Tumor associated macrophages are strongly related to vascular invasion, non-luminal subtypes and interval breast cancer

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Running head: Tumor associated macrophages in breast cancer

Summary

Tumor associated macrophages (TAM) resemble M2 macrophages, promote tumor invasion and show strong expression of CD163 in breast cancer. We here investigated the association between CD163 positive macrophages and vascular invasion, molecular subgroups, mode of detection, and patient outcome. We performed a population-based, retrospective study of invasive breast cancer from the Norwegian Breast Cancer Screening Programme in Vestfold County (2004-2009), including 200 screen-detected and 82 interval cancers. Immunohistochemically CD163 positive macrophages were counted in the most active areas (hot-spots) and dichotomized as high (upper quartile) and low counts. Lymphatic vessel involvement (LVI) and blood vessel invasion (BVI) were recorded separately based on immunohistochemical staining (D2-40 and CD31 antibodies). High levels of CD163 positive macrophages were associated with blood vessel invasion and lymphatic involvement as well as interval cancer detection when compared to screening detected tumors. In addition, the presence of high CD163+ TAM levels was more often observed in HER2 positive, basal-like and triple negative breast cancers and was associated with several features of aggressive tumors. In survival analyses, cases with combined high CD163 counts and BVI showed a significantly reduced recurrence free survival (RFS) and disease specific survival (DSS) ($P<0.001$ for both) compared with all other cases. The presence of CD163 positive tumor associated macrophages is strongly related to aggressive features of breast cancer such as vessel invasion, detection between screening intervals, non-luminal molecular subgroups and reduced survival.

Keywords: Tumor associated macrophages; CD163 counts; Breast cancer, Vessel invasion; Molecular subtypes; Interval breast cancer

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1. Introduction

Vascular invasion (VI) in breast cancer is associated with increased risk of recurrence, metastases and death of the disease (1, 2). In a recent study we demonstrated that BVI was strongly related to a basal-like phenotype, interval cancer presentation and poor prognosis (3).

Tumor associated macrophages (TAM) resemble M2 macrophages in established tumors, contributing to tumor progression, probably because neoplastic tissues show similarities to wound healing (4-7). M1 macrophages are pro-inflammatory and suppress tumor cells, unlike M2 macrophages, which are immunosuppressive and promote tumor growth (4, 6). Intra-vital imaging of rodent mammary tumors have shown that intravasation of tumor cells into blood vessels can be enhanced by perivascular TAMs through secretion of epidermal growth factor (EGF) and colony-stimulating factor (CSF-1) (8). Macrophages and tumor cells co-migrate along collagen fibers to vessels where cancer cells intravasate at sites enriched with perivascular macrophages (9, 10). TAMs also produce matrix-degrading enzymes such as matrix metalloproteinases which facilitate migration of tumor cells, degradation of blood vessel basal membranes and intravasation (11).

M2 macrophages express high levels of CD163, and this marker has been used to discriminate between M1 and M2 macrophages (12). To our knowledge, CD163 as a macrophage marker in breast cancer has only been evaluated in a few studies (13-15), where high levels of CD163+ macrophages positively correlated with unfavorable prognostic factors. However, the relationship between high levels of TAM, VI and detection mode (screen-detected versus interval breast cancer) has not been investigated.

The aim of our study was to establish whether high CD163 macrophage content and VI are associated in human breast cancer, and whether CD163 is related to the mode of detection. Furthermore, we wanted to investigate if this marker is related to specific molecular subgroups of breast cancer and if it is a marker of poor prognosis.

2. Material and Methods

2.1 Patient series

The cohort of invasive breast cancer utilized in this study consists of 282 cases with 200 screen-detected and 82 interval cancers from Vestfold County in Eastern Norway between 2004 and 2009. The Norwegian Breast Cancer Screening Program (NBCSP), which started in 1996, is population based and covers women aged 50-69 years. The screening interval is two years, and two-view mammograms and independent double readings are standard. As part of the NBCSP, we here studied a consecutive population-based series from Vestfold County in Eastern Norway. Vestfold comprises 5% of the Norwegian population with around 230,000 inhabitants. A total of 37,977 women participated during the study period 2004 - 2009, with attendance rates 71% and 76%, respectively, during the first two screening rounds. During this period, 204 invasive screen-detected cancers and 85 interval breast cancers were diagnosed after the prevalent and subsequent rounds. Three cases were excluded from this series: 1 screen-detected cancer had no residual tumor tissue for further investigation; 1 screen-detected tumor was diagnosed as a malignant phyllodes tumor, and 1 patient with interval cancer suffered from multiple metastases at the time of diagnosis, and no biopsy or surgery was performed. Four patients (two screening- and two interval-patients) had simultaneous tumors in both breasts. To represent these cases for the present study, the Nottingham Prognostic Index (NPI) was used for three cases, to select the tumor with characteristics of the worst prognosis. The fourth patient had identical NPI in both tumors; the tumor with the highest Ki67 score was subsequently selected for our study. Thus, a total of 282 invasive cancers were available for this population-based study, 200 screen-detected and 82 interval cancers.

Regarding primary treatment, 204 resections (72%) and 78 mastectomies (26%) were performed; 218 patients received radiation (77%), 156 received endocrine treatment (55%), and 77 received chemotherapy (27%). In 12 patients, no information on radiation, endocrine treatment or chemotherapy was available. Distant metastases were observed at follow–up in 34 cases (12%), and 24 patients (8%) died of breast cancer. The study was approved by the Regional Ethics Committee of Eastern Norway (REK #S-08685d).

