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A disintegrin and metalloprotease 23 hypermethylation predicts decreased disease-free survival in low-risk breast cancer patients

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A Disintegrin And Metalloprotease 23 (ADAM23), a member of the ADAM family, is involved in neuronal differentiation and cancer. *ADAM23* is considered a possible tumor suppressor gene and is frequently downregulated in various types of malignancies. Its epigenetic silencing through promoter hypermethylation was observed in breast cancer (BC). In the present study, we evaluated the prognostic significance of *ADAM23* promoter methylation for hematogenous spread and disease-free survival (DFS). Pyrosequencing was used to quantify *ADAM23* methylation in tumors of 203 BC patients. Presence of circulating tumor cells (CTC) in their peripheral blood was detected by quantitative RT-PCR. Expression of epithelial (*KRT19*) or mesenchymal (epithelial-mesenchymal transition [EMT]-inducing transcription factors *TWIST1*, *SNAI1*, *SLUG* and *ZEB1*) mRNA transcripts was examined in CD45-depleted peripheral blood mononuclear cells. *ADAM23* methylation was significantly lower in tumors of patients with the mesenchymal CTC ($P = .006$). It positively correlated with Ki-67 proliferation, especially in mesenchymal CTC-negative patients ($P = .001$). In low-risk patients, characterized by low Ki-67 and mesenchymal CTC absence, *ADAM23* hypermethylation was an independent predictor of DFS ($P = .006$). Our results indicate that ADAM23 is likely involved in BC progression and dissemination of mesenchymal CTC. *ADAM23* methylation has the potential to function as a novel prognostic marker and therapeutic target.

KEYWORDS
ADAM23 gene, breast cancer, disease-free survival, hematogenous dissemination, mesenchymal circulating tumor cell

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1 | INTRODUCTION

Breast cancer (BC), accounting for 15.6% of all cancer deaths in the European Union (EU), is the most common female malignancy. In 2015, Slovakia recorded the second highest standardized death rate for BC among the EU Member States (40.6 per 100 000 women).[1] Despite relatively effective treatment, metastatic disease is responsible for most patient deaths with distant metastases preferentially localized in bone, lung, liver and brain.[2,3]

It is known that epigenetic deregulation, including aberrant DNA methylation, plays an important role in BC development and progression, mainly by affecting expression of specific genes. Therefore, aberrant cancer-specific methylation patterns can serve as diagnostic and prognostic markers.[4-6]

In addition, increased proliferative activity characterized by nuclear antigen Ki-67 determination is typical for malignant breast tumors.[7] Several studies confirmed the prognostic value of Ki-67 expression when high rates of Ki-67 were associated with reduced survival and aggressive tumor features.[8,9]

One of the key invasive tumor characteristics is the ability to release cancer cells from the primary tumor site. Circulating tumor cells (CTC) are individual cells or cell clusters that have detached and entered the vascular system, although only very few aggressive subpopulations have the potential to initiate the formation of metastases.[10,11] The presence of CTC in peripheral blood of BC patients represents a strong prognostic and predictive factor and is associated with development of distant metastases, decreased survival and response to treatment.[12-16.]

Accumulating evidence points to a critical role of an epithelial-mesenchymal transition (EMT) in metastatic progression.[17,18] During EMT, epithelial cancer cells lose epithelial (E-cadherin, desmoplakin, cytokeratins etc.) and gain mesenchymal (N-cadherin, vimentin, fibronectin etc.) marker expression. They turn into motile mesenchymal cells with invasive and metastatic properties, and some of them also gain cancer stem cell (CSC) characteristics.[19-23] Therefore, commonly used CTC identification strategies exclusively based on epithelial marker detection omit patients who have the most aggressive CTC subpopulations with the mesenchymal phenotype in their peripheral blood.[24] It has been shown in several studies that mesenchymal CTC presence strongly correlates with worse prognosis and risk of developing distant metastases compared to epithelial CTC.[25,26]

A Disintegrin And Metalloprotease (ADAM) proteins, consisting of multiple domains, operate in various processes such as ectodomain shedding of growth factors, cytokines or receptors, cleavage and remodeling of extracellular matrix proteins and adhesive activities through their disintegrin and cysteine-rich domains.[27] Among 21 human ADAM proteins, ADAM23 belongs to a non-catalytic group because of its inactive metalloprotease domain.[28,29] However, ADAM23 has been shown to have specific binding affinity for $\alpha_V\beta_3$ integrin, which is mediated by a short amino acid sequence present in its putative disintegrin loop. Through its disintegrin-like domain, ADAM23 may function as an adhesion molecule involved in $\alpha_V\beta_3$-mediated cell interactions occurring in normal and pathological processes.[30] In addition to $\alpha_V\beta_3$, the disintegrin domain of ADAM23 also ensures specific binding to other integrins such as $\alpha_4\beta_1$ and $\alpha_4\beta_7$.[31,32] Based on its adhesion function, *ADAM23* is considered a possible tumor suppressor gene which is frequently downregulated in various types of malignancies including breast, head and neck, lung, gastric, brain and ovarian cancers.[33-38] Its epigenetic silencing through promoter hypermethylation[31,33] and association between *ADAM23* methylation and clinical characteristics has been previously observed in BC patients.[31,33,39,40]

In the present study, we evaluated the prognostic significance of *ADAM23* promoter methylation for hematogenous spread and disease-free survival (DFS).

