

The genetic landscape of extramedullary plasmacytoma: a comparative analysis with extramedullary disease of multiple myeloma

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Received: November 10, 2025.
Accepted: March 27, 2026.
Early view: April 9, 2026.

<https://doi.org/10.3324/haematol.2025.300201>

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Supplemental methods

Immunohistochemistry

Specimens were fixed in 4% buffered formalin and embedded in paraffin for histopathological analysis. Sections were stained with hematoxylin and eosin (H&E) and Giemsa. Immunostaining and EBV in situ hybridization were performed on an automated immunostainer (Ventana Benchmark XT, Ventana, Tucson, AZ, USA). All cases were stained for CD138 (ready-to-use [rtu], Roche, Basel, Switzerland), Cyclin D1 (rtu, Roche), CD56 (1:300, Menarini, Florence, Italy), c-MYC (rtu, Roche), immunoglobulin (IG) Kappa (1:25,000, Dako, Jena, Germany), IG Lambda (1:25,000, Dako), MIB1 (1:200, Dako), MUM1 (1:400, Dako), p53 (1:400, Novocastra, Newcastle upon Tyne, UK) and EBV-encoded RNA (rtu, Roche). In addition, a subset of cases was stained for IG heavy chains IgA (1:80,000, Roche), IgG (1:30,000, Dako), and IgM (1:10,000, Dako) as well as for CD3 (1:100, DCS, Hamburg, Germany) and CD20 (1:500, Dako).

Aberrant p53 staining was defined as either a uniform overexpression or a complete absence of p53 expression in the tumor cells. MYC expression was assessed quantitatively as described by Xiao et al., with each sample assigned a score of 0 (no expression), 1 (<30% positive cells), 2 (30–70% positive cells), or 3 (>70% positive cells).¹ Ki67 was assessed in one high-power field (diameter 0.55 mm) at the proliferation hotspot using automated image analysis with aetherAI software (version 105122, Taipei City, Taiwan), following digitization of the slides with the Pathology Scanner SG300 (Philips, Hamburg, Germany). Histological images were processed with Adobe Photoshop CS4 (version 11.0, Adobe Systems, San José, CA, USA).

Fluorescence *in situ* hybridization (FISH) and copy number (CN) analysis

FISH analysis was generally performed using the ZytoLight SPEC IGH Dual Color Break Apart Probe (ZytoVision, Bremerhaven, Germany), the ZytoLight SPEC CKS1B/CDKN2C Dual Color Probe (ZytoVision), the ZytoLight SPEC TP53/CEN 17 Dual Color Probe (ZytoVision), as well as the 5p15/q22/15q22 Hyperdiploidy Amplification Probe (MetaSystems, Altlußheim, Germany) and was evaluated using the Axio Imager.M2 microscope equipped with the integrated ApoTome.2 system (Zeiss, Oberkochen, Germany). Extramedullary plasmacytoma (EMP) samples with an IGH translocation, as well as case 41, in which several spatially and temporally distinct manifestations were studied, were additionally analyzed using the Vysis LSI CCND1 Break Apart Rearrangement Probe (Abbott GmbH, Wiesbaden, Germany), the ZytoLight SPEC FGFR3/IGH Dual Color Dual Fusion Probe (ZytoVision), the ZytoLight SPEC MAF/IGH Dual Color Dual Fusion Probe (ZytoVision), and the ZytoLight SPEC MAFB/IGH Dual Color Dual Fusion Probe (ZytoVision). At least 50 nuclei were evaluated per sample and probe. Due to the use of FFPE tissue sections, which result in a higher background of aberrant fluorescence patterns, the threshold for numerical alterations was conservatively set at 40% of cells. In contrast, the threshold for IGH breaks was set at 10%, based on extensive prior validation of the IGH break-apart probe for routine diagnostics. A gain of both *CKS1B* (1q21.3–q22) and *CDKN2C* (1p32.3) was interpreted as a gain of chromosome 1 as a whole. Similarly, concurrent gains of *TP53* (17p13.1) and the centromeric reference probe were interpreted as polyploidy of chromosome 17. Amplification of 1q was defined by FISH as a gain with >3 fluorescence signals per nucleus.²

Concerning the CN analysis, CN alterations (CNA) with a minimum size of 100 kb and CN neutral losses of heterozygosity (CNN-LOH) larger than 5 Mb were considered informative, given that somatic CNA typically involve larger genomic regions than constitutional CN variations (CNV). To exclude larger constitutional CNV, we assessed the percentage of overlap with known CNV for alterations larger than 100 kb but smaller than 500 kb using the Database of Genomic Variants (DGV).³ The human reference genome was GRCh37/hg19.