2.2 Tissue categories

TMA (tissue microarray) blocks were used; three tissue cores (diameter 1.0 mm) were extracted from tumor samples in paraffin embedded blocks and inserted into TMA recipient blocks using a semi-automated precision instrument (Minicore 3 Tissue Arrayer, Alphelys, France). TMA was available for 229 (81%) cases. In 40 (14%) cases, TMA cores had too limited tissue for evaluation, and whole sections (WS) were therefore selected. In 13 cases (5%), preoperative core needle biopsies (CNB) were used as tissue source because TMA cores and surgical specimens were limited or of insufficient quality for evaluation. Notably, there were no significant differences when these 13 CNB cases, or when the 53 CNB and whole tissue section cases combined, were compared with all other cases, for standard variables, i.e. tumor diameter, histologic grade, lymph node status, expression of ER, PR, HER2 and Ki67, and there was no significant difference regarding detection mode. Thus, we consider these 13 CNB cases and the 40 whole section cases as random subgroups of the series, and they were included for further studies.

2.3 Immunohistochemistry

All slides were dewaxed with xylene/ethanol before antigen retrieval in a pressure cooker (Decloaking Chamber Plus) in TRS buffer (pH6). The slides were incubated for 1 hour with a mouse monoclonal CD163 antibody (S1699, clone 10D6; Dako, Glostrup, Denmark), diluted 1:200. The staining was performed using the ENVision-labelled polymer method.

CD163 staining was detected in the cytoplasm, and was evaluated in a quantitative manner. Staining was examined in the most active areas with respect to presence of TAMs (hot-spot areas), most often situated in the periphery of the tumor **(Figure 1),**

in stromal bands or between tumor cells. The target area was evaluated by using an eye-piece graticule (10 x 10 grid-lines; 0.31 x 0.31 mm; total 0.096 mm^2), and the number of positive cells were counted within one such area per case. For estimation of inter-observer agreement for these CD163 counts, 50 cases were examined by two pathologists (TAK, YC), showing good agreement with a kappa-value of 0.72.

Additionally, we also evaluated the proportion of CD163 positive cells relative to the total amount of macrophages. CD163 positive cells and negative cells with a macrophage like morphology crossing horizontal grid lines were included, and this CD163 score was expressed as the percentage of positively stained cells among the total number of cells with macrophage like morphology (sum of CD163 positive and negative cells). This CD163 score (or proportion) was examined in the same area and as part of the same procedure as the CD163 count. At least 50 cells (in total) were examined per case.

Immunohistochemistry and evaluation of staining for LVI (D2-40) and BVI (CD31) have been described previously (3, 16), using whole sections. VI was most often found in peritumoral areas. Evaluations of estrogen receptor (ER), progesterone receptor (PR) and HER2 status have also been reported (17). Positivity for CK5/6 and/or P-cadherin was used to define basal-like differentiation (18, 19).

Surrogate markers for molecular subtypes of breast cancer were defined and applied according to the St Gallen consensus from 2013 (20). The cut-off point for estrogen and progesterone receptors was 1% in the present study.

2.4 Statistics

All statistical analyses were performed by IBM SPSS Statistics, version 23.0 (Armonk, NY: IBM Corp.). Two-sided p-values of <0.05 were considered statistically significant. Associations between different categorical variables were assessed by Pearson's χ^2 test. Univariate survival analysis of time to death due to breast cancer (disease specific survival) and time to recurrence for patients without metastases at the time of diagnosis (recurrence free survival) were performed using the Kaplan-Meier method (log-rank test for differences). Entry date was the time of diagnosis. Patients who died from other causes were censored at the date of death in the

analyses of disease specific survival. The Cox´ proportional hazards method was used for multivariate survival analyses. Variables were visually examined by log-minus-log plots to check proportionality assumptions. A multivariate analysis was conducted for combinations of high CD163 and presence of BVI or LVI together with standard prognostic variables. For statistical analysis, CD163 counts were dichotomized; high CD163 count (upper quartile) and low CD163 count (others). CD163 levels as a continuous variable (%) was used to compare molecular subgroups by the Kruskal-Wallis H-test. A total of 282 patients were accessible for survival analysis in the current study.

3. Results

3.1 Presence of CD163 positive macrophages in breast cancer tissue

CD163 positive macrophages were present in the tumor stroma in 257 cases (91%), within tumor cell nests in 224 cases (79%), and in both compartments in 200 cases (71%). The median CD163 count, irrespective of the compartment, was 72 (range 2-347) (i.e. per target area as previously defined, see above). The median score of CD163 positive macrophages from all cases was 44% (range 10-85) based upon hot-spot assessment and 39% (range 8-75) for average counts. Distribution of CD163 counts and scores was not different between tissue categories (TMA, CNB, WS) (Kruskal-Wallis test, P= 0.4 and P=0.6, respectively) as shown in **Table S-1** and **S-2.**

3.2 High levels of CD163 positive macrophages are associated with adverse clinico-pathologic features

CD163 counts were quantified and dichotomized as high (by upper quartile) and low levels. Associations of CD163 counts are shown in **Table 1**. High counts of CD163 in hot-spot areas were significantly more frequent with tumor size ≥ 2cm (OR=1.8; P<0.049) and grade 2-3 versus grade 1 (OR=5.3; P<0.001). A trend was also present for positive nodal status compared to lymph node negative tumors (OR=1.7; P=0.054). Furthermore, high CD163 counts were strongly associated with negative ER (OR=11.8; P<0.001), PR (OR= 2.7; P<0.001), Ki67-high tumors (by upper quartile) (OR=5.4; P<0.001) and positive HER2 status (OR=4.5; P<0.001).