2 | MATERIALS AND METHODS

2.1 | Patients

All analyses were carried out on samples of 203 invasive BC patients, TNM stages I-III, collected between March 2012 and March 2014. The Institutional Review Board of the National Cancer Institute of Slovakia approved this study, and written informed consent was obtained from all participants before study enrolment. Formalin-fixed paraffin-embedded (FFPE) tumor tissue and peripheral blood were collected from each patient. Sampling for CTC detection was carried out in the morning on the day of surgical procedure. Patients who suffered from a concurrent malignancy other than non-melanoma skin cancer in the previous 5 years were excluded. Relevant clinicopathological data from an identical sample set were recorded and described previously.[41] Briefly, 76.8% (n = 156) of 203 patients were older than 50 years, 32.5% (n = 66) were diagnosed with T-stage II or III and 40.6% (n = 82) with lymph node positivity. Valid percentages are presented for all parameters, missing data are excluded from calculations. Histological subtypes consisted of 86.2% (n = 175) invasive ductal, 11.3% (n = 23) invasive lobular and 2.5% (n = 5) tubular or mucinous carcinomas. Histological grade 3 was diagnosed in 37.2% (n = 74), hormone receptor negativity in 16.5% (n = 33), human epidermal growth factor receptor 2 (HER2) positivity (HER2+) in 15.8% (n = 32) and high Ki-67 proliferation (cut-off 20%) in 42.6% (n = 86) of patients. CTC positivity was detected in 22.9% (n = 44) of patients, of which epithelial CTC were present in 9.2% (n = 14), mesenchymal CTC in 15.8% (n = 27) and both epithelial and mesenchymal CTC in 1.6% (n = 3) of patients.

2.2 | Detection of CTC in peripheral blood

RosetteSep Human CD45 Depletion Cocktail (StemCell Technologies, Vancouver, BC, Canada) was used for peripheral blood mononuclear cell depletion from peripheral blood samples. RNA was extracted from CD45-cell population and mixed with TRIzol® LS Reagent (Invitrogen Corporation, Carlsbad, CA, USA). CTC were detected

by the presence of mRNA transcripts of epithelial (*KRT19*) or EMT-inducing transcription factors *TWIST1*, *SNAI1*, *SLUG* and *ZEB1* by quantitative real-time PCR (qRT-PCR).[42] The following TaqMan assays from Life Technologies (Carlsbad, CA, USA) were used for qRT-PCR: *TWIST1*: Hs00361186_m1; *SNAI1*: Hs00195591_m1; *SLUG*: Hs00161904_m1; *ZEB1*: Hs01566408_m1; *GAPDH* Hs99999905_m1; *KRT19*: Hs00761767_s1. Values of 60 age-matched healthy donors were used as a "cut-off" to determine CTC positivity.[42] Patient samples with higher epithelial and/or mesenchymal gene transcripts than those of healthy donors were considered CTC positive.

2.3 | DNA extraction and sodium bisulfite modification

Genomic DNA was extracted from FFPE tumor tissues by the MagneSil Genomic Fixed Tissue System (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA yield and purity were determined by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Bremen, Germany). EpiTect Bisulfite kit (Qiagen, Hilden, Germany) was used for sodium bisulfite treatment of genomic DNA isolated from the FFPE blocks, following the provided protocol. Modified DNA aliquots were stored at −18°C. Sodium bisulfite treatment of genomic DNA converts all unmethylated cytosine to uracil, while methylated cytosines remain unaffected.

2.4 | Pyrosequencing

The *ADAM23* promoter methylation level was measured by the quantitative pyrosequencing method as published elsewhere.[43] In brief, the region of interest was amplified from bisulfite-modified DNA using amplification primers (Forward Biotin-GCGTCGTTTTAGTATTTTTAGGTT; Reverse TCCCCAACCACTACTCCCT) and PyroMark PCR kit (Qiagen). After purification and denaturation, the biotinylated PCR product (89 bp) was sequenced (Pyrosequencing primer: ACTACTCCCTCCCCC) by Pyromark Q24 Pyrosequencing System (Qiagen). Obtained raw data were analyzed using PyroMark Q24 2.0.6 software (Qiagen). The results are presented as percentage of average methylation in eight CpG sites. The cut-off for *ADAM23* hypermethylation (9.53%) was established in our previous study as the mean methylation level determined in normal mammary glands plus 2 SD.[39]

2.5 | Immunohistochemistry

Immunohistochemistry (IHC) analysis was used to detect ADAM23 protein expression in the FFPE tumor tissues. Representative tumor areas were identified by hematoxylin and eosin staining and inserted into donor and recipient blocks. The 5-μm sections were transferred to coated slides and, after deparaffinization and rehydration, they were incubated overnight with specific antibody against the ADAM23 protein (ab101638; Abcam, Cambridge, UK) and immunostained as described previously.[43] Evaluation of IHC results was carried out in parallel by two pathologists. In brief, the percentage of positive cells was scored according to the following standard: 0 (0%), 1 (1%-10%), 2 (11%-50%), 3 (51%-80%) and 4 (81%-100%). Staining intensity of the cytoplasm was graded as follows: 0 (no staining), 1 (weakly stained, 1%-30%), 2 (moderately stained, 31%-70%), and 3 (strongly stained, 71%-100%). The results were obtained by multiplying the intensity and proportion scores and are expressed as an immunoreactive score (IRS), also known as the German IRS.[44] Based on this method, the IHC scores (0-12) were finally classified into four IRS categories: negative (0); weak (1-4); moderate (5-8); and high (9-12) protein expression.