The cancer cell fraction (CCF) for each CNA was determined using ASCAT (v3.2.0). For each case (n=22) carrying CNA, the B-allele frequency (BAF) and logR values were derived from OncoScan data via Nexus Copy Number Software v9.0 (Bionano Genomics, San Diego, CA, USA). Tumor purity and ploidy estimates derived from ASCAT were used to adjust the predicted CCF, as previously reported and outlined below.^{4,5} Although these formulas were defined for near-diploid tumors, they were adapted accordingly for polyploid cases.

$$\begin{aligned}
\text{CCF}_{\text{CN LOSS}} &= \frac{(-2 * \text{BAF} + 1)}{(1 - \text{BAF})} \longrightarrow \boxed{\text{Corrected CCF}_{\text{CN LOSS}} = \frac{\text{CCF}_{\text{CN LOSS}}}{\text{Purity}}} \\
\text{CCF}_{\text{CN GAIN}} &= \frac{(2 * \text{BAF} + 1)}{(\text{major} - 1) - ((\text{major} - 1) * \text{BAF}) - ((\text{minor} - 1) * \text{BAF})} \longrightarrow \boxed{\text{Corrected CCF}_{\text{CN GAIN}} = \frac{\text{CCF}_{\text{CN GAIN}}}{\text{Purity}}} \\
\text{CCF}_{\text{CN LOH}} &= \frac{(2 * B + 1)}{(1 - \text{BAF})} \longrightarrow \boxed{\text{Corrected CCF}_{\text{CNN-LOH}} = \frac{\text{CCF}_{\text{CNN-LOH}}}{\text{Purity}}}
\end{aligned}$$

For two samples (cases 11 and 33) that ASCAT predicted as polyploid, manual normalization was performed, assigning the diploid region at the level of chromosomes 6/13 and 12, respectively.⁶ Hyperdiploidy was defined as trisomy of three or more of the odd-numbered chromosomes 3, 5, 7, 9, 11, 15, 19, and/or 21.⁷ For the genomic complexity analysis, hyperdiploid chromosomes were counted as separate chromosomal aberrations. For samples analyzed by FISH only, hyperdiploidy was defined as gains of any two of the chromosome 5, 9, or 15 probes.^{8,9} The final CN profile reflects the integration of FISH and CN analyses. In cases with discrepancies, the FISH results served as ground truth, owing to their single-cell resolution and higher reliability in degraded samples.^{10,11}

Targeted next-generation sequencing analysis

Library preparation and sequencing

Amplicon library preparation and semiconductor sequencing were performed according to the instructions of the manufacturer using the Ion AmpliSeq Library Kit 2.0/Plus, the Ion Library TaqMan Quantitation Kit, the Ion 510 & Ion 520 & Ion 530 Kit - Chef/the Ion 540 Kit - Chef, and the Ion 530/540 Chip Kit (Thermo Fisher Scientific, Waltham, MA, USA). In brief, for each primer pool, 10 ng of DNA were combined with the AmpliSeq HiFi Mix (Thermo Fisher Scientific) and the respective primer sets to amplify the target regions. Subsequently, the primer end sequences were partially digested using FuPa reagent (Thermo Fisher Scientific), followed by the ligation of barcoded sequencing adapters (Ion Xpress Barcode Adapters; Thermo Fisher Scientific). The final libraries were purified using AMPure XP magnetic beads (Beckman Coulter, Brea, CA, USA), quantified using qPCR on the QuantStudio 5 Dx Real-Time PCR System (Thermo Fisher Scientific) and then pooled. Processing of the library pool, including attachment of DNA fragments to Ion Sphere Particles (ISPs), clonal amplification, and enrichment of template-positive ISPs, was performed with the Ion Chef platform (Thermo Fisher Scientific). Sequencing was performed on the Ion GeneStudio S5 Prime using the Ion 530/540 Chip Kit (Thermo Fisher Scientific).

Alignment, variant calling, and filtering

Raw data analysis was performed using the Ion Torrent Software Suite (version 5.16.1, Thermo Fisher Scientific). The reads were aligned to the human reference genome GRCh37/hg19. Bioinformatic analysis was conducted using the Ion Reporter Software (version 5.18.2.0, Thermo Fisher Scientific). Intronic and synonymous variants, as well as known polymorphisms, identified via public databases and online tools such as dbSNP, gnomAD, and VarSome, were excluded.^{12,13} Panel-specific and fixation-related artifacts were excluded by visualizing all variants using the Integrative Genomics Viewer (Broad Institute, Cambridge, MA, USA). Furthermore, in cases with limited DNA integrity, the detection threshold, generally set at an allele frequency of 5%, was adjusted individually to account for the increased number of sequencing artifacts.

Supplemental results

Concordance of FISH and OncoScan

FISH and OncoScan were concordant for hyperdiploidy and chromosome 1 and 17 alterations in 86% of calls, including both aberrant and wild-type findings. Differences could largely be attributed to methodological characteristics of the respective platforms. Very focal CN losses of 1p32.3 and 17p13.1 detected by OncoScan were not detected by FISH, whereas FISH showed higher sensitivity in samples with tumor cell purity <50% (Supplemental Table S4).

