cancers

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Article

Granulocyte colony stimulating factor expression in breast cancer and its association with carbonic anhydrase IX and immune checkpoints

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Supplementary data: cohort I:

G-CSF expression in breast cancer and its association with clinicopathological features and prognosis:

Overall, 76.7% (253/330) cases were evaluable for G-CSF expression. Of these, 46.6% tumours were high expressers. After Bonferroni corrections for multiple comparisons, no significant associations were observed between expression of G-CSF and major clinicopathological features in this cohort (Supplementary Table S2). Univariable analysis revealed that cases with G-CSF$_{high}$ expression demonstrated a favorable trend for breast cancer specific survival (HR 0.58, CI 0.32–1.06; $p = 0.07$) and a significantly better overall survival (HR 0.51, CI 0.32–0.82; $p = 0.004$) (Supplementary Figure 1). In multivariable analysis tumours with G-CSF$_{high}$ expression maintained the prognostic associations with primary and secondary endpoints, independent of the traditional clinicopathological features (Supplementary Table 3)

Supplementary Tables:

Table S1. Details of the antibodies and staining protocols.

Antibodies	Clone	Supplier	Antigen retrieval	Dilution	Detection	Positive controls
Rabbit polyclonal G-CSF	HPA001412	Sigma	HIER x 64 minutes **	1:25 × 2 h (RT)	ChromoMap DAB (Ventana)	Normal pancreas
Mouse monoclonal CD163	NCL-L-CD163	Novocastra	HIER **	1:500 × 2 h (RT)	ChromoMap DAB (Ventana)	Placenta
Mouse monoclonal CAIX	M75	Bioscience Slovakia	HIER ×30 min ***	1:50	Vector Im-PRESS	Breast cancer tissue

* For all protocols, array sections at 4μm were mounted on poly-L-lysine coated glass slides and baked for an hour at 60 °C to prepare for staining on the Ventana Discovery XT automated Stainer (Ventana Medical Systems, Tucson, AZ). For negative controls, primary antibodies were replaced with PBS; ** Antigen retrieval was performed by using Cell Conditioning 1 (Ventana); HIER = heat induced epitope retrieval; *** Antigen retrieval was performed using citrate buffer.

Table S2. Cohort I: Correlation of G-CSF expression with clinicopathological features & other biomarkers.

Clinicopathological variables	G-CSF Expression		*p*-value *
	Low (≤1)	High (>1)	
Age at diagnosis			
< 50	40 (29.6)	51 (43.2)	0.03
≥ 50	95 (70.4)	67 (56.8)	
Menstrual status			0.03

Clinicopathological variables	G-CSF Expression		*p*-value *
	Low (≤1)	High (>1)	
Premenopausal	46 (34.8)	56 (48.3)	
Postmenopausal	86 (65.2)	60 (51.7)	
Tumour size (cm)			
≤ 2	76 (56.3)	65 (55.1)	0.85
> 2	59 (43.7)	53 (44.9)	
Tumour grade			
1 & 2	83 (62.4)	59 (50)	0.05
3	50 (37.6)	59 (50)	
Axillary lymph node status			
Negative	56 (42.7)	35 (34.7)	0.21
Positive	75 (57.3)	66 (65.3)	
Lymphovascular invasion			
Negative	34 (26.2)	25 (21.7)	0.42
Positive	96 (73.8)	90 (78.3)	
ER expression			
Negative	26 (19.4)	30 (25.4)	0.25
Positive	108 (80.6)	88 (74.6)	
PR expression			
< 1%	46 (34.3)	38 (32.5)	0.76
≥ 1%	88 (65.7)	79 (67.5)	
HER2 overexpression/amplification			
Negative	126 (94.7)	104 (92.9)	0.54
Positive	7 (5.3)	8 (7.1)	
CK5/6 expression			
Negative	129 (96.3)	105 (89)	0.03
Positive	5 (3.7)	13 (11)	
EGFR expression			
Negative	125 (92.6)	98 (83.1)	0.02
Positive	10 (7.4)	20 (16.9)	
Ki-67 proliferation index			
< 14%	82 (61.7)	66 (56.9)	0.45
≥ 14%	51 (38.3)	50 (43.1)	
Breast cancer subtypes (IHC based)			
Luminal-NOS	1 (0.8)	2 (1.9)	0.09
Luminal A	69 (57)	60 (57.7)	
Luminal B	38 (31.4)	20 (19.2)	
HER2	5 (4.1)	6 (5.8)	
Basal	8 (6.6)	16 (15.4)	
Treatment			
No systemic therapy	28 (20.7)	25(21.2)	0.21
Tamoxifen only; No chemotherapy	45 (33.3)	30 (25.4)	
Chemotherapy only; no hormonal therapy	21 (15.6)	30 (25.4)	
Chemotherapy + Tamoxifen	41 (30.4)	33 (28)	

