

Promoter hypermethylation as a reversible mechanism of resistance to GPRC5D-directed therapy in multiple myeloma

GPRC5D-directed immunotherapies, such as the GPRC5D×CD3 bispecific antibody talquetamab (TAL) and the anti-GPRC5D chimeric antigen receptor (CAR) T-cell products MCARH109 and Arlocabtagene autoleucel (Arlo-cel), have shown significant clinical activity in heavily pretreated multiple myeloma (MM) patients.¹⁻³ Most patients however eventually develop resistance, and while biallelic loss of *GPRC5D* on chromosome 12p13.1 is now established as a resistance mechanism in GPRC5D-exposed patients,⁴⁻⁶ Derrien and colleagues have recently described long-range chromatin closure and loss of promoter accessibility at the *GPRC5D* locus as a novel form of epigenetic resistance in talquetamab-resistant patients.⁷ Similarly, Ma and colleagues demonstrated that altered *GPRC5D* promoter methylation results in antigen loss in patients relapsing after anti-GPRC5D CAR T-cell therapy.⁸ Although both studies offered novel mechanistic insights, direct functional implications of *GPRC5D* promoter hypermethylation as a

reversible and putatively druggable mechanism of target evasion have yet to be demonstrated.

Building on our previously published findings,⁹ we first analyzed an expanded whole-genome sequencing (WGS) dataset comprising 402 TAL-naïve patients. All patients provided informed consent in accordance with the Declaration of Helsinki (ethics vote 8/21, University of Wuerzburg). Monoallelic *GPRC5D* alterations (including deletions, frameshift or missense mutations) were detected in 12.9% of cases, whereas the frequency of *GPRC5D* loss, defined as either deletion or copy-neutral loss of heterozygosity (CN-LOH), was substantially enriched at 61.5% (8/13) in a second cohort of TAL-exposed patients with more extensive pretreatment (Figure 1A). Of these TAL-exposed patients, four of eight patients exhibited clonal biallelic *GPRC5D* alterations (biallelic deletions or CN-LOH with a clonal mutation), whereas the remaining four patients carried isolated monoallelic deletions or monoallelic loss

