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LETTER

High levels of global hydroxymethylation predict worse overall survival in MDS patients treated with azacitidine

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Myelodysplastic syndromes (MDS) are a heterogeneous group of hematological malignancies characterized by cytopenia, dysplasia, and a risk of progressing to acute myeloid leukemia (AML).[1] Using the international prognostic scoring systems (IPSS, IPSS-R, and recently IPSS-M), patients can be categorized into different risk groups for overall and leukemia-free survival.[2–4] In combination with fitness and individual preferences, the therapeutic strategy for each patient is determined.[5] Currently, the strategies most commonly used are best supportive care (BSC) with or without EPO/G-CSF in lower-risk MDS, lenalidomide (LEN) in patients with a del(5q), or luspatercept in patients with ring sideroblasts/*SF3B1* mutations. In higher-risk MDS, hypomethylating agents (HMAs), chemotherapy, and/or stem cell transplantation can be considered. MDS patients carry mutations in genes involved in DNA methylation including *TET2* (20%–30%), *DNMT3A* (10%), and *IDH1/2* (5%–10%).[6] DNMT3A is a DNA methyltransferase that converts cytosine (C) into 5-methylcytosine (5mC). Methylated DNA can in turn be actively demethylated by TET enzymes (including TET2), converting 5mC into 5-hydroxymethylcytosine (5hmC) which is further converted into cytosine by subsequent actions of TET proteins, thymidine DNA glycosylase (TDG), and the base excision repair (BER) pathway. Mutations in *TET2* result in defective enzymatic activity and significantly decreased levels of 5hmC. TET proteins need vitamin C, $Fe^{2+,}$ and alpha-ketoglutarate (α-KG) as cofactors for proper enzymatic activity. The latter is produced by IDH1/2 enzymes. Mutations in *IDH1* and *IDH2* result in the aberrant production of 2-hydroxyglutarate instead of α-KG, which inhibits TET activity. Therefore, also in *IDH1/2* mutated cells, decreased 5hmC levels can be observed.[7]

Cancer cells often show hypermethylation, which may result in silencing of tumor suppressor genes.[8] The methylation process is reversible and can be influenced by the administration of HMAs like azacitidine (AZA) and decitabine. Both compounds have shown important activity in MDS and AML.[9] HMAs are analogs of the nucleoside cytidine and they are incorporated into the DNA during DNA replication, inhibiting the DNA methylation process and causing hypomethylation. In addition, 80%–90% of azacitidine is incorporated into the RNA. As not all patients respond to HMAs and the response may take several courses of therapy before an effect becomes apparent,[10] the identification of markers that predict response is warranted. Recently, a set of 39 methylation sites was found significantly different in MDS patients responding to AZA, compared to non-responders.[11] We previously demonstrated that in AML patients receiving high-dose chemotherapy, high 5hmC was an independent prognostic marker for poor overall survival (OS).[12] In this study, we assessed the impact of global 5mC and 5hmC on OS in MDS patients receiving BSC, LEN, or AZA.

