Macrophage-Colony Stimulating Factor (CSF1) Predicts Breast Cancer Progression and Mortality

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Abstract. *Background: Macrophage colony-stimulating factor (CSF1), also known as colony-stimulating factor-1 (CSF1), and its receptor CSF1R have been correlated with poor prognosis in many cancer types including breast cancer. Herein, we investigated the prognostic impact of CSF1 and CSF1R expression in tumor epithelial and stromal compartments in primary breast cancer and axillary lymph node metastases. In addition, the density of CD68+ tumor-associated macrophages (TAMs) and CD3+ T-lymphocytes was examined. Materials and Methods: Tumor tissue was obtained at the time of primary surgery from 68 prior treatment breast cancer patients, 38 with axillary lymph node metastases and 30 patients without metastases. Digital video analysis was performed on immunohistochemically stained slides. Results: The expression of CSF1, CSF1R and the density of TAMs and CD3+ T-lymphocytes were then correlated to metastases and disease-specific mortality. Metastasized primary cancers had higher tumor epithelial and stromal expressions of CSF1 ($p<0.001$ and $p=0.002$, respectively) and CSF1R (both $p=0.03$) compared to non-metastatic cancers. Similar findings were made for the density of CD68+ ($p=0.003$) and CD3+ cells in the tumor epithelium ($p<0.001$). In multivariate analysis, a high tumor epithelial expression of CSF1 in primary breast cancer predicted mortality (hazard ratio (HR)=8.6, $p=0.039$). Conclusion: High expression of CSF1 and CSF1R and high density of TAMs and CD3+ T-lymphocytes were related to breast cancer progression. CSF1 expression in tumor epithelium predicted breast cancer mortality.*

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Breast cancer is the most common neoplasm in women. Several studies have documented that these tumors are infiltrated by a heterogeneous populations of immune cells (1, 2). It is also well-established that the tumor microenvironment plays a major role in aggressive behaviour of malignant solid tumors, including breast cancer (3). Tumor-associated macrophages (TAMs) have powerful effects in the neoplastic process because they can adopt tropic roles and are educated by the tumor microenvironment to facilitate angiogenesis, matrix breakdown and tumor cell motility, thus enhancing invasiveness and metastasis (1, 4-6). Monocytes migrate to sites of tissue injury guided by a wide range of chemotactic factors. Macrophage-colony stimulating factor (CSF1) is a haematopoietic growth factor synthesized by fibroblasts, endothelium, macrophages and CD3-activated T-cells (7, 8). CSF1 binds to a specific cell-surface tyrosine kinase, the macrophage-colony stimulating factor 1 receptor (CSF1R). The CSF1/CSF1R, which is the product of the *c-fms* proto oncogene, regulates proliferation and differentiation of the monocytes-macrophage lineage (9-11). Most solid tumors contain a large number of inflammatory cells, including TAMs (4, 9). CSF1 stimulates macrophages to secrete cytokines and proteases, thereby enhancing the macrophages' combat abilities (10). Activated macrophages are the main source of growth factors and cytokines that attract and stimulate the T-lymphocytes (8, 11). In breast cancer, tumor-infiltrating lymphocytes (CD3+ cells) predict response to chemotherapy (12-14). High circulating and/or high tissue expression of CSF are found in breast, ovarian, endometrial and metastatic prostate cancer (15-19).

Immunohistochemical (IHC) and *in situ* hybridization studies have shown that normal, non-lactating breast tissue expresses a low level of CSF1 (20, 21). In breast cancer, CSF1 and CSF1R expressions are correlated with histological grade, progression and clinical outcome (23-25). In the present study on human breast cancer tissues, we investigated whether the expression of the macrophage markers CSF1 and CSF1R were related to breast cancer

death. The density of macrophages (CD68) and T-lymphocytes (pan T-cell marker; CD3) were also assessed and related to CSF1/CSF1R and clinicopathological variables. We compared primary breast cancer tumors with and without metastases, as well as the corresponding axillary lymph node metastases. In all specimens, both tumor epithelial cells and tumor stromal areas were examined.

Materials and Methods

Clinicopathological variables. Primary tumor tissues from 68 patients diagnosed with breast cancer at the University Hospital of Northern Norway (UNN) from 1985 to 2003 were used in this study. Tumor tissue was obtained at the time of primary surgery and prior to any treatment. Diagnoses were made by routine diagnostic pathology and based on fine-needle biopsies, lumpectomy specimens and resection specimens from the archives of the Department of Pathology, UNN. To be included, the diagnosis of breast cancer was confirmed by representatively formalin-fixed in paraffin-embedded tissue blocks. Histological classification and grading of breast cancer was made according to the WHO criteria; Tumor of the Breast and Female Genital Organs, 2003, ISBN 92 832 24124, TMN – classification of Malignant Tumor, 6th ed, UICC 2002, ISBN 0 471 22288 7 (26, 27) and the Nottingham grading system, based on the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system (28).

The metastatic group consisted of 38 patients that had axillary lymph nodes metastases at the time of surgery. Histological diagnoses were ductal carcinoma (n=34) and lobular carcinoma (n=4). Hormone receptor status was recorded at the time of initial diagnoses. The non-metastatic group consisted of 30 patients with no clinical, biochemical or radiological evidence of tumor progression during follow-up. The histological diagnoses in this group were: ductal carcinoma (n=27), lobular carcinoma (n=2) and tubular carcinoma (n=1). Follow-up time was assigned from the date of diagnosis until the date of breast cancer death or through February 2010 (median 200 months), whichever came first. In the event of death, information from the National Causes of Death Registry was used to collect relevant information about the event. The Norwegian National Cancer Data Inspection board and The Regional Committee for Medical and Health Research Ethics (2009/1393), the Data Protection Official for Research (NSD) and the National Data Inspection Board approved the study. The data were analyzed anonymously.