When CNBs were excluded from the analyses, similar associations were found for most features, like tumor size ≥ 2cm (OR=1,6; P= 0.12), histologic grade 2-3 versus grade 1 (OR=4.5; P<0.001), negative ER (OR=12.7; P<0.001), PR (OR=3.0; P<0.001), Ki67-high tumors (by upper quartile) (OR=4.7; P< 0.001) and positive HER2 status (OR=4.0; P=0.001), but with a borderline significance for positive nodal status (OR 1.7; P=0.063) and tumor size (OR 1.6; P=0.129).

High CD163 scores (in %) in hot-spot areas were also significantly more frequent with tumor size ≥ 2cm (OR=3.2; P<0.001), grade 2-3 versus grade 1 (16.7; P<0.001), and positive nodal status, compared to lymph node negative tumors (OR=2.0; P=0.013).

3.3 High levels of CD163 positive macrophages are associated with non-luminal breast cancer subgroups

When looking at molecular subtypes in this population-based cohort, we found 51% Luminal A, 32% Luminal B (HER2-negative), 6% luminal B (HER2-positive), 4% HER2-type, and 7% Triple negative tumors (**Table 2**). Median CD163 counts were 58 and 68 in Luminal A and Luminal B/HER2-negative tumors. Median counts in Luminal B/HER2-positive tumors, Triple negative tumors and the HER2- type were 115, 149 and 177, respectively (Kruskal-Wallis test, P<0.001) **(Figure 2).**

Nearly identical results for CD 163 counts were found when excluding CNB, with 52% Luminal A, 31% Luminal B (HER2-negative), 6% Luminal B (HER2-positive), 3% HER2-type, and 8% Triple negative tumors. Median CD163 counts without CNB were 57 and 66 for Luminal A and Luminal B/HER2-negative tumors, whereas Luminal B/HER2-positive tumors, Triple negative tumors and the HER2 type showed the same median counts with 115, 149 and 177 respectively (Kruskal-Wallis test, P<0.001).

Both Luminal A and Luminal B/HER2-negative tumors had a median CD163 score of 42%. The median hot-spot scores of CD163 in Luminal B/HER2-positive tumors and the HER2-type were 50% and 59%, respectively, whereas the Triple negative

subgroup demonstrated the highest CD163 score of 62% (Kruskal-Wallis test, P<0.001) (**Figure S-1**).

3.4 High levels of CD163 positive macrophages are strongly associated with VI, triple negative tumors, a basal-like phenotype and interval breast cancer

VI by combined CD31 and D2-40 immuno-histochemistry for all tumors was present in 88 (31%) of the cases. Of these, 45 tumors (16%) showed LVI only, 18 (6%) had BVI only, and 25 (9%) showed both BVI and LVI, giving overall frequencies of LVI and BVI of 25% and 15%. High CD163 counts (by upper quartile) showed a strong association with both BVI (OR=4.2; p<0.001) and LVI (OR=4.6; p<0.001) compared to tumors without VI (**Table 3**). Notably, when we excluded cases with combined BVI and LVI, high CD163 counts still had a strong association with both BVI (OR=3.2; P=0.016) and LVI (OR=3.9; P<0.001). Furthermore, high CD163 counts were more frequent in Triple-negative (OR=9.4; p<0.001) and Basal-like tumors, as defined (OR=6.1; p<0.001), compared with other subgroups. High CD163 counts were more frequent in interval cancers versus screen-detected tumors (OR=9.4; p<0.001). High CD163 scores (by upper quartile) for all cases and high CD163 counts (by upper quartile) without CNB both showed a similar and significant relationship to VI, Triple negative tumors, the Basal-like phenotype and interval breast cancer (data not shown).

3.5 Survival analysis

The median follow-up period among survivors was 71 months (range 2-117 months). Among the 282 patients, distant metastases or local tumor recurrence were observed at follow up in 34 cases (12%), and 24 patients (8%) died of breast cancer.

In univariate survival analysis, a high CD163 macrophage count (by upper quartile) was associated with reduced recurrence-free survival (RFS) and disease specific survival (DSS), compared to low CD163 counts (P=0.006 and P=0.060, respectively) **(Figure 3)**. However, there was no significant association with survival for CD163 counts within the five St. Gallen subtypes when studying them separately (data not shown). By multivariate analysis of RFS, CD163 counts showed a significant

influence (HR=2.2; P=0.022) in addition to lymph node status (HR=4.6; P<0.001) when compared with tumor diameter, histological grade, and lymph node status.

Cases with combined high CD163 counts and BVI showed a significantly reduced RFS and DSS (P<0.001 for both) compared with all other cases. Similar trends were seen for high CD163 counts and LVI for RFS (P=0.15) and DSS (P=0.30) (**Figure S-2**). Additionally, the whole patient series was divided into three categories: 1) BVI+ and high CD163 counts, 2) BVI- and low CD163 counts, and 3) other cases (either BVI+ or high CD163 counts). Univariate survival analysis showed three significantly different survival curves for RFS (P<0.001) and DSS (P<0.001), with intermediate prognosis for cases with either BVI or high CD163 expression (by counts), whereas those with both BVI positivity and high CD163 count had the worst prognosis (**Figure 4**).

Basic histopathologic markers such as tumor diameter, histologic grade and lymph node status in addition to high CD163 counts and VI were included in multivariate analysis of RFS. High CD163 counts in combination with BVI were significantly associated with shorter RFS (HR=3.8, p=0.001) as was also lymph node metastases (HR=4.4, p<0.001), whereas high CD163 in combination with LVI was not statistically significant (**Table 4**).

