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PRECLINICAL STUDY

Infiltration by myeloperoxidase-positive neutrophils is an independent prognostic factor in breast cancer

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Abstract

Purpose Myeloperoxidase (MPO) is an enzyme secreted by neutrophil granulocytes as a result of phagocytosis during inflammation. In colorectal cancer, tumour infiltration by MPO expressing cells has been shown to be independently associated with a favourable prognosis. In this study, we explored the role of MPO-positive cell infiltration and its prognostic significance in invasive breast cancer.

Methods We performed immunohistochemical staining for MPO on multiple tissue microarrays comprising a total of 928 human breast cancer samples with detailed clinical-pathological annotation and outcome data.

Results MPO-positive cell infiltration (≥ 5 cells/tissue punch) was found in 150 (16%) of the 928 evaluable breast cancer cases. In univariate survival analyses, infiltration by MPO-positive cells was associated with a significantly better overall survival ($p < 0.001$). In subset univariate analyses, the infiltration by MPO-positive cells was associated with significantly better overall survival in the Luminal B/HER2-negative subtype ($p = 0.005$), the HER2 enriched subtype ($p = 0.011$), and the Triple Negative subtype ($p < 0.001$). In multivariate analysis, MPO expression proved to be an independent prognostic factor for improved overall survival ($p < 0.001$).

Conclusions This is the first study to show that infiltration of MPO-positive cells is an independent prognostic biomarker for improved overall survival in human breast cancer.

Keywords Breast cancer · Myeloperoxidase · Prognostic factor · Tumour-associated neutrophils

Introduction

Breast cancer is the most common malignant cancer among women worldwide and accounts for 14% of all cancer-related deaths [1]. Over the past decades, important progress has been made in developing ever better treatment options resulting in a considerable increase in both overall and recurrence-free survival rates for almost all types of breast cancer [2]. A significant part of that success is due to the identification of prognostic factors, which provide the basis for clinical decision-making regarding breast cancer treatment, especially neoadjuvant treatment [2]. While various prognostic factors in breast cancer are determined from classical pathophysiological and clinical data, recent evidence suggests that markers expressed in the stroma surrounding neoplastic cells are also of prognostic value [3, 4].

It is well known that fibroblasts, dendritic cells, macrophages and lymphocytes interact in complex ways with

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breast cancer cells [5]. Aside from these key players, neutrophils are also part of the tumour microenvironment (TME) in breast cancer [6, 7]. Tumour-associated neutrophils (TAN) are defined as neutrophils which have migrated into tumours [7]. A meta-analysis conducted in 2014 investigating the presence of TAN across different cancer types showed an association with worse overall and recurrence-free survival [8]. The first study investigating the presence of TAN in breast cancer subtypes in 2017 observed the occurrence of TAN almost exclusively in hormone-receptor-negative ductal adenocarcinoma (Her2-positive and triple negative), with 88% of triple negative breast cancers being TAN positive [9]. However, the small sample size of 105 breast cancer cases and the lack of determination of TAN polarization and therefore distinction between pro-tumourigenic and anti-tumourigenic subtype of TANs limit the informative value of this study [9].

When interpreting the literature, it is very important to consider which of the two existing polarization phenotypes of TANs are predominantly present in the tumour [10]. Due to the presence of cytokines, TAN undergo polarization into a pro-tumourigenic (N2) or pro-inflammatory and anti-tumourigenic (N1) phenotype [10]. The polarization towards the N2 phenotype is transforming growth factor β (TGFβ)-dependent, while blockade of TGF-β leads to a recruitment and activation of the anti-tumourigenic N1 phenotype [10].

