Abstract. MMP9 is involved in extracellular matrix degradation during various physiological and pathological conditions, including tumorigenesis. The present study aimed to assess the prognostic role of intratumoral MMP9 and to determine its association with circulating tumor cells (CTCs) in patients with early breast cancer. A total of 318 patients with primary breast cancer (PBC) were enrolled into the present study. Specimens were subjected to immunohistochemistry analysis, using the MMP9 monoclonal antibody. MMP9 expression was scored using a weighted histoscore (WH). The results demonstrated that the mean WH ± SEM for MMP9 expression was significantly higher in breast tumor cells compared with tumor associated stromas (132.0±5.2 vs. 50.8±3.7; P<0.00001). Furthermore, a positive association was observed between MMP9 expression, the hormone positive status and proliferation index of analysed breast cancer tumour cells. Notably, the prognostic role of MMP9 was not observed in tumor cells [hazard ratio (HR) =0.96; 95% confidence interval (CI), 0.58‑1.59; P=0.864] or tumor associated stroma (HR=1.29; 95% CI, 0.60‑2.78; P=0.547). Subgroup analysis demonstrated that patients that were HR negative or triple negative, with low MMP9 expression in tumor cells and stroma had a significantly improved disease‑free survival than patients with high MMP9 expression. Taken together, the results of the present study demonstrated that high MMP9 expression in PBC was associated with favorable tumor characteristics. However, the prognostic value of MMP9 was limited to only the HR negative and CTC epithelial‑to‑mesenchymal transition positive subgroups. Thus, analyzing MMP9 tumor expression may help identify patients with increased risk of disease recurrence in these subgroups.

Introduction

Breast cancer is the most common malignancy in women worldwide, whereby 2,088,849 new cases of invasive breast cancer and 626,679 mortalities were reported in 2018 (1,2). Tumor invasion and metastasis affect >90% of patients with breast cancer, and thus notably contribute to the high mortality rate (3‑5). This metastatic disease remains incurable, and effective treatment for end‑stage metastatic breast cancer are yet to be determined (6‑8). The aggressiveness of a tumor is closely associated with its ability to evade natural barriers, to invade adjacent tissues and metastasize distant sites (9). The metastatic cascade is a multistep process where cancer cells escape from the primary tumor site to distant locations, where they can potentially establish new cancer colonies (10,11). Under optimal conditions, epithelial cancer cells detach from the primary tumor site, penetrate and migrate via peripheral circulation, and invade secondary sites, where they ultimately undergo extravasation and populate distant organs (11,12).
Proteolytic degradation of the basement membrane and extracellular matrix (ECM) is considered a crucial aspect of metastatic growth, which enables low anchorage of neoplastic cells (13-17). Several cell-secreted proteolytic enzymes, including matrix metalloproteinases (MMPs) are implicated in the cleavage of ECM (13,18,19). Matrix metalloproteinase 9 (MMP9) is a member of the gelatinase subfamily of MMPs and is secreted by a variety of cell types in an inactive form that undergoes activation upon cleavage by different types of extracellular proteases (18,20). MMP9 activity is modulated via different biochemical molecules, including growth factors and cytokines (19,21). Notably, MMP9 is actively involved in the degradation of type IV collagen, which is a crucial component of the basement membrane (19,22). In addition, MMP9 facilitates the dissemination machinery, and is particularly involved in tumor invasion, tumor-induced angiogenesis, and immunomodulation of the tumour microenvironment, where it is implicated in the formation of so-called premetastatic niches (23,24). Previous studies have focused on the association between high MMP9 expression and the number of distant metastases in patients with breast cancer (25-27), as well as poor prognosis (28,29). It has been speculated that circulating tumor cells (CTCs), which are responsible for distant metastasis formation, use MMPs to form new metastatic sites (19,30). In addition, a previous study demonstrated that elevated MMP1 expression is significantly associated with the presence of CTC_ epithelial-to-mesenchymal transition (EMT) cells in the peripheral blood of patients with primary breast cancer (PBC), as well as with poor prognostic features of their primary tumors (31). The present study aimed to assess MMP9 expression in tumor cells as well as tumor associated stroma of patients with PBC, and determine its association with the presence of CTCs in the peripheral blood of these patients and other clinicopathological characteristics. The prognostic value of MMP9 in patients with PBC was also assessed.

Patients and methods

Study patients. The present study (Protocol TRU-SK 002; Chair: Michal Mego) enrolled 318 patients with stages I-III PBC who underwent definitive surgery. The samples were collected from the National Cancer Institute (Bratislava, Slovakia) between March 2012 and February 2015. The paraffing embedded tumor tissue and CTCs status in peripheral blood were available for all patients included in the present study. Complete diagnostic evaluation was performed in all patients to exclude the presence of distant metastasis. Patients to exclude the presence of distant metastasis. Patients with concurrent malignancy in the last 5 years, other than non-melanoma skin cancer, were excluded from the present study. The clinicopathological data including age, tumor stage, histology, regional lymph node involvement, hormone receptor status and HER2 status were retrieved and tabulated from the patients' records after obtaining all the relevant ethical approvals. Breast cancer subtypes were identified by immunohistochemical staining (see below) and classified according to the ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up for early breast cancer (32).