2.6 | Statistical analysis

IBM SPSS statistics version 23.0 software for Windows (IBM) was used for statistical analyses of the data. Pearson chi-squared or Fisher's exact tests were carried out to find association between *ADAM23* hypermethylation and CTC status. For continuous variables, the normality of distribution was tested by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed data were tested by Student's *t* test or analysis of variance (ANOVA) with Bonferroni's corrections. Non-normally distributed data were tested by non-parametric Mann-Whitney *U* or Kruskal-Wallis *H* tests. Pearson's or Spearman's correlation tests were used to assess the correlations between *ADAM23* methylation and continuous data. Multivariate analysis methods, logistic regression and Cox proportional hazard regression were used to search for predictors independently associated with adverse outcomes. A multivariate logistic regression was applied to determine the effect of the independent variables on the presence of CTC in peripheral blood. This determination included computation of the risk estimate presented as estimated odds ratio (OR) and 95% confidence interval (CI) for the OR. Each model included age and clinicopathological characteristics; for example, Ki-67 proliferation (cut-off 20%), hormone receptor status (negative for both or positive for either one with cut-off of 10%), HER2 status (negative or overexpressed), tumor grade (1 and 2 vs 3), tumor size (T1 vs T2 and higher). A backward model selection was conducted, and the final fitted model is presented. Estimates of the cumulative survival distributions were calculated by the Kaplan-Meier method, and the differences were compared using the log-rank test. DFS was defined as the time interval from the date of sampling (usually date of surgery) to the date of disease recurrence, death or last follow up. A Cox proportional hazard model using stepwise regression procedure was applied to evaluate the significance of *ADAM23* hypermethylation and individual clinicopathological variables as defined above, including auxiliary lymph node involvement (N0 vs N+) for DFS. *P* value <.05 was considered to indicate statistical significance.

3 | RESULTS

3.1 | *ADAM23* promoter methylation and protein expression

Negative ADAM23 protein expression was identified in 24.5% (n = 49) of tumor tissues, and weak, moderate or high expression

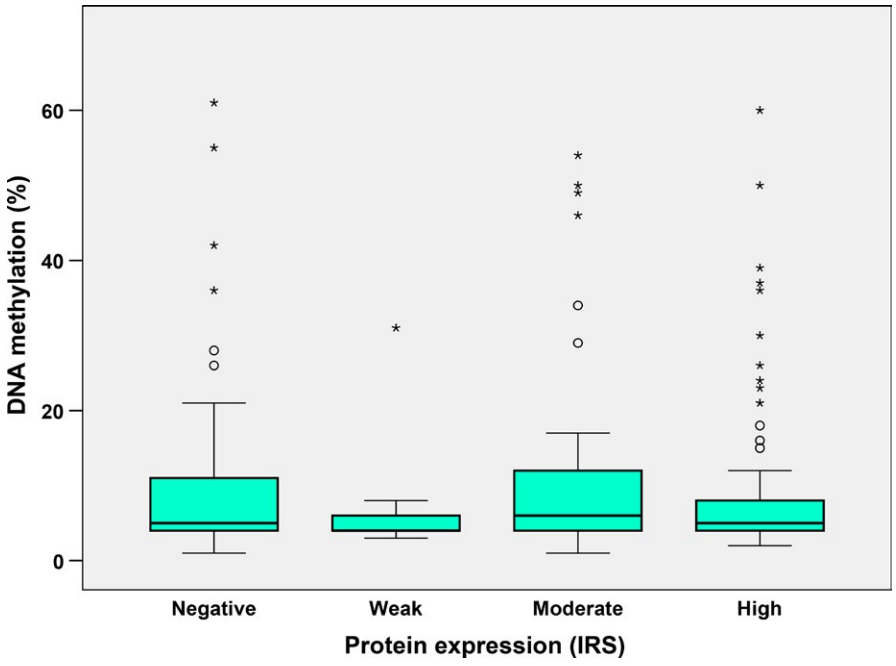

FIGURE 1 Distribution of *ADAM23* methylation in different protein expression categories in breast cancer patient tumors. ADAM23, A Disintegrin And Metalloprotease 23; IRS, immunoreactive score. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than 3 IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line