Myeloperoxidase (MPO) is one of the main enzymes secreted by neutrophils as a result of phagocytosis during an inflammatory reaction and serves as an immunohistochemical marker for neutrophils [11, 12]. MPO is part of the Reactive Oxygen Species (ROS) and converts hydrogen peroxide (H_2O_2) to the cytotoxic and bacteria killing hypochlorous acid (HOCl) [13, 14]. MPO-derived oxidants induce tumour cell lysis through HOCl delivered directly at the membrane of the cell [15–17] and also activate cell-signalling pathways [18–20]. Pro-inflammatory neutrophils (N1 phenotype) produce more ROS and higher levels of H_2O_2, NO and tumour necrosis factor alpha (TNF-α) than N2 TAN [21, 22]. N1 TANs are also able to recruit and activate CD8 + T cells, which are key contributors in any tumour-immune response, while N2 TANs inhibit their functions [10, 17, 23].

In colorectal cancer (CRC), tumour infiltration by MPO expressing cells has been shown to be an independent prognostic factor for better overall survival [24, 25]. In light of these findings, we explored the role of MPO-positive cell infiltration in invasive breast cancer and correlate its expression with survival data.

Materials and methods

Patients

Tissue from a total of 1476 patients was included in this study. The mean follow-up time was 80.8 months (range 1–263). No patients were lost to follow-up in this cohort. According to the current TNM classification, 50.0% of tumours were of pT2 ($n = 738$) and 49.1% were of pN0 stage ($n = 725$). Detailed demographic information of all 1476 patients can be found in Table 1.

Breast cancer tissue microarray

We used a tissue microarray (TMA) platform of 1476 primary human invasive breast cancers, collected between 1985 and 2007 [26]. Breast cancer tissue punches originating from formalin-fixed and paraffin-embedded tumour tissue were assembled into a TMA format as previously described [27]. The paraffin blocks of the TMA were stored in the certified biobank of the institute of Medical Genetics and Pathology at the University Hospital Basel. Histopathological data was obtained from the individual pathology reports, while clinical and survival data were extracted from the hospital database, Cancer Registry of Basel or from the patients' attending physicians. Ethical standards and patients' confidentiality were ensured and the study was approved by the local ethical committee (Ethikkommission Nordwest- und Zentralschweiz, EKNZ 2014-397, 26.12.2014) and in line with data safety laws.

Immunohistochemistry

For the immunohistochemical staining, we used standard indirect immunoperoxidase assay (IHC; ABC-Elite, Vector Laboratories, Burlingame, CA). All slides were temporarily dewaxed and rehydrated in distilled water, while 0.5% H_2O_2 was used to block endogenous peroxidase activity. Subsequently, the sections were incubated with 10% normal goat serum (DakoCytomation, Carpinteria, CA) and primary MPO-specific antibody (clone 59A5 Novocastra, Newcastle, UK) for 20 min at room temperature. Finally, after being immersed in 3-amino-9-ethylcarbazole plus substrate-chromogen (DakoCytomation) for 30 min, the sections were counterstained with Gill's hematoxylin. For each punch, the MPO-positive tumour infiltrating cells were counted by a trained medical research fellow (F.A.), blinded to the histopathological, clinical and survival data, and difficult cases were discussed with an experienced breast pathologist (S.M) until consensus was reached. Ki-67, ER, PR and HER2 were stained and scored

Table 1 Basic demographic data of all evaluable breast cancer cases ($n = 1476$)

Median tumour size (mm) ± SD	25 ± 18	
Mean age at diagnosis (years) ± SD	64 ± 14	
	Number (*n*)	Percentage
Tumour stage		
pT1	385	26.1
pT2	738	50.0
pT3	107	7.2
pT4	172	11.7
NA	74	5.0
Lymph node involvement		
pN0	725	49.1
pN1	540	36.6
pN2	134	9.1
pN3	0	0.0
NA	77	5.2
Tumour grade		
1	324	22.0
2	576	39.0
3	502	34.0
NA	74	5.0
Histologic subtype		
Invasive ductal	993	67.3
Invasive lobular	203	13.8
Mucinous	38	2.6
Apocrine	17	1.2
Cribriform	41	2.8
Others	117	7.9
NA	67	4.5
Intrinsic subtype		
Luminal A-like (ER^+ and/or PR^+, $HER2^-$, Ki-67 < 14%)	208	14.1
Luminal B-like (HER2-negative) (ER^+ and/or PR^+, $HER2^-$, Ki-67 ≥ 14%)	683	46.3
Luminal B-like (HER2-positive) (ER^+ and/or PR^+, $HER2^+$)	156	10.6
HER2-enriched (ER^-, PR^-, $HER2^+$)	111	7.5
Basal-like (ER^-, PR^-, $HER2^-$)	227	15.4
NA	91	6.2