4. Discussion

In this study, we found a strong and novel association between CD163 positive tumor-associated macrophages and vascular invasion. This relationship applied to both BVI and LVI, possibly indicating that intravasation into blood vessels and lymphatic channels may have a similar dependence on M2 macrophages. This is in keeping with previous experimental observations that macrophages promote migration and intravasation of breast cancer cells into both blood and lymphatic vessels (10). Interestingly, a recent study of syngeneic mice indicated that mammary tumor cells undergoing epithelial-mesenchymal transition (EMT) preferentially migrated toward lymphatic vessels compared with blood vessels (21). Transforming growth factor-β1 produced by macrophages and regulatory T-cells may promote this EMT in mammary tumor cells (21-23).

In survival analysis, high levels of CD163 positive M2 macrophages were significantly associated with reduced recurrence-free and breast cancer specific survival. Studies have shown that aggressive breast cancer is associated with TAM accumulation in hypoxic areas, and vascular endothelial growth factor (VEGF) production from these macrophages stimulates tumor angiogenesis (11, 24, 25). Furthermore, we have previously reported that aggressive breast cancers of a basal-like phenotype appear to have increased angiogenesis with more microvessel proliferation and higher frequency of the glomeruloid microvascular pattern (GMP) (19, 26)This may indicate a link between TAM accumulation with high CD163 content, angiogenesis and BVI in aggressive breast cancer.

Notably, we have earlier reported a strong association between BVI and tumor detection during breast cancer screening intervals following mammography. Our study demonstrates that this strong relationship also seems to include high CD163 counts in interval cancers compared to screening-detected tumors. To our knowledge, this is the first time an association between high TAM level (CD163+) and interval detected breast cancer has been reported. Follow-up studies from different countries indicate that screen-detected cancers have more low-grade features and better outcome than predicted from tumor-size, histological grade, and lymph node status, and molecular subtyping of tumors can only partly account for this difference (27-31). The presence of CD163 positive macrophages together with BVI can potentially explain some of these observations.

Interestingly, we found that triple negative tumors showed a strong association with high CD163+ tumor associated macrophages. A recent study demonstrated that triple-negative breast cancer promotes monocyte differentiation into M2 like macrophages with increased CCL2 secretion, a chemokine with tumor promoting properties (32). In addition, we also found higher CD163 levels in basal-like tumors compared to other cases.

We have previously shown a correlation between interval presenting cases and the basal-like phenotype (17). Basal-like breast cancer cell lines express a broader range of receptors for macrophage-derived cytokines than luminal cell lines, and many of

the most highly expressed growth factors, such as epidermal growth factor (EGF), transforming growth factor (TGF), and CD44 are associated with tumor invasion and metastasis in the basal-like cell lines (33). Furthermore, basal-like tumors recruit monocytes and polarize macrophages more effectively than luminal cancers (34). Our present data are consistent with these previous findings.

A limitation to our study may be the use of TMA sections. Due to tumor heterogeneity, CD163 levels evaluated on TMAs will not accurately reflect CD163 levels from whole sections. To reduce this potential confounding factor, care was taken to select areas for TMA with high tumor purity and to include the periphery of the tumor. Additionally, three tissue cores (diameter 1.0 mm) were included to increase the amount of tissue being evaluated.

We decided to include cases represented by CNB tissue in this study, since we found that these were not significantly different with respect to standard features of the primary tumors, like diameter, histologic grade, lymph node status, ER, PR, HER2 and Ki67 expression, as well as mode of detection. In these cases, TMA cores were lacking and the remaining tissue was too limited or of insufficient quality for evaluation. Conversely, the essential associations were similar when CNB cases were excluded, and there was no statistical difference between the CD163 count on whole sections, CNB tissues and TMA slides. In the final analyses, CNB cases were therefore included in our series.

In conclusion, this study demonstrates that high levels of CD163+ tumor associated macrophages were strongly related to VI as well as tumor detection in screening intervals. In addition, the presence of high CD163+ TAM levels was more often observed in non-luminal molecular subgroups and basal-like breast cancer, and was associated with adverse prognosis.

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Figure legends

Figure 1: **Histological images of CD163 by immunohistochemistry.**
A histological image of tumor tissue with clusters of CD163 positive cells in the periphery with low magnitude (1A) (X100) and high magnitude 1B (X400). Another image is shown without CD163 positive cells (1C) (X100).

Figure 2: **Median CD163 counts in molecular breast cancer subgroups.**
Data represent median values with error bars denoting the limits of the 95% confidence intervals.

Figure 3: **Estimated recurrence free (A) and disease specific (B) survival according to high (upper quartile) or low CD163 counts.**
Survival curves are estimated by the Kaplan-Meier method (with log-rank test differences). For each category, number of events / total number of cases are given.

Figure 4: **Estimated recurrence free (A) and disease specific (B) survival according to high (upper quartile) or low CD163 counts in a combination with BVI positive or negative cases.**
Survival curves are estimated by the Kaplan-Meier method (with log-rank test for differences). For each category, number of events / total number of cases are given.

Figure S-1: **Median CD163 scores in molecular breast cancer subgroups.**
Data represent median values with error bars denoting the limits of the 95% confidence intervals.

Figure S-2: **Estimated recurrence free (A and C) and disease specific (B and D) survival according to high (upper quartile) CD163 counts in a combination with LVI or BVI positivity.**
Survival curves are estimated by the Kaplan-Meier method (with log-rank test for differences). For each category, number of events / total number of cases are given.