* Denotes differences between low and high G-CSF groups that are significant at the Bonferroni-corrected *p*-value of <0.003 (= 0.05/14). G-CSF, granulocyte colony stimulating factor; ER, estrogen receptor; PR, progesterone receptor; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; CK, cytokeratin; NOS, not otherwise specified; IHC, immunohistochemistry.

Table S3. Cohort I: Multivariable analysis for breast cancer specific survival and overall survival.

Covariates	Cohort I (*n* = 253)			
	Breast cancer specific survival		Overall survival	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Age at diagnosis				
< 50	1	0.15	1	0.12
≥ 50	1.65 (0.83–3.28)		2.03 (1.13–3.66)	
Tumour size (cm)				
≤ 2	1	0.007	1	0.10
> 2	2.72 (1.32–5.62)		1.55 (0.92–2.62)	
Tumour grade				
1 & 2	1	0.07	1	0.19
3	1.85 (0.96–3.56)		1.41 (0.85–2.35)	
Axillary lymph node status				
Negative	1	0.08	1	0.03
Positive	0.50 (0.24–1.08)		0.80 (0.45–1.43)	
Lymphovascular invasion				
Negative	1	0.008	1	0.45
Positive	0.39 (0.20–0.78)		0.53 (0.30–0.95)	
G-CSF expression				
Low (≤1)	1	0.02	1	<0.001
High (>1)	0.43 (0.21–0.87)		0.32 (0.18–0.58)	

Table S4. Cohort II: Multivariable analysis for prognostic significance of immune biomarkers within non-luminal cases with CAIX positive expression.

Covariates	BCSS	
	Non-luminal cases /CAIX+ cases	
	HR (95% CI)	*p*-value
Age at diagnosis		
< 50	1	0.22
≥ 50	0.72 (0.42–1.22)	
Tumour size (cm)		
≤ 2	1	0.01
> 2	2.16 (1.20–3.87)	
Tumour grade		
1 & 2	1	0.31
3	0.68 (0.32–1.44)	
Axillary lymph node status		
Negative	1	0.63
Positive	1.16 (0.63–2.13)	
LVI		
Negative	1	0.27
Positive	1.41 (0.77–2.58)	
H & E stromal TILs (%)		
< 10	1	0.001
≥ 10	0.31 (0.16–0.60)	
Age at diagnosis		
< 50	1	0.41
≥ 50	0.79 (0.46–1.37)	
Tumor size (cm)		0.02

Covariates	BCSS	
	Non-luminal cases /CAIX+ cases	
	HR (95% CI)	*p*-value
≤ 2	1	
> 2	2.01 (1.12–3.60)	
Tumor grade		
1 & 2	1	0.19
3	0.59 (0.27–1.29)	
Axillary lymph node status		
Negative	1	0.55
Positive	1.20 (0.65–2.24)	
LVI		
Negative	1	0.13
Positive	1.60 (0.87–2.98)	
CD8 iTIL count		
< 1	1	0.04
≥ 1	0.57 (0.32–0.99)	
Age at diagnosis		
< 50	1	0.69
≥ 50	0.88 (0.49–1.60)	
Tumour size (cm)		
≤ 2	1	0.13
> 2	1.60 (0.87–2.98)	
Tumour grade		
1 & 2	1	0.51
3	0.74 (0.31–1.79)	
Axillary lymph node status		
Negative	1	0.35
Positive	1.37 (0.71–2.66)	
LVI		
Negative	1	0.38
Positive	1.35 (0.69–2.66)	
PD1 iTIL count		
< 1	1	0.05
≥ 1	0.48 (0.23–1.01)	
Age at Diagnosis		
< 50	1	0.27
≥ 50	0.73 (0.41–1.29)	
Tumour size (cm)		
≤ 2	1	0.03
> 2	1.94 (1.06–3.54)	
Tumour grade		
1 & 2	1	0.21
3	0.59 (0.25–1.36)	
Axillary lymph node status		
Negative	1	0.30
Positive	1.40 (0.75–2.61)	
LVI		
Negative	1	0.33
Positive	1.37 (0.73–2.57)	
FOXP3 iTIL count	1	0.20