on the corresponding whole-mounted sections according to the ASCO/CAP guidelines as previously described [28] and were used as surrogate markers for the molecular subtyping [29–31]. According to the classification system for breast cancer subtypes of the St. Gallen Consensus Conference, we divided the cases into Luminal A, Luminal B/HER2-negative, Luminal B/HER2-positive, HER2 enriched and basal-like (triple negative) subtypes [32].

Study design

This is a retrospective cohort study. We selected 1476 primary human invasive breast cancers, which were contained in our TMA platform. The clinical endpoint was overall survival, defined as percentage of patients in our study group alive after the follow-up period. The initially collected variables were median tumour size, mean age at diagnosis,

tumour stage, lymph node involvement, tumour grade, histologic subtype, intrinsic subtype, Ki-67, hormone receptor expression, HER2 status and time of death.

Statistical analysis

5 cells/TMA-punch for MPO infiltration was set as specific cut-off value. All stained cells on the TMA core (intra- and peritumoural) were scored. We defined 2 subgroups (MPO-high and MPO-low). To correlate MPO infiltration with clinicopathological features, we used Chi-Square or Mann–Whitney *U* tests, respectively, for categorical and non-categorical variables. Overall survival was calculated using the Kaplan–Meier method and differences between groups assessed using log-rank tests. Univariate analyses of the effect of MPO positivity and of MPO positivity stratified by intrinsic subtypes were performed using the Cox proportional hazards regression model. For multivariate Cox proportional hazards regression analyses, we evaluated the effects of clinicopathological parameters (age, tumour stage, lymph node involvement, tumour grade), intrinsic subtype and MPO positivity on overall survival. Hazard ratios and corresponding 95% confidence intervals were estimated. All tests were two-sided. *P* values < 0.05 were considered statistically significant. All analyses were performed using R v3.4.2. Any missing clinicopathological information was assumed to be missing at random.

Results

Patient characteristics

The mean age of all 1476 patients was 64 ± 14 years (S.D.) and mean follow-up time was 80.8 months (range 1–263). According to the current TNM classification, 50.0% of tumours were of pT2 ($n = 738$) and 49.1% were of pN0 stage ($n = 725$). Only 22% of the tumours had a BRE grade 1 ($n = 324$). The majority of patients (67.3%) had an invasive ductal carcinoma ($n = 993$), with Luminal B/HER2-negative (46.3%) being the most common molecular subtype ($n = 683$). This high percentage of Luminal B subtypes might be due to the chosen original threshold of Ki-67at $\geq 14\%$. Detailed demographic information of the patients can be found in Table 1.

Association between MPO expression and clinicopathological parameters

Because of loss of tissue on the TMA, MPO expression was evaluable in 928 of the 1476 breast cancer patients.

MPO-positive cell infiltration (≥ 5 cells/tissue punch) was found in 150 (16%) of the 928 evaluable breast cancer cases. Mean tumour size was similar between the two groups (MPO-positive, defined as ≥ 5 cells/punch, and MPO-negative), as shown in Table 2. 56% and 50.6% of the MPO-positive and MPO-negative patients, respectively, presented with tumour stage pT2. In contrast, only 6% of the MPO-positive patients presented with tumour stage 4 compared to 14.3% of the MPO-negative cases. Presence of more than 5 MPO-positive cells was significantly associated with less lymph node involvement seen ($p = 0.002$) with 64% of the MPO-positive cases being of stage pN0 compared to 49.9% of the MPO-negative population. A significant difference could also be observed in the oestrogen receptor expression. Loss of oestrogen receptor expression was more often associated with MPO positivity (45.3% vs. 23.3%, $p > 0.001$). Interestingly, high Ki-67 expression was significantly more often in MPO-positive cases (90.6% vs. 80.6%, $p = 0.005$). Detailed information can be found in Table 2. The association of MPO expression with the breast cancer intrinsic subtype was also significant ($p < 0.001$). Tumours with presence of MPO-positive cells were more often of triple negative (28.7% vs. 15.6% of the MPO-negative cases) and Her2 enriched subtype (14.7% vs. 6.8% of the MPO-negative cases). The complete data can be found in Table 3.