Immunohistochemistry (IHC). IHC was performed on phosphate-buffered saline (PBS)-formalin fixed and paraffin-embedded tissue sections (4 μm) using the standard protocol. The sections were de-paraffinised with xylene and rehydrated through a graded series of ethanol. The applied antibodies had been in-house validated by the manufacturer for IHC analysis on paraffin-embedded material. For antigen retrieval for CSF1 (Clone H-300, sc-13103; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and CSF1R (Clone H-300, sc-13949; Santa Cruz Biotechnology) were diluted 1:50 in PBS. The sections were placed in a microwave oven with Tris/EDTA buffer, pH 9.0 for intervals of 2×10 min at 450 W. For CD68 and CD3 (diluted 1:100 in PBS), citrate buffer, pH 7.0, was used for intervals of 2×10 min at 450 W. The slides were transferred to a Ventana Benchmark XT automated slide stainer (Ventana Medical System, Illkirch, France). The tissue sections were incubated with primary antibodies recognizing CSF1 and CSF1R (both rabbit polyclonal antibodies), as well as the macrophage marker CD68 and the T-lymphocyte marker CD3 (both mouse monoclonal antibody; Ventana). Biotinylated goat anti-mouse IgG and mouse anti-rabbit IgM, both 200 μg/ml, were used as the secondary antibodies. The Peroxidase Block from a DAKO EnVision + System, peroxidase (DAKO Envision + System, HRP) was used for endogenous peroxidase blocking. This system is based on an HRP labelled polymer, which is conjugated with secondary antibodies. In summary, to quench endogenous peroxidase activity, the specimens were incubated with DAKO Peroxidase Block for 5-10 min (room temperature). Then, the specimens were incubated with primary mouse or rabbit antibody, followed by incubation with labelled polymer for 30 min. Finally, the specimens were incubated by diaminobenzidine (DAB) + substrate-chromogen for 5-10 minutes, which resulted in a brown-coloured precipitate at the antigen site. All the slides were then counterstained with hematoxylin to visualize the nuclei. As negative staining controls, the primary antibodies were replaced with the primary antibody diluents. The stromal areas were defined as stromal tissue surrounding the tumor epithelial cells. For stromal cell characterization, the slides were stained for Masson Trichrome (collagen fibres), Giemsa (granulocytes), CD34 (vessels), CD20 (B-lymphocytes), CD3, CD8 and CD4 (T-lymphocytes), CD68 (macrophages), CD56 (NK-cells) and CD1a (dendritic cells); all antibodies were from Ventana. All Ventana antibodies were prediluted by the manufacturer. Estrogen receptors (ER) were visualized by the 1D15 antibody (Dako) and progesterone receptors (PRs) by the NCL-PGR antibody (Abbott Laboratories, Maidenhead, UK) according to a previously published protocol. (26) The staining of ER and PRs was estimated using the "quick score" technique as follows: slides were assessed for both the proportion of cells stained and staining expression. Proportions were scored as 0, no cell staining; 1, 1-25%; 2, 26-50%; 3, 50-75%; or 4, >75% stained cells. The expression was scored as 0, (no staining); 1, (weak); 2, (moderate); or 3, (strong staining). The two scores were added to give a final score of 0-7. A final score <3 was regarded as negative. HER2/neu expression was examined with rabbit monoclonal antibodies, clone 4B5; #790-4493, prediluted by the manufacturer (Ventana). The immunostaining was read in a semiquantitative manner and graded as follows: 0, 1+, 2+ and 3+. Intensity scores of 0 or 1+ were designated as negative expression and 3+ were designated as positive expression.

Digital video analysis. Microscopic images for quantitative analysis were recorded with a Leitz Aristoplane microscope equipped with a Leica DFC 320 digital camera. The Leica QWin V3 Image Analyzing System (Leica Microsystems Digital Imaging Solutions Ltd, Cambridge, UK) was used for morphometric analysis. Leica DFC320 is based on a 3.3 megapixel sensor. Immunoreactivity of CSF1/CSF1R and the number of CD68+ and CD3+ cells was quantified by measuring the colour value of red, green and blue colours (RGB), expressed in composite units. The density threshold of RGB was set to quantify immunopositivity of the RGB colour components; these thresholds were fixed during the study. These measurements were done for both cell areas and tumor stromal areas. The number of pixels falling within each threshold (1 pixel=0.168 μm) indicated the immunoreactivity of each field and was recorded quantitatively by the analysing system. Each whole mount slide was initially examined with light microscopy ×10 to