Detailed CNA profiles of EMP and EMD

CNA in EMP consisted of 35 CN losses, 1 homozygous deletion, 28 gains, 1 amplification, and 7 CNN-LOH events, whereas the extramedullary disease of multiple myeloma (EMD) samples harbored 84 losses, 7 homozygous deletions, 84 gains, 18 amplifications, and 30 CNN-LOH events (Supplemental Figures S3 and S4; Supplemental Tables S4 and S5). The most recurrent CNA in EMD were losses of 13q14.2–q21.31 (8/10 samples), followed by gains of 5p15.33–p14.1, 15q11.2–q21.1, and 19p (each in 7/10), gains of 3q22.1–q26.31 and 9p (6/10) and gains of 1q21.1–q31.2, 6p, 7q, and 9q (5/10). The most frequent CNN-LOH regions in EMD were located at 20p12.1–p11.21 and 9q22.2–q22.33 (each in 3/10).

CNA comparison with multiple myeloma (MM)

The comparison of the EMD samples with previously published CN data from two MM cohorts (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70399>; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31339>) revealed higher genomic complexity in EMD, with significantly more CNN-LOH events (mean 3.0 vs. 1.3 per case; $p < 0.05$)

and a trend toward a higher number of CNA (mean 19.3 vs. 12.9; $p = 0.065$).^{14,15} No individual CNA region was significantly enriched in either group (Supplemental Figure S6).

Supplemental references

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Supplemental tables

The following tables are provided in a separate file:

Supplemental Table S1. First AmpliSeq custom panel used for next-generation sequencing analysis.

Supplemental Table S2. Second AmpliSeq custom panel used for next-generation sequencing analysis.

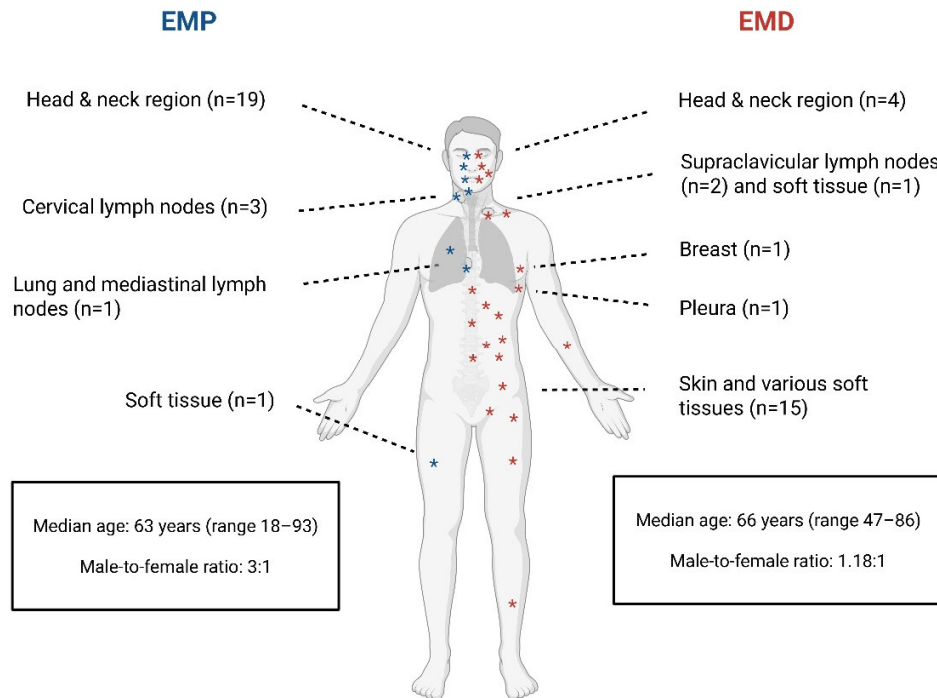
Supplemental Table S3. Clinical characteristics of the EMD cohort.

Supplemental Table S4. Overview of copy number alterations and copy number neutral losses of heterozygosity.