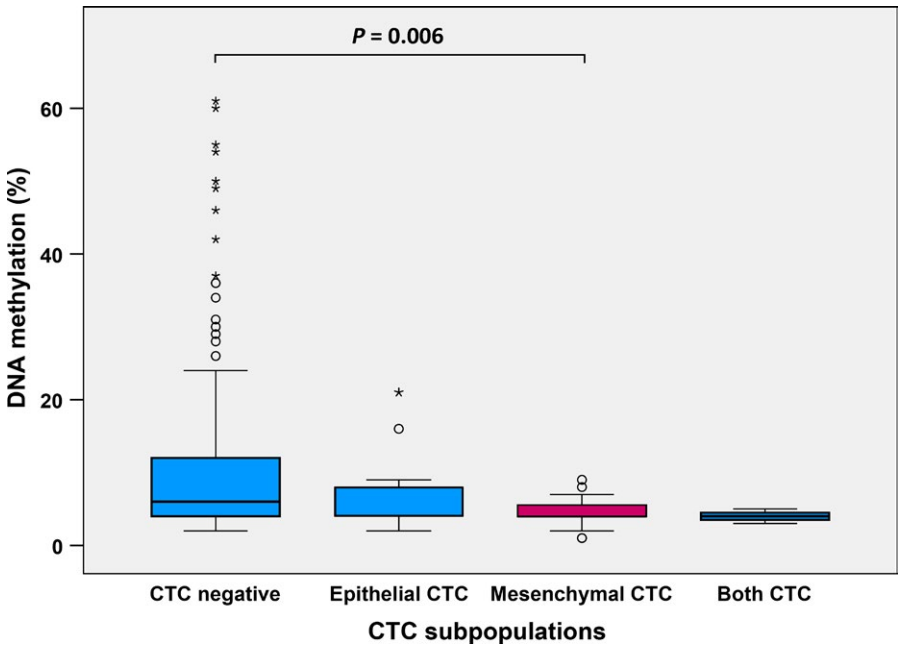

FIGURE 2 *ADAM23* promoter methylation stratified by the presence of individual circulating tumor cell (CTC) subpopulations in peripheral blood. ADAM23, A Disintegrin And Metalloprotease 23. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than 3 IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line

was present in 7.5% (n = 15), 25.5% (n = 51) and 42.5% (n = 85) of tumors, respectively. As shown in Figure 1, DNA methylation levels were approximately equally distributed across the individual protein expression categories (10.6 ± 13.2% for negative, 6.8 ± 7.0% for weak, 11.1 ± 13.0% for moderate and 9.5 ± 10.7% for high expression). Mean *ADAM23* promoter methylation was 9.8 ± 11.7%.

3.2 | *ADAM23* methylation and hematogenous dissemination

Based on the presence of individual CTC subpopulations in patient peripheral blood, these were stratified into four groups: CTC negative (n = 146); epithelial CTC (expression of epithelial marker *KRT19* in peripheral blood, n = 14); mesenchymal CTC (expression of either *TWIST1* or *SLUG; SNAI1* and *ZEB1* mRNA transcripts were not expressed, n = 27); and both CTC (expression of both, epithelial and EMT markers, n = 3). DNA methylation of *ADAM23* significantly varied between studied groups ($P = .002$), with the highest level in CTC-negative patients (11.3 ± 12.9%) and decreasing mean methylation values ranging from 6.5 ± 5.5% for epithelial CTC, 4.4 ± 2.0% for mesenchymal CTC, and 4.0 ± 1.0% for both CTC (Figure 2). There was strong evidence of a substantial difference between the CTC-negative and mesenchymal CTC-positive patients ($P = .006$, adjusted using the Bonferroni correction). In the CTC-negative group and in

TABLE 1 Risk estimation of *ADAM23* methylation and clinical status for the presence of mesenchymal CTC in peripheral blood of breast cancer patients (logistic regression adjusted for age)

Risk factor	Variable	*P* value	OR	95% CI
Mesenchymal CTC positivity	↑ *ADAM23* methylation	< .001	0.683	0.606-0.770
	Ki-67 proliferation index (cut-off 20%)	.009	2.995	1.322-6.784

ADAM23, A Disintegrin And Metalloprotease 23; CTC, circulating tumor cells.
−2 Log likelihood 134.03; *R* squared (Cox & Snell) 0.48; *R* squared (Nagelkerke) 0.64. Variables entered on step 1: age, *ADAM23* methylation, Ki-67 proliferation (cut-off 20%), hormone receptor status (negative for both or positive for either with cut-off 10%), human epidermal growth factor receptor 2 (HER2) status (negative vs amplified), tumor size (T1 vs T2 and higher), grade (1 and 2 vs 3).

the epithelial-CTC group, hypermethylation was present in 30.1% (n = 44) and in 14.3% (n = 2) of patients, respectively, whereas none of the patients positive for mesenchymal CTC or both CTC had a hypermethylated *ADAM23* gene promoter (*P* = .004).

Based on these findings, we aimed our attention at unveiling the role of *ADAM23* promoter methylation in hematogenous spread of cancer cells. Logistic regression analysis was used to predict the probability with which the presence of mesenchymal CTC was affected by the level of *ADAM23* promoter methylation in primary tumors. For risk estimation, patients were divided into two groups: mesenchymal CTC negative (n = 160) and mesenchymal CTC positive (n = 30). The predictor variables were age, *ADAM23* methylation, Ki-67 proliferation, hormone receptor and HER2 status, tumor size and histological grade. The model correctly classified 99.4% of mesenchymal CTC-negative and 6.9% of mesenchymal CTC-positive patients, with an overall success rate of 84.7%. Table 1 shows OR, *P* values and 95% CI for each of the significant variables. The OR for *ADAM23* methylation indicates that mesenchymal CTC positivity negatively correlated with *ADAM23* methylation (OR = 0.683; 95% CI, 0.606-0.770; *P* < .001), whereas it was nearly threefold more likely in patients whose Ki-67 proliferation was above 20% (OR = 2.995; 95% CI, 1.322-6.784; *P* = .009; Table 1). Because patient stratification by mesenchymal CTC negativity/positivity versus complete CTC negativity/mesenchymal CTC positivity yielded almost the same results (Table S1), stratification by mesenchymal CTC was chosen for the next analyses with the intention not to omit patients with epithelial CTC.