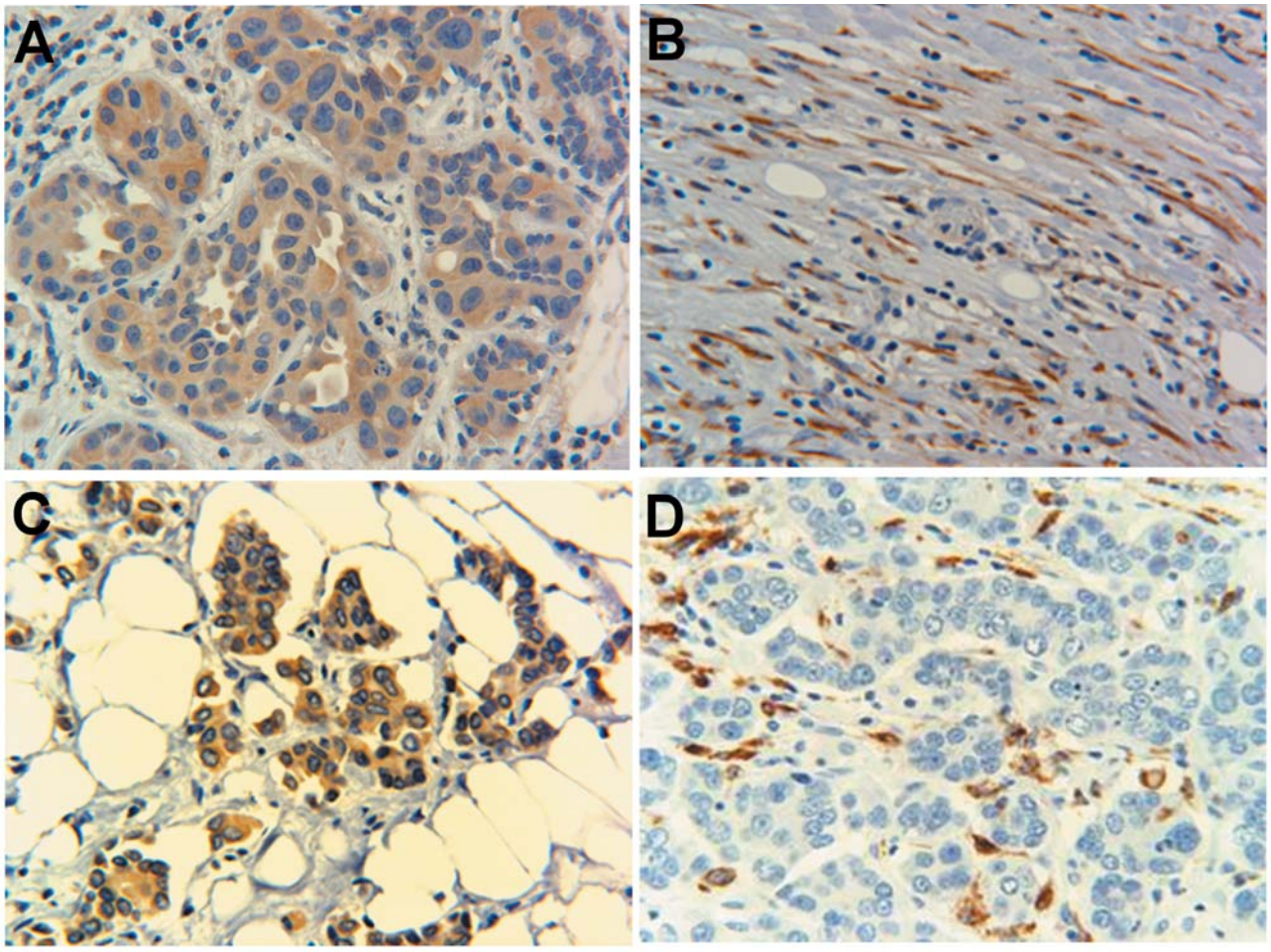

Figure 1. *Immunohistochemical staining. A) Diffuse cytoplasmic expression of M-CSF in groups of tumor epithelial cells in primary metastatic tumor. (B) Strong expression of M-CSF in tumor stromal areas. (C) The expression of CSF-1R in primary metastatic tumor composed of clusters of glands, ill-defined glands with poorly-formed glandular lumina or strands of single cells. (D) CD3-positive cells in between cluster of epithelial cells in tumor cell areas of the metastases.*

×20 magnifications for an overall view. This allowed an area to be chosen as the most representative, with no tissue folding or overlapping and minimal background reactivity, before pictures were taken. This real-time live preview allowed us to adjust and focus directly to the computer monitor without the need of the microscopic eyepiece. Ten different areas along a projected Z-line at ×400 magnification from both the epithelial tumor cells and the tumor surrounding stromal areas in each slide were systematically evaluated for the expression of CSF1 and CSF1R, as well as the number of CD68+ and CD3+ cells. Immunopositivity of CSF1 and CSF1R was identified by the presence of marked diffuse brown cytoplasm in breast tumor cells and in tumor surrounding stromal areas (Figure 1). The staining expression of CSF1 and CSF1R was scored as 0 (no staining), 1 (weak), 2 (moderate) or 3 (strong) and the proportion of positive stained cells within each group was assessed. Score 1-3 was considered as positive staining. Immunopositivity of CD68 and CD3 was seen in cells with morphology as macrophages (histiocytes) and lymphocytes, respectively. The number of CD68+ and CD3+ cells was recorded in single non-epithelial cells within groups of epithelial cancer cells (epithelial tumor areas) and in tumor surrounding stroma in central parts of the tumor. The number of CD68+ and CD3+ cells were scored as 0 (no cells), 1 (1-5 cells), 2 (6-19 cells) and 3 (≥ 20 cells). Stromal areas at the periphery of the tumor were not investigated. All samples were anonymized and scored independently by three investigators (ER, RDU, LTB). When in disagreement, the slides were re-examined and consensus was reached by the observers. The investigators were blinded for all clinical and pathological data.

Statistical analysis. Differences between the primary tumor and their corresponding metastasis were analyzed by the Wilcoxon signed rank test. The Mann-Whitney *U*-test was used to analyze differences between primary tumor of the metastatic and the non-metastatic groups. The risk of death from breast cancer in high (above median) and low (below median) staining groups was compared by the Kaplan-Meier survival analysis and log-rank

Table I. *Patients' characteristics (n=68).*

	Histological grade			Overall
Metastatic group	1 n=6	2 n=20	3 n=12	 n=38
Mean age (range), years ears	52 (39-60)	62 (45-88)	64 (45-89)	59 (39-89)
Hormone therapy	6	20	12	38†
Hormone receptor status				
ER+	5	19	5	29*
ER–	1	1	7	9
PGR+	3	10	0	13
PGR–	3	8	6	17
Her2-Neu+	1	0	6	7
Her2-Neu–	2	4	2	8
Tumor size, mm				
0-10	-	1	1	2
11-20	2	9	3	14*
21-30	1	6	4	11
31-40	-	3	-	3
>41	3	1	4	8*
Mean	2	4	3	9
	Histological grade			Overall
Non-metastatic group	1 n=7	2 n=14	3 n=9	 n=30
Mean age (range), years	57 (38-79)	57 (29-78)	60 (44-75)	58 (29-79)
Hormone therapy				1
Hormone receptor status				
ER+	-	1	-	1
ER–	-	3	1	4
PGR+	-	1	-	1
PGR–	-	-	1	1
Her2-Neu+	-	-	-	0
Her2-Neu–	-	-	-	0
Tumor size, mm				
0-10	3	7	2	12†
11-20	-	4	3	7
21-30	2	2	2	6
31-40	1	-	1	2
>41	-	1	-	1
Mean	2	3	2	7

ER, Estrogen; PGR, progesterone; *$p<0.05$, †$p<0.0001$ for difference between metastatic and non-metastatic group.

test. Cox proportional hazards regression models were used to model the outcome of breast cancer death as a function of staining expression. Age, histological grade (1, 2 and 3), tumor size, hormone therapy (yes/no) and metastasis (yes/no) were included in the models in separate analyses to adjust for possible confounding. Due to the limited numbers of patients and few deaths, the survival analysis was performed for all primary tumor pooled together. Disease-specific survival was determined from the date of diagnosis to the date of breast cancer death. A two-sided *p*-value <0.05 was considered statistically significant. The SPSS 20.0 software package was used in all analyses (SPSS Inc., Chicago, IL, USA).