Covariates	BCSS	
	Non-luminal cases /CAIX+ cases	
	HR (95% CI)	*p*-value
< 2	0.69 (0.40–1.22)	
≥ 2		
Age at diagnosis		
< 50	1	0.33
≥ 50	0.76 (0.43–1.32)	
Tumour size (cm)		
≤ 2	1	0.03
> 2	2.04 (1.09–3.80)	
Tumour grade		
1 & 2	1	0.37
3	0.69 (0.30–1.59)	
Axillary lymph node status		
Negative	1	0.24
Positive	1.46 (0.78–2.75)	
LVI		
Negative	1	0.69
Positive	1.14 (0.59–2.19)	
TIM3 iTIL count		
< 1	1	0.02
≥ 1	0.38 (0.17–0.85)	
Age at diagnosis		
< 50	1	0.44
≥ 50	0.79 (0.43–1.44)	
Tumour size (cm)		
≤ 2	1	0.02
> 2	2.11 (1.13–3.93)	
Tumour grade		
1 & 2	1	0.10
3	0.50 (0.22–1.55)	
Axillary lymph node status		
Negative	1	0.34
Positive	1.37 (0.71–2.63)	
LVI		
Negative	1	0.23
Positive	1.50 (0.79–2.91)	
LAG3 iTIL count		
< 1	1	0.02
≥ 1	0.44 (0.21–0.89)	
Age at diagnosis		
< 50	1	0.17
≥ 50	0.65 (0.35–1.21)	
Tumour size (cm)		
≤ 2	1	0.07
> 2	1.81 (0.95–3.45)	
Tumour grade		
1 & 2	1	0.20
3	0.58 (0.25–1.34)	
Axillary lymph node status		0.34

Covariates	BCSS	
	Non-luminal cases /CAIX+ cases	
	HR (95% CI)	*p*-value
Negative	1	
Positive	1.42 (0.69–2.91)	
LVI		
Negative	1	0.24
Positive	1.55 (0.75–3.24)	
PD-L1+ tumor cells (%)		
0	1	0.02
≥ 1	0.35 (0.15–0.83)	
Age at diagnosis		
<50	1	0.37
≥50	0.77 (0.44–1.35)	
Tumour size (cm)		
≤ 2	1	0.04
> 2	1.80 (1.02–3.18)	
Tumour grade		
1 & 2	1	0.16
3	0.58 (0.27–1.23)	
Axillary lymph node status		
Negative	1	0.68
Positive	1.14 (0.62–2.08)	
LVI		
Negative	1	0.10
Positive	1.67 (0.91–3.08)	
CD163+M2 macrophages		
Sparse	1	
Moderate	0.98 (0.47–2.10)	0.97
Dense	0.83 (0.40–1.71)	0.61

Supplementary Figures:

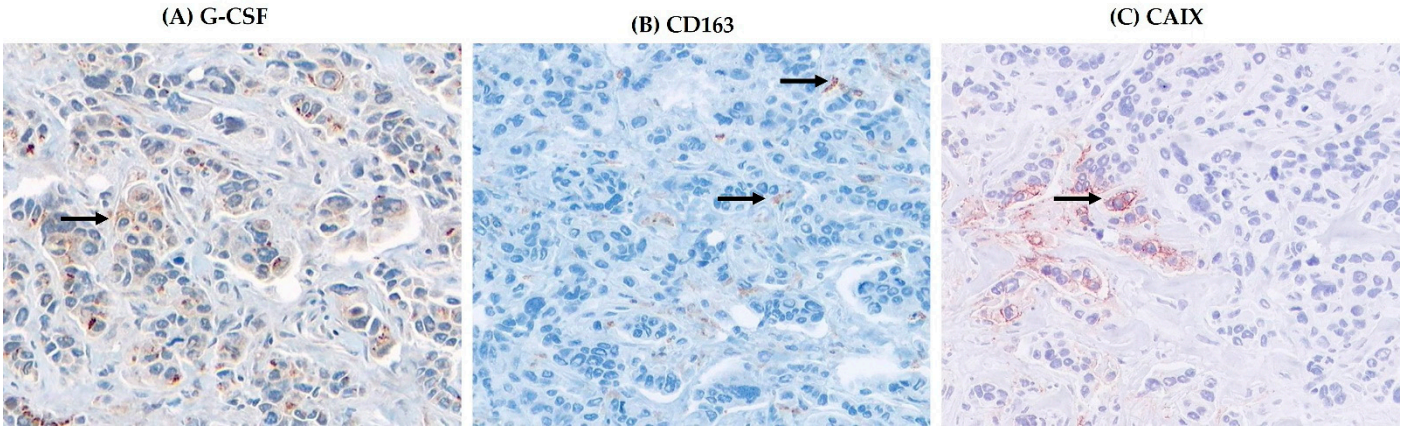

Figure S1. Representative photomicrographs for immunohistochemical staining of G-CSF, CD163 and CAIX in serial sections in core # 1715 from breast carcinoma tissue microarray (cohort II). Black arrows indicate (A) cytoplasmic expression of G-CSF (>1) on breast carcinoma cells; (B) membranous or cytoplasmic expression of CD163 on tumor associated macrophages (>5 but ≤25); and (C) membranous expression of CAIX on breast carcinoma cells (Images acquired at 200×).

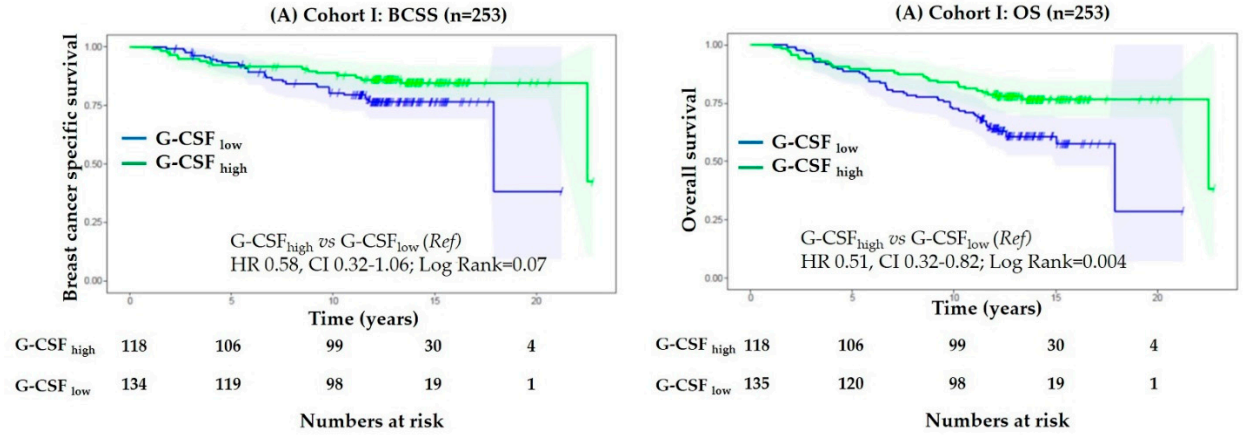

Figure S2. Cohort I: Kaplan Meier curves: Association of G-CSF expression with breast cancer specific survival (A) and overall survival (B).