Table 1: Associations between high and low counts of CD163 positive TAM`s compared with various clinicopathological variables (n=282)

Variable		CD163 counts			
	N	Low	High	OR	P-value
Histologic type					
Others	54	45 (21%)	9 (13%)	1	0.123
Ductal	228	167 (79%)	61 (87%)	1.8 (0.8-3.9)	
Tumor size					
< 2cm	214	167 (79%)	47 (67%)	1	0.049
≥ 2cm	68	45 (21%)	23 (33%)	1.8 (0.99-3.3)	
Histologic grade					
1	76	70 (33%)	6 (9%)	1	<0.001
2-3	206	142 (67%)	64 (91%)	5.3 (2.1-12.7)	
Lymph node status*					
Negative	187	147 (70%)	40 (57%)	1	0.054
Positive	94	64 (30%)	30 (43%)	1.7 (0.98-3.0)	
ER					
Positive	249	203 (96%)	46 (66%)	1	<0.001
Negative	33	9 (4%)	24 (34%)	11.8 (5.1-26.9)	
PR					
Positive	186	152 (72%)	34 (49%)	1	<0.001
Negative	96	60 (28%)	36 (51%)	2.7 (1,5-4.7)	
HER 2 status					
Negative	255	200 (94%)	55 (79%)	1	<0.001
Positive	27	12 (6%)	15 (21%)	4.5 (2.0-10.3)	
Ki67					
Low	211	177 (84%)	34 (49%)	1	<0.001
High	71	35 (16%)	36 (51%)	5.3 (2.9-9.7)	

P-values were obtained using Pearson´s Chi square test. High CD 163 counts are given by the upper quartile. *One case excluded due to missing information on lymph node status. Abbreviations: BVI, blood vessel invasion; LVI, lymph vessel invasion; N, number of cases; OR, odds ratio; TAM, tumor associated macrophages

Table 2: Associations between high and low counts of CD163 positive TAM`s by tumour subtypes (n=282)

	Luminal A	Luminal B HER2-	Luminal B HER2+	HER2 type	Triple negative		Total
	N (%)	N (%)	N (%)	N (%)	N (%)	p-value	
	145 (51%)	89 (32%)	18 (6%)	9 (4%)	21 (7%)		282
CD163							
High counts	20 (14%)	20 (22%)	8 (44%)	7 (78%)	15 (71%)	<0.001	70
Low counts	125 (86%)	69 (78%)	10 (56%)	2 (22%)	6 (29%)		212

Number of cases (N) and % within molecular subgroups is given according to the St Gallen consensus 2013. P values were obtained using Pearson`s Chi square test. High CD163 counts are given by the upper quartile. Abbreviation: TAM, tumor associated macrophages.

Table 3: Association between high and low counts of CD163 positive TAM`s by vessel invasion, basal-like and triple negative tumors.

Variable			CD163 counts			
		N	Low	High	OR	P-value
BVI	Negative	239	191 (90)	48 (69)	1	<0.001
	Positive	43	21 (10)	22 (31)	4.2 (2.1-8.2)	
LVI	Negative	212	176 (83)	36 (51)	1	<0.001
	Positive	70	36 (17)	34 (49)	4.6 (2.6-8.3)	
Basal-like	No	229	189 (89)	40 (57)	1	<0.001
	Yes	53	23(11)	30 (43)	6.1 (3.2-11.7)	
Triple negative	No	261	206 (97)	55 (79)	1	<0.001
	Yes	21	6 (3)	15 (21)	9.4 (3.5-25.3)	

P-values were obtained using Pearson´s Chi square test. High CD 163 counts are given by the upper quartile.
Abbreviations: BVI, blood vessel invasion; LVI, lymph vessel invasion; N, number of cases; OR, odds ratio.

Table 4: Univariate and multivariate recurrence free survival analysis (Cox`proportional hazard method) of pathological variables and combinations of CD163 counts and VI by immunohistochemistry (n=282)

Variables	Categories	Univariate analysis		Multivariate analysis	
		HR (95%CI)	p-value	HR (95% CI)	p-value
High CD163 and BVI	No	1		1	
	Yes	5.0 (2.2- 11.2)	<0.001	3.4 (1.4-8.5)	0.009
High CD163 and LVI	No	1		1	
	Yes	1.8 (0.8-4.2)	0.156	0.9 (0.4-2.2)	0.795
Tumor diameter	< 2cm	1		1	
	≥2cm	2.7 (1.4-5.4)	0.004	1.3 (0.6-2.7)	0.518
Histologic grade	1	1		1	
	2-3	2.9 (1.1-8.5)	0.040	1.9 (0.6-5.7)	0.264
Lymph node status	Negative	1		1	
	Positive	5.0 (2.4-10.6)	<0.001	4.1(1.9-8.7)	<0.001

Abbreviations: HR, hazard ratio; 95% CI; 95% confidence interval;

Table S1. CD163 counts according to tissue categories

	N (%)	CD163 hotspot counts		
		Median	Range	Mean
All cases	282 (100)	72	2-347	86
TMA	229 (81)	67	2-347	84
WS	40 (14)	85	13-250	89
CNB	13 (5)	90	20-237	109