Results

Clinicopathological variables. Table I provides clinical details of the patients. Mean age in the metastatic and non-metastatic groups was 61.3 years (range=39-89) and 57.7 years (range=29-79). In the metastatic group, 52% were of histological grade 2 and 3. Estrogen and progesterone receptor status was examined in all patients, whereas Her2-neu receptor status was checked in 15 patients. In the non-metastatic group, 47 % were histological grade 2. Estrogen

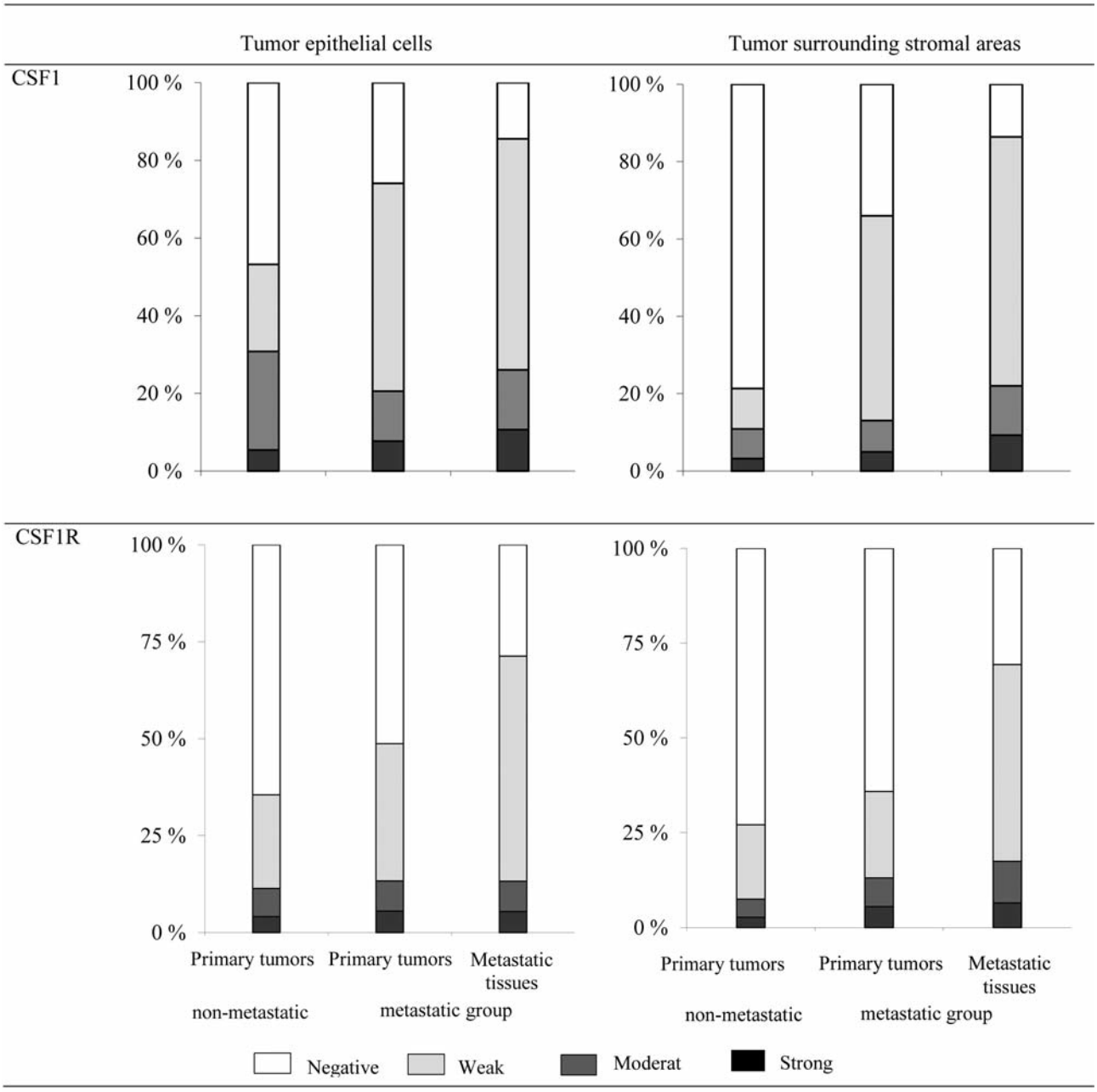

Figure 2. *Percentage distribution of staining intensity of CSF1/CSF1R.*

and progesterone receptor status was performed in 7 patients, whereas Her2- status in none. Tumor size was >10 mm in 36 (95 %) patients with metastasis and in 16 (57 %) patients without metastasis.

Immunohistochemistry and tissue specificity of CSF1, CSF1R, CD68+ and CD3+. Figure 1 and Table II demonstrate the IHC staining of CSF1 and CSF1R in tumor epithelium and tumor surrounding stroma of non-metastatic and metastatic cancers. The expression of CSF1 and CSF1R were predominantly cytoplasmic with no membrane or nuclear staining (Figure 1). There were higher expressions of CSF1 and CSF1R in the metastatic primary cancers compared to the non-metastatic cancers and this difference was significant for both tumor epithelium and tumor surrounding stroma (Figure 2) (CSF1; $p<0.001$ and $p=0.002$,