The present study was reviewed and approved by the Institutional Review Board of the National Cancer Institute of Slovakia, Bratislava, Slovakia (TRUSK002, 20.6.2011). Written informed consent was provided by all patients prior to the study start.

Tumor pathology. Pathological review was performed at the Department of Pathology, Faculty of Medicine, Comenius University, (Bratislava, Slovakia) by an experienced pathologist (ZC).

Tumor samples and tissue microarray construction. Tumor specimens used in the present study were classified according to the 2019 World Health Organization classification (33). According to the tumor histology results, one or two representative areas containing the most representative part of the hematoxylin and eosin (H&E) stained tumor tissues were observed under a light microscope, original magnification×400. The identified sections were matched to their corresponding wax blocks (donor blocks). The 3-mm diameter cores of the tumors were removed from the donor blocks using the multipurpose sampling tool HarrisUni-Core (Sigma-Aldrich; Merck KGaA) and inserted into the recipient master block. The recipient block was cut into 5-μm-thick sections, which were transferred onto coated slides.

Immunohistochemical (IHC) staining. Deparaffinized slides were rehydrated in phosphate buffered saline solution (10 mM, pH 7.2). Tissue epipettes were demasked using the automated water bath heating process in Dako PT Link (Dako; Agilent Technologies, Inc.) and the slides were incubated in pH 6.0 citrate retrieval buffer at 98°C for 20 min. The slides were subsequently incubated for 1 h at room temperature with primary mouse monoclonal antibody against MMP9 (Abcam; MMP9 (SB15c); cat. no. ab51203) diluted 1:200 in Dako REAL antibody diluent (Dako; Agilent Technologies, Inc.) and immunostained with anti-mouse/anti-rabbit immuno-peroxidase polymer (EnVision FLEX/HRP, Dako; Agilent Technologies, Inc.) for 30 min at room temperature, according to the manufacturer's instructions. The reaction was visualized using diaminobenzidine substrate-chromogen solution (Dako; Agilent Technologies, Inc.) for 5 min, and the slides were counterstained with hematoxylin. The human clone tissue served as the positive control, and colon tissue subjected to the same procedure omitting the primary antibody was used as the negative control.

IHC evaluation. Tumor scores were blindly assessed by a pathologist (ZC). The results of the IHC analyzes were scored using a weighted histoscore (WH), assessing both the percentage of positive cells (PP) and the staining intensity (SI) of the cytoplasm as follows: The proportion of cells with nuclear staining was multiplied by the intensity of staining to provide a histoscore ranging from 0-300. The histoscore was calculated as follows: Score=(0x percentage not stained) + (1x percentage weakly stained) + (2x percentage moderately stained) + (3x percentage strongly stained) (34). The mid-point of WH histoscore was used as the cut-off criterion simillary as previously (35,36). MMP9 expression was stratified as low vs. high, according to the cut-off value of WH histoscore (150).

Detecting CTCs in peripheral blood. CTCs were identified via reverse transcription-quantitative (RT-q)PCR analysis.
Enrichment of CTC from peripheral blood by depleting CD45+ cells was performed using the Rosette Sep™ kit (15162; Stemcell Technologies, Inc.), as previously described (37,38). Briefly, RNA isolated from CD45-depleted peripheral blood samples were transcribed into cDNA, which was subjected to RT-qPCR analysis to assess the expression levels of epithelial-to-mesenchymal transition (EMT-TF) genes, including TWIST1, SNAIL1, SLUG and ZEB1. Compared with healthy donors, patient samples with higher EMT-TF gene transcript levels were classified as CTC EMT positive, based on the preclinical study and human sample testing. The highest expression values in healthy donors were used as a cut-off value to determine CTC positivity (39).

Statistical analysis. Patient characteristics were summarized using the median (range) values for continuous variables and frequency (percentage) for categorical variables. The distribution of MMP9 histoscore was significantly different from the normal distribution (Shapiro-Wilk test), thus non-parametric tests were used for analyses. Mann-Whitney U test was used to compare the differences in distributions of MMP9 expression between two groups of patients with PBC, whereby MMP9 expression was categorized as absent or present. Fisher’s exact test or the χ² test were used where appropriate.

The median follow-up period was estimated as a median observation time among all patients and among those still alive at the time of their last follow-up. Disease-free survival (DFS) was calculated from the date of CTCs measurement to the date of disease recurrence (locoregional or distant), secondary cancer, death or last follow-up. DFS was estimated using the Kaplan-Meier product limit method and log-rank test. Two-sided P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using NCSS 11 statistical software (2016; NCSS, LLC.; ncss.com/software/ncss).

Results

Patient characteristics. The present study enrolled 318 patients with PBC. The median age of the assessed cohort was 60 years (age range, 25-83 years). The majority of patients had node negative (60.1%) and hormone positive (83.6%) tumors; 48/318 patients (15.1%) had a HER-2/neu amplified status. Patient characteristics are summarized in Table I.

CTCs detection. To establish overexpression of the EMT-inducing TF gene transcripts in patients with PBC, the expression levels were compared between patient samples and healthy donors, as previously described (39). Among the patient samples, CTCs were detected in 83 patients (26.1%). CTCs with only epithelial markers were detected in the peripheral blood of 34 patients (10.7%), while CTCs with an EMT phenotype were present in 56 patients (17.6%).