Prognostic significance of high MPO-positive cell infiltration

928 of the 1476 breast cancer samples were evaluable for multivariate survival analysis. Infiltration by MPO-positive cells (≥ 5 cells/punch) was found in 150 (16.1%) of the 928 evaluable breast cancer cases. Representative pictures of high and low MPO-positive cell infiltration can be found in Fig. 1. In univariate survival analysis, infiltration by MPO-positive cells was associated with a significantly better overall survival (Hazard ratio 0.2736, 95% CI 0.1723–0.4346, $p < 0.001$, Table 4 and Fig. 2). In subset univariate analyses, the infiltration by MPO-positive cells was associated with significantly better overall survival in the Luminal B/HER2-negative subtype (Hazard ratio 0.3982, 95% CI 0.2097–0.7562, $p = 0.005$), the HER2 enriched subtype (Hazard ratio 0.2111, 95% CI 0.0637–0.6992, $p = 0.011$), and the triple negative subtype (Hazard ratio 0.0927, 95% CI 0.0339–0.2534, $p < 0.001$, Table 4 and Fig. 2). In multivariate analysis, MPO expression proved to be an independent prognostic factor for improved overall survival (Hazard ratio 0.2376, 95% CI 0.1476–0.3807, $p < 0.001$, Table 5).

Discussion

Our results clearly show that infiltration of MPO-positive cells is an independent prognostic biomarker for better overall survival in human breast cancer. To our knowledge, this

Table 2 Association between MPO expression (pos defined as ≥ 5) and clinicopathological parameters

Clinicopathologic parameter		MPO-positive		MPO-negative		*p* value
Mean tumour size (mm) ± SD		25 ± 16		25 ± 18		0.679
Mean age at diagnosis (years) ± SD		60.5 ± 13		65.0 ± 14		0.012
Clinicopathologic parameter	MPO-positive			MPO-negative		*p* value
	(*n*)	(%)		(*n*)	(%)	
Tumour stage						0.051
pT1	45	30.0		221	28.4	
pT2	84	56.0		394	50.6	
pT3	12	8.0		52	6.7	
pT4	9	6.0		111	14.3	
Lymph node involvement						0.002
pN0	96	64.0		385	49.5	
pN1	47	31.3		306	39.4	
pN2	7	4.7		86	11.1	
pN3	0	0.0		0	0.0	
Tumour grade						< 0.001
1	22	14.7		181	23.2	
2	49	32.7		318	40.9	
3	79	52.7		279	35.9	
Oestrogen receptor						< 0.001
ER^+	82	54.7		594	76.7	
ER^-	68	45.3		180	23.3	
HER2						0.501
$HER2^+$	32	21.3		144	18.6	
$HER2^-$	118	78.7		631	81.4	
Ki67						0.005
$Ki67^{-high}$	135	90.6		620	80.6	
$Ki67^{-low}$	14	9.4		149	19.4	

Table 3 Association between MPO expression (positivity defined as ≥ 5 cells/punch) and breast cancer intrinsic subtype

Intrinsic subtype	MPO-positive		MPO-negative		*p* value
	(*n*)	(%)	(*n*)	(%)	< 0.001
Luminal A (ER^+ and/or PR^+, $HER2^-$, Ki-67 < 14%)	10	6.7	117	15.2	
Luminal B with HER2-negative (ER^+ and/or PR^+, $HER2^-$, Ki-67 ≥ 14%)	65	43.3	388	50.4	
Luminal B with HER2-positive (ER^+ and/or PR^+, $HER2^+$)	10	6.7	92	12.0	
HER2-enriched (ER^-, PR^-, $HER2^+$)	22	14.7	52	6.8	
Triple negative (ER^-, PR^-, $HER2^-$)	43	28.7	120	15.6	

is the first study to investigate MPO-positive cell infiltration in human breast cancer. The fact that MPO expression is associated with negative prognostic factors such as loss of oestrogen receptor, high Ki-67 as well as triple negative and Her2 enriched subtype, but still remains an independent positive prognostic factor for overall survival suggests a potent role of MPO expressing cells in the tumour microenvironment.