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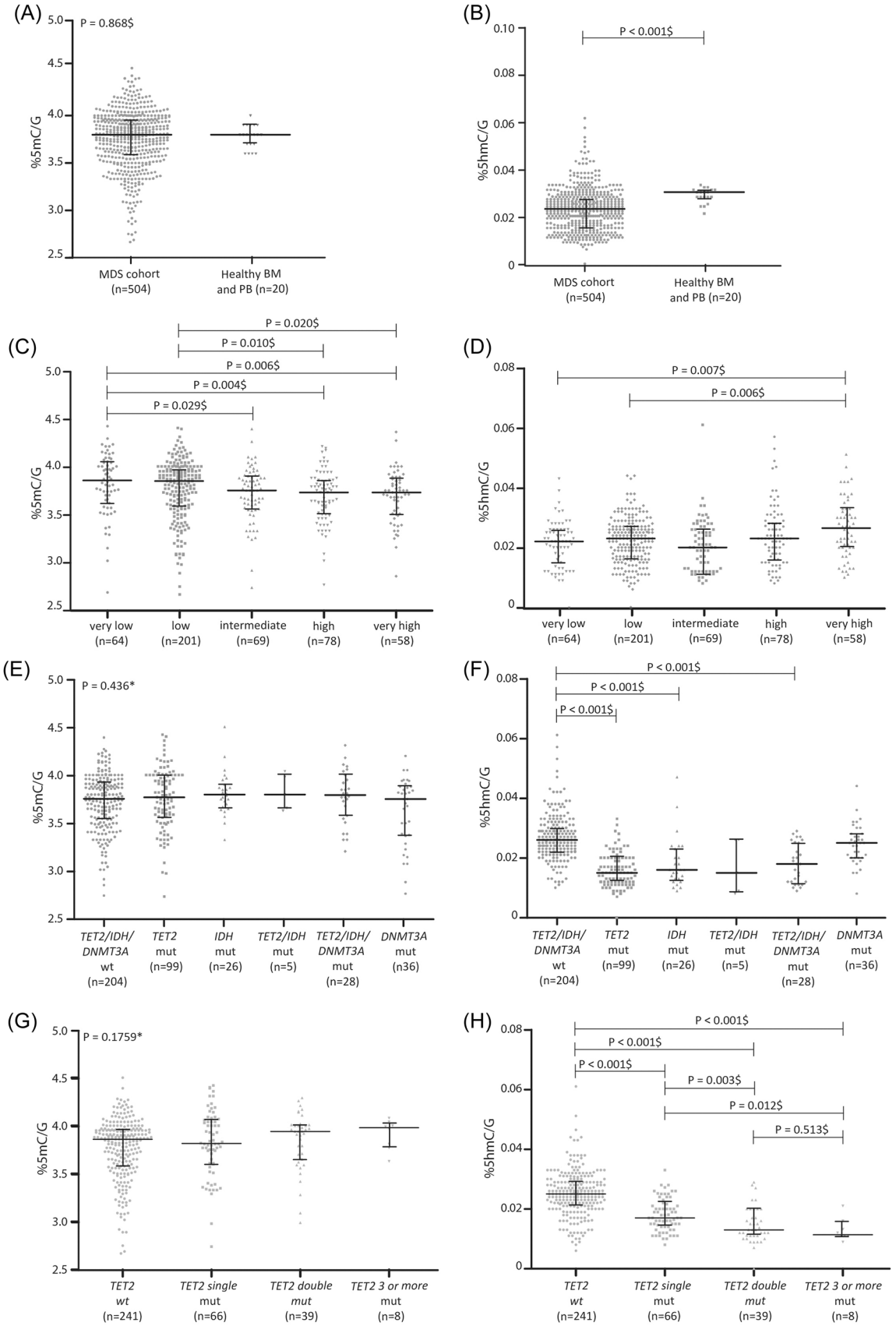


FIGURE 1 (See caption on next page).

FIGURE 1 **5hmC and 5mC levels. (A)** 5mC levels in MDS patients ($n = 504$) are comparable to healthy controls. **(B)** 5hmC levels in MDS patients ($n = 504$) are significantly lower compared to healthy controls. **(C)** 5mC is higher in patients with very low and low IPSS-R compared to the intermediate, high, and very high IPSS-R risk groups. **(D)** 5hmC is higher in patients with very high IPSS-R scores, compared to very low and low IPSS-R patients. **(E)** 5mC levels are not affected by mutations in any of the genes involved in the (de)methylation pathway. **(F)** 5hmC levels are lower in patients carrying a *TET2* or *IDH1/2* mutation. **(G)** 5mC levels are not impacted by the number of *TET2* mutations. **(H)** 5hmC decreases when there are two mutations in *TET2*, compared to only one. "$" symbol indicates that the *p*-value is obtained using the Mann–Whitney test and "*" indicates *p*-values obtained in a Kruskal–Wallis test. The bars in the plots indicate the mean values and the interquartile ranges.

To do so, we measured 5mC and 5hmC in 504 MDS patients (demographics in Table S1) and 20 healthy controls. We isolated DNA from bone marrow or peripheral blood samples, collected before treatment. We measured 5mC and 5hmC using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), as described.[12] To perform the analyses, we grouped the patients into three cohorts, based on the received treatment (BSC, LEN, or AZA). To assess the impact of 5mC and 5hmC on OS, we divided the cohorts into quartiles based on 5mC and 5hmC levels. In addition, we analyzed the mutational profile using a panel of frequently mutated genes in myeloid malignancies.