Association between MMP9 expression, and patients/tumor characteristic and CTCs. MMP9 protein expression in tumor cells was assessed in all patients (n=318) (Fig. 1). However, pathologists were unable to detect stromal cells in 9/318 tumor tissues due to the small sample size, which only constituted tumor cells. Thus, MMP9 expression in stroma was only assessed in 309 patients. MMP9 expression intensity at least 1+...
and higher was detected in 255 samples (80.2%) in breast tumor cells and in 307 samples (99.4%) of tumor associated stroma (P<0.00001). The mean WH ± standard error of the mean (SEM) for MMP9 expression was significantly higher in breast tumor cells compared with tumor associated stroma (132.0±5.2 vs. 50.8±3.7, P<0.00001). The association between MMP9 expression in tumor cells and clinicopathological characteristics, as well as its association with CTCs are presented in Table II. The results demonstrated that elevated MMP9 expression was significantly associated with EP/PR positive breast cancer cells (mean WH ± SEM=137.6±5.6 vs. 103.4±12.8, P=0.011) and low proliferating tumors (Ki67 <20%) (mean WH ± SEM=141.1±6.7 vs. 117.9±8.1, P=0.018), while elevated MMP9 expression in tumor associated stroma was associated with hormone receptor (EP/PR) status (mean WH ± SEM=54.6±4.0 vs. 30.7±9.1, P=0.021) (Table III). In our analysis, there was found no association between MMP9 expression in breast cancer cells, or in tumor associated stroma and CTCs.

Prognostic value of MMP9 in PBC. The median follow-up time was 54.9 months (range, 0.2-76.6 months). In the assessed cohort, 61 patients (19.2%) experienced a disease progression during follow-up. Among the subgroup of patients where MMP9 expression in tumor associated stroma was assessed (n=309), the median follow-up time was 55.3 months (range, 0.2-76.6), and 59 patients (19.1%) experienced a DFS event. Due to insufficiency of overall survival data, only DFS data are presented in the present study.

Univariate analysis was performed to determine the prognostic value of MMP9 in PBC cells [hazard ratio (HR)=0.96; 95% confidence interval (CI), 0.58-1.59; P=0.864; Fig. 2A], as well as in tumor associated stroma (HR=1.29; 95% CI, 0.60-2.78; P=0.547; Fig. 2B). Exploratory subgroup analysis was performed to determine a potential subgroup-related prognostic value of MMP9 (Tables IV and V). In addition, also the univariate analysis in group of patients with concomitant high MMP9 expression in tumor and stromal cells was carried out. However, no prognostic value was found using this analysis (HR=1.27, 95% CI 0.59-2.75, P=0.573) (Fig. 2C). The results demonstrated that low MMP9 expression in tumor cells was associated with better DFS in hormone receptor (ER/PR) negative and triple negative patients with PBC (HR=0.33; 95% CI, 0.12-0.93; P=0.025; Fig. 3A) and (HR=0.17; 95% CI, 0.05-0.57; P=0.003; Fig. 3B), respectively. Notably, the prognostic value of MMP9 in tumor cells was also observed in the CTC_EMT-positive subgroup of patients (HR=0.40; 95% CI, 0.16-0.95; P=0.047; Fig. 3C). Among the subgroup of patients where MMP9 expression in tumor associated stroma was assessed, the prognostic value of MMP9 was observed in the hormone receptor (ER/PR) negative subgroup of patients (HR=0.14; 95% CI, 0.00-4.81; P=0.002; Fig. 4A), triple negative (HR=0.12; 95% CI, 0.00-4.89; P=0.001; Fig. 4B). In addition, among the subgroup of CTC_EMT positive patients
was progression of the disease documented in 1 of 2 patients with high MMP9 expression in stromal cells compared to 22 of 51 patients with low MMP9 expression within 4-years follow up. In subgroup of the CTC_EMT positive patients 2 of 4 patient with high MMP9 expression in stromal cells experienced progression of disease compared to 6 of 22 patient with low MMP9 expression after 4-years follow up.

Notably, combinatorial survival analysis of CTC_EMT and MMP9 expression in tumor cells demonstrated that CTC_EMT positive patients with high MMP9 expression had a significantly shorter DFS compared with CTC_EMT negative patients (P<0.00001; Fig. 5).

Discussion
MMPs represent a large family of proteolytic enzymes of the extracellular matrix that are involved in extracellular matrix degradation, tumor cell invasion, metastasis and
### Table I. Patient characteristics.