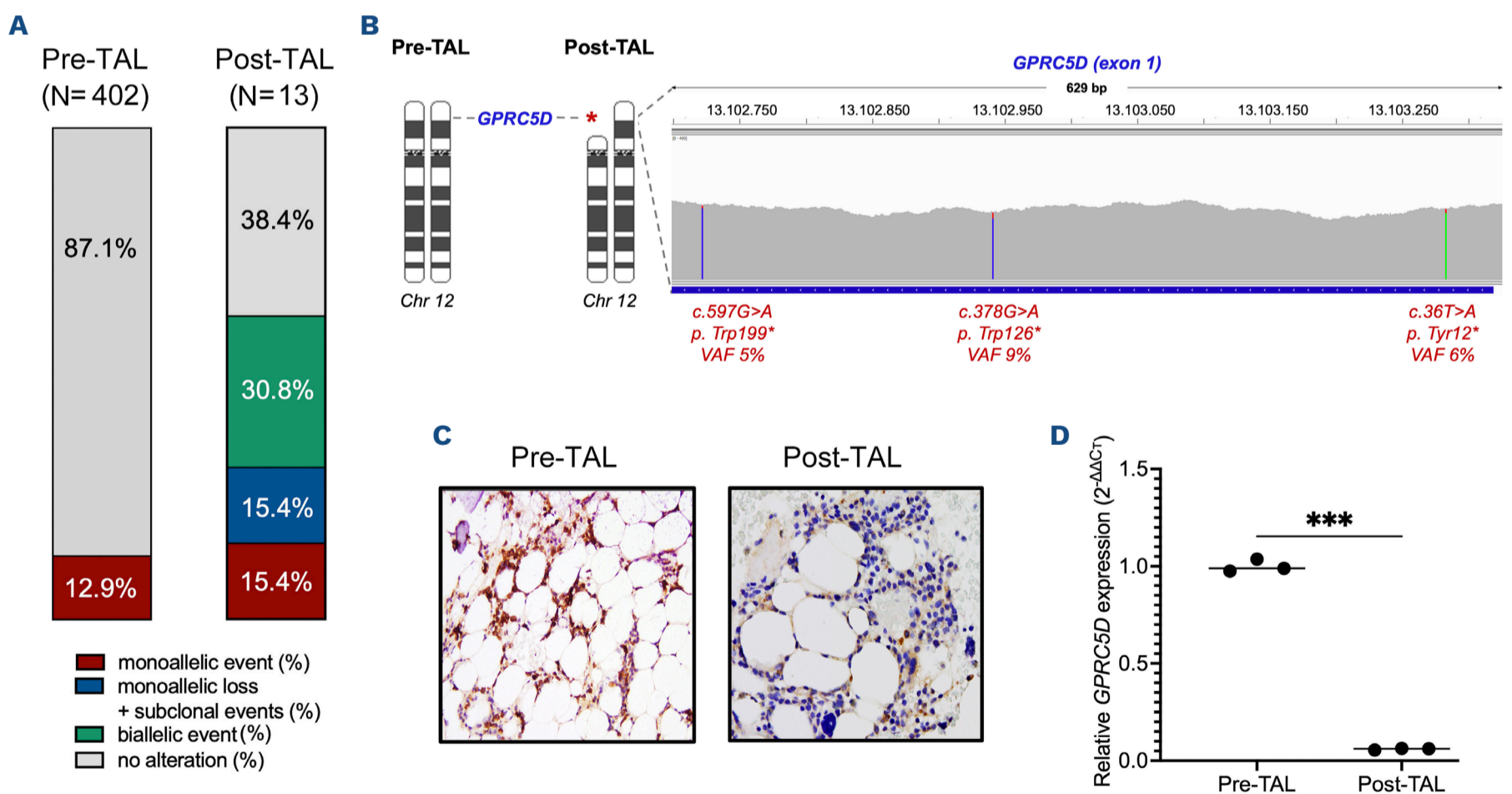


Figure 1. Clinical and functional evidence of monoallelic *GPRC5D* loss. (A) Bar chart demonstrating frequency of *GPRC5D* alterations in talquetamab (TAL)-naïve (N=402) versus TAL-exposed multiple myeloma (MM) patients (N=13). (B) 23.3 Mb monoallelic deletion on chromosome 12p including the G-protein coupled receptor class C group 5 member D (*GPRC5D*) gene and 3 subclonal *GPRC5D* mutations detected in MM1 by whole-genome sequencing. (C) Immunohistochemistry showing complete loss of GPRC5D at relapse. (D) Quantitative polymerase chain reaction analysis showing a 94% reduction of *GPRC5D* mRNA expression at TAL relapse versus baseline.

combined with subclonal events.

From the subset of patients with monoallelic *GPRC5D* alterations, we here report on a 58-year-old patient (MM1) with penta-refractory high-risk MM, including del17p and high-risk gene expression profiling by Sky-92 testing.¹⁰ In his tenth line of therapy, the patient was treated with TAL

and daratumumab as part of the phase Ib TRIMM-2 clinical trial (*clinicaltrials.gov*. Identifier: NCT04108195) and achieved a stringent complete remission (sCR) according to International Myeloma Working Group criteria lasting for a total of 15 months. At relapse, WGS of bone marrow-derived CD138⁺ plasma cells revealed an acquired monoallelic 12p deletion