DNA methylation of *ADAM23* promoter was significantly higher in mesenchymal CTC-negative than in mesenchymal CTC-positive patients (10.9 ± 12.5% vs 4.4 ± 1.9%; *P* = .001) (Figure 3). This difference was even more pronounced in subjects with high Ki-67 (15.9 ± 17.0% vs 4.3 ± 1.6%; *P* = .001). *ADAM23* promoter methylation positively correlated with Ki-67 proliferation (*r* = 0.163, *P* = .020), especially in mesenchymal CTC-negative patients (*r* = 0.272, *P* = .001).

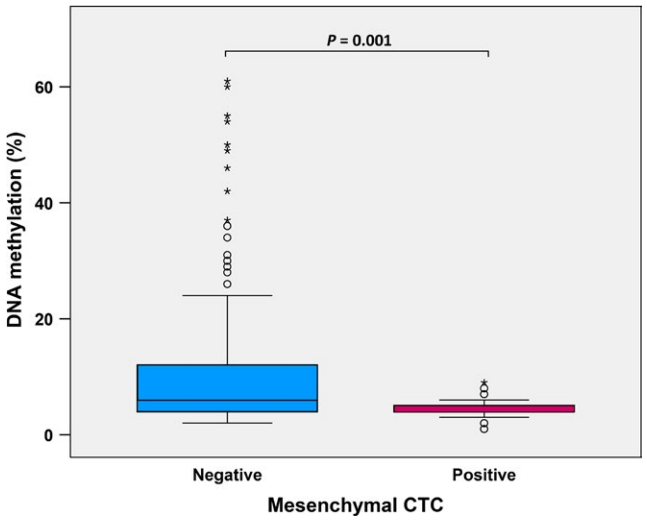

FIGURE 3 *ADAM23* methylation in mesenchymal circulating tumor cell (CTC)-negative and -positive breast cancer patients irrespective of Ki-67 proliferation index. ADAM23, A Disintegrin And Metalloprotease 23. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than 3 IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line

3.3 | *ADAM23* methylation and disease-free survival

At a mean follow-up time of 60.2 ± 16.1 months (range, 0.2-76.6 months), 21.5% of patients (n = 41) had experienced a DFS event, and 10.8% of patients (n = 22) had died. As a result of immaturity of overall survival data, only the results of DFS analyses are presented.

To assess the role of *ADAM23* methylation in disease recurrence, we further stratified patients by Ki-67 proliferation and presence of mesenchymal CTC in their peripheral blood (Figure 4). Although *ADAM23* was hypermethylated in 28.7% (n = 46) of mesenchymal CTC-negative, Ki-67-low patients and in 45.0% (n = 27) of mesenchymal CTC-negative, Ki-67-high patients, in mesenchymal CTC-positive patients, *ADAM23* hypermethylation did not occur (*P* = .001). Regardless of Ki-67 expression, mesenchymal CTC-negative patients with recurrent disease had higher levels of *ADAM23* promoter methylation than those without recurrence (Figure 4A,B) (16.0 ± 15.5% vs 10.2 ± 11.8%; *P* = .017). No differences in generally extremely low *ADAM23* promoter methylations were found in tumors of mesenchymal CTC-positive patients (Figure 4C,D) (4.5 ± 2.0% for patients with recurrent disease vs 4.3 ± 2.0% for patients without recurrence).

As expected, high Ki-67 proliferation ([DFS time 60.3 months [95% CI: 54.8-65.8] vs 72.7 months [95% CI: 70.6-75.0] in Ki-67-low patients, *P* < .001]) and presence of mesenchymal CTC ([DFS time 52.8 months [95% CI: 44.6-61.1] vs 70.1 months