| Characteristic                     | n (%)          |
|-----------------------------------|----------------|
| All patients                      | 318 (100.0)    |
| **Histology**                     |                |
| Invasive ductal carcinoma         | 272 (85.5)     |
| Invasive lobular carcinoma        | 32 (10.1)      |
| Other histological subtypes       | 14 (4.4)       |
| **Grade**                         |                |
| Low and intermediate              | 200 (62.9)     |
| High grade                        | 110 (34.6)     |
| Unknown                           | 8 (2.5)        |
| **T stage**                       |                |
| T1                                | 218 (68.6)     |
| T2 and more                       | 100 (31.4)     |
| **N stage**                       |                |
| N0                                | 191 (60.1)     |
| N1mi                              | 10 (3.1)       |
| N1                                | 68 (21.4)      |
| N2                                | 27 (8.5)       |
| N3                                | 19 (6.0)       |
| Unknown                           | 3 (0.9)        |
| **Hormone receptor status (cut-off 1%)** |            |
| Negative for both                 | 52 (16.4)      |
| Positive for either               | 266 (83.6)     |
| **HER2 status**                   |                |
| Negative                          | 270 (84.9)     |
| Positive                          | 48 (15.1)      |
| **Ki67 status**                   |                |
| <20%                              | 189 (59.4)     |
| ≥20%                              | 128 (40.3)     |
| Unknown                           | 1 (0.3)        |
| **Molecular subtype**             |                |
| Luminal A                         | 166 (52.2)     |
| Luminal B                         | 99 (31.1)      |
| HER2+                             | 13 (4.1)       |
| Triple negative                   | 39 (12.3)      |
| Unknown                           | 1 (0.3)        |
| **P53 status**                    |                |
| Negative                          | 193 (60.7)     |
| Positive                          | 124 (39.0)     |
| Unknown                           | 1 (0.3)        |
| **BCL-2 status**                  |                |
| Negative                          | 92 (28.9)      |
| Positive                          | 225 (70.8)     |
| Unknown                           | 1 (0.3)        |
| **CTC EP**                        |                |
| Negative                          | 235 (73.9)     |
| Positive                          | 27 (8.5)       |
| **CTC EMT**                       |                |
| Negative                          | 235 (73.9)     |
| Positive                          | 56 (17.6)      |
| **CTC any**                       |                |
| Negative                          | 235 (73.9)     |
| Positive                          | 83 (26.1)      |

CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial-positive.

### Table II. Association between MMP9 expression in tumour cells, patients, tumour characteristics and circulating tumor cells.

| Characteristic                     | N | Mean | SEM | Median | P-value |
|-----------------------------------|---|------|-----|--------|---------|
| MMP9 expression weighted histoscore | 318 | 132.0 | 5.2 | 150 NA |         |
| Histology                         |   |      |     |        |         |
| Invasive ductal carcinoma         | 272 | 136.0 | 5.6 | 150   | 0.081   |
| Other                             | 32  | 108.6 | 13.6| 150   |         |
| T stage                           |   |      |     |        |         |
| T1                                | 218 | 136.4 | 6.3 | 150   | 0.163   |
| T2 and more                       | 100 | 80.0  | 32.7| 0     |         |
| N stage                           |   |      |     |        |         |
| N0                                | 191 | 135.4 | 6.5 | 150   | 0.468   |
| Positive                          | 266 | 137.6 | 5.6 | 150   |         |
| **Hormone receptor status (cut-off 1%)** |    |      |     |        |         |
| Negative for both                 | 52  | 103.4 | 12.8| 100   | 0.011   |
| Positive for either               | 266 | 137.6 | 5.6 | 150   |         |
| **HER2 status**                   |    |      |     |        |         |
| Negative                          | 270 | 131.4 | 5.6 | 150   | 0.792   |
| Positive                          | 48  | 135.7 | 13.4| 150   |         |
| **Ki67 status**                   |    |      |     |        |         |
| <20%                              | 189 | 141.0 | 6.7 | 170   | 0.018   |
| ≥20%                              | 128 | 117.9 | 8.1 | 110   |         |
| Unknown                           | 1   | 250.0 | 92.3| 250   |         |
| **Molecular subtype**             |    |      |     |        |         |
| Luminal A                         | 166 | 138.6 | 7.2 | 160   | 0.0711  |
| Luminal B                         | 99  | 134.8 | 9.3 | 150   |         |
| HER2+                             | 13  | 106.2 | 25.6| 100   |         |
| Triple negative                   | 39  | 102.4 | 14.8| 100   |         |
| Unknown                           | 1   | 250.0 | 92.3| 250   |         |
| **P53 status**                    |    |      |     |        |         |
| Negative                          | 193 | 131.1 | 6.7 | 150   | 0.632   |
| Positive                          | 124 | 134.1 | 8.3 | 150   |         |
| Unknown                           | 1   | 50.0  | 92.9| 50    |         |
| **BCL-2 status**                  |    |      |     |        |         |
| Negative                          | 92  | 126.0 | 9.7 | 110   | 0.229   |
| Positive                          | 225 | 134.0 | 6.2 | 150   |         |
| Unknown                           | 1   | 250.0 | 92.7| 250   |         |
| **CTC EP**                        |    |      |     |        |         |
| Negative                          | 235 | 134.1 | 6.0 | 150   | 0.851   |
| Positive                          | 27  | 138.1 | 17.5| 150   |         |
| **CTC EMT**                       |    |      |     |        |         |
| Negative                          | 235 | 134.1 | 6.1 | 150   | 0.300   |
| Positive                          | 56  | 120.4 | 12.4| 153   |         |
| **CTC any**                       |    |      |     |        |         |
| Negative                          | 235 | 134.1 | 6.1 | 150   | 0.472   |
| Positive                          | 83  | 126.1 | 10.2| 150   |         |

CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial-positive.
angiogenesis (19,40–42). The results of the present study demonstrated that elevated MMP9 expression levels in tumor cells and tumor associated stroma were significantly associated with favorable tumor characteristics. Hormone-positive tumors exhibited significantly higher MMP9 expression in tumor cells, as well as in tumor associated stromal cells. In addition, the results demonstrated an association between increased MMP9 expression and low proliferation index of Ki67. Although the role of MMP9 and its association with breast cancer has been extensively studied, data regarding the prognostic value of MMP9 are inconsistent. On one hand, it has been reported that MMP9 expression is associated with a shorter relapse-free survival time in patients with primary breast tumours (26,29,43,44). The association between upregulated MMP9 expression and an increased risk of overall survival and relapse-free survival in breast cancer has also been confirmed via meta-analyses by Song et al (45) and Ren et al (46). Conversely, some studies have identified MMP-9 as a favourable prognostic marker for breast cancer (9,47).

The results of the present study demonstrated a significant association between high MMP9 expression in tumour cells and poor DFS in hormone receptor negative, triple negative, as well as in the CTC_EMT-positive subgroup of patients with early breast cancer. Analysis of stromal cells exhibited this association in the hormone receptor negative and triple negative subgroups of patients.

These results are in concordance with previous studies, confirming the association between MMP9 expression and a shorter progression time, particularly in patients with basal-like or triple negative breast cancer (48,49). Controversy regarding the association between MMP9 expression and clinical outcomes in different types of malignant tumors, including breast cancer, suggests the presence of active and inactive forms of MMP9. MMPs are secreted in the form of inactive proenzymes, whose activation is mediated via different molecular mechanisms (20,21). Thus, the level of active MMP9 in stromal cells and tumor cells may vary, which will subsequently account for the differences in clinical outcomes (50).

Table III. Association between MMP9 expression in stromal cells, patients, tumour characteristics and circulating tumor cells.

| Characteristic                        | N    | Mean  | SEM  | Median | P-value |
|--------------------------------------|------|-------|------|--------|---------|
| MMP9 expression weighted histoscore  |      |       |      |        |         |
| Histology                            |      |       |      |        |         |
| Invasive ductal carcinoma            | 266  | 51.9  | 4.0  | 30     | 0.434   |
| Other                                | 43   | 44.2  | 9.8  | 20     | 0.489   |
| Grade                                |      |       |      |        |         |
| Low and intermediate                 | 194  | 50.9  | 4.6  | 30     | 0.469   |
| High grade                           | 108  | 51.7  | 6.2  | 20     |         |
| Unknown                              | 7    | 34.3  | 24.4 | 0      |         |
| T-stage                              |      |       |      |        |         |
| T1                                   | 213  | 52.6  | 4.4  | 25     | 0.536   |
| >T1                                  | 96   | 46.8  | 6.6  | 25     |         |
| N stage                              |      |       |      |        |         |
| N0                                   | 197  | 54.9  | 4.6  | 30     | 0.021   |
| N1                                  | 110  | 43.5  | 6.1  | 20     |         |
| Unknown                              | 2    | 50.0  | 45.6 | 50     |         |
| Hormone receptor status (cut-off 1%) |      |       |      |        |         |
| Negative for both                    | 49   | 30.7  | 9.1  | 5      | 0.872   |
| Positive for either                  | 260  | 54.6  | 4.0  | 30     |         |
| HER2 status                          |      |       |      |        |         |
| Negative                             | 263  | 50.7  | 4.0  | 20     | 0.137   |
| Positive                             | 46   | 51.6  | 9.5  | 30     |         |
| Ki67 status (cut-off 20%)            |      |       |      |        |         |
| <20%                                 | 183  | 56.3  | 4.8  | 30     | 0.094   |
| ≥20%                                 | 126  | 42.9  | 5.7  | 20     |         |
| Molecular subtype                    |      |       |      |        |         |
| Luminal A                            | 163  | 57.0  | 68.7 | 30     |         |
| Luminal B                            | 97   | 50.5  | 62.0 | 30     |         |
| HER2+                                | 12   | 15    | 24.3 | 0      |         |
| Triple negative                      | 37   | 35.8  | 55.7 | 5      |         |
| P53 status                           |      |       |      |        |         |
| Negative                             | 188  | 48.1  | 4.7  | 30     | 0.537   |
| Positive                             | 120  | 55.5  | 5.9  | 20     |         |
| Unknown                              | 1    | 0.0   | 64.5 | 0      |         |
| BCL-2                                |      |       |      |        |         |
| Negative                             | 88   | 47.9  | 6.9  | 30     | 0.995   |
| Positive                             | 221  | 52.0  | 4.3  | 20     |         |
| CTC EP                               |      |       |      |        |         |
| Negative                             | 229  | 54.4  | 4.1  | 30     | 0.350   |
| Positive                             | 27   | 41.3  | 12.8 | 20     |         |
| CTC EMT                              |      |       |      |        |         |
| Negative                             | 229  | 54.4  | 4.1  | 30     | 0.400   |
| Positive                             | 53   | 40.3  | 10   | 10     |         |
| CTC any                              |      |       |      |        |         |
| Negative                             | 229  | 54.4  | 4.1  | 30     | 0.168   |
| Positive                             | 80   | 40.6  | 7.2  | 20     |         |

CTC, circulating tumor cells; EP, epithelial; EMT, epithelial-to-mesenchymal transition; NA, not applicable.