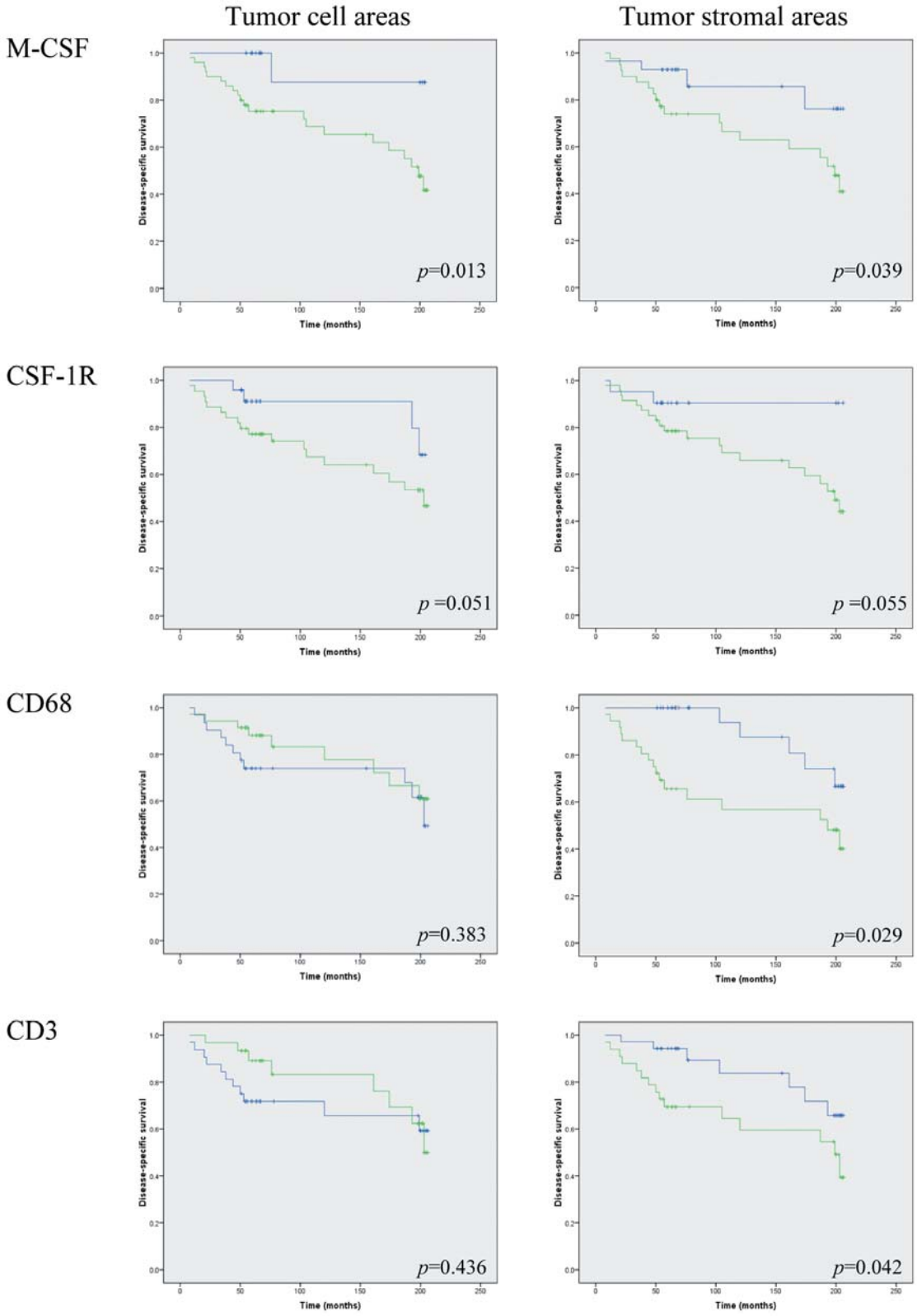

Figure 3. *Disease-specific survival in tumor cell areas and tumor stromal areas of metastatic tumors by assessment of M-CSF, CSF-1R, CD68 and CD3 expression.*

Table II. *Median (IQR) distribution of CSF1 and CSF1R in metastatic versus non-metastatic group.*

		Primary tumors non-metastatic group		Primary tumors metastatic group		Metastatic tissues	
		Median	IQR	Median	IQR	Median	IQR
CSF	Negative	466.1*	377.7-549.2	239.7	7.45-424.5	28.8	0.0-207.3
Tumor epithelium	Weak	22.5	181.8-265.2	554.1†	406.3-686.7	565.6	500.5-875.9
	Moderate	262.3†	208.6-314.4	59.9	67.3-195.3	100.8	75.4-198.1
	Strong	51.5	43.3-60.0	70.7	27.0-110.9	85.1	19.9-145.8
Tumor stroma	Negative	806.4†	754.2-909.3	270.5	2.11-639.3	1.8	0.0-271.0
	Weak	103.1	49.4-142	483.4†	255.3-812.6	675.1	420.6-824.5
	Moderate	57.8	33.4-196.1	64.6	33.9-119.8	96.1	56.5-184.7
	Strong	21.1	8.8-56.1	40.7	18.4-61.1	77.5	34.3-110.8
CSF1R	Negative	587.3	367.1-677.9	640.0	516.2-832.5	177.7	65.7-556.0
Tumor epithelium	Weak	220.7	240.4-411.4	316.6*	116.0-351.4	627.1	331.7-791.0
	Moderate	45.8	36.1-71.0	58.4	31.3-102.9	58.1	24.9-127.7
	Strong	49.2	23.9-70.0	34.7	16.1-70.0	17.0	4.3-66.8
Tumor stroma	Negative	761.6*	547.1-741.0	655.7	632.0-843.8	192.8	53.4-360.6
	Weak	237.0*	160.8-309.1	154.3	96.1-199.3	523.0	380.3-724.0
	Moderate	34.3	48.8-104.7	76.6†	15.0-62.7	103.8	52.5-205.9
	Strong	25.8	9.54-28.2	28.8	12.1-68.1	50.0	20.2-99.3

Medians per 1,000 counted cells. IQR=Interquartile range. *$p<0.005$, †$p<0.0001$ for differences between metastatic- and non-metastatic primary tumors.

CSF1R; both, $p=0.003$). In the axillary lymph node metastases, CSF1 expression was even higher (both $p<0.001$). For CSF1R, higher expression was restricted to the tumor epithelium only ($p=0.007$).

Also, the number of CD68+ cells in tumor epithelial areas and tumor surrounding stroma (both $p=0.003$) was higher in primary tumor of the metastatic group and highest in axillary lymph nodes ($p<0.001$ for both) (Table III). The highest density of CD3+ cells was found in the metastatic group in tumor epithelial cells ($p=0.003$).

There were no correlations between the investigated markers and hormone receptor status.