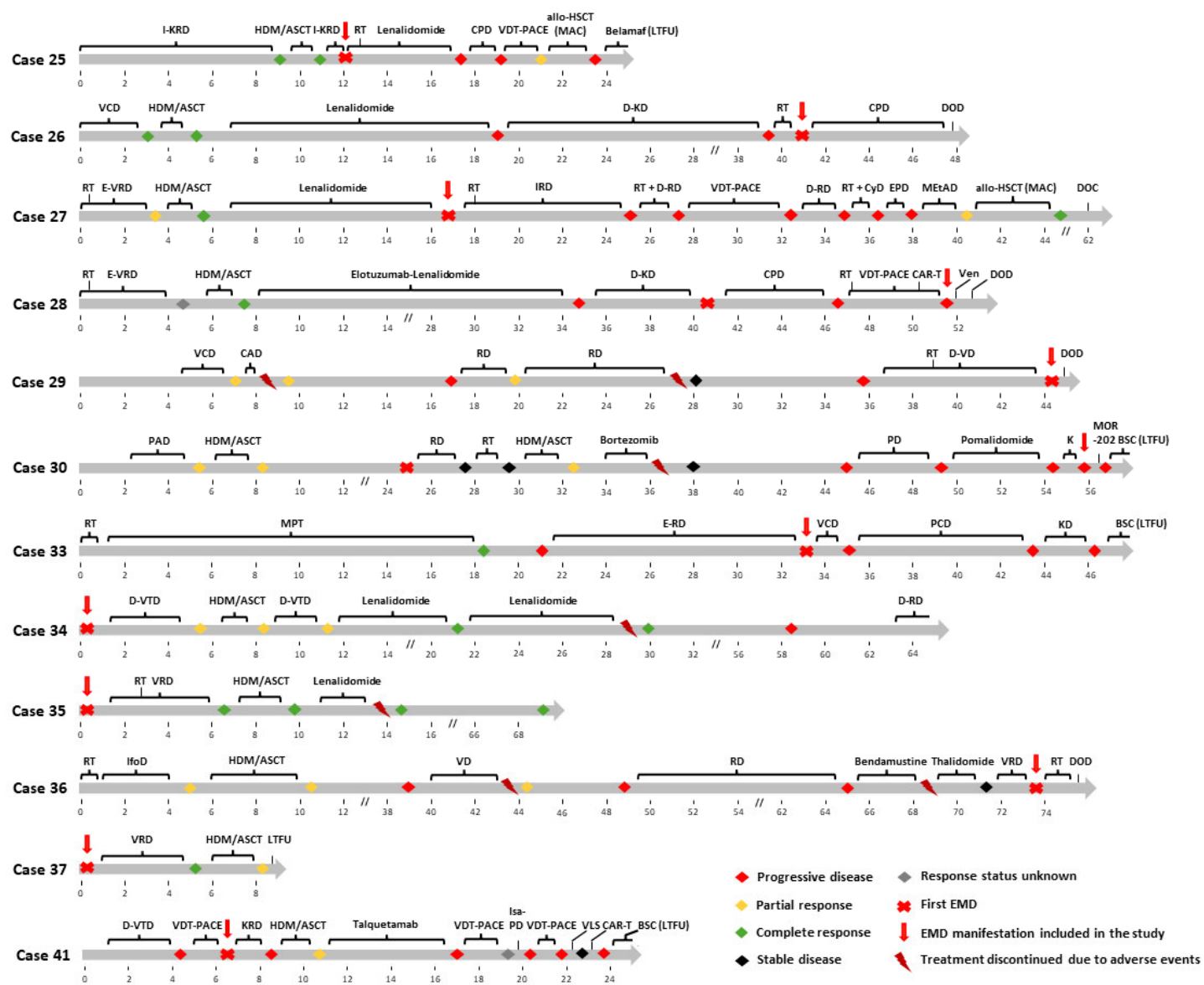
Supplemental Table S5. Summary of copy number alterations and copy number neutral losses of heterozygosity.

Supplemental Table S6. Detailed results of the mutational analysis.