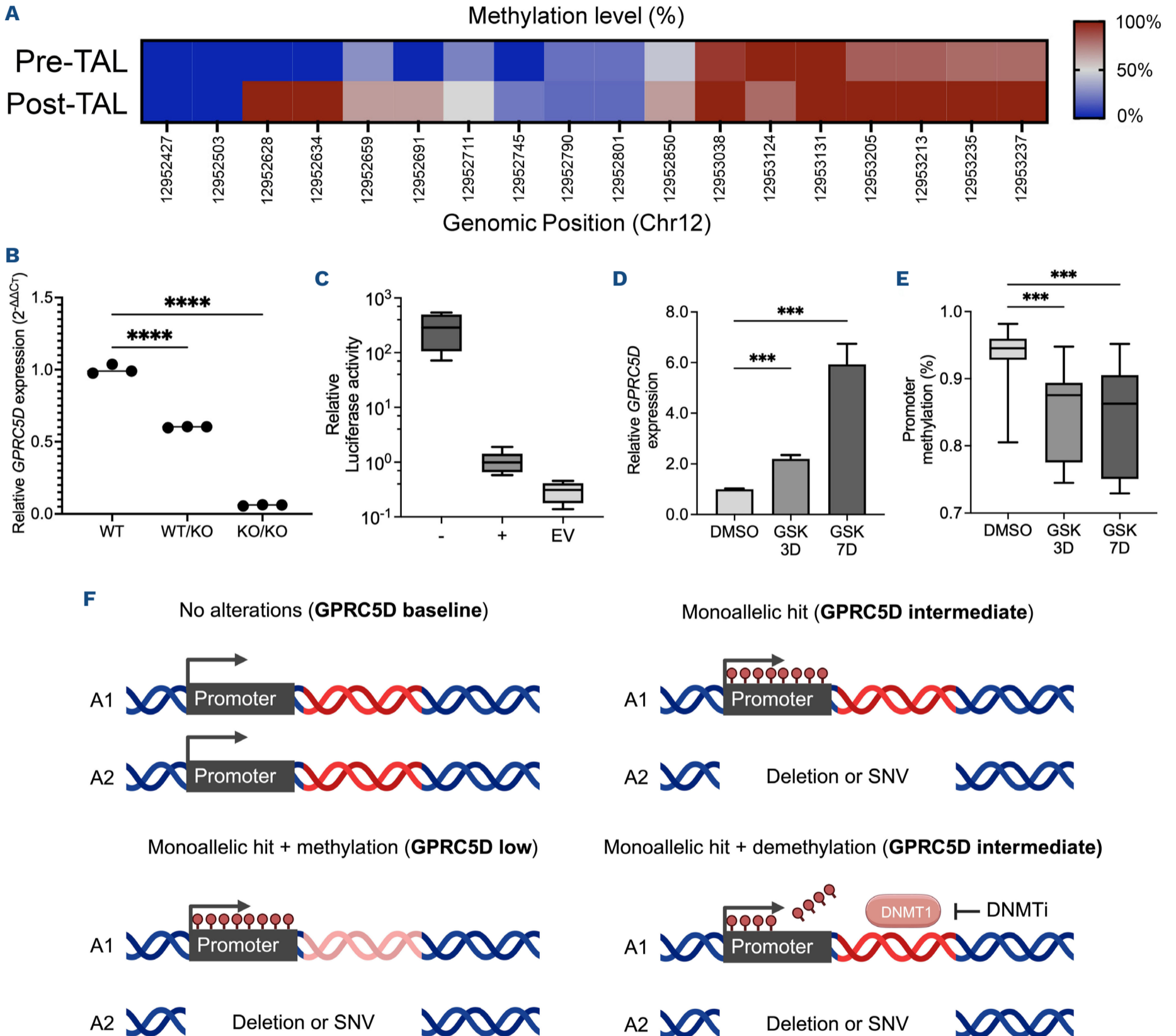


Figure 2. Functional validation of promoter hypermethylation and therapeutic intervention *in vitro*. (A) Whole-genome bisulfite sequencing (WGBS) in patient MM1 at relapse *versus* baseline visualizes increase in G-protein coupled receptor class C group 5 member D (*GPRC5D*) promoter methylation. (B) Quantitative polymerase chain reaction (qPCR) analysis comparing *GPRC5D* mRNA expression in OPM2 with wild-type *versus* monoallelic *versus* biallelic knockout status. (C) Dual-luciferase reporter assay using CpG-free constructs containing the unmethylated (-) *GPRC5D* promoter region *versus* methylated construct (+) *versus* empty vector (EV). (D) qPCR analysis for *GPRC5D* mRNA expression in AMO1 cells treated with the DNA methyltransferase 1 inhibitor (DNMTi) GSK-3484862 after 3 and 7 days of treatment. (E) Promoter methylation in AMO1 cells treated with GSK-3484862 after 3 and 7 days of treatment. (F) Proposed mechanism of *GPRC5D* regulation in patients with pre-existing monoallelic alterations, suggesting promoter hypermethylation as a second hit and potential target for DNMTi. TAL: talquetamab; DMSO: dimethyl sulfoxide. Figure 2F created in BioRender under publication license <https://BioRender.com/hv0ij0d>. Statistical significance is indicated as follows: *** $P < 0.001$; **** $P < 0.0001$.

spanning 23.3 Mb, which included the *GPRC5D* gene, along with three subclones carrying *GPRC5D* mutations (p.Tyr12*, variant allele frequency [VAF] 6%; p.Trp126*, VAF 9%; p.Trp199*, VAF 5%) (Figure 1B). Immunohistochemistry (IHC) analysis demonstrated complete loss at protein level (Figure 1C) and a 94% reduction of *GPRC5D* mRNA expression ($P < 0.0001$) by quantitative polymerase chain reaction (qPCR) (Figure 1D). Given the subclonal nature of the single-nucleotide variants (SNV) which affected the remaining allele, we concluded that these additional genomic events were unlikely to fully account for the near-complete loss of *GPRC5D* expression, but rather support a scenario in which a second epigenetic hit, e.g., by promoter methylation, may have contributed to inactivation of the residual allele.¹¹