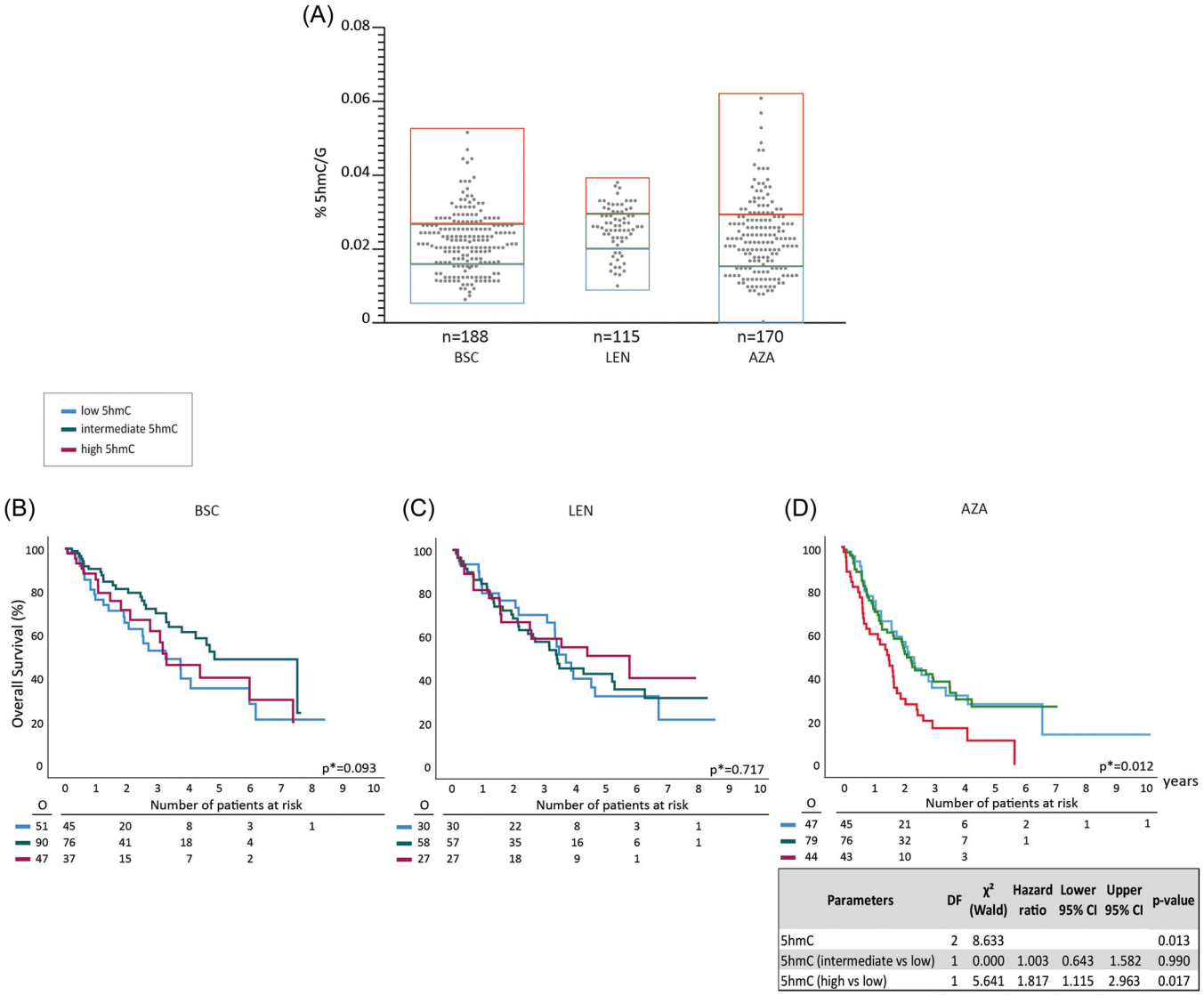
Median 5mC levels were comparable between MDS patients and healthy controls, but in MDS patients, the values were distributed across a broader range (Figure 1A; median MDS = 3.8, range = 2.665–4.500; median healthy donors = 3.8, range = 3.600–4.000, $p = 0.868$). In contrast, the median value of the demethylation intermediate 5hmC was significantly lower in MDS patients compared to healthy controls (Figure 1B; median MDS = 0.023, range = 0–0.061; median healthy controls = 0.030, range = 0.021–0.033, $p < 0.001$). Overall methylation (5mC) was lower in higher IPSS-R categories (Figure 1C) whereas 5hmC levels were increased in very high-risk patients (Figure 1D). *TET2* mutations were found in 31% of the patients, *DNMT3A* in 13%, and *IDH1/2* in 8% (Figure S1 and Table S2), which is in line with previous studies.[6,13,14] 5mC was not significantly influenced by the presence of *TET2/IDH* mutations or by *DNMT3A* mutations (Figure 1E). As expected, 5hmC was significantly decreased in patients carrying mutations in *TET2* and *IDH1/2*, compared to patients with wild type *TET2* and *IDH1/2* (Figure 1F; median *TET2/IDH/DNMT3A*wt = 0.026; median *TET2*mut = 0.015, $p < 0.001$; median *IDH1/2*mut = 0.016, $p < 0.001$). Mutations in *TET2* or *IDH1/2* can co-occur with mutations in *DNMT3A*. In these patients, the 5hmC levels were comparable to the 5hmC in patients with solitary *TET2* or *IDH1/2* mutations (median *TET2/IDH/DNMT3A*mut = 0.018, $p < 0.001$). The overall 5mC values did not increase significantly in patients with single or more *TET2* mutations (Figure 1G); 5hmC levels decreased in the case of a single *TET2* mutation, which was further decreased in the case of two mutations (Figure 1H). In patients with three or more mutations (suggestive of the presence of separate *TET2* mutated clones), the 5hmC levels did not further decrease. Mutations in *TET2* are considered to cause a loss of function,[15] irrespectively of the type of mutation. This was confirmed by the observation that no significant differences were found in 5hmC depending on the type of mutation (frameshift versus nonsense or missense mutations) (data not shown).