All slides were also stained for Masson Trichrome, Giemsa, CD34, CD20, CD3, CD8 and CD4 CD68, CD56 and CD1a, for the purpose of characterizing stromal cells and to ensure that we only were measuring stromal cells and compared the staining expression of CSF1/CSF1R with these cell markers. These markers were not further scored.

Table III. *Distribution of CD68- and CD3-positive stained cells in metastatic and non-metastatic cancers.*

	Tumor cell areas		Tumor stromal areas	
	CD68	CD3	CD68	CD3
Metastatic group				
Primary tumours				
Median	643*	534*	373*	487
Interquartile range	532-776	282-717	233-477	275-738
Metastases				
Median	556†	270*	828†	917†
Interquartile range	294-810	84-507	641-915	724-945
Non-metastatic group				
Median	453	104.5	173	761
Interquartile range	166-704	32-370	88-68	545-900

Medians per 1,000 counted cells. *$p=0.003$, †$p<0.001$ for differences between metastatic- and non-metastatic primary tumor.

Survival analyses. During follow-up, death from breast cancer was registered in 24 persons (35.3%). Disease-specific survival, according to high *versus* low staining expression in primary tumors, is presented as Kaplan-Meier plots (Figure 3). High expression (above median) of CSF1 in both tumor epithelial and stromal areas was associated with breast cancer mortality ($p=0.013$ and $p=0.039$, log rank test), whereas high CSF1R expression was borderline associated ($p=0.051$ for tumor epithelium, $p=0.055$ for stromal areas). There were significantly more breast cancer deaths in the groups with high stromal density of CD68+ and CD3+ cells ($p=0.029$ and $p=0.042$, respectively).

Table IV displays hazard ratios (HR) for breast cancer mortality in high *versus* low staining of primary tumors. High CSF1 expression in the tumor epithelium predicted about eight times higher risk of death compared to low CSF1 expression (HR 7.9, 95% CI 1.1-59.7, $p=0.005$). After adjustment for age, histological grade, tumor size, hormone therapy and metastasis, this risk remained significant (HR 8.6, 95% CI 1.1-

Table IV. *Risk of death from breast cancer in high versus low staining intensity of M-CSF, CSF-1R, CD68 and CD3 of primary tumors (n=68).*

	Tumor	*p*-Value	Stroma	*p*-Value
M-CSF				
Unadjusted HR (95% CI)	7.9 (1.1-59.7)	0.005	2.5 (0.8-7.7)	0.072
Multivariate adjusted* HR (95% CI)	8.6 (1.1-66.9)	0.039	2.2 (0.7-6.9)	0.157
CSF-1R				
Unadjusted HR (95% CI)	3.1 (0.9-10.8)	0.066	2.2 (0.6-7.4)	0.214
Multivariate adjusted* HR (95% CI)	2.8 (0.7-10.0)	0.100	3.9 (0.9-17.5)	0.391
CD68				
Unadjusted HR (95% CI)	0.6 (0.2-1.5)	0.304	4.2 (1.1-15.7)	0.034
Multivariate adjusted* HR (95% CI)	0.9 (0.1-2.0)	0.308	1.1 (0.2-5.6)	0.891
CD3				
Unadjusted HR (95% CI)	0.3 (0.1-1.2)	0.436	4.3 (1.1-15.9)	0.031
Multivariate adjusted* HR (95% CI)	0.4 (0.1-2.0)	0.308	4.0 (0.7-21.1)	0.100

HR, Hazard ratio; CI, confidence interval. *Adjusted for age, tumor size, histological grade, hormone therapy and metastases.

66.9, p=0.039). No association was found between stromal CSF1 expression and breast cancer mortality. For CSF1R, neither tumor epithelial nor stromal expression predicted mortality. In univariate analyses, high stromal density of CD68+ and CD3+ cells was associated with increased breast cancer death (p=0.034 and p=0.031, respectively). However, after adjustment, the associations were no longer significant.

Discussion

We found higher expressions of CSF1 and CSF1R, as well as higher density of CD68+ and CD3+ cells in metastatic breast cancer. These findings were apparent in both tumor epithelium areas and the surrounding stroma. For all the markers, the expressions were even higher in metastatic tissue. This may indicate a dose-response relationship between these markers and tumor aggressiveness. The higher stromal expression in metastatic tumors suggests that the surrounding microenvironment plays an important role in breast cancer progression. Moreover, CSF1 expression in the neoplastic epithelium of primary cancers predicted breast cancer mortality.

A large number of studies have focused on the prognostic value of TAMs in solid tumors. Several studies suggest that TAMs are the most abundant inflammatory cells in solid tumor microenvironment, beneficial for tumor growth and correlated with poor disease outcome in breast cancer (29-33). However, there are some studies showing that high density of macrophages correlate with increased survival. A meta-analysis including 144 studies on different solid cancers reported that TAMs showed antitumorigenic properties in studies on colorectal cancer (35).

CSF1R is an important regulator of proliferation and differentiation of monocytes and macrophages regulating most of the tissue macrophages and is expressed in breast epithelial tissue during physiological stages like pregnancy and lactation (20). Data indicate that the oncogenic potential of CSF1 and CSF1R in epithelial cancer cells is due to co-expression of the receptor and its ligand (23); however, cancer cells produce various cytokines and chemokines that attract leucocytes in a similar manner as in a site of tissue injury (31). Both transgenic and xenograft mouse models have shown that macrophages are essential for tumor invasion (21). Furthermore, TAMs have high impact on cancer development because they are educated by tumor microenvironment to facilitate matrix invasion, angiogenesis and tumor cell motility (1, 31, 35). This demonstrates the autocrine manner in tumor cells in which CSF1 and CSF1R are co-expressed or the paracrine manner when CSF1R is stimulated by CSF1 release by the tumor microenvironments. An experimental study by Beck *et al*. showed that CSF1 deficient mice had fewer tumor metastases than those producing normal levels of CSF1 (31). Furthermore, local expression of CSF1 in the primary breast tumors in these mice led to accumulation of monocytes and macrophages in the tumor and promoted metastases equivalent to those seen in wild-type mice with normal levels of CSF1 (32). Maher *et al*. (1998) found a relationship between CSF1R and ipsilateral recurrence (33). However, it is debated whether circulating CSF1 is a tumor marker for breast cancer or predictive of breast cancer risk. Our findings suggest the former.