Figure 5. Kaplan-Meier DFS analysis for a combination of CTC EMT and MMP9. CTC EMT positive patients with MMP9 expression had a worse DFS than patients that are CTC EMT negative. P<0.00001. DFS, disease-free survival; CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition.
ETM is considered a developmental process, facilitating the resistance to apoptosis and increased invasion, and is closely associated with development of a cancer stem cell phenotype (19,51). This machinery can be directly induced by MMPs in the target epithelial cells. Expression of proteases, including MMPs, is upregulated during reorganization of ECM in EMT. In addition, the process of MMP-induced EMT has been best characterized in mammary epithelial cells (52,53). According to the results of the present study, there was no significant association between any subpopulations of CTCs and MMP9 expression. Contrary to MMP1, MMP9 does not actively participate in the release of CTCs into the blood stream of patients with PBC (31). However, these changes may result in the resistance to therapy, and development of a cancer stem cell phenotype closely associated with poor DFS. Given the limited treatment options for these subgroups of patients (triple-negative and CTC_EMT-positive PBC), MMP9 may potentially offer a novel therapeutic target. In addition, the results from the combinatorial survival analysis demonstrated that CTC_EMT

| Characteristic | N | HR Low CI | High CI | P-value |
|---------------|---|-----------|---------|---------|
| Overall       |   | 0.96 0.58 | 1.59    | 0.864   |
| Low MMP9      | 156| 1.47      |         |         |
| High MMP9     | 162|           |         |         |
| Invasive ductal carcinoma |   | 0.84 0.48 | 1.47    | 0.550   |
| Low MMP9      | 126|           |         |         |
| High MMP9     | 146|           |         |         |
| Other histology |   | 2.12 0.55 | 8.17    | 0.335   |
| Low MMP9      | 30 |           |         |         |
| High MMP9     | 16 |           |         |         |
| Intermediate/low grade |   | 0.95 0.44 | 2.07    | 0.901   |
| Low MMP9      | 93 |           |         |         |
| High MMP9     | 107|           |         |         |
| High grade 5 to NA | | | | |
| Low MMP9      | 58 | 0.80 0.40 | 1.58    |         |
| High MMP9     | 52 |           |         |         |
| T1 stage      |   | 0.97 0.48 | 1.98    | 0.936   |
| Low MMP9      | 99 |           |         |         |
| High MMP9     | 119|           |         |         |
| T2 stage and higher | | 0.77 0.37 | 1.60    |         |
| Low MMP9      | 57 |           |         |         |
| High MMP9     | 43 |           |         |         |
| N0 stage      |   | 0.92 0.41 | 2.09    | 0.844   |
| Low MMP9      | 99 |           |         |         |
| High MMP9     | 102|           |         |         |
| N+ stage      |   | 1.04 0.54 | 2.00    | 0.909   |
| Low MMP9      | 55 |           |         |         |
| High MMP9     | 59 |           |         |         |
| ER/PR positive for either | | 1.20 0.66 | 2.18    | 0.539   |
| Low MMP9      | 122|           |         |         |
| High MMP9     | 144|           |         |         |
| ER/PR negative for both | | 0.33 0.12 | 0.93    | 0.025   |
| Low MMP9      | 34 |           |         |         |
| High MMP9     | 18 |           |         |         |
| HER positive  |   | 1.22 0.42 | 3.49    | 0.712   |
| Low MMP9      | 22 |           |         |         |
| High MMP9     | 26 |           |         |         |
| HER negative  |   | 0.91 0.51 | 1.62    | 0.741   |
| Low MMP9      | 134|           |         |         |
| High MMP9     | 136|           |         |         |
| Ki67 low (<20%) | | 1.30 0.57 | 2.99    | 0.523   |
| Low MMP9      | 84 |           |         |         |
| High MMP9     | 105|           |         |         |
| Ki67 high (≥20%) | | 0.62 0.33 | 1.19    | 0.149   |
| Low MMP9      | 72 |           |         |         |
| High MMP9     | 57 |           |         |         |
| Triple negative | | 0.17 0.05 | 0.57    | 0.003   |
| Low MMP9      | 26 |           |         |         |
| High MMP9     | 13 |           |         |         |
| P53 negative  |   | 0.90 0.49 | 1.65    | 0.735   |
| Low MMP9      | 96 |           |         |         |
| High MMP9     | 97 |           |         |         |