As expected, the OS of our cohort was highly influenced by the IPSS-R risk group (Figure S2A; $n = 459$, $p < 0.001$). Patients receiving AZA performed worse compared to patients receiving BSC or treated with LEN (Figure S2B; $p < 0.001$), which is in line with the higher IPSS-R risk patients present in this treatment group (Figure S2C).

It was previously reported that *TET2* mutations independently predict better response to HMA treatment,[16,17] but no effect was seen on overall survival. In our study, we found an improved OS in *TET2* mutated patients receiving AZA (Figure S3A; $n = 170$, $p = 0.021$); however, this was not significant in multivariate analysis including IPSS-R (Figure S3B; HR = 0.797, 95% HR = 0.512–1.239, $p = 0.313$).

To investigate whether pre-treatment 5mC/5hmC levels have an effect on OS in patients receiving different treatment modalities, we divided the cohort based on the treatments received by the patients: BSC ($n = 194$), LEN ($n = 115$), and AZA ($n = 170$); for each cohort, we divided the patients into quartiles based on the levels of 5mC (Figure S4A) and 5hmC (Figure 2A). The 5mC status (from the time of sampling, before the start of the treatment) did not have an impact on the OS of MDS patients who received any of these three different regimes (Figure S4B–D). Our results do not confirm data from a previous study, in which it was reported that the level of 5mC was predictive of overall survival.[18] However, to measure the global methylation, the authors used enzyme-linked immunosorbent assay (ELISA), reported to be less accurate and sensitive compared to HPLC-MS/MS.[19] This difference makes it hard to compare the group of patients defined as low or high 5mC in the two studies. Furthermore, the studied cohort was smaller, and the majority of the patients had lower or intermediate IPPS-R scores, which might have influenced the definition of high/low 5mC and therefore the results. Next, we analyzed the effect of the demethylation intermediate 5hmC on OS. The 5hmC levels did not have an impact on MDS patients who received supportive care or on patients treated with LEN (Figure 2B,C). We also did not observe any impact of 5hmC on the OS of patients treated with LEN, neither when performing the analysis separately in del(5q-) ($n = 21$) and non del(5q-) patients ($n = 82$) (data not shown). Interestingly, in patients receiving AZA, high 5hmC levels (≥0.0290) correlated with a significantly worse OS (Figure 2D; $p = 0.012$) compared to low (≤0.0150) or intermediate 5hmC levels (5-year OS low 5hmC = 27.9%, intermediate 5hmC = 26.8%, and high 5hmC = 11.2%). This could not be explained by a difference in the number of AZA cycles that was received (median = 7 in all groups). Since patients with low and intermediate 5hmC levels did not show a significantly different OS, they were considered as one group in further assessments. In the multivariate analysis, together with the IPSS-R, the effect of the 5hmC was less striking, but the same trend was still observed (Figure 2E; HR = 1.477, 95% CI = 0.977–2.233, $p = 0.064$).