Supplemental figures

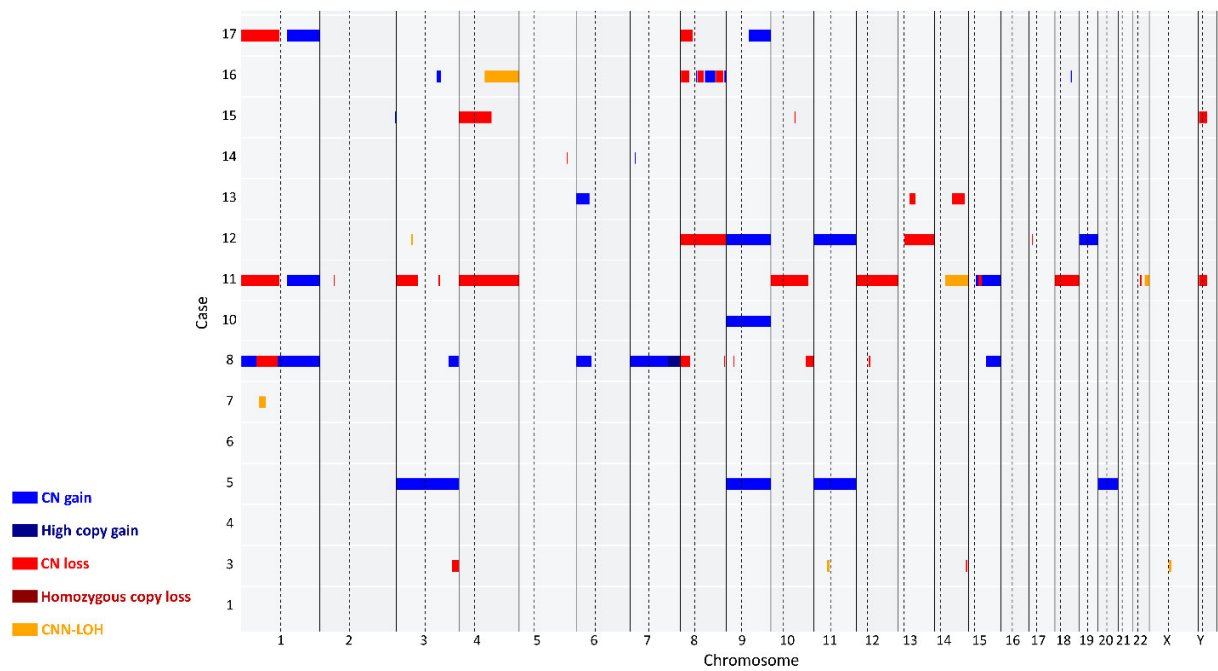


Supplemental Figure S1. Anatomical distribution and patient characteristics. Schematic body map with asterisks indicating sites of manifestation. Extramedullary plasmacytoma (EMP) is shown on the left and extramedullary disease of multiple myeloma (EMD) on the right. In EMP, cervical lymph node involvement is only depicted when it represented the primary site of manifestation. Created in BioRender. Vogelsberg, A. (2026) <https://BioRender.com/bx2fgh8>.

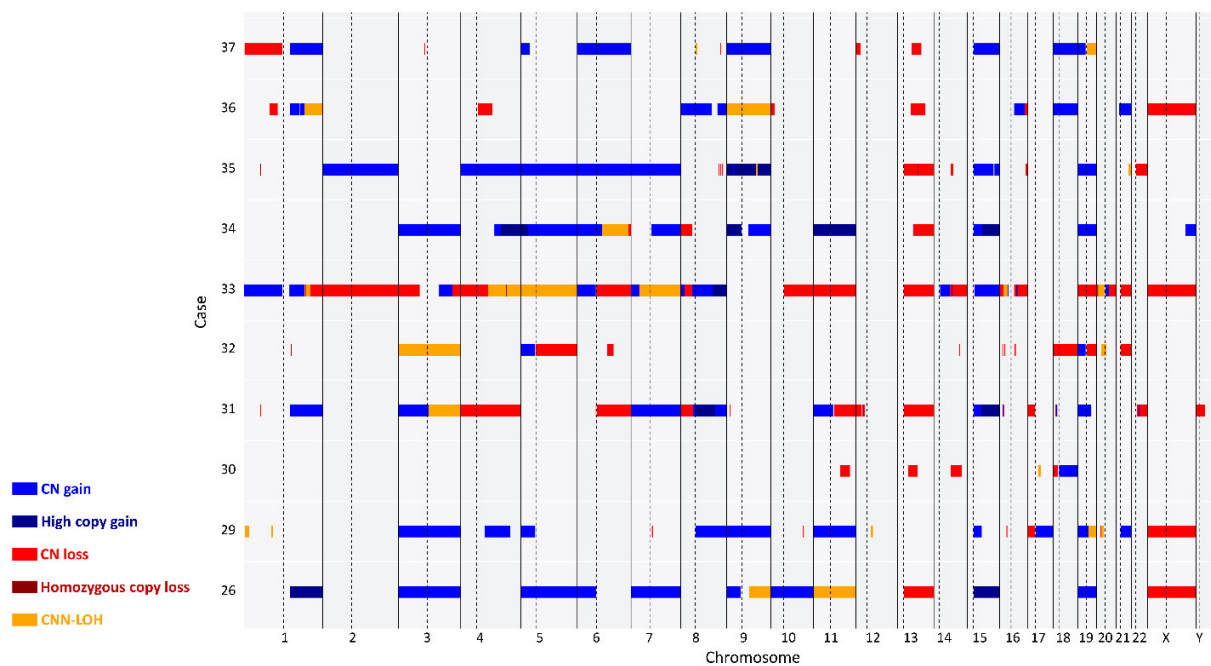


Supplemental Figure S2. Swimmer plot of clinical course and treatments in EMD patients.