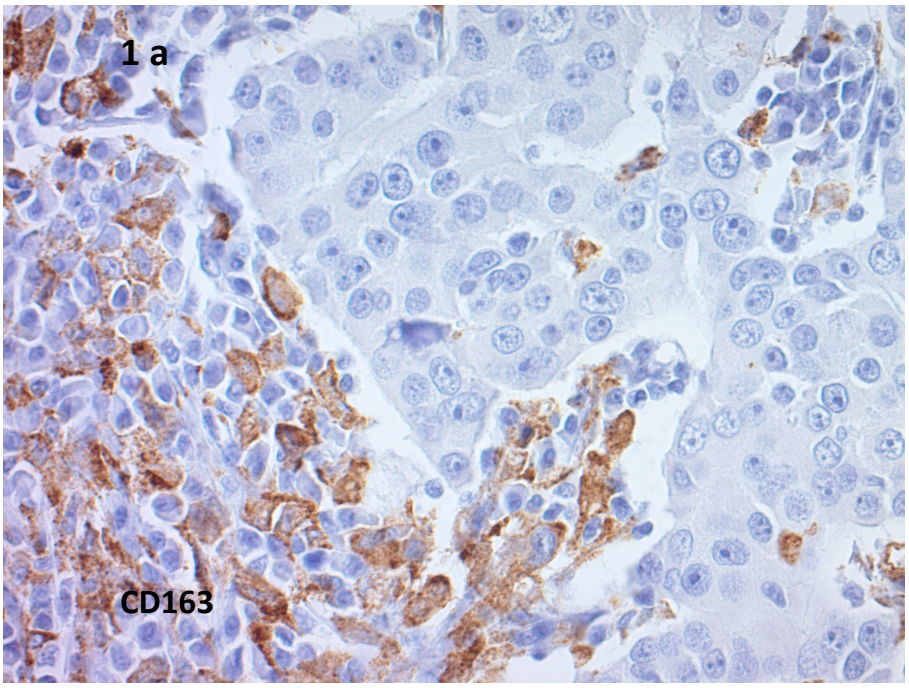
TMA, tissue microarrays; WS, whole sections; CNB, core needle biopsies

Table S2. CD163 scores according to tissue categories

	N (%)	CD 163 hotspot scores		
		Median	Range	Mean
All cases	282 (100)	44	10-85	45
TMA	229 (81)	44	10-85	45
WS	40 (14)	49	15-71	48
CNB	13 (5)	43	25-72	46