Over the last couple of years, there has been an increasing scientific interest in the role of neutrophils in the TME [7, 10, 33]. A meta-analysis across different cancer types conducted in 2014 found a worse overall and recurrence-free survival in the presence of TAN [8]. As mentioned before, TAN undergo polarization into 2 phenotypes, pro-tumourigenic (N2 phenotype), and pro-inflammatory and anti-tumourigenic (N1 phenotype) [10].

N1 TANs express immune-activating chemokines and cytokines and have the capability to kill tumour cells [34]. They also activate and recruit CD8+ T cells by producing T cell attracting chemokines and pro-inflammatory cytokines,

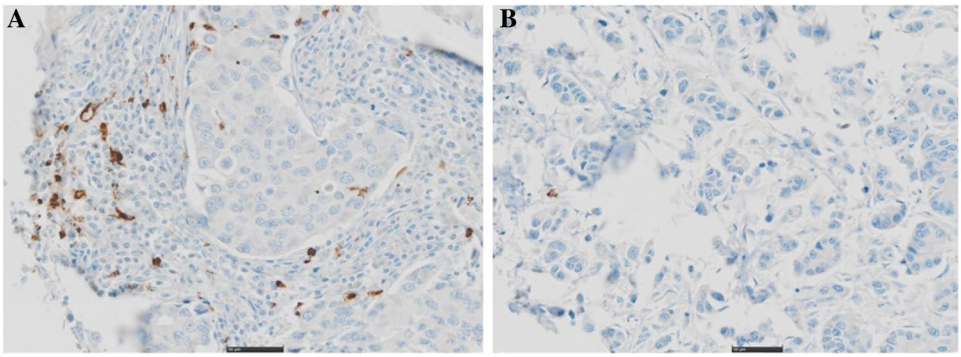

Fig. 1 Representative pictures of high and low MPO-positive cell infiltration. A breast cancer case with high MPO-positive cell infiltration (**a**) and an image with low MPO-positive cell infiltration (**b**). Both cases were stained with immunohistochemical staining for MPO. Scale bars represent 50 µm. Magnification is ×200

Table 4 Univariate analyses for all cases, and by intrinsic subtype, for the effect of MPO expression (positivity defined as ≥ 5 cells/punch) on overall survival

MPO expression, all cases	Hazard ratio (95% CI)	*p* value
MPO-positive	0.2736 (0.1723–0.4346)	< 0.001
MPO expression, by intrinsic subtype		
Luminal A (ER^+ and/or PR^+, $HER2^-$, Ki-67 < 14%)	0.4736 (0.0642–3.4940)	0.464
Luminal B with HER2-negative (ER^+ and/or PR^+, $HER2^-$, Ki-67 ≥ 14%)	0.3982 (0.2097–0.7562)	0.005
Luminal B with HER2-positive (ER^+ and/or PR^+, $HER2^+$)	0.2196 (0.0302–1.5990)	0.134
HER2-enriched (ER^-, PR^-, $HER2^+$)	0.2111 (0.0637–0.6992)	0.011
Triple negative (ER^-, PR^-, $HER2^-$)	0.0927 (0.0339–0.2534)	< 0.001

leading to a lasting immune response against the tumour [35]. It is thus clear that N1 TANs are necessary for developing a tumour-specific primary and memory CD8+ T cell response [36]. Importantly, direct release of HCOl produced by MPO to the tumour cell membrane is responsible for the antibody-dependent cell-mediated cytotoxic (ADCC) effect of N1 TANs [37]. The summary of these findings suggests that MPO is strongly involved in the anti-tumourigenic mechanisms of TANs and that MPO-positive cells mainly correspond to anti-tumourigenic N1 TAN which explains their association with better overall survival.