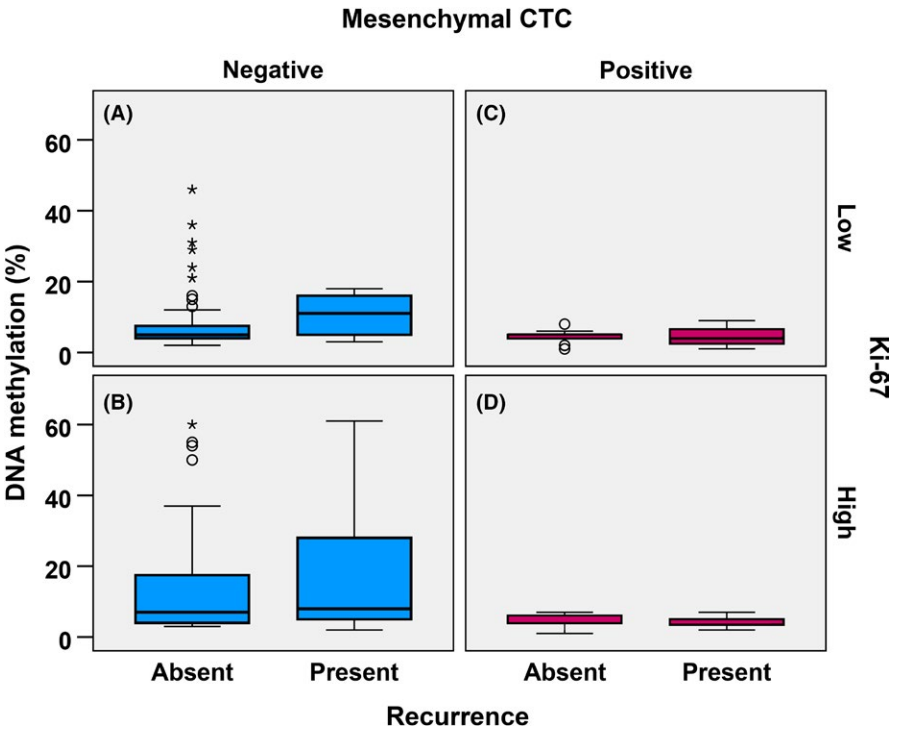

FIGURE 4 Association of *ADAM23* methylation with disease recurrence, stratified by the presence of mesenchymal circulating tumor cells (CTC) (A, B vs C, D) and Ki-67 proliferation (A, C vs B, D). Differences between patients with recurrent disease and those without recurrence were not significant. ADAM23, A Disintegrin And Metalloprotease 23. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than 3 IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line

[95% CI: 67.5-72.7] in mesenchymal CTC-negative patients, $P < .001$]) were the two most significant risk factors affecting DFS. Furthermore, we assessed the prognostic significance of *ADAM23* methylation. Although mean DFS in mesenchymal CTC-negative, hypermethylation-free patients was 72.5 months (95% CI: 70.0-75.0), in high-risk, mesenchymal CTC-positive, Ki-67-high patients, we found the shortest mean DFS in all studied groups (44.1 months; 95% CI: 33.1-55.2). Surprisingly, in mesenchymal CTC-negative patients with hypermethylated *ADAM23* promoter, we identified nearly identical mean DFS as in mesenchymal CTC-positive, Ki-67 low patients (64.1; 95% CI: 58.0-70.1 vs 63.6; 95% CI: 54.0-73.2 months, respectively). Kaplan-Meier DFS estimates for mesenchymal CTC-negative and CTC-positive patients are plotted in Figure 5. In mesenchymal CTC-negative patients, log-rank test showed a significantly reduced DFS in those with *ADAM23* hypermethylation in tumors ($P = .003$) whereas in mesenchymal CTC-positive patients, it was high Ki-67 proliferation that significantly affected DFS ($P = .014$). Prognostic value of *ADAM23* hypermethylation was seen mainly in mesenchymal CTC-negative, Ki-67-low patients ($P = .002$) where mean DFS time in patients without *ADAM23* hypermethylation was 75.0 months (95% CI: 73.4-76.5), whereas in those with *ADAM23* hypermethylation in tumors, it was only 66.3 months (95% CI: 58.8-73.8). Differences in mesenchymal CTC-negative, Ki-67-high patients did not reach statistical significance.

Cox logistic regression was used to predict the recurrence probability in mesenchymal CTC-negative, Ki-67-low BC patients, with the following predictor variables: age, *ADAM23* hypermethylation, hormone receptor and HER2 status, tumor size and auxiliary lymph

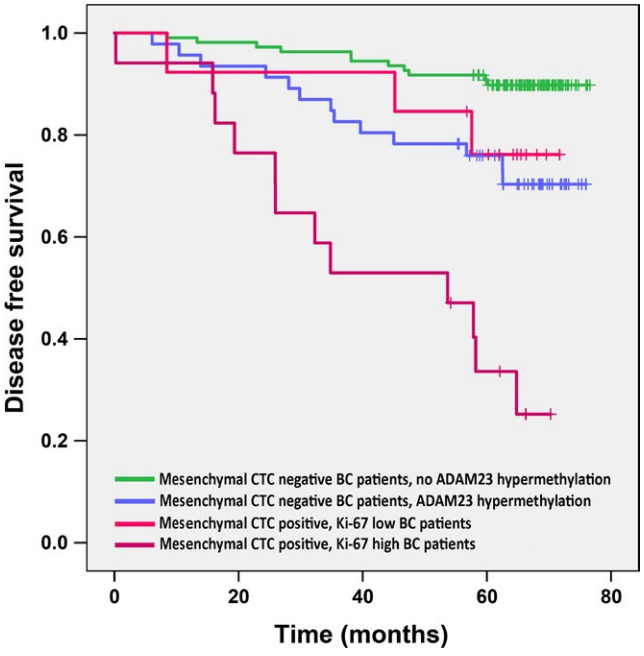

FIGURE 5 Disease-free survival stratified by *ADAM23* hypermethylation (cut-off 9.53%)[39] in mesenchymal circulating tumor cell (CTC)-negative and by Ki-67 proliferation in mesenchymal CTC-positive patients. ADAM23, A Disintegrin And Metalloprotease 23. BC, breast cancer. The length of the boxes is the interquartile range (IQR) that represents values between the 75th and 25th percentiles. Values more than 3 IQRs from the end of a box are labeled as extreme (*). Values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (O). The median is depicted by a horizontal line