TMA, tissue microarrays; WS, whole sections; CNB, core needle biopsies

Figure 1



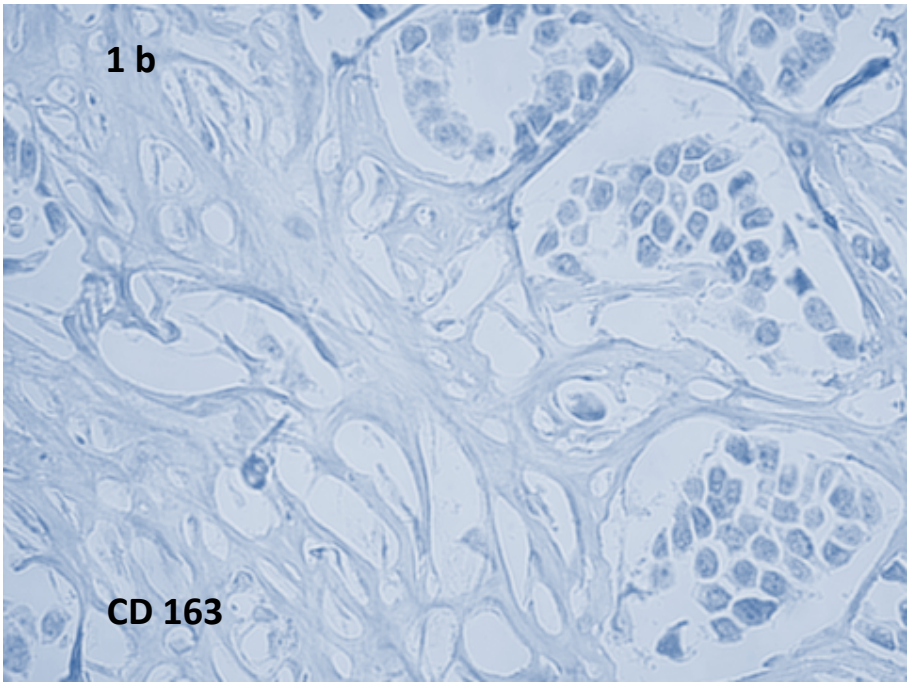


Figure 2

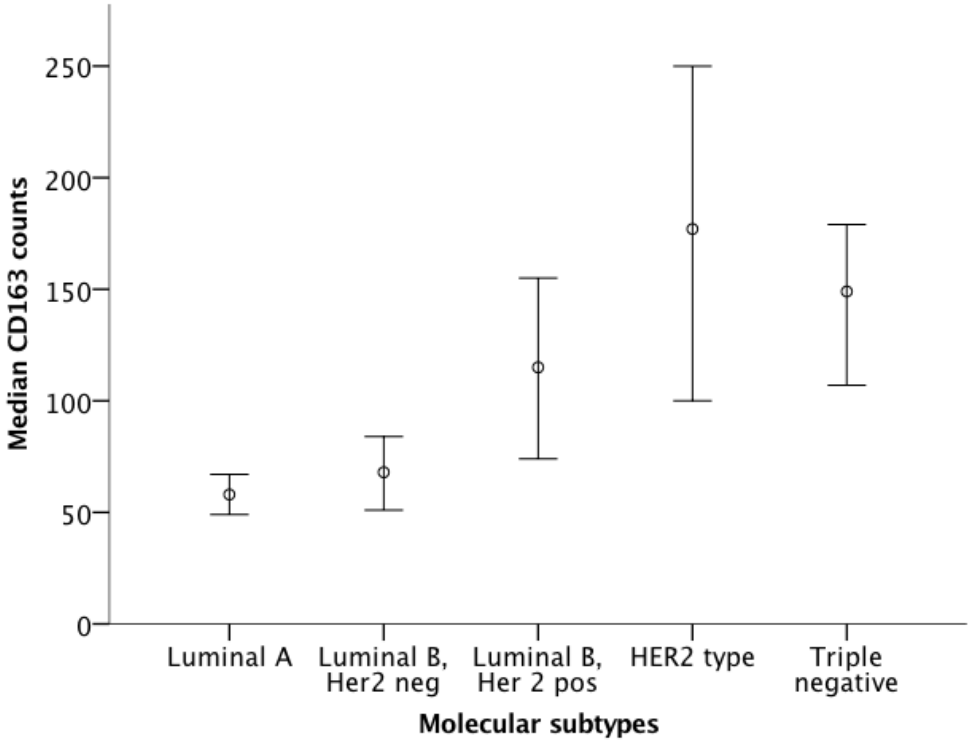

Median CD163 counts
250
200
150
100
50
0
Luminal A
Luminal B, Her2 neg
Luminal B, Her 2 pos
HER2 type
Triple negative
Molecular subtypes

Figure 3

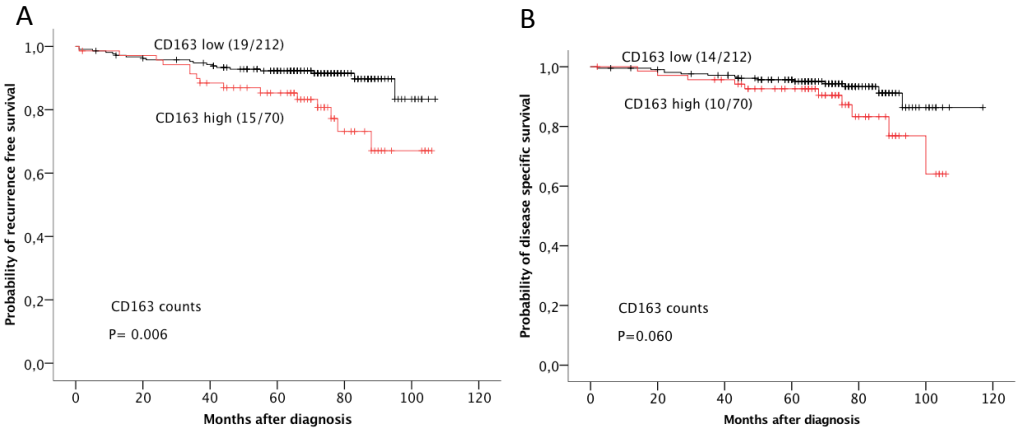

A
CD163 low (19/212)
CD163 high (15/70)
CD163 counts
P= 0.006
Probability of recurrence free survival
Months after diagnosis
B
CD163 low (14/212)
CD163 high (10/70)
CD163 counts
P=0.060
Probability of disease specific survival
Months after diagnosis

Figure 4

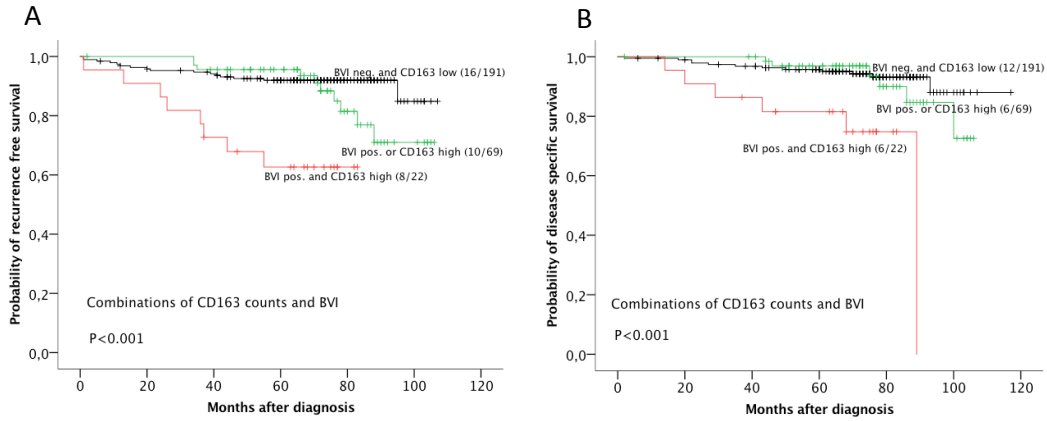

A
Probability of recurrence free survival
1,0
0,8
0,6
0,4
0,2
0,0
BVI neg. and CD163 low (16/191)
BVI pos. or CD163 high (10/69)
BVI pos. and CD163 high (8/22)
Combinations of CD163 counts and BVI
P<0.001
0
20
40
60
80
100
120
Months after diagnosis
B
Probability of disease specific survival
1,0
0,8
0,6
0,4
0,2
0,0
BVI neg. and CD163 low (12/191)
BVI pos. or CD163 high (6/69)
BVI pos. and CD163 high (6/22)
Combinations of CD163 counts and BVI
P<0.001
0
20
40
60
80
100
120
Months after diagnosis