Studies conducted in CRC show that MPO-positive cell infiltration is also associated with a favourable prognosis [24, 25]. Interestingly, MPO-positive cell infiltration in CRC is characterized by a prognosis as favourable as that of colorectal cancers with high CD8+ T cell infiltration. However, MPO-positive and CD8+ CRC infiltrating cells did not synergize in determining a more favourable outcome, compared with $MPO_{high}/CD8_{low}$ or $MPO_{low}/CD8_{high}$ infiltrating cells [24]. In the past, tumour infiltration by TAN has usually been associated with poor prognosis [38]. The findings in CRC [24, 25] as well as our results in breast cancer suggest a predominance of the N1 phenotype [34–36].

In contrast to our findings, MPO-positive cell infiltration is described as beneficial for tumour development in other cancer types [39]. Polymorphisms in the *MPO* gene leading to higher MPO expression are associated with higher oxidative stress and correlated with a higher susceptibility to lung, ovarian, bladder and liver cancers [40]. Oxidative stress and ROS act as oncogenic factors and lead to genomic instability [41], with HCOl promoting DNA crosslinks and inhibiting DNA repair [42, 43], and MPO inducing DNA damage [41, 44]. Furthermore, MPO leads to activation and inactivation of various proteins, which can contribute to cancer progression and metastasis [45]. These findings suggest that MPO is associated with the anti-tumourigenic features as well as the pro-tumourigenic features of TANs.

Therapeutic approaches are being investigated to support the anti-tumour potential of N1 TANs. In murine cancer models, inhibition of TGF-β promotes the development of N1 TANs, making this a possible therapeutic approach for human cancer patients [10]. Other investigators have tried to block the negative effect of pro-tumourigenic N2 TANs by inhibition of neutrophil infiltration into the tumour [46–48] or inhibition of neutrophil-specific enzymes [49–51]. Another more promising therapeutic approach is the activation of ADCC by the use of anti-tumour monoclonal antibodies (mAbs). Through this activation, TANs develop direct anti-tumourigenic effects through production of ROS [52].