The exact mechanism behind the difference in response to AZA is not clear, but it can be hypothesized that in patients with low and moderate 5hmC levels, the tumor cells may be dependent on the silencing of specific tumor-suppressor genes by hypermethylation, which may be corrected by hypomethylating agents such as AZA. Conversely, tumor cells with already very active demethylation (high 5hmC) may be transformed in a different manner, being less dependent on the hypermethylation of specific tumor suppressor genes, and therefore less responsiveness to hypomethylating agents. It would be interesting to identify the crucial genomic areas and associated genes and test their methylation status before and during treatment with AZA. We conclude that the pre-treatment, global 5hmC level is a prognostic marker and that lower 5hmC levels can help to identify MDS patients who are more likely to respond to AZA treatment. These results should be confirmed in an independent MDS cohort treated with AZA, as well as in a patient cohort treated with decitabine.



Parameters	DF	χ^2 (Wald)	Hazard ratio	Lower 95% CI	Upper 95% CI	p-value
5hmC	2	8.633				0.013
5hmC (intermediate vs low)	1	0.000	1.003	0.643	1.582	0.990
5hmC (high vs low)	1	5.641	1.817	1.115	2.963	0.017

(E)

Parameters	DF	χ^2 (Wald)	Hazard ratio	Lower 95% CI	Upper 95% CI	p-value
5hmC (low+intermediate vs high)	1	3.425	1.477	0.977	2.223	0.064
IPSS-R	4	35.841				< 0.001
IPSS-R (very low vs very high)	1	1.721	0.421	0.116	1.536	0.190
IPSS-R (low vs very high)	1	15.904	0.139	0.052	0.368	< 0.001
IPSS-R (intermediate vs very high)	1	21.799	0.256	0.144	0.455	< 0.001
IPSS-R (high vs very high)	1	16.796	0.420	0.275	0.642	< 0.001

FIGURE 2 **Impact of 5hmC status on the overall survival of MDS patients undergoing different treatment regimes. (A)** 5hmC divided into the highest quartile (red), intermediate 50% (green), and lowest quartile (light blue) in patients receiving BSC, LEN, or AZA. **(B)** In MDS patients receiving BSC, the level of 5hmC does not have an impact on OS. **(C)** In MDS patients treated with LEN, the level of 5hmC does not have an impact on the overall survival. **(D)** In MDS patients receiving AZA, the highest quartile of 5hmC levels correlates to worse overall survival when compared to patients with low and intermediate 5hmC levels. The symbol "*" indicates that the p-value was calculated using the log-rank test. **(E)** Multivariate Cox regression analysis. In the multivariate analysis, the effect of 5hmC was analyzed after grouping the low and intermediate 5hmC quartiles, together with IPSS-R (as categorical value, taking IPSS-R very high as the reference category).

AUTHOR CONTRIBUTIONS

Francesca Tiso, Florentien E. M. in 't Hout, and Joop H. Jansen designed the research, analyzed data, and wrote the paper. Ruth Knops and Arno van Rooij performed the experiments. Arjan A. van deLoosdrecht, Theresia M. Westers, Saskia M. C. Langemeijer, Claude Preudhomme, Nicolas Duployez, Pierre Fenaux, Olivier Kosmider, Didier Bouscary, Lionel Adès, and Michaela Fontenay provided patient material and the clinical data. Aniek O. deGraaf contributed

to the sequencing data and all authors discussed the results and commented on the manuscript at all stages.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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