| Characteristic | N | HR Low CI | High CI | P-value |
|---------------|---|-----------|---------|---------|
| P53 positive  |   | 1.11 0.43 | 2.82    | 0.829   |
| Low MMP9      | 59 |           |         |         |
| High MMP9     | 65 |           |         |         |
| BCL2 negative |   | 0.53 0.24 | 1.18    | 0.124   |
| Low MMP9      | 51 |           |         |         |
| High MMP9     | 41 |           |         |         |
| BCL2 positive |   | 1.29 0.67 | 2.49    | 0.445   |
| Low MMP9      | 105|           |         |         |
| High MMP9     | 120|           |         |         |
| CTC EP negative | | 1.33 0.69 | 2.57    | 0.387   |
| Low MMP9      | 115|           |         |         |
| High MMP9     | 120|           |         |         |
| CTC EP positive | | 1.52 0.20 | 11.24   | 0.675   |
| Low MMP9      | 12 |           |         |         |
| High MMP9     | 15 |           |         |         |
| CTC EMT negative | | 1.33 0.69 | 2.47    | 0.387   |
| Low MMP9      | 115|           |         |         |
| High MMP9     | 120|           |         |         |
| CTC EMT positive | | 0.40 0.16 | 0.95    | 0.047   |
| Low MMP9      | 29 |           |         |         |
| High MMP9     | 27 |           |         |         |
| CTC any negative | | 1.33 0.69 | 2.57    | 0.387   |
| Low MMP9      | 115|           |         |         |
| High MMP9     | 120|           |         |         |
| CTC any positive | | 0.51 0.23 | 1.14    | 0.113   |
| Low MMP9      | 41 |           |         |         |
| High MMP9     | 42 |           |         |         |

aData not available in 8 patients; bdata not available in 3 patients; cdata not available in one patient; ER, estrogen receptor; PR, progesterone receptor; CTC, circulating tumor cells; EMT, epithelial-to-mesenchymal transition; EP, epithelial; HZ, hazard ratio; CI, confidence interval; NA, not applicable.
positive patients with MMP9 expression in tumor cells had a significantly lower DFS compared with CTC_EMT negative patients, suggesting that EMT acts as a negative prognostic marker only in subgroups of patients with high MMP9 expression, while the subgroup of CTC_EMT positive patients, with low MMP9 expression exhibited no effects.

The spectrum of synthetized MMP inhibitors (MMPIs) assessed in clinical trials have demonstrated poor effectiveness and serious side effects (54,55). The limited clinical effect of MMPIs may be due to their poor selectivity. Previous studies have focused on a broad spectrum of MMPs, most of which exert tumorigenic activity. However, it is necessary to take into consideration that some MMPs are characterized by antitumorigenic effects. Another reason for MMPIs inefficacy can be due to their administration to unselected groups of patients (44,56).

In conclusion, this prospective translational study demonstrated the protective role of MMP9 in patients with breast cancer, whereby its increased expression was associated

| Characteristic | N   | HR  | 95% CI Low | 95% CI High | P-value |
|---------------|-----|-----|------------|-------------|---------|
| Overall       |     |     |            |             | 0.547   |
| Low MMP9 expression | 276 | 1.29 | 0.60 0.60  | 2.78        |
| High MMP9 expression | 33  |     |            |             |         |
| Invasive ductal carcinoma | 237 | 1.41 | 0.63 0.63  | 3.18        |
| Low MMP9 expression | 29  |     |            |             |         |
| High MMP9 expression |     |     |            |             |         |
| Other histology |     |     |            |             | 0.825   |
| Low MMP9 expression | 39  | 0.79 | 0.08 0.08  | 7.84        |
| High MMP9 expression | 4   |     |            |             |         |
| Intermediate/low gradea | 174 | 1.02 | 0.31 0.31  | 3.38        |
| Low MMP9 expression | 20  |     |            |             |         |
| High MMP9 expression |     |     |            |             |         |
| High gradea |     |     |            |             | 0.458   |
| Low MMP9 expression | 96  | 1.56 | 0.57 0.57  | 4.24        |
| High MMP9 expression | 12  |     |            |             |         |
| T1 stage |     |     |            |             | 0.974   |
| Low MMP9 expression | 189 | 1.02 | 0.36 0.36  | 2.90        |
| High MMP9 expression | 24  |     |            |             |         |
| T2 stage and higher |     |     |            |             | 0.457   |
| Low MMP9 expression | 87  | 1.71 | 0.54 0.54  | 5.43        |
| High MMP9 expression | 9   |     |            |             |         |
| N0 stageb |     |     |            |             | 0.460   |
| Low MMP9 expression | 174 | 1.71 | 0.53 0.53  | 5.55        |
| High MMP9 expression | 23  |     |            |             |         |
| N+ stageb |     |     |            |             | 0.871   |
| Low MMP9 expression | 100 | 0.92 | 0.31 0.31  | 2.70        |
| High MMP9 expression | 10  |     |            |             |         |
| ER/PR positive for either |     |     |            |             | 0.323   |
| Low MMP9 expression | 229 | 1.67 | 0.71 0.71  | 3.88        |
| High MMP9 expression | 31  |     |            |             |         |
| ER/PR negative for both |     |     |            |             | 0.002   |
| Low MMP9 expression | 47  | 0.14 | 0.00 0.00  | 4.81        |
| High MMP9 expression | 2   |     |            |             |         |
| HER positive |     |     |            |             | 0.242   |
| Low MMP9 expression | 39  | 3.12 | 0.83 0.83  | 11.74       |
| High MMP9 expression | 7   |     |            |             |         |
| HER negative |     |     |            |             | 0.900   |
| Low MMP9 expression | 237 | 1.06 | 0.43 0.43  | 2.64        |
| High MMP9 expression | 26  |     |            |             |         |
| Ki67 low (<20%) |     |     |            |             | 0.486   |
| Low MMP9 expression | 159 | 1.67 | 0.50 0.50  | 5.53        |
| High MMP9 expression | 24  |     |            |             |         |
| Ki67 high (≥20%) |     |     |            |             | 0.679   |
| Low MMP9 expression | 117 | 0.80 | 0.26 0.26  | 2.50        |
| High MMP9 expression | 9   |     |            |             |         |
| Triple negative |     |     |            |             | 0.001   |
| Low MMP9 expression | 35  | 0.12 | 0.00 0.00  | 4.89        |
| High MMP9 expression | 2   |     |            |             |         |
| P53 negativec |     |     |            |             | 0.901   |
| Low MMP9 expression | 171 | 1.07 | 0.39 0.39  | 2.92        |
| High MMP9 expression | 17  |     |            |             |         |