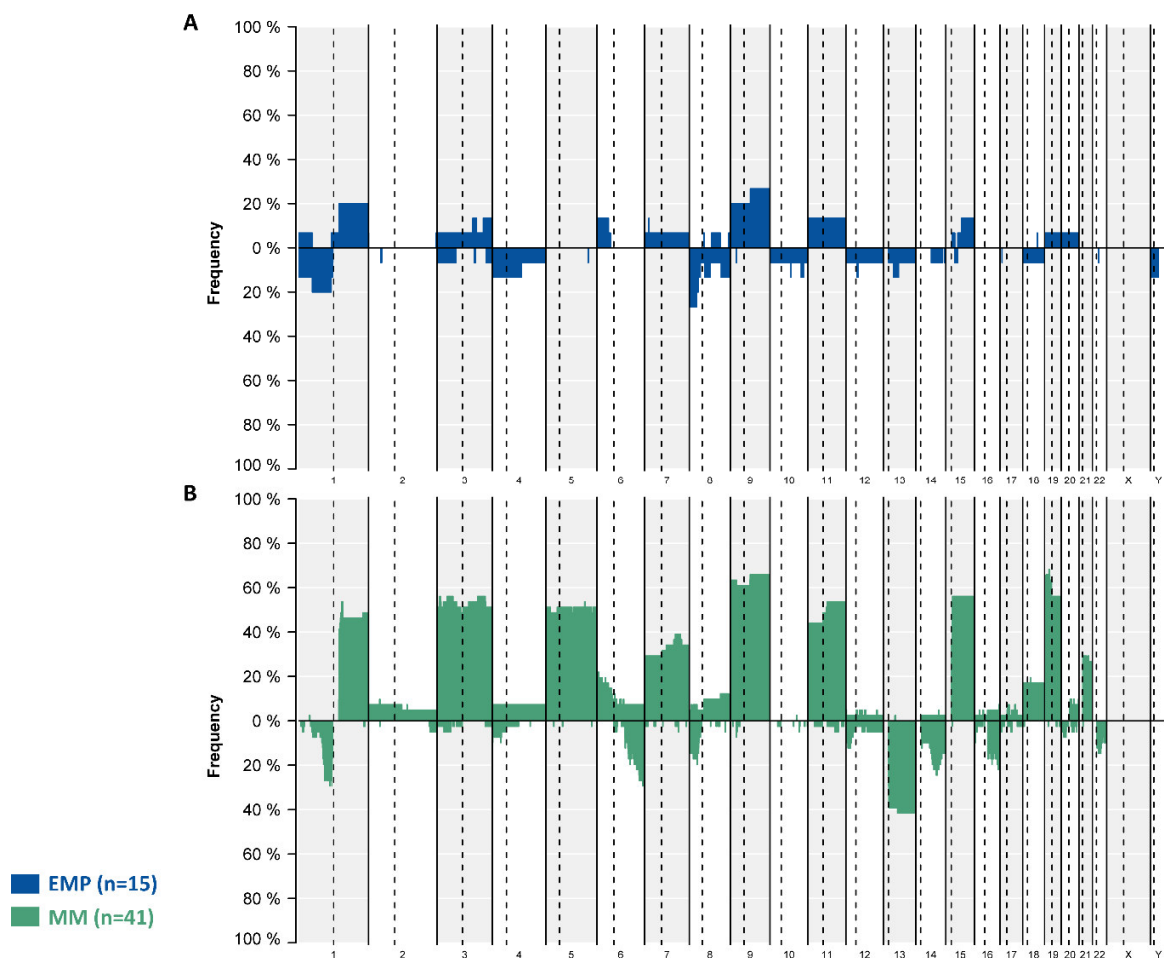
Only EMD patients with sufficient longitudinal clinical data were included. General abbreviations: LTFU, lost to follow-up; EMD, extramedullary disease; DOD, died of disease; DOC, died of other causes. Abbreviations of treatment regimens and agents (regimen abbreviations indicate the presence of agents and do not reflect dose or schedule): allo-HSCT (MAC), allogeneic hematopoietic stem cell transplantation with myeloablative conditioning; Belamaf, belantamab mafodotin; BSC, best supportive care; CAD, cyclophosphamide, doxorubicin, dexamethasone; CAR-T, B-cell maturation antigen–directed chimeric antigen receptor T-cell therapy; CPD, cyclophosphamide, pomalidomide, dexamethasone; CyD, cyclophosphamide and dexamethasone; D-KD, daratumumab, carfilzomib, dexamethasone; D-RD, daratumumab, lenalidomide, dexamethasone; D-VD, daratumumab, bortezomib, dexamethasone; D-VTD, daratumumab, bortezomib, thalidomide, dexamethasone; EPD, elotuzumab, pomalidomide, dexamethasone; E-RD, elotuzumab, lenalidomide, dexamethasone; E-VRD, elotuzumab, bortezomib, lenalidomide, dexamethasone; HDM/ASCT, high-dose melphalan followed by autologous stem cell transplantation; I-KRD, isatuximab, carfilzomib, lenalidomide, dexamethasone; IfoD, ifosfamide and dexamethasone; IRD, ixazomib, lenalidomide, dexamethasone; Isa-PD, isatuximab, pomalidomide, dexamethasone; K, carfilzomib; KD, carfilzomib and dexamethasone; KRD, carfilzomib, lenalidomide, dexamethasone; MEtAD, methotrexate, etoposide, cytarabine, dexamethasone; MOR202, anti-CD38 monoclonal antibody; MPT, melphalan, prednisone, thalidomide; PAD, bortezomib, doxorubicin, dexamethasone; PCD, pomalidomide, cyclophosphamide, dexamethasone; PD, pomalidomide and dexamethasone; RD, lenalidomide and dexamethasone; RT, radiotherapy; VCD, bortezomib, cyclophosphamide, dexamethasone; VD, bortezomib and dexamethasone; VDT-PACE, bortezomib, dexamethasone, thalidomide, cisplatin, doxorubicin, cyclophosphamide, etoposide; Ven, venetoclax; VLS, venetoclax, lenalidomide, selinexor; VRD, bortezomib, lenalidomide, dexamethasone; VTD, bortezomib, thalidomide, dexamethasone.



