ABL OR bcr-abl)) OR Immunoglobulin OR (tcr OR (t cell receptor)) OR (immune AND system)) AND (exome OR whole OR deep OR high-throughput OR (next AND generation) OR (massively AND parallel)) AND sequencing)

Sequencing costs source:

National Human Genome Research Institute (NHGRI) (Wetterstrand 2016).

List of abbreviations:

BCR	B-cell receptor
CDR3	Complementarity determining region 3
CH1	Constant region one of the heavy chain
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
gDNA	Genomic Deoxyribonucleic acid
FACS	Fluorescence-activated cell sorting
fw	Forward
rev	Reverse
HCDR3	Heavy chain complementarity determining region 3
HSCT	Hematopoietic stem cell transplantation
IgA1/2	Immunoglobulin alpha subclass 1/2
IgG1/2/3/4	Immunoglobulin gamma subclass 1/2/3/4
IR Seq	Immune repertoire Sequencing
MB	Mega base (1.000.000 bases)
M-PC	Malignant plasma cell
MRD	Minimal residual disease
NGS	Next-generation sequencing
PBMCs	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Seq-Costs	Sequencing costs
TCR	T-cell receptor
V,D,J gene	Variable, joining, diversity gene

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VI. Authorship Contribution

"Next-generation sequencing of peripheral B-lineage cells pinpoints the circulating clonotypic cell pool in multiple myeloma"

Benjamin Thiele[*], Marie Kloster[*], Malik Alawi, Daniela Indenbirken, Martin Trepel, Adam Grundhoff, and Mascha Binder.

[*]authors contributed equally

I planned the general BCR amplification strategy for BCR deep sequencing, including: reverse transcription PCR assay outline and utilization with Evrogen's Mint-2 kit, immunoglobulin class primer design for BCR deep sequencing, PCR amplification steps and establishing of PCR protocols. Also, I performed and established downstream deep sequencing data processing and quality checks by QC reports. Furthermore, I utilized the IMGT High/V-Quest platform for data analysis, clonotype identification and aggregation and analyzed resulting data. Additionally, I developed and applied visualization concepts for displaying BCR repertoires utilizing R statistical language and the ggplot2 and treemap package. Besides the general protocol for BCR deep sequencing, I also designed, established and carried out primer design, RNA extraction and reverse transcription as well as PCR amplification for BCR subclass deep sequencing. I performed BCR subclass data analysis including quality checks, IMGT High/V-Quest analysis and identification of subclasses by clonotypes with the Biostrings package for R statistical language. Furthermore, I visualized the data with the treemap package and interpreted the data.

Marie Kloster performed patient work up, data collection and patient library preparation for deep sequencing according to the developed protocol. She also carried out multicolor FACS analysis and interpreted data in the context of clinical outcome information. Marie Kloster cloned, sanger sequenced and analyzed individual subclass clonotypes. She furthermore interpreted the deep sequencing results in the context of her literature research for the clonotypic pre-switch B-cell concept in multiple myeloma. Marie Kloster designed tables and the BCR amplification pipeline figure.

Marie Kloster, Mascha Binder and I wrote the manuscript. Proof reading was carried out by all authors. Marie Kloster and I wrote figure descriptions as well as the supplementary material section. Malik Alawi, Daniela Indenbirken und Adam Grundhoff loaded the the flowcells and operated the MiSeq sequencing machine.

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VIII. Lebenslauf

entfällt aus datenschutzrechtlichen Gründen

VIII. Publikationsverzeichnis/ Wissenschaftliche Beiträge

"A Transplant-Immunome Deep Sequencing and Screening Platform Defines a Unique Targetable Epitop Fingerprint of Multiple Myeloma."
Schieferdecker, Oberle*, Thiele*, Hofmann, Göthel, Miethe, Hust, Braig, Voigt, Koch-Nolte, Haag, Alawi, Indenbirken, Grundhoff, Bokemeyer, Bacher, Kröger, Binder.*
Blood: 31/03/2016.

"Next-Generation Sequencing of Peripheral B-Lineage Cells Pinpoints the Circulating Clonotypic Cell Pool in Multiple Myeloma."
Thiele, Kloster*, Alawi, Indenbirken, Trepel, Grundhoff, Binder.*
Blood: 05/06/2014.

"Multidimensional Scaling Analysis Identifies Pathological and Prognostically Relevant Profiles of Circulating T-Cells in Chronic Lymphocytic Leukemia."
Rissiek, Schulze, Bacher, Schieferdecker, Thiele, Jacholkowski, Flammiger, Horn, Haag, Tiegs, Zirlik, Trepel, Tolosa, Binder.
International Journal of Cancer: 15/11/2014.

**contributed equally*

In review:
"Early T-cell repertoire diversification reflects an imprint of antigen selection in the blood of patients on immune checkpoint inhibition and may be exploited as liquid biopsy response prediction biomarker."
Akyüz, Brandt, Stein, Schliffke, Mährle, Quidde, Gökkurt, Loges, Haalck, Ford, Asemissen, Thiele, Radloff, Thenhausen, Krohn-Grimberghe, Bokemeyer, Binder.
International Journal of Cancer: 09/08/2016.

Submitted:
"Dynamic changes of the normal B lymphocyte repertoire in chronic lymphocytic leukemia in response to ibrutinib or FCR chemo-immunotherapy."
Schliffke, Sivina, Kim, Thiele, Akyüz, Falker-Gieske, Thenhausen, Krohn-Grimberghe, Bokemeyer, Jain, Estrov, Ferrajoli, Wierda, Keating, Burger, Binder.
Leukemia: 28/09/2016

"Mutational landscape reflects the biological continuum of plasma cell disorders."
Rossi, Voigtländer, Janjetovic, Thiele, Alawi, März, Brandt, Hansen, Radloff, Hegenbart, Schönland, Langer, Bokemeyer, Binder.
British Journal of Haematology: 09/11/2016

In submission:
"Monitoring multiple myeloma by next-generation sequencing of V(D)J rearrangements from circulating myeloma cells and cell-free myeloma DNA."
Oberle, Brandt, Voigtlaender, Thiele, Radloff, Schulenkorf, Akyüz, März, Ford, Krohn-Grimberghe, Binder.
Blood: 11/2016

Short talk: "Detektion klonotypischer B-Vorläuferzellen des Multiplen Myeloms mittels nextgeneration Sequencing." Selected for best abstract session.
Jahrestagung der Deutschen Gesellschaft für Hämatologie und Onkologie, Hamburg 2014

Short talk: "Next-generation immunosequencing reveals humoral anti-cancer immunity in patients undergoing T-cell targeted immunotherapy."
Immune Profiling in Health and Disease - Nature Conferences, Seattle 2016

IX. Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

Unterschrift: .....