Figure S-1

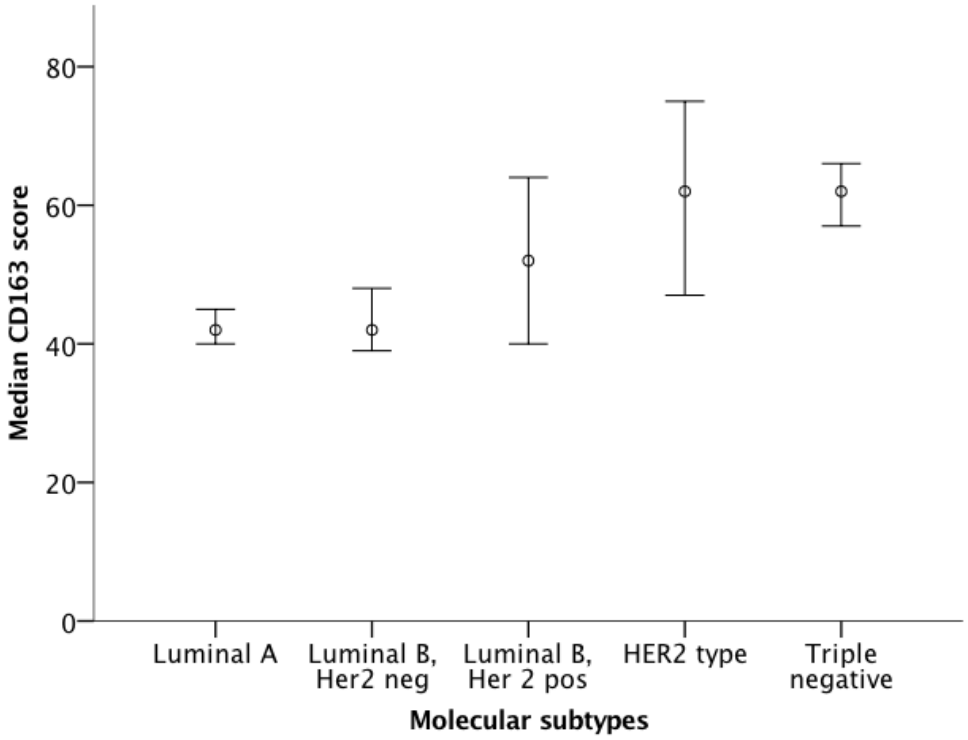
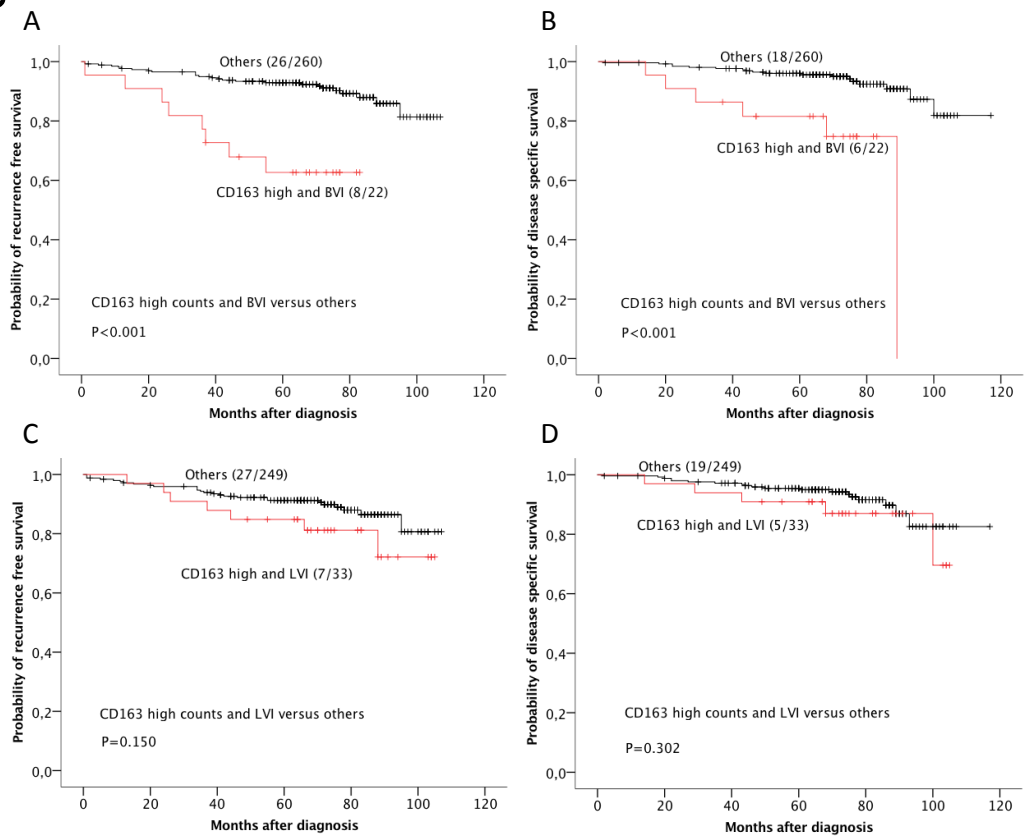

80
60
40
20
0
Median CD163 score
Luminal A
Luminal B, Her2 neg
Luminal B, Her 2 pos
HER2 type
Triple negative
Molecular subtypes

Figure S-2


A
Others (26/260)
CD163 high and BVI (8/22)
CD163 high counts and BVI versus others
P<0.001
Probability of recurrence free survival
Months after diagnosis
B
Others (18/260)
CD163 high and BVI (6/22)
CD163 high counts and BVI versus others
P<0.001
Probability of disease specific survival
Months after diagnosis
C
Others (27/249)
CD163 high and LVI (7/33)
CD163 high counts and LVI versus others
P=0.150
Probability of recurrence free survival
Months after diagnosis
D
Others (19/249)
CD163 high and LVI (5/33)
CD163 high counts and LVI versus others
P=0.302
Probability of disease specific survival
Months after diagnosis