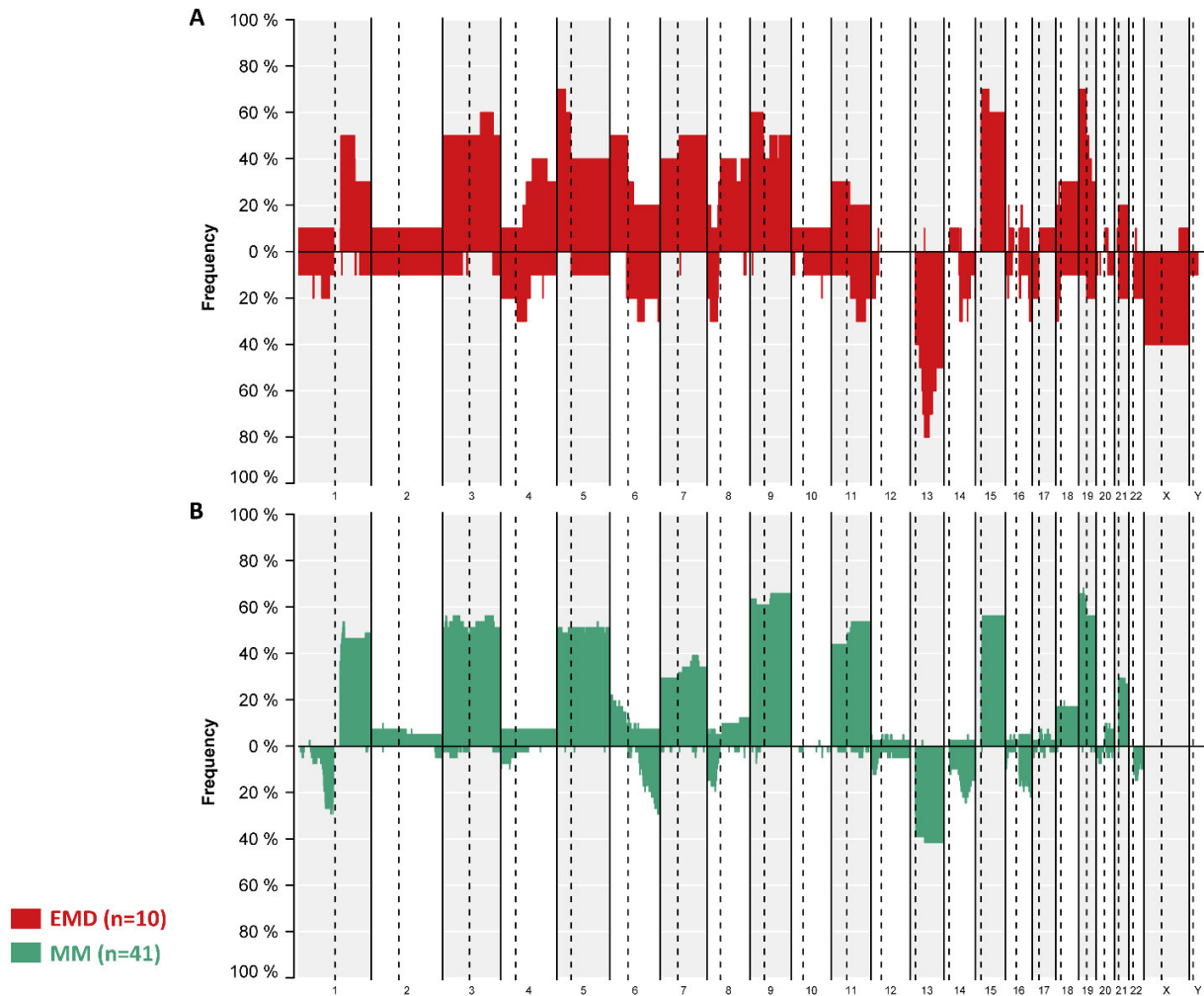
Supplemental Figure S3. Individual CN and CNN-LOH profiles of the EMP cases. Each row corresponds to a single case of extramedullary plasmacytoma (EMP). The presence of each alteration, i.e., copy number (CN) gains and losses, as well as copy number neutral loss of heterozygosity (CNN-LOH), is depicted along the X-axis across chromosomes 1 through Y, from the p- to the q-arm.