To further explore this hypothesis, we assessed promoter methylation in MM1 using whole-genome bisulfite sequencing (WGBS). This analysis revealed a 26.5% increase in *GPRC5D* promoter methylation in the TAL relapse sample of MM1 compared to baseline (Figure 2A). In further validation of this observation, we generated CRISPR-Cas9 OPM2 MM cell line models with either monoallelic or biallelic *GPRC5D* knockouts (KO). Biallelic KO models showed an expected 91.5% reduction in *GPRC5D* mRNA levels, whereas monoallelic alterations reduced *GPRC5D* expression to 57.2% of wild-type (WT) (42.8% reduction) (Figure 2B), confirming that monoallelic gene loss was indeed not sufficient to fully abrogate *GPRC5D* expression. We next investigated if altered promoter methylation may account for the loss of *GPRC5D* expression in MM1. To this end, regulatory promoter activity was studied by Firefly/Renilla dual-luciferase assay¹² using a CpG-free vector (CpGL) containing the *GPRC5D* promoter and the differentially methylated segment for chromosome 12 (12,952,081-12,952,935). *In vitro* methylation reduced the reporter activity by ~278-fold as compared to the unmethylated construct (Figure 2C), providing direct evidence that hypermethylation of this regulatory element suppresses the transcriptional activity of *GPRC5D*. Next, AMO1 MM cells, intrinsically characterized by a hypermethylated *GPRC5D* promoter, were treated with the pre-clinical non-covalent DNA methyltransferase 1 inhibitor (DNMTi) GSK-3484862. DNMT1 inhibition led to a 2.2-fold increase in *GPRC5D* expression by day 3 and a 5.9-fold increase at day 7 after treatment (Figure 2D) along with a significant decrease of promoter methylation (mean methylation difference: -8.6%; $P < 0.001$; Figure 2E).

Taken together, the functional and clinical data presented in this report support an epigenetic second-hit model, in which promoter hypermethylation contributes to *GPRC5D* down-regulation under the selective pressure of *GPRC5D*-targeted therapy.

Over the past years, cellular plasticity and epigenetic adaptation, either through permissive chromatin accessibility or enhancer rewiring, have emerged as key mechanisms of resistance in cancer which enable rapid cellular adaptation without the need for genetic alterations.^{13,14} In an elegant study, Derrien and colleagues previously combined WGS with

single-nucleus RNA- and ATAC-sequencing to describe loss of promoter accessibility together with long-range chromatin closure (~1.15 Mb) around the *GPRC5D* locus in TAL-resistant patients. These observations indicate that epigenetic repression of the promoter may contribute to genetic silencing and subsequent antigen loss.⁷ Beyond chromatin accessibility, Ma and colleagues provided further single-base-resolved DNA methylation data to describe multiple hypermethylated CpGs across *GPRC5D* regulatory regions in post-CAR T relapses lacking biallelic genetic lesions.⁸ The methylation levels of these regions showed an inverse correlation with *GPRC5D* mRNA expression across MM cell lines. However, the specific regulatory elements demethylated by treatment, as well as a direct causal relationship at the promoter site, were not identified. Our study adds to this gap in knowledge by demonstrating a direct link between epigenetic alterations at the *GPRC5D* promoter and reduced *GPRC5D* expression. Using CRISPR gene edited mono- versus biallelic *GPRC5D* KO models, we further delineate that monoallelic *GPRC5D* loss does not fully abolish expression but rather implicates the need for a second epigenetic hit for full transcriptional loss. These findings propose a model in which MM patients with baseline monoallelic *GPRC5D* deletions may be particularly susceptible to secondary promoter hypermethylation (Figure 2F). Moreover, our data provide first evidence that promoter hypermethylation can be overcome by targeted inhibition of DNA methyltransferases. This co-treatment strategy, which aims at preventing target evasion, may have broader implications for overcoming epigenetic resistance to immunotherapies across other targets, including the BCMA-coding gene *TNFRSF17*¹⁵, as well as for other malignancies beyond MM.

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Contributions

SH, UM, JMW, and KMK conceptualized and designed the study. Experimental work and data acquisition were conducted by SH, MT, EG, LP, AH, SK, SN, CV, EB, NR, JL and MK. Data processing and statistical analyses were performed by SH, UM, MT, EG, FS, YT, XZ, TS, CH, AR, JMW, and KMK. The manuscript was drafted by SH, MT, JMW, and KMK, and revised and approved by all authors.

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Data-sharing statement

De-identified methylation data from whole-genome bisulfite sequencing (WGBS) are available under access number 10.5281/zenodo.17709806. Adherence to the Datenschutz-Grundverordnung (<https://dsgvo-gesetz.de/> and [https://data.consilium.europa.eu/doc/document/ST-5419-2016-REV\[1\]1/en/pdf](https://data.consilium.europa.eu/doc/document/ST-5419-2016-REV[1]1/en/pdf)) is mandatory for sharing genome-wide sequencing data. Thus, the raw genome-wide sequencing data of a single patient cannot be shared according to European law. Processed data from which the identification of a patient is not possible can be made available upon reasonable request.

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