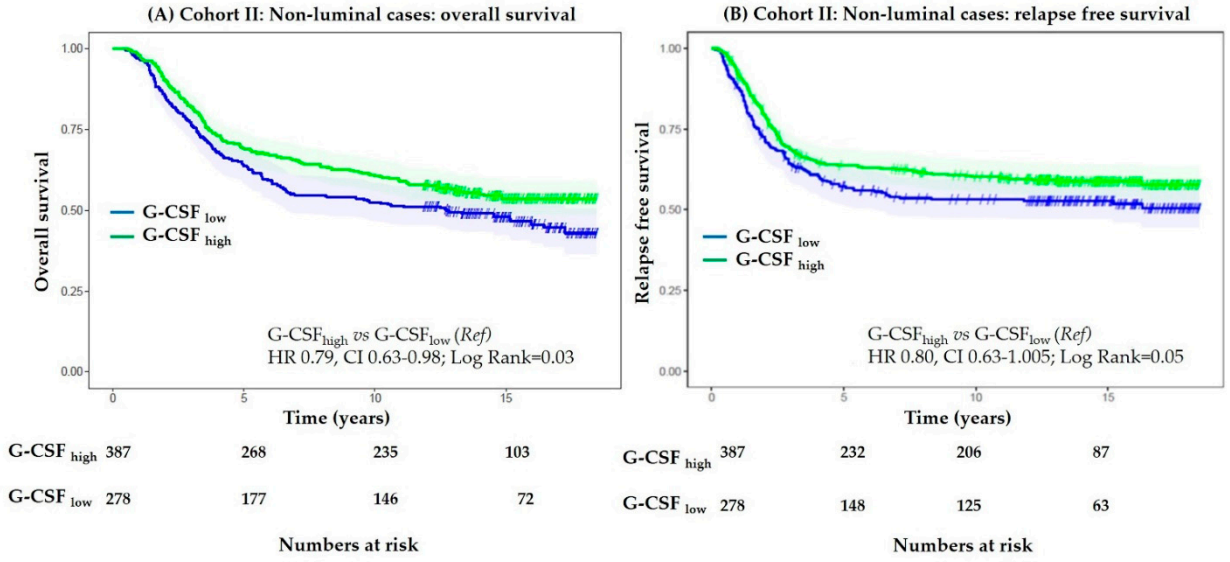

Figure S3. Cohort II: Kaplan Meier curves: Association of G-CSF expression with overall survival (A) and relapse free survival (B) in non-luminal cases.

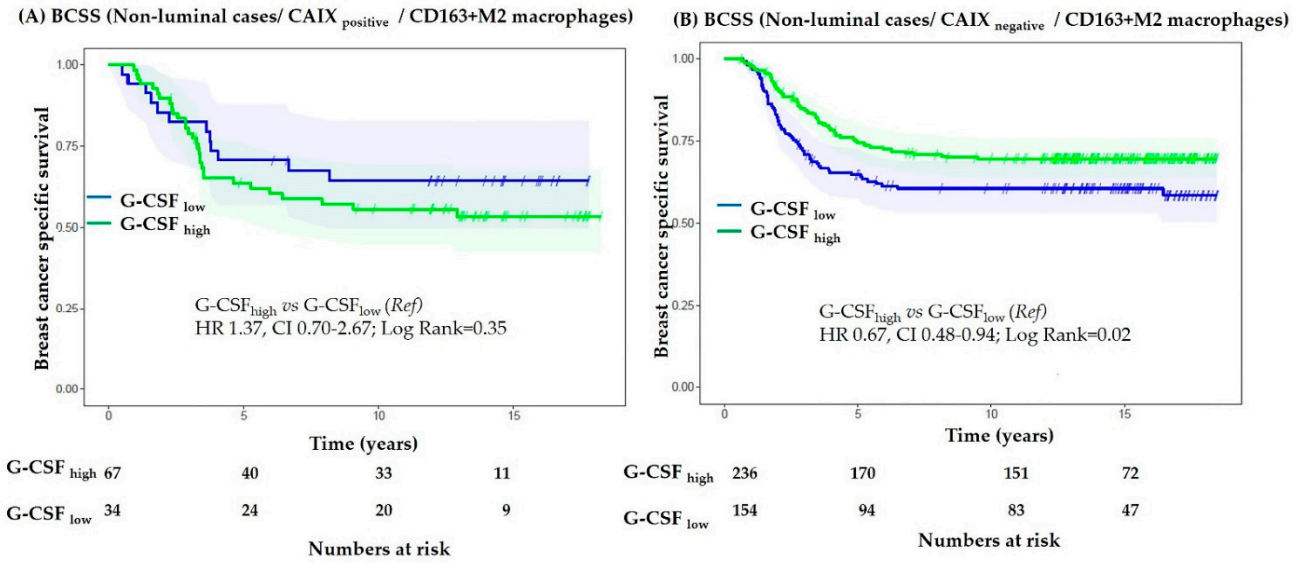

Figure S4. Kaplan Meier curves for association of G-CSF in CD163+ non-luminal tumors with positive (A) and negative (B) expression of CAIX.