Table V. Continued.

| Characteristic | N   | HR  | 95% CI Low | 95% CI High | P-value |
|---------------|-----|-----|------------|-------------|---------|
| P53 positivec |     |     |            |             | 0.482   |
| Low MMP9 expression | 104 | 1.68 | 0.50 0.50  | 5.69        |
| High MMP9 expression | 16  |     |            |             |         |
| BCL2 negative |     |     |            |             | 0.078   |
| Low MMP9 expression | 83  | 0.35 | 0.05 0.05  | 2.29        |
| High MMP9 expression | 5   |     |            |             |         |
| BCL2 positive |     |     |            |             | 0.238   |
| Low MMP9 expression | 193 | 2.00 | 0.81 0.81  | 4.96        |
| High MMP9 expression | 28  |     |            |             |         |
| CTC EP negative |     |     |            |             | 0.143   |
| Low MMP9 expression | 202 | 2.76 | 1.08 1.08  | 7.09        |
| High MMP9 expression | 27  |     |            |             |         |
| CTC EP positive |     |     |            |             | 0.053   |
| Low MMP9 expression | 23  | 0.18 | 0.01 0.01  | 2.75        |
| High MMP9 expression | 4   |     |            |             |         |
| CTC EMT negative |     |     |            |             | 0.143   |
| Low MMP9 expression | 202 | 2.76 | 1.08 1.08  | 7.09        |
| High MMP9 expression | 27  |     |            |             |         |
| CTC EMT positive |     |     |            |             | 0.168   |
| Low MMP9 expression | 51  | 0.37 | 0.04 0.04  | 3.50        |
| High MMP9 expression | 2   |     |            |             |         |
| CTC any negative |     |     |            |             | 0.143   |
| Low MMP9 expression | 202 | 2.76 | 1.08 1.08  | 7.09        |
| High MMP9 expression | 27  |     |            |             |         |
| CTC any positive |     |     |            |             | 0.128   |
| Low MMP9 expression | 74  | 0.44 | 0.10 0.10  | 1.91        |
| High MMP9 expression | 6   |     |            |             |         |

aData not available in 7 patients; bdata not available in 2 patients; cdata not available in patient; ER, estrogen receptor; PR, progesterone receptor; CTC, circulating tumor cells; EP, epithelial; EMT, epithelial-to-mesenchymal transition; HZ, hazard ratio; CI, confidence interval; NA, not applicable.
with favourable tumour characteristics. Thus, as it has been proposed by Pozzi et al (57), inhibition of MMP9 antitumori-
genic and antiangiogenic activities may result in a paradoxical increase of tumor angiogenesis and tumor growth. Conversely, the results of the present study demonstrated the association between high MMP9 expression and poor DFS in selected subgroups of patients with PBC, particularly hormone receptor negative and triple negative tumors, as well as in CTC_EMT positive patients. These results suggest that MMP9 exerts different biological roles in HR positive vs. negative tumors, further supporting the concept of different biology of breast cancer subtypes according to their HR status. Thus, assessing MMP9 tumor expression may help identify individuals with increased risk of disease recurrence within the aforementioned subgroups of patients with PBC. However, there were certain limitations to the present study, such as the retrospective design of the study and semi-quantitative IHC analysis used for investigating of MMP9 expression. In addition, the study population represent a homogenous cohort of patients, treatment-naïve, without metastatic disease, in order to avoid the effect of the metastatic site heterogeneity factor on analysed variables.

Further studies are required to develop selective MMPIs against the specific protumorigenic MMPs or protumori-
genic activities of selected MMPs. Another strategy may be anticancer therapy with antitumorigenic MMPs or with their antitumorigenic subparts. An example of this phenomenon involves the MMP8 enzyme, whereby high MMP8 expression supresses metastasis, while MMP8 silencing induces tumour progression and metastasis (58–60).

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Availability of data and materials

All datasets generated and analyzed during the present study are included in this published article.

Authors' contributions

KK, ZC, JM, MM and GM conceived and designed the present study. KK performed statistical analysis. ZC and IM performed immunohistochemical analysis. GM, TS and DK were involved in CTC detection. MK, JB and DP were involved in patient accrual and performed breast surgery. KK and MM drafted the initial manuscript, and all authors reviewed it critically for important intellectual content. All authors participated in the acquisition, analysis and interpretation of data. All authors have read and approved the final version of the manuscript for publication.

Ethics approval and consent to participate

The present study was reviewed and approved by the Institutional Review Board of the National Cancer Institute of Slovakia, Bratislava, Slovakia (approval no. TRU-SK 002; Chair: Professor Michal Mego). Written informed consent was provided by all patients prior to study commencement.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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