Most solid tumors contain large numbers of infiltrating macrophages in the surrounding stromal areas (30), which is correlated to poor prognosis (12, 23). CSF1 also stimulates macrophages to cytokine production, especially interleukin-10 (IL-10) (1), which is present in high levels in metastatic tumors (18), also correlated with tumor progression. The high density of CD68+ cells in metastasizing cancers reflect the important role cells of the macrophage lineage exert in tumor aggressiveness.

As CD3+ cells do not express the CSF1 or CSF1R, the CSF1 they produce acts locally or by humoral route on other cells, such as monocytes, participating in the regulation of the inflammatory and immune response (36, 37). There is increasing evidence that both local and systemic inflammatory responses play an important role in the progression of solid tumors. Especially, the presence of CD8+ T cells in breast cancer is associated with a significant reduction in risk of death in both the ER+/ER- Her2-positive subtypes (34). Our findings support the hypothesis that macrophages and T-cell activity in the stromal microenvironment play an important role in breast cancer progression.

Recent studies have highlighted differential roles for distinct TAM subsets in promoting metastasis in breast cancer. The blockade of CSF1R signalling decreases the number of macrophages. However, additional mechanistic insights are needed in order to understand how macrophages are depleted and the global effects of CSF1R inhibition on other tumor-infiltrating immune cells. These findings, taken together with the antitumor effects of CSF1R blockade, implicate the CSF1R pathway as a key regulator in the maintenance of an immunosuppressive tumor microenvironment (38).

Instead of evaluating the protein expression in hotspots areas, we evaluated ten consecutively chosen fields along a projected Z-line in each tumor specimen. We also distinguished between tumor epithelial cells and tumor stromal areas. This approach, chosen for both, achieved a more representative picture of the tumor specimens and emphasized on the stromal tissue's role in tumourigenesis. We used digital video image analysis for quantification of the immunostaining. In general, this method is regarded as being more objective with higher sensitivity and reproducibility than using a light microscope and with better responsiveness to changes in cell counts (39, 40).

A shortcoming to this study is the limited number of patients and archived materials. Even if some risk estimates are strong, the confidence intervals are wide. However, our study supports that the investigated markers are potentially therapeutic targets and should warrant larger prospective studies.

Conclusion

In metastasizing primary breast cancers, we found higher expressions of CSF1 and CSF1R, as well as higher density of CD68+ and CD3+ cells compared to non-metastasizing cancers. CSF1 expression in tumor epithelium was an independent predictor of breast cancer death.

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References

1 Pollard JW: Trophic macrophages in development and disease. Nat Rev Immunol *9*: 259-270, 2009.

2 Campell MJ, Toonlar NY, Garwood ER, Huo D, Moore DH, Kramtsov AL, Au A, Chen Y, Malaka DO, Lin A, Adeyanju OO, Li S, Gong C, McGrath M, Olopack OI and Essermann LJ: Proliferating macrophages associated with high grade hormone receptor negative breast cancer and poor clinical outcome. Breast Cancer Res Treat *128(3)*: 703-711, 2011.

3 Bingle L, Brown NJ and Lewis CE: The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol *196*: 254-265, 2002.

4 Qian BZ and Pollard JW: Macrophage diversity enhances tumor progression and metastasis. Cell *141*: 39-51, 2010.

5 Pixley F and Stanley ER: CSF-1 regulation of the wandering macrophage: Complexity in action. Trends Cell Biol *14(11)*: 628-638, 2004.

6 Ide H, Hatake K, Terado Y, Tsukino H, Okegawa T, Okegawa T, Nutahara K, Higashihara E and Horie SH: Serum level of macrophage colony-stimulating factor is increased in prostate cancer patients with bone metastasis. Hum Cell *21(1)*: 1-6, 2008.

7 Stanley ER, Berg KL, Einstein DB, Lee PS, Pixley FJ, Wang Y and Yeung YG: Biology and action of colony-stimulating factor-1. Mol Reprod Dev *46(1)*: 4-10, 1997.

8 Fretiér S, Besse A, Delwail A, Garcia M, Morel F, leprivey-Lorgeot V, Wijdenes J, Praloran V and Lecron JC: Cyclosporin A inhibition of macrophage colony-stimulating factor (M-CSF) production be activated human T Lymphocytes. J Leucoc Biol *71(2)*: 289-294, 2002.

9 Hume DA: The mononuclear phagocyte system. Curr opin Immunol *18(1)*: 49-53, 2006.

10 Pollard JW: Tumor-educated macrophages promote tumor progression and metastasis. Nat Rev Cancer *4(1)*: 71-78, 2004.

11 Condeelis J and Pollard JW: Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. Cell *124(2)*: 263-266, 2006.

12 Dermaria S, Volm MD, Shapiro RL, Yee HT, Oratz R, Formenti SC and Muggia F: Development of tumor-infiltrating lymhocytes in breast cancer after neoadjuvant Paclitaxel chemotherapy. Clin cancer Res *7(10)*: 3025-3030, 2001.

13 Denkert C, Loibl S, Roller M, Muller BM, Komor M, udczies J, Darb-Esfahani S; Kronenwett R, Hanusch C, von Torne C, Weichert W, Engels K, Solbach C, Schrader I, Dietel M and von Minckwitz G: Tumor-associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol *28*: 105-113, 2010.