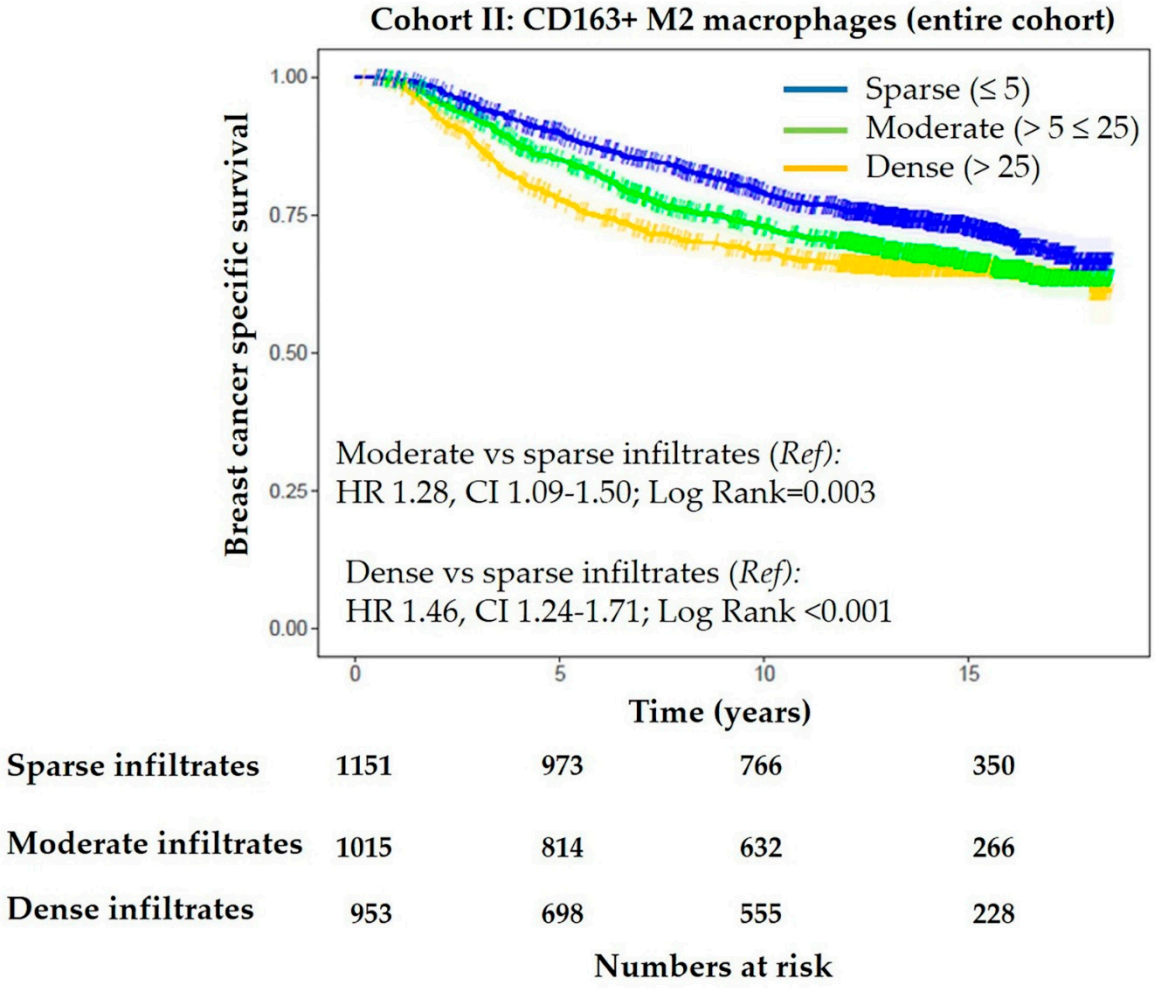

Figure S5. Kaplan Meier curves: Presence of moderate and dense infiltrates of CD163+ M2 tumor associated macrophages is associated with poor breast cancer specific survival.