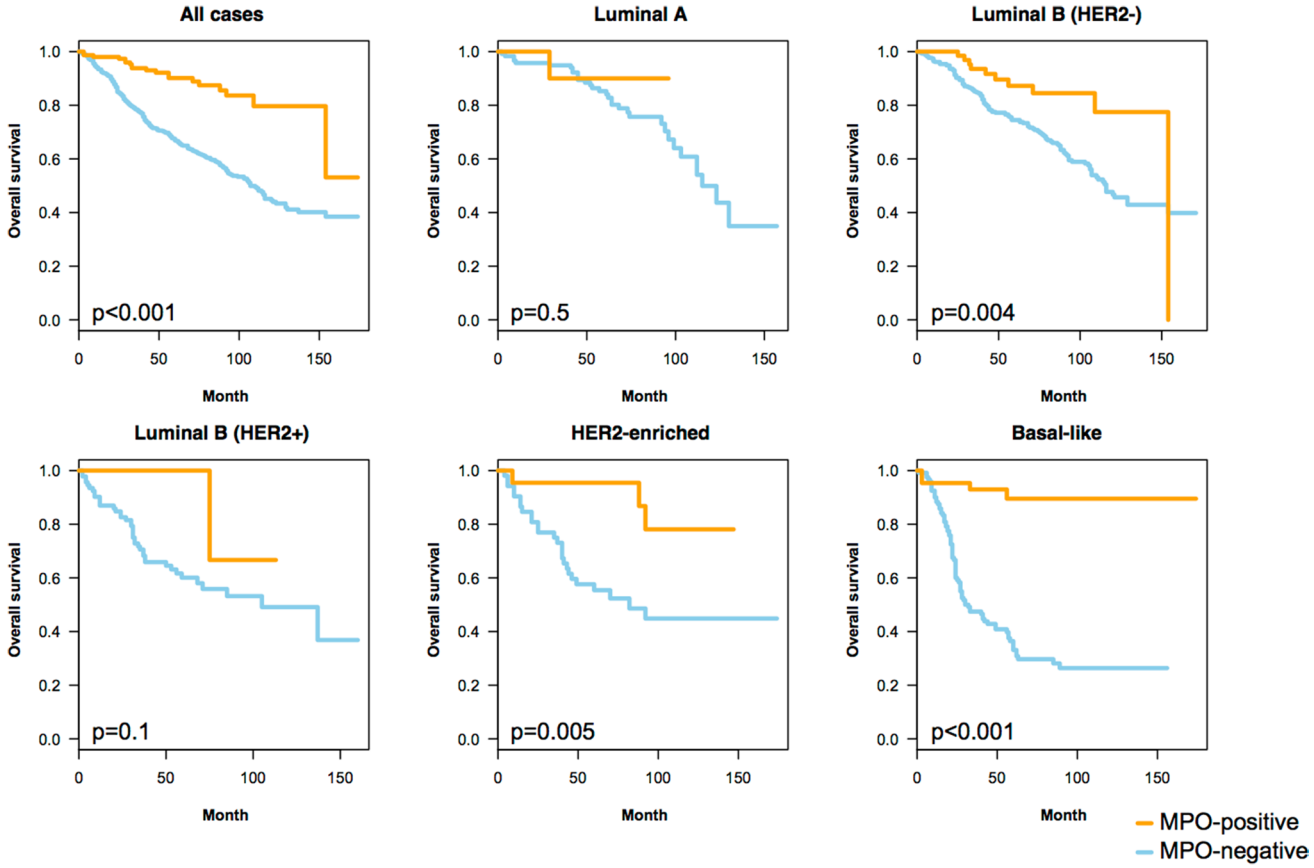

Fig. 2 Correlation of infiltration by MPO-positive cells and overall survival. Kaplan–Meier plots depicting the impact of high MPO-positive cell infiltration (defined as ≥ 5 cells/punch) on overall survival of the different molecular breast cancer subtypes. Statistical analyses were performed using log-rank tests

Table 5 Multivariate analysis for the effect of clinicopathologic parameters and MPO expression (positivity defined as ≥ 5 cells/punch) on overall survival

Clinicopathologic parameter	Hazard ratio (95% CI)	*p* value
Age (per 1-year)	1.0306 (1.0214–1.0398)	< 0.001
Tumour stage	1.4050 (1.2482–1.5815)	< 0.001
Lymph node involvement	1.3428 (1.1350–1.5885)	< 0.001
Tumour grade	1.8637 (1.5672–2.2163)	< 0.001
Intrinsic subtype		
Luminal A	1	
Luminal B with $HER2^-$	0.9736 (0.6631–1.4295)	0.891
Luminal B with $HER2^+$	1.1854 (0.7407–1.8971)	0.478
HER2-enriched	0.9004 (0.5259–1.5414)	0.702
Triple negative	2.4230 (1.5834–3.7078)	< 0.001
MPO expression		
MPO-positive	0.2371 (0.1476–0.3807)	< 0.001

While presenting important findings, our study also has several limitations. First, even though the cohort is well characterized, it is based on a retrospective analysis, and thus more prone to bias, especially selection bias, and other confounders. Secondly, we did not investigate the relation of infiltration by MPO-positive cells and the presence of CD8+ T cells as well as the polarization of the TANs (presence of N1 or N2 phenotype) in breast cancers. A further point which needs to be mentioned is the method of defining the molecular subtypes. We used the definition of the 2011 St. Gallen Consensus Conference, which only provide an approximate definition of intrinsic subtypes and might lead to an underestimation of the impact of MPO in the triple negative subtype [53, 54]. Moreover, the exact molecular mechanisms of the anti-tumourigenic effect of MPO-positive cell infiltration in breast cancer are not completely established and seem to be multifactorial. Further studies are needed to study these interplays. Even so, our data provide new and insightful information into the significance of MPO-positive cell infiltration in breast cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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