TABLE 2 Cox regression analysis in the group of low-risk (Ki-67-low and mesenchymal CTC-negative) breast cancer patients

Risk factor	Variable	Risk value	*P* value	Odds ratio	95% Confidence interval
Disease recurrence	*ADAM23* hypermethylation	>9.53%	.006	6.220	1.669-23.181

ADAM23, A Disintegrin And Metalloprotease 23; CTC, circulating tumor cells.
−2 Log likelihood 74.42. Variables entered in step 1: age, *ADAM23* hypermethylation, hormone receptor status (negative for both or positive for either with cut-off 10%), human epidermal growth factor receptor 2 (HER2) status (negative vs amplified), tumor size (T1 vs T2 and higher), auxiliary lymph node involvement (N0 vs N+).

node involvement (Table 2). Disease recurrence was 6.2-fold more likely in patients with *ADAM23* hypermethylation in tumor tissue than in those with promoter methylation below threshold value (OR = 6.220; 95% CI: 1.669-23.181; *P* = .006).

4 | DISCUSSION

A Disintegrin And Metalloprotease proteins have been shown to play a significant role in tumor growth and metastasis.[29] In comparison with other family members, the biological role of ADAM23, especially in cell adhesion and cell-matrix interactions, largely depends on its disintegrin domain, whereas its metalloprotease domain remains inactive.[45] The *ADAM23* gene is epigenetically silenced in various cancers, including BC, where it correlates with disease progression[33,46] and lower survival rates.[31] In our previous study, carried out on almost the same set of BC patients (including three metastatic patients), we analyzed the association of DNA methylation changes in 11 genes, including *ADAM23*, with clinicopathological characteristics. We identified a twofold higher level of *ADAM23* promoter methylation in BC patient tumors than in normal mammary glands (10.01% vs 4.89%).[39] In primary breast tumors, *ADAM23* hypermethylation was associated with higher proliferation of cancer cells measured through Ki-67 expression (OR 5.23, 95% CI: 2.27-12.05, *P* < .001). In addition to high Ki-67 proliferation, significantly higher mean *ADAM23* methylation was also identified in tumors of estrogen receptor (ER) and HER2-positive patients (12.24% vs 7.19% in Ki-67-low, *P* = .008; 11.25% vs 6.37% in ER-negative, *P* = .001; and 16.81% vs 8.76% in HER2-negative, *P* = .001, respectively). The presence of *ADAM23* hypermethylation in tumors and lymph nodes was equally common (24.2% vs 21.6%) and positively correlated (*r* = 0.442, *P* = .001). Higher *ADAM23* promoter methylation in lymph nodes was identified in histological grade 3, HER2-positive and progesterone receptor (PGR)-negative patients (12.64% vs 6.41% in grades 1 and 2, *P* < .001; 15.00% vs 7.02% in HER2-negative, *P* < .001; and 15.00% vs 7.81% in PGR-positive, *P* < .001, respectively). However, we did not find any association between *ADAM23* methylation and protein expression.[39] In our recent study, we detected an association between low *ADAM23* promoter methylation and hematogenous spread.[40]

Based on these findings and given the time that has passed since the end of the sampling, we have now focused on identifying the factors that have affected DFS. We confirmed positive correlation between Ki-67 proliferation and *ADAM23* methylation, with the most significant impact in the mesenchymal CTC-negative patient group. We did not find DNA hypermethylation in tumors of mesenchymal CTC-positive patients, in which Ki-67 proliferation was the most significant independent predictor of DFS. Epithelial CTC positivity was neither significantly associated with *ADAM23* promoter methylation nor DFS. The EMT process, endowing epithelial tumor cells with enhanced motility and invasiveness, is an important prerequisite for intravasation and release of CTC. EMT is mediated by several EMT-inducing transcription factors, including SNAI1, SNAI2 (also termed SLUG), TWIST1/2 and ZEB1/2. These suppress expression of key epithelial genes, such as *CDH1*, coding for adherence junction protein E-cadherin.[47] CTC with mesenchymal phenotype can be detected in the peripheral circulation of BC patients through mRNA expression of EMT-inducing transcription factors.[42,48,49] Their presence in BC patient peripheral blood was associated with worse prognosis[25,26] (Mego et al, in preparation). Herein, we have shown an association between *ADAM23* promoter methylation and mesenchymal CTC, detected by the above-mentioned method. We identified lack of *ADAM23* methylation in tumors of patients with mesenchymal CTC in their peripheral blood. However, we have not found correlation between ADAM23 protein expression and DNA methylation or hematogenous spread.[39,41] One of the possible explanations could be the recently described intratumoral heterogeneity of ADAM23 protein expression observed in BC.[46] As shown by these authors, the topographically distinct invasive tumor areas within undifferentiated invasive ductal carcinomas are composed of a mosaic cluster of ADAM23-positive and ADAM23-negative tumor cells. Furthermore, diverse ADAM23 expression in tumor-derived endothelium could be another confounding factor explaining the negative findings.[50] As DNA methylation is cell type-specific, the results of DNA methylation assessment are currently based on bulk analyses of heterogeneous cell populations interpreting their mixture averages. Moreover, it was shown that a threshold of 40%-60% of methylated CpG dinucleotides was required for complete *ADAM23* mRNA expression silencing in BC cell lines, whereas in colorectal cancer cells, it was 70%-90%.[33,51] However, only 4.4% (n = 9) of our patients had a mean level of DNA methylation in tumor above 40% (42%-61%).