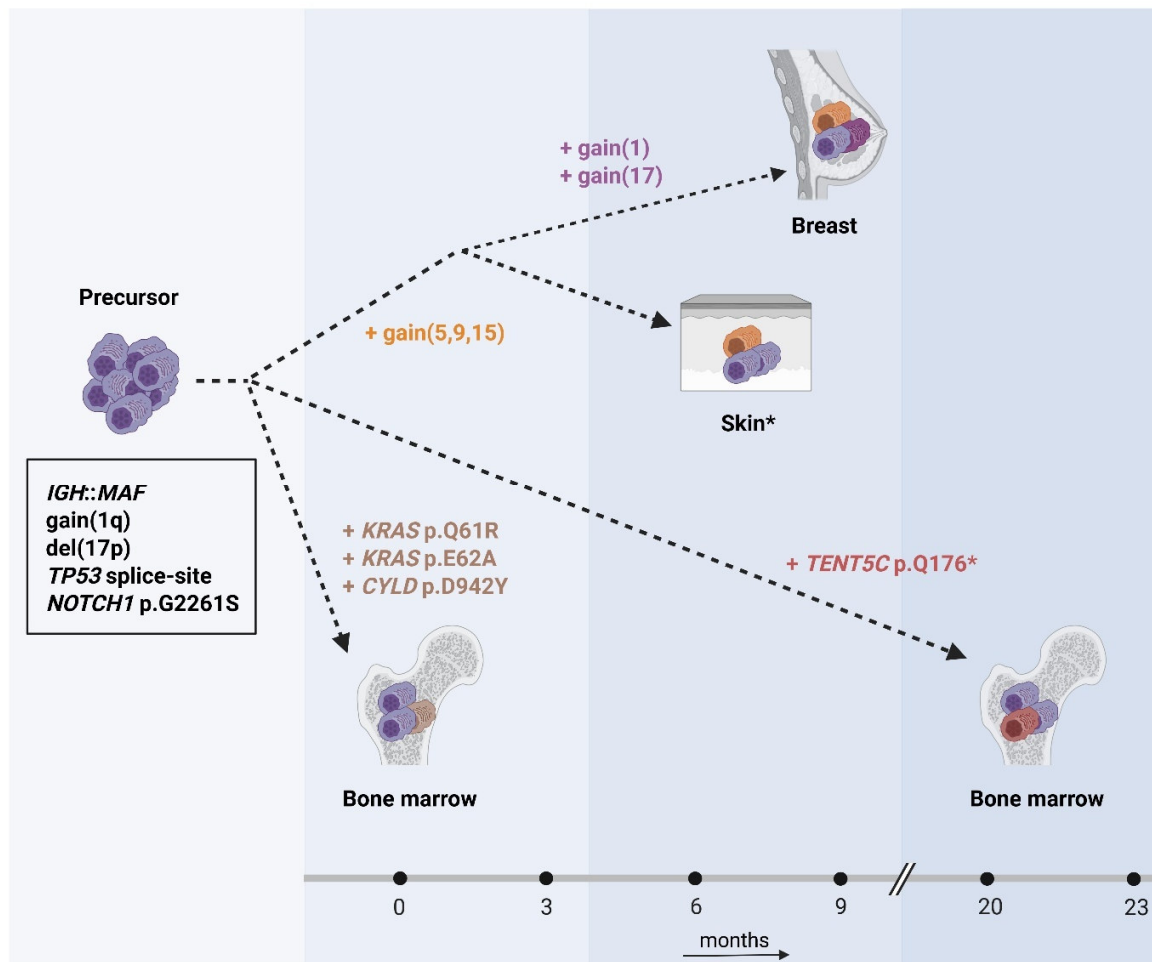
Supplemental Figure S4. Individual CN and CNN-LOH profiles of the EMD cases. Each row corresponds to a single case of extramedullary disease of multiple myeloma (EMD). The presence of each alteration, i.e., copy number (CN) gains and losses, as well as copy number neutral loss of heterozygosity (CNN-LOH), is depicted along the X-axis across chromosomes 1 through Y, from the p- to the q-arm.



Supplemental Figure S5. CNA frequency plots of the EMP cohort and two previously published MM series. The frequency of copy number alterations (CNA), i.e., gains and losses, is shown on the Y-axis, with the X-axis depicting chromosomes 1 through Y, from the p- to the q-arm. Alterations of the extramedullary plasmacytoma (EMP) samples are shown in blue in the top panel (A), whereas those of two previously published series of multiple myeloma (MM) are shown in green in the bottom panel (B).



Supplemental Figure S6. CNA frequency plots of the EMD cohort and two previously published MM series. The frequency of copy number alterations (CNA), i.e., gains and losses, is shown on the Y-axis, with the X-axis depicting chromosomes 1 through Y, from the p- to the q-arm. Alterations of the extramedullary disease of multiple myeloma (EMD) samples are shown in red in the top panel (A), whereas those of two previously published series of multiple myeloma (MM) are shown in green in the bottom panel (B).



Supplemental Figure S7. Illustration of intraclonal heterogeneity in MM. Reconstruction of the clonal evolution of different multiple myeloma (MM) manifestations occurring at different time points and anatomical sites in patient 41. The asterisk (*) marks the sample included in the comparative analyses with extramedullary plasmacytoma. Chromosomal alterations are based on fluorescence in situ hybridization (FISH). All MM manifestations shared an *IGH::MAF* fusion, gain(1q), del(17p), as well as mutations in *TP53* and *NOTCH1*, indicating that these alterations were acquired in a common precursor clone, with the fusion presumably representing the initiating event. Both extramedullary manifestations additionally demonstrated hyperdiploidy (gains of chromosomes 5, 9, and 15) and therefore most likely originated from the same subclone, with the breast manifestation harboring further gains of chromosomes 1 and 17. In contrast, the bone marrow samples harbored private mutations in *KRAS* and *CYLD*, as well as *TENT5C*, respectively, but did not show additional gains of the chromosomes analyzed by FISH. Created in BioRender. Vogelsberg, A. (2026) <https://BioRender.com/v39b5af>.