14 Loi S1, Sirtaine N, Piette F, Salgado R, Viale G, Van Eenoo F, Rouas G, Francis P, Crown JP, Hitre E, de Azambuja E, Quinaux E, Di Leo A, Michiels S, Piccart MJ and Sotiriou C: Prognostic and predicitive value of tumor-infiltrating lymphocytes in phase III randomized adjuvant breast cancer trial in node-positive

breast cancer comparing the addition of docetaxel to doxorubicin with doxorubicin-based chemotherapy: BIG 02-98. J Clin Oncol *31(7)*: 860-867, 2013.
15 Ham M and Moon A: Inflammatory and microenvironmental factors involved in breast cancer progression. Arch Pharm Res *36(12)*: 1419-1431, 2013.
16 Pollard JW: Macrophages define the invasive microenviroment in breast cancer. J Leucocyte Biol *84(3)*: 623-630, 2008.
17 Toy EP and Azodi M: Enhanced ovarian cancer tumorigenesis and metastasis by the macrophage colony-stimulating factor. Neoplasia *11(1)*: 136-144, 2009.
18 Richardsen E, Uglehus RD, Due J, Busch and Busund LT: The prognostic impact of M-CSF, CSF-1R, CD68 and CD3 in prostatic carcinoma. Histopathology *53(1)*: 30-38, 2008.
19 Lewis CE and Pollard JW: Distinct role of macrophages in different tumor microenvironments. Cancer Res *66(2)*: 605-612, 2006.
20 Sapi E: The role of CSF-1 in normal physiology of mammary gland and breast cancer. Exp Biol Med *229(1)*: 1-11, 2004.
21 Kacinski BM, Scata KA, Carter D, Yee LD, Sapi E, King BL, Chambers SK, Jones MA, Pirro MH and Stanley ER: FMS (CSF-1R) and CSF-1 transcripts and protein are expressed by human breast carcinomas *in vivo* and *in vitro*. Oncogene *6(6)*: 941-952, 1991.
22 Tamimi RM, Brugge JS, Freedman ML, Miron A, Iglehart JD, Colditz GA and Hankinson SE: Circulating colony stimulating factor-1 of mammary gland and breast cancer. Cancer Res *68(1)*: 18-21, 2008.
23 Lin EY and Pollard JW: Role of infiltrated leucocytes in tumor growth and spread. Br J Cancer *90(11)*: 2053-2058, 2004.
24 Barrios CH, Liu MC, Lee SC, Vanlemmens L, Ferrero JM, Tabei T, Pivot X, Iwata H, Aogi K, Lugo-Quintana R, Harbeck N, Brickman MJ, Zhang K, Kern KA and Martin M: Phase III study of bevacicumab plus docetaxel compared with placebo plus docetaxel for first-line treatment of human epidermal growth factor receptor 2-negative metastatic breast cancer. J Clin Oncol 10; *28(20)*: 3239-3227, 2010.
25 Zhang QW, Liu L, Gong CY, Shi HS, Zeng YH, Wang XZ, Zhao YW, and Wei YQ: Prognostic significance of tumor-associated macrophages in solid tumor: A meta-analysis of the literature. PLoS ONE *7(12)*: e50946, 2012.
26 World Health Organizations: Tumors of the Breast and female Genital Organs. ISBN 92 832 24124, 2003.
27 World Health Organizations: TMN – classification of Malignant Tumors. 6th ed, UICC, ISBN 0 471 22288 7, 2002.
28 Elston CW and Ellis IO: Pathologic prognostic factors in breast cancer. I. The value of histological grades in breast cancer. Experience from a large study with long-term follow-up. Histopathology *19*: 403-410, 1991.
29 Aharinejad S, Salama M, Paulus P, Zins K, Berger A and Singer CF: Elevated CSF1 serum concentration predicts poor overall survival in women with early breast cancer. Endocr Relat Cancer 14; *20(6)*:777-783, 2013.
30 Tang RP, Kacinski B, Validire P, Beuvon F, Sastre X, Benoit P, dela Rochefordière A, Mosseri V, Pouillart P and Scholl S: Oncogene amplification correlates with dense lymphocyte infiltration in human breast cancers: a role for hematopoietic growth factor release by tumor cells. J Cell Biochem *44(3)*: 189-198, 1990.
31 Beck AH, Espinosa I, Edris B, Li R, Montgomery K, Zhu S, Varma S, Marinelli RJ, van de Rijn M and West RB: The macrophage colony-stimulating factor 1 response signature in breast carcinoma. Clin Ca Res *15(3)*: 778-787, 2009.
32 Lin EY, Gouon-Evans V, Nguyen AV and Pollard JW: The macrophage growth factor csf-1 in mammary gland development and tumor progression. J Mammary Gland Biol Neoplasia *7(2)*: 147-162, 2002.
33 Maher MG, Sapi E, Turner B, Gumbs A, Perotta PL, Carter D, Kacinski BM and Haffty BG: Prognostic significance of colony-stimulating factor receptor expression in ipsilateral breast recurrence. Clin Cancer Res *4(8)*: 1851-1856, 1998.
34 Ali HR, Provenzano E, Dawson SJ, Blows FM, Liu B, Shah M, Earl HM, Poole CJ, Hiller L, Dunn JA, Bowden SJ, Twelves C, Bartlett JM, Mahmoud SM, Rakha E, Ellis IO, Liu S, Gao D, Nielsen TO, Pharoah PD and Caldas C: Association between CD8+ T-cell infiltration and breast cancer survival in 12,439 patients. Ann Onco*l 25(8)*: 1536-1543, 2014.
35 Qian BZ and Pollard JW: New tricks for metastasis-associated macrophages. Breast Cancer Res *14(4)*: 316, 2012.
36 Qian BZ, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, Kaiser EA, Snyder LA and Pollard JW: CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature *475(7355)*: 222-2225, 2011.
37 Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, Lang RA and Pollard JW: A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS ONE *4*: e6562, 2009.
38 Rolny C, Mazzone M, Tugues S, Laoui D, Johansson I, Coulon C, Squadrito ML, Segura I, Li X, Knevels E, Costa S, Vinckier S, Dresselaer T, Åkerud P, De Mol M, Salomäki H, Phillipson M, Wyns S, Larsson E, Buysschaert I, Botling J, Himmelreich U, Van Ginderachter JA, De Palma M, Dewerchin M, Claesson-Welsh L, and Carmeliet P: HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell *19*: 31-44, 2011.
39 De Boer WI, Hiemstra PS, Sont JK, De Heer E, Rabe KF, Van Krieken JH and Sterk PJ: Image analysis and quantification in lung tissue. Clin Exp Allergy *31(3)*: 504-508, 2001.
40 Faul JL, Demers EA, Burke CM and Poulter LW: The reproducibility of repeated measures of airway inflammation in stable atopic asthma. Am J Respir Crit Care Med *160(5 Pt 1)*: 1457-1461, 1999.

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