Our current findings show that *ADAM23* hypermethylation increases the risk of disease recurrence in mesenchymal CTC-negative patients with low Ki-67 proliferation. We can only speculate that low *ADAM23* methylation levels in tumors of mesenchymal CTC-positive patients is a result of gradual accumulation of *ADAM23* methylation in distinct tumor cell subclones and subsequent release of

ADAM23-negative cells into circulation. We assume that the effect of *ADAM23* hypermethylation is overlapped by the action of other significant prognostic risk factors such as high Ki-67 proliferation rate or presence of mesenchymal CTC alone. Consequently, we are able to detect the prognostic role of *ADAM23* methylation only in low-risk patients. Because we were unable to address *ADAM23* methylation in the mesenchymal CTC subpopulation, we cannot exclude the possibility that differences in *ADAM23* methylation in tumors of mesenchymal CTC-positive and CTC-negative patients could be associated with additional factors. In agreement with our hypothesis, Costa and coauthors showed in vitro and in vivo that ADAM23 intra-allelic and intratumoral heterogeneity is critical for tumor growth and metastasis.[33,46] They suppose that during metastatic progression, epigenetic instability may provide tumor cells with a growth advantage, leading to selection of clones with the densest methylation profiles.[33] They also showed that ADAM23 silencing can elicit a phenotypic switch from a proliferative to an invasive phenotype of tumor cells. Moreover, they showed that in ADAM23 heterotypic environments, cells without ADAM23 expression promote tumor growth and metastasis by enhancing the proliferation and invasivity of adjacent ADAM23-positive cells.[46]

This type of epigenetic plasticity was recently also observed in one of the EMT hallmarks – E-cadherin loss.[52] It was shown that E-cadherin loss may promote invasion, allowing cells with the highest methylation density and the most diminished protein expression to dissociate from the primary tumor and disseminate through the vascular system to a distal, secondary organ site. In contrast, partial restoration of E-cadherin expression mediated by the evolution of cells with reduced methylation increases intercellular adhesion and may facilitate cell survival within metastatic deposits.[52] The intratumoral heterogeneity described for ADAM23 expression,[46] as well as the dynamic epigenetic heterogeneity during metastatic progression described for *CDH1*,[52] highlights the importance of DNA methylation changes and fine-tuning of the methylome leading to corresponding alterations in individual steps of the metastatic cascade.

Although direct association between ADAM23 and EMT has not yet been published, ADAM23 has been shown to be a key suppressor for cardiac hypertrophy and fibrosis by blocking the focal adhesion kinase-protein kinase B (FAK-AKT) signaling cascade.[53] The authors hypothesize that ADAM23 can directly affect the pathogenesis of fibrosis through the same downstream target. FAK, identified as one of the first integrin signaling molecules, is a non-receptor tyrosine kinase, required for the transcriptional regulation of several mesenchymal markers and for the delocalization of E-cadherin.[54] The $\alpha_V\beta_3$ integrin is an β3 integrin subtype that was associated with EMT in cancer cells and shows the widest range of functions, including FAK-induced actin cytoskeleton remodeling and FAK-dependent cytokine activation, affecting cell migration and invasion.[55] Activation of $\alpha_V\beta_3$ has been reported to upregulate Slug and facilitate anchorage-independent growth in pregnant and neoplastic mammary glands[56] whereas ADAM23 knockdown cells showed increased migration and adhesion to classic $\alpha_V\beta_3$ integrin ligands.[31] Additionally, downregulation of ADAM23 in a side population of lung adenocarcinoma cells contributed to the CSC phenotype, supposedly by promoting activity of $\alpha_V\beta_3$ integrin.[57] These findings support the hypothesis that by binding to integrins, namely $\alpha_V\beta_3$ and α4 and/or other cell-surface molecules, ADAM23 can act as a mediator of intercellular association and participate in facilitating EMT in cancer cells.[31,32]

To the best of our knowledge, this is the first study showing strong association of *ADAM23* hypermethylation with DFS in supposedly low-risk, mesenchymal CTC-negative and Ki-67-low BC patients. In tumors of mesenchymal CTC-positive patients, *ADAM23* methylation was extremely low, suggesting involvement of this protein in EMT. ADAM23 may represent a new therapeutic target, enabling dissemination of the most aggressive mesenchymal CTC subpopulations. However, as a result of the complexity of this process, future studies are warranted to verify the role of ADAM23 in EMT and hematogenous spread.

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CONFLICTS OF INTEREST

Authors declare no conflicts